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Efficacy of immunization with a recombinant *S. aureus* vaccine formulated with liposomes and ODN-CpG against natural *S. aureus* intramammary infections in heifers and cows

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ABSTRACT

The aims of the present study were to evaluate the ability of a subunit vaccine composed of recombinant molecules of α-toxin, β-toxin, FnBPA and ClfA, formulated with cationic liposomes and CpG-ODN, to confer protection against natural *S. aureus* intramammary infection (IMI) and to assess the antibody response against the vaccine components. A stringent criterion based on molecular identification of the isolates was used to define IMI. The proportion of animals that developed new *S. aureus* IMI was higher in the Control group compared with the Vaccine group (reduction of 60.7%), and time to new *S. aureus* IMI was higher for animals in the Vaccine group compared with animals in the Control group, although not statistically significant. Molecular identification of the isolates allowed the detection of *S. aureus* pulsotypes that appeared transiently in milk and others that were able to establish IMI, providing a new perspective to define parameters related to the definition of new IMI and cures. Specific IgG, IgG1 and IgG2 levels against the four recombinant proteins included in the vaccine were significantly increased in the vaccinated group and the recombinant α-toxin included in the vaccine generated antibodies that reduced significantly the haemolytic activity of native α-toxin. Data reported in the present study indicate a possible effect on both the proportion of animals developing new IMI and the time to new *S. aureus* IMI, but the incidence of disease within the study was too low to provide statistical confirmation.

INTRODUCTION

Staphylococcus aureus is one of the most frequently isolated pathogens from bovine intramammary infections (IMI) in Argentina and worldwide (Dieser et al., 2014; Persson et al., 2011). Traditional mastitis control measures against this pathogen include milking-time hygiene, antibiotic therapy for clinical cases during lactation and for subclinical cases at drying off, and culling of chronically infected cows. However, the chronic nature of most *S. aureus*

IMI, and the limited cure rate following classic antibiotic therapy make this disease difficult to control (Zecconi et al., 2006). Due to these limitations, several practices, new developments and strategies have been proposed to complement classical measures to control *S. aureus* mastitis (Rainard et al., 2018); among them, vaccination has been for many years a subject of considerable interest for the dairy sector (reviewed by Middleton, 2008).

In the last decades, several *S. aureus* mastitis vaccines have been developed and their efficacy was evaluated in field trials (reviewed by Pereira et al., 2011). Among them, only a few immunogens based on lysates or inactivated organisms formulated with traditional adjuvants, are currently commercially available worldwide. However, they have shown limited efficacy for preventing *S. aureus* IMI (Freick et al., 2016; Landin et al., 2015; Middleton et al., 2009; Schukken et al., 2014). This has been attributed, among other factors, to the fact that bacterins or lysates obtained at fixed culture times may not be representative of the most relevant *S. aureus* virulence factors exposed to the host during an IMI. Hence, it has been proposed that to overcome some of these limitations a *S. aureus* vaccine should include not only a variety of immunogenic antigens known to be involved in different stages of staphylococcal pathogenesis, but also that these antigens maintain the structure as expressed by the bacteria *in vivo* (Middleton, 2008; Scali et al., 2015).

S. aureus expresses a family of cell wall-anchored (CWA) proteins. Among them, fibronectin-binding proteins (FnBPs) are considered crucial components in early host-pathogen interactions (Fowler et al., 2000) due to their involvement in host cells invasion (Speziale & Pietrocola, 2020). Another CWA protein is Clumping factor (Clf). This protein interacts with plasma fibrinogen allowing an instantaneous agglomeration of bacterial cells. In addition, Clf can interact directly with the host cell through its binding to different ligands, favoring the subsequent internalization of the microorganism (Josse et al., 2017). These CWA were early explored as candidate vaccine antigens both in murine and bovine mastitis models since the 2000s (reviewed by Scali et al., 2015). In addition, *S. aureus* is also capable of secreting various

toxins. Among them, α-toxin is a protein toxic to a wide range of mammalian cells, including erythrocytes and epithelial cells, and its main function is to convert host tissue into nutrients for the bacteria that express it. Alpha-toxin has been described as a key factor in the intracellular survival of the pathogen since it is capable of inducing dysfunctional autophagy in the infected cell (Grumann et al., 2014). Beta-toxin is a sphingomyelinase, capable of hydrolysing the sphingomyelin present in the plasma membrane of the host cells. Although it would not be able to lyse most of the host's cells alone, it would render them susceptible to the action of other lytic agents, such as α-toxin. It was suggested that its primary activity is to modulate host processes that affect pathogenesis, rather than directly killing the cell (Bien et al., 2011). All these virulence factors are widely distributed among *S. aureus* isolated from bovine IMI, highlighting the importance of these molecules in staphylococcal pathogenesis. These toxins were early included as toxoids in experimental vaccines for bovines since the 1980s (Opdebeeck and Norcross, 1982) (reviewed by Scali et al., 2015). In recent studies, we have demonstrated the antigenicity of three recombinant *S. aureus* proteins: β-toxin, ClfA and FnBPA, formulated with a commercial new generation adjuvant, the *in vitro* functional role of the antibodies generated against these proteins in heifers and the lack of specific antibodies against rClfA and FnBP in heifers vaccinated with a lysate of a *S. aureus* strain (Camussone et al., 2014; Pujato et al., 2018; Renna et al., 2014).

