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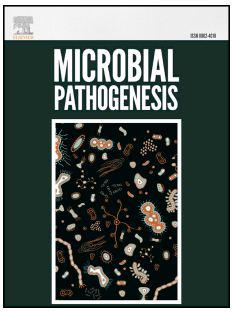

Differential immune response to two *Staphylococcus aureus* strains with distinct adaptation genotypes after experimental intramammary infection of dairy cows

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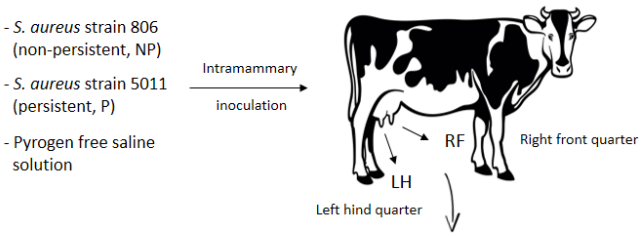
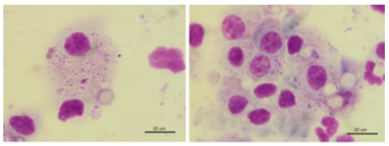
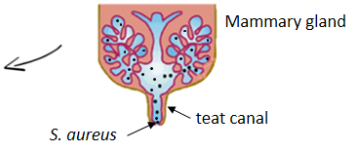




Cytospins from a pool of milk samples from quarters (RF+LH) of animals 1 and 2 showed stained cocci within macrophages between 1 and 3 d pi (1000x).



Milk at different days (d) post inoculation (pi)

	Bacterial isolation	Somatic cell count	Lactoferrin	Nitrites	IL-1β	IL-6	IL-4	IgGs
- Strain 806 (NP)	d 1 pi and up to d 3 pi	↑ 2, 3, 4 and 7 d pi	↑ 3 to 21 d pi	↑ d 3 pi	↑ 1 to 21 d pi	↑ 1 to 4 and 14 to 21 d pi	↑ d1 and 7 to 21 d pi	806 > 5011
- Strain 5011 (P)	d 7 pi and up to d 56 pi	↑ 14 and 21 d pi	↑ 4 and 21 d pi	↑ d 21 pi			↑ d 1 pi	

Differential immune response to two *Staphylococcus aureus* strains with distinct adaptation genotypes after experimental intramammary infection of dairy cows

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Abstract

The aim of this study was to evaluate and compare the ability of two *S. aureus* strains with different adaptation genotypes (low and high) to the bovine mammary gland (MG) to establish an intramammary infection (IMI) and induce an immune response after an experimental challenge in lactating cows. Two isolates (designated 806 and 5011) from bovine IMI with different genotypic profiles, harboring genes involved in adherence and biofilm production, belonging to different capsular polysaccharide (CP) type, accessory gene regulator (*agr*) group, pulsotype (PT) and sequence type/clonal complex (ST/CC) were selected. Strains 806 and 5011 were associated with low (nonpersistent-NP) and high (persistent-P) adaptation to the MG, respectively. Strain 806 (NP) was characterized as *agr* group II, *cap5* positive and ST350; strain 5011 (P) *agr* group I, *cap8* positive and CC188. Three groups of clinically healthy cows, 4 cows/treatment group, were inoculated by the intramammary route with strain 806 (NP), strain 5011 (P) and pyrogen-free saline solution. All mammary quarters challenged with strain 806 (NP) developed mild clinical mastitis between 1 and 7 d post inoculation (pi). Quarters challenged with strain 5011 (P) developed a persistent IMI; bacteria were recovered from milk from d 7 pi and up to d 56 pi. In quarters inoculated with strain 806 (NP) the inflammatory response induced was greater and earlier than the one induced by strain 5011 (P), since a somatic cell count (SCC) peak was observed at d 2 pi, while in quarters inoculated with strain 5011 (P) no variations in SCC were observed until d 4 pi reaching the maximum values at d 14 pi; indicating a lower and delayed initial inflammatory response. The highest levels of nitric oxide (NO) and lactoferrin (Lf) detected in milk from quarters inoculated with both *S. aureus* strains coincided with the highest SCC at the same time periods, indicating an association with the magnitude of inflammation. The high levels of IL-1β induced by strain 806 (NP) were associated with the highest SCC detected (d 2 pi); while quarters inoculated with strain 5011 (P) showed similar IL-1β levels to those found in control quarters. In quarters inoculated with strain 806 (NP) two peaks of IL-6 levels on d 2 and 14 pi were observed; while in quarters inoculated with strain 5011 (P) IL-6 levels were similar to those found in control quarters. The

strain 806 (NP) induced a higher total IgG and IgG_1 response; while strain 5011 (P) generated a higher IgG_2 response (even against the heterologous strain). The present study demonstrated that *S. aureus* strains with different genotype and adaptability to bovine MG influence the local host immune response and the course and severity of the infectious process.

Key words: Bovine mastitis; experimental intramammary infection; *Staphylococcus aureus*; immune response; persistent and nonpersistent strains

1. Introduction

Staphylococcus aureus is still one of the most prevalent pathogens associated with bovine intramammary infection (IMI) worldwide, causing important economic losses to dairy farming [1,2]. Early contact of *S. aureus* with the bovine mammary gland is associated with a delayed and moderate inflammatory response compared with mastitis caused by Gram negative bacteria (e.g. *Escherichia coli*) [3,4]. As a result these infections may very often establish and become persistent, leading not only to increased transmission between cows during milking time, but also to a loss of mammary function and fibrosis [5,6,7] which can in turn lead to culling of chronically affected cows. Classic antibiotic treatment in chronic cases is ineffective [8] and so far commercially available vaccines have shown limited success to prevent *S. aureus* IMI [2].

While significant advances have been made towards understanding the mechanisms employed by *S. aureus* to persist within the mammary gland (MG) [4], information available about host immune mechanisms evoked during chronic staphylococcal IMI is limited [9-12]. Chronic *S. aureus* IMI induces a sustained innate and adaptive immune response during lactation [9,10] or active involution [11,12] in bovine mammary tissue, however this response appears to be insufficient to eliminate the pathogen. It has been suggested that several phenotypic and genotypic characteristics are linked to *S. aureus* long-term persistence in the MG, including the capacity to form biofilms and to invade cells and/or survive intracellularly, the production of capsular polysaccharides (CP) and

the accessory gene regulator (*agr*) type of the strain [13,14]. In a previous study we used 20 selected *S. aureus* isolates from bovine IMI categorized as persistent (P) and non-persistent (NP) based on clinical behavior and presence of different genetic profiles. We demonstrated that *S. aureus* internalization into bovine mammary epithelial cells, which is considered an early bacterial-host interaction, was strain-dependent and that internalized bacteria overexpressed adherence and biofilm-forming genes, particularly those encoding FnBPs and IcaD, compared with organisms that remained in coculture supernatants [15].

Bacterial factors that contribute to intracellular persistence and host factors leading to *S. aureus* clearance or survival during IMI are poorly documented. To gain insights into the mechanism allowing *S. aureus* to successfully persist intracellularly in the MG, in a recent study, we evaluated and compared the ability of two *S. aureus* isolates from IMI with different adaptation genotypes (low and high) to the bovine MG to adhere/internalize, persist, and induce damage in a bovine mammary epithelial cell line (MAC-T). In addition, we evaluated the phagocytic and bactericidal capacity induced after the interaction between mammary macrophages with both *S. aureus* strains [16]. These isolates harbored genes involved in adherence and biofilm production and belonged to different CP type, *agr* group, pulsotype (PT) and sequence type/clonal complex (ST/CC) [16]. The non-persistent (NP) strain, selected for its low adaptation to bovine MG and characterized as *agr* group II, *cap5* positive, ST350 and weak biofilm producer, showed a low adhesion/internalization and intracellular persistence capacity in MAC-T cells. Conversely, the persistent (P) strain, selected for its high adaptation to bovine MG and characterized as *agr* group I, *cap8* positive, CC188 and strong biofilm producer showed high adhesion/internalization and persistence capacity in MAC-T cells. Although the P strain was recognized and phagocytized with greater efficiency by mammary secretion macrophages compared with the NP strain, it showed greater resistance to microbicidal mechanisms. From these results we hypothesized that the *in vitro* behavior of NP and P *S. aureus* strains regarding the ability to invade, persist and induce damage in MAC-T cells, could be associated with their *in vivo* behavior during a natural bovine IMI [16].

Mastitis outcome is determined by pathogen virulence and cow's immune response [17]. According to several *in vivo* studies different *S. aureus* strains trigger differential innate immune responses which can influence the course and severity of bovine mastitis [18,19]. In this regard, in a previous study we investigated the immune response in a mouse mastitis model induced by two *S. aureus* strains isolated from bovine IMI with different clinical manifestations (P and NP), phenotypic and genotypic profile [20]. This study revealed that the host immune response was different for each *S. aureus* strain throughout the course of infection, showing in general a greater initial response to strain NP compared with strain P and then a further immune response, mainly stimulated by strain P and consistent with the development of a chronic inflammatory process. Strain P, compared with strain NP, showed a greater adaptation to the MG, inducing a higher immune response in the advanced stages of IMI but with lower bacterial clearance from tissue suggesting differential bacterial strategies for overcoming host immune response. In this model, considering that the animals used were similar, the immune response observed against strains bearing specific pathogenic traits implied that pathogen factors, rather than host factors, could influence the host response to achieve persistence in the MG [21].

