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Immune response of heifers against a *Staphylococcus aureus* CP5 whole cell vaccine formulated with ISCOMATRIX™ adjuvant

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The shortcomings of Staphylococcus aureus vaccines to control bovine mastitis have been attributed to insufficient capacity of the vaccines to induce opsonizing antibodies and to stimulate cellular immune responses. Types of antigen, administration route and adjuvant used in a vaccine formulation have been identified as critical factors for the development of opsonic antibodies. Current commercially available vaccines for Staph. aureus bovine mastitis control are formulated with $Al(OH)_3$ and oil-based adjuvants. The aim of this study was to evaluate the immune response of heifers immunized with a Staph. aureus CP5 whole cell vaccine formulated either with $Al(OH)_3$ or ISCOMATRIX™. Twenty primigravid Holstein dairy heifers in the last trimester of gestation were immunized either with a vaccine formulated with ISCOMATRIX™ ($n=6$), $Al(OH)_3$ ($n=7$), or saline solution (placebo) ($n=7$). Immunization was carried out 38 and 10 d before calving. Heifers vaccinated with Staph. aureus adjuvanted with ISCOMATRIX™ responded with significantly higher levels of anti-bacterin and anti-CP5 IgG and IgG_2 in sera than animals in the $Al(OH)_3$ or control groups. Animals in the ISCOMATRIX™ group responded with significantly higher anti-bacterin specific IgG in whey than animals in the $Al(OH)_3$ and control groups, detected from the first week post calving until 60 d of lactation. Sera from animals inoculated with Staph. aureus in ISCOMATRIX™, obtained 7 d post partum, significantly increased both the number of neutrophils ingesting bacteria and the number of bacteria being ingested by the neutrophils, compared with sera obtained from heifers vaccinated with $Al(OH)_3$ or non-vaccinated controls. These features coupled to safety of the ISCOMATRIX™ formulation, warrant additional studies.

Keywords: Mastitis, *Staphylococcus aureus*, immunization, ISCOMATRIX™.

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections (IMI) worldwide (Zecconi et al. 2006). Control of *Staph. aureus* IMI is based on milking-time hygiene; antibiotic therapy and culling of chronically infected cows (Neave et al. 1969). However, despite the application of these practices, the chronic nature of most *Staph. aureus* IMI and the limited cure rate following antibiotic therapy make this disease difficult to control (Zecconi et al. 2006). Hence, other approaches to control *Staph. aureus* IMI, such as vaccination, have been proposed to complement classical measures. Phagocytosis by polymorphonuclear neutrophils (PMN) is considered the main mammary gland line of defence against invading pathogens (Paape et al. 2003). However, PMN phagocytic activity diminishes owing to reduction of energy reserves during diapedesis across mammary epithelium and ingestion of fat and casein upon entering the mammary gland (Paape et al. 2003). Enhancement of mammary gland defences through vaccination aims to improve PMN phagocytic efficacy by increasing opsonizing antibodies in mammary secretions, especially IgG_2 subclass (O'Brien et al. 2001;

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Tollersrud et al. 2001; Middleton, 2008), and to stimulate cytotoxic responses, able to reach intracellular bacteria (Wallemacq et al. 2012).

Staph. aureus produces extracellular capsular polysaccharides (CP) that 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 *Staph. aureus*, enhancing neutrophil phagocytosis (O'Brien et al. 2001). Capsular polysaccharides 5, 8 and the surface polysaccharide 336 have been identified among *Staph. aureus* isolated from bovine IMI. However, considerable variation between countries in the distribution of isolates expressing these antigens has been reported (Han et al. 2000; Tollersrud et al. 2000; Camussone et al. 2012). The anti-phagocytic characteristics of CP and prospects of stimulating a specific immune response against them, aiming to facilitate clearance of *Staph. aureus* from the mammary gland, turned these components into important targets for vaccine development (Tollersrud et al. 2000; O'Brien et al. 2001). Capsular polysaccharides have been included in different vaccines, such as whole bacteria capsulated strains (Tollersrud et al. 2001; Lee et al. 2005), lysates from capsulated strains (Middleton et al. 2006, 2009) and capsular conjugates (Gilbert et al. 1994; Tollersrud et al. 2001). Among those vaccines, long-standing bacterins showed a certain effect by reducing the incidence of subclinical IMI and severity of clinical mastitis in challenge and field trials; however, none of the formulations evaluated have completely prevented new IMI in dairy cattle (Middleton, 2008; Pereira et al. 2011). The limited efficacy obtained has been attributed, among other factors, to shortcomings of vaccines to induce sufficient opsonizing antibodies (Luby et al. 2007; Middleton et al. 2009) and to stimulate a cellular immune response (Pereira et al. 2011). These observations point up the need for exploring alternatives to improve currently available bacterins.

