Liposomal and CpG-ODN formulation elicits strong humoral immune responses to recombinant *Staphylococcus aureus* antigens in heifer calves

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ABSTRACT

Bovine mastitis caused by *Staphylococcus aureus* is a serious problem in dairy production and effective immunoprophylaxis is an unmet goal so far. The objective of this work was to assess the humoral immune response of heifer calves against two recombinant *S. aureus* antigens: Clumping factor A (ClfA) and Fibronectin Binding Protein A (FnBPA), formulated with a novel adjuvant based on cationic liposomes (Lip) and CpG oligodeoxynucleotides (CpG-ODN). Six groups of 6–8 months old heifer calves received three doses biweekly of antigens, formulated with $Al(OH)_3$, liposomes, CpG-ODN or Lip + CpG-ODN. Animals also received a fourth dose after a year (day 410) and a booster before calving. The administration of $Al(OH)_3$ + FnBPA/ClfA and Lip + FnBPA/ClfA + CpG-ODN induced the highest specific IgG levels, after the first 3 doses and induced a fast increase of antibodies after the fourth dose. All the formulations stimulated the production of specific IgG1, after the third and fourth dose. Specific IgG2 for both proteins was only stimulated after the fourth dose by Lip + FnBPA/ClfA + CpG-ODN. Pre-calving immunisation with Lip + FnBPA/ClfA + CpG-ODN led to the highest IgG levels during the calving period and to the production of the IgG2 subclass. The formulation was also able to stimulate the highest antibody levels in milk, 30 and 45 days after pre-calving booster. The combination of liposomes and CpG-ODN as adjuvant for a subunit vaccine, together with the immunisation schedule described, induced a strong humoral immune response with production of specific IgG2. The formulation demonstrated to induce immune memory allowing the application of a single pre-calving booster to maintain high antibody levels throughout the period of increased susceptibility to intramammary infections.

1. Introduction

Bovine mastitis is the disease of dairy cattle that causes the greatest economic losses to dairy farmers and industry worldwide (Petrovski et al., 2006). *Staphylococcus aureus* is the major pathogen most frequently isolated from bovine intramammary infections (IMI) over the world (Zecconi et al., 2006). Current control methods directed to control *S. aureus* IMI are based on hygiene during milking time and antibiotic therapy. However, the particular pathogenic characteristics of this organism usually determine the lack of effectiveness of traditional preventive and therapeutic methods, tending to produce chronic infections that may cause permanent damage to the mammary tissue

(Zhao and Lacasse, 2008). In addition, *S. aureus* causes IMI in heifer calves and heifers before its first calving (Calvinho et al., 2007; Oliver et al., 2005), which can compromise mammary gland development, affect milk production after calving and shorten the productive life of the animal (Piepers et al., 2009). Therefore, complementary actions directed to reduce the prevalence of *S. aureus* IMI from early stages of the animal's life have been encouraged (Oliver et al., 2005; Zecconi et al., 2006). Among them, the use of non-antibiotic approaches strengthening the host immune mechanisms through vaccination has been proposed (Rainard et al., 2018). However, although several vaccines against *S. aureus* mastitis have been evaluated (Pereira et al., 2011), only a few based on lysates or inactivated organisms are

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commercially available worldwide and have shown limited efficacy for preventing IMI (Middleton et al., 2009; Schukken et al., 2014).

There is a need for exploring new alternatives for immunizing against *S. aureus* both improving the antigens as well as the adjuvants included in the formulations. There is an agreement among authors about the need of combining multiple antigens in vaccines, in order to generate immune responses against molecules intervening in different stages of staphylococcal pathogenesis (Middleton, 2008; Anderson et al., 2012; Scali et al., 2015). However, there are few reports on the use of multicomponent immunogens conformed by defined antigens destined to control *S. aureus* IMI. This pathogen possesses multiple virulence factors, which facilitate immune response evasion and survival within phagocytic cells (Fournier et al., 2008; Camussone and Calvinho, 2013). The ability of *S. aureus* to internalize in phagocytic and epithelial cells is considered one of the key steps of staphylococcal pathogenesis. Despite the presence of numerous adhesins in the microorganism, FnBPs are considered crucial components in early host-pathogen interactions (Fowler et al., 2000), since strains that do not express these molecules, show a significant reduction in the capacity of adhesion and internalization in bovine mammary epithelial cells *in vitro* (Dziewanowska et al., 1999; Pereyra et al., 2016). In addition, ClfA promotes the aggregation of *S. aureus* to host platelets and is also able to interact with complement factor I, affecting the phagocytic capacity as a result of an augmented C3b degradation (Scali et al., 2015). In previous reports by our group, pregnant heifers were inoculated with multicomponent vaccines containing recombinant peptides of the fibronectin binding domains D1D2D3 of FnBPA and the fibrinogen binding region A of ClfA. This immunisation stimulated the production of specific antibodies that significantly reduced bacterial binding to fibronectin or fibrinogen and inhibited bacterial internalization into mammary epithelial cells *in vitro* (Camussone et al., 2014; Pujato et al., 2018). Based on these results, the recombinant proteins FnBPA and ClfA, previously evaluated, were used in the present study as vaccine model antigens, in order to evaluate the performance of a new generation adjuvant based on liposomal formulations, to induce a specific humoral immune response in heifer calves.