Results from both previous studies carried out *in vitro* with MAC-T cells and bovine macrophages [16] and *in vivo* in a mouse mastitis model [21] gave rise to the need to explore aspects of the immune response induced by distinct *S. aureus* strains using an experimental challenge model for inducing bovine mastitis. A better understanding both of the pathogen and the immune response aspects is crucial to delineate alternatives to classic control practices and to refine current strategies to intervene in the disease progress. Therefore, the aim of this study was to evaluate and compare the ability of two *S. aureus* strains with different adaptation genotypes (low and high) to the bovine MG to establish an IMI and induce an immune response after an experimental challenge in lactating cow.

2. Materials and methods

2.1. Cows

Twelve clinically healthy Holstein dairy cows (4 cows/treatment group) in mid lactation (weeks 31–36) from the School of Agriculture and Livestock of Universidad Nacional del Litoral (UNL) were used. Cows were from parity 2 to 3, milked twice daily in a herringbone milking parlor at 6:00 a.m. and 4:00 p.m., kept under grazing conditions (alfalfa pasture in the morning, ryegrass in the afternoon and corn silage and alfalfa at night), received concentrates in the milking parlor (6.5 kg per cow per day (18% CP) administered twice, 60% in the morning and 40% in the afternoon) and produced an average of 16 kg milk/day at the beginning of the study. The cows had no previous history of clinical mastitis and had not received antibiotic or anti-inflammatory medication forty days before the initiation of the experiment. Selection of each group of cows was based on milk somatic cell counts (SCC) < 150 × 10^3/mL, available from the local dairy herd improvement system, and negative bacterial culture in all quarters. Quarter foremilk samples were aseptically collected (21, 14 and 7 days prior to challenge) and 48 h before challenge according to standard procedures [21]. The first two streams of milk from each teat were discarded, the next 5 ml were collected in sterile plastic vials for bacteriological analysis and finally ~20 ml were collected for SCC.

2.2. Bacterial strains and preparation of S. aureus inocula

Two *S. aureus* strains (designated 806 and 5011) isolated from milk samples from two Holstein cows with subclinical mastitis that belonged to two different dairy herds, were used for the intramammary challenge. These strains were used in previous *in vitro* studies with MAC-T cells and bovine macrophages [16]. The *S. aureus* strain 806 was isolated only once from a mammary quarter of a cow in lactation and not re-isolated in three consecutive milk samplings (7, 14 and 21 days) following standard treatment with a beta lactam antibiotic for 3 days. It was considered to

153 have low adaptation to the bovine mammary gland and designated as NP. The *S. aureus* strain 5011
154 was isolated from the same mammary quarter of a cow in consecutive monthly milk samplings over
155 a period of six months during lactation. It was considered highly adapted to the bovine MG and
156 designated as P. To confirm that it was the same strain of *S. aureus* that was isolated over a 6-month
157 period, the genotypic profiles of *S. aureus* isolates obtained from the same quarter were compared
158 using pulse field gel electrophoresis (PFGE) [15]. Table 1 shows a summary of phenotypic,
159 genotypic and functional characteristics of *S. aureus* strains used in this study.

160 Before intramammary challenge, bacteria were activated from frozen stocks (−80 °C) by culture
161 on Columbia agar base (CAB) (Britania, Buenos Aires, Argentina) and incubated at 37 °C for 24 h
162 under aerobic conditions. Three colonies of each strain were inoculated into 5 ml of trypticase soy
163 broth (TSB) (Britania) and incubated for 16 h at 37 °C on a rotary shaker (150 rpm). Culture was
164 vortexed and diluted 1:100 in TSB (Britania) and incubated to mid-log phase for 2 h at 37 °C on a
165 rotary shaker (150 rpm). Immediately before intramammary challenge, bacteria were diluted to
166 reach approximately 250 colony forming units (CFU)/ml in pyrogen-free saline solution based on
167 previous direct plate counts carried out for each strain.

168

169 ***2.3. S. aureus intramammary challenge***

170 A schematic overview of the *S. aureus* intra-mammary challenge model is given in Fig. 1. Of the
171 12 selected cows, 4 were inoculated in the contralateral quarters (right front-RF and left hind-LH)
172 with 1 ml of the suspension containing *S. aureus* strain 806 (NP) (~250 CFU), 4 cows were
173 inoculated in the contralateral quarters (RF and LH) with 1 ml of the suspension containing *S.*
174 *aureus* strain 5011 (P) (~250 CFU) and 4 cows were inoculated with 1 ml of pyrogen-free saline
175 solution in the contralateral quarters (RF and LH). We used a separate group of control animals that
176 were independent from any of the *S. aureus* challenged animals as we also aimed to evaluate the
177 effect of the experimental infection on the systemic immune response (manuscript in preparation).
178 Intramammary inoculation of different bacterial suspensions and PBS was performed after the

afternoon milking as follows: teat ends were thoroughly swabbed with 70% ethanol and then a sterile plastic cannula attached to a disposable syringe was inserted through the teat canal. Following infusion, the teat was gently massaged in a dorsal direction [22]. Teats were dipped in 1% iodine teat dip after the infusion process. An aliquot of the bacterial inocula and the pyrogen-free saline solution were used for viable bacterial counts to determine the actual bacterial inocula and sterility check, respectively.

2.4. Progress of infection and sample collection

The animals were monitored daily until 21 d post-inoculation (pi), registering the general condition, MG clinical inflammation, appetite and milk production. Local inflammatory changes of MG and milk were detected following the scoring scheme proposed by Middleton et al. [23]: 0 = no overt changes in gland or milk, 1 = overt changes in milk with no observed MG inflammation, 2 = local inflammation of the MG accompanied by overt changes in milk and 3 = severe clinical mastitis with systemic symptoms. Milk samples were taken from each inoculated mammary quarter, pre-inoculation (time 0) and 0.5, 1, 2, 3, 4, 7, 14 and 21 days pi. Following this experimental period, the mammary quarters inoculated with *S. aureus* strains 806 and 5011 were further evaluated by bacterial culture for 5 weeks at 7 day intervals (28, 35, 42, 49, and 56 days pi) to monitor the development of chronic infections. General condition and presence of clinical mastitis was daily evaluated during this period. After the whole observation period was over (day 56), challenged animals were treated with intramammary antibiotics.

2.5. Bacteriological examination, SCC and cell microscopic observation

Milk samples were analyzed by standard bacteriological methods and identity of challenge strains was confirmed by molecular analysis. Milk samples (10 μl) were streaked onto blood agar plates supplemented with 5% bovine blood and incubated for 48 h aerobically at 37°C. Plates were examined for bacterial growth at 24 h and 48 h. *S. aureus* was presumptively identified based on the

hemolytic pattern on blood agar, catalase and coagulase tests and differentiated from other coagulase-positive staphylococci by acetoin production and selective growth on P agar with 7 μg/ml acriflavine [24]. The presence of one colony of *S. aureus* on blood agar was considered as a positive identification; therefore, detection limit was 100 CFU/ml. *S. aureus* colony counts (CFU/ml) were performed from the challenged mammary quarters immediately prior to infusion (d 0) and on days 0.5, 1, 2, 3, 4, 7, 14, 21, 28, 35, 42, 49, and 56 pi. Identity of *S. aureus* isolates recovered following experimental challenge was confirmed by PFGE of *SmaI*-digested chromosomal DNA fragments using a CHEF-DR II apparatus (BioRad Laboratories, CA, USA) as described previously [25].

Quarter milk samples for SCC determination were preserved with azidiol (0.3%) at 4°C and analyzed within 24 h. The SCC was performed by a commercial laboratory (Laboratorio Regional de Servicios Analíticos, Esperanza, Santa Fe, Argentina) using an automated counter (Somacount 300, Bentley Instruments, Minesotta, USA).

In order to detect the presence of *S. aureus* inside milk cells, cytospins from a pool of milk samples from mammary quarters (RF+LH) of each animal were prepared. Only the cytospins of animals with positive microbiological isolation in milk were stained. First, milk samples were defatted by two centrifugations at 600 x g 10 min. Pellet was resuspended in 1 ml of PBS and 100 µl of this solution was used per slide. Cytospins were centrifuged at 30 x g 10 min using a cytocentrifuge Cyto Tek 2500 (Sakura). Then, were dried and frozen at -20 °C until staining with May-Grünwald Giemsa Stain Kit (Abcam). Briefly, slides were fixed with methanol 2 min, stained for 3 min in May-Grünwald and by 1 min in distilled water. Then, the cytospin was incubated in Giemsa (one drop per 1 ml of distilled water) for 15 min. Finally, slides were examined by light microscopy for the presence of stained cocci.

