Vaccine 34 (2016) 3356–3362

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Staphylococcus aureus avirulent mutant vaccine induces humoral and cellular immune responses on pregnant heifers

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ARTICLE INFO

Article history:
Received 4 December 2015
Received in revised form 4 May 2016
Accepted 6 May 2016
Available online 17 May 2016

Keywords:
Staphylococcus aureus
Bovine mastitis
Avirulent mutant

ABSTRACT

Bovine mastitis produces economic losses, attributable to the decrease in milk production, reduced milk quality, costs of treatment and replacement of animals. A successful prophylactic vaccine against *Staphylococcus aureus* should elicit both humoral and cellular immune responses. In a previous report we evaluated the effectiveness of a live vaccine to protect heifers against challenge with a virulent strain. In the present study the immunological response of heifers after combined immunization schedule was investigated. In a first experimental trial, heifers were vaccinated with 3 subcutaneous doses of avirulent mutant *S. aureus* RC122 before calving and one intramammary dose (IMD) after calving. Antibodies concentration in blood, bactericidal effect of serum from vaccinated animals and lymphocyte proliferation was determined. The levels of total IgG, IgG1 and IgG2 in colostrum and the lymphocyte proliferation index were significantly higher in vaccinated respect to non-vaccinated group throughout the experiment. The second trial, where animals were inoculated with different vaccination schedules, was carried out to determine the effect of the IMD on the level of antibodies in blood and milk, cytokines (IL-13 and IFN-γ) concentration and milk's SCC and bacteriology. The bacterial growth of the *S. aureus* strains was totally inhibited at $1–3 \times 10^6$ and $1–3 \times 10^3$ cfu/ml, when the strains were mixed with pooled serum diluted 1/40. The results shown that IMD has not a significant effect on the features determinate. In conclusion, a vaccination schedule involving three SC doses before calving would be enough to stimulate antibodies production in milk without an IMD. Furthermore, the results showed a bactericidal effect of serum from vaccinated animals and this provides further evidence about serum functionality. Immune responses, humoral (antigen-specific antibodies and Th2 type cytokines) and cellular (T-lymphocyte proliferation responses and Th1 type cytokines), were augmented by administration of the avirulent mutant which represent an antigenic pool.

1. Introduction

Bovine mastitis occurs when pathogenic bacteria are able to enter to the udder, establish an infection there and produce inflammation of udder secretory tissue [1,2]. This disease produces economic losses that are attributable to the decrease in milk production, reduced milk quality, costs of treatment and replacement of animals [3].

In Argentina, as in many other countries, *Staphylococcus aureus* is still one of the major causes of clinical and subclinical mastitis, especially due to resistance to antibiotic treatment and its ability to persist in a herd in an undetected form [4–6].

Dry cow therapy is one of the recommended methods to cure existing intramammary infections and to prevent new ones at the peripartum period where, animals are most susceptible to new infections [7,8]. These treatments are only partly successful because of the intracellular biological face characteristic of *S. aureus* and

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http://dx.doi.org/10.1016/j.vaccine.2016.05.014
0264-410X/

because the maximum efficacy of antibiotic therapy is only 50% [9,10]. Moreover, there is a global pressure to limit antibiotic therapy in dairy cattle with the aim of reducing the incidence of drug residues in milk to make safe food [11–13].

Numerous strategies have been developed in order to increase the resistance of herd to *S. aureus* mastitis and to reduce the clinical and economic consequences of this disease. Although vaccination is a common practice for the control of many infectious diseases, it is not yet a major prophylactic measure against bovine mastitis [14,15]. A successful prophylactic vaccine should elicit both humoral and cellular immune responses in immunized individuals. The potential of immunization to induce an effective immune response is directly related to the titters of antigen-specific antibodies and T-lymphocyte proliferation [8,16]. Specific and innate immune factors associated with mammary gland tissues and secretion also play a vital role in protecting the gland from infectious disease [7]. During mastitis, CD4+ T lymphocytes prevail, are activated in response to recognition of an antigen and activate macrophages through the production of cytokines. Depending on the repertoire of cytokines produced, the T helper cells were divided in type Th1 or type Th2 [17,18]. Th1 clones were characterized by the production of IFN-γ and IL-2, whereas Th2 clones produced IL-4 [19,20], IL-5, IL-6, IL-10 and IL-13 [21]. Th1 cytokines activate macrophages, which are responsible for cell mediated immunity and phagocyte dependent protective responses such as the production of opsonizing b-complement-fixing antibodies [22]. In contrast, Th2 cytokines are responsible for strong antibody production and eosinophil activation as well as inhibition of several macrophage functions, thus resulting in phagocyte-independent protective responses [22–24].

