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Immune response of heifers against a *Staphylococcus aureus* CP5 whole cell and lysate vaccine formulated with ISCOM Matrix adjuvant

Cecilia M. Camussone [a,b], Carolina M. Veaute [c], Nazarena Pujato [b,c], Bror Morein [d], Iván S. Marcipar [b,c], Luis F. Calvinho [a,e,*]

[a] E.E.A. Rafaela, INTA, Santa Fe, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina
[c] Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[d] Department of Clinical Virology, Uppsala University, Sweden
[e] Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Esperanza, Santa Fe, Argentina

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ABSTRACT

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections worldwide. Commercially available vaccines for mastitis control are composed either of *S. aureus* lysates or whole-cells formulated with traditional adjuvants. We recently showed the ability of a *S. aureus* CP5 whole-cell vaccine adjuvanted with ISCOM Matrix to increase specific antibodies production in blood and milk, improving opsonic capacity, compared with the same vaccine formulated with $Al(OH)_3$. However, there is no information about the use of ISCOM Matrix for the formulation of bacterial lysates. The aim of this study was to characterize the innate and humoral immune responses induced by a *S. aureus* CP5 whole-cell or lysate vaccine, formulated with ISCOM Matrix after immunization of pregnant heifers. Both immunogens stimulated strong humoral immune responses in blood and milk, raising antibodies that increased opsonic capacity. Lysate formulation generated a higher and longer lasting antibody titer and stimulated a higher expression of regulatory and pro-inflammatory cytokines compared with the whole-cell vaccine.

1. Introduction

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections (IMI) worldwide (Zecconi et al., 2006). Control of *S. aureus* IMI is based on milking-time hygiene; antibiotic therapy and culling of chronically infected cows. However, despite the application of these practices, the chronic nature of most *S. aureus* IMI and the limited cure rate following antibiotic therapy makes this disease difficult to control (Zecconi et al., 2006). Hence, other approaches to control *S. aureus* IMI, such as vaccination, have been proposed to complement classical measures.

During the last two decades several experimental vaccines including whole cells, lysates and defined antigens for *S. aureus* mastitis control have been evaluated (reviewed by Pereira et al., 2011). However, only two vaccines are currently commercially available worldwide. One is a long-standing bacterin containing *S. aureus* whole-cell lysates of capsular polysaccharide (CP) serotypes 5, 8 and 336 (Ma et al., 2004), adjuvanted with $Al(OH)_3$ (Lysigin™, Boehringer Ingelheim Vetmedica Inc., St Joseph MO, USA); while the other is a recently marketed vaccine, Startvac® (Laboratorios Hipra, S.A. authorized by the European Medicines Agency), containing a *S. aureus* strain expressing slime associated antigen complex (SAAC) formulated with an oil-based adjuvant. Capsular polysaccharides are considered important components for vaccine development since antibodies against them opsonize *S. aureus*, enhancing neutrophil phagocytosis (Guidry et al., 1991, 1994); while bacterins from strong biofilm-producing bacteria were shown to trigger a high antibody production against poly-N-acetyl β-1,6 glucosamine, major constituent of the staphylococcal biofilm matrix (Pérez et al., 2009). Regarding the efficacy of these vaccines, a study carried out in heifers in which multiple doses of Lysigin™ were administered led to a significant reduction in new *S. aureus* IMI (Nickerson et al., 1999). However, in later studies, even when immunization stimulated production of specific antibodies in milk (Luby et al., 2007), field trials or experimental challenge carried out in adult cows have not provided conclusive results, showing only some effectiveness in reducing infection duration and severity (Middleton et al., 2006, 2009). Immunization of pregnant heifers with the formulation containing

* Corresponding author at: E.E.A. Rafaela, INTA, Santa Fe, Argentina. Tel.: +54 3492 440121; fax: +54 3492 440114.
E-mail addresses: calvinho.luis@inta.gob.ar, lcalvinho@fcv.unl.edu.ar (L.F. Calvinho).

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a *S. aureus* strain expressing high SAAC content stimulated strong humoral responses in blood and milk, and was associated with reduced bacterial cell count and less severe clinical signs after experimental challenge (Prenafeta et al., 2010). However, no peer reviewed articles about this vaccine efficacy in field trails are available.

The adjuvant used in the formulation is a critical factor for vaccine efficacy improvement. Even when diverse adjuvants and antigen delivery systems in immunogens directed to *S. aureus* mastitis have been evaluated (Cui et al., 2010; Hu et al., 2003; O'Brien et al., 2001; Tollersrud et al., 2001), commercially available vaccines are formulated with classical adjuvants. During the last years, new immune-stimulating complexes like ISCOMs, that formulate antigens and adjuvant in one and the same nanoparticle, have been developed (Morein et al., 2004; Sjölander et al., 1998). These compounds are able to produce a balanced humoral/cellular response, through the induction of multiple innate and adaptive mediators, cellular processes and the interplay between these elements (Pearse and Drane, 2004). A diversity of experimental vaccines have been formulated with ISCOMs and ISCOMATRIX™ and evaluated for the capability of inducing strong humoral and cellular responses in a variety of animal species as recently reviewed (Sun et al., 2009). However, these formulations included defined antigens; but there is little experience in the usage of these complexes with whole bacteria (Camussone et al., 2013), whereas its use with bacterial lysates has not been explored (Sun et al., 2009). The objective of this study was to characterize the innate and humoral immune responses induced by a *S. aureus* CP5 whole-cell vaccine and compare that with a CP5 lysate vaccine, formulated with ISCOM Matrix, after immunization of pregnant heifers.