The adjuvant used in the formulation is a critical factor for the improvement of vaccine efficacy. During the last years, new immune-stimulating complexes like ISCOM, that formulates antigens and adjuvant in one and the same nanoparticle, have been developed (Sjölander et al. 1998; Morein et al. 2004). These are 40-nm nanoparticles composed of saponins, cholesterol and phospholipids, that are kept together through hydrophobic interactions (Sjölander et al. 1998; Morein et al. 2004). These compounds are not only more efficient as vehicles, but are also able to produce a balanced humoural/cellular response, through the induction of multiple innate and adaptive mediators, cellular processes and the interplay between these elements (Pearse & Drane, 2004). Later the ISCOM Matrix was formulated as an adjuvant particle in a separate entity from the antigen with the simplicity just to be added to the antigen solution (Morein et al. 2007b). The diversity of experimental vaccines formulated with ISCOMATRIX™ that have been evaluated up to now and the capability of inducing strong humoural and cellular responses in a variety of animal species has been reviewed recently (Sun et al. 2009). However, these formulations included defined antigens; but there is no experience in the usage of these complexes with whole or lysed bacteria. In addition, it has to be considered that the commercially available vaccine for *Staph. aureus* mastitis most extensively studied (Lysigin®, Boehringer Ingelheim Vetmedica, Inc.) contains whole-cell lysates of polysaccharide serotypes 5, 8 and 336 formulated with $Al(OH)_3$, while a recently marketed vaccine contains a *Staph. aureus* strain expressing slime-associated antigen complex formulated with an oil-based adjuvant (Startvac®, Laboratorio Hipra S.A. authorized by the European Medicines Agency). The aim of this study was to compare the innate and humoural immune responses induced by a *Staph. aureus* CP5 whole cell vaccine formulated either with $Al(OH)_3$ or ISCOMATRIX™ adjuvants, when immunizing pregnant heifers.

Materials and methods

Formulation of vaccines

Staph. aureus CP type 5 strain (Reynolds) (Fournier et al. 1987) 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), inactivated with 0·5% formalin, washed with PBS and adjusted to a final concentration of 1×10^9 cfu/ml. Capsular polysaccharide expression of the vaccine strain was evaluated by CP extraction, purification and detection with anti-CP5 antisera as described previously (Camussone et al. 2012). The vaccines were formulated with 15% $Al(OH)_3$ (Alhydrogel™) or with 2 mg/dose of immune stimulating complexes (ISCOMATRIX™). A placebo consisting of sterile saline solution 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.

Animals and treatments

Twenty primigravid Holstein dairy heifers in the last trimester of gestation belonging to the dairy herd of INTA Rafaela Experiment Station were used in the experiment. Animals were randomly allocated to three groups; ISCOMATRIX™ ($n = 6$), $Al(OH)_3$ ($n = 7$) and control ($n = 7$). To detect statistical differences in 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 7 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 45 and 15 d before the expected calving date. Heifers were bled by puncture of the coccygeal vein before each inoculation, and at 1, 7, 14, 21, 30 and 60 d after calving; blood was allowed to clot and sera were collected via centrifugation. At 14 d before expected calving date udders of all heifers were clinically examined by palpation and samples of pre-partum

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mammary secretion were taken to determine presence of *Staph. aureus* IMI. After parturition, aseptic quarter foremilk samples were collected every week from the first week after calving to 60 d after calving, according to standard procedures (Oliver et al. 2004). An aliquot of each quarter sample was subjected to bacterial culture and composite samples, composed of 500 µl of milk from each quarter, were used for antibody determinations. These samples were centrifuged at 300 ***g*** for 15 min; supernatants were collected and stored at −20 °C until processed. Only animals free of *Staph. aureus* IMI 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).

Bacteriological examination

Mammary secretion samples were cultured for mastitis pathogens according to standard procedures (Oliver et al. 2004). Briefly, 10 µl of mammary secretion was 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. Presence of one colony of *Staph. aureus* on blood agar was considered as a positive identification; therefore, the detection limit was 100 cfu/ml. Heifers yielding a positive culture in any quarter sample along the trial were excluded from the study.

