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ORIGINAL ARTICLE

# Evaluation of a new vaccine against *Staphylococcus aureus* mastitis in dairy herds of southern Chile. I. Challenge trial

**Armin Mella[a,b], Fernando Ulloa[a,c], Iván Valdés[d], Nicolás Olivares[a], Alejandro Ceballos[e], Juan Kruze[a*]**

**ABSTRACT.** *S. aureus* is the most frequently isolated mastitis pathogen in southern Chile. Hygiene during milking time and DCT have not been successful to control this pathogen. Vaccination has been suggested as a useful tool to combat mastitis and several vaccines have been developed and evaluated worldwide. This study presents the results of a pilot trial to evaluate the effects of a new vaccine produced in Chile against *S. aureus* mastitis on udder health, humoral immune response, and milk production after an experimental intramammary challenge with a heterologous *S. aureus* strain. Four 2 mL doses of the vaccine were administered intramuscularly to five pregnant heifers free of IMI infections, using another five heifers as controls. Ten days after last immunisation two mammary quarters of all heifers were challenged with a pathogenic strain of *S. aureus*. Heifers were monitored for clinical signs, bacterial count, SCC, differential leukocyte count in blood, *S. aureus* antibody, and milk yield during 14 days after challenge. No systemic effects were observed in any of the challenged heifers, and severe clinical cases were only observed in the control group. The SCCs were always higher in heifers of the control group. The challenged quarters of the control group yielded the highest counts of *S. aureus*, but no significant difference was observed between vaccinated and non-vaccinated groups. Serum antibody titres were twice as high in the vaccinated group compared to the control group, and average milk yield reduction was lower in the vaccinated group. In conclusion, this vaccine was able to stimulate the humoral immune response of vaccinated heifers which could have a beneficial effect against new IMI by *S. aureus* and help to combat this mastitis pathogen.

*Key words:* mastitis, vaccine, *Staphylococcus aureus*, challenge trial.

**RESUMEN.** *S. aureus* es el patógeno mamario más frecuentemente aislado en el sur de Chile. La higiene durante la ordeña y la terapia de secado no han sido exitosas para controlar estos patógenos y la vacunación podría ser una alternativa. Varias vacunas se han desarrollado en otros países. Este estudio presenta los resultados de un ensayo piloto para evaluar los efectos de una nueva vacuna producida en Chile contra mastitis bovina por *S. aureus* sobre la salud de la glándula mamaria, la respuesta humoral, y la producción de leche, posterior al desafío experimental intramamario con una cepa heteróloga de *S. aureus*. Se vacunaron intramuscularmente cinco vaquillas preñadas con 4 dosis de 2 mL de la vacuna, dejando otras cinco vaquillas como control. Diez días después de la última inmunización, dos cuartos mamarios de cada vaquilla fueron desafiados con una cepa patógena de *S. aureus*. Los animales fueron monitoreados durante 14 días postdesafío por signos clínicos, recuento bacteriano, RCS, recuento diferencial de leucocitos, anticuerpos anti-*S. aureus,* y producción de leche. No hubo alteraciones sistémicas en los animales vacunados y un caso severo de mastitis clínica se presentó en el grupo control; los animales controles siempre tuvieron los RCS más elevados y los cuartos desafiados del grupo control arrojaron los recuentos bacterianos más elevados, pero sin diferencia significativa; los títulos de anticuerpos fueron más elevados en el grupo vacunado, y la reducción promedio de la producción de leche fue más baja en el grupo vacunado. Se concluye que la vacuna estimuló la respuesta inmune humoral en las vaquillas vacunadas, lo que podría tener un efecto beneficioso para la glándula mamaria contra nuevas infecciones por *S. aureus*.

*Palabras clave:* mastitis, vacuna, *Staphylococcus aureus,* desafío experimental.

## INTRODUCTION

Bovine mastitis is one of the most frequent and costly infectious diseases present in all dairy operations worldwide. Several microorganisms have been described to produce bovine mastitis but most cases are caused by different species of *Streptococcus*, *Staphylococcus* and coliform bacteria (Blowey and Edmondson 2010). *S. aureus* is considered the most contagious mastitis pathogen of dairy cattle in most countries and it is normally related to subclinical or chronic mastitis. In Chile, *S. aureus* has been the most frequently mastitis pathogen isolated from clinical and subclinical mastitis during the last decades in dairy herds, accounting for 30-50% of all mastitis cases (San Martín *et al* 2002, Reyes-Jara *et al* 2016). The major reservoir for infection is the udder and transmission occurs during milking time (Middleton *et al* 2006), therefore control has been focused on milking hygiene and dry cow therapy (Dodd *et al* 1969). However, the cure rate of antibiotic treatment for this mastitis pathogen is very low and the excessive use of antibiotics for the treatment of mastitis and other infectious diseases in domestic animals have induced the emergence of highly resistant bacterial strains to antibacterial drugs, making more difficult to control *S. aureus* mastitis (San Martín *et al* 2002). Consequently, the disease has not been effectively

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[a]Mastitis Laboratory, Biochemistry and Microbiology Department, Faculty of Sciences, Universidad Austral de Chile, Valdivia, Chile.

[b]PhD Program in Veterinary Sciences, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile.

[c]MSc Program in Microbiology, Faculty of Sciences, Universidad Austral de Chile, Valdivia, Chile.

[d]Veterquímica S.A., Santiago, Chile.

[e]Department of Agricultural Sciences, Universidad de Caldas, Manizales, Caldas, Colombia.

*Corresponding author: J Kruze; POB 567, Valdivia, Chile; jkruze@uach.cl

controlled and in many herds continues to be the most prevalent infection.

During the last years, vaccination has been introduced to combat bovine mastitis by *S. aureus* (Pereira *et al* 2011, Schukken *et al* 2014, Bradley *et al* 2015). Vaccination against mastitis pathogens is a good control strategy and can be used as a complement of traditional control programs based upon hygiene and management. Because *S. aureus* responds poorly to antibiotic therapy, vaccines against this mastitis pathogen have been studied and suggested as an important tool in the management of staphylococcal infections in dairy cows (Pereira *et al* 2011). Numerous attempts to develop a vaccine against *S. aureus* using different approaches have been made including whole organism vaccines, DNA vaccine encoding clumping factor A, live attenuated *S. aureus*, capsular polysaccharide CPS-protein conjugate vaccines, and DNA recombinant *S. aureus* protein (Pereira *et al* 2011). Currently in the USA there are two commercial mastitis vaccines available to control bovine mastitis against *S. aureus,* Somato-Staph and Lysigin (Middleton *et a*l 2009). More recently, the Spanish Laboratory HIPRA has developed a new vaccine against *S. aureus* and *E. coli* (Startvac) which is also directed to coagulase-negative staphylococci (Schukken *et al* 2014). The effectiveness of theses vaccines is variable and contradictory. It is generally accepted that commercially available *S. aureus* vaccines have limited ability to prevent new infections, do not significantly affect somatic cell count in milk although the vaccine could enhance the spontaneous cure rates of intramammary infections and decrease the development of clinical symptoms (Pankey *et al* 1983, Middleton *et al* 2009, Schukken *et al* 2014). In Chile only the J5 vaccine against *E. coli* is commercially available as Enviracor J5 (Pfizer Animal Health) to combat bovine mastitis by this pathogen.