In addition, the adjuvant used in the formulation, as well as the route of administration of the immunogen, have been identified as critical factors for increasing the effectiveness of vaccines, through the production of opsonic antibodies and the recruitment of activated neutrophils (Yancey, 1999; Boerhout et al., 2018). Among new generation adjuvants are liposomes, composed of biocompatible phospholipid bilayers. Liposomes can mimic pathogens by transporting large quantities of antigens to antigen presenting cells (APCs) and are able to induce both humoral and cellular immune responses to protein and polysaccharide antigens (Gregoriadis, 1990; Sivakumar et al., 2011). Another advantage of liposomes is that they are capable of loading and delivering hydrophilic and hydrophobic molecules, making possible the co-delivery of antigens and immunostimulatory agents, with the consequent improvement of specific immune responses. Oligodeoxynucleotides with non-methylated CpG motifs (CpG-ODN) are currently under study as immunostimulants. In previous reports, CpG-ODN 2007 was biologically capable of stimulating both innate and adaptive immune responses in different bovine models (Ioannou et al., 2002; Nichani et al., 2004; Pontarollo et al., 2002; Wedlock et al., 2005). However, there are no reports about the use of these molecules in the formulations of experimental bovine mastitis vaccines. Moreover, the CpG-ODN used is commercially available as a sequence containing phosphorothioate bonds (PS CpG-ODN), which renders the internucleotide linkage resistant to nuclease digestion, resulting in a longer *in vivo* half-life than phosphodiester ODNs (PO CpG-ODN) (Mutwiri et al., 2004). However, the cost of production of PS CpG-ODN is higher than PO CpG-ODN, which is a relevant factor to consider in vaccine production, commercialization, and consumer acceptance. Moreover, the long-term use of modified links has been associated with adverse side effects in mice and primates (Shargh et al., 2012). Previous work in

a mouse model showed that the combination of liposomes with CpG-ODN stimulated more powerful immune responses than those obtained with both adjuvants separately with a marked tendency to a Th1 profile (Kim et al., 2011). In recent work from our group using a model antigen, combination of liposomes and CpG-ODN stimulated both humoral immune response with high antibody titers and production of IgG2a, and cellular immune responses with IFN-γ production in mice, lacking adverse effects (Reidel et al., 2017).

Given the liposomes ability to act as vehicle and the immunostimulant effect of CpG-ODN, this study was aimed to obtain a new generation adjuvant through combination of both components, and to evaluate the immune response elicited in heifer calves when used for the formulation of a subunit vaccine, administering multiple doses from 6 to 8 months of age to pre-calving period.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol) and stearylamine (SA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The CpG-ODN used in this study was a 23-mer 5′-tcgtcgtttgtcgttttgtcgtt-3 (Mulongo et al., 2013), synthesized with phosphodiester bonds by Invitrogen (Waltham, MA, USA). Aluminium hydroxide (Alhydrogel™) was a kind gift from Dr. G. Lagioia (Biogénesis Bagó).

Unless specified, chemicals were of analytic grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Formulation of vaccines

The experimental immunogen consisted in 200 μg/dose of each of the recombinant *S. aureus* antigens, Fibronectin Binding Protein A (FnBPA) and Clumping Factor A (ClfA) obtained as previously described with minor protocol modifications (Camussone et al., 2014). Briefly, *Escherichia coli* BL21(DE3) cells bearing the different plasmid constructions pET32a ClfA and pET32a FnBPA, were grown overnight (ON) in LB medium, supplemented with 0.1 mg/ml of ampicillin at 37 °C, with shaking. Protein expression was induced ON with 2.5 mg/ml of lactose. The respective ClfA and FnBPA antigens were purified with a Ni-nitrilotriacetic acid column (GE), as described elsewhere (Aguirre et al., 2006) and purity was analysed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining (Laemmli, 1970).

Vaccines were formulated alternatively with a control adjuvant, aluminium hydroxide (Al(OH)$_3$ + FnBPA/ClfA), or three experimental formulations: Liposomes (Lip + FnBPA/ClfA), CpG-ODN (CpG-ODN + FnBPA/ClfA), or the combination of Lip and CpG-ODN (Lip + FnBPA/ClfA + CpG-ODN). Liposomes were prepared using DPPC:Chol:SA, in 7:2:2 mol/mol ratio and 4 mM as final lipid concentration by Ethanol Injection method (Wagner et al., 2006). CpG-ODN was used at 7.5 nmol/mL concentration.

2.3. Animals and vaccination scheme

Fifty-three heifer calves of 6–8 months old belonging to the dairy herd of INTA Rafaela Experiment Station (Santa Fe, Argentina) were selected. Before the beginning of the trial, the presence of anti-*S. aureus* antibodies was evaluated in sera from all heifer calves through ELISA assay (Nickerson et al., 1999). Forty-four animals free of anti-*S. aureus* antibodies were finally included in the experiment. Animals were randomly allocated to 6 groups; Al(OH)$_3$ (n = 7), Lip + CpG-ODN (n = 7), Al(OH)$_3$ + FnBPA/ClfA (n = 7), Lip + FnBPA/ClfA (n = 8), CpG-ODN + FnBPA/ClfA (n = 7) and Lip + FnBPA/ClfA + CpG-ODN (n = 8). Heifer calves were vaccinated subcutaneously in the neck with

4 doses of 2 ml of vaccine. A three-dose series was administrated at 0, 15 and 45 days after the beginning of immunisation protocol. The fourth dose was administrated after one year (day 410) by the same immunisation route. This scheme was referred to as "four doses scheme". Three weeks approximately before the expected calving date, a booster was administered subcutaneously in the supra-mammary lymph node area. This dose was referred to as "pre-calving booster". At this moment, udders of all heifers were clinically examined by palpation and samples of pre-partum mammary secretion were taken following standard procedures (Oliver et al., 2004) to determine the presence of *S. aureus* IMI at prepartum. Following sample collection, heifers' teats were dipped in a 0.5% iodophor solution.