The adjuvant used in the formulation has also been identified as a critical factor for vaccine efficacy improvement (Boerhout et al., 2018; Camussone et al., 2013). An effective *S. aureus* vaccine would probably need to both induce high titers of opsonizing and neutralizing antibodies and stimulate cellular immunity, particularly Th1 and Th17 profiles (Wang & Lee, 2015). Commercially available vaccines, as well as the majority of veterinary vaccines, are mostly formulated with aluminum salts or oil-based adjuvants (Burakova et al., 2018). Although Al(OH)3 is able to enhance strong humoral responses, it fails to stimulate cellular Th1 immune responses. Oil in water emulsions induce a more balanced immune response but

remain weak inducers of Th1 profiles (Maisonneuve et al., 2014). Consequently, there is a need to develop and test new, safe, non-toxic adjuvants able to direct immune responses to a protective profile for inclusion in the formulation of *S. aureus* vaccines. Among new generation adjuvants, liposomes are phospholipid vesicles able to promote the development of specific immune responses by carrying the antigen together with immunostimulant molecules. Our group has recently designed a cationic liposome-based adjuvant supplemented with CpG motifs oligonucleotides (ODN-CpG) as immunostimulant. This adjuvant was evaluated in BALB/c mice using a model protein antigen, resulting in high antibody titers generation, IgG1 and IgG2a production, stimulation of T lymphocytes with IFN-γ production and no adverse effects (Reidel et al., 2017). Further studies were performed immunizing heifer calves with recombinant ClfA and FnBPA molecules as model antigens formulated with the newly developed liposomes and a bovine-specific CpG-ODN which induced a strong serum response of specific IgG1 and IgG2, as well as of IgG1 in milk upon their first lactation. The presence of anti-ClfA neutralizing antibodies *in vitro* and induction of memory immune response was demonstrated (Reidel et al., 2019); however, the ability of these immunogens to confer protection against natural *S. aureus* IMI has not been addressed.

The aims of the present study were to evaluate the ability of a subunit vaccine composed of recombinant molecules of α-toxin, β-toxin, FnBPA and ClfA, formulated with cationic liposomes and CpG-ODN, to confer protection against natural *S. aureus* IMI and to assess the antibody response against the vaccine components.

MATERIAL AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from AvantiPolar 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) (Reidel et al., 2019). Unless specified, chemicals were of analytic grade and purchased from Sigma-Aldrich (St. Louis, MO, USA)

Vaccine components

The α-toxin gene was amplified from *S. aureus* Reynolds strain by PCR using specific primers (Fwmat: 5′-catatgGCAGATTCTGATATTAAT-3′, Rvmat: 5′-ctcgagATTTGTCATTTCTTCTTT-3′). Amplicon was sequenced using an ABI3130xl DNA sequencer (Applied Biosystems) and the obtained sequence was deposited in the GenBank database under Accession Number MZ398129. Fragment was cloned into pET32a vector (Merck Millipore, Germany) by NdeI/XhoI restriction and used to transform competent *E. coli* BL21(DE3) cells.

The other recombinant antigens were obtained as previously described for β-toxin, ClfA and FnBPA (Camussone et al., 2014). Briefly, *E. coli* BL21(DE3) cells bearing plasmid constructions pET32a-α-toxin, 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 shaking. Protein expression was induced ON with 0.1mM isopropil-β-D-1-thiogalactopyranoside at 25°C and the respective antigens were purified with a Ni-nitrilotriacetic acid column (Thermo Scientific). Protein quantification was performed using a spectrophotometer (NanoDrop 2000, Thermo Scientific) and purity was analysed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Brilliant Blue staining (Laemmli, 1970).

The experimental immunogen consisted of 100 μg/dose of each recombinant β-toxin, FnBPA and ClfA, as previously described (Camussone et al., 2014) together with 100 μg/dose of recombinant α-toxin. Vaccines were formulated with a combination of liposomes and CpG-ODN (Reidel et al., 2019). Positively charged liposomes were prepared using DPPC:Chol:SA, in 7:2:2 mol/mol ratio and 8 mM as lipid concentration by the ethanol injection method (Wagner et al., 2006). Briefly, the lipids were dissolved in an ethanol/isopropanol mixture (1:1 v/v)

considered as organic phase. The aqueous phase consisted of 50 mM acetate buffer (AcB, pH=4.3). While the aqueous phase was kept in continuous stirring, the organic phase was incorporated by injection in a 1:9 (v/v) proportion achieving a liposomal suspension with an 8 mM lipid concentration. CpG-ODN were dissolved in AcB at 30 µM. Antigens were diluted in AcB at 200 µg/ml each. Final formulation was prepared by mixture of liposomes 8mM, CpG-ODN 30 µM and antigens 200 µg/ml each, in 2:1:1 v/v ration, resulting the following concentration: lipids 4mM, antigen 50 µg/mL, CpG 7.5µM. Animals were immunized with 2 ml of final formulation.

Dairy herd

The study was carried out in a dairy herd located in Santa Fe province of Argentina that reported mastitis problems due to *S. aureus* during the four years prior to the initiation of the experiment. In addition, selection of the herd was defined based on the following criteria: location near to the laboratory, willingness of the farmer to collaborate, availability of animal restraint facilities to allow vaccination and sampling of pre partum heifers and *S. aureus* IMI prevalence at cow level higher than 15%. Mastitis control practices consisted of administration of dry cow antibiotic therapy on all quarters of all cows at drying off, treatment of clinical mastitis cases with a macrolide antibiotic as first choice protocol, and a beta-lactam antibiotic in case of clinical failure of first choice protocol. No commercial *S. aureus* vaccine was applied in the herd as preventive measure. Pre milking routine consisted of forestripping and teat washing with water before attaching the milking units. Milkers used inconsistently latex gloves. Post milking teat disinfection was carried out with a 0.5% iodophor formulation. Culling decisions were made by the farm owner based on fertility, chronic mastitis, lameness, or other health problems.

To determine the initial prevalence of *S. aureus* IMI in the herd, composite milk samples from all lactating cows (n=163) were taken before starting the vaccination protocol and cultured for

detection of mastitis pathogens according to standard procedures (Oliver et al., 2004). At the time of sampling 24.7% were first lactation cows, 26% second lactation and 49.3% third lactation or higher. The prevalence of *S. aureus* IMI in lactating cows was also determined one year after the study began following the same procedures. At the time of sampling 37.3% were first lactation cows, 18.2% second lactation and 44.5% were third lactation or higher.

The herd had 163 crossbreed (Holstein x Jersey) lactating cows with an average milk yield per cow of 22.4 litres. The average somatic cell count (SCC) was 790 x 10^3 cells/ml in the year prior to the start of this study and 765 x 10^3 cells/ml at the beginning of the experiment. Cows were kept under grazing conditions on alfalfa-based pastures, milked twice daily in a single 10 herringbone parlour and received concentrates in the milking parlour. Approximately 20 days before the expecting calving date, cows were moved to a group maternity area where they were fed a balanced TMR, alfalfa hay and had free access to water.