The main objective of this study was to evaluate the effects of a new vaccine produced in Chile against *S. aureus* mastitis on udder health (clinical signs, bacterial shedding in milk, SCC), the humoral immune response, and milk production after an experimental intramammary challenge with a heterologous *S. aureus* strain, as part of a large research project involving experimental and commercial dairy herds in southern Chile.

## MATERIAL AND METHODS

### BACTERIAL STRAIN FOR VACCINE DEVELOPMENT

Twenty field strains of *S. aureus* isolated from subclinical bovine mastitis in southern Chile were used for the selection of the vaccine isolate. The strains were analised at the Research and Development Laboratory of Veterquímica S.A. by means of RFLP-PFGE, REP-PCR, and ERIC-PCR. It was observed that all characterised strains belonged to the same group with no significant genetic differences. Furthermore, the degree of virulence was determined in 6 of the 20 field strains tested selecting the most representative and most pathogenic strain for vaccine development (*S. aureus UACh-68*) (Valdés *et al* 2013). The vaccine, an inactivated bacterin, was developed at the same Laboratory following own company´s production protocols, and formulated as an injectable suspension in an aqueous polymer at a concentration of $8\times10^8$-$1.6\times10^9$ cfu/mL.

### CHALLENGE STRAIN

*S. aureus UACh-70*, a pathogenic field strain isolated from clinical mastitis in a dairy herd in southern Chile was used for the challenge trial. This strain was grown in Heart Brain Infusion Broth (Difco, USA) for 24 h at 37 °C and harvested by centrifuging at 3000 x *g* for 15 min at 4 °C. The pellet was resuspended in non-pyrogenic PBS (pH 7.6) and washed, and the bacterial concentration determined by serial dilutions on blood-agar plates according to methodology described by Leitner *et al* (2003). The capacity of this strain to cause clinical mastitis in dairy cows was previously evaluated in a short experimental trial (data not shown). Two mammary quarters of four lactating cows free of intramammary infections and with < 200,000 cells/mL were challenged via intracisternal administration with 1000 cfu/mL of this strain. Cows were monitored daily for clinical signs, bacterial count, somatic cell count (SCC), and milk yield during 14 days post challenge. Linear mixed models used for data analysis included the fixed effects of the independent variable treatments (intramammary challenge or not) and time (0 to 14 days post challenge).

### ANIMALS

The trial was conducted in a dairy herd at the Experimental Research Station, Universidad Austral de Chile, using 10 healthy Holstein Friesian pregnant heifers (7 mo.), free of intramammary infections determined by three negative bacterial cultures. The heifers were milked twice a day in a separate milking unit and milk yield automatically recorded daily. The assay was performed in accordance with the Bioethics Committee for the use of animals in research currently in force at Universidad Austral de Chile. All animals were kept in the same management system of the experimental herd, with the same food regime on pasture and concentrate supplements given during milking time.

### EXPERIMENTAL DESIGN

Heifers were randomly allocated to 2 experimental groups of 5 animals each; heifers of one group were immunised intramuscularly (rump) with 4 doses of 2 mL of the vaccine, 45 and 15 days before expected calving and 15 and 35 days after calving; the remaining 5 heifers were used as control non-vaccinated group and received 2 mL PBS as a placebo. Ten days after the last immunisation, two mammary quarters (LF and LR) of all heifers (vaccinated

and controls) were challenged via intracisternal with 1 mL of a bacterial suspension containing 1000 cfu/mL of *S. aureus UACh-70*; 1 mL of PBS were intramammary inoculated into the remaining two quarters of each animal and were used as controls. Two days before challenge, and thereafter daily during 14 days, animals were monitored for clinical signs, bacterial count, quarter SCC (qSCC), differential leukocyte count in blood, *S. aureus* antibody in serum, and milk yield.

### CLINICAL EXAMINATION

Two days before challenge and daily after challenge the udder of each heifer was carefully examined after each milking by manual palpation of each mammary quarter to detect any abnormalities such as swelling, indurations, pain, redness, and body temperature rise (rectal temperature recorded daily with a digital thermometer). Abnormalities of milk secretion were detected in foremilk samples prior to each milking using a strip cup and the secretion was classified as normal, aqueous, viscous, with clots, blood, or pus. The scoring system (0-7) based only on local udder signs described by Wenz *et al* (2006) was used to evaluate the severity of clinical signs of vaccinated and unvaccinated heifers after the challenge. Animals with a total score of 0-2, 3-4, and 5-7 were classified as mild, moderate, and severe clinical mastitis, respectively.

### BACTERIOLOGICAL ANALYSIS

Aseptic quarter foremilk samples were taken from all heifers according to the International Dairy Federation (1985) procedures during three consecutive occasions before experimental challenge for bacteriological examination. Only heifers with all three negative cultures were eligible to enter the trial. After challenge, daily quarter foremilk milk samples from all heifers were aseptically collected for *S. aureus* isolation during the 14-days evaluation period. Bacteriological analyses were carried out following accepted standards recommended by the National Mastitis Council, USA (Hogan *et al* 1999). A 0.01 mL aliquot from each fresh milk sample was cultured on blood-agar plates (Oxoid Diagnostic Reagents, UK) containing 5% sheep blood cells. Plates were incubated at 37 °C and examined for growth at 24-48 h. Colonies suspected to be staphylococci based on morphology were sub-cultured on a new blood-agar plate and tested for coagulase production by the tube test with rabbit plasma with EDTA (Bactident Coagulase, Merck, Germany); for the identification of *S. aureus* the rapid kit STAPHYTECT PLUS (Oxoid Diagnostic Reagents, UK) was used.

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### SOMATIC CELL COUNT

Two days before challenge, and daily after challenge during 14 days, individual quarter foremilk samples from all heifers were collected into 50 mL plastic, disposable bottles with bronopol as preservative, and submitted to a commercial laboratory (Labsur, Osorno, Chile) for qSCC. Cell counting was performed using flow cytometry technology in the Fossomatic™ FC 5000 equipment (Foss, Denmark).