2.4. Blood sampling

Animals were bled by puncture of the coccygeal vein, every 15 days from day 0 to day 75, 270 days after the first dose and 0, 15 and 60 days after the fourth dose. Four additional samples were collected, every 15 days from day 0 to day 45, after the pre-calving booster. Blood was allowed to clot, sera were collected via centrifugation and stored at −20 °C until processed.

2.5. Milk sampling

After parturition, heifers were milked daily at 12-h intervals. Quarter milk samples were collected aseptically 30 and 45 days after the pre-calving booster (15 and 30 days approximately after calving, respectively), following standard procedures (Oliver et al., 2004). 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 x *g* and 4 °C; whey was collected and stored at −20 °C until processed.

All the procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies (FASS, 2010).

2.6. Serological methods

The antibody responses (IgG, IgG1 and IgG2) to FnBPA and ClfA were determined by indirect ELISA (Camussone et al., 2014). Briefly, 96-well flat bottom plates (Greiner Bio-One, Frickenhausen, Germany) were coated for 2 h at 37 °C with 0.5 μg/well of FnBPA or ClfA in sodium carbonate buffer (pH 9.6). The coated plates were first incubated with 5% (w/v) low-fat goat milk powder dissolved in PBS at pH 7.4, then with animals sera or whey diluted 1/1000 and 1/100, respectively, in 1% (w/v) low-fat goat milk powder dissolved in PBS at pH 7.4, and finally, with horseradish peroxidase conjugate anti-bovine IgG, anti-bovine IgG1, or non-conjugated mouse anti-bovine IgG2 (Sigma-Aldrich) followed by incubation with anti-mouse IgG (Jackson InmunoResearch Inc., West Grove, PA, USA). All incubations were carried out for 1 h at 37 °C. Between each step, plates were washed three times with 0.05% Tween 20 in PBS. Lastly, hydrogen peroxide and tetramethylbenzidine (TMB; Zymed, San Francisco, CA, USA) was added and the colour reaction was stopped by the addition of 2 N H_2SO_4 to the wells. Optical density was measured at 450 nm using a microplate reader (EmaxMicroplate Reader, Molecular Devices, Sunnyvale, CA, USA). Antibody levels were expressed as optical density (OD).

2.7. Blocking assay

Ability of antibodies to block fibrinogen (Fb) binding was tested as previously described (Schennings et al., 1993) with modifications. Briefly, 96-well flat-bottom plates were coated with 5 μg/well of bovine Fb, in sodium carbonate buffer (pH 9.6). Fibrinogen was purified from bovine plasma by ethanol precipitation (Ismail and Ayman, 2012). The remaining sites were blocked with 5% (w/v) low-fat goat milk powder dissolved in PBS at pH 7.4. Sera obtained 15 days after pre-calving booster were diluted 1/200 in a 1×10^9 CFU/ml *S. aureus* Reynolds suspension and incubated for 1 h at 37 °C with gentle shaking. As a positive control, a 1×10^9 CFU/ml *S. aureus* Reynolds suspension was incubated without sera. 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-Aldrich). All incubations were carried out for 1 h at 37 °C. Plates were washed three times with 0.05% Tween 20 in PBS between each step. Lastly, the chromogenic substrate (TMB, Zymed) 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 the percentage of binding with respect to positive control considered as maximum binding ability.

2.8. Statistical analysis

Normal distribution was confirmed by the Kolmogorov-Smirnov test. Statistical differences between groups were analysed by ANOVA test, followed by the Bonferroni test to detect differences between pairs (p < 0.05). Results are reported as mean ± Standard Error of the Mean (SEM) for each group. In case of not normal distribution, Kruskal-Wallis test followed by Mann-Whitney test were applied and results reported as median ± Interquartile Range (IQR) for each group. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad. com).

3. Results

3.1. Antibody response to fourth doses scheme

3.1.1. IgG anti-FnBPA and anti-ClfA levels in serum

Immunoglobulin G levels against FnBPA (Fig. 1-A) and ClfA (Fig. 1-B) in serum were assessed. FnBPA or ClfA antibodies were detected neither in pre-immune sera samples of any animal included in the study, nor in sera from animals in Al(OH)$_3$ and Lip + CpG-ODN groups, during the whole study. Regarding the use of control adjuvant, the co-administration of FnBPA and ClfA with Al(OH)$_3$, induced specific humoral immune responses to both proteins. Levels of IgG in Al(OH)$_3$ + FnBPA/ClfA group were higher than those in Al(OH)$_3$, between days 30 and 75 for FnBPA (p < 0.001) and between days 30 and 60 for ClfA (p < 0.001).

Regarding experimental formulations, animals in Lip + FnBPA/ClfA + CpG-ODN group maintained higher levels of antibodies to both proteins, compared with Lip + CpG-ODN, Lip + FnBPA/ClfA, and CpG-ODN + FnBPA/ClfA groups, from day 30–75 (p < 0.001). No group presented specific antibodies 270 days after the beginning of the study. Finally, Lip + FnBPA/ClfA + CpG-ODN formulation induced similar IgG levels to those obtained with the control adjuvant (Al(OH)$_3$ + FnBPA/ClfA) for both antigens, except for day 45 when anti-ClfA IgG levels were higher in Lip + FnBPA/ClfA + CpG-ODN group (p < 0.01).