Animals and sampling

Vaccinations and sampling collection data were performed on cohorts during a 26-months period starting in June 2018 until August 2020. Considering a reduction of 70% in new *S. aureus* IMI, a confidence of 95%, and a power of 80%, it was calculated that a total of 89 animals should be included per group.

Forty-four cows with one previous calving and no previous reports of *S. aureus* IMI, and 123 primigravid heifers, all of them in the last trimester of gestation, were used. Animals were randomly allocated in two groups: Vaccine (n=85; 65 heifers and 20 second-lactation cows) and Control (n=82; 58 heifers and 24 second-lactation cows). Animals in the Vaccine group were injected subcutaneously with two doses of 3 ml of vaccine in the supramammary lymph node area at 45 and 15 days before the expected calving date. Animals in the Control group received no treatment. Neither the herd staff nor the milking personnel were aware of the vaccination status of the animals. Fifteen days before the expected calving date heifers were

immobilized in a chute, udders were clinically examined by palpation, and samples of pre-partum mammary secretion were taken following standard procedures (Oliver et al., 2004). All samples were subjected to bacterial culture to determine the presence of *S. aureus* IMI. After sampling, the teats were dipped in a 0.5% iodophor solution. Only animals free of *S. aureus* IMI and with no clinical signs of inflammation continued in the experiment. After parturition, aseptic quarter foremilk samples were collected monthly for nine months and subjected to bacterial culture. Any gross abnormality of heifers' mammary glands or teats at sampling time was recorded. All milk and mammary secretion samples were immediately refrigerated, transported to the laboratory, and cultured within 24 h. Animals that ceased lactation before the third sampling for causes not related to the trial, were excluded from the study. When a case of clinical mastitis occurred (defined as macroscopic changes in milk with or without swelling and/or general symptoms), a mammary quarter sample was obtained by the farm staff using aseptic procedures and submitted to the laboratory for bacteriological culture.

Twenty-four animals in the Control group and 21 animals in the Vaccine group were also studied during the next lactation. Fourteen animals in Vaccine group received a third dose of vaccine in the supramammary lymph node area at approximately 10 days before the expected calving date. After parturition, aseptic quarter foremilk samples were collected every two months for 9 months and subjected to bacterial culture (5 samples per cow). Clinical examination and sampling was carried out as described above.

All procedures used in this study were consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 2010) and approved by the Ethics and Security Committee of the Santa Fe Regional Center from the National Institute of Agricultural Technology (CICUAE, Nº18-006).

Bacteriological examination and definition of intramammary infection

Intramammary infection was defined based on standard bacteriological methods, confirmed by molecular analysis and further classification of the isolates at clone level. Milk and mammary secretion samples were cultured for mastitis pathogens according to standard procedures seeding 10 µl of sample in a quarter of a blood agar plate (Oliver et al., 2004). Presumptive phenotypical identification was performed based on colony morphology, Gram staining, catalase test and tube coagulase test using rabbit plasma. Presence of one colony of coagulase-positive *Staphylococci* on blood agar was considered as a positive identification; therefore, the detection limit was 100 CFU/ml. Isolates were kept at -80°C on BHI-glycerol 20%. Coagulase-positive *Staphylococci* were confirmed as *S. aureus* through amplification of *nuc* gene by PCR (Brakstad et al., 1992). Further, clonality of *S. aureus* isolates was assessed by Pulse field gel electrophoresis (PFGE) of *Sma*I-digested chromosomal DNA fragments using a CHEF-DR II apparatus (BioRad Laboratories, CA, USA) as described previously (Camussone et al., 2020). A dendrogram was constructed based on UPGMA algorithm with TREECON 1.3b.

A new *S. aureus* IMI was defined as two consecutive or interspersed samples with positive identification (Sears et al., 1990), positivity of *nuc* gene PCR, and the presence of identical pulsotype by PFGE in every isolate obtained from the same mammary quarter of a cow. A cure was defined as 2 consecutive monthly milk samples that did not show the presence of the causative organism after being previously identified as an IMI. The isolation of *S. aureus* from one mammary quarter milk sample of a cow at only one sampling time was considered a transient IMI. Milk samples with 3 or more colony types were considered contaminated.

Milk yield and somatic cell count

Milk yield and milk samples for SCC from all cows enrolled in the study were obtained monthly during the whole experimental period from the regional dairy herd improvement system. For determination of milk SCC, milk samples were preserved with azidiol (0.3%) at 4ºC and

analysed within 24 h by Laboratorio Regional de Servicios Analíticos (Esperanza, Santa Fe, Argentina) using an automated counter (Somacount 300, Bentley Instruments, Minesotta, USA).

Antibody response

Eleven animals from the vaccinated group and 12 animals from the control group were randomly selected for specific antibodies evaluation. Animals were bled by coccygeal vein venipuncture before each inoculation, and 14, 28 and 42 days after the second dose. Blood was allowed to clot; sera were collected via centrifugation and stored at −20 °C until processed. Specific IgG, IgG1, and IgG2 were determined by indirect ELISA. Flat bottom 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 0.5µg/well of recombinant α-toxin, β-toxin, FnBPA or ClfA in sodium carbonate buffer (pH 9.6). ELISA was performed as previously described (Camussone et al., 2014). In the case of α-toxin, which had not been used in previous research from our group, different amounts of antigen were tested to set up the technique, selecting 0.5ug/well. Working dilution was set at 1/1000 on the basis of previous optimization to achieve values into the dynamic range of this technique (Camussone et al., 2014). In order to standardize the process, all plates needed for the analysis were coated at the same time, and stored at −20 °C until use. Three positive and negative controls were analysed in each assay. In order to diminish between assays variations, a 10% tolerance in OD values of control samples was accepted.

Haemolytic activity inhibition of native α-toxin (nα-toxin)

Native α-toxin was partially purified from culture supernatants (Lind et al., 1987) of a *S. aureus* isolated from a mastitis case found to produce only α-toxin *in vitro*, as previously described

(Calvinho and Dodd, 1994). The obtained extract was analysed by 15% SDS-PAGE, followed by Coomassie Brilliant Blue staining (Laemmli, 1970) and stored at −20°C until needed.