2. Materials and methods

2.1. Formulation of vaccines

S. aureus CP type 5 strain (Reynolds) was grown on Columbia agar added with 2.5% NaCl and incubated overnight at 37 °C. The culture was resuspended in PBS (pH 7.4), adjusted to a final concentration of 1×10^9 CFU/ml and divided into two fractions. One fraction was inactivated with 0.5% formalin for 24 h at 37 °C, washed two times with PBS and adjusted to a final concentration of 1×10^9 CFU/ml (Bacterin), while the other fraction was treated with 35 U of lysostaphin for 6 h at 37 °C with shaking at 100 rpm. The enzyme was inactivated by incubation for 15 min in water bath at 75 °C (Lysate). Vaccines were formulated with 2 mg/doses of ISCOM Matrix. The adjuvant was kindly supplied by Isconova, Uppsala, Sweden. A placebo consisting of sterile saline solution and adjuvant (2 mg ISCOM Matrix/doses) was used as control. Sterility of these formulations was evaluated by plating 100 µl on blood agar plates in duplicate and incubating overnight at 37 °C.

2.2. Animals and treatments

Twenty-five Holstein dairy heifers in the last trimester of gestation belonging to the dairy herd of INTA Rafaela Experiment Station were used in the experiment. Only animals free of *S. aureus* IMI and with no clinical signs of inflammation were included in the experiment. All the procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999). Animals were randomly allocated in three groups; bacterin ($n = 8$), lysate ($n = 9$) and control ($n = 8$). To detect statistical differences in ELISA OD between vaccinated groups of 0.75 (SD 0.4) with a two-side 5% significance level and a power of 80%, a sample size of seven heifers per group was necessary. Each group received one of the different formulations. Heifers were injected subcutaneously with 1 ml of vaccine in the supramammary lymph node area 6 and 2 weeks before the expected calving date, and 31 weeks after calving. Animals were bled by puncture of the coccygeal vein before each inoculation, and 24 h, 1, 2, 3, 4, 8, 32, and 33 weeks after calving. Blood was allowed to clot and sera were collected via centrifugation. At 14 days before expected calving date udders of all heifers were clinically examined by palpation and samples of pre-partum mammary secretion were taken to determine presence of *S. aureus* IMI. After parturition, aseptic quarter foremilk samples were collected every week from the first week after calving to 8 weeks after calving, according to standard procedures (Oliver et al., 2004). An aliquot of each quarter sample was subjected to bacterial culture and composite samples, conformed by 500 µl of milk from each quarter, were used for antibody determinations. These samples were centrifuged for 15 min at 300 g, supernatants were collected and stored at −20 °C until processed.

Milk samples for somatic cell count (SCC) from all the cows in the different treatment groups were collected monthly during lactation (9 months) into non-sterile vials containing 0.3% azidiol, stored at 4 °C, and shipped overnight to a commercial laboratory (Laboratorio Regional de Servicios Analíticos, Esperanza, Santa Fe, Argentina).

2.3. Bacteriological examination and somatic cell count determination

Mammary secretion samples were cultured for mastitis pathogens according to standard procedures (Oliver et al., 2004). Briefly, 10 µl of mammary secretion samples were streaked onto agar plates supplemented with 5% calf blood and incubated aerobically for 48 h. Plates were examined for bacterial growth at 24 and 48 h and isolated colonies were identified according to standard procedures. The presence of one colony of *S. aureus* on blood agar was considered as a positive identification; therefore, detection limit was 100 CFU/ml. Heifers yielding a positive culture in any quarter sample along the trial were excluded from the study.

Milk SCC determinations were performed within 24 h of sample collection using an automated counter (Somacount 300, Bentley Instruments, Minesotta, USA). Prior to processing, milk samples were warmed up at 39–40 °C to facilitate homogenization.

2.4. Serological methods

Antibodies (IgG and IgG_2 subtype) against *S. aureus* CP5 lysate, *S. aureus* CP5 bacterin or purified CP5 were measured by enzyme-linked immunosorbent assay (ELISA). IgG_2 was quantified since this subclass is considered the most effective opsonin promoting neutrophils paghocytosis (Burton and Erskine, 2003). Production and purification of CP5 from *S. aureus* Reynolds was carried out according to Fattom et al. (1990). Flat-bottomed 96-well microtitre plates were coated with a suspension of *S. aureus* CP5 bacterin (1×10^8 CFU/well), *S. aureus* CP5 lysate (corresponding to 1×10^8 CFU/well), or purified CP5 (5 µg/well) in PBS (pH 7.2). Between each step, plates were washed three times with 0.05% Tween 20 in PBS. The coated plates were first incubated with PBS with low fat goat milk 5%, then with heifer's sera or whey diluted in PBS, and finally with mouse-anti cow IgG or IgG_2 (Sigma). After washing, peroxidase-conjugated rabbit anti-mouse IgG (Sigma) was added. All incubations were 1 h at 37 °C. Lastly, enzyme substrate (H_2O_2/tetrametylbenzidine) was added and the reaction was stopped by the addition of 0.5 N H_2SO_4. The absorbance was read at 450 nm. Antibody levels were expressed as optical density (OD).

2.5. Neutrophil phagocytosis

Bovine polymorphonuclear neutrophils (PMN) were obtained from a healthy animal as described previously (Siemsen et al., 2007). A suspension of *S. aureus* Reynolds was labelled with FITC and resuspended in Hanks Balanced Salt Solution to a final concentration of 1×10^8 CFU/ml. One hundred microlitre of bacterial suspension was incubated with sera from each animal included in the study, for 30 min at 37 °C with gentle shaking. Then, 100 μl of a 1×10^7 cells/ml suspension of bovine PMN were added to each well and incubated for another 30 min at 37 °C with gentle shaking. Phagocytosis was stopped by the addition of NaCl 0.85%/EDTA 0.04%. Finally, the mixture was stained with ethidium bromide to quench extracellular fluorescence (Weingart et al., 1999). Fluorescence intensity was read by flow cytometry (FACSCanto II, BD Biosciences). On the basis of forward and side light scatter, PMN were gated in the region 1 (R1). Fluorescence intensity was depicted on a four-decade logarithmic scale and single-parameter analysis as histograms. Data were analysed with WinMDI software. The percentage of PMN with associated bacteria was assessed and the mean fluorescence intensity (MFI) was used to estimate the number of bacteria internalized per positive cell (Zetterlund et al., 1998).