2.6. Nitric oxide production

Evaluation of NO production in milk was carried out by measuring its most stable metabolite, nitrite (NO_2), following the methodology of Renna et al. [12] with modifications. First, milk

samples were defatted by centrifugation at 1500 x g 10 min. After removing the fat layer, 1 ml of supernatant was treated with 1.5 ml of cold sodium acetate 0.1 M, pH 4.0 to precipitate caseins. Samples were clarified by centrifugation (4000 x g 10 min) and supernatants were incubated 10 min in a boiling bath to inactivate proteases. After samples reached room temperature (RT), two more centrifugations were carried out at 10000 x g for 10 and 5 min. Once clarified supernatants were obtained, pH was adjusted to 7 with sodium hydroxide 5N. Processed samples were aliquoted and stored at -80°C until use.

This method allows for detection of nitrites formed by spontaneous oxidation of NO under physiological conditions [26]. Nitrite concentration was determined by Griess kit (Thermo Fisher Scientific, MA, USA). Briefly, 150 µl of clarified supernatants were placed in a 96-well plate, 150 µl of Griess reagent was added and incubated for 30 min at RT in the dark. Finally, absorbance at 550 nm was measured using the SPECTROstar Nano equipment (BMG Labtech). Nitrite concentration was calculated using a nitrite standard (sodium nitrite, provided in the kit).

2.7. Lactoferrin concentration

Milk samples were defatted by two centrifugations (1500 x g, 10 min; 20800 x g 30 min) at 4°C. Supernatants were aliquoted and frozen at -80°C until use. Lf production was evaluated by a commercial ELISA kit (Bethyl Laboratories. Inc., Montgomery, USA). Briefly, microplate was sensitized for 1 h at RT (20-25°C) with the capture antibody diluted 1/100 in carbonate/bicarbonate buffer 0.05 M, pH 9.6. After three washings, the plate was covered with blocking solution (Tris-NaCl buffer, 0.05% Tween 20, pH 8) for 30 min at RT. Then, processed samples (diluted 1/1600) and reagents for constructing a standard curve were placed in duplicate and incubated at RT for 1 h. After three washings, samples were incubated with the peroxidase-conjugated detection antibody (diluted 1/100000) for 1 h at RT. Reaction was revealed with the incubation of chromogen 3,3', 5,5'-tetramethylbenzidine (TMB) for 15 min and stopped with HCl 1N. Absorbance was read at 450 nm

256 in SPECTROstar Nano equipment. Lf concentrations for each sample were obtained by
257 extrapolation from the standard curve made with recombinant bovine Lf provided in the kit.
258
259 ***2.8. Cytokine immunoassay***
260 Samples were defatted following the same protocol as in Lf. To determine levels of IL-1β, IL-6
261 and IL-4, commercial ELISA kits were used following manufacturer's instructions (Thermo Fisher
262 Scientific, MA, USA). Briefly, the plate was sensitized overnight at RT with the capture antibody
263 diluted 1/100 in carbonate/bicarbonate buffer 0.2 M, pH 9.4 and subsequently blocked for 1 h at RT
264 using D-PBS containing BSA 4% and sucrose 5%. Then, samples were incubated in duplicate at RT
265 for 1 h (IL-1β and IL-6) or 1 h 30 min (IL-4). To perform the quantification, a standard curve was
266 constructed from known recombinant bovine IL-1β, IL-6 and IL-4 concentrations provided in the
267 kits. After three washings with D-PBS Tween, samples were incubated with the detection antibody
268 diluted 1/100 for 1 h at RT. Subsequently, three washings were carried out and samples were
269 incubated with streptavidin-peroxidase (1/400) for 30 min at RT. The reaction was evidenced by
270 incubation with TMB for 20 min and stopped with HCl 1N. Absorbance was read at 450 nm in a
271 SPECTROstar Nano equipment.
272
273 ***2.9. Analysis of immunoglobulins levels***
274 Total IgG and IgG_1 and IgG_2 subtypes against *S. aureus* strains 806 (NP) and 5011 (P) were
275 determined following the procedure described by Renna et al. [12] with modifications. A 96-well
276 microplate was sensitized with *S. aureus* lysates of these strains (10 µg/well) in bicarbonate buffer
277 0.1M, pH 9 and incubated overnight at 4°C. *S. aureus* 806 (NP) and 5011 (P) strains lysates were
278 obtained as described by Camussone et al. [27]. After three washings performed with PBS-Tween
279 (0.05%) the plate was blocked for 1 h at 37°C with 5% of goat milk in PBS. Defatted milk samples
280 were diluted 1/50 and incubated 1 h at 37°C. Then, three washings were carried out, and the plate
281 was covered with the detection antibodies: anti cow IgG-HRP (1/20.000) (Abcam), anti cow IgG_1-

HRP (1/15.000) (Abcam), anti cow IgG_2-HRP (1/1.000) (Bethyl Laboratories, Inc) and incubated 1 h at 37°C. Finally, after five washings TMB was added and the reaction was stopped after 5 min by the addition of 1N HCl. Absorbance at 450 nm was read in a SPECTROstar Nano equipment. Results were expressed as optical density (OD). In all cases for each immunoglobulin studied, plates were sensitized with both *S. aureus* strains (806 and 5011) and cross reactivity effects between both strains were evaluated.

2.10. Statistical analysis

Statistical analysis of data was performed using SPSS 25.0 Software (SPSS Inc., Chicago, IL). To determine the effect of IMI with different *S. aureus* strains on variables evaluated, data obtained were statistically analyzed using repeated measures ANOVA (RMANOVA). ANOVA assumptions, such as normality of the distribution and homogeneity of variances, were verified by the Kolmogorov-Smirnov and Levene tests, respectively. One-way ANOVA was performed for each time evaluated, followed by Duncan's multiple comparison test. Differences between time 0 (pre-i) and the rest of sampling times from quarters inoculated with both *S. aureus* strains were analyzed using one-way ANOVA followed by Duncan´s multiple comparison test. Differences of $p<0.05$ were considered significant. Results were expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. S. aureus intramammary challenge inocula

Based on viable bacterial counts of the prepared bacterial inocula, quarters challenged with *S. aureus* strain 806 (NP) received ~350 CFU while quarters challenged with the strain 5011 (P) received ~400 CFU/quarter. Similar numbers were recovered from the remainder of the *S. aureus* inocula that were used for the intramammary challenge. No bacterial growth was detected on pyrogen-free saline solution used for control quarters.

3.2. Bacteriological examination, SCC and cell microscopic observation

Bacterial isolation and identification was carried out from milk samples of quarters inoculated with *S. aureus* strains 806 (NP) and 5011 (P) and controls inoculated with saline solution at different times pi. Results are detailed in Table 2.

From quarters inoculated with strain 806 (NP) typical *S. aureus* yellowish colonies with double hemolysis halo were isolated at d 1, d 2 and at d 3 pi from RF quarter of animal 1, at d 1, d 2 and d 3 pi in LH quarter of animal 1 and only at d 1 pi in RF quarter of animal 2. Although microbiological cultures were negative between days 4 and 7 in RF quarter of animal 1, isolation was positive at 14 and 21 d pi and the morphology of colonies was similar to that observed in the first 3 d. Complementary biochemical tests confirmed the presence of *S. aureus* in the inoculated mammary quarters and the same original pulsotype was recovered from these post-challenge samples (Fig 1.; supplementary material). Quarters RF and LH of animal 3 did not yield a positive microbiological result at any of the evaluated times. In RF quarter of animal 4, isolation was positive at d 1 and d 2 pi and from LH quarter no bacteriological growth was observed. Between 1 and 7 d pi, in quarters with both positive and negative microbiological results, mastitis score 1 was observed (i.e. small flakes and lumps without apparent inflammatory signs at MG palpation; Table 2). Cytospins from a pool of milk samples from quarters (RF+LH) of animals 1 and 2 showed stained cocci inside macrophages between days 1 and 3 pi (Fig.2; supplementary material).

From mammary quarters inoculated with strain 5011 (P), typical *S. aureus* slightly yellowish colonies with double hemolysis halo were observed at 14 d pi in all quarters evaluated (average of 360 CFU/ml). In addition, bacterial growth was observed at 7 d pi in RF quarter and in LH quarter of animal 5, and at 21 d pi in LH quarter of animals 6 and 7. The biochemical tests carried out confirmed the presence of *S. aureus* and the same original pulsotype was recovered from these post-challenge samples (Fig. 1; supplementary material). Mastitis score 1 was only observed in milk of two LH quarters in animals 6 and 7 at 14 d pi, (i.e. small flakes and lumps without presence of local

inflammatory signs; Table 2). Cytospins from a pool of milk samples from quarters (RF+LH) of animals 5 and 6 showed stained cocci inside macrophages at 14 d pi (Fig.2; supplementary material).

For further monitoring the development of IMI, additional bacterial cultures from quarters inoculated with *S. aureus* strains 806 (NP) and 5011 (P) were carried out for 5 weeks with 7 d intervals. Bacterial growth was not detected in any of the samples taken from cows inoculated with strain 806 (NP). Whilst from animals inoculated with strain 5011 (P) *S. aureus* colonies were obtained from LH quarter (animal 5) at 28 d pi, in RF quarter (animal 6) at 28, 42 and 49 d pi and in LH quarter (animal 6) at 28, 35 and 56 d pi; without macroscopic changes in milk or apparent signs of inflammation (mastitis score 0). In every case identification was carried out by standard biochemical tests and original pulsotype identity was confirmed. Microbiological analysis from control mammary quarters did not show bacterial growth, macroscopic changes in milk or apparent signs of inflammation (mastitis score 0) at any of the sampling times evaluated.