Haemolytic activity against rabbit red blood cells (RBC) was assessed as previously described (Wadström and Möllby, 1971), with some modifications. Briefly, 100 µl of 1/3200 dilutions of native α-toxin were incubated in round-bottomed 96-well microtiter plates with 100 µl 1/50 dilutions of sera collected 14 days after the second dose, for 1 h at 37 °C. Then, 100 µl of 1/20 dilution of RBC were added and incubated for 2 h at 37°C, and finally ON at 4 °C. After incubation, supernatants were collected, and optical density was measured at 545 nm. Results were expressed as percentage of haemolysis with respect to the control without sera. Dilutions used for each reagent were chosen based on the results of previous set-up tests for β-toxin (Camussone et al., 2014).

Statistical analysis

Firstly, a descriptive analysis of the bacteriological findings and the pulsotypes detected among *S. aureus* isolated from IMI was performed. Then, the proportion of quarters that developed a new *S. aureus* IMI were compared between groups using the Chi-square or Fisher test, according to the frequency of cases. Time to new *S. aureus* IMI was compared between groups using a Kaplan–Meier analysis. Time was measured in months and was censured at nine months if no new *S. aureus* IMI was detected.

The mean SCC and average milk yield were compared between the Control and Vaccinated groups using a generalized model with repeated measures (gamma distribution and log link function). In addition, an analysis by sampling time was performed in order to look for differences between groups (generalized model with gamma distribution and log link function). The rate of new infections per month at risk was calculated for vaccinated and control cows. The rates were calculated on a monthly basis (calendar months) for the duration of the study. For comparative analysis of IgG response between groups, a generalized linear

model (with gamma distribution and log link function) for each antigen and sampling time was performed. For comparative analysis of IgG1 and IgG2 responses between groups at day 14 post calving, a generalized linear model (with gamma distribution and log link function) for each antigen was performed. The native α-toxin haemolytic activity inhibition between groups was assessed by means of a Mann Whitney test. Finally, a Spearman correlation analysis between percentage of haemolysis and antibody levels (IgG, IgG1, and IgG2) was performed.

In all cases, the distribution of the scale variables was studied and adjusted to the model that best fitted. GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used to make figures 1 to 5. Significance for each test was $P<0.05$. All the statistical analyses were carried out using InfoStat software (Universidad Nacional de Córdoba, Argentina).

RESULTS

Thirty-one heifers and 7 cows were excluded from the study due to presence of IMI at pre-partum detected by bacteriological culture (8 Vaccine, 6 Control), abortion (3 Vaccine, 6 Control), death (1 Vaccine, 4 Control), early birth (1 Vaccine) and cull off (4 Vaccine, 5 Control). Cows were culled due to chronic mastitis (2 Vaccine, 2 Control), fertility problems (1 Vaccine, 1 Control), lameness and other health problems (1 Vaccine, 2 Control). Hence, data from 63 animals (46 heifers and 17 cows) from the Vaccine group (252 mammary quarters) and 62 animals (42 heifers and 20 cows) from the Control group (248 mammary quarters) free of *S. aureus* IMI at the beginning of the study were used for vaccine efficacy analysis. On average the first vaccine dose was administered at 38 days prepartum and the second dose at 10 days prepartum. No adverse reactions were detected at the inoculation site in the Vaccine group during the whole study. A similar number of clinical mastitis cases (n =9), attributed to non- *S. aureus* isolates, were detected in both groups, respectively. In the control group 5 cases yielded no growth, one non *agalactiae Streptococcus*, one *Escherichia coli* and one *Bacillus*

cereus. In the Vaccine group two yielded no growth, two non *agalactiae Streptococci*, two *E. coli*, one yeasts and two *Trueperella pyogenes*. One of these latter cases was detected at a monthly sampling and *T. pyogenes* was the predominant organism in mixed culture with few S. *aureus* colonies. All clinical cases but one were detected during the first experimental year and were equally distributed among heifers and cows among both groups (results not shown).

Bacteriological findings and pulsotypes. Initial *S. aureus* IMI prevalence in lactating cows (cow level) was 18.4%. During the study, 4,899 quarter milk samples were obtained and subjected to bacteriological culture. A total of 4,083 (83.34%) samples were culture-negative and 189 (3.86%) were contaminated. From 135 primigravid heifers sampled at prepartum, 12 (8.9%) yielded a coagulase-positive *Staphylococci* culture. The identity of the isolates was confirmed by PCR as *S. aureus* and these heifers were therefore excluded from the study. Fifty one coagulase-positive *Staphylococci* were isolated from quarter milk samples of lactating heifers and cows. Among them, 41 were confirmed as *S. aureus* by PCR. Five different pulsotypes (PT) were found among isolates from lactating cows. The frequency of these PT was: 26 PT1, 6 PT2, 3 PT3, 5 PT4 and 1 PT5. Regarding the treatment group, 29 isolates were from the Control group (21 PT1, 6 PT2, 1 PT3 and 1 PT5) and 12 from the Vaccinated group (5 PT1, 2 PT3 and 5 PT4).

Incidence of new S. aureus IMI during the first year. Thirteen lactating quarters were positive at least once for *S. aureus* (41 isolates), but in only 7 cases *S. aureus* was isolated more than once. In every case, isolates obtained more than once (range 2 to 6 times) from the same quarter showed identical PT, meeting the criterion for defining a *S. aureus* IMI. From the 7 cows with confirmed IMI 35 isolates were obtained. These isolates belonged to three PT: Five cows had IMI by PT1, one by PT2 and one by PT4. Regarding the treatment group, 5 from these 7 IMI isolates belonged to the Control group (4 PT1 and one PT2) and two to the Vaccinated

group (1 PT1 and 1 PT4). Among the 6 transient isolates, 3 were PT3, 2 PT1, and 1 PT5. Regarding the treatment group, 4 transient isolates were from the Control group (2 PT1, 1 PT3 and 1 PT5) and 2 from the Vaccinated group (2 PT3). Figure 1 shows clonal relationship between isolates.