2.6. Cytokine expression

Blood samples were collected aseptically from four animals of each group, 24 h after the second inoculation of vaccines, by jugular venipuncture. Samples were transferred to sterile tubes containing EDTA and centrifuged for 25 min at 1000*g* at room temperature. Plasma was removed; the buffy coat was collected and resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the manufacturer's directions and dissolved in RNase-free water. RNA was quantified using a spectrophotometer (NanoDrop 2000, Thermo Scientific) at 260 nm, and quality was assessed by determining the OD ratio 260/280. Samples were stored at −80 °C until processed.

One micrgram of total RNA of each sample was retrotranscribed to cDNA with MMLV reverse transcriptase (Fermentas, Germany), according to manufacturer's directions. cDNA samples were kept at −20 °C until processed. IL-4, IL-10, IL-12 p-40, IFN-γ and TNF-α relative mRNA levels were determined by quantitative Real Time PCR with specific primers (Riollet et al., 2001). Each PCR was performed in triplicate in a total volume of 10 μl containing the following: 1 μl of cDNA, 5 μl of 2× SYBR green PCR master mixture (Real Mix, Biodynamics), 0.15 μl of each 10 μM sense/antisense primers and 3 μl of sterilized deionized H_2O. PCR reactions were performed on a Rotor Gene Q (Qiagen, Hilden, Germany) using a basic program as follows: one hold of initial denaturation of 3 min at 94 °C, followed by 40 cycles of 20 s at 95 °C, 15 s at 55 °C, 30 s at 72 °C, and a final extension hold of 3 min at 72 °C. Results were analyzed with the software REST 2009 V2.0.13, which determines the difference between samples and controls, taking into account issues of reaction efficiency and reference gene normalization (β-actin) (Pfaffl et al., 2002).

2.7. Statistical analysis

A statistical software package (SPSS version 17.0) was used to perform statistical analysis. A design with data collected in a sequence of unequally spaced points in time was used for comparative analysis of antibody responses of different groups through time. A 3 × 8 or 3 × 5 factorial model for factors treatment (control, bacterin, lysate) and time (−45, −30, −15, 7, 15, 30, 60 days relative to calving for serum and 7, 15, 21, 30, 60 days relative to calving for whey) was used for serum and whey, respectively. OD medium values obtained for IgG and IgG_2 by ELISA for different groups, and neutrophil phagocytosis results, were compared by non-parametric test Kruskal–Wallis followed by Mann–Whitney's test to detect differences between pairs ($p < 0.05$). SCC data were log transformed and analysed by repeated-measures ANOVA.

3. Results

Immunization did not cause any adverse reaction at the injection site in either group. Bacteriological analyses were negative for *S. aureus* in all animals during the whole study. No clinical mastitis cases were detected during the experiment. Mean milk SCC for the control, lysate and bacterin groups were 138,675; 133,352 and 201,372 cells/ml, respectively. Mean SCC did not differ between treatment groups along the lactation.

3.1. Antibody response in serum

3.1.1. IgG anti- S. aureus bacterin and anti – S. aureus lysate

No *S. aureus* antibodies were detected in pre-immune samples of any animal included in the study (Fig. 1 A and B). Both vaccinated groups showed IgG anti-*S. aureus* bacterin levels in sera significantly higher than those in control group ($p < 0.05$). These levels remained significantly augmented from the day of calving for bacterin group and the first week post-calving for lysate group, until the eighth week post-calving ($p < 0.05$). The highest IgG anti-bacterin concentration was observed in both vaccinated groups 1 week after calving, and exceeded about six times concentration in control group (Fig. 1A).

Regarding IgG anti-*S. aureus* lysate in sera, at calving day both vaccinated groups showed antibody levels significantly higher than those observed for control group ($p < 0.05$). However, animals immunized with *S. aureus* lysate had the highest levels from the first week until the eighth week post-calving ($p < 0.05$). IgG anti-*S. aureus* lysate in sera from bacterin-immunized animals remained significantly elevated with respect to control animals from the first to the eighth week post-calving ($p < 0.05$) (Fig. 1B).

3.1.2. IgG_2 anti- S. aureus bacterin and anti- S. aureus lysate

No *S. aureus* IgG_2 was detected in pre-immune samples of any animal included in the study (Fig. 1 C and D). From 24 h until 8 weeks post-calving, both immunized groups developed similar IgG_2 anti-bacterin levels in sera (Fig. 1C); which were significantly higher than those detected in the control group ($p < 0.05$). Maximum antibody production was observed in both vaccinated groups at the first week post-calving, exceeding about 10 times the levels detected in control animals.

Regarding IgG_2 anti-*S. aureus* lysate determination in sera, in both immunized groups antibody levels exceeded significantly those observed in control animals ($p < 0.05$), from the day of calving to the eighth week post-calving. However, after the first week post calving, this subclass levels in animals immunized with *S. aureus* lysate were significantly higher than those detected in bacterin-immunized animals ($p < 0.05$) (Fig. 1D).

3.1.3. IgG anti- S. aureus CP5

ELISA to detect IgG anti- *S. aureus* CP5, was performed with sera samples obtained the first week post-calving. Both immunized groups developed similar antibody levels, which were significantly higher than those detected in control animals ($p < 0.05$) (Fig. 2).

