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Research paper

Immune response and functional role of antibodies raised in heifers against a *Staphylococcus aureus* CP5 lysate and recombinant antigens vaccine formulated with Iscom Matrix adjuvant

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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 inactivated whole-cells formulated with traditional adjuvants. We recently showed the ability of a *S. aureus* CP5 lysate vaccine adjuvanted with Iscom Matrix to generate a longer lasting specific antibody response in blood and milk, with improved opsonic capacity, compared with a *S. aureus* CP5 whole-cell formulation. The aim of the present study was to obtain an experimental immunogen composed of lysed cells of a CP5 *S. aureus* strain supplemented with recombinant clumping factor A, fibronectin binding protein A and β-toxin formulated with Iscom Matrix, characterize the immune response generated when immunizing pregnant heifers and assess the functional role of antibodies raised against this immunogen in experimental models. Both a lysate vaccine and a lysate + recombinant antigens vaccine elicited antibodies that promoted neutrophil phagocytosis and inhibited internalization into mammary epithelial cells, *in vitro*. Incorporation of defined antigenic molecules to the lysate formulation elicited a strong specific humoral immune response against both lysate and recombinant antigens and was associated with higher expression of regulatory and pro-inflammatory cytokines. In addition, antibodies were efficient for blocking *S. aureus* binding to bovine fibrinogen and fibronectin, and neutralizing β-toxin effect *in vitro*, placing these antigens as candidates to be included in a formulation directed to prevent staphylococcal bovine mastitis.

1. Introduction

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections (IMI) worldwide (Zecconi et al., 2006). Lack of effectiveness of traditional control measures based on milking time

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hygiene and antibiotic therapy against this organism (Zecconi et al., 2006), has led to the development of complementary alternatives directed to prevent the disease. Among them, manipulation of the host immune mechanisms through vaccination has been explored. During the last two decades several experimental immunogens for *S. aureus* mastitis control have been evaluated (Pereira et al., 2011). However, only two vaccines, composed of lysates of different *S. aureus* strains (Lysigin™, Boehringer Ingelheim Vetmedica Inc., St Joseph, MO, USA) and inactivated *S. aureus* expressing slime associated antigenic complex (Startvac® Laboratorios Hipra, Spain), are currently commercially available worldwide. However, field trials or experimental challenge carried out with Lysigin™ in adult cows showed only some effectiveness in reducing infection duration and severity (Middleton et al., 2006, 2009), whereas Startvac® efficacy was estimated in two herds based on infection transmission and duration parameters, resulting in a relatively low reduction of new IMI and a moderate efficacy for cure (Schukken et al., 2014).

S. aureus has multiple virulence factors that interact with the host at different stages of an IMI; hence, a successful *S. aureus* vaccine will probably need to include a variety of antigens to stimulate antibody production against key pathogen factors involved in IMI (Middleton, 2008). In addition, increasing evidence of the importance of cell-mediated immunity in protection against *S. aureus* infections indicates that a vaccine should be capable of T-cell stimulation for inducing a superior protective efficacy (Gómez et al., 2002; Middleton, 2008; Proctor, 2012). Several molecules that contribute to pathogenesis and also stimulate mammary gland defences were identified and proposed as vaccine candidates. The ability of the organism to internalize in epithelial and phagocytic cells is considered one of the key steps in staphylococcal pathogenesis, and this mechanism is mainly attributed to the presence of fibronectin (Fn) binding protein (FnBP) A and B (Dziewanowska et al., 1999; Lammers et al., 1999; Fowler et al., 2000). The *S. aureus* clumping factors A and B (Clf) allow interaction between bacteria and plasmatic fibrinogen (Fb) leading to an instantaneous clumping of bacterial cells (Hawiger et al., 1982). It was also demonstrated that these factors have anti-phagocytic activity *in vitro* (Higgins et al., 2006). Capsular polysaccharides (CP) 5 and 8 are considered important components for vaccine development since antibodies against them opsonize *S. aureus*, enhancing polymorphonuclear neutrophil (PMN) phagocytosis (Guidry et al., 1991, 1994). Beta toxin (β-toxin) is a sphingomyelinase secreted by most *S. aureus* strains (Aarestrup et al., 1999). This toxin lyses red blood cells (RBCs), PMN (Marshall et al., 2000) and proliferating T-cells (Huseby et al., 2007), and it may be involved in endosomal membrane lysis resulting in the escape of bacteria into the cytoplasm (Shompole et al., 2003).

The combination of *S. aureus* whole or lysed cells with inactivated toxoids and capsular or extracellular products has been a widely used strategy for vaccine formulation (Pereira et al., 2011). However, most of these components were used as crude extracts and therefore not completely characterized (Pereira et al., 2011). In addition, there is scarce information about the use of multicomponent vaccines formulated with defined antigens against *S. aureus* IMI and only one of them has been evaluated in a bovine model (Shkreta et al., 2004). In previous studies, we have shown the ability of experimental immunogens composed by *S. aureus* whole or lysed cells formulated with Iscom Matrix to stimulate strong humoral immune responses in blood and milk, with production of opsonic antibodies and expression of regulatory and pro-inflammatory cytokines (Camussone et al., 2013, 2014). We hypothesized that inclusion of key antigenic components involved in different steps of staphylococcal IMI in vaccine formulations would extend the immune response reached by our previous immunogen (Camussone et al., 2014). The aim of this study was to obtain an experimental immunogen composed of lysed cells of a CP5 *S. aureus* strain supplemented with recombinant β-toxin, FnBPA and ClfA, formulated with Iscom Matrix, characterize the immune response generated when immunizing pregnant heifers and assess the functional role of antibodies raised against this immunogen in experimental models.