The proportion of animals that developed new *S. aureus* IMI was higher, although not statistically significant, in the Control group (5/62; 8.06%) compared with the Vaccine group (2/63, 3.17%) (*P*=0.234), representing a reduction of 60.7%. The proportion of quarters that developed new *S. aureus* IMI was higher, although not statistically significant, in animals from Control group (5/248; 2.01%) compared with animals in Vaccine group (2/252; 0.79%) (*P*=0.245; Figure 2 A, B), representing a reduction of 60.7% (Table 1). One year after starting the study, prevalence of *S. aureus* IMI in lactating cows was 15.7%.

The monthly incidence of IMI up to the third month of lactation was similar in both groups (Vaccinated = 0.031; Control = 0.032). During the two last trimesters of lactation animals in the Vaccinated group maintained a null incidence while new cases were detected in the Control group. According to the criteria used, no cures of IMI were detected.

Time to new S. aureus IMI.

Time to new *S. aureus* IMI was higher, but not statistically significant, for animals in the Vaccine group in comparison to animals in the Control group (*P* = 0.140; Figure 2A). Observation of risk function showed that the probability of acquiring an IMI at a given sampling time was higher for those animals that were not vaccinated , but not statistically significant (*P* = 0.140; Figure 2B).

Incidence of new S. aureus IMI during the second year

Twenty-four cows from the Control group and 20 in the Vaccine group were evaluated during the ensuing lactation. Among them, fourteen animals from Vaccine group received a third dose of vaccine before the next calving. All of them were in their second or third lactation.

Nineteen *S. aureus* isolates were obtained from 7 quarters. Two isolates were obtained only once, and belong 1 to Control group and 1 to Vaccine without booster group. Seventeen isolates were obtained from 5 quarters more than once, meeting the criterion of *S. aureus* IMI. Two cows from the Control group and one from the Vaccinated with booster group had an IMI in one mammary quarter that persisted from the previous lactation. Both cows in the Control group presented a new IMI in another mammary quarter during the second experimental year. No animals in both subgroups of Vaccine (with or without booster) presented a new *S. aureus* IMI during the second experimental year. The proportion of quarters that developed new *S. aureus* IMI during the second experimental year was higher in animals from the Control group (2/96; 2.08%) in comparison to animals in the Vaccine group (0/80; 0%) (with or without booster) (P=0.194), although not statistically significant. The proportion of animals that developed new *S. aureus* IMI was higher in animals from the Control group (2/24; 8.33%) in comparison to animals in the Vaccine group (with or without booster) (0/20; 0%) (P=0.186), although not statistically significant (Table 1). Both isolates from Control group (obtained 3 and 4 times, respectively) belonged to PT1 (Figure 1).

Milk yield and SCC

The mean SCC for the Vaccinated and Control groups during the experimental period were 473.35 x 10^3 (IC95% 414.89-540.05 x 10^3) and 480.96 x 10^3 (IC95% 412.43-560.68 x 10^3), respectively. No differences between groups were detected along the study (P= 0.877) or at any lactation month. The average milk yields for the Vaccinated and Control groups during the experimental period were 21.56 (IC95% 20.21-22.91) and 22.35 liters/day (IC95% 21.20-23.49),

respectively. No differences between groups were detected along the study (*P*=0.382) or at any lactation month.

Antibody response

IgG anti- α-toxin, β-toxin, ClfA and FnBPA levels in serum. Specific IgG levels for each protein included in the formulation were assessed. Results obtained for each of the evaluated antigens are shown individually, in Figures 3 A, C, E, G, to facilitate their interpretation. Before immunization, no differences in specific antibody levels were found between groups, for the 4 antigens evaluated. In pre-immune samples, only small levels of anti-α-toxin IgG (0.165≤OD≤0.474) were detected in 3 control and in 3 vaccinated animals (Fig. 3 E). Similarly, anti-β-toxin IgG (0.165≤OD≤0.616) was detected in 5 control and in 6 vaccinated animals (Fig. 3 G).

Cows in the Control group did not show an increase of specific IgG during the whole study, maintaining the pre-immune antibody levels. Regarding the Vaccine group, after application of the first dose, IgG levels were higher than those in the Control group for the 4 antigens (*P*< 0.001). These levels reached their maximum after application of the second dose and remained higher than those in the Control group until the end of the evaluation period (*P*< 0.001).

A third dose was administered 10 days before the second calving, and specific IgG levels for each protein were evaluated (Fig. 3 B, D, F, H). Before the third dose, no differences in antibodies levels were observed between groups. However, this single dose was able to rapidly rise the antibody levels in the Vaccine group, reaching higher specific IgG levels than the Control group against the 4 antigens at day 4 post calving (*P*< 0.001). Increased antibody levels in the Vaccine group were detected up to the end of the evaluation period (*P*< 0.001).

- ***IgG1 and IgG2 anti- α-toxin, β-toxin, ClfA and FnBPA levels in serum.*** Levels of IgG1 and IgG2 specific for each recombinant protein were determined 14 days after the administration of the second dose (Fig. 4 A and C, respectively) and 14 days after the third dose (Fig. 4 B and D, respectively). Regarding IgG1, Vaccine group showed higher specific antibody levels than Control group against the 4 antigens at both evaluation times (P<0.001). An increase in specific IgG2 was also detected, but to a lesser extent than IgG1. The IgG2 levels of cows from the Vaccine group were higher than those in cows from the Control group (rFnBPA: P<0.01; rClfA; α-toxin, β-toxin: P<0.001).

Native α- toxin inhibition assay

Capability of antibodies generated by immunization to inhibit native α- toxin was evaluated in serum samples obtained at day 14 after application of second dose of vaccine. Pre-incubation of native α- toxin with sera from the Vaccine group reduced the percentage of haemolysis in comparison to pre-incubation with sera from the Control group (P<0.01) (Fig 5-A).

A matrix grouping specific anti-α-toxin antibody levels at day 4 and percentage of haemolysis was performed (Fig. 5.B) and Spearman correlation coefficients were calculated. The color matrix evidenced that sera from the 3 animals in the Control group that presented less than 50% of haemolysis corresponded to animals with moderate IgG anti-α-toxin levels (ODs: 0.294; 0.302; 0.476). Regarding Spearman's correlation analysis, all coefficients were negative, indicating that higher antibody levels corresponded to lower percentage of haemolysis (IgG: R=-0.806, P<0.01; IgG1: Rs=-0.644, P<0.001; IgG2: Rs=-0.660, P<0.001).