3.2. Antibody response in milk

IgG and IgG_2 anti-*S. aureus* bacterin, *S. aureus* lysate, or purified CP5 levels were analysed in milk samples of all animals included in the study.

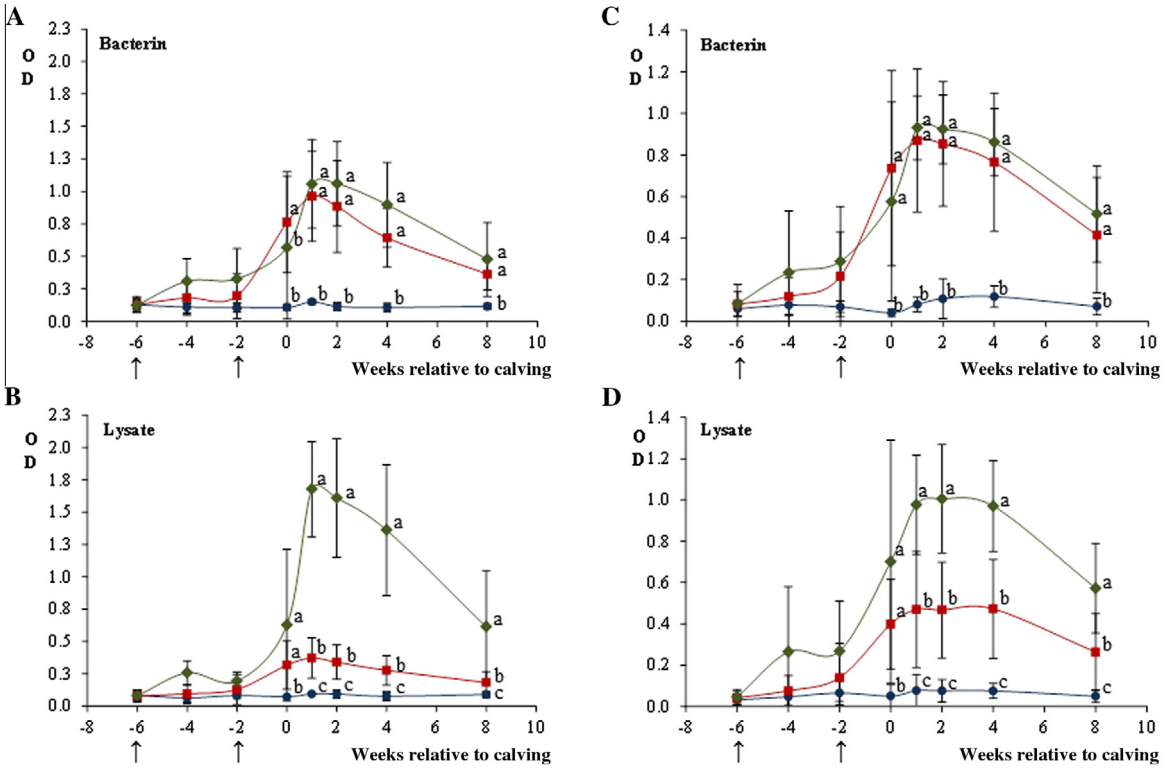

Fig. 1. Antibody response in serum. (A) Mean IgG anti-*S. aureus* bacterin, (B) Mean IgG anti-*S. aureus* lysate, (C) Mean IgG_2 anti-*S. aureus* bacterin, (D) Mean IgG_2 anti- *S. aureus* lysate; in heifers immunizated with *S. aureus* bacterin (■), *S. aureus* lysate (♦) or control (●). Arrows represent immunization times. Different letters correspond to statistically significant differences ($p < 0.05$).

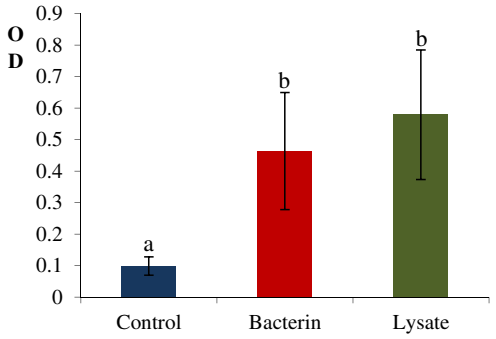

Fig. 2. Serum mean IgG anti-CP5 at 7 days post calving, in heifers immunized with *S. aureus* bacterin, *S. aureus* lysate or control. Different letters correspond to statistically significant differences ($p < 0.05$).

Animals immunized with *S. aureus* lysate presented the highest IgG anti-bacterin levels in whey. These values doubled the ones detected in animals immunized with *S. aureus* bacterin; being statistically significant for samples taken on weeks 2 and 3 post-calving ($p < 0.05$). In addition, maximum values detected the first week post-calving for animals in lysate group exceeded more than 10 times those observed in control animals. IgG anti-bacterin levels in whey from animals in bacterin group were significantly superior to those detected in the control group at every sampling time ($p < 0.05$) (Fig. 3A).