2. Materials and methods

2.1. Vaccine components

Construction of expression plasmids: The nucleotide sequence of *S. aureus* β-toxin, ClfA and FnBPA genes were obtained from the GenBank database (Accession numbers X13404, Z18852 and AJ629121.1, respectively). Genomic DNA was extracted using a commercial kit (Genomics DNA extraction kit, Real Biotech Corporation), from either a *S. aureus* isolated from a mastitis case for β-toxin coding sequence or from Reynolds reference strain for ClfA and FnBPA coding sequences. PCR was performed in a total volume of 50 μl containing: 1× PCR buffer, 2 mM $MgCl_2$, 1 U/μl *Thermus aquaticus* DNA polymerase (Fermentas, Germany), 0.2 mM dNTPs (Genbiotech, Argentina), 0.3 μM primers, and 50 ng of genomic DNA. Specific primers for full length β-toxin protein were: BTfw (5′-ggattcAAAGGAGTGATAATGATG-3′) and BTrv (5′-gtcgacCTATTTACTATAGGCTT-3′). ClfA primers were designed to obtain the Region A containing the Fb binding domain being: ClfAfw (5′-gaattcGAAAATAGTGTT ACGCAATCT-3′) and ClfArv (5′-gtcgacCTCTGGAATTGGTT CAATTTC-3′). FnBPA primers were designed to obtain the Fn binding domains D1D2D3 being: FnBPAfw (5′-gaattcGGTGGCCAAAATAGCGGTA-3′) and FnBPArv (5′-gtcgacTTGGTGGCACGATTGGAG-3′). Lower case sequences correspond to restriction enzymes sites used for cloning. Primers were purchased from PB-L (Argentina). Amplification was performed in GeneAmp PCR System (Applied Biosystems, USA) using the following program: an initial 3 min denaturation step at 95 °C, followed by 10 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 50 °C for β-toxin or 51 °C for ClfA or FnBPA, and 1 min of extension at 72 °C, and 20 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 58 °C for β-toxin or 65 °C for ClfA or FnBPA, and 1 min of extension at 72 °C; with a final extension step at 72 °C for 5 min. PCR products were analyzed by electrophoresis on GelRed (Biotium, USA)-stained 1% agarose gels (Biodynamics, Argentina), and sequenced

using an ABI3130xl DNA sequencer (Applied Biosystems). The obtained sequences were deposited in the GenBank database under Accession Numbers KC242859, KJ001294, and KJ001293 for β-toxin, ClfA and FnBPA, respectively. PCR products were cloned into pET32a expression vector (Merck Millipore, Germany) and transformed into competent *Escherichia coli* BL21(DE3) cells.

Protein expression and purification: *E. coli* BL21(DE3) cells bearing the different plasmidic constructions pET32a_β-toxin, pET32a_ClfA and pET32a_FnBPA, were grown overnight (ON) in LB medium, supplemented with 0.1 mg/ml of ampicillin at 37 °C, with agitation. Protein expression was induced ON with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside and the respective rβ-toxin, rClfA and rFnBPA antigens were purified with a Ni-nitrilotriacetic acid column (GE), as described elsewhere (Aguirre et al., 2006). Protein quantification was performed using a spectrophotometer (NanoDrop 2000, Thermo Scientific) and purity was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining (Laemmli, 1970).

S. aureus lysate: *S. aureus* CP type 5 strain (Reynolds) was obtained as described previously (Camussone et al., 2014).

2.2. Formulation of vaccines

The experimental immunogens consisted of a *S. aureus* Reynolds lysate suspension corresponding to 1×10^9 CFU/dose (Lysate), or a *S. aureus* Reynolds lysate suspension (corresponding to 1×10^9 CFU) supplemented with 200 μg of each rβ-toxin, rClfA and rFnBPA antigens/dose (Lysate + Recombinant antigens). Vaccines were formulated with 2 mg/dose of Iscom Matrix. The adjuvant was kindly supplied by Isconova, Uppsala, Sweden. A placebo consisting of sterile PBS and adjuvant (2 mg/dose Iscom Matrix) was used as control. Sterility of these formulations was evaluated by plating 100 μl on blood agar plates in duplicate and incubating 48 h at 37 °C.

2.3. Animals and treatments

Twenty seven Holstein dairy heifers in the last trimester of gestation belonging to the dairy herd of INTA Rafaela Experiment Station were used. Animals were randomly allocated in three groups; Lysate (Lys; $n = 9$), Lysate + Recombinant antigens (Lys + Rec; $n = 9$) and control ($n = 9$). 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 2.3 ml of vaccine in the supramammary lymph node area at approximately 45 and 14 days before the expected calving date. At 14 days before expected calving date udders of all heifers were clinically examined by palpation, samples of pre-partum mammary secretion were taken following standard procedures (Oliver et al., 2004) and subjected to bacterial culture to determine the presence of *S. aureus* IMI. Only animals free of *S. aureus* IMI and with no clinical signs of inflammation continued in the experiment. Animals were bled by puncture of the coccygeal vein before each inoculation, and 7, 14, 21, 30 and 60 days after calving. Blood was allowed to clot and sera were collected *via* centrifugation. After parturition, aseptic quarter foremilk samples were collected every week from the first week after calving to 60 days after calving. 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 acidified and centrifuged for 15 min. at 300 × *g*; whey was collected and stored at −20 °C until processed. From the third month post-calving to the end of lactation period, milk samples were taken monthly and subjected to bacterial culture. 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).

2.4. Bacteriological examination

Mammary secretion samples were cultured for mastitis pathogens according to standard procedures (Oliver et al., 2004). Gram positive, coagulase positive isolates were subjected to Multiplex PCR for *S. aureus* identification, as previously described (Martineau et al., 1998). The presence of one colony of *S. aureus* on blood agar was considered as a positive identification; therefore, detection limit was 100 CFU/ml. IMI was defined as two consecutive samples yielding the same organism (Smith et al., 1990).

2.5. Serological methods

Specific antibody production (IgG and IgG_2 subtype) was measured by enzyme-linked immunosorbent assay (ELISA). IgG_2 was quantified since this subclass is considered the most effective opsonin promoting PMN phagocytosis (Burton and Erskine, 2003). Flat-bottomed 96-well microtitre plates were coated with a suspension of *S. aureus* Reynolds lysate (corresponding to 1×10^8 CFU/well), 5 μg/well of purified CP5, or 0.5 μg/well of rβ-toxin, rClfA or rFnBPA, in PBS (pH 7.2). ELISA was performed as previously described (Camussone et al., 2013). Working dilutions of sera and whey were 1/2000 and 1/200, respectively.