Serological methods

Antibodies (IgG and IgG_2 subclass) against *Staph. aureus* bacterin or purified CP5 were measured by ELISA. IgG_2 was quantified since this subclass is considered the most effective opsonin promoting neutrophil paghocytosis (Burton & Erskine, 2003). Production and purification of CP5 from *Staph. aureus* Reynolds was carried out according to Fattom et al. (1990). Flat-bottomed 96-well microtitre plates were coated with a suspension of CP5 bacterin (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% free from anti-*Staph. aureus* antibodies, then with heifers' 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 0·25 m-H_2SO_4. The absorbance was read at 450 nm. Antibody levels were expressed as optical density (OD). Confirmation of the goat milk as being free of anti-*Staph. aureus* antibodies was performed by ELISA, using the blocking solution (PBS + 5% goat milk) as a sample. OD values for this solution were less than negative controls. This procedure was repeated for every test. OD values obtained for these controls were subtracted in order to reduce plate to plate variation.

Neutrophil phagocytosis

Bovine PMN were obtained from a healthy animal as described previously (Siemsen et al. 2007). A suspension of inactivated *Staph. aureus* Reynolds was labelled with FITC and resuspended in PBS to a final concentration of 1×10^8 cfu/ml. One-hundred µl of bacterial suspension was incubated with pooled sera from each group of animals (O'Brien et al. 2001; Lee et al. 2005) for 30 min at 37 °C with gentle shaking. Then, 100 µl of a 1×10^7 cells/ml suspension of bovine PMN was added to each well and incubated for a further 30 min at 37 °C with gentle shaking. Phagocytosis was stopped by the addition of 0·85%NaCl/0·04% EDTA. 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.

Cytokine expression

Blood samples were collected aseptically 24 h after the second vaccine inoculation by jugular venipuncture and transferred to sterile tubes containing EDTA. Tubes were centrifuged at 1000 ***g*** for 25 min at room temperature. Plasma was removed; the buffy coat was collected and resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was obtained according to the manufacturer's directions. Samples were incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex and 100 µl of chloroform was added. Then, the tube was shaken vigorously by hand for 15 s and incubated for 2–3 min at room temperature. Samples were centrifuged at 12 000 ***g*** for 15 min at 4 °C. The aqueous phase of the sample was removed and transferred into a new tube. Two-hundred-and-fifty µl of 100% isopropanol (Merk) were added to the aqueous phase and incubated at room temperature for 10 min. Samples were centrifuged at 12 000 ***g*** for 10 min at 4 °C. The supernatant was removed from the tube, leaving only the RNA pellet. The pellet was washed with 1 ml of 75% ethanol (Merck) and vortexed for 15 s. Then, the tube was centrifuged at 7500 ***g*** for 5 min at 4 °C. Supernatant was discarded and the RNA pellet was air dried for 5–10 min and then resuspended in 20 µl of RNA-free water (Gibco). Total RNA was quantified using a spectrophotometer (NanoDrop 2000, Thermo Scientific) and samples were stored at −80 °C until

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processed. One microgram of total RNA of each sample was retrotranscribed to cDNA with MMLV reverse transcriptase (Fermentas, Germany). cDNA samples were kept at −20 °C until processed. IL-4, IL-10, IL-12 p-40, IFN-γ and TNF-α mRNA relative expression 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 ul of 2X green fluorochrome PCR master mixture (Real Mix, Biodynamics), 0·5 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, 30 s at 55 °C, 30 s at 72 °C, and a final extension hold of 3 min at 72 °C. Results were analysed 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 (beta actin) (Pfaffl et al. 2002).

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, followed by Duncan's test to compare different groups against control group.

Results

Heifers calved, on average, 38 d after the first dose of vaccine and 10 d after the second dose. Immunization did not cause any adverse reaction at the injection site in either group. Bacteriological analyses were negative for *Staph. aureus* in all animals during the whole study.

Antibody response to bacterin in sera

No *Staph. aureus* antibodies were detected at the beginning of the trial (Fig. 1a, b). After immunization, heifers vaccinated with *Staph. aureus* in ISCOMATRIX™, presented the highest levels of anti-bacterin IgG in sera (Fig. 1a). These levels increased after the second vaccine dose and remained significantly higher than those in the *Staph. aureus* in $Al(OH)_3$ or control groups, during the whole study ($P<0·05$). Remnant antibodies at 60 d post calving were almost 50% of the maximum level at day 7 and six-times higher than pre-immune sera. Animals vaccinated with *Staph. aureus* in $Al(OH)_3$ only showed significantly higher IgG titres with respect to the control group at day 7 post calving ($P<0·05$). Regarding IgG_2 subclass specific against bacterin in sera, antibody titres in the ISCOMATRIX™ group were significantly higher than those in the control and $Al(OH)_3$ groups ($P<0·05$) after the second vaccine dose until the end of the trial (Fig. 1b). IgG_2 titres in heifers vaccinated with *Staph. aureus* in $Al(OH)_3$ were significantly higher than those in the control group only at days 7, 30 and 60 post calving ($P<0·05$).