The development of vaccines for protection against *S. aureus* is of considerable interest in the milk production industry. While some formulations have demonstrated promise in ameliorating the disease, few *S. aureus* vaccines have adequately prevented new infections [2,9,15,25,26]. The use of an attenuated vaccine could enhance both cellular and humoral protective responses by simulating natural infection without causing the disease. Attenuated vaccines may contain native antigens (proteins, polysaccharides, lipids, nucleic acids, etc.) that are expressed for extended periods of time [27].

In a previous report [27], we investigated the response of heifers after combined immunization schedule (three subcutaneous doses before calving and one intramammary dose (IMD) after calving) with the avirulent mutant *S. aureus* vaccine. The results of this study demonstrated that immunization of dairy heifers with the strain *S. aureus* RC122 was able to elicit a specific and significant opsonic antibody response in blood and milk and to provide protection by a significant reduction in post challenge milk bacterial shedding. These results could be due to activation and proliferation of blood memory cells previously stimulated. In order to demonstrate this, we investigate the blood memory cell proliferation and immunoglobulin isotype concentration in colostrum. The effect of IMD was determined by evaluation of antibodies in blood, bactericidal effect of serum from vaccinated animals and by the type of lymphocytes that proliferate through the quantification of IL-13 and IFN-γ in heifers immunized with different schemes.

2. Materials and methods

2.1. Experimental trial I

2.1.1. Animals, vaccine and immunization

Eleven clinically healthy Holtein heifers, free of antibodies for *S. aureus* after specific serological testing, were randomly divided into two groups. One group, the vaccinated group (VG) ($n = 8$), was inoculated subcutaneously with three doses of vaccine preparation (subcutaneous dose, SCD) containing 5×10^8 cfu/ml of *S. aureus* RC122, lyophilized and homogenized in phosphate-buffered saline (PBS) pH 7.2 at the original concentration, as described by Pellegrino et al. [25]. The first vaccine dose was administered to 14–16-month-old heifers immediately after they arrived to dairy farm. Heifers were inseminated artificially approximately 30 days after the first dose. The second dose was administrated 30 days after pregnancy was diagnosed. Ten days before calving, a third dose was administrated. Twenty days after calving, all quarters of the vaccinated group were inoculated intramammarily with 1 ml of 10^9 cfu of formol-killed avirulent mutant. The other group of animals, non-vaccinated group (NVG) ($n = 3$), was used as control.

Table 1
Immunization schedule of heifers during trials I and II.

Treatment (T)/no. of cows	Subcutaneous dose (SCD)	Intramammary dose (IMD)
T1/8[a]	Yes	Yes
T2/2	Yes	No
T3/2	No	Yes
T4/3[a]	No	No

[a] Animals from experimental trial I.

2.2. Experimental trial II

2.2.1. Animals and immunization

The level of antibodies on blood and milk after the intramammary dose (IMD) was determined. Four additional clinically healthy Holstein heifers were included to animal trial I and inoculated subcutaneously or intramammarily as described in Table 1.

2.3. Blood sampling

For ELISA assay, blood samples were collected from heifers immediately before the administration of each vaccine dose and 20, 21 and 26 days after calving. Approximately 30 ml of blood were obtained from the tail vein and placed into sterile tubes. Bactericidal assay was performed using 21 day pool serum. Samples were maintained at room temperature, centrifuged at $1200 \times g$ for 10 min and blood sera was collected and stored at −20 °C.

For lymphocyte proliferation assay, heparinized whole blood samples were obtained from the jugular vein (approximately 30 ml) immediately before the administration of each subcutaneous dose of vaccine and 27 days after calving. To avoid blood coagulation, syringes were rotated slowly and immediately processed in the laboratory for the isolation of lymphocytes. An additional sample was taken at day 27, after calving, for cytokines quantification.

2.4. Milk sampling and analysis

Heifers were milked daily at 12-h intervals and individual milk samples collected aseptically 20 (immediately before the administration of IMD), 21 and 26 days after calving. Colostrum samples were collected 24–48 h after calving. Samples were collected according to the National Mastitis Council procedure [28]. For ELISA assay, the serum obtained after centrifugation of whole milk or colostrum at $3700 \times g$ for 15 min was stored at −20 °C.