SCC in milk samples from mammary quarters inoculated with both *S. aureus* strains and control quarters inoculated with saline solution at different sampling times was evaluated (Fig. 2). A significant effect of *S. aureus* challenge was observed over time ($p<0.001$), finding differences in the SCC between the experimental groups. Regarding quarters inoculated with strain 806 (NP), the highest SCC values were observed at d 2 pi, reaching a peak (mean) of 3.7×10^6 cells/ml. From d 7 pi there was a marked decrease that lasted until d 21 pi reaching basal values. From mammary quarters inoculated with strain 5011 (P), no variations were observed until d 3 pi; maximum values were reached at d 14 (3.8×10^6 cells/ml) and remained high at 21 d pi. In control quarters, no variations were observed during the sampling times studied ($\bar{x}$ 3.06×10^4 cells/ml).

3.3. Nitrites and lactoferrin production

Nitrites and Lf production were evaluated in milk samples from mammary quarters inoculated with *S. aureus* strains 806 (NP) and 5011 (P) and control quarters inoculated with saline solution at

different sampling times (Fig. 3 A-B). A significant effect of *S. aureus* challenge was observed over time ($p=0.041$), finding differences in nitrites concentration between the experimental groups (Fig. 3A). Statistical analysis showed a significant difference only at 21 d pi, when an increase in nitrite levels was found in mammary quarters inoculated with strain 5011 (P). Additionally, the *S. aureus* challenge effect in quarters inoculated with strain 806 (NP) was evaluated over time, showing an increase in nitrite production at d 3 pi compared with time 0 (pre-i). Control mammary quarters did not show variations throughout the experimental period (Fig. 3A).

A significant effect of *S. aureus* challenge was observed over time ($p=0.004$) in Lf levels between the experimental groups (Fig. 3B). Maximum Lf concentrations were detected in mammary quarters inoculated with strain 806 (NP), showing an increase from d 3 pi. At d 21 pi, Lf levels began to decline; however, they remained higher than those observed in control quarters ($p<0.05$). Although in quarters inoculated with strain 5011 (P) Lf levels were higher than in control mammary quarters, significant differences between groups were not detected at most of the evaluated times. At 4 and 21 d pi, in quarters inoculated with strain 5011 (P) a significant increase of Lf levels was observed ($p<0.05$); reaching at d 21 similar values to those found in milk from quarters inoculated with strain 806 (NP). Control mammary quarters did not show variations throughout the test (Fig 3B).

3.4. Cytokine levels

IL-1β, IL-6 and IL-4 concentrations were evaluated in milk samples from mammary quarters inoculated with *S. aureus* strains 806 (NP) and 5011 (P) and control quarters inoculated with saline solution at different sampling times (Fig. 4 A-C). A significant effect of *S. aureus* challenge was observed over time ($p<0.001$) finding differences in the concentrations of IL-1β between experimental groups (Fig. 4A). In mammary quarters inoculated with strain 806 (NP) the maximum concentrations of IL-1β were observed at d 2 pi and were higher than those observed in control quarters and quarters inoculated with strain 5011 (P) ($p<0.05$). From d 3 pi a decrease was observed

386 that lasted until d 21 pi; however, this cytokine concentration remained significantly higher
387 ($p<0.05$) during the observation period. In mammary quarters inoculated with strain 5011 (P), IL-1β
388 concentrations were similar to those found in the control quarters (Fig. 4A).

389 A significant effect of *S. aureus* challenge was observed over time ($p<0.001$) in the
390 concentrations of IL-6 between the different experimental groups (Fig. 4B). In mammary quarters
391 inoculated with strain 806 (NP) a significant increase of IL-6 was observed from d 1 and continued
392 until d 2 pi reaching the maximum concentrations ($p<0.05$). A marked decrease was observed on d
393 3 pi reaching basal values on d 4 pi. A second significant increase was observed on d 14 pi that
394 lasted until day 21 pi ($p<0.05$). In mammary quarters inoculated with strain 5011 (P) IL-6
395 concentrations were similar to those found in the control quarters (Fig. 4B).

396 A significant effect of *S. aureus* challenge was observed over time ($p<0.001$) in the
397 concentrations of IL-4 between the different experimental groups (Fig. 4C). In mammary quarters
398 inoculated with strain 806 (NP) a significant increase was observed at d 1 pi ($p<0.05$); however, the
399 concentration decreased on d 2 reaching basal values. At d 7 pi a second increase was observed and
400 remained higher than control and quarters inoculated with strain 5011 (P) until d 21 pi ($p<0.05$). In
401 mammary quarters inoculated with strain 5011 (P) a significant increase was observed at d 1
402 compared with control quarters ($p<0.05$); however, these values did not reach the IL-4
403 concentrations found in quarters inoculated with strain 806 (NP). At the remaining sampling times
404 evaluated IL-4 concentrations were similar to those found in the control quarters (Fig. 4C).

405

406 ***3.5. Levels of total and specific immunoglobulins***

407 The levels of total IgG, IgG_1 and IgG_2 were evaluated in milk samples from mammary quarters
408 inoculated with *S. aureus* strains 806 (NP) and 5011 (P) and control quarters inoculated with saline
409 solution at different sampling times (Fig. 5 A-F).

410 In plates sensitized with strain 806 (NP), a significant effect of *S. aureus* challenge was observed
411 over time finding differences ($p=0.030$) in the total IgG levels between the experimental groups

(Fig. 5A). Higher levels of total IgG were observed in the mammary quarters inoculated with strain 806 (NP) than in the control quarters at all sampling times evaluated ($p<0.05$). In mammary quarters inoculated with strain 5011 (P) the total IgG levels did not differ from control quarters and quarters inoculated with strain 806 (NP).

In plates sensitized with strain 5011 (P), a significant effect of *S. aureus* challenge was observed over time finding differences ($p=0.015$) in the total IgG levels between the different experimental groups (Fig. 5D). In mammary quarters inoculated with strain 806 (NP) higher total IgG levels were observed at all sampling times evaluated ($p<0.05$) compared with control quarters. In mammary quarters inoculated with strain 5011 (P) total IgG levels did not differ from control quarters and quarters inoculated with strain 806 (NP).

In plates sensitized with strain 806 (NP), a significant effect of *S. aureus* challenge was observed over time finding differences ($p=0.011$) in the IgG_1 levels between the different experimental groups (Fig. 5B). In mammary quarters inoculated with strain 806 (NP) higher levels of IgG_1 were observed at all sampling times evaluated ($p<0.05$) compared with control quarters. In mammary quarters inoculated with strain 5011 (P) a significant increase was observed at d 14 pi compared with the levels of control quarters and was maintained until d 21 pi ($p<0.05$).

In plates sensitized with strain 5011 (P), a significant effect of *S. aureus* challenge was observed over time finding differences ($p=0.018$) in the IgG_1 levels between the different experimental groups (Fig. 5E). In mammary quarters inoculated with strain 806 (NP) a significant increase was observed at d 7 and 21 pi compared with control quarters ($p<0.05$). In mammary quarters inoculated with strain 5011 (P) a significant increase was observed at d 7 and 21 pi compared with control quarters ($p<0.05$). In plates sensitized with strain 5011 (P), the IgG_1 levels for both *S. aureus* strains were lower to those observed in plates sensitized with strain 806 (NP).

In plates sensitized with strain 806 (NP), a significant effect of *S. aureus* challenge was observed over time finding differences ($p=0.039$) in the IgG_2 levels between the different experimental groups (Fig. 5C). In mammary quarters inoculated with strain 806 (NP) a significant increase was

438 observed at d 7 and 21 pi compared with control quarters ($p<0.05$). In mammary quarters inoculated
439 with strain 5011 (P) a significant increase was observed on d 21 pi compared with control quarters
440 ($p<0.05$).

441 In plates sensitized with strain 5011 (P), a significant effect of *S. aureus* challenge was observed
442 over time finding differences ($p=0.005$) in the IgG_2 levels between the different experimental
443 groups (Fig. 5F). In both mammary quarters inoculated with strain 806 (NP) and strain 5011 (P) a
444 significant increase was observed at d 7 pi and continue until d 21 pi compared with control
445 quarters ($p<0.05$). The IgG_2 levels for both *S. aureus* strains in plates sensitized with strain 5011 (P)
446 were higher than the levels found in plates sensitized with strain 806 (NP).