Antibody response to CP5 in sera

Prior to vaccination, no antibodies against CP5 were detected in any of the animals included in the study (Fig. 1c, d). IgG titres to CP5 in sera of animals vaccinated with *Staph. aureus* in ISCOMATRIX™ were the highest among the three evaluated groups ($P<0·05$), from the day of calving until the end of the trial, while serum IgG levels of animals in the $Al(OH)_3$ group were significantly higher than the ones in the control group from weeks 1 to 4 after calving ($P<0·05$) (Fig. 1c). IgG_2 serum titres to CP5 of animals in the ISCOMATRIX™ group were significantly higher than titres in the other two evaluated groups ($P<0·05$); except for day 7 post calving, where no significant differences were found between titres of the ISCOMATRIX™ and $Al(OH)_3$ group. Significant differences ($P<0·05$) in IgG_2 levels between $Al(OH)_3$ group and control group were found at 7 d post calving (Fig. 1d).

Antibody responses in milk

Anti-bacterin specific IgG in whey of animals in the ISCOMATRIX™ group showed significantly higher titres than the ones in the control and Al(OH)3 groups, from the first week post calving until the end of the trial ($P<0·05$) (Fig. 1e). No significant differences were found between the antibody levels of $Al(OH)_3$ and control group. Neither anti-bacterin IgG_2 nor anti- CP5 IgG or IgG_2 were detected in whey of any of the three treatment groups.

Neutrophil phagocytosis

Neutrophil phagocytosis of opsonized *Staph. aureus* Reynolds was evaluated with pooled sera from day 7 post calving, since this was the period in which the highest specific antibody levels were detected. Based on light scatter properties, Region 1 (R1) was defined to further analyse bacterial incorporation. Sera from animals vaccinated with *Staph. aureus* in ISCOMATRIX™ augmented the percentage of bacteria-containing neutrophils (71·43%), compared with sera from animals vaccinated with *Staph. aureus* in $Al(OH)_3$ or placebo, that yielded 59·40 and 42·89%, respectively. The MFI parameter was also higher when pooled sera from the *Staph. aureus* ISCOMATRIX™ vaccinated group were used for phagocytosis assay (1711), compared with that of heifers vaccinated with $Al(OH)_3$ (1426) and placebo (227) group (Fig. 2).

Cytokine expression

Transcripts for IL-4, IL-10, IL-12 p-40, IFN-γ and TNF-α were detected in all animals (Fig. 3). Quantitative analysis showed significantly increased IL-10 levels in the ISCOMATRIX™

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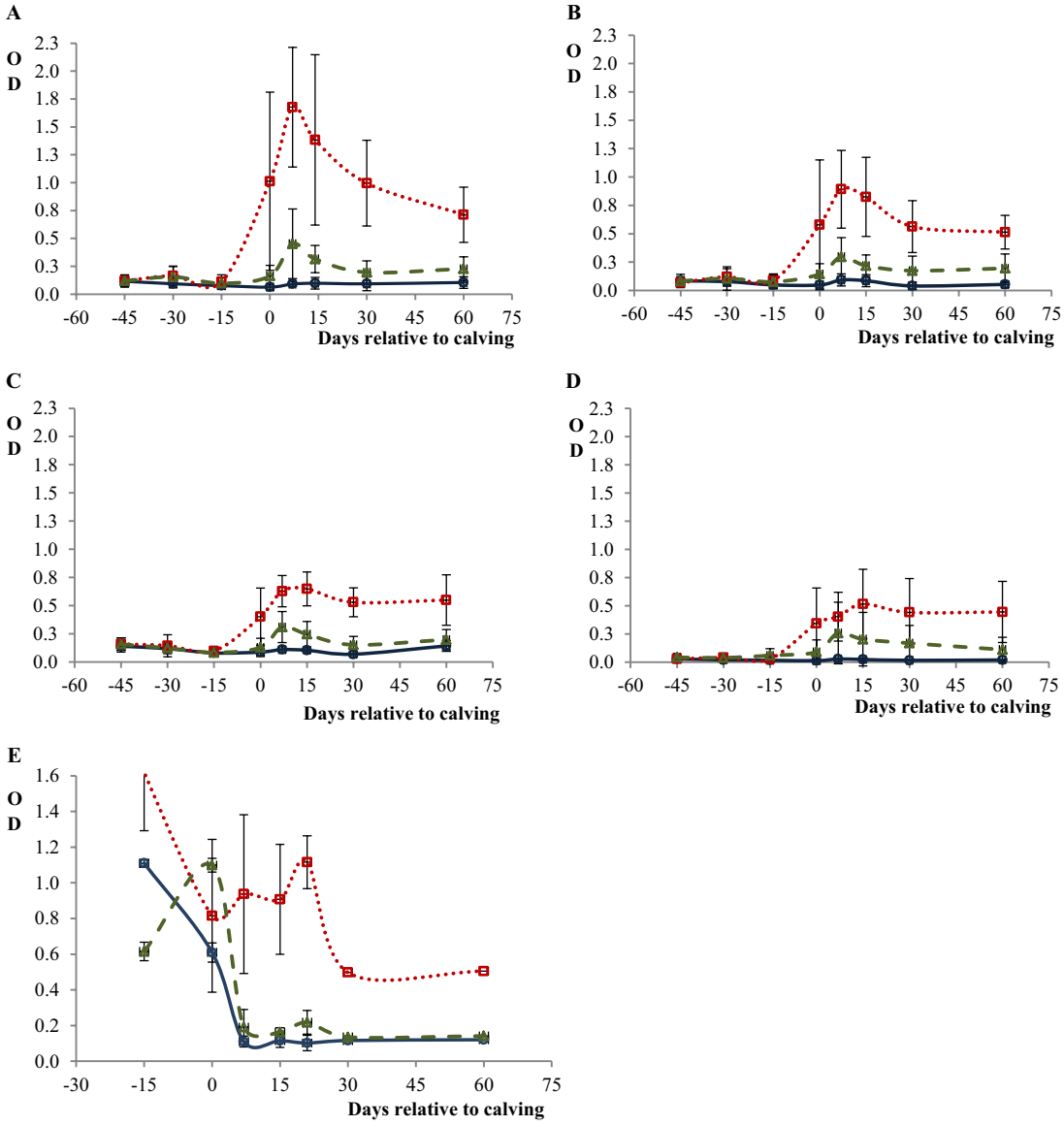