A fourth dose was administrated at day 410, and specific IgG levels for each protein were evaluated. After 15 days (day 425), Lip + FnBPA/ClfA + CpG-ODN group was able to generate higher IgG levels against both proteins, compared with Lip + CpG-ODN (p < 0.001) and CpG-ODN + FnBPA/ClfA (p < 0.001) groups. In the same way, IgG levels in Al(OH)$_3$ + FnBPA/ClfA group were higher than those in Al(OH)$_3$ for both FnBPA (p < 0.01) and ClfA (p < 0.05). No significant differences were found between Al(OH)$_3$ + FnBPA/ClfA, Lip + FnBPA/ClfA and Lip + FnBPA/ClfA + CpG-ODN at this time point. At day 470, antibody levels in animals from group Lip + FnBPA/ClfA + CpG-ODN tended to be higher compared with other evaluated groups.
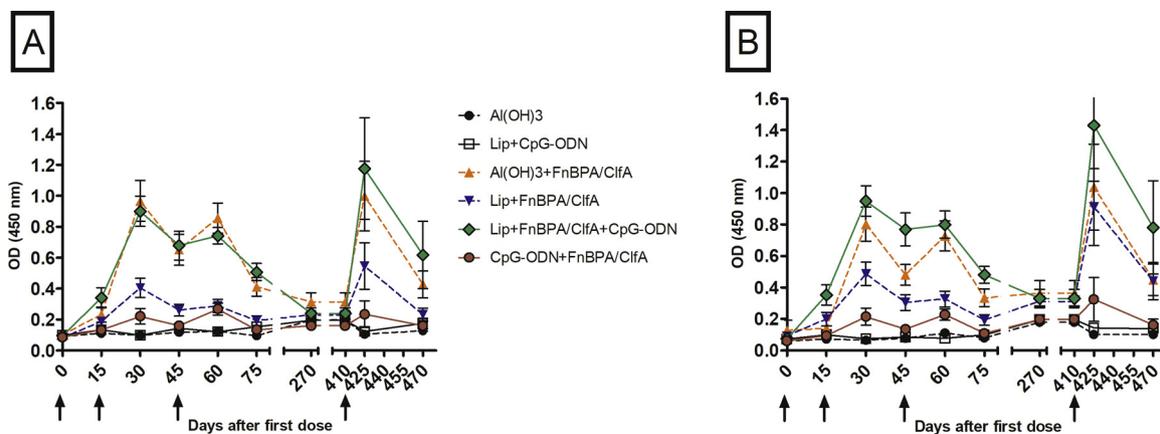
Fig. 1. Specific IgG detection in sera of calves immunised with different formulations containing FnBPA and ClfA, by indirect ELISA, during four doses immunisation scheme. Sera were tested in a 1/1000 dilution against single antigens: A- IgG anti-FnBPA; B- IgG anti-ClfA. Sera from calves immunised with formulations without antigens were used as controls. Optical density (OD) at 450 nm was measured. A Two-Ways ANOVA statistical analysis was done. Mean ± SEM of seven or eight animal sera from each group (according to specifications in *Materials and Methods: Animals and vaccination scheme*) is shown. Arrows indicate immunisation times.

3.1.2. IgG1 and IgG2 anti-FnBPA and anti-ClfA in serum

Levels of IgG1 and IgG2 specific for each protein were determined (Fig. 2), 15 days after the third dose (day 60) and 15 days after the fourth dose (day 425).

Regarding IgG1 production, all formulations were able to increase the specific antibodies levels to both proteins, FnBPA (Fig. 2-A) and

ClfA (Fig. 2-C), at day 60, being $Al(OH)_3$ + FnBPA/ClfA the group with the highest levels. At day 425, the groups Lip + FnBPA/ClfA + CpG-ODN and Lip + FnBPA/ClfA reached similar levels of specific IgG1 compared with $Al(OH)_3$ + FnBPA/ClfA. Moreover, the groups Al $(OH)_3$ + FnBPA/ClfA and Lip + FnBPA/ClfA + CpG-ODN had significantly higher IgG1 levels than those detected in their corresponding
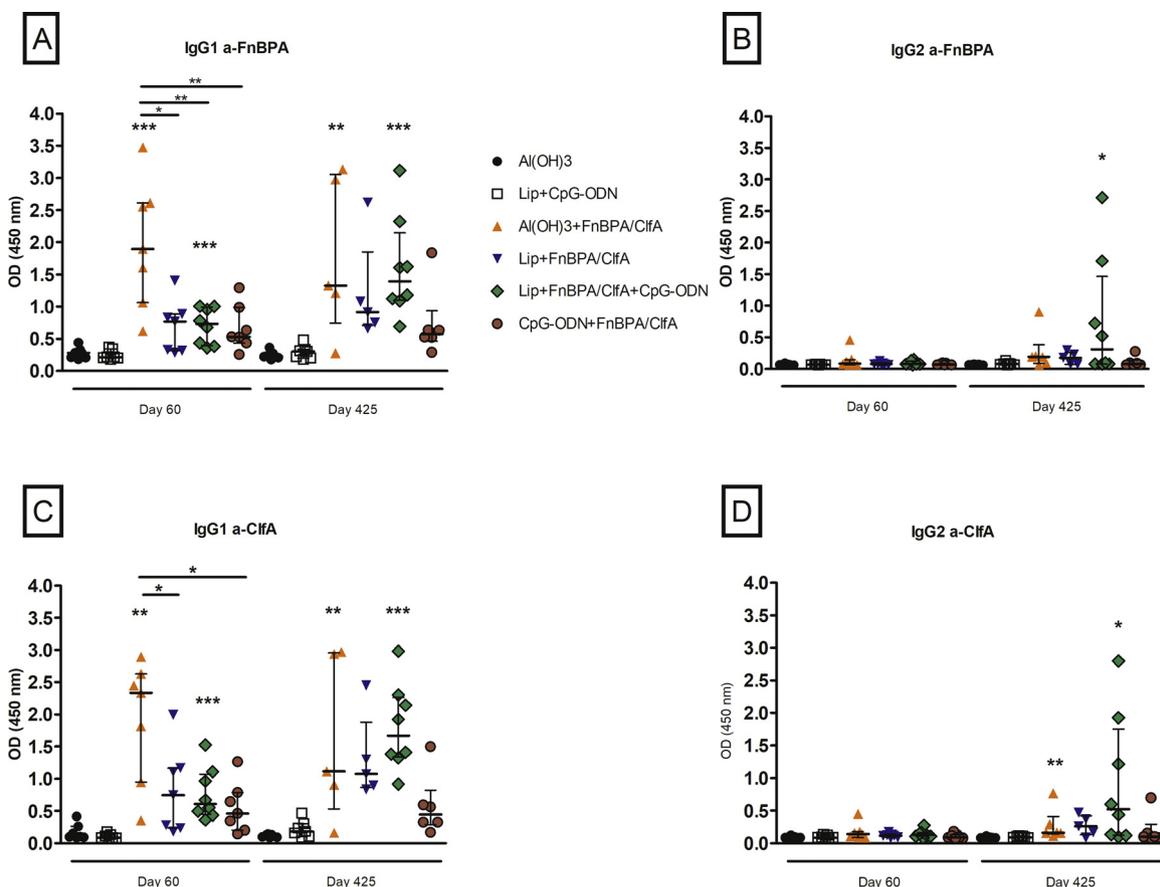