447

448 **4. Discussion**

449 This study focused on two *S. aureus* isolates collected from bovine IMI with different adaptation
450 genotypes (low and high) to the bovine MG using an experimental challenge model for bovine
451 mastitis to gain insights into the mechanism of the immune response induced by distinct strains of
452 this organism. *In vitro* studies of host-pathogen interactions have demonstrated that differences
453 exist between *S. aureus* strains and lineages in their ability to invade and/or survive intracellularly
454 and to elicit expression of pro-inflammatory mediators in bovine mammary cells [28,29,16]. In a
455 recent study bovine experimental IMI using *S. aureus* strains belonging to the main bovine lineages
456 detected in Ireland (CC97 and CC151) were performed inducing a differential immune response in
457 the host demonstrating that the outcome of mastitis induced by this pathogen was strain dependent
458 [19]. CC 97 has been identified as the most prevalent genotype around the world [30]. However, a
459 wide variety of genotypes have been detected in different countries, as well as in regions within
460 each country [30]. Although some genotypes are considered to have higher pathogenic potential, a
461 clear link between presence of virulence factors and clinical outcome or mastitis severity has not
462 been established [31, 30]. In this study, we used two isolates from bovine mastitis cases with

463 different genotypic profiles characterized by microarray analysis as unusual bovine lineages: strain
464 806 (NP) that belonged to ST350-MSSA and is a rare ST that has been isolated from humans, dogs,
465 horses, a wild deer and cows with mastitis (Monecke, S; data not published) and strain 5011 (P) that
466 belonged to CC188-MSSA and is considered mostly a human lineage although it has also been
467 found in association with bovines [32,33]. The ST used in the present study have also been detected
468 in bovine milk in other countries [30]. Although direct comparisons cannot be made with the study
469 by Niedziela et al. [19] since different lineages of *S. aureus* were used, both studies agreed that the
470 genetic characteristics of the strains were associated with differential local immune response that
471 determined the course and severity of the infection.

472 In the last decades, experimental *in vivo* infection models have been extensively used and
473 constitute an effective tool for the investigation of the host immune response against *S. aureus*
474 causing bovine mastitis [22,34-38]. Although animal conditions and experimental designs vary
475 between studies, experimental challenges with *S. aureus* generally range from mild or moderate
476 acute clinical to subclinical mastitis depending on the number of challenge organisms and the
477 anatomical inoculation site [39-41], triggering a slight local immune reaction of the MG and
478 generally no systemic involvement. As a result, these experimental infections may very often
479 become persistent [42,35]. Besides, the *S. aureus* strain effect on clinical/sub-clinical mastitis has
480 recently been demonstrated [43,44,19].

481 There are numerous experimental IMI studies in cattle in which different *S. aureus* strains and
482 bacterial concentrations have been used: ~40 CFU/ml [39]; ~300 CFU/ml [24]; ~1000 CFU/5 ml
483 [18]; ~2000 CFU/ml [34]; $5x10^4$ CFU/2 ml [36]. In the present study, a bacterial suspension
484 containing ~400 CFU/ml was used. The selection of the bacterial concentration was based on
485 previous studies in which using a low number of bacteria (~72 CFU of the *S. aureus* strain
486 Newbould 305 in 2 ml) in mid-lactation cows with low SCC a mild clinical IMI in all inoculated
487 quarters was established [39]. We also selected this concentration to avoid overwhelming the MG
488 immune system. In this study, using MGs with low SCC, *S. aureus* experimental IMI were

successfully established for both strain. Detection of *S. aureus* in milk from challenged quarters had the characteristics of intermittent and cyclical shedding [45] and bacteria were not detected simultaneously in all challenged quarters at a given sampling time. In the quarters challenged with strain 806 (NP), bacteria were recovered from milk at 24 to 72 h pi in half of the challenged quarters. However, in this period the highest SCC were detected in all challenged quarters irrespective of the bacteriological status, reaching a mean of 3.7 x 10^6 cells/ml at d 2 pi. The lack of isolation in four of the eight challenged quarters could have been due both to the contribution of the innate immune response to eliminate the inoculum and to the presence of organisms in milk below the detection limit of the methodology used. From d 28 to d 56 pi this *S. aureus* strain was no longer detected in milk of challenged cows. All mammary quarters challenged with strain 806 (NP) developed mild clinical mastitis (score 1) between 1 and 7 d pi and high SCC during this period. In quarters challenged with strain 5011 (P), bacteria were recovered from milk from d 7 pi and up to d 56 pi in one of the challenged quarters, confirming the ability of this strain to adapt to the MG microenvironment and develop a persistent IMI as in the natural case from which it was isolated [27]. In all quarters challenged with this strain, only small lumps in milk were observed on day 14 pi without presence of local inflammatory signs and coinciding with the highest SCC values. Our results agree with previous studies where, high SCC were reported even when *S. aureus* was not isolated from the milk of the challenged MG [46,39]. The fact that *S. aureus* could not be isolated at all sampling periods after challenge with both strains reflects the previously described cyclical shedding pattern of *S. aureus* in milk [45,47]. Collectively these findings demonstrated that, although both *S. aureus* strains were capable of establishing an IMI, strain 806 (NP) triggered a more rapid and intense immune response than strain 5011 (P) at early stages (24 to 72 h pi) persisting until day 7 pi; while strain 5011 (P) multiplied initially at a lower rate but from 7 d pi to the end of the study was detected more frequently in milk probably associated to an increased ability to evade host defenses and adapt to the MG environment.

514 The differences observed in this study on the persistence of *S. aureus* strains in the MG could be
515 related to the carriage and expression of certain virulence factors, as shown by Buzzola et al. [48] in
516 experimental IMI in murine MG challenged with *S. aureus* strains of different *agr* type. These
517 authors, demonstrated that *S. aureus agr*-type II or IV strains were more efficiently eliminated from
518 the MG than those of type I, suggesting that *agr*-type I strains may persist in greater numbers in
519 mammary tissue than *agr* types II, III and IV strains. In the present study, strain 806 (NP) classified
520 as *agr*-type II was eliminated from MG with greater efficiency than strain 5011 (P) (*agr*-type I),
521 which evaded the immune system and persisted in the MG until 56 days pi. These results are in
522 agreement with previous research from our laboratory [20], in which the immune response induced
523 in mice MG challenged with two *S. aureus* strains isolated from bovine mastitis with different
524 phenotype, genotype and adaptation to the MG (P and NP) was evaluated. The *S. aureus* strain
525 isolated from a NP IMI showed a greater ability to multiply in mammary tissue in the early stages
526 of the IMI compared with the P strain, while the P strain multiplied initially at a lower rate, but
527 increased its replication capacity from 120 h pi to the end of the study (11 days pi), indicating a
528 greater ability to evade the immune system and thus persist in the MG [20]. In addition to the *agr*
529 type, the *cap* gene type and capsular polysaccharide (CP) expression has also been shown to play an
530 important role in *S. aureus* intracellular survival. In this context, in *in vitro* studies with bovine
531 mammary epithelial cells, Bardiau et al. [13] demonstrated that isolates belonging to *agr* group II,
532 *cap8* positive and expressing CP8, were less likely to survive intracellularly than isolates belonging
533 to *agr* group I not expressing any CP. In our study, although both *S. aureus* strains carried *cap*
534 genes, the expression of CP5 or CP8 *in vitro* was not determined, therefore direct comparisons with
535 previous research [13] cannot be made.

536 The SCC has long been used as an indicator of inflammation for bovine mastitis diagnosis based
537 on the increase in the number of cells due to the infiltration of neutrophils that gain access to the
538 milk as a consequence of the inflammation [49]. In experimental challenges with *S. aureus* the time
539 to the appearance of detectable signs of mastitis is variable depending on the inoculum size and cow

factors and can be limited to a gradual increase in the SCC over a period of 48 to 72 h after challenge, with often concomitant isolation of *S. aureus* in milk [50,39-41]. In this study, the SCC response elicited by the two *S. aureus* strains differed and showed variations over time. In mammary quarters inoculated with strain 806 (NP) the inflammatory response induced was greater and earlier than the one induced by strain 5011 (P), since a SCC peak was observed at d 2 pi, while in mammary quarters inoculated with strain 5011 (P) no variations in SCC were observed until d 4 pi reaching the maximum values at d 14 pi; indicating a lower and delayed initial inflammatory response. This delay in response to strain 5011 (P) compared with strain 806 (NP), could be associated both with (1) less initial stimulation of macrophages to release cytokines and recruit neutrophils to the MG minimizing the inflammatory response and (2) increased capacity to survive intracellularly. Differential adhesion/internalization and intracellular persistence capacities were observed for both *S. aureus* strains in mammary epithelial cells in a previous *in vitro* study [16]; in which strain 5011 (P) showed higher adhesion/internalization and persistence capacity in MAC-T cells and greater resistance to microbicidal mechanisms than strain 806 (NP) [16]. Internalization might protect bacteria from clearance by the immune system and allow for long-term persistence in chronically infected hosts [51]. Accordingly, it is possible that induction of inflammatory response, as indicated by a noticeable increase in milk SCC from d 4 pi occurred once the strain 5011 (P) reached a minimum threshold to trigger recognition by the innate immune system. Although this period of delay to trigger the immune response could be associated with the greater capacity of this strain to internalize in mammary cells *in vitro*, the experimental setting of the present study do not allow to confirm this hypothesis. Results obtained in this study are indicative that the SCC response was directly influenced by the characteristics of the *S. aureus* strain and are in line with previous findings from Niedziela et al. [19].