DISCUSSION

Numerous studies to determine the efficacy of vaccines based on bacterins, toxoids and attenuated strains were carried out previous to the 1990s and over the following decades with varying results (reviewed by Pereira et al. (2011). Only few developments based on bacterins

and lysates are commercially available (Middleton, 2008). In the last two decades subunit vaccines composed by *S. aureus* virulence factors that interact with the host at different stages of an IMI have been proposed to overcome the shortcomings of currently commercially available bacterin-based vaccines for this disease (Middleton, 2008; Scali et al., 2015). In recent years both the immunogenicity (Boerhout et al., 2015; Camussone et al., 2014; Leitner et al., 2011; Pujato et al., 2018; Ster et al., 2010) and the protection against experimental challenge (Alabdullah et al., 2020; Chang et al., 2008) of several candidate recombinant antigens formulated with different adjuvants have been evaluated in bovines. In this study we assessed the antigenicity and the efficacy of an experimental immunogen composed of selected recombinant antigens of *S. aureus* formulated with liposomes and -CPG-ODN to prevent natural *S. aureus* IMI in primigravid heifers and second lactation cows in a high prevalence *S. aureus* dairy herd.

In the present study identification of *S. aureus* causing an IMI was confirmed by molecular methods. To the best of our knowledge, no previous studies assessing efficacy of a *S. aureus* vaccine under natural exposure conditions have confirmed identity of the isolates by molecular methods. Routine phenotypical methods based on haemolysis pattern and coagulase test can identify correctly most *S. aureus* present in bovine milk since is the most frequently isolated species among coagulase-positive *Staphylococci* (Oliver et al., 2004). However, other Staphylococcocal species that produce constant or inconstantly a coagulase positive test result and a variable haemolysis pattern have been isolated with varying frequencies from IMI, producing effects in the mammary gland that differ from those caused by *S. aureus* (Adkins et al., 2018; González-Martín et al., 2020). In the present study from 51 coagulase-positive, 41 (80.4%) were confirmed as *S. aureus*, thus avoiding incorrect classifications that might have led to misinterpretation of results. In addition, PFGE was performed on *S. aureus* isolates both to get an insight of the distribution of clones within treatment groups and to confirm establishment of an IMI based on the presence of the same PT in more than one sample from a

mammary quarter of a cow. Five different PT were detected; being PT1 the most predominant (63%), while the rest were detected with varying frequencies (from 2.4 to 14.6% of isolates). This agrees with previous studies that have found a small number of PT within a herd with one or two predominating PT, which has been associated with *S. aureus* contagious transmission in the herd (Capurro et al., 2010; Haveri et al., 2008). Three PT were found in established (persistent) IMI (PT 1, 2 and 4), two PT (PT 3 and 5) were only found in transient IMI and PT 1 was found in both cases, which can reflect different adaptation capabilities inherent to the PT to establish and maintain an IMI (Grunert et al., 2018; Haveri et al., 2007; Sacco et al., 2020). PT1 was found in 4 IMI in the Control group versus one IMI in the vaccinated group. Although this fact could suggest an effect of vaccination on the establishment of this PT, characterization of the isolates to determine the presence of the antigens included in the vaccine was beyond the scope of this study. Although the antigens included in this vaccine are conserved in most *S. aureus* isolated from IMI, since variations in the virulence gene profiles among PT has been reported (Haveri et al., 2007, 2008), further studies of the distribution of these antigens in different PT can provide an insight of the potential breadth of coverage of this vaccine.

The stringent criteria to define an IMI used in the present study allowed differentiation from transient infections caused in most cases by PT that can be considered as less adapted to the mammary environment and therefore more likely to be eliminated from the mammary gland by the innate immune response (Haveri et al., 2007). Definition of an IMI based on the presence of the same PT in a mammary quarter at least twice can be particularly relevant since it contributes to determine the duration of an IMI and affects the definition of cure which is an important parameter to evaluate protection of a vaccine (Schukken et al., 2104). Although the use of genotypic methods has a significant impact on the experimental costs, the use of phenotypic methods alone might not be the most appropriate to discriminate between effects attributable to vaccine efficacy to prevent establishment of a *S. aureus* IMI or to different PT capabilities to establish within the mammary gland.

Vaccine protective effects in animals are mainly evaluated through challenge studies with the target pathogen under controlled experimental conditions or through randomized control trials under natural exposure conditions. For the latter, the study group that represents the population of interest will preferably be in a condition of high incidence of the disease (Knight-Jones et al., 2014). In the present study, initial prevalence in the herd at the cow level was 18.4%. Several trials conducted under natural exposure conditions that estimated initial *S. aureus* cow prevalence through phenotypic methods found this figure to range from 5 to 30% (Hoedemaker et al., 2001; Leitner et al., 2003; Middleton et al., 2009; Nordhaug et al., 1994a; Schukken et al., 2014). It has to be considered that definitions of low- and high-prevalence *S. aureus* herds vary substantially between studies (Crespi et al., 2015; Roberson et al., 1998). In the present study, a lower incidence of new *S. aureus* IMI, representing a reduction of 60.7% and a tendency to higher time to new *S. aureus* IMI were observed in vaccinated cows compared with controls which is indicative of a potential protective effect of the vaccine; however, differences were not statistically significant. These results partially agree with those of a previous study that evaluated a bacterin-based vaccine direct efficacy against *S. aureus* in lactating heifers using natural exposure conditions (Nordhaug et al., 1994a); however, differences in the definitions of IMI preclude direct comparisons. Other similar studies that evaluated direct protective effect of vaccines under natural exposure conditions were conducted in herds where the low level of IMI in the study group did not allow evaluating the potential protective action of the vaccine (Leitner et al., 2003, Middleton et al., 2009). Conversely, a significant reduction in the frequencies of *S. aureus* IMI in heifers (Giraudo et al., 1997) and cows (Calzolari et al., 1997) vaccinated with a multivalent bacterin compared to unvaccinated control groups was reported. In the present study, among other factors, lack of statistical significance in the analysed parameters could be in part a consequence of the low number of isolations that met the stringent defined criteria for IMI. In addition, despite that almost 94% of the calculated needed animals were enrolled, approximately 20% of them had

to be excluded due to different reasons not related to the study. Although enrolment of more animals would have contributed to gain statistical power as defined at the beginning of the study, the COVID-19 pandemics restrictions during 2020 made enrolment of more animals not possible. Although the prevalence of *S. aureus* IMI at the beginning of the second year was 15.7%, showing a slight reduction compared with the initial prevalence, it was beyond the objectives of this study to determine the effect of vaccinating a group of 1st and 2nd lactation animals on the prevalence of *S. aureus* IMI in the whole herd.