### DIFFERENTIAL LEUKOCYTE COUNT

Blood samples (5 mL) were collected by coccygeal venipuncture into Vacutainer tubes with heparin sodium (Becton Dickinson, USA) right before challenge ($T_0$) and then at day 3 ($T_3$), day 7 ($T_7$) and day 10 ($T_{10}$) after challenge. The samples were submitted to the Pharmacology Department of the Faculty of Veterinary Sciences, Universidad Austral de Chile, for leukocyte count using flow cytometry with an Accuri C6 flow cytometer (BD Biosciences, USA), according to the procedure described by Leitner *et al* (2000). Tubes containing 200 µL of total blood were added with 2 mL of lysing buffer (BD Pharm Lyse) and incubated for 15 min at room temperature (RT). The tubes were centrifuged at 200 *g* for 5 min, the supernatant discarded, the pellets resuspended in 1 mL of stain buffer (BD Pharmingen) and washed again by centrifugation at 200 *g* for 5 min. Staining was performed as follows: after washing, 50 µL of each mAb or PBS (as a negative control) were added to cell pellets in each tube, mixed gently and incubated for 1 h at 4 ºC. After further washing (three times), 50 µL of conjugated antibodies were added, mixed gently, incubated for 30 min at 4 ºC, washed again, and resuspended in Phosphate Buffered Saline (PBS) to a final volume of 500 µL. Differential leukocyte subsets were determined using the following monoclonal antibodies (Veterinary Medical Research & Development, VMRD Inc., Pullman, WA): anti-lymphocyte (anti-CD4+: CACT 138A, anti-CD8+: CACT 80C and anti-B: BAQ44A); and (3) anti-granulocyte [CH138A (G1) and MM20A (G2)]. All mAbs were species-reactive with bovine cells. The polyclonal antibody used was goat F(ab´) 2 anti-mouse IgG (H + L) conjugated with fluorescein isothiocyanate (FITC) (Jackson Immuno Research Laboratory, West Grove, PA, USA). To calculate the percentages of the various leukocytes, 10,000 events were read per sample. The relative percentage for each cellular subset was determined in the forward scatter (FSC) versus side scatter (SSC) plot. The results are given as the percentage of positive cells labeled by each relevant mAb.

Because differential leukocyte count by flow cytometry does not disclose the number of different types of blood cells but rather establishes percentage differences between different cell lines, it was decided to count the total leucocyte number in blood in parallel with the count by flow cytometry, according to the methodology described elsewhere (Wittwer 2012), using an automated hematology analyzer (Sysmex KX-21N, USA).

### SERUM ANTIBODY DETECTION

*S. aureus* antibody in serum was determined in all animals before immunisation ($T_0$) and ten days after each immunisation using a homemade ELISA test, and performed according to a modified protocol described by Toro *et al* (2007). Flat-bottomed 96-well microtitre plates (Nunclon, Denmark) were coated with 100 µL (100 µg/mL) of a suspension in PBS (pH 7.4) of a fibronectin binding protein (FnBP), a specific *S. aureus* superficial polypeptide. The plates were incubated at 4 °C overnight and then washed 2x with PBS 1x plus 0.1% Tween 20. Then the remaining free-binding sites were blocked with 200 µL/well of blocking solution (3% horse serum + 3% chicken albumin + 0.5% Tween 20 in PBS 1x), incubated at 37 °C for 2 h, and then washed 2x with the same washing solution. For sample preparation, test and control sera were diluted 1:200 in an antibody solution (PBS 1x plus 1% horse serum plus 0.1% Tween 20) followed by two-fold dilutions up to a final dilution 1: 51,200; 10 serum samples were tested per plate leaving the remaining two columns of the plate for control samples (antibody solution alone). After incubation at 37 °C for 2 h the plates were washed 5x with a washing solution B (PBS 1x + 0.5% Tween 20), and the plates with the last washing solution were left at RT for 5 min. Plates were dried by hitting several times onto an absorbent paper, and then 100 µL/well of a conjugate antibody anti-bovine-HRP diluted 1: 10,000 was added. Plates were incubated at 37 °C for 2 h and washed five times with the washing solution B, and the plates with the last washing solution were left at RT for 5 min. Plates were dried as before and 100 µL/well of TMB solution (Thermo Fisher Scientific Inc., USA) were added. After incubation for 10 min at RT in a dark room, 100 µL/well of $H_2SO_4$ 3N were added to stop reaction. Plates were read in a microtitre reader (Synergy 2, BioTek Instruments, Inc., USA), and the optical density value of each test sample was compared with the average OD of control samples. The final antibody titres of each test sample was calculated considering the OD of the dilution exceeding at least 3 times the average OD values of control samples.

### DAILY MILK PRODUCTION

To evaluate the effect of the experimental challenge with *S. aureus* on milk production, daily milk yield was recorded on 3 consecutives occasions before challenge ($T_{-5}$, $T_{-3}$, $T_0$), and then daily for 14 days after challenge. The average milk production per cow before and after challenge was obtained and the percentage reduction in each experimental group was calculated.

### STATISTICAL ANALYSIS

The statistical program STATA V.14.1 (Stata Corp., College Station, TX, USA) was used to determine significant differences between vaccinated and non-vaccinated animals. Significant results were set at $P<0.05$. Data analysis for somatic cell count, bacterial count, and milk production were conducted using lineal regression mixed models, where the cow was considered a random variable to account for clustering of the observations within the cow. Data related to clinical signs of the mammary gland were analysed using descriptive statistics, such as mean and standard deviation for the variable of interest (Dohoo *et al* 2003). To analyse the survival time of *S. aureus* intramammary infection after challenge the Cox regression model was applied (Walters 2012). In order to normalise the bacterial count data of *S. aureus* shed in milk after challenge, the values obtained were transformed to $log_n$ scale. Because numerous bacterial counts resulted in zero values, 0.5 was added to each bacterial count and the adjusted mean of each group was calculated. For this purpose, a mammary quarter was considered infected only when a bacterial culture of such quarter was positive.

## RESULTS

### CLINICAL EXAMINATION

Throughout the study period vaccinated animals showed no adverse reactions due to vaccination, except a mild local swelling (<1 cm diameter) at the injection site (rump) in some animals that lasted only 2-3 days. There were no calving problems among vaccinated heifers or disturbances of calves at birth. Throughout the experimental period no systemic effects (fever) were observed in any of the heifers after challenge. No significant difference in rectal temperature was observed between control group and vaccinated group after challenge ($P>0.05$). Averages rectal temperature post challenge was 37.7 °C for the vaccinated group and 37.9 °C for the control group.