Bacteriological assays were performed according to the criteria of the National Mastitis Council [28]. The presence of bacterial strains of the genus *Staphylococcus* was determined using 10 μl of milk plated onto blood-agar (Tryptic Soy agar with 5% of sheep blood) and incubated at 37 °C for 24 h. The Gram-positive cocci were characterized by standard biochemical tests [29].

The somatic cell count (SCC) was performed with a Somacount 300 (Bentley, USA, 1997), according to the revised protocol of the

148A method C, fluoro-opto-electronic, International Dairy Federation Laboratory (1995).

2.5. Detection of antibodies

The levels of specific antibodies (total IgG, IgG_1 and IgG_2) in blood, milk and colostrum were determined by enzyme-linked inmunosorbent assay (ELISA) as described by Pellegrino et al. [27].

2.6. In vitro bactericidal assay

In vitro evaluation of the bactericidal activity of bovine serum from vaccinated animals was tested for three *S. aureus* strains: *S. aureus* RC108 (virulent parental), *S. aureus* 101 (isolated from clinical bovine mastitis) and *S. aureus* ATCC® 23925. Colonies of each *S. aureus* strains were cultured in brain heart infusion broth (BHI; Difco) for 18 h at 37 °C. Bacteria were harvested by centrifugation and suspended in saline solution and optical density (OD) was recorded. The suspension was diluted in Mueller Hinton broth (MH, Difco) containing different serum dilutions (1/2, 1/20, 1/40 and 1/80) into a final bacterial concentration of $1–3 \times 10^8$, $1–3 \times 10^6$ and $1–3 \times 10^3$ cfu/ml. Aliquots of 100 μl, from each dilution, were added by triplicate in 96-well microplates (Nunc-Immuno Plate Maxi Sorb™). After incubation for 24 h at 37 °C, plates were read at $OD_{600\,nm}$ to determine bacterial growth inhibitions. For each dilution, the following wells were added: (1) bacteria without serum (positive control), (2) serum without bacteria (negative control) and (3) medium without bacteria and serum (negative control).

2.7. Lymphocyte proliferation assay

Lymphocytes from whole blood were obtained from control and immunized animals. Briefly, blood was transferred into centrifuge tubes prefilled with 5 ml of Histopaque® 1077 (Sigma–Aldrich) and centrifuged at $1000 \times g$ for 30 min. The mononuclear cells were removed and washed with 10 ml of Completed Lymphocyte Medium (CLM) (RPMI-1640 supplemented with 10% bovine fetal serum, 25 mM HEPES buffer, 2 mM 2-mercaptoethanol, 100 UI/ml penicillin and 100 μg/ml streptomycin). Cells (2×10^5 cells in 200 μl of CLM) were cultured in 96-wells round bottom plates (Nunc-Immuno Plate Maxi Sorb™) in presence of the whole heat-killed *S. aureus* RC122 strain (5×10^8 cfu/ml) in quintupled. Cultures were made in CLM along (negative control) or containing 2 μg of concavalin A (Sigma–Aldrich)/ml (positive control) in quintuple too. Plates were incubated for 2 days at 37 °C in 5% CO_2. After incubation, 1 μCi of [methyl-H^3] thymidine (NEN-Perkin Elmer Life Sciences) was added per well and the plates were cultured for other 24 h. Cells were harvested on glass-fiber filters (Glass microfiber filters 934AH, Whatman, England) using a semiautomatic cell harvester (Skatron Micro 96S, England). Incorporation of [methyl-H^3] thymidine was measured in an automatic liquid scintillation counter (Beckam LS 6000IC, USA). The proliferation response was calculated as a stimulation index (SI), whereby the average count per minute (CPM) for the five wells cultured with the whole heat-killed *S. aureus* RC122 strain was divided by the average CPM for the five wells cultured with CLM.

2.8. Cytokine quantification by enzyme-linked immunosorbent assay

Culture supernatants obtained from the lymphocyte proliferation assay were sampled after the first 48 h of incubation, to quantify gamma interferon (IFN-γ) and interleukin-13 (IL-13) through enzyme-linked immunosorbent assay. The IFN-γ concentration in culture supernatants was determined using Bovine IFN-γ Screening Set (Thermo Scientific) according to the original protocol described by the manufacturer. The IL-13 concentration in culture supernatants was determinate using the Bovine IL-13 ELISA Kit (Bethyl Laboratories, Inc.) according to the original protocol described by the manufacturer. For both cytokines, a standard curve was made and IFN-γ and IL-13 concentrations in each sample were determined by regression analysis. The sensitivity of the assays was 0.03125 ng/ml for IFN-γ and 0.39 ng/ml for IL-13. This assay was made with the objective of establishing the activation of different subsets of lymphocyte T stimulated is produced by the vaccine. IFN-γ is produced principally by lymphocytes Th1 and IL-13 by helper T lymphocytes of the Th2 phenotype (Trigona et al. [39]).