Fig. 2. Specific IgG1 and IgG2 detection in sera of calves immunised with different formulations containing FnBPA and ClfA, by indirect ELISA, evaluated at days 60 and 425 of the immunisation scheme (corresponding to 15 days after third and fourth dose, respectively). Sera were tested in a 1/1000 dilution against single antigens: A- IgG1 anti-FnBPA; B- IgG2 anti-FnBPA; C- IgG1 anti-ClfA; D- IgG2 anti-ClfA. Sera from calves immunised with formulations without antigens were used as controls. OD at 450 nm was measured. A Mann-Whitney statistical analysis was done. Median ± IQR of seven or eight animal sera from each group (according to specifications in *Materials and Methods: Animals and vaccination scheme*) is shown. *p < 0.05; **p < 0.01; ***p < 0.001, compared with control groups. Between-groups comparisons are indicated with lines.
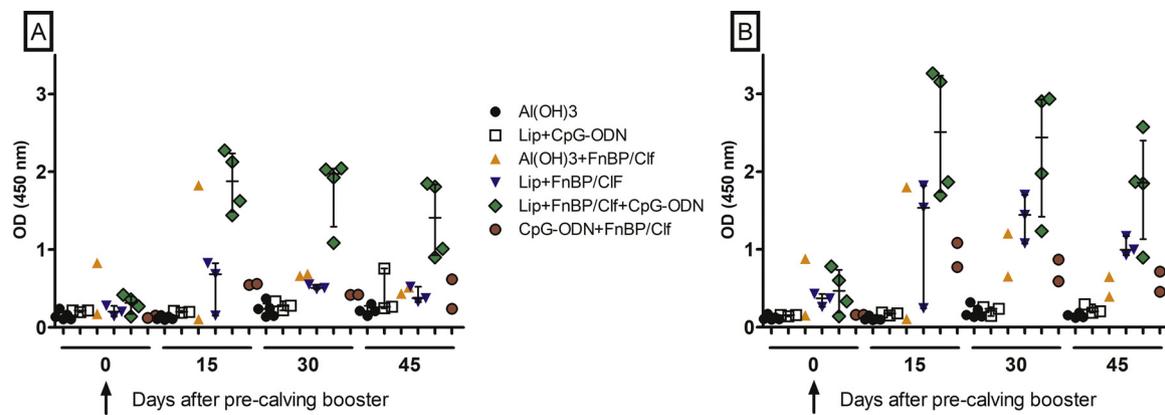
Fig. 3. Specific IgG detection in sera of primiparous heifers after a pre-calving booster with different formulations containing FnBPA and ClfA, by indirect ELISA. The booster was administrated approximately 3 weeks before the expected calving date. Sera were tested in a 1/1000 dilution against single antigens: A- IgG anti-FnBPA; B- IgG anti-ClfA. Sera from calves immunised with formulations without antigens were used as controls. OD at 450 nm was measured. A Mann-Whitney statistical analysis was done to make a comparison between those groups with an $n \geq 3$, at the same time point. Median $\pm$ IQR of three to five animal sera from each group (according to specifications in *Results: Humoral immune response after pre-calving booster. IgG anti-FnBPA and anti-ClfA in serum*) is shown. The arrow indicates pre-calving booster time.

controls, for FnBPA and ClfA, at days 60 and 425. Concerning the production of IgG2 specific for FnBPA (Fig. 2-**B**) or for ClfA (Fig. 2-**D**), no group showed significant differences compared with their controls at day 60. At day 425, Lip + FnBPA/ClfA + CpG-ODN formulation was able to significantly increase the IgG2 levels for both proteins, while, Al $(OH)_3$ + FnBPA/ClfA inoculation induced a significant increase of IgG2 levels only against ClfA (Fig. 2-**D**).