Throughout the evaluation period, 9 out of 10 mammary quarters of the vaccinated group developed mild mastitis and only one quarter developed a moderate inflammation three days after challenge; in the control group, however, 4 out of 10 mammary quarters developed mild mastitis, 5 out of 10 moderate clinical mastitis starting three days after challenge, and only one severe case was observed at day 5 after challenge.

### BACTERIOLOGICAL ANALYSIS

The challenged mammary quarters of the unvaccinated control group yielded the highest counts of *S. aureus* throughout the follow-up period except for the day 1 post challenge (figure 1); the average values considering both positive and negative cultures obtained for each group were 200.5 cfu/mL for the vaccinated group and 520.0 cfu/mL for the unvaccinated control group. However, there was no significant difference between vaccinated and controls ($P>0.05$). *S. aureus* was not isolated from

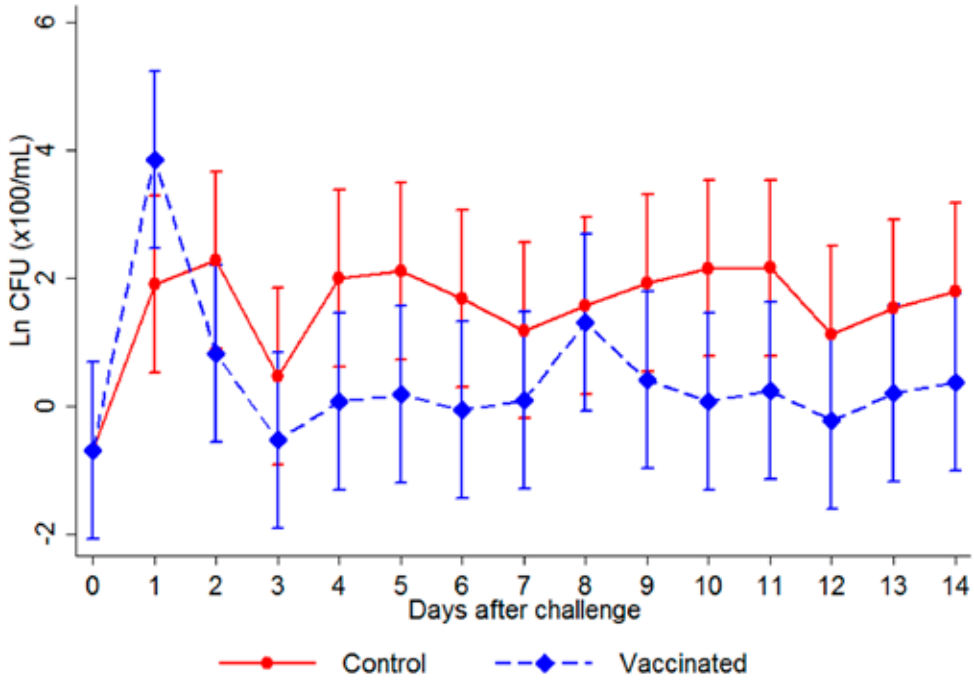



**Figure 1.** Adjusted mean of daily counts (cfu/mL) of *S. aureus* shed in milk after challenge in vaccinated and control animals.

the milk of non-inoculated quarters. Among vaccinated heifers, *S. aureus* was isolated at the beginning (day 1) of the follow-up period only one day after challenge in 3 out of 10 challenged quarters; however, among the challenged quarters of control heifers *S. aureus* was isolated for several days after challenge and, in most cases, the pathogen was isolated until the end of the follow-up period.

Regarding the survival of *S. aureus* in the infected mammary glands, it is interesting to note that most intramammary infections in vaccinated and control heifers occurred within the first days post challenge. The Cox regression model to analyse time to event outcomes showed that at day 3 after challenge more than half of all heifers were infected with *S. aureus*. All mammary quarters of the control group were infected at day 5, however, among vaccinated heifers, infection in all mammary quarters occurred only 9 days after challenge (figure 2).

## SOMATIC CELL COUNT

Before challenge all mammary quarters of vaccinated and non-vaccinated heifers had less than 50,000 cells/mL, however, somatic cell counts increased from day 2 post challenge and began to decrease from day 6 only among vaccinated heifers; cell counts were always higher in the control group. At the end of the 14-day period, the average qSCC in the vaccinated and control groups were, respectively, 296,200 cells/mL (CI 95%, 138.4-634.0) and 634,100 (CI 296.2-1357.2) (table 1). Significant differences were observed between vaccinated and control groups when evaluating the interaction time/treatment ($P<0.001$); however, no significant difference was observed between vaccinated and non-vaccinated heifers when comparing the adjusted means of daily qSCC after challenge (figure 3).

## DIFFERENTIAL LEUKOCYTE COUNT

The effect of vaccination on the different cellular subsets evaluated is presented in table 2. Results show slight variations on the cellular percentages, particularly an increase on granulocytes (G1 and G2) at $T_3$ sampling time. However, there was no significant difference between vaccinated and control heifers (figure 4). The total leukocyte counts also showed no differences between the total number of leukocytes before and after challenge in the two experimental groups. The leukocyte counts before challenge and the average count after challenge were, respectively, 8,200 and 8,646 cells/ µL in the vaccinated group and 7,440 and 7,740 cells/µL, in the control group.

## SERUM ANTIBODY DETECTION

All animals showed antibody titres against *S. aureus* >1600 before the first immunisation, probably due to high natural exposure to this pathogen in the experimental herd. However, titres increased significantly after immunisation, especially in vaccinated animals after the first immunisation. Antibody titres in unvaccinated control animals remained relatively stable until the end of the observational period. At the end of the study, serum antibody titres against *S.*