2.9. Clinical observations and animal care

Clinical signs were monitored throughout the experiment by a veterinarian. This was done after each vaccine inoculation (during 48 h), and subsequently every time the cows were milked. General attitude and appetite were observed. The udders were palpated for soreness, swelling, hardness and heat, and the appearance of milk was assessed visually for clots and changes in color or composition every time the cows were milked. All animals involved in this investigation were cared for in accordance with The International Guiding Principles for Biomedical Research Involving Animals (1985).

2.10. Statistical analysis

For SCC and antibody level, comparisons among treatments were made at each time point, using the mixed model analysis of variance (ANOVA) for repeated measurements (SAS Institute, Cary, North Carolina, USA). A p-value < 0.05 was considered as indicative of a statistically significant difference.

3. Results

3.1. Field trial I

3.1.1. Colostrum antibody determination

The levels of total IgG, IgG_1 and IgG_2 in colostrum were significantly higher ($p < 0.05$) in VG than in NVG (Fig. 1). With respect to IgG isotypes present in cattle, similar kinetics curves were found between the levels of IgG_1 and IgG_2, compared to total IgG in both conditions (VG and NVG).

3.2. Lymphocyte proliferation

After the lympho-proliferation assay was done, the corresponding wells from each sample were observed at 24 h of incubation to confirm the correct cell growth, the formation of clusters

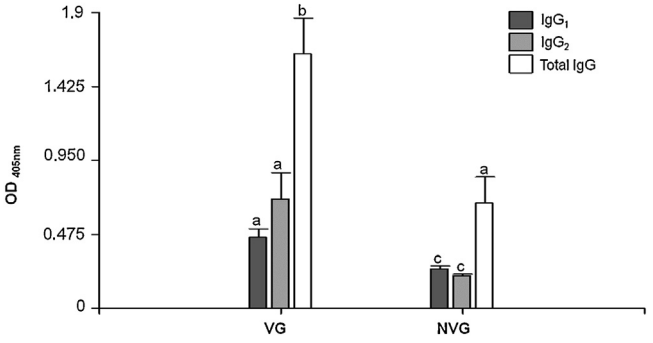

Fig. 1. Specific antibody levels of total IgG, IgG_1 and IgG_2 in colostrum from heifers vaccinated with the strain *S. aureus* RC122 determined by ELISA. Colostrum samples were diluted 1/10. Data are expressed as mean ± S.E. values of optical density (OD) read at 405 nm. Significant differences between groups were considered with a $p < 0.05$ and are indicated by distinct letters.VG: vaccinated group, NVG: non-vaccinated group.

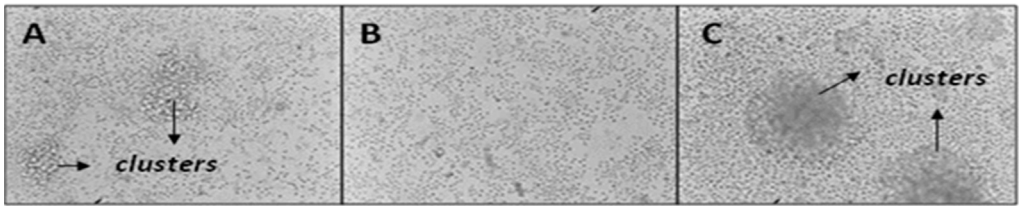

Fig. 2. Photomicrograph inverted microscope (40×) of lymphocytes from vaccinated (A) and non-vaccinated (B) heifers, stimulated with *S. aureus*, strain RC122, and positive control (C) stimulated with Concanavalin A (ConA).

(indicating proliferation) and the absence of contamination. Fig. 2 shows a confluent growth of homogeneous cells and the formation of clusters in lymphocyte isolates from VG, stimulated with strain RC122 (Fig. 2A) and ConA-stimulated lymphocytes (positive control) (Fig. 2C). No cluster formation was observed on samples of lymphocytes isolated from NVG (Fig. 2B). This preliminary microscopic observation allowed having a general idea about the in vitro response of lymphocytes from vaccinated heifers, which have been in contact with the same antigenic stimulus (*S. aureus* RC122) by SC dose administration.