2.6. Neutrophil phagocytosis

Bovine PMN were obtained from a healthy animal as described previously (Siemsen et al., 2007). Briefly, FITC-labeled *S. aureus* Reynolds (1×10^8 CFU/ml) was incubated with heat inactivated sera obtained at day 7 post-calving from each animal included in the study or Hanks Balanced Salt Solution (HBSS), for 30 min at 37 °C with gentle shaking. Bovine PMN (1×10^7 cells/ml) were added 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). PMN were gated and fluorescence intensity was depicted on a

four-decade logarithmic scale and single-parameter analysis as histograms. Data were analyzed 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).

2.7. Mammary epithelial cells 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, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), insulin (5 μg/ml), hydrocortisone (1 μg/ml), penicillin (100 U/ml) and streptomycin sulphate (100 μg/ml) (Sigma Chemical Co., St. Louis, MO). The bacterial invasion assay was performed as previously described (Almeida et al., 1996) with modifications. *S. aureus* Reynolds was pre-incubated with 1/10 dilution of whey samples obtained at day 7 post-calving from each animal included in the study, for 60 min at 37 °C with gentle shaking. After incubation, bacterial suspension was co-cultured with confluent monolayer of MAC-T cells in DMEM at a multiplicity of infection of 100. After incubation, monolayers were washed three times with PBS (pH 7.4) and treated with gentamicin (100 μg/mL, Sigma) in DMEM at 37 °C in 5% CO_2 for 2 h to kill extracellular bacteria. Supernatants were collected and streaked onto blood agar to verify the killing by gentamicin. Monolayers were then washed three times with PBS, treated with 0.25% trypsin-0.1% EDTA (Gibco BRL), and further lysed with Triton X-100 (Amersham, Arlington Heights, IL, USA) 0.025% (v/v) in sterile distilled water to release intracellular bacteria. Cell lysates were 10-fold serially diluted, streaked onto blood agar and incubated ON at 37 °C to determine *S. aureus* CFU/ml. Each assay was run in triplicate with three observations per assay. Data were expressed as percentage of control group (100%).

2.8. Recognition and haemolytic activity inhibition of native β-toxin (nβ-toxin)

Partial purification of native β-toxin: nβ-toxin was partially purified from culture supernatants of a *S. aureus* isolated from a mastitis case and found to produce only β-toxin *in vitro*, as previously described (Calvinho et al., 1993). The obtained extract was stored at −20 °C until needed. Haemolytic activity against sheep red blood cells (RBC) was assessed as previously described (Wadström and Möllby, 1971). The end point was taken as the dilution showing 100% haemolysis. The inverted value of this dilution indicated the number of haemolytic units of undiluted extract/ml (HU/ml).

Western blot: Ten microliters of each nβ-toxin or rβ-toxin were separated by SDS-PAGE and transferred to 0.45 μm nitrocellulose membranes (Mini Trans-Blot Cell, BioRad). Membranes were first incubated with PBS with low fat goat milk 5%, and then with pooled sera obtained at 7 days post-calving from each group of animals, diluted in PBS with low fat goat milk 1%. Finally membranes were incubated with peroxidase-conjugated rabbit-anti-cow IgG (Sigma). Between each step, plates were washed five times with 0.05% Tween 20 in PBS. All incubations were 1 h at 37 °C. Lastly, enzyme substrate (H_2O_2/diaminobenzidine, Sigma) was added and the reaction was stopped by submerging the membranes in distilled water.

Native β-toxin activity inhibition: one hundred microliters of 1/50 dilution of extract containing nβ-toxin was incubated with 100 μl of serial 2-fold dilutions of sera obtained at 7 days post-calving from each animal included in the study, for 1 h at 37 °C. Then, 100 μl of sheep RBC were added and incubated for another hour at 37 °C, for 1 h at room temperature, and finally ON at 4 °C. The end point was taken as the dilution showing 100% haemolysis. The inverted value of this dilution indicated the haemolysis inhibition titer.

2.9. Blocking assays

For Fn binding assays, IgG specific for vaccine components was purified from sera obtained at 7 days post-calving in order to remove soluble Fn or other plasma proteins that might interfere with binding (Schennings et al., 1993). Briefly, pieces of nitrocellulose membranes were coated with a suspension of *S. aureus* lysate or *S. aureus* lysate + recombinant antigens (1 mg of total protein content). After three washes with 0.05% Tween 20 in PBS, membranes were incubated for 1 h with 2 ml of sera from either Lys or Lys + Rec groups, respectively, and washed again. Finally, antibodies were desorbed by 5 min incubation with 2 ml of Glycine–HCl 0.1 M, pH 2.8, and then neutralized with NaOH 0.1 N. Nitrocellulose membranes coated with rβ-toxin were used to purify specific antibodies to be used as control group, since these antibodies do not block FnBPA-Fn binding. Purified antibodies were conserved at −20 °C.

Ability of antibodies to block Fn/Fb binding was tested as described previously (Schennings et al., 1993) with modifications. Briefly, flat-bottomed 96-well microtitre plates were coated with 5 μg/well of bovine Fb or 2 μg/well of bovine Fn (Invitrogen), in PBS (pH 7.2). Fibrinogen was purified from bovine plasma by ethanol precipitation (Ismail, 2012). The remaining sites were blocked by incubating with bovine serum albumin 5% in PBS. A suspension of 1×10^9 CFU/ml of *S. aureus* Reynolds was pre-incubated with sera (Fb assays) or purified antibodies (Fn assays) obtained at day 7 post-calving from each animal included in the study, for 1 h at 37 °C with gentle shaking. The coated plates were first incubated with 100 μl of pre-incubated bacteria, then with mouse anti-*S. aureus* Reynolds serum and finally with peroxidase-conjugated rabbit anti-mouse IgG (Sigma). All incubations were 1 h at 37 °C. Plates were washed three times with 0.05% Tween 20 in PBS between each step. Lastly, enzyme substrate (Tetramethylbenzidine, Invitrogen) was added and the reaction was stopped by the addition of 0.5 N H_2SO_4. The absorbance was read at 450 nm and results were expressed as optical density.