Fig. 1. Antibody levels in pregnant heifers immunized with *Staph. aureus* in ISCOMATRIX™ (. . .), *Staph. aureus* in $Al(OH)_3$ (- - -) and control (—). (a and b), serum mean IgG and IgG_2 anti-bacterin, respectively; (c and d), serum mean IgG and IgG_2 anti-CP5, respectively; (e), whey mean IgG anti-bacterin.

group relative to controls (Expression Ratio 4·011; $P<0·05$). However, this difference was not observed for the $Al(OH)_3$ group. Even when no significant differences were found in IL-12 expression for any of the vaccinated groups relative to controls, high levels of IL-12 were found in 3 out of 6 animals from the ISCOMATRIX™ group, but in only one animal in the $Al(OH)_3$ group. The ISCOMATRIX™ formulation induced an increase of TNF-α in 2 heifers and of IFN-γ in 1 heifer, while immunization with $Al(OH)_3$ was associated with high levels of these cytokines in 1 and 2 heifers, respectively. No significant differences were observed in either vaccinated group with respect to the controls for any of these cytokines. IL-4 expression was not affected by either treatment.

Discussion

In this study, we compared the immune response in pregnant heifers to a *Staph. aureus* CP5 whole cell vaccine formulated with the adjuvant $Al(OH)_3$ or with the new generation adjuvant ISCOMATRIX™. There is little information available about the use of ISCOMs for vaccination against *Staph. aureus* bovine mastitis. In a preliminary study, Nelson et al. (1991) immunized cows with a fusion fibronectin binding protein (FnBP) formulated with ISCOMs, resulting in protection against mastitis following experimental challenge compared with cows in a control group. In addition, ISCOM Matrix was added to the FnBP antigen (US/PCT/SE2006/