Regarding IgG anti-lysate in whey, animals immunized with *S. aureus* lysate had the highest antibody levels during the first 4 weeks following calving ($p < 0.05$). Maximum values exceeded about 10 times the ones detected for bacterin or control groups. Statistically significant differences between IgG levels in animals from bacterin and control groups were only detected in milk

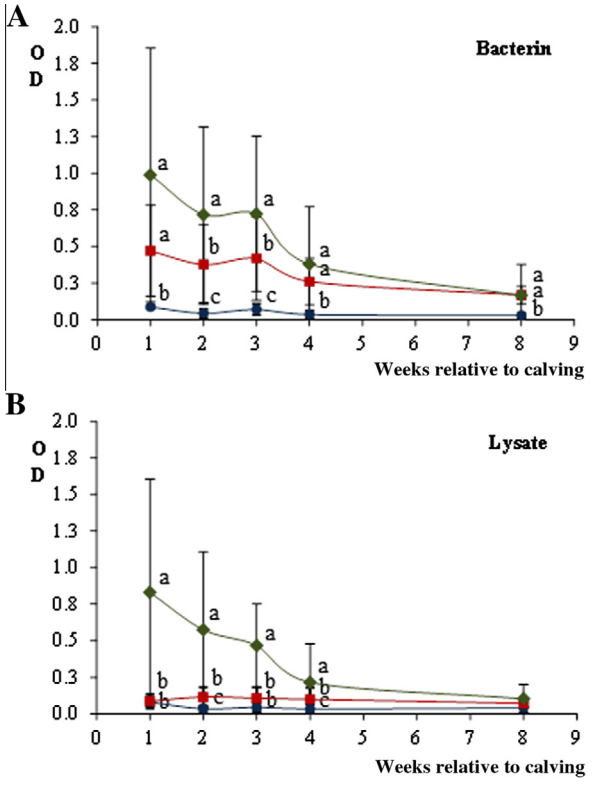

Fig. 3. Antibody response in milk. Whey mean IgG anti-*S. aureus* bacterin (A) and anti- *S. aureus* lysate (B) in heifers immunizated with *S. aureus* bacterin (■), *S. aureus* lysate (♦) or control (●). Different letters correspond to statistically significant differences ($p < 0.05$).

samples taken the second and fourth week post-calving ($p < 0.05$). By the end of the observation period (8 week post-calving) no differences in IgG anti-lysate levels were observed among groups (Fig. 3B).

Neither anti-bacterin nor anti-lysate IgG_2 were detected in whey of any of the three treatment groups. No anti-CP5 IgG or IgG2 were detected in whey of any of the three treatment groups.

3.3. Serum antibody response to a vaccine booster

In order to evaluate immune response of heifers after a booster vaccine dose, by the end of the lactating period, animals were inoculated with a third dose of immunogens at week 31 post-calving. Blood samples were taken before inoculation and once a week for the two following weeks. IgG anti-*S. aureus* bacterin and anti-*S. aureus* lysate were evaluated (Fig. 4).

Before inoculation, animals in lysate group had higher IgG anti-bacterin antibody levels remnant from primary immunization compared with bacterin ($p = 0.074$) and control groups ($p < 0.05$). Anti-bacterin IgG levels in both immunized groups were significantly higher than those in the control group ($p < 0.05$) for the following 2 weeks. These levels were almost 70% of maximum levels detected in both groups at the first week post-calving (Fig. 4A).

Regarding IgG anti-lysate response, antibody levels in animals immunized with *S. aureus* lysate were the highest at every sampling time ($p < 0.05$). Antibody levels 2 weeks post-immunization were about 90% of maximum levels detected the first week post-calving. Animals immunized with bacterin formulation developed IgG anti-lysate levels significantly higher than those in the control group during the 2 weeks following the administration of a third vaccine dose (Fig. 4B).

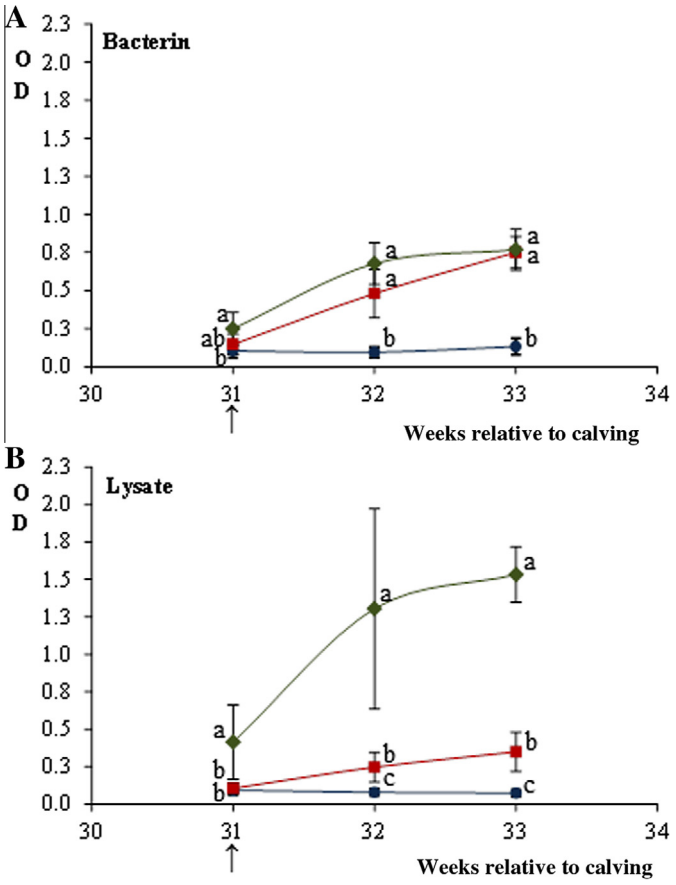

Fig. 4. Serum antibody response to a vaccine booster. Serum mean IgG anti-*S. aureus* bacterin (A) and anti- *S. aureus* lysate (B) in heifers after the third immunization with *S. aureus* bacterin (■), *S. aureus* lysate (♦) or control (●). Arrows represent immunization times. Different letters correspond to statistically significant differences ($p < 0.05$).