2.10. Cytokine expression

Blood samples were collected aseptically 24 h after the second inoculation of vaccines, by jugular venipuncture from seven animals in control group, four animals in Lys group and six animals in Lys + Rec group. RNA extraction and cDNA preparation were carried out as previously described (Camussone et al., 2014). IL-4, IL-10, IL-12 p-40, IL-17A and IFN-γ mRNA relative expression levels were determined by quantitative Real Time PCR with specific primers, using β-actin as normalization gene as described (Camussone et al., 2014). IL-17A forward/reverse primers (Ta = 62 °C) were those previously used by Maeda et al. (2013). Each PCR was performed in duplicate in a total volume of 10 μl containing the following: 2 μl of 1/8 diluted cDNA, 5 μl of 2× SYBR green PCR master mixture (Real Mix, Biodynamics), 0.25 μl of each 10 μM sense/antisense primers and 2.5 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 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at Ta (°C), and 30 s at 72 °C. Relative expression levels were calculated using REST 2009 V2.0.13 software (Pfaffl et al., 2002).

2.11. S. aureus isolates characterization

Pulse field gel electrophoresis (PFGE): the clonality of *S. aureus* isolated from animals included in the study was assessed by PFGE of SmaI-digested (Invitrogen) chromosomal DNA fragments using a CHEF-DR II apparatus (BioRad Laboratories, CA, USA) as previously described (Barbagelata et al., 2012), including *S. aureus* Reynolds strain. The similarity between PFGE types was evaluated by the Dice coefficient. The resultant similarity matrix was analyzed by the unweighted pair group method using arithmetic averages (UPGMA), and data were analyzed with PyElph 1.4 software (Pavel and Vasile, 2012).

Capsular genotype: The presence of *cap*5k and *cap*8I loci in *S. aureus* isolates was evaluated by PCR as described (Camussone et al., 2012).

2.12. 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, Lys, Lys + Rec) and time (−45, −30, −15, 0, 7, 15, 30, 60 days relative to calving for serum and 7, 15, 21, 30, 60 days relative to calving for whey) was used. Results were compared by non-parametric test Kruskal–Wallis followed by Mann–Whitney's test to detect differences between pairs ($p < 0.05$).

3. Results

Immunization did not cause any adverse reaction at the injection site in neither group. One animal belonging to control group was excluded from the study because of pre-partum *S. aureus* IMI. One heifer belonging to control group and other from Lys group were excluded because of abortion.

3.1. Antibody response in serum

3.1.1. Antibody response to S. aureus lysate

IgG and IgG_2 against *S. aureus* lysate in serum were assessed. No *S. aureus* antibodies were detected in pre-immune samples of any animal included in the study. After the application of two doses of vaccine and until the end of the observation period (60 days post-calving), both immunized groups showed significantly higher IgG anti-*S. aureus* lysate levels in serum than those observed in control group ($p < 0.05$). Among immunized animals, those in Lys group developed higher IgG anti-lysate levels compared with animals in Lys + Rec group, at every sampling time during the first 60 days post-calving ($p < 0.05$). Maximum IgG anti-*S. aureus* lysate absorbances in serum were observed in both Lys and Lys + Rec groups 1 week after calving, and exceeded about 13 and 8 times, respectively, levels in control group (Fig. 1A).

Regarding IgG_2 anti-*S. aureus* lysate in serum, after inoculation with two doses of vaccines, both immunized groups significantly augmented their antibody levels compared with those observed in control animals ($p < 0.05$). These levels reached their maximum at 7 days post-calving and remained augmented during the first 60 and 30 days post-calving for Lys and Lys + Rec groups, respectively, in comparison with levels in control group ($p < 0.05$). Among immunized animals, this subclass levels were significantly higher in animals from Lys group than those detected in animals from Lys + Rec group ($p < 0.05$) (Fig. 1B).

3.1.2. Antibody response to S. aureus virulence factors

Capability of formulations to induce antibodies against selected *S. aureus* virulence factors was also evaluated. From the first week post-calving and until the end of the observation period, both immunized groups showed similar anti-CP5 antibody levels, which were significantly higher than those detected in control animals ($p < 0.05$). Only animals in Lys + Rec group developed specific IgG against rβ-toxin, rClfA and rFnBPA. For the three recombinant antigens, specific antibody levels reached their maximum around the first week post-calving and remained augmented for 2 months, in comparison with levels in Lys or control groups ($p < 0.05$) (Fig. 1C–F).

3.2. Antibody response in milk

3.2.1. Antibody response to S. aureus lysate

IgG and IgG_2 anti-*S. aureus* lysate levels were analyzed in whey samples of all animals included in the study. Both immunized groups developed significantly higher IgG anti-*S. aureus* lysate levels in whey, in comparison with those in control group ($p < 0.05$). These levels remained augmented from the first week post-calving until 21 days post-calving for Lys + Rec group, and until 60 days post-calving for Lys group ($p < 0.05$) (Fig. 2A). No anti-*S. aureus* lysate IgG_2 was detected in whey of any of the three treatment groups.

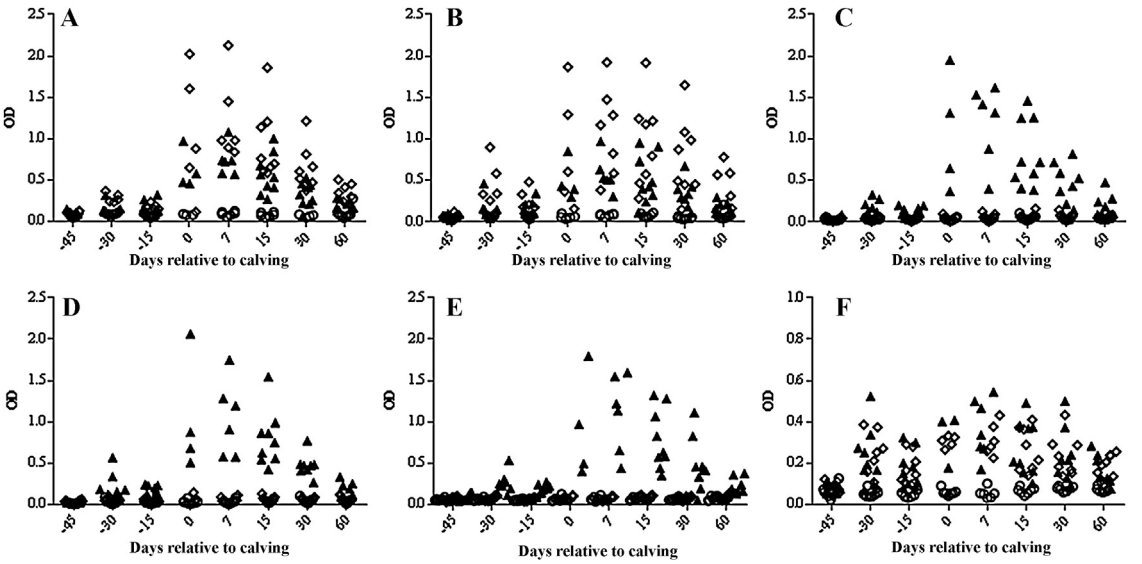

Fig. 1. Antibody response in serum. IgG anti-*S. aureus* lysate (A), Mean IgG_2 anti-*S. aureus* lysate (B), IgG specific to *S. aureus* selected virulence factors, rβ-toxin (C), rClfA (D), rFnBPA (E) and CP5 (F); in sera of heifers immunized with *S. aureus* lysate (◊), *S. aureus* lysate + recombinant antigens (▲), or control (○).