3.2. Humoral immune response after pre-calving booster

3.2.1. IgG anti-FnBPA and anti-ClfA in serum

A booster was administered at day 21 approximately before the expected calving date. Twenty-five animals were excluded from the study due to insemination failure, abortion or death. Experimental groups were conformed as follows: Al(OH)$_3$ (n = 5), Lip + CpG-ODN (n = 3), Al(OH)$_3$ + FnBPA/ClfA (n = 2), Lip + FnBPA/ClfA (n = 3), CpG-ODN + FnBPA/ClfA (n = 2) and Lip + FnBPA/ClfA + CpG-ODN (n = 4).

After pre-calving booster, IgG levels against FnBPA (Fig.3-**A**) and ClfA (Fig.3-**B**) in serum were assessed. Specific antibody levels, for both proteins, in group Lip + FnBPA/ClfA + CpG-ODN tended to increase, from 15 days after pre-calving booster until the end of the observation period.

3.2.2. IgG1 and IgG2 anti-FnBPA and anti-ClfA in serum

Levels of IgG1 and IgG2 against both proteins after 15 days of pre-calving booster were studied (Fig.4). Specific IgG1 for both proteins (Fig.4-**A**) was stimulated by Lip + FnBPA/ClfA and Lip + FnBPA/ClfA + CpG-ODN formulations. However, the increase was only significant for Lip + FnBPA/ClfA + CpG-ODN group compared with its control group (p < 0,05, Mann-Whitney). Concerning IgG2 levels (Fig.4-**B**), only Lip + FnBPA/ClfA + CpG-ODN formulation was able to stimulate this subclass in 2 out of 4 animals, and this increase was significant for anti-ClfA IgG2 (p < 0,05, Mann-Whitney). In addition, a trend towards stimulation of specific IgG1 by Al(OH)$_3$ + FnBPA/ClfA and CpG-ODN + FnBPA/ClfA was observed for both proteins.

3.2.3. IgG anti-FnBPA and anti-ClfA in milk

Milk samples were collected, 30 and 45 days after pre-calving booster, and whey IgG levels against FnBPA (Fig.5-**A**) and ClfA (Fig.5-**B**) were analysed. Animals from the Lip + FnBPA/ClfA + CpG-ODN group tended to present the highest IgG levels for both proteins. However, no statistical differences were found among groups.

3.2.4. IgG1 and IgG2 anti-FnBPA and anti-Clf in milk

Levels of whey specific IgG1 (Fig.5-**C**) and IgG2, 30 days after pre-calving booster, were studied. Only the Lip + FnBPA/ClfA + CpG-ODN group showed a trend towards an increase of IgG1 levels, specific to both proteins. No specific IgG1 was detected in whey from animals from other experimental groups. Regarding IgG2, no group presented detectable levels of this immunoglobulin in milk whey (data not shown).

3.2.5. Blocking assay

An ELISA assay was designed to evaluate the capability of antibodies generated by immunisation to inhibit *S. aureus* binding to bovine Fb *in vitro* (Fig. 6). Pre-incubation of *S. aureus* with antibodies obtained 15 days after pre-calving booster from animals in Lip + FnBPA/ClfA and Lip + CpG-ODN + FnBPA/ClfA groups tended to decrease the binding of bacteria to Fb.

4. Discussion

In the present work, a novel adjuvant formulation based on cationic liposomes in combination with CpG-ODN 2007 as immunostimulant was used to induce immunity against two staphylococcal proteins in heifer calves. The ODN sequence corresponding to CpG-ODN 2007 is specific for bovine and porcine TLR9, and in this case, it contains natural phosphodiester bonds. As demonstrated by a DNAse protection assay, liposomes can protect the CpG-ODN from nuclease degradation (results not shown) and provide a valid method to prolong the oligonucleotide half-life. Liposome use allows all molecules included in the formulation to be captured by the same APC, with the consequent enhancement of the specific immune response (Krishnamachari and Salem, 2009; Song et al., 2014). Although the use of formulations using cationic liposomes or CpG-ODN as adjuvants in cattle is well known (reviewed by Heegaard et al., 2011), there is only scarce information about the use of a combination of both components in this species. The only report, to the best of our knowledge, is the commercial immunostimulant Zelnate™ (Bayer HealthCare LLC, USA) indicated for use as an aid in the treatment of Bovine Respiratory Disease (BRD) due to *Mannheimia haemolytica*.

As far as we know, there is only one report of an immunisation study with animals before its first calving. Nickerson et al. (1999) inoculated 2 doses, at 15 days interval, of Lysigin® (Boehringer Ingelheim Vet-medica, USA), to 6 months old calves, followed by booster vaccinations every 6 months, resulting in a considerable reduction of new *S. aureus* IMI, during pregnancy and calving, and in the percentage of quarters with chronic or persistent *S. aureus* IMI during the observation period.
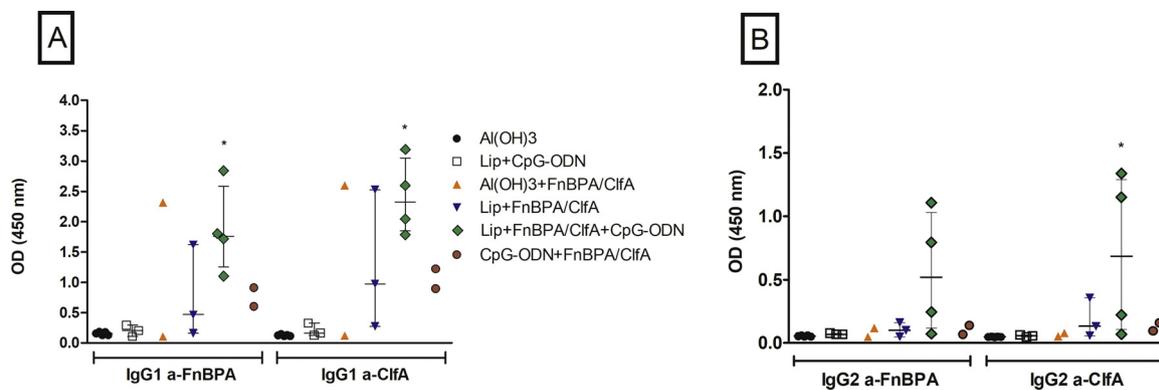
Fig. 4. Specific IgG1 and IgG2 detection in sera of primiparous heifers after a pre-calving booster with different formulations containing FnBPA and ClfA, by indirect ELISA (day 15 post-booster). The booster was administered approximately 3 weeks before the expected calving date. Sera were tested in a 1/1000 dilution against single antigens: A- IgG1 anti-FnBPA and anti-ClfA; B- IgG2 anti-FnBPA and anti-ClfA. A Mann-Whitney statistical analysis was done to make comparison between those groups with an n ≥ 3. Median ± IQR of three to five animal sera from each group (according to specifications in *Results: Humoral immune response after pre-calving booster. IgG anti-FnBPA and anti-ClfA in serum*) is shown. *p < 0.05, compared with control groups.