3.4. Neutrophil phagocytosis

Neutrophil phagocytosis of opsonized *S. aureus* Reynolds was evaluated with sera collected the first week post calving, since this was the period when the highest specific antibody levels were detected. Based on light scatter properties, R1 was defined (Fig. 5A) to further analysing bacterial incorporation (Fig. 5B). Sera from animals vaccinated with *S. aureus* bacterin or *S. aureus* lysate augmented significantly the percentage of bacteria-containing neutrophils (56.3% ± 10.2 and 54.2% ± 17.5; respectively), compared with sera from control animals (22.6% ± 4.3; $p < 0.05$) (Fig. 5C). The MFI parameter was also higher when sera from the *S. aureus* bacterin or lysate groups were used for phagocytosis assay (337 ± 95 and 317 ± 124, respectively), compared with that of heifers in the control group (117 ± 29; $p < 0.05$) (Fig. 5D).

3.5. Cytokine expression

Transcripts for IL-4, IL-10, IL-12 p-40, IFN-γ and TNF-α were detected in all animals (Fig. 6). Both immunized groups showed significantly higher TNF-α mRNA levels compared with control group (Expression Ratio [ER] bacterin 21.807, $p < 0.05$; ER lysate 25.438, $p < 0.05$). Even when both formulations induced an increase in IL-12 expression relative to controls, only for animals in lysate group this difference was statistically significant (ER 5.210; $p < 0.05$). Interleukin 10 expression was augmented in both immunized groups with respect to controls, but this increase was not statistically significant. Lysate formulation stimulated a non-statistically significant increment on IL-4 mRNA levels. IFN-γ expression was not affected by either treatment; immunization with *S. aureus* lysate was associated with high levels of this cytokine in only one heifer.

4. Discussion

There is scarce information about the use of ISCOMs in the formulation of whole cell vaccines against *S. aureus* bovine mastitis. In a recent study we reported the ability of a CP5 *S. aureus* bacterin formulated with ISCOM Matrix to strengthen humoral immune response, opsonic capacity and cytokine production in pregnant heifers, compared with a vaccine formulated with $Al(OH)_3$ (Camussone et al., 2013), a classical adjuvant used in veterinary vaccines (Spickler and Roth, 2003). In the current study, immune responses generated by a *S. aureus* bacterin and a lysate obtained from the same strain formulated with ISCOM Matrix, when immunizing pregnant heifers, were characterized.

Previous studies reported the ability of immunogens prepared with *S. aureus* lysates and formulated with different adjuvants, to induce antibody production against vaccine components (Luby et al., 2007; O'Brien et al., 2001). In the current study, two vaccine doses were administered before calving to reach high antibody levels during the first month postcalving, since there is an increased susceptibility to IMI in this period (Burton and Erskine, 2003; Sordillo and Streicher, 2002). The lysate-based immunogen not only stimulated a greater specific antibody production, but also antibodies from animals immunized with *S. aureus* lysate were able to recognize whole bacteria, which agrees with a previous report where antibodies produced in heifers following Lysigin™ immunization recognized *S. aureus* whole cells (Nickerson et al., 1999). Moreover, even when the difference in IgG and IgG_2 anti-bacterin

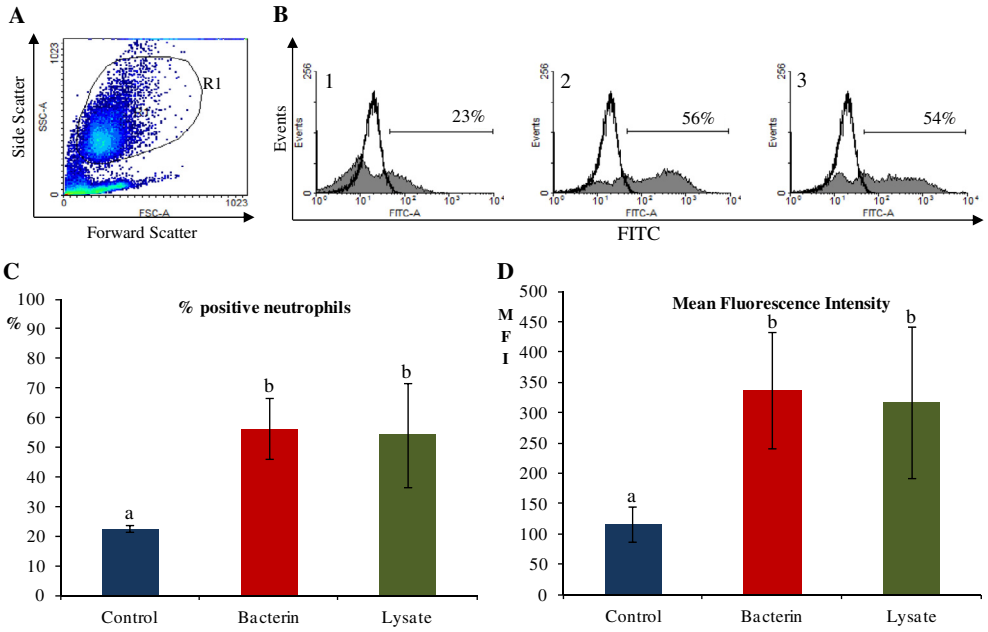

Fig. 5. Neutrophil phagocytosis of FITC positive *S. aureus* Reynolds opsonized measured by flow cytometry. (A) PMN population selected on the basis of forward and side light scatter. (B) Representative histograms showing fluorescence intensity for control PMN incubated with HBSS (empty) or PMN incubated with FITC positive *S. aureus* Reynolds opsonized with sera from *S. aureus* bacterin (2) *S. aureus* lysate (3) or control (1) groups (gray filled). Percentages of FITC+ PMN (C) or MFI (D) are shown.

levels between both evaluated groups was not statistically significant, a tendency to a higher antibody production in animals vaccinated with *S. aureus* lysate was observed.

In the present study, IgG_2 was determined, since this subclass is considered to be the main opsonin supporting neutrophil phagocytosis in milk of the infected mammary gland (Paape et al., 2003; Sordillo and Streicher, 2002). Both immunogens stimulated specific IgG_2 production in serum, compared with control animals, agreeing with previous reports about ISCOMS upregulation of antigen-specific production of different IgG subclasses (Sjölander et al., 1997; Morein et al., 2007; Morein and Bengtsson, 1999).