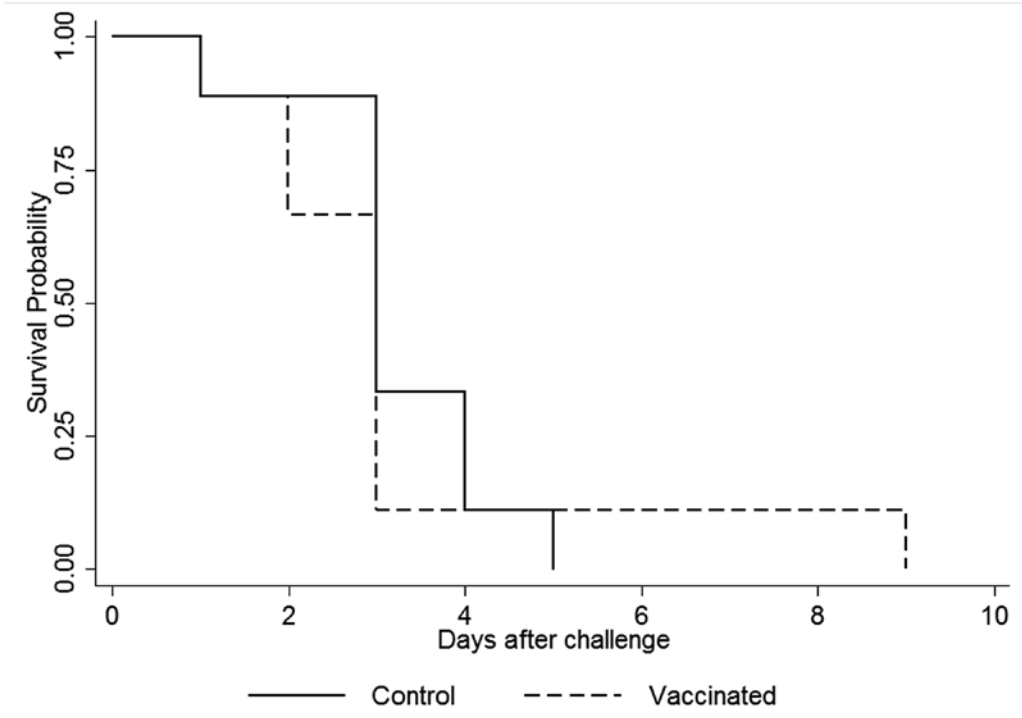



**Figure 2.** Survivor curve of *S. aureus* in vaccinated and control heifers estimated from Cox time to event regression analysis.

**Table 1**. Adjusted means of daily qSCC after challenge with *S. aureus* in vaccinated and control heifers.

| Group | Days after challenge with *S. aureus* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | Av |
| | Somatic cell counts (cells/mL x 1000) | | | | | | | | | | | | | | | |
| Vaccinated | 48 | 55 | 3327 | 2221 | 1523 | 1006 | 358 | 215 | 184 | 251 | 346 | 235 | 144 | 77 | 115 | 296 |
| Control | 47 | 102 | 1287 | 1779 | 1116 | 1927 | 675 | 1170 | 740 | 469 | 1017 | 525 | 899 | 496 | 683 | 634 |

**Table 2.** Differential leukocyte count in blood by flow cytometry in vaccinated and control heifers before ($T_0$) and after challenge ($T_3$, $T_7$, $T_{10}$) with *S. aureus.*

| Cells | Vaccinated group | | | | | |
|---|---|---|---|---|---|---|
| | $T_0$ (%) | $T_3$ (%) | $T_7$ (%) | $T_{10}$ (%) | Mean ($T_{3\text{-}10}$) (%) | Difference (%) |
| CD4+ | 16.23 | 7.93 | 17.12 | 11.48 | 12.18 | -4.05 |
| CD8+ | 7.95 | 4.38 | 8.03 | 6.69 | 6.37 | -1.58 |
| B | 1.24 | 0.73 | 0.72 | 0.71 | 0.72 | -0.52 |
| G1 | 22.14 | 32.01 | 21.25 | 27.46 | 26.91 | +4.77 |
| G2 | 26.94 | 38.04 | 21.99 | 30.52 | 30.78 | +3.24 |
| | Control group | | | | | |
| CD4+ | 17.84 | 10.56 | 16.43 | 13.38 | 13.60 | -4.24 |
| CD8+ | 7.57 | 5.26 | 6.88 | 6.32 | 6.15 | -1.42 |
| B | 1.37 | 1.04 | 0.86 | 0.93 | 0.94 | -0.36 |
| G1 | 22.41 | 32.08 | 18.01 | 24.63 | 24.91 | +2.50 |
| G2 | 27.18 | 34.61 | 26.04 | 25.88 | 28.80 | +1.62 |