Previous studies have indicated that NO is a key mediator of the inflammatory responses caused by IMI [52]. Atakisi et al. [53] have observed higher concentrations of NO in milk from MG with subclinical mastitis compared with non-infected MG, indicating a relationship between elevated

levels of NO and inflammation. In agreement with Atakisi et al. [53], the highest levels of NO detected in milk from quarters inoculated with both *S. aureus* strains were associated with the time periods when highest SCC were detected. The ability of macrophages to kill microbial pathogens has been linked to their capability to generate NO, a highly bactericidal moiety [54]. In the present study, the highest concentrations of NO observed at d 3 pi in quarters challenged with strain 806 (NP) could have contributed to the faster bacterial clearance of the MG compared with strain 5011 (P). Levels of NO and SCC began to increase from day 4 prior to the detection of the organisms in milk and remained high until day 21 in quarters challenged with strain 5011 (P). This suggests that strain 5011 (P) weakly stimulated macrophages during the first 4 days inducing a more gradual and delayed inflammatory reaction than strain 806 (NP), favoring the adaptation of the microorganism to the microenvironment of the MG and further establishment of IMI.

Levels of Lf in milk vary according to the age of the cow, stage of lactation, parity, SCC and presence of pathogenic organisms [55]. Hagiwara et al. [55] have reported Lf levels in milk samples from healthy quarters and with subclinical mastitis of approximately 170 and 501 μg/ml, respectively. In the present study, in accordance with the aforementioned, maximum Lf concentrations were detected in mammary quarters inoculated with strain 806 (NP) on d 3 pi (average maximum value ~375 μg/ml). In mammary quarters inoculated with strain 5011 (P) the maximum Lf concentrations were detected on d 21 pi (average maximum value ~285 μg/ml), while in control quarters Lf concentration did not vary through the experimental period (~120 μg/ml). During inflammation, Lf production by MG epithelial cells is not only intensified, but also released from neutrophils secondary granules [56]. Lactoferrin increases production of inflammatory cytokines and chemokines, and migration of leukocytes to the MG [57]. Kawai et al. [58] demonstrated that high Lf concentrations in mastitis milk were associated with the severity of the mammary inflammation. In this study the highest levels of Lf detected in milk from quarters inoculated with both *S. aureus* strains coincided with high SCC in the same time periods, indicating an association with the magnitude of inflammation.

592 The IL-1β response during experimental IMI has been shown to be highly variable compared to
593 other cytokines. Following an experimental infection of mid lactation cows with ~ 40 CFU of *S.*
594 *aureus* strain Newbould 305, Banneman et al. [39] observed an increase in the IL-1β concentration
595 in milk after 32 h which was maintained for an additional 8 h. Riollet et al. [59], after experimental
596 inoculation of cows in mid lactation with ~ 100 CFU of a *S. aureus* strain isolated from a natural
597 bovine mastitis case (strain 107-59), did not detect this cytokine in milk at any of the evaluated
598 times (from d 1 to d 28 pi). Differences in results could have been related to strain characteristics,
599 inoculum preparation and size, site of inoculation, lactation period and other cow factors as well as
600 sensitivity of the ELISA. In this study, the IL-1β response depended on the *S. aureus* strain infused.
601 In mammary quarters inoculated with strain 806 (NP) IL-1β concentration in milk increased sharply
602 at d 2 pi reaching a peak and remained at higher levels than those detected for strain 5011 (P) or
603 control during the observation period. During the inflammatory response, IL-1β regulates the
604 expression of adhesion molecules in epithelial cells and the chemotaxis of neutrophils in the early
605 stages of infections caused by *S. aureus* [60]. The high levels of IL-1β induced by strain 806 (NP)
606 were associated with the highest SCC detected, demonstrating the ability of this strain to induce a
607 rapid local inflammatory response, which in turn contributed to control the multiplication of the
608 microorganism. In mammary quarters inoculated with strain 5011 (P), although higher IL-1β levels
609 were detected at day 4 pi, concentrations did not differ from those found in control quarters at every
610 sampling time. This strain induced a lower and more gradual local inflammatory reaction,
611 demonstrated by the low levels of IL-1β and NO in milk, as well as by the low SCC detected until
612 day 4 pi.

613 IL-6 is considered one of the key mediators of the acute phase response in inflammation [61]. In
614 addition, this cytokine is involved in differentiation, activation of lymphocytes and production of
615 immunoglobulins [62]. Increased concentrations of IL-6 have been detected in milk and blood of
616 cows with naturally acquired [63] or experimentally induced mastitis during lactation [64] and in
617 mammary secretion of chronically infected *S. aureus* quarters during involution [12]. In this study,

618 in mammary quarters inoculated with strain 806 (NP) two IL-6 peaks on days 2 and 14 pi were
619 observed. IL-6 plays a crucial anti-inflammatory role in both local and systemic acute inflammatory
620 responses by controlling the level of pro-inflammatory, but not anti-inflammatory cytokines [65].
621 The first increase on day 2 may have been due to an inflammatory effect since it coincided with the
622 high SCC, which would indicate a role in the recruitment of cells to the site of infection. In murine
623 experimental models, when the inflammation resolves, the recruited leukocyte population shifts
624 primarily from neutrophils to monocytes and this transition is regulated by IL-6 [66]. In the present
625 study, the second increase on day 14 could be related to an anti-inflammatory effect which
626 coincided with an abrupt decrease in SCC. Additional studies are necessary to confirm the
627 association of these cytokines and their action as part of the innate immune response against
628 infection. In mammary quarters inoculated with strain 5011 (P), IL-6 concentrations were similar to
629 those found in the control quarters. Hagiwara et al. [63] confirmed high levels of IL-6 in the first
630 stage of natural infections, with a mean concentration of IL-6 on the first day of the disease 25
631 times higher in milk samples and 5 times higher in serum samples in cows with acute clinical
632 mastitis compared with normal cows. These authors observed that sera and whey samples from
633 cows infected with *E. coli, K. pneumoniae, S. aureus* or *Streptococcus sp.* contained significantly
634 higher concentrations of IL-6 than those of normal cows, suggesting that the levels of IL-6 in milk
635 and serum depend not only on the stage of infection but also on the type of microorganisms that
636 causes mastitis. In agreement with these findings, the variation in levels of IL-6 detected in the
637 present study could have been due to the inherent characteristics of the two *S. aureus* strains.
638 However, further studies are needed to elucidate the mechanisms of IL-6 production and action in
639 the MG infected with different strains of the same pathogen.

640 IL-4 is a multifunctional pleiotropic cytokine produced mainly by activated T cells, but also by
641 mast cells, basophils and eosinophils. Functionally, IL-4 is best known for defining the Th2-type
642 response profile of CD4 T cells and for regulating cell proliferation, apoptosis, and expression of
643 numerous genes in various cell types [67]. In this study, mammary quarters inoculated with strain

806 (NP) showed a significant increase on d 1 pi, a second increased on d 7 pi and higher levels than the other experimental groups until d 21 pi. Previous research from our laboratory [12] showed significantly higher IL-4 levels in mammary secretions from quarters chronically infected with *S. aureus* compared with control quarters at 24 h post-drying off, with a decrease until day 14 and a return to high levels at day 21 of involution. IL-4 participates in the alternative activation of macrophages towards a type 2 tolerogenic profile (M2) to counteract inflammation through the release of IL-10 and transforming growth factor (TGF)-β, promoting wound healing and tissue repair [68]. To the best of our knowledge, there is no direct evidence available about the role of IL-4 in *S. aureus* bovine mastitis [69] and no studies have quantified the IL-4 concentration in milk of an experimentally induced *S. aureus* mastitis up to 21 d after challenge. In the present study, the high levels of IL-4 detected in milk from quarters inoculated with strain 806 (NP) on d 1 pi, previous to peak concentrations of both IL-1β and IL-6 is difficult to explain within the general knowledge of the functions of this IL [69,67]. Further studies are needed to evaluate other anti-inflammatory cytokines (IL-10, TGF-β) that could contribute to elucidate the anti-inflammatory role of IL-4 in bovine experimental infections by *S. aureus*. The second increase in IL-4 levels observed at d 7 pi coincided with high SCC in milk and could be explained as an attempt to counteract the inflammation caused by *S. aureus* in this period. This coincides with an increase in IL-6 levels to reach a peak at d 14 pi acting together with IL-4 modulating the inflammatory process, exerting an anti-inflammatory role. However, this putative association needs to be confirmed by further studies. On the other hand, in quarters inoculated with strain 5011 (P), an increase in IL-4 levels was observed on day 1 pi, while at the rest of the evaluated times the levels of this cytokine were similar to those found in the control quarters. Bochniarz et al. [70], in a study in Holstein-Friesian cows during lactation, determined that the concentration of IL-4 was significantly lower in both serum and milk from cows with mastitis caused by coagulase-negative staphylococci compared with control animals, highlighting the importance of type and characteristics of pathogen on the inflammatory response induced in MG. For all the aforementioned, additional studies with a greater

number of animals are needed to characterize and understand the functions of IL-4 in the immune response to *S. aureus*.