Taking these considerations into account, another novel approach incorporated in the present work was the application of a vaccination schedule starting at an early age, seeking for prevention of IMI in heifers before the beginning of lactation.

There is no information about the combined use of cationic liposomes and PO CpG-ODN for antigen-specific immunisation in heifers. The results obtained here with PO CpG-ODN, in combination with liposomes warrant the use of unmodified CpG-ODN in the formulation of cattle vaccines. Besides the problems associated with costs and possible adverse effects, as previously stated, there is evidence that CpG-ODN with natural structure activates TLR9 more efficiently than their PS modified counterpart (Haas et al., 2008; Kindrachuk et al., 2007).

Specific humoral immune responses were significantly elevated in serum for Lip + FnBPA/ClfA + CpG-ODN and Al(OH)$_3$ + FnBPA/ClfA groups after the second dose with no or only a minor effect after the third dose. Similar results were previously reported for a subunit vaccine in calves using a cationic liposomal adjuvant (CAF01) and a cocktail of recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins (Thakur et al., 2013). The authors observed that application of the second dose of vaccine strongly induced specific antibody responses towards all five vaccine antigens, meanwhile application of a third dose had no effect in the increment of specific antibody levels (Thakur et al., 2013). In this work, the serum IgG levels in Lip + FnBPA/ClfA + CpG-
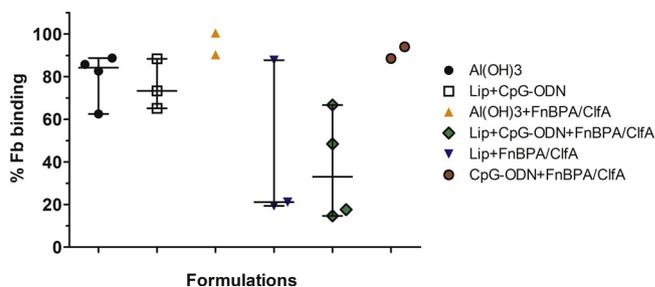

Fig. 6. *In vitro* functional analysis of antibodies in sera of primiparous heifers after a pre-calving booster with different formulations containing FnBPA and ClfA. The ability of antibodies to block *S. aureus* Reynolds binding to fibrinogen (Fb) was tested at day 15 after pre-calving booster. The booster was administrated approximately 3 weeks before the expected calving date. Sera were tested at 1/200 dilution in a 1 × 10^9 CFU/ml *S. aureus* Reynolds suspension. Bacterial suspension without sera was used as positive control. Results were expressed as the percentage of binding with respect to positive control considered as maximum binding ability. A Mann-Whitney statistical analysis was done to make a comparison between those groups with an n ≥ 3. Median ± IQR of three to five animal sera from each group (according to specifications in *Results: Humoral immune response after pre-calving booster. IgG anti-FnBPA and anti-ClfA in serum*) is shown.
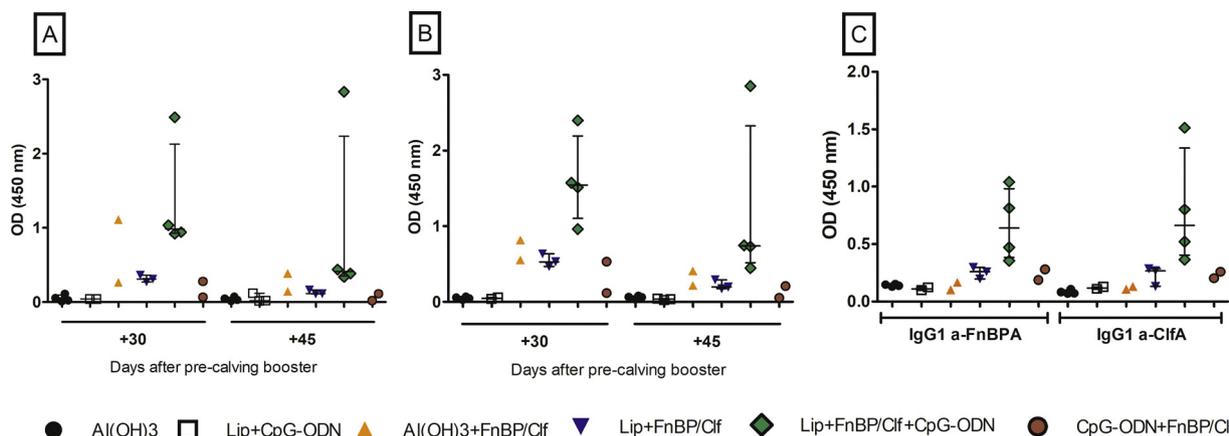