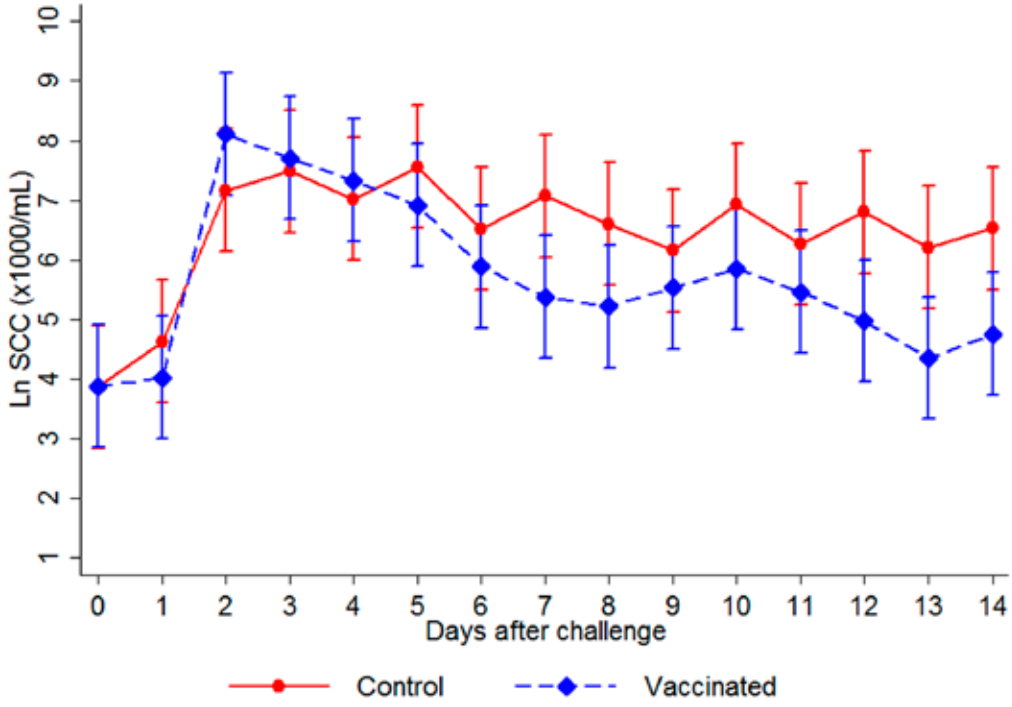



**Figure 3.** Adjusted means of daily qSCC after challenge with *S. aureus* of vaccinated and control heifers.

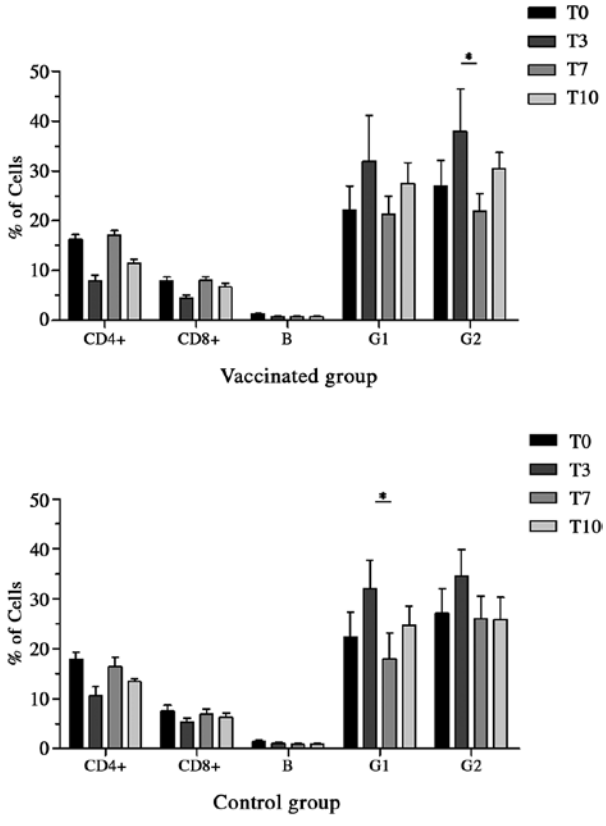



**Figure 4.** Differential leukocyte count in blood by flow cytometry in vaccinated and control heifers before ($T_0$) and after challenge ($T_3$, $T_7$, $T_{10}$) with *S. aureus.*

*aureus* were twice much higher in the vaccinated group than in the control group ($P$<0.05) (table 3, figure 5).

### DAILY MILK PRODUCTION

Milk production decreased in all animals (vaccinated and unvaccinated) after challenge with *S. aureus*, particularly in unvaccinated heifers where the milk yield reduction reached to 30.88% compared to only 3.47% of the vaccinated group (table 4). Statistical analysis of these results showed that there were significant differences between the vaccinated group and the control group when evaluating the interaction time/treatment ($P$<0.001); also the adjusted means of daily milk production after challenge showed significant differences between groups on days 3, 10, and 13 post challenge ($P$<0.05) (figure 6).

## DISCUSSION

Common mastitis control measures, such as hygiene during milking time, post-milking teat dipping, dry-cow therapy, lactation treatment of clinical cases, and disinfection of the milking machine at the end of the milking, have been applied in Chile for more than 40 years, however, mastitis still remains as one of the most frequent and costly diseases of dairy herds, with *S. aureus* being the most frequent mastitis pathogen, particularly in southern Chile (San Martín *et al* 2002, Reyes-Jara *et al* 2016). Most infections with this pathogen are subclinical and of long duration and the major economic effect of this type of

**Table 3.** Geometric mean of antibody titres against *S. aureus* FnBP before and after each immunisation in vaccinated and control heifers.

| Group | 1st Immunisation (45 d pre-calving) | | 2nd Immunisation (15 d pre-calving) | | 3rd Immunisation (15 d post-calving) | | 4th Immunisation (35 d post-calving) | |
|---|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After | Before | After |
| Vaccinated | 8.320 | 17.920 | 16.640 | 14.080 | 12.000 | 16.640 | 19.200 | 28.320 |
| Control | 3.840 | 4.160 | 5.120 | 4.160 | 8.960 | 5.120 | 4.480 | 10.880 |

$P<0.05$.

**Table 4.** Milk yield reduction after challenge with *S. aureus* in vaccinated and control heifers.

| Experimental group | Average milk yield (Lt/cow/day) | | % Reduction |
|---|---|---|---|
| | Pre challenge | Post challenge | |
| Vaccinated | 15.51 | 14.97 | 3.47 |
| Control | 16.32 | 11.28 | 30.88 |

$P<0.05$.

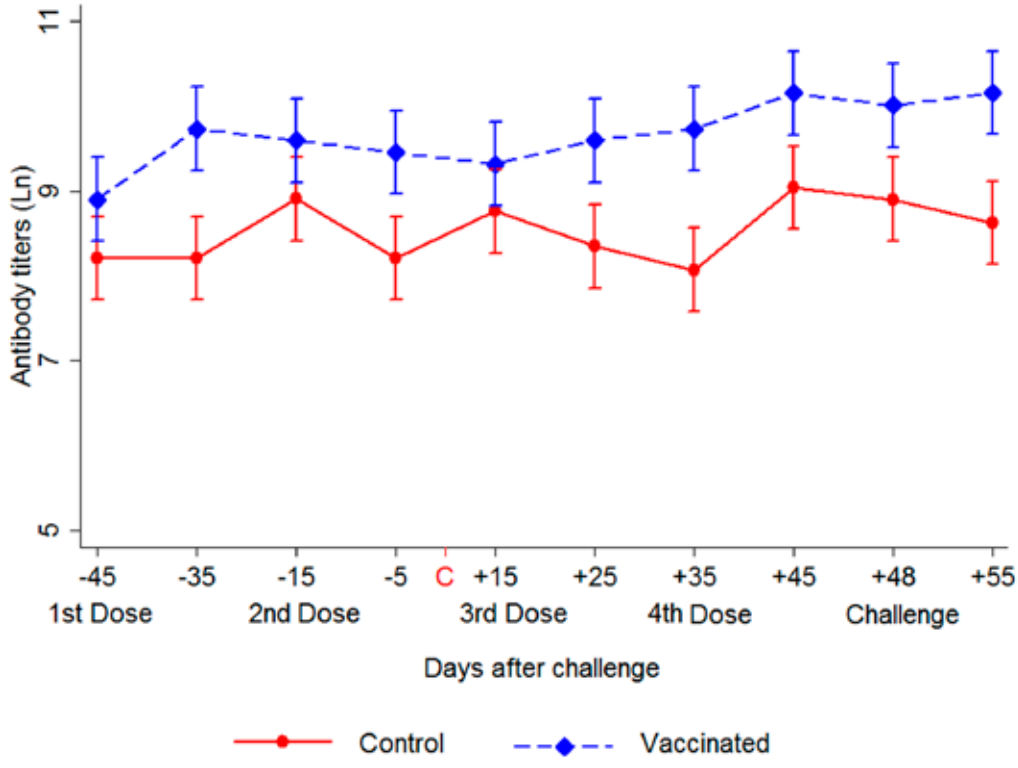



**Figure 5.** Adjusted means of antibody titres against *S. aureus* before and after each immunization in vaccinated and control heifers ($P<0.05$).

mastitis is reduced milk yield. The infected udder is the main reservoir of *S. aureus* and the cure rate of chronically infected cows is very low, therefore, culling these animals has been highly recommended to lower the prevalence of intramammary infection. Unfortunately, producers are reluctant to cull these chronic cows because they are high producing cows and it is costly due to the marked difference between the sale value and the replacement costs (Blowey and Edmonson 2010).