S. aureus CP mask recognition of antibodies directed against the cell wall by PMN and prevent complement activation (O'Brien et al., 2001; Paape et al., 2003). However, antibodies against CP opsonize *S. aureus*, enhancing neutrophil phagocytosis (O'Brien et al., 2001). Considering that cell integrity disruption can affect antigen structure, serum IgG against purified CP5 was also evaluated. Both immunized groups developed similar anti-CP5 IgG levels, which were higher than those in control animals, in accordance with previous studies where immunization of heifers with a *S. aureus* CP2 lysate incorporated into micro-spheres induced specific humoral response against CP2 (O'Brien, 2001). These results suggest that a formulation composed of strains expressing different capsular serotypes would induce antibody responses against each CP.

Numerous attempts have been made during the last decades to stimulate opsonising antibody production in the mammary gland (reviewed by Pereira et al., 2011). In the current study, immunization with *S. aureus* bacterin or lysate, formulated with ISCOM Matrix, induced specific IgG production in whey compared with control animals. However, no IgG_2 against *S. aureus* whole cells, *S. aureus* lysate, or CP5 were detected. These findings are in accordance with previous reports, since intramammary vaccination with heat-killed *S. aureus* CP5 and CP8 (Barrio et al., 2003), subcutaneous administration of Lysigin™ in the neck (Luby et al., 2007) and subcutaneous administration in the supramammary linph node area of a formalin inactivated *S. aureus* CP5 formulated with ISCOM Matrix or $Al(OH)_3$ (Camussone et al., 2013) failed to produce detectable amounts of IgG_2 in whey. Milk from healthy mammary glands has a low content of IgG_2 (Caffin and Poutrel, 1988); but it increases substantially during mammary gland inflammation (Sordillo and Streicher, 2002) 6–12 h earlier than the peak neutrophil response (Burton and Erskine, 2003). Therefore, the synergism between high levels of specific serum IgG_2 and migrating neutrophils should result in the stimulation of a specific effector response, enhancing phagocytic capacity and bacterial clearance in the infected mammary gland (Burton and Erskine, 2003; Paape et al., 2003). However, a potential passage of this subclass into milk needs to be evaluated following experimental challenge.

In order to evaluate the presence of memory cells by the end of lactation and before cessation of milking, a third dose of vaccine was given about 8 months after the beginning of the trial. Booster dose stimulated specific IgG response in both vaccinated groups, which reached in the subsequent 15 days more than 70% of the highest antibody levels observed at one week post-calving. In a previous study where immune responses generated in heifers after immunization with inactivated CP5 *S. aureus* or human serum albumin-conjugated CP5 were compared, application of a third dose of immunogens 11 months after the second dose, generated a secondary antibody response that reached after 21 days similar levels to those observed during primary response (Tollersrud et al., 2001). These observations suggest that an immunization schedule based on the application of two vaccine doses before first calving, followed by a booster vaccination by the end of lactation would enhance natural protective immunity that can be beneficial to control *S. aureus* IMI during early mammary gland involution. This stage is characterized by increased susceptibility to IMI, since changes that take place in the mammary gland may facilitate bacterial penetration of the streak canal, interfere with natural defence mechanisms and enhance bacterial growth (Oliver and Sordillo, 1988).

One of the main virulence mechanisms of *S. aureus* is the ability to avoid PMN phagocytosis through the production of capsular polysaccharides (O'Riordan and Lee, 2004). The opsonic capacity of antibodies generated through vaccination was evaluated