A reduction in the severity of mastitis (clinical vs subclinical) is beneficial since it can lessen severe potential damage to mammary tissue and economic losses (Middleton et al., 2006; Nordhaug et al., 1994a). In the present study, clinical mastitis cases were mainly caused by environmental pathogens that were evenly distributed among groups, which do not allow conclusions to be drawn about a vaccine effect. Milk SCC and milk yield were similar in both experimental groups during lactation. This result is not unexpected since the lower incidence of *S. aureus* IMI detected in the Vaccine group was not significant and, therefore, could not reflect changes in mammary health parameters that are the indirect result of an IMI. Similar results regarding these parameters were obtained in several trials evaluating *S. aureus* vaccines efficacy under natural exposure conditions in which no differences in the incidence of IMI were detected between experimental groups (Freick et al., 2016; Middleton et al., 2009; Nordhaug et al., 1994a).

Humoral immune response was evaluated in sera from randomly selected animals in order to assess the capacity of the experimental vaccine to stimulate specific antibodies production against recombinant antigens. Although vaccine first dose was able to induce specific IgG against the 4 antigens, administration of the second dose increased the antibodies levels, reaching maximum values at 4 days post calving date and maintaining high levels during at least one month at a period of high susceptibility to IMI (Sordillo & Streicher, 2002). This result is in accord with previous reports from our group using recombinant antigens formulated with

ISCOM-Matrix (Camussone et al., 2014; Pujato et al., 2018). Although a slight drop was observed, antibodies in vaccinated animals maintained significantly higher levels than those in control group up to 30 days post-second dose. Regarding IgG subclasses at 4 days post calving date, animals in Vaccine group presented higher specific IgG1 and IgG2 levels than Control group against the 4 antigens, with an IgG1 predominance. A balanced response between the two subtypes was also found in previous research using a similar subunit vaccine formulated with a different immunostimulant adjuvant (Pujato et al., 2018). Moreover, we also reported a balanced IgG1/IgG2 stimulation at calving date in primiparous heifers immunized with ClfA and FnBPA formulated with liposomes and CpG-ODN as adjuvant, using a vaccination schedule that started at an early age (6-8 months old) (Reidel et al., 2019). The induction of IgG1 is associated to a Th2 profile, while IgG2 associates to a Th1 profile and its production is dependent on IFNγ (Estes & Brown, 2002). Both isotypes are able to fix complement *in vivo*, and in addition, IgG2 is the main opsonin promoting neutrophil phagocytosis in the mammary gland during an IMI (Burton & Erskine, 2003). In cattle, responses to vaccination are predominantly IgG1-mediated, independently of the type of immunization, and this isotype is the predominant immunoglobulin in milk (Estes & Brown, 2002). Therefore, induction of the IgG2 isotype with the formulation described here is relevant and suggests the stimulation of Th1 differentiated T-cells. Moreover, immunization of BALB/c and C57BL/6 mice with rClFA formulated with this adjuvant stimulated the production of IL17 and IFNγ by CD4+ T-cells, and IFNγ by CD8+ T-cells (manuscript in preparation). Similar studies will be conducted in cattle receiving this vaccine.

In the present study, low levels of specific antibodies against α- and β-toxins were found in heifers pre-immune sera from both the Vaccine and Control groups indicating previous natural exposure to these *S. aureus* antigens. Previous studies have also shown the presence of antibodies against various staphylococcal antigens prior to immunization (Boerhout et al., 2015, 2018; Hayakawa et al., 2000; Pujato et al., 2018). Antibodies against *S. aureus* immune

evasion proteins, like extracellular fibrinogen-binding protein and leukocidin subunit M were detected in calves from week 12 onwards as a result of natural exposure to this organism (Benedictus et al. 2019). These antibody levels tend to increase after week 12; however, they were significantly lower than those achieved following vaccination at early age and do not seem to play an important role in protecting against new infections (Benedictus et al., 2019).

Finally, a single booster administered 10 days before the second calving increased rapidly and significantly the specific IgG levels in serum, compared with pre-boost levels, which remained high at parturition and for at least 3 weeks postcalving, demonstrating the vaccine capacity to generate a memory immune response. Similar results were obtained by our group using liposomes and CpG-ODN as adjuvant in a subunit vaccine against *S. aureus* in heifer calves, demonstrating consistency in the ability of this adjuvant to generate immunological memory (Reidel et al., 2019). In the present study, these high antibody titers did not seem to be associated with the ability of the vaccine to prevent establishment of new IMI since the majority of IMI were detected during the first trimester of lactation. Similar observations were made by Nordhaug et al (1994b) evaluating a bacterin formulated with α and β *S. aureus* toxoids; however, they observed less cases of clinical mastitis in the vaccinated group suggesting an amelioration of clinical signs. In the present study *S. aureus* was isolated from only one case of clinical mastitis and in mixed culture which prevents establishing an association between these two parameters. Additional research will be needed to characterize further aspects of the immune response to the vaccine in experimental challenge models including evaluation of cell-mediated immunity which was beyond the scope of the present study.

The vaccine formulation included α-toxin since it has been early identified as a virulence factor in *S. aureus* causing bovine IMI and its toxoid was part of several experimental vaccines showing potential as an immunogen (reviewed by Middeton, 2008; Rainard et al., 2018; Scali et al., 2015). In previous reports, we have shown the ability of specific antibodies against

FnBPA, ClfA, and β-toxin to block *S. aureus* binding to fibronectin and fibrinogen and to inhibit haemolytic activity of native β-toxin *in vitro* (Camussone et al., 2014; Pujato et al., 2018). However, the ability of antibodies directed to recombinant α-toxin for inhibiting haemolytic activity of native α-toxin was not previously addressed. Reduction of haemolytic activity of native α-toxin was associated with increased levels of specific immunoglobulins against the recombinant toxin used in the formulation. Boerhout et al (2018) reported that immunization of cows with recombinant genetically detoxified α-toxin formulated with an oil-based adjuvant induced the production of neutralizing antibodies against the recombinant toxin. Results obtained in the present study were similar to those obtained by Boerhout et al (2018) showing an association between IgG1 levels and α-toxin neutralization.