The failure to control the contagious *S. aureus* mastitis and the public concern about the emergence of antimicrobial resistance and drug residues in milk stress the need for alternatives to antibiotic therapy, and vaccination is emerging as an attractive alternative

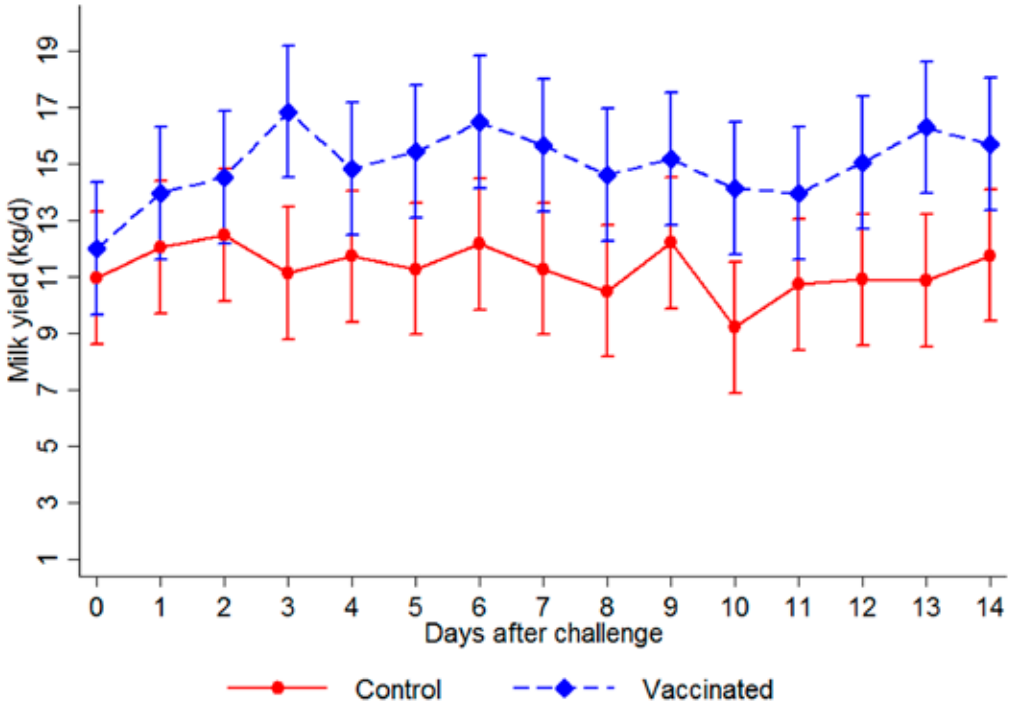



**Figure 6.** Adjusted means of daily milk production after challenge with *S. aureus* in vaccinated and control heifers ($P<0.001$).

approach for mastitis prevention and control (Middleton *et al* 2009).

In the present study four doses of the vaccine were administered, two doses before calving and two doses after calving to reach high antibody levels during the first month post calving, the period with higher susceptibility to intramammary infections (Sordillo and Streicher 2002).

No adverse effects were observed in vaccinated animals and, therefore, we can assure that the vaccine was safe; a similar result was observed by Pellegrino *et al* (2008) in an experimental challenge trial involving four pregnant heifers vaccinated subcutaneously 30 and 10 days before calving with two doses of a *S. aureus* avirulent mutant strain. No systemic effects (fever or loss of appetite) were observed in any of the vaccinated or non-vaccinated heifers after challenge with *S. aureus* and, therefore, in this study fever cannot be considered indicative of intramammary infection. Most mammary quarters of vaccinated animals showed no clinical signs of mastitis; however, in the unvaccinated animals most quarters developed clinical o subclinical mastitis. These results are in agreement with other studies which have demonstrated that vaccination can reduce both the development of clinical symptoms and the development of new subclinical mastitis infections (Pankey *et al* 1985, Watson 1992, Watson *et al* 1996, Middleton *et al* 2006), but disagree with others which have shown that vaccination did not significantly affect the rate of clinical mastitis (Tenhagen *et al* 2001). According to Rainard and Poutrel (1991), reduced severity of symptoms is probably mediated via antibodies neutralizing the *S. aureus* toxins, and this effect is easiest to generate with vaccination.

Although there was no significant difference between vaccinated and controls regarding the number *S. aureus* shed in milk post-challenge, starting on day 2 post-challenge control animals excreted always a larger number of cfu/mL throughout the study period. These results are consistent with those described by Pellegrino *et al* (2008) who in a similar challenge trial found that bacterial counts in the milk of vaccinated heifers was slightly lower than in the non-vaccinated group, but there was no significant difference. According to these authors, the heavy inoculum used for challenge (1000 cfu/mL), as it was the case in our study, is very different from natural infection and it may have obscured the protective effect of vaccination. A different inoculation regime was used by Middleton *et al* (2006) who inoculated 250 cfu/mL on three consecutive days based on a reported 49.7% failure rate of a single intramammary infusion of *S. aureus* to establish an intramammary infection (Schukken *et al* 1999); however, these authors found no evidence that cfu/mL of *S. aureus* recovered from milk post-challenge was lower in any of the vaccinated groups than control. The high count of *S. aureus* observed on day 1 after challenge in the vaccinated group (figure 2) can be explained because there were two mammary quarters shedding very high numbers of cfu (uncountable) which increased the average count. Regarding the survival of *S. aureus* in the infected mammary glands, it is interesting to note that most intramammary infections

in vaccinated and control heifers occurred within the first days post challenge (figure 3). The Cox regression model to analyze time to event outcomes showed that quarters of vaccinated heifers had a better health condition as they needed more time to get infected than the control ones. In fact, while in the control group all challenged quarters were infected on day 5 after challenge, in the vaccinated group all challenged quarters got infected on day 9 post challenge. These results suggest that the vaccine does not completely protect the mammary gland against *S. aureus* infections.

The effect of vaccination on somatic cells in milk has been controversial. Neutrophils, the main component of somatic cells in milk, are the second defense mechanism of the udder after the teat barrier to control an intramammary infection following a microbial invasion. Consequently, it was logical to expect an increase in somatic cell counts immediately after the challenge with *S. aureus* in both groups of animals; in the vaccinated group somatic cell counts returned to normality ($< 200\times10^3$ cells/mL) after day 6 post challenge, however in the control group somatic cells remained relatively high ($> 600\times10^3$ cells/mL) until the end of the follow-up period, probably because the udders of vaccinated animals were in a better health condition (table 1). Our results are consistent with those reported by Pankey *et al* (1985), Leitner *et al* (2003), and Pellegrino *et al* (2008). Although there was no significant difference in SCC between vaccinated and non-vaccinated groups, control animals ended the evaluation period with a numerically higher average count than vaccinated animals. Although there was no evidence for a reduction of SCC in the vaccinated group as a single effect, the vaccination had an effect on the reduction depending on the interaction time/treatment ($P<0.001$) (figure 3), this lower inflammatory response in immunised animals could be interpreted as a protective effect of the vaccine against intramammary infection by *S. aureus*. Several other trials have found contradictory results regarding vaccination effect on somatic cell count in milk, without evidence that vaccinated groups had a lower mean somatic cell count than controls (Middleton *et al* 2009).