Fig. 3 shows the results obtained by analyzing blood samples taken before administration of each SCD, until 27 days postpartum. Before administration of D2 (day −240), there was a significant increase ($p < 0.05$) in SI in comparison to day −300 in the VG. Before administration of D3 (10 days before calving), a great decrease in lymphocyte proliferation was observed in VG. Twenty seven days after calving, a similar SI was observed to that found after administration of the D2.

With respect to blood lymphocytes from NVG, no significant differences ($p > 0.05$) were found between SI at all times assayed. The animals also showed considerably lower SI ($p < 0.05$) than those found for VG throughout the experiment.

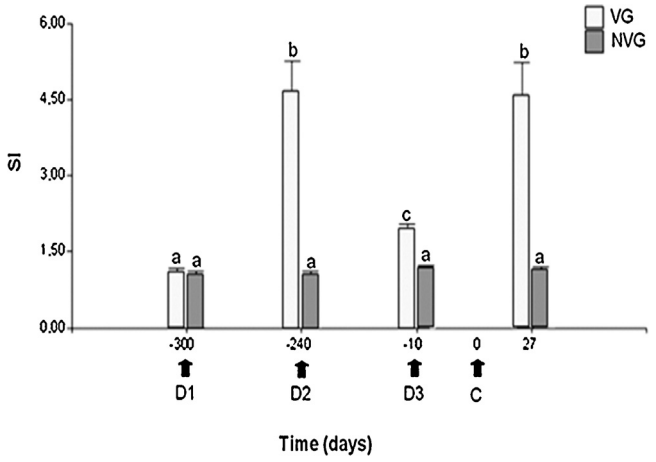

Fig. 3. Stimulation index (SI) of blood lymphocytes isolated from vaccinated (VG) and non-vaccinated groups (NVG) stimulated with the mutant strain of *S. aureus* RC122. Differences between groups were considered significant at $p < 0.05$ and are indicated by distinct letters. D: dose, C: calving.

3.3. Field trial II

3.3.1. Level of antibodies in blood and milk

With the aim of determining the effect of IMD on antibody levels in blood and milk, heifers were treated as described in Table 1.

Total IgG levels obtained from blood and milk before IMD (20 d after calving) and 21 and 26 days after calving are shown in Fig. 4.

With respect to antibody levels in blood from T1 samples, no significant ($p > 0.05$) difference was observed in total IgG one day after intramammary inoculation (Fig. 4A). At day 26 after calving, there was an increase in total IgG level similar to those observed before inoculation. On the other hand, in T2, no relevant decrease on the level of antibodies was found at any assayed times (Fig. 4A). No significant differences ($p > 0.05$) were found in total IgG level between T1 and T2 at days 20 and 26 after calving. T3 and T4 had a total IgG level significantly lower ($p < 0.05$) to that found for T1 and