3.2.2. Antibody response to S. aureus virulence factors

Antibody response directed to selected *S. aureus* virulence factors was evaluated in whey samples of all animals included in the study. Only animals belonging to Lys + Rec group showed significantly elevated IgG anti-*S. aureus* rβ-toxin, rClfA and rFnBPA levels in whey, from the first week post-calving and until the end of the observation period, in comparison with levels in Lys or control groups ($p < 0.05$) (Fig. 2B–D). No IgG anti-CP5 was detected in whey samples from any of the three treatment groups.

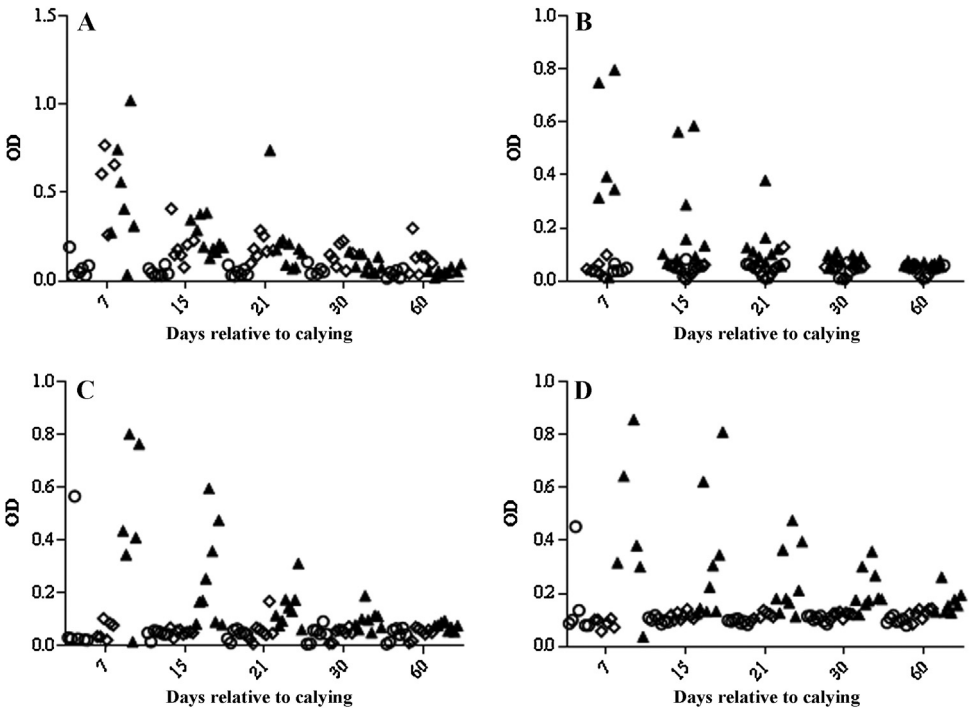

Fig. 2. Antibody response in whey. IgG anti-*S. aureus* lysate (A), IgG specific to *S. aureus* selected virulence factors, rβ-toxin (B), rClfA (C) and rFnBPA (D); in whey of heifers immunized with *S. aureus* lysate (◊), *S. aureus* lysate + recombinant antigens (▲), or control (○).

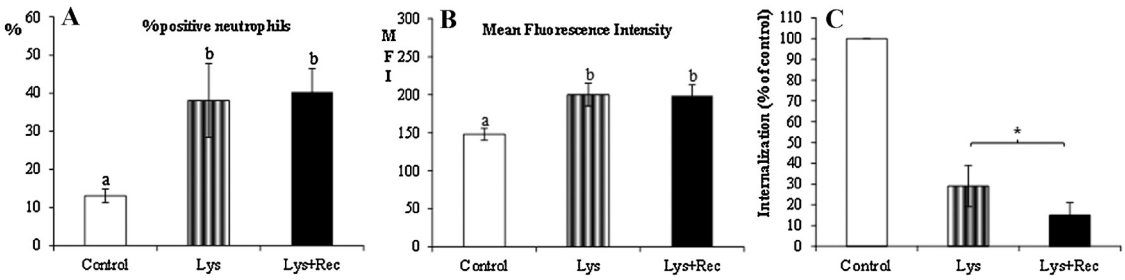

Fig. 3. *In vitro* functional analysis of immune serum and whey. (A)/(B) Neutrophil phagocytosis of *S. aureus* Reynolds opsonized with sera obtained at 7 days post-calving from heifers immunized with *S. aureus* lysate, *S. aureus* lysate + recombinant antigens or control, measured by flow cytometry. Data are expressed as percentage of FITC + PMN or MFI, respectively. Bars represent the mean of eight individual sera per group run in duplicate. Error bars represent the standard deviation of the mean (S.D.M.). Different letters correspond to statistically significant differences ($p < 0.05$). (C) Internalization of *S. aureus* Reynolds into bovine mammary epithelial cells. Percentage of *S. aureus* Reynolds internalization into bovine mammary epithelial cells after pre-treatment of bacteria with whey from heifers immunized with *S. aureus* lysate, *S. aureus* lysate + recombinant antigens, or PBS (as control group). Data are expressed as percentage of controls (100%). Bars represent the mean of eight individual whey samples per group, run in triplicate. Error bars represent the standard deviation of the mean (S.D.M.). $^{*}p < 0.05$ *vs* control group.