IgG is the main effector of the humoral immune response of the MG responsible for promoting phagocytosis of neutrophils [50]. The IgG_1 subclass is the predominant type of antibody in milk from healthy quarters due to its selective transfer across the blood-mammary barrier [71]. In mastitic milk IgG_2 becomes the dominant antibody subclass and is considered the major opsonin that supports neutrophil phagocytosis in bovine MG [72]. In the present study, in both plates sensitized with strains 806 (NP) and 5011 (P), the highest values of total IgG were observed in mammary quarters inoculated with strain 806 (NP). In mammary quarters inoculated with strain 5011 (P) values of IgG did not differ from the other experimental groups. The possible explanations for these finding are that (1) immunoglobulins could be opsonizing the bacteria present in milk leading to a decrease in detection of free immunoglobulins and (2) considering that in previous studies the strain 5011 (P) showed a high capacity for adherence/internalization and persistence in MAC-T cells [16] and that intracellular *S. aureus* can curb the immune response of the MG [73], the reduction in specific IgG levels in quarters challenged with 5011 (P) strain reflects a lower capacity of this strain to stimulate a humoral immune response.

In the case of IgG_1 in both plates, sensitized with strains 806 (NP) and 5011 (P), a significant increase was observed in mammary quarters inoculated with strains 806 (NP) and 5011 (P) compared with control quarters. However, the values of IgG_1 for both strains (NP and P) in plates sensitized with strain 5011 (P) were lower than the values from plates sensitized with strain 806 (NP). In the case of IgG_2 in both plates, sensitized with strains 806 (NP) and 5011 (P), a significant increase was observed in mammary quarters inoculated with strains 806 (NP) and 5011 (P) compared with control quarters. However, the values of IgG_2 for both strains (NP and P) in plates sensitized with strain 806 (NP) were lower than the values from plates sensitized with strain 5011 (P). These results could indicate a specific humoral immune response against *S. aureus* during the experimental challenge that could have contributed to opsonophagocytosis and the elimination of

bacteria by phagocytic cells. However, highest levels of specific opsonic IgG_2 are expected to be achieved during inflammation 6 to 12 h before the peak neutrophil response [72], which could be effective in previously immunized rather than in naïve animals. In addition, clearance of strain 806 (NP) took place in most inoculated quarters mainly before day 14 pi; while clearance of strain 5011 (P) did not occur in most of the inoculated quarters. Therefore, the value of IgG_2 for the clearance of bacteria in this study is doubtful. Although an adaptive humoral immune response was developed against challenge with both *S. aureus* strains, a more effective total IgG and IgG_1 response was induced by strain 806 (NP), while strain 5011 (P) was more effective in generating an IgG_2 response (even against the heterologous strain).

In this study, the sensitization of the ELISA plates to evaluate the levels of immunoglobulins was carried out with lysates of the *S. aureus* strains 806 (NP) and 5011 (P), observing similar results in the levels of specific IgG for both antigens in the different sampling times evaluated. This demonstrates cross-immunity against the antigens used, indicating that immunoglobulins generated against one strain of *S. aureus* could respond against other heterologous strains. To confirm these findings, functionality tests of the generated antibodies should be performed.

Conclusion

The present *in vivo* study confirms previous *in vitro* observations about differential behavior of *S. aureus* strains with distinct adaptation capabilities to the MG demonstrating their ability to trigger, modulate and evade the host immune response influencing the course and severity of IMI. These features should be taken into account both in the diagnosis of intramammary infections within the framework of the classic control programs for this organism, and in the design of experimental studies aimed at generating new control alternatives.

Conflict of Interest

There are no conflicts of interest to declare.

Ethics approval

All procedures with animals were conducted under protocols approved by the Ethics and Security Committee of the Facultad de Ciencias Veterinarias, UNL (protocol N° 293) and were consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 2010).

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954

955 **References figures**

956 **Figure 1**: Schematic overview of the *S. aureus* intramammary challenge model. Two of four
957 quarters per animal (right front-RF and left hind-LH) were inoculated with 1 ml of the suspension
958 containing *S. aureus* strain 806 (NP) (~400 CFU) or *S. aureus* strain 5011 (P) (~350 CFU) or with 1
959 ml of pyrogen-free saline solution (controls). The animals were monitored daily until 21 d post-
960 inoculation (pi), registering the general condition, local inflammatory changes in MG and milk,
961 appetite and milk production. Milk samples were taken from each inoculated mammary quarter,
962 pre-inoculation (time 0) and 0.5, 1, 2, 3, 4, 7, 14 and 21 days pi. Following this experimental period,
963 the mammary quarters inoculated with *S. aureus* strains 806 (NP) and 5011(P) were further
964 evaluated by bacterial culture for 5 weeks at 7 day intervals (28, 35, 42, 49, and 56 days pi).

965 **Figure 2:** Somatic cell count (SCC) in milk samples from mammary quarters inoculated with *S.*
966 *aureus* strains 806 (NP) and 5011 (P) and controls inoculated with saline solution at different times
967 pi. The data are presented as means ± standard error of mean (SEM). Results of a repeated measure
968 ANOVA (RMANOVA) are indicated, different letters correspond to statistically significant
969 differences ($p<0.05$).

970 **Figure 3:** A) Nitrite concentration (generated by spontaneous oxidation of NO). B) Lactoferrin
971 concentration. Assays performed in milk samples from mammary quarters inoculated with *S. aureus*
972 strains 806 (NP) and 5011 (P) and controls inoculated with saline solution at different times pi. The
973 data are presented as means ± standard error of mean (SEM). Results of a repeated measure

974 ANOVA (RMANOVA) are indicated. Different letters correspond to statistically significant
975 differences ($p<0.05$). Asterisk represent significant difference between time 0 (pre-i) and the rest of
976 sampling times from quarters inoculated with 806 (NP) *S. aureus* strain (*$p<0.05$).

977 **Figure 4:** Cytokines concentrations in milk samples from mammary quarters inoculated with *S.*
978 *aureus* strains 806 (NP) and 5011 (P) and controls quarters inoculated with saline solution at
979 different times pi. The concentrations of A) IL-1β, B) IL-6 and C) IL-4 were all determined by
980 ELISA. The data are presented as means ± standard error of the mean (SEM). Results of a repeated
981 measure ANOVA (RMANOVA) are indicated. Different letters correspond to statistically
982 significant differences ($p<0.05$).

983 **Figure 5:** A) Total IgG, B) IgG_1 and C) IgG_2. Assays performed in milk samples from mammary
984 quarters inoculated with *S. aureus* strains 806 (NP) and 5011 (P) and controls quarters inoculated
985 with saline solution at different times pi. Plates were sensitized with 806 (NP) strain. D) Total IgG,
986 E) IgG_1 and F) IgG_2. Assays performed in milk samples from mammary quarters inoculated with *S.*
987 *aureus* strains 806 (NP) and 5011 (P) and controls quarters inoculated with saline solution at
988 different times pi. Plates were sensitized with strain 5011 (P). Assays were all carried out by
989 ELISA. The data are presented as means ± standard error of the mean (SEM). Results of a repeated
990 measure ANOVA (RMANOVA) are indicated. Different letters correspond to statistically
991 significant differences ($p<0.05$).

992

993 **Supplementary material**

994

995 **Figure 1:** Representative banding patterns obtained from *SmaI* digested DNA and pulsed field gel
996 electrophoresis (PFGE) of *S. aureus* strains 806 (NP) and 5011 (P) isolated from milk during
997 different period post challenge. RF: Right front quarter. LH: Left hind quarter. PT: Pulsotype.

998

Figure 2: Light microscope of cytospins from milk samples of animals challenged with *S. aureus* strains. A) Cytospin of animal 1 challenged with strain 806 (NP) at 2 d pi. B) Cytospin of animal 5 challenged with strain 5011 (P) at 14 d pi. Arrows indicate bacteria (cocci) within the cytoplasm of macrophages. May Grünwald Giemsa stain. Magnification 1000x.

Table 1: Summary of phenotypic, genotypic and functional characteristics of *S. aureus* strains used in this study.[†] Full characterization of strains in [16].

***S. aureus* strains**[†]	**Biofilm (MPA)**	***agr* type**	**Capsule type**	**Adhesion genes**	**Biofilm producing genes**	**Penicillin resistance**	**Pulsotype (PFGE)**	**Sequence type (ST)**[*]	**Adherence/invasion and persistence capacity#**
Strain NP (806)	Weak	*agrII*	*cap5*	*clfA, clfB, fnbpA, fnbpB (-), fib, cna*	*icaA, icaC, icaD, bap (-)*	*blaZ (-)*	D	ST350	Low
Strain P (5011)	Strong	*agrI*	*cap8*	*clfA, clfB, fnbpA, fnbpB (-), fib, cna*	*icaA, icaC, icaD, bap (-)*	*blaZ*	O	ST188 (CC188)	High