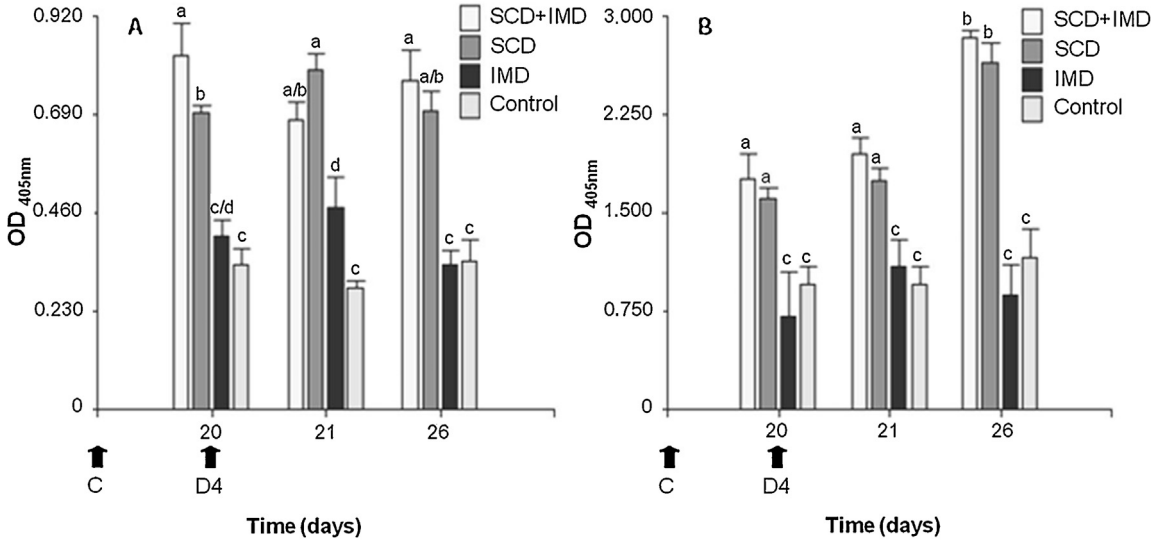

Fig. 4. Antigen specific immunoglobulin (Ig)G response in blood (A) and milk (B) as determined by enzyme-linked immunosorbent assay (ELISA). Samples were diluted 1/80 and 1/10 for blood and milk, respectively. Data are expressed as mean ± S.E. of the optical density (OD) values read at 405 nm. Treatment with different letters at the same time point are significantly different ($p < 0.05$). SCD + IMD = Treatment 1, SCD = Treatment 2, IMD = Treatment 3 and Control = Treatment 4.

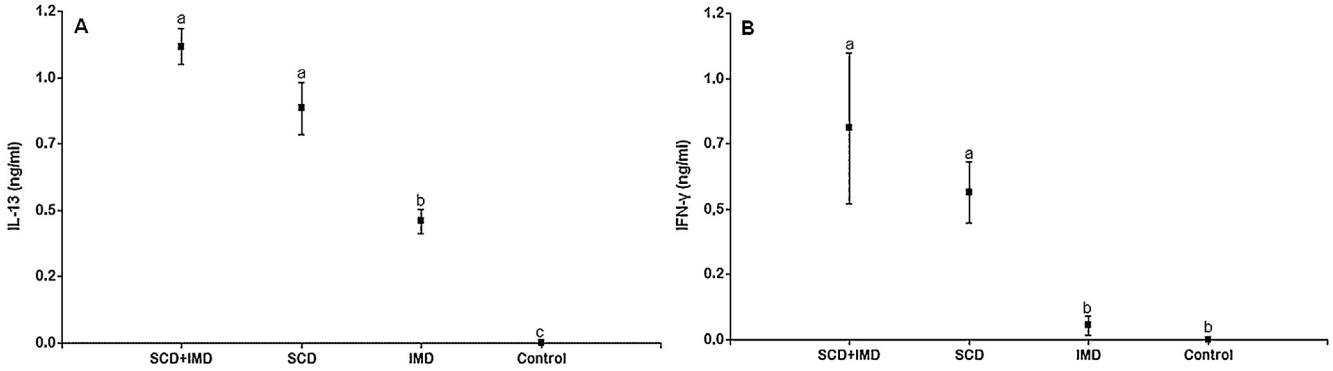

Fig. 5. Mean serum concentrations (ng/ml) of (A) IL-13 and (B) IFN-γ at 27 d after calving. SCD + IMD = Treatment 1, SCD = Treatment 2, IMD = Treatment 3 and Control = Treatment 4. Data are expressed as mean ± S.E. of ng/ml concentrations. Significant differences between treatments were considered with a $p < 0.05$ and are indicated by distinct letters.

T2. One day after IMD, a higher level of antibodies ($p < 0.05$) was observed in T3 respect to T4. Six days after IMD, in T3, the level of total IgG decreased, similar values to those found in T4 (Fig. 4A). In addition, total IgG levels were determined in milk samples (Fig. 4B). No significant differences were found ($p > 0.05$) at any time between T1 and T2. Even though no significant differences were observed ($p > 0.05$) between T3 and T4, total IgG levels for these treatments were lower than those found for T1 and T2 (Fig. 4B).

3.4. Bactericidal assay

The intrinsic activity of bovine serum against *S. aureus* was evaluated. This methodology was proposed as a complementary assay in order to provide further evidence about the functionality of serum. The bacterial growth of the *S. aureus* strains was totally inhibited at $1–3 \times 10^6$ and $1–3 \times 10^3$ cfu/ml, when the strains were mixed with pooled serum diluted at 1/2, 1/20 and 1/40. Serum dilution of 1/80 delayed growth when this reaction mix was compared to the positive control (bacteria alone). A concentration of $1–3 \times 10^8$ cfu/ml of each bacterial strain was not inhibited by serum dilution of 1/80, but bacterial growth was lower compared to the positive control.