Vaccination had no systemic effect neither on subsets of leukocytes nor on leukocyte direct counts because the measurement was performed on peripheral blood lymphocytes (PBL). In PBL only a super-antigen could increase by ten percent a population of cells (Janeway 2005). However, there was an increase in granulocyte populations (G1 and G2) in peripheral blood at $T_3$ sampling time followed by a significant decrease at $T_{10}$. This increment in $T_3$ seems to be due to the local challenge with *S. aureus*, and probably because of the stimulation of bone marrow as a result of the local inflammation in the mammary gland.

In order to know if the immune response of vaccinated animals was specific against *S. aureus*, serum IgG antibody titres were detected in all animals using a homemade ELISA test prepared with a peptide of 30 amino acids specific against fibronectin binding protein (FnBP), an important virulence factor of the bacterium. In this way, it was possible to know whether the immune response was specific against *S. aureus*, not being muddied by natural bovine antibodies against other structures of the bacterium homologous to other gram-positive bacteria. In addition, to explain how the vaccine works, which could be, among others, to generate antibodies to block this important mechanism of bacterial adhesion. In this study it was shown that vaccinated heifers had serum antibody titres significantly higher than control animals after each immunisation ($P<0.05$), which was particularly marked when comparing the antibody titres obtained before the first immunisation with those obtained after the last immunisation (table 3). These results demonstrate that the tested vaccine could provide some level of immunity due to increased blood antibodies since there is a close association between serum antibody titres and the status of infection. This association was demonstrated in Canada by Wagter *et al* (2000) when evaluating the humoral immune response in three Holstein dairy herds and finding that cows with a high antibody-mediated immune response had a lower incidence of mastitis. Later, this finding was confirmed by Thompson-Crispi *et al* (2012) with a larger number of animals, demonstrating that dairy cows with high immune response have a lower risk of developing infections such as mastitis and metritis, indicating that cows with an improved antibody response are better equipped to respond immunologically to the constant challenge of mastitis pathogens. Hypothetically, the protection conferred by the vaccine could be the result of an increased opsonization of bacteria via a higher antibody concentration in both blood and milk combined with more efficient phagocytosis and killing of bacteria by PMN. Enhancement of the PMN viability and activity could be another potential mechanism. However, the immunological basis of vaccination has not been yet elucidated (Piepers *et al* 2017).

Contradictory results have been reported regarding the efficacy of Lysigin™, a commercial vaccine against *S. aureus* available for several years in the USA market. In one study, heifers were intramammary challenged in one quarter with a heterologous strain of *S. aureus* in early lactation; all heifers were infected with *S. aureus* after challenge and the vaccinated group developed mostly mild mastitis cases which recovered faster compared to the control heifers. However, there were no significant differences in SCC or milk production between vaccinated and non-vaccinated animals (Middleton *et al* 2006). In an early study with the same vaccine, Nickerson *et al* (1999), vaccinated heifers at 6 months of age followed by a booster 2 weeks later and subsequent booster vaccinations every 6 months until calving. Vaccinated heifers had a 45% reduction in *S. aureus* IMI during pregnancy and new *S. aureus* IMI at calving relative to controls. These results suggest that administration of more doses of the vaccine can maintain adequate levels of circulating antibodies that can help to

eliminate an infection. This correlates well with our results, because significant differences in antibody titres between vaccinated and non-vaccinated groups were observed only after the administration of the third dose of the vaccine. On the other hand, in another work with the same Lysigin™ vaccine, anti-*S. aureus* antibody levels in milk were not different from control animals suggesting that vaccination provided minimal immune protection (Luby *et al* 2007). Finally, Prenafeta *et al* (2010) evaluated a new commercial vaccine produced in Spain (Startvac, HIPRA Laboratories) based on extracellular slime associated antigenic complex (SAAC) from *S. aureus*. Immunisation of pregnant heifers with the formulation containing a *S. aureus* strain expressing high SAAC content stimulated strong humoral responses in blood and milk, and was associated with reduced bacterial cell count and less severe clinical signs after experimental challenge. In our study, like most other vaccine assessment works, increased antibody titres and a lower but no significant bacterial count in the vaccinated group was achieved; however, there was no complete prevention of intramammary infection by *S. aureus*. Despite not having performed a study of correlation between antibody titres and bacterial counts, our results coincide with those of Prenafeta *et al* (2010), who suggest that antibodies from vaccinated animals may be involved in the elimination of bacteria and in reducing clinical signs after intramammary *S. aureus* infection.

The most important effect of the vaccine observed in this challenge trial was the reduced milk yield in the control non-vaccinated heifers compared to the vaccinated ones. As shown in table 4, the average reduction of milk yield after challenge was 10 times higher in controls animals compared to the vaccinated heifers. Statistical analysis of the data showed that there was a significant difference between vaccinated and control heifers only at day 3, 10, and 13 post challenge. It is well known that intramammary inflammation affects milk producing cells of the udder causing a decrease of milk production, and the greater the severity of inflammation, the greater the loss of production. As previously mentioned, severity of clinical signs among control heifers was higher than in the vaccinated group and which might result in a higher milk yield due to a reduced inflammation of the udder after vaccination. Vaccination effect on milk production has been reported by other researchers as well. In a similar challenge trial conducted in Argentina, Pellegrino *et al* (2008) reported a mean daily milk yield from inoculated quarters 20% higher than the non-vaccinated group 21 days after challenge, although this difference was not statistically significant. A significantly higher milk yield in vaccinated cows when compared to non-vaccinated cows has also been reported by several other authors (Watson 1992, Bradley *et al* 2015). However, Middleton *et al* (2006, 2009) when evaluating the efficacy of different Lysigin™ formulations in dairy heifers in the USA found no evidence that vaccinates had greater milk yield than controls post-challenge. As with the SCC, the results with milk production are controversial.

According to the results obtained in this study it can be concluded that the new vaccine was able to stimulate the humoral immune response in vaccinated heifers inducing a high level of serum antibodies against *S. aureus,* which could have a beneficial effect on the mammary gland against *S. aureus* new intramammary infections. However, it is important to recognise the limitations of this study considering the small number of animals and the short evaluation period.

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