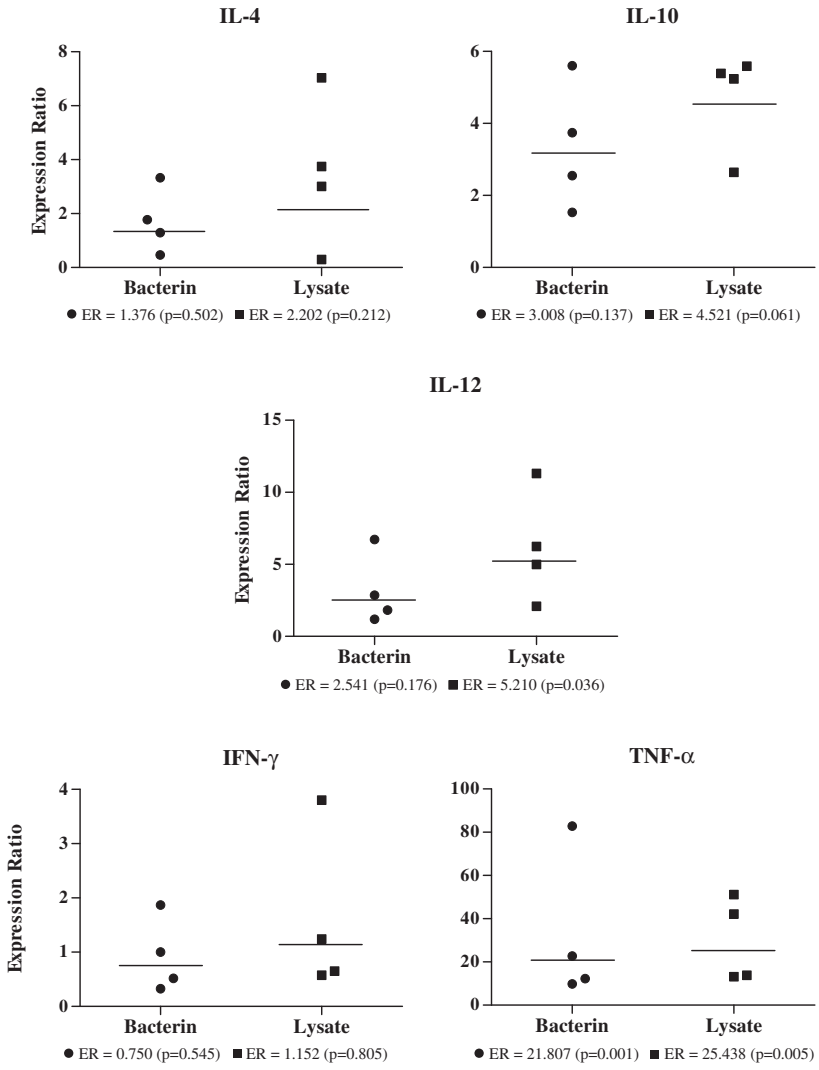

Fig. 6. Cytokine expression levels relative to the control group (Expression ratio, ER) observed *in vivo* 24 h after the second dose of vaccine, in animals immunized with *S. aureus* bacterin or with *S. aureus* lysate. Median ER values are represented as horizontal bars and shown below each graph.

in vitro by phagocytosis assays with bovine PMN. Serum samples from the first week postpartum were used, since they yielded the highest antibody titers for both experimental groups. Antibodies generated by both immunogens enhanced *S. aureus* phagocytosis by PMN, through an increment in the percent of PMN positive for *S. aureus*/FITC and in the number of bacteria internalized by positive cell, compared with antibodies from control animals. No significant differences in the opsonic capacity of sera from both immunized groups were observed. In previous studies, a lysate incorporated to microspheres (O'Brien et al., 2001) or a formalin inactivated *S. aureus* CP5 formulated with ISCOM Matrix (Camussone et al., 2013), stimulated antibody production with improved opsonic capacity. Former research showed that only antibodies against CP were able to increase phagocytic activity by bovine PMN (Guidry et al., 1991, 1994). In the present study, a simultaneous increase in PMN phagocytosis and anti-CP5 antibody levels developed by both formulations was observed.

Stimulation of innate immune system has an important role in evolution to adaptive immune response (Hoebe et al., 2004). Hence, inclusion of adjuvants which trigger early innate immune response, and cooperate in the development of robust and long-lasting adaptive immune responses is crucial to improving vaccine effectiveness. In this study, innate and T helper activation was evaluated through the assessment of IL-4, IL-10, IL-12 p-40, TNF-α and IFN-γ mRNA relative levels (Zhu et al., 2010) 24 h following the administration of the second dose of vaccines. Both formulations up regulated relative expression of IL-10, IL-12 and TNF-α. These findings are in agreement with recent observations from our laboratory where immunization of heifers with a CP5 *S. aureus* bacterin formulated with ISCOM Matrix stimulated higher relative expression levels of IL-10 and IL-12, 24 h following the administration of a second vaccine dose, compared with the same immunogen formulated with $Al(OH)_3$ (Camussone et al., 2013). There is scarce information about cytokine stimulation after immunization with staphylococcal vaccines in cattle. In a previous study, subcutaneous immunization of lactating cows with two doses of α-toxin formulated with Freund incomplete adjuvant, failed to induce detectable IL-10, IL-12, or TNF-α mRNA levels in milk cells (Riollet et al., 2000b). However, evaluation time following vaccination (3 months after the last vaccine dose) and origin of cells examined do not enable comparisons with the present study. IL-10 is now known to be expressed not only by innate immune cells, like dendritic cells, macrophages, and natural killer cells, but also by many acquired immune cells including, TH1, TH2 and TH17 cells, Treg cells, CD8+ T cells and B cells (Kubo and Motomura, 2012). In a recent study, intramuscular inoculation of ISCOM Matrix without

antigen in pigs, up-regulated IL-10 expression assessed in muscle at the injection site 24 h post-inoculation (Ahlberg et al., 2012). IL-10 expression plasticity is not only controlled by cytokine microenvironment, but also by sustained antigen presentation (Kubo and Motomura, 2012), which is in accordance with ISCOM Matrix mechanism of action (Morelli et al., 2012). IL-12 plays a central role in ISCOMs action mechanism. Smith et al. (1999) demonstrated that ISCOMs immunogenicity relies on an IL-12-dependent cascade of innate immune responses. These complexes can stimulate dendritic cells either by their particulate nature or by the presence of molecules, like Quil A, enhancing expression of IL-12 and type I-IFN (Robson et al., 2003). Failure of early induction of inflammatory mediators, such as TNFα in milk after experimental *S. aureus* IMI was associated with low cell recruitment at the infection site and establishment of chronic mastitis (Riollet et al., 2000a). This observation suggests that immunogens capable of up regulating early expression of pro-inflammatory cytokines would be of importance to *S. aureus* IMI control. However, it has to be taken into account that there are no previous data about kinetics of cytokine expression after immunization against *S. aureus* mastitis in heifers (Pereira et al., 2011), and that cytokine expression was evaluated at a single time point in this study. In spite of these limitations, a tendency to a higher stimulation of pro-inflammatory cytokines, as IL-12 and TNF-α, and a regulatory one, as IL-10, was observed for both formulations, although early induction of these cytokines was more noticeable after immunization with *S. aureus* lysate. Complementary assays *in vitro*, evaluating cytokine production kinetics should be carried out to delineate potential mechanisms activated by these formulations.

In conclusion, immunization of pregnant heifers with two immunogens composed by *S. aureus* whole or lysed cells formulated with ISCOM Matrix, stimulated strong humoral immune responses in blood and milk, raising antibodies that increased opsonic capacity. Lysate formulation generated a higher and longer lasting antibody titer than the bacterin formulation. In addition, this formulation stimulated a higher expression of regulatory and pro-inflammatory cytokines.

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