3.5. Cytokine quantification

To establish whether subcutaneous immunization with the avirulent mutant induced an adaptive response in heifers, production of IFN-γ and IL-13 was determined as an indirect measurement of different subsets of T cell (Th1 and Th2) activation. Levels of both, IL-13 and IFN-γ, were significantly higher in blood samples of T1 and T2 at levels ($p < 0.05$) than in T3 and T4. For T3, only IL-13 concentration was significantly higher ($p < 0.05$) than T4 (control group) (Fig. 5). No considerable differences were found between T1 and T2 with respect to concentration of IL-13 and IFN-γ.

3.6. Milk sample analysis: SCC and bacteriology

The SCC from milk samples of inoculated udders (T1 and T3) was 40 times higher, 24 h after IM inoculation (T1 = 7.5×10^4 to 4.0×10^6 cells/ml and T3 = 1.25×10^5 to 4.25×10^6 cells/ml). On T3, SCC reached a 2-fold increase at day 6 after inoculation (T3 = 8.0×10^6 cells/ml). On the other hand, in T1, the SCC decreased significantly six days after inoculation and reached a value four times lower than that found one day after inoculation (T1 = 1.0×10^6 cells/ml).

No bacteria identified as *S. aureus* were isolated from the milk of animals during the experience.

4. Discussion

The application of immunomodulatory strategies for the control of outbreaks of *S. aureus* mastitis in dairy herds during the calving period is critical. It constitutes an alternative to antibiotic therapy, which may lead to the entrance of antibiotic resistant bacteria into the food chain. In a previous report, we evaluated the effectiveness of a live vaccine to protect heifers against challenge with a virulent strain [27]. In this study, the response of heifers after combined immunization schedule (three subcutaneous doses before calving and one intramammary dose after calving) was investigated. The results demonstrated that immunization of dairy heifers with the RC122 avirulent strain induce specific and significant opsonic antibody responses in blood and milk and provides protection through a significant reduction of post challenge milk bacterial shedding. Furthermore, a bactericidal effect of serum from vaccinated animals was observed and this provides further evidence about serum functionality.

In our present study, the immunoglobulin isotype concentration in colostrum was first determined. Colostrum is the first milk available in the mammary gland and is produced within the first 24 h after birth. Colostrum differs from the milk in that it contains composition and levels of Igs, nutrients, vitamins and minerals, higher than those found in milk [30]. At the VG, the level of IgG_2 in colostrum was higher than IgG_1, although this difference was not significant. Similar results were observed in blood levels of IgG_2 in an artificial challenge in heifers, where this isotype was predominant at calving [27]. With respect to milk, the results of this trial are in agreement with those found in a previous report, where VG animals had levels of total IgG significantly higher than those found for NVG within 47 days postpartum [27]. These results allow us to infer that 3 SC doses of vaccine administered before calving, cause an increase in the levels of the two principal isotypes of IgG which are found in concentrations extremely low in normal milk and colostrum. This piece of information is very revealing, since IgG_2 is the major opsonic antibody in blood and milk and an increase in their level stimulates phagocytosis by neutrophils and could streamline the removal.

With respect to lymphocyte proliferation, in vitro studies have shown that *S. aureus* is able to internalize itself within host cells and survive in macrophages and in alveolar cells isolated from chronically infected cows with mastitis [31,32]. Internalization and survival of *S. aureus* could explain why the humoral immune response alone is inefficient to combat the infection and why the failure of antibiotic treatments to eliminate this pathogen is also ineffective [33]. A vaccine which is able to awaken cellular immune responses and opsonic antibodies could be a very effective tool for the prevention of bovine mastitis. The results obtained in a

previous challenge trial showed an increase of opsonic antibodies in blood and milk at peripartum (time of increased susceptibility to IM infections) and a less likelihood of infection in vaccinated heifers compared to that obtained in unvaccinated heifers [27]. For this reason, it was interesting to determine the ability of the vaccine to stimulate cellular immunity. The increase of the proliferation of lymphocytes, observed before the administration of D2 in VG, indicates that lymphocytes isolated from this group proliferate to in vitro second exposure of the same antigen. This property would be related to the ability of lymphocytes to acquire immunological memory. A decrease in proliferation of lymphocytes was observed 10 days before calving. This could be related to hormonal changes and stress that occur in pregnancy and calving. Glucocorticoids have been shown to cause a decrease in blood T lymphocytes [8,34] and the changes in concentration of progesterone, estradiol 17β, and growth hormone modify some essential functions of lymphocytes [35,36]. A proved advantage in using live attenuated strains for the prevention of various diseases is that they have the ability to induce both, humoral and cellular immune responses by activating B and T cells and by allowing the presentation of antigen for long terms [27,37]. These features allow T lymphocytes from immunized animals to respond quickly and effectively while being in contact with the same antigen, even several months after vaccination. These findings may explain the significant increase in the SI observed 26 days after calving.