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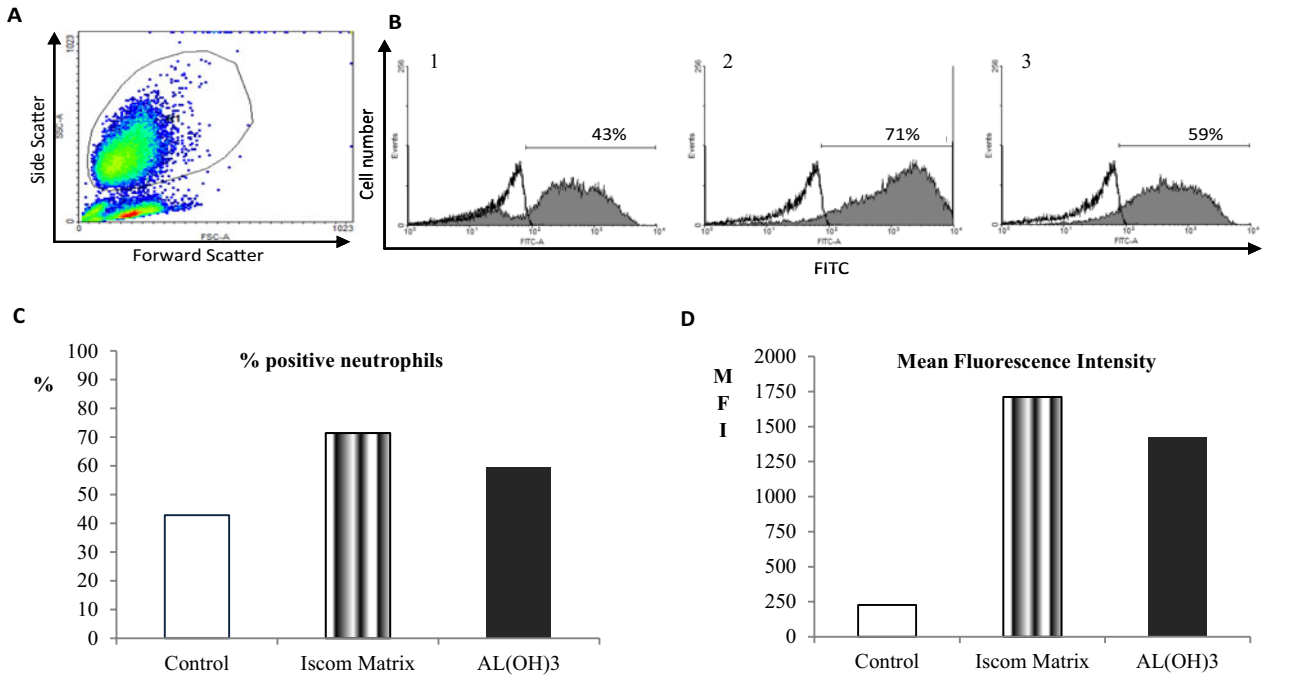

Fig. 2. Neutrophil phagocytosis of FITC positive *Staph. aureus* Reynolds opsonized measured by flow cytometry. (a) PMN population selected on the basis of forward and side light scatter. (b) Representative histograms showing fluorescence intensity for control PMN incubated with HBSS (empty) or PMN incubated with FITC positive *Staph. aureus* Reynolds opsonized with pooled sera from ISCOMATRIX™ (2) $Al(OH)_3$ (3) or control (1) groups (grey filled). Percentages of FITC+ PMN (c) or MFI (d) are shown.

000082) generating an immune response against FnBP peptide both in serum and whey of primiparous heifers that lasted over the lactation period (Morein et al. 2007b). Moreover, the gravid heifers responded with potent serum antibody responses against ISCOM Matrix adjuvanted α and β *Staph. aureus* haemolysins (Morein et al. 2007b, US/PCT/SE2006/000082). The present study is the first report of the use of this new generation adjuvant for the formulation of a *Staph. aureus* whole cell vaccine.

Two vaccine doses were administered before calving to reach high antibody levels during the first month post calving, since there is an increased susceptibility to IMI in this period (Sordillo & Streicher, 2002; Burton & Erskine, 2003). Antibody kinetics in sera were similar in both vaccinated groups showing a peak level during the first and second week after calving for whole cell and CP5 antibodies, respectively. However, the vaccine formulated with ISCOMATRIX™ induced total IgG and IgG_2 responses in sera against whole bacteria and CP5 significantly higher than those obtained with $Al(OH)_3$, maintaining these differences until the end of the observation period. Immunization of cows with a trivalent *Staph. aureus* whole cell vaccine formulated with Freund incomplete adjuvant or $Al(OH)_3$ increased IgG_2 responses against CP8 and CP5, but this effect only lasted for 14 d after calving (Lee et al. 2005).

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 (Sordillo & Streicher, 2002; Paape et al. 2003). Although IgG_1 is the predominant antibody subclass in normal bovine milk, owing to selective transfer from blood into milk, the actual roles of this antibody subclass in immune defence against IMI are ill defined and its concentration diminishes during inflammation (Burton & Erskine, 2003). IgG_2 increases substantially during mammary gland inflammation enhancing phagocytosis, since neutrophils possess specific high-affinity Fc receptors for pathogen-bound IgG_2 (Burton & Erskine, 2003; Paape et al. 2003). The significantly higher levels of IgG_2 in heifers vaccinated with ISCOMATRIX™, in comparison with those in animals immunized with $Al(OH)_3$, agrees with previous observations about ISCOMS up-regulation of antigen-specific production of different IgG subclasses (Morein & Bengtsson, 1999; Morein et al. 2007a). Although particulate antigen presentation by ISCOMS has been extensively studied (Sun et al. 2009), the mechanisms involved in presentation and adjuvant effect of a whole cell vaccine formulated with this immunostimulant complex have not been explored.