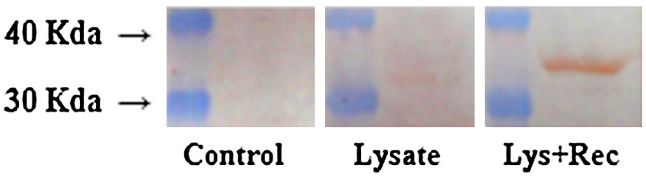

Fig. 4. Western blot. Native β-toxin recognition by pools of sera obtained at 7 days post-calving from heifers immunized with *S. aureus* lysate, *S. aureus* lysate + recombinant antigens, or control.

3.3. *In vitro functional analysis of immune serum and whey*

3.3.1. *Neutrophil phagocytosis*

Sera from animals in Lys or Lys + Rec groups augmented significantly the percentage of bacteria-containing neutrophils (38.1% ± 9.7% and 40.3% ± 6.2%; respectively) compared with sera from control animals (13.1% ± 1.8%; $p < 0.05$) (Fig. 3A). The MFI parameter was also significantly higher when sera from Lys or Lys + Rec groups were used for phagocytosis assay (200 ± 15 and 199 ± 14, respectively), compared with that of heifers in the control group (148 ± 8; $p < 0.05$) (Fig. 3B).

3.3.2. *Internalization by MAC-T cells*

Whey from Lys and Lys + Rec vaccinated groups inhibited internalization of *S. aureus* into bovine mammary epithelial cells (29% ± 10% and 15% ± 6% of internalization, respectively) compared with PBS-treated controls ($p < 0.05$), whereas no significant differences were observed between both vaccinated groups (Fig. 3C).

3.3.3. *Native β-toxin inhibition assays*

The extract containing *n*β-toxin partially purified from *S. aureus* culture supernatant showed a haemolysis titer of 1/2187 HU/ml. Western blot assays were performed in order to confirm the ability of antibodies generated by immunization of heifers with recombinant β-toxin to recognize the native antigen (*n*β-toxin). Only pooled sera obtained at 7 days post-calving from heifers in Lys + Rec group were able to recognize a 35 kDa band corresponding to *n*β-toxin (Fig. 4). In addition, sera obtained at 7 days post-calving from animals in Lys + Rec group showed higher *n*β-toxin inhibition titers in hemolytic activity assays with sheep RBC than sera from animals in Lys or control groups ($p < 0.05$) (Table 1).

3.3.4. *Blocking assays*

Table 1 shows the results obtained in the ELISA designed to evaluate capability of antibodies generated by immunization to inhibit *S. aureus* binding to bovine Fb and Fn *in vitro*. Pre-incubation of *S. aureus* with antibodies obtained at 7 days post-calving from animals in Lys + Rec group significantly reduced binding of bacteria to Fb or Fn

Table 1
In vitro functional analysis of antibodies obtained at 7 days post-calving from heifers immunized with *S. aureus* lysate, *S. aureus* lysate + recombinant antigens, or control. Different letters correspond to statistically significant differences ($p < 0.05$) among groups.

	Control	Lys	Lys + Rec
Haemolysis inhibition titer	108 ± 74[a]	320 ± 268[a]	1440 ± 1152[b]
Fb binding (%)	94.0 ± 11.9[a]	57.2 ± 23.1[b]	16.2 ± 7.8[c]
Fn binding (%)	114.0 ± 6.37[b]	129.5 ± 6.1[a]	26.7 ± 11.2[c]

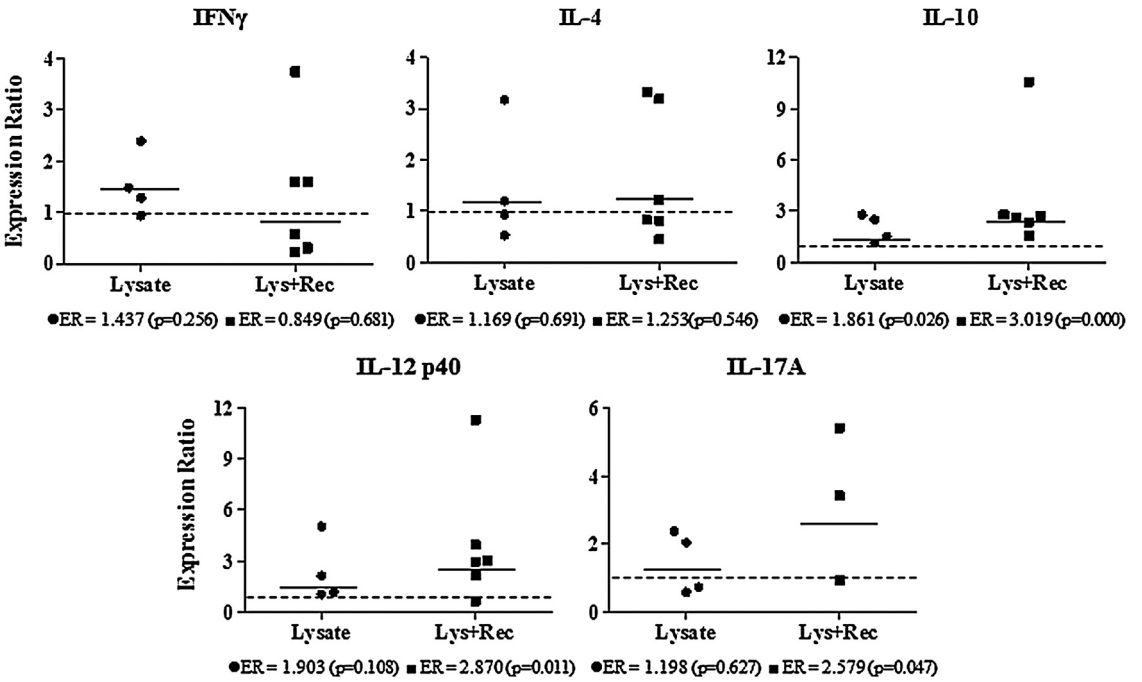

Fig. 5. Cytokine expression levels relative to the control group (Expression ratio, ER) in blood observed *in vivo* 24 h after the second dose of vaccine, in animals immunized with *S. aureus* lysate or *S. aureus* lysate + recombinant antigens. Median ER values are represented as horizontal bars and shown below each graph. Control group value is represented as dashed line (ER 1).

compared with antibodies from animals in Lys or control group ($p < 0.05$).