With the aim of determining the effect of IM dose on the levels of antibodies in blood and milk, four heifers were treated differentially (Table 1). No considerable decrease was observed in total IgG one day after IM inoculation in the level of antibodies in blood from T1. This could be due to the passage of antibodies from blood to milk in response to local stimulus caused by the inoculation of inactivated bacteria in the udder. At day 26 after calving, there was an increase in the total IgG level similar to those observed before inoculation. This increase could be related to the activation of antibody-producing B lymphocytes present in the lymph nodes. A similar result was observed in the total IgG level in blood samples obtained from heifers in a previous challenge trial reported [27]. On the other hand, no significant differences were found in the total IgG level between T1 and T2 at days 20 and 26 after calving. T3 and T4 had a total IgG level significantly lower than that found at T1 and T2. This effect could be due to a short-acting local stimulus caused by IM inoculation with the inactivated RC122 strain in an animal that had never been exposed to this antigen. In milk, no significant differences were found at any times between T1 and T2 and the antibody levels in T3 and T4 were lower than those found in T1 and T2. The results obtained in the present study show that the IM dose does not have a significant effect on blood and milk antibody levels. This could be explained by the discovery of a decrease in total IgG in blood. An increase in milk at day 21 was not significant compared to T1 and T4. To establish whether immunization with the avirulent mutant induced adaptive responses in cows, production of IFN-γ and IL-13 was determined as an indirect measurement of activation of different subsets of T cells (Th1 and Th2, respectively). The results suggest that immunization with 3 SC doses before calving, induced activation of Th1 and Th2 cell subsets in the mammary gland. Previous evidence suggests that IFN-γ could elicit functional changes in phagocytic cells of the mammary gland that could make it effective in the control of bovine mastitis [38]. In a previous report, we observed that opsonization was enhanced after vaccination and the phagocytosis was significantly higher in vaccinated animals with respect to control animals [27]. IL-13 mediates its effects on several immune cells including B lymphocytes, by stimulating their proliferation and differentiation into antibody-producing plasma cells [39]. This is in agreement with the antibody levels found in the present work in blood, milk and colostrum of animals inoculated with the RC122 strain. Riollet et al. [40] suggests that an orientation toward a Th1-type response was induced by immunization with a single staphylococcal component, such as α-hemolysin. Immunization with the RC122 avirulent mutant induced high levels of both IFN-γ and IL-13, which agrees with the fact that multiple antigens are involved in the adaptive response to a live attenuated vaccine. This is supported by the early and exacerbated immune response found in animals inoculated with SC and IM doses (T1). This could be due to acquired immunity that is rapidly activated upon contact with the antigens. This response could stimulate the production of antibodies and increase the number of neutrophils in the mammary gland, which would eliminate microorganisms rapidly. This is shown by a marked decrease in the SCC and by a fast restoration of udder health. Moreover, in those animals inoculated only with IM dose (T3), the response was much slower and mainly mediated by innate immunity. This could cause an increase in the number of neutrophils in the mammary gland even after 6 days after inoculation, in order to eliminate the inoculated microorganisms. In these animals, which were not previously in contact with the antigen, the response would be less efficient due to the absence of opsonic antibodies which make more effective the neutrophil-mediated phagocytosis. The results found in this study, where animals inoculated with different vaccination schedules (T1, T2, T3 and T4) were used, corroborate that IM dose (D4) has not a significant effect on the increase of the level of antibodies in blood and milk. This was demonstrated by a decrease in blood antibodies at day 1 after IM inoculation (T1) and by non-significant increase in the level of antibodies in milk between T1 and T2. It can be inferred that a vaccination schedule, involving three SC doses before calving, would be enough to stimulate the production of antibodies in milk without an IM dose that cause the passage of the antibodies from blood into the udder. On the other hand, the results showed that immune responses, whether humoral (antigen-specific antibodies and Th2 type cytokines) or cellular (T-lymphocyte proliferation responses and Th1 type cytokines), were augmented by the administration of the avirulent mutant which represents an antigenic pool.

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