Regarding detection of specific antibodies in milk, heifers that received whole cell vaccine formulated with ISCOMATRIX™ showed significantly higher levels of IgG against whole cells than those receiving whole cell vaccine formulated with $Al(OH)_3$ or placebo. However, these antibodies were IgG class while IgG_2 against whole cells or CP5 were not detected. These results agree with previous findings, since neither intramammary vaccination with heat-killed *Staph. aureus* CP5 and CP8 (Barrio et al. 2003),

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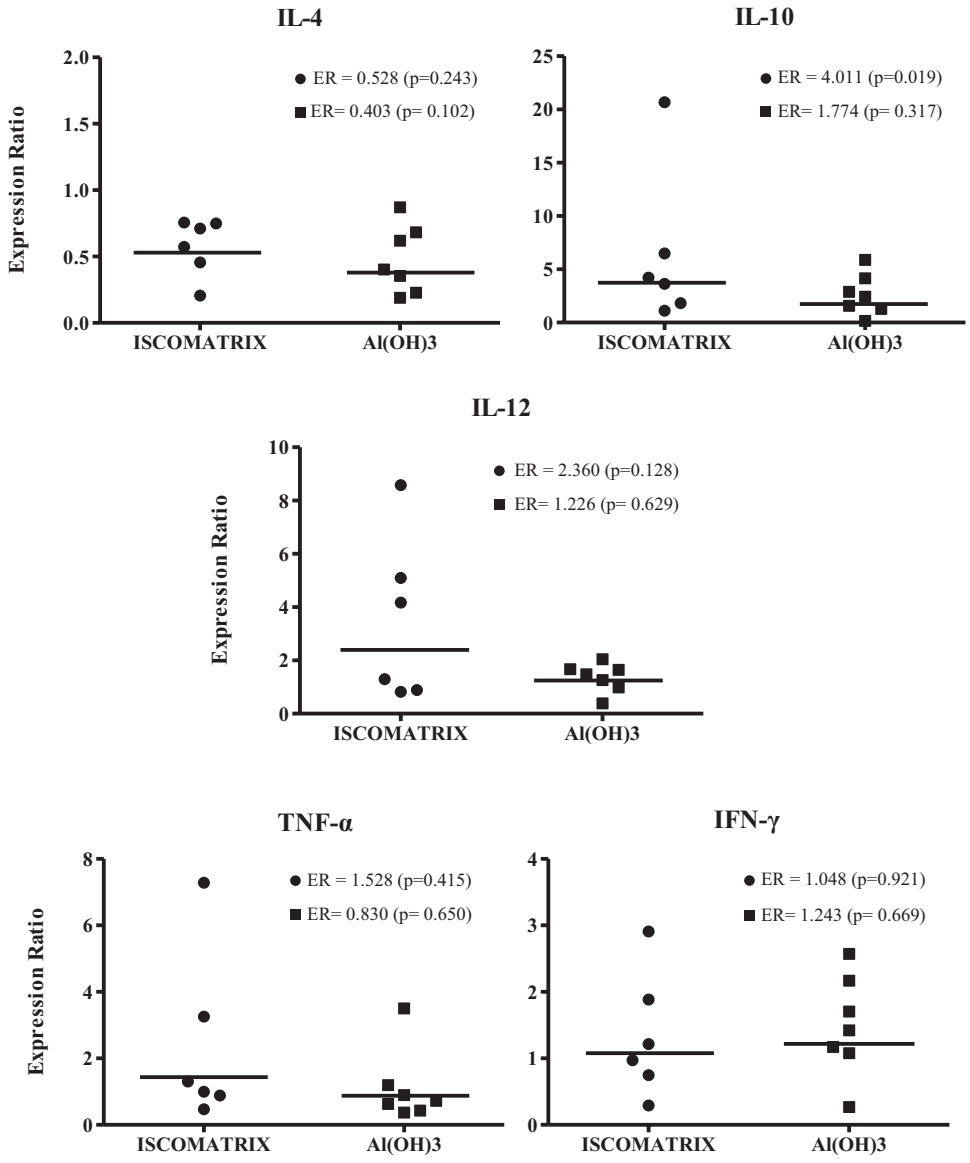

Fig. 3. Cytokine mRNA expression levels relative to the control group [Expression ratio (ER) observed in vivo 24 h after the second dose of vaccine] in animals immunized with *Staph. aureus* in ISCOMATRIX™ or with *Staph. aureus* in $AL(OH)_3$. Median ER values are represented as horizontal bars and shown in the top right of each graph.