3.4. Cytokine expression

Both immunized groups showed significantly higher IL-10 mRNA levels compared with control group 24 h after inoculation with second dose of vaccine (Expression ratio [ER] Lys 1.861, $p < 0.05$; ER Lys + Rec 3.019, $p < 0.05$). Both formulations also induced an increase in IL-12 p40 mRNA levels relative to non-immunized controls, but this difference was statistically significant only for animals in Lys + Rec group (ER 2.870; $p < 0.05$). Only the lysate + recombinant antigens formulation stimulated a significant increment on IL-17 mRNA levels (ER 2.579; $p < 0.05$). Interleukin-4 and IFN-γ expression was not affected by either treatment (Fig. 5).

3.5. Characterization of *S. aureus* isolated from natural IMI

Milk samples for bacteriological culture were obtained monthly during the whole lactation period (10 months). *S. aureus* was isolated from the front right quarter of an animal belonging to control group at 2 months post-calving and was recovered from the same quarter until the end of lactation. The presence of *cap*5k and *cap*8l loci was not detected in this isolate. One heifer from Lys group showed *S. aureus* IMI in the rear right and left quarters and the front right quarter at 2, 5 and 6 months post-calving, respectively. These quarters remained infected until the end of lactation. This isolate was genotyped as CP5. Isolates from heifers of control and Lys group expressed different PFGE patterns; while within groups, the isolates obtained more than once from the same quarter or cow expressed identical PFGE, confirming establishment of IMI.

4. Discussion

There is scarce information about the immune response to bacterial lysates and defined antigens formulated with ISCOMs for vaccination against *S. aureus* bovine mastitis (Camussone et al., 2014; Nelson et al., 1991). In the present study, administration of two doses of both Lysate and Lysate + Recombinant antigens formulations elicited strong IgG and IgG_2 responses in blood and IgG in milk, in comparison with control animals. However, no specific IgG_2 was found in whey of heifers included in the study. In previous reports, no detectable amounts of IgG_2 were produced in whey after 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) or subcutaneous administration in the supramammary lymph node area of a formalin inactivated or lysate *S. aureus* CP5 formulated with Iscom Matrix (Camussone et al., 2013, 2014). These findings are in accordance with the low IgG_2 levels found in milk from healthy quarters (Caffin and Poutrel, 1988). However, IgG_2 becomes the dominant antibody subclass in milk 4–12 h post-bacterial infection of the mammary gland due to massive transport from blood into milk during inflammation prior to the peak neutrophil response (Sordillo and Streicher, 2002), thus enhancing phagocytic capacity and bacterial clearance in the infected gland (Burton and Erskine, 2003; Paape et al., 2003). The

potential passage of this subclass into milk in immunized animals as well as its contribution to *S. aureus* IMI clearance still needs to be evaluated following experimental challenge.

Only heifers in Lys + Rec group developed significant levels of IgG specific for rβ-toxin, rClfA and rFnBPA in blood and milk. Antibody levels were similar for the three recombinant antigens, suggesting a balanced host response against each component of the formulation. In a previous study where heifers received two doses of plasmids encoding *S. aureus* Clf and FnBP, followed by a booster with the recombinant antigens formulated with incomplete Freund's adjuvant, IgG responses against Clf were at least five times higher than the ones directed to FnBP (Shkreta et al., 2004). In addition, IgG production following mice intranasal immunization with two doses of a plasmid mixture coding for four *S. aureus* adhesins was not equally efficient against every antigen. Authors suggested that this biased response might have depended on a difference in the immunodominance of expressed antigens and its ability to interact with antigen presenting cells (Castagliuolo et al., 2006). It has been proposed that DNA vaccines are often less effective in large animals than in mice, probably due to a transfection deficiency and a low level of expression elicited by plasmid vectors (Babiuk et al., 2003). Results in the present study suggest that administration of two doses of recombinant antigens formulated with an appropriate adjuvant would be more effective than DNA vaccines for the stimulation of an adequate humoral response in dairy cattle. Lastly, in accordance with previous findings (O'Brien et al., 2001; Camussone et al., 2014), both formulations elicited high levels of anti-CP5 antibodies in blood, confirming that enzymatic lysis of *S. aureus* cells does not affect CP structure.

Functionality of blood and milk antibodies generated through vaccination was evaluated *in vitro* in phagocytosis assays with bovine PMN and internalization assays into mammary epithelial cells, respectively; using experimental models representing early interactions of the infectious process. Antibodies generated by both immunogens enhanced *S. aureus* phagocytosis by PMN, through an increment in the PMN that internalized bacteria 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. This similarity in the opsonization efficiency induced by both formulations can be explained by the analogous anti-CP5 IgG levels detected in animals from both vaccinated groups, agreeing with previous observations that only antibodies directed to CP were able to increase *S. aureus* phagocytosis by bovine PMN (Guidry et al., 1991, 1994). *S. aureus* ClfA anti-phagocytic properties, including formation of aggregates as a result of interaction with plasma Fb (Higgins et al., 2006) and inactivation of complement cascade (Hair et al., 2010) have been reported. However, since these mechanisms are not explored in the PMN phagocytosis assays performed in the present study, the potential contribution of antibodies anti-ClfA in sera of animals from Lys + Rec group cannot be determined. Mammary epithelial cell invasion was evaluated to determine the effect of antibodies raised by vaccination on adherence and internalization of *S. aureus* in mammary epithelial cells. Pre-incubation of *S. aureus* Reynolds with whey from animals in Lys or Lys + Rec groups significantly reduced the number of bacteria internalized by MAC-T cells, in comparison with whey from control animals. In a previous study, whey from heifers immunized with a DNA vaccine coding *S. aureus* Clf and FnBP, followed by a booster with the recombinant antigens, was not able to significantly inhibit binding of *S. aureus* to MAC-T cells compared with their respective pre-immune whey samples (Shkreta et al., 2004). More recently, it was demonstrated that milk from cows immunized with a plasmid containing the coding sequence for ClfA followed by a protein boost decreased *S. aureus* adherence to MAC-T cells in a concentration-dependent manner, compared with milk from non-immunized animals (Nour El-Din et al., 2006). In the present study, the higher antibody titer specific for the recombinant adhesion molecules in whey from heifers in Lys + Rec group, was not associated with significant *S. aureus* internalization reduction compared with whey from Lys group, although a tendency to a higher inhibition of bacterial internalization for whey in Lys + Rec group was observed. It has to be taken into account that even though the FnBP plays a major role in *S. aureus* adhesion/internalization into epithelial cells, other *S. aureus* molecules involved in cell adhesion that can function in concert with the former to promote cell invasion have been identified (Sinha and Fraunholz, 2010; Foster et al., 2014)).