In conclusion, data reported in the present study indicate a possible effect on both the proportion of animals developing new IMI and the time to new *S. aureus* IMI, but the incidence of disease within the study was too low to provide statistical confirmation. Molecular identification of the isolates allowed the detection of PT of transient appearance in milk while other PT were capable of establishing IMI, providing a new perspective to define parameters related to the definition of new IMI and cures. Specific antibody levels against the four recombinant proteins included in the vaccine were significantly increased in the vaccinated group during the observation period although they could not be associated with a protective effect of the vaccine. Further research will be needed to characterize the cellular-based immune response against the vaccine and to test the vaccine in a higher number of animals from dairy herds with distinct *S. aureus* populations.

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Declarations of interest

None

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Figure 1. Dendrogram of *S. aureus* isolated from lactating vaccinated and control cows. SmaI PFGE typing discriminated 5 different pulsotypes. References: Transient indicates that was isolated only once from a mammary quarter. IMI: intramammary infection indicates that was isolated more than once from a mammary quarter (number between brackets indicates times that was isolated).

Figure 2: Image A shows the time in months until a new *S. aureus* IMI in each of the treatment groups. Image B shows the cumulative risk to be positive to a new *S. aureus* IMI.

Figure 3: Specific IgG detection in sera of heifers and cows immunized with recombinant FnBPA, ClfA, α-toxin and β-toxin, formulated with a combination of liposomes and CpG-ODN, by indirect ELISA, during two doses immunization scheme. Sera were tested in a 1/1000 dilution against single antigens: A, C, E, G: IgG anti-FnBPA, anti-ClfA, anti-α-toxin and anti-β-toxin during two doses immunization scheme, respectively. B, D, F, H: IgG anti-FnBPA, anti-ClfA, anti-α-toxin and anti-β-toxin after third dose, respectively. Sera from no immunized heifers and cows were used as negative controls. A generalized linear model (with gamma distribution and log link function) for each antigen and sampling time was performed. Mean ± SD of sera from each group (according to specifications in Materials and Methods: Antibody response) is shown. ***p<0,001 compared with control group. Arrows indicate immunization times.

Figure 4: Specific IgG1 and IgG2 detection in sera of heifers and cows immunized with recombinant FnBPA, ClfA, α-toxin and β-toxin, formulated with a combination of liposomes and CpG-ODN, by indirect ELISA. Sera were tested in a 1/1000 dilution against single antigens: A and B- IgG1 anti-FnBPA, anti-ClfA, anti-α-toxin, anti-β-toxin, evaluated at 14 days after second dose and 14 days after third dose, respectively. C and D- IgG2 anti-FnBPA, anti-ClfA, anti-α-toxin, anti-β-toxin, evaluated at 14 days after second dose and 14 days after third dose, respectively. Sera from no immunized heifers and cows were used as negative controls. A generalized linear model (with gamma distribution and log link function) for each antigen was performed. Mean ± SD of sera from each group (according to specifications in Materials and Methods: Antibody response) is shown. **p<0,01; ***p<0,001 compared with control group.

Figure 5: A- Haemolytic activity inhibition of native α-toxin by sera of heifers immunized with recombinant FnBPA, ClfA, α-toxin and β-toxin, formulated with a combination of liposomes and CpG-ODN. Sera obtained 14 days after second dose (day 4 post calving) were used. An

extract containing α-toxin was pre-incubated with 1/50 dilutions of sera and then incubated with rabbit red blood cells (RBC) overnight. Apha-toxin extract incubated with RBC without sera pre-incubation was considered 100% of haemolysis. Percentage of haemolysis for each serum is shown. A Mann-Whitney statistical analysis was performed. Median and RI of sera from each group (according to specifications in Materials and Methods: Haemolytic activity inhibition of native α-toxin) is shown. **p<0,01; compared with control group. B- Matrix diagram presenting the humoral immune response against recombinant α-toxin in sera obtained 14 days after the second dose (day 4 post calving). Rows represent animals in each group and columns represent determinations. The first 3 columns correspond to IgG, IgG1 and IgG2 OD values, respectively. The red, yellow and green colors represent low, medium and high OD values corresponding to antibodies levels. The fourth column corresponds to percentage of haemolysis. The red and green colors represent high (near 100%) and low (near 0%) percentage of haemolysis. At the bottom of the matrix are shown the Spearman correlation results for each immunoglobulin vs percentage of haemolysis. Rs: Spearman's rank correlation coefficient. ***p<0,001, correlation probability.

Table 1. Incidence of new *Staphylococcus aureus* IMI in Vaccine and Control groups during the first and second year.

	Quarters with new *S. aureus* IMI (first year)	Animals with new *S. aureus* IMI (first year)	Quarters with new *S. aureus* IMI (second year)	Animals with new *S. aureus* IMI (second year)
Vaccine	2/252 (0.79%)	2/63 (3.17%)	0/80 (0%)	0/20 (0%)
Control	5/248 (2.01%)	5/62 (8.06%)	2/96 (2.08%)	2/24 (8.33%)

1. The proportion of animals that developed new *S. aureus* IMI was higher in the Control group compared with the Vaccine group, representing a reduction of 60.7%, although not statistically significant.
2. Time to new *S. aureus* IMI higher for animals in the Vaccine group in comparison to animals in the Control group, although not statistically significant.
3. Molecular identification of the isolates allowed differentiation of PT causing transient and persistent intramammary infections.
4. Specific antibody levels against the four recombinant proteins included in the vaccine were significantly increased in the vaccinated group during the observation period.
5. Recombinant α-toxin included in the vaccine generated antibodies that reduced significantly the haemolytic activity of native α-toxin.