nor subcutaneous administration of a commercial vaccine containing *Staph. aureus* CP5, CP8 and 336 (Luby et al. 2007; Middleton et al. 2009), produced detectable amounts of IgG_2 in milk. Although lack of detection of IgG_2 suggests that it may not be present in sufficient concentration in milk during early interactions between *Staph. aureus* and host defences, this subclass increases substantially during mammary gland inflammation owing to massive leakage of serum into milk and binds to the Fcγ2 receptors of recruited neutrophils (Zhang et al. 1995; Sordillo & Streicher, 2002). Considering that significantly higher levels of IgG_2 were detected in serum against whole bacteria and CP5 in the ISCOMATRIX™ vaccinated group, serum IgG_2 in the presence of migrating neutrophils should result in the stimulation of a specific effector response, enhancing phagocytic capacity and bacterial clearance in the infected mammary gland (Burton & Erskine, 2003; Paape et al. 2003). However, this protective effect remains to be proven.

The opsonic capacity of antibodies generated through vaccination was evaluated in vitro by phagocytosis assays with bovine PMN. Serum samples from the first week post partum were used, since they yield the highest antibody titers. Pooled sera from groups of animals vaccinated with different formulations have been used to evaluate the opsonic capacity for bovine neutrophils (O'Brien et al. 2001; Lee et al. 2005), although the behaviour of individual members within the group cannot be analysed. A higher percent of PMN positive for *Staph. aureus*/FITC was

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observed with sera from heifers immunized with whole cells formulated with ISCOMATRIX™. In addition, MFI generated for each group was evaluated as an indirect measure of the amount of bacteria phagocytosed by positive cell (Zetterlund et al. 1998). This factor was higher for the group immunized with vaccine formulated with ISCOMATRIX™, indicating that not only a higher number of PMN could phagocytose bacteria, but also that PMN had an increased capacity to ingest bacteria. This augmented activity can be attributed to presence of a higher concentration of specific antibodies against CP5, since previous research showed that only antibodies against CP were able to increase phagocytic activity by bovine PMN (Guidry et al. 1991, 1994). In previous studies, using a lysate incorporated to microspheres (O'Brien et al. 2001) or an avirulent mutant vaccine (Pellegrino et al. 2010), IgG_2 blood levels found were not directly related to *Staph. aureus* phagocytosis. Conversely, in the present study we observed a simultaneous increase of opsonic activity and antibody titre. Absence of anti-CP5 IgG and IgG_2 in whey in the present study suggests a reduced opsonic capacity in milk. However, since IgG_2 increases in milk during mammary gland inflammation (Sordillo & Streicher, 2002), a potential passage of this subclass into milk needs to be evaluated following experimental challenge.

Adjuvants not only enhance the levels of immune responses, but have immunomodulatory properties that can influence the type and character of induced immune responses (Morein et al. 1996, 2004). In this study, innate and T helper activation were evaluated through the assessment of mRNA relative expression levels of TNF-α, IL-12 p-40, IFN-ϒ, IL-4, and IL-10 (Zhu et al. 2010; Murphy, 2011), 24 h after the administration of the second dose of vaccines. Findings were in agreement with earlier experience of the ISCOM formulations (Morein et al. 2004, 2007b). Heifers immunized with *Staph. aureus* in ISCOMATRIX™ showed, on average, higher levels of IL-12, and IL-10 with respect to controls, compared with those receiving the vaccine in $Al(OH)_3$. IL-12 plays a central role in the mechanism of action of ISCOMs. These complexes can stimulate dendritic cells either by their particulate nature o by the presence of molecules, like Quil A, enhancing expression of IL-12 and type I-IFN (Robson et al. 2003). These authors and others (Smith et al. 1999) also demonstrated that immunogenicity of ISCOMs relies on an IL-12-dependent cascade of innate immune responses. Regarding IL-10, an increase of this cytokine was described in a mouse model following immunization against *Leishnmania* using ISCOMs (Papadopoulou et al. 1998). However, it has to be taken into account that there are no previous data about kinetics of cytokine expression after immunization for *Staph. 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 in a pro-inflammatory cytokine as IL-12 and a regulatory one as IL-10 in heifers vaccinated with ISCOMATRIX™ was observed. Complementary assays in vitro, evaluating cytokine production kinetics should be carried out to delineate potential mechanisms activated by these formulations.

In conclusion, immunization with a whole cell vaccine formulated with ISCOMATRIX™ strengthened humoural response, through production of specific IgG in blood and milk, and a significant increase in IgG_2 serum titres, which improved opsonic capacity compared with a vaccine formulated with a classical adjuvant. These features, coupled to safety of the formulation, warrant additional studies. Experimental challenge with homologous and heterologous strains, as well as field trials will be needed to explore the protective effect of such vaccines.

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