Fig. 5. Specific IgG and IgG1 detection in whey of primiparous heifers after a pre-calving booster with different formulations containing FnBPA and ClfA, by indirect ELISA. IgG was evaluated at days 30 and 45 after pre-calving booster, and IgG1 at day 30. The booster was administrated approximately 3 weeks before the expected calving date. Whey samples were tested in a 1/100 dilution against single antigens: A- IgG anti-FnBPA; B- IgG anti-ClfA; C- IgG1 anti-FnBPA and anti-ClfA. A Mann-Whitney statistical analysis was done to make a comparison between those groups with an n ≥ 3, at the same time point. Median ± IQR of three to five animal sera from each group (according to specifications in *Results: Humoral immune response after pre-calving booster. IgG anti-FnBPA and anti-ClfA in serum*) is shown. *p < 0.05, compared with control groups.

ODN and Al(OH)$_3$ + FnBPA/ClfA groups continued higher than controls for one month after the third dose, in both groups. On the contrary, antibody levels in Lip + FnBPA/ClfA and CpG-ODN + FnBPA/ClfA groups declined to pre-immune levels at that stage. Although no group presented specific antibodies 270 days after the beginning of the study, a single dose at day 410 induced a rapid increase of IgG levels in Lip + FnBPA/ClfA, Lip + FnBPA/ClfA + CpG-ODN and Al(OH)$_3$ + FnBPA/ClfA groups, showing the capacity of these formulations to induce immunological memory.

Regarding the production of IgG subclasses, all experimental groups presented antigen-specific IgG1 in serum 15 days after both, third and fourth dose. However, IgG2 specific for both proteins were only detected after the fourth dose in Lip + FnBPA/ClfA + CpG-ODN group. The difference in IgG2 response between the third and fourth doses could be due to the difference in animal ages since some authors suggest that levels of IgG2 could increase as cattle grow (Williams and Millar, 1978). Furthermore, the dispersion observed in specific IgG2 levels among animals could be explained by the inter-individual variability in response to CpG-ODN found in outbred populations, suggesting that CpG sensitivity is partly genetically controlled (Kamstrup et al., 2001; Mena et al., 2003). On the other hand, taking into account that on days 60 and 425 similar levels of IgG were detected in the groups Al(OH)$_3$ + FnBPA/ClfA and Lip + FnBPA/ClfA + CpG-ODN, and based on the levels of IgG1 and IgG2 subtypes, we cannot discard another subclass contribution. However, there is a lack of consensus among authors in the identification of other than IgG1 and IgG2 bovine IgG subclasses and, consequently, a lack in commercially available reagents for its determination (Butler and Kehrli, 2005; Fernández et al., 2011; Rabbani et al., 1997).

After the pre-calving booster, Lip + FnBPA/ClfA + CpG-ODN group showed a rapid increase in specific IgG levels in sera for both FnBPA and ClfA, that lasted during calving period and until, at least, 45 days after booster administration. Although the number of animals still in the protocol was small, these results suggest that a single booster, approximately 21 days before parturition, would be enough to quickly increase specific IgG levels in serum in a period of high susceptibility to IMI (Sordillo and Streicher, 2002). Moreover, the *in vitro* blocking assay to bovine Fb demonstrated the capacity of liposomal formulations to induce antibodies able to inhibit *S. aureus* binding. In previous work of our group, two doses of a subunit vaccine containing recombinant peptides of FnBPA and ClfA formulated with immune-stimulating complexes were highly immunogenic in pregnant heifers, triggering a high humoral response during the first month after calving (Pujato et al., 2018). The vaccination schedule evaluated here proposes only one pre-calving dose leading to simpler handling of the animals. Concerning IgG subclasses after pre-calving booster in serum, Lip + FnBPA/ClfA + CpG-ODN formulation induced a significant increment in IgG1 level and a trend to higher IgG2 production.

Regarding the presence of antibodies after pre-calving booster in milk, Lip + FnBPA/ClfA + CpG-ODN group IgG levels tend to be higher, even one month after parturition, with high IgG1 levels and absence of IgG2. Immunoglobulin G2 is considered the main opsonin supporting neutrophil phagocytosis in the milk of the infected mammary gland (Paape et al., 2003; Sordillo and Streicher, 2002). The concentration of this subclass is extremely low in milk on healthy mammary glands (Caffin and Poutrel, 1988). However, the opsonic activity of mastitic milk increases during the acute phase of mammary gland inflammation due to a massive transport of IgG2 from blood into milk 4 h post-IMI, 6–12 h prior to the peak of neutrophil response (Sordillo and Streicher, 2002). Presence of IgG2 in serum of Lip + FnBPA/ClfA + CpG-ODN group suggests an increment in phagocytic capacity and bacterial clearance in the mammary gland of these animals, under inflammatory conditions. Lack of proper statistical analysis in most antibody determinations in this part of the study is due to the reduced number of animals included, which is logical considering the long-term nature of the study.

In conclusion, immunisation of 6–8 months old calves with two recombinant proteins formulated with an adjuvant based on cationic liposomes and a CpG-ODN, induced a strong humoral immune response and was able to stimulate the production of specific IgG2. In addition, the formulation induced memory immune response, allowing the application of a single pre-calving booster able to maintain high antibody levels throughout the period of increased susceptibility to IMI.

Declarations of interest

None

Acknowledgements

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