There is scarce information about the use of recombinant *S. aureus* antigens in the formulation of vaccines directed to prevent *S. aureus* mastitis in cattle (Pereira et al., 2011). Previous reports demonstrated that a recombinant β-toxin expressed in *E. coli* cells had biological activity against sheep RBC (Huseby et al., 2007). However, this molecule was not evaluated as immunogen for *S. aureus* mastitis in cattle. Therefore, to the best of our knowledge, the ability of antibodies specific for this recombinant toxin to recognize and block native β-toxin haemolytic activity was evaluated for the first time in the present study. Sera from heifers in Lys + Rec group significantly inhibited sheep RBC haemolysis by *n*β-toxin *in vitro* confirming its potential value as an immunogen. Although toxoids (Watson, 1992; Nickerson et al., 1993; Nordhaug et al., 1994a,b; Hwang et al., 2000) and exosecretion material (Leitner et al., 2003a,b) have been included in vaccines to immunize cattle against *S. aureus* mastitis, these components were used as crude extracts and therefore not completely characterized. In a field trial, where pregnant heifers were immunized with inactivated *S. aureus* bacteria with pseudocapsule and α and β toxoids, antibodies specific to partially purified β-toxin were only detected in serum, whereas no differences in antibody levels were found between vaccinated and control animals in milk (Nordhaug et al., 1994b). Recombinant peptides containing the Fn binding domains D1D2D3 of FnBPA and the Fb binding Region A of ClfA, were obtained in the present study based on previous results showing the antigenicity of these molecules in cattle (Nelson et al., 1991; Nour El-Din et al., 2006). Antibodies from heifers in Lys + Rec group significantly reduced bacterial binding to Fn or Fb *in vitro*. These

results suggest that immunization with peptides containing the active domains of these *S. aureus* adhesins would induce antibodies able to block the organism binding to their target molecules. However, contribution of these antibodies to the inhibition of early host–*S. aureus* interactions conducive to IMI clearance needs to be evaluated in experimental *in vivo* models.

It was proposed that vaccines that solely stimulate humoral immunity will probably not be sufficient to prevent new *S. aureus* IMI (Middleton, 2008). Establishment of chronic *S. aureus* infection in the mammary gland may only be prevented through vaccination if both humoral and cell-mediated immune responses against the pathogen are stimulated (Gómez et al., 2002). In the present study, innate and T helper activation was indirectly evaluated through the assessment of IL-4, IL-10, IL-12 p-40, IL-17A and IFN-γ mRNA relative levels (Zhu et al., 2010) in PBMC 24 h following the administration of the second dose of vaccines. In accordance with recent observations in our laboratory with Iscom Matrix formulations, both immunogens up-regulated the expression of IL-10 and IL-12 p40 relative to controls (Camussone et al., 2013, 2014). Interleukin-10 is a regulatory cytokine that acts limiting inflammation and influencing the nature of the adaptive immune response to infection. Recent studies (reviewed by Kubo and Motomura, 2012) demonstrated that IL-10 can be expressed by many cells of both adaptive and innate immunity, like dendritic cells, macrophages and natural killer cells. Plasticity of IL-10 production by T cells is not only the result of cytokine microenvironment, but also of sustained antigen presentation (Kubo and Motomura, 2012), which is one of the main properties of Iscom Matrix mechanism of action (Morelli et al., 2012). IL-12 appears to be essential for the immunogenicity of ISCOMs. Early studies suggested that ISCOMS prime antigen-specific immune responses activating, at least in part, IL-12-dependent aspects of the innate immune system (Robson et al., 2003; Smith et al., 1999). This cytokine bridges the innate and adaptive arms of the immune system and promotes cellular immunity stimulating IFN-γ production by cells involved in innate immune responses as well as proliferation and cytolytic activity of natural killer and T cells (Del Vecchio et al., 2007). Only animals in Lys + Rec group showed significant increases in IL-17A expression relative to controls. It was suggested that IL-17 can play a protective role in bovine mastitis, since an up-regulation of IL-17 mRNA levels in milk leukocytes from cows suffering from *S. aureus* IMI has been reported (Riollet et al., 2006; Tao and Mallard, 2007). *In vitro* stimulation of mammary epithelial cells with recombinant bovine IL-17, or IL-17 in combination with TNF-α, resulted in up-regulation of a number of genes related to innate immune defences, providing evidence of a functional IL-17A receptor in these cells (Riollet et al., 2006; Bougarn et al., 2011). Although there are no previous reports about cytokine expression kinetics in heifers vaccinated with *S. aureus* formulations and that cytokine expression was evaluated at a single time point in this study, a tendency to a higher stimulation of pro-inflammatory cytokines as IL-12 and IL-17A and a regulatory one as IL-10 was observed in the Lys + Rec group. Complementary assays *in vivo* and *in vitro* are needed to delineate potential mechanisms activated by these formulations.

One of the main goals of *S. aureus* mastitis vaccination is prevention of new IMI in order to reduce contagious spread (Middleton, 2008). In the present study, one heifer in each control and Lys groups, developed *S. aureus* IMI with isolates that showed different capsular genotype and PFGE patterns. In contrast, none of the animals in Lys + Rec group developed *S. aureus* IMI in natural exposure conditions. Evaluation of the vaccine efficacy to prevent *S. aureus* IMI was beyond the scope of this study. Although is tempting to speculate that the multicomponent formulation can exert a protective effect, experimental challenge and field trials are granted to characterize immune response of vaccinated heifers and to evaluate the efficacy of the formulation to prevent new *S. aureus* IMI.

In conclusion, incorporation of defined antigenic molecules to a *S. aureus* lysate formulation adjuvanted with Iscom Matrix strengthened specific antibodies production which was efficient in neutralizing *S. aureus* virulence factors *in vitro* and was associated with expression of regulatory and pro-inflammatory cytokines, placing them as potential candidates to be included in a formulation directed to prevent bovine mastitis.

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