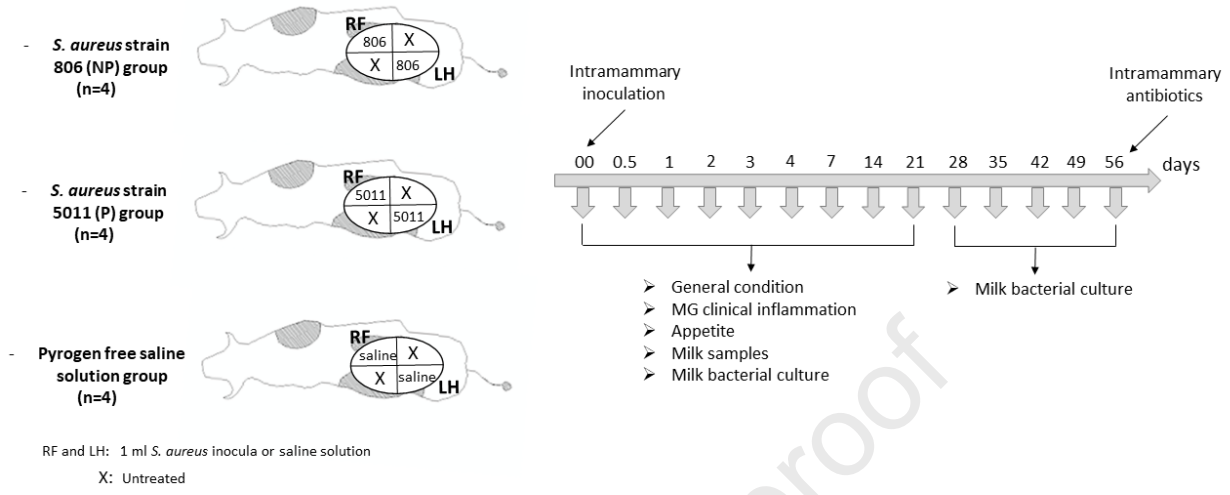
References: NP: nonpersistent. P: persistent. MPA: microtiter plate assay. PFGE: pulse-field gel electrophoresis. (*) Evaluated by DNA microarrays. (#) Evaluated in a bovine mammary epithelial cell line (MAC-T). Presence of capsular polysaccharaide genes 5 and 8 (*cap5*, *cap8*). Presence of clumping factor A and B genes (*clfA, clfB*); fibronectin binding proteins A and B genes (*fnbpA, fnbpB*); fibrinogen binding protein gene (*fib*); collagen adhesion gene (*cna*). Presence of intercellular adhesion genes A, C and D (*icaA, icaC, icaD*) and biofilm-associated protein gene (*bap*). Presence of beta-lactamase gene (*blaZ*).

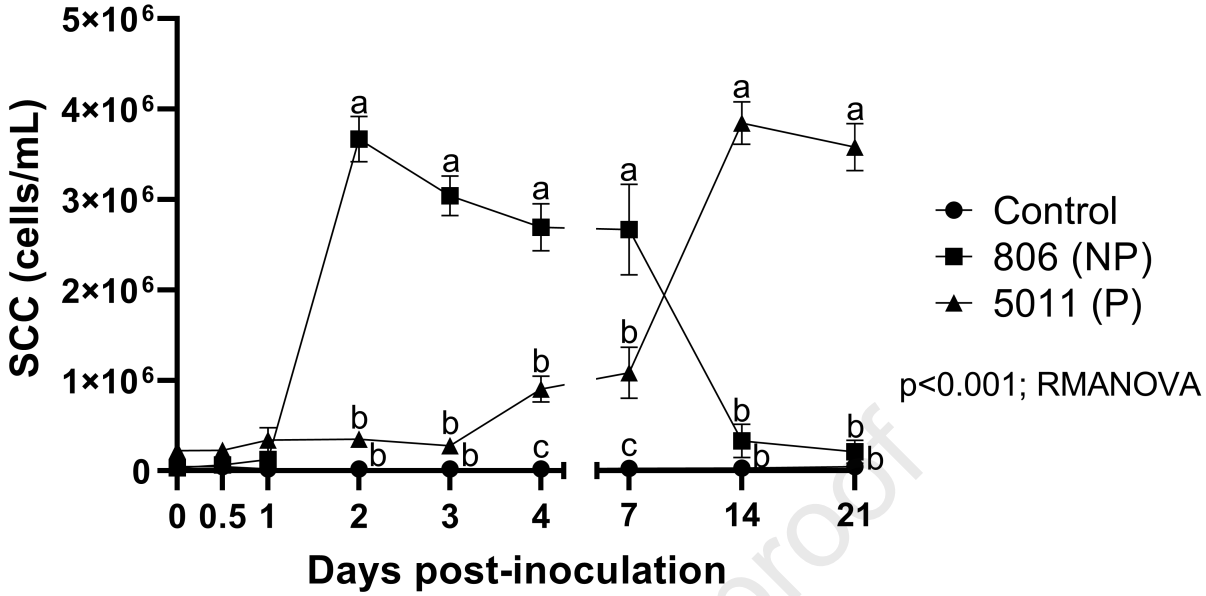
Table 2: Isolations from mammary quarters experimentally inoculated with two selected *S. aureus* strains and mastitis score (0-1) at different times post inoculation.

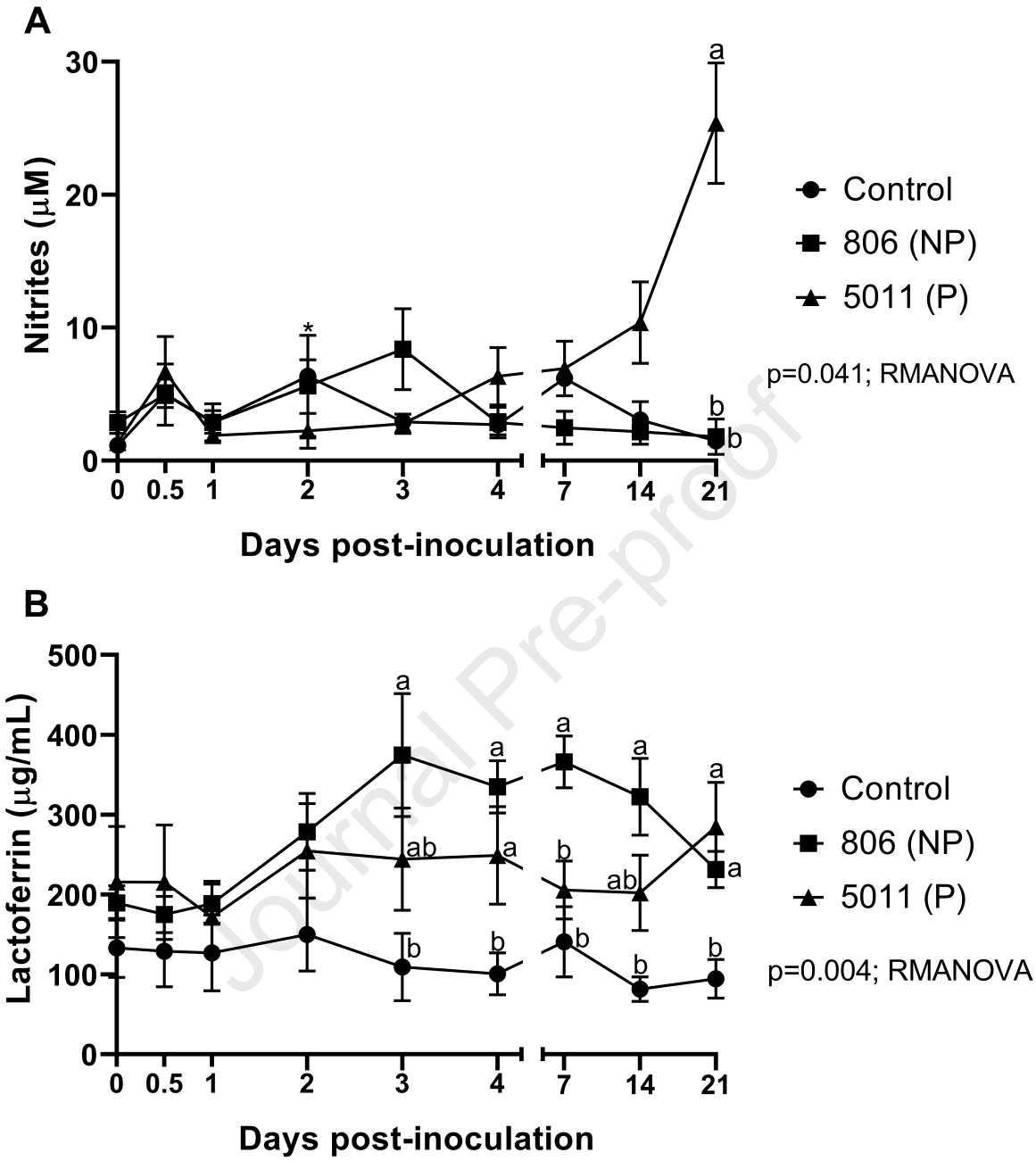
Strain (animal – inoculated quarter)	**0**	**0.5 d**	**1 d**	**2 d**	**3 d**	**4 d**	**7 d**	**14 d**	**21 d**	**28 d**	**35 d**	**42 d**	**49 d**	**56 d**
806 (NP) (Animal 1 RF)	(-) (0)	(-) (0)	**5,000 CFU/ml (1)**	**1,500 CFU/ml (1)**	**500 CFU/ml (1)**	(-) **(1)**	(-) **(1)**	**100 CFU/ml** (0)	**300 CFU/ml** (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
806 (NP) (Animal 1 LH)	(-) (0)	(-) (0)	**1,500 CFU/ml (1)**	**500 CFU/ml (1)**	**500 CFU/ml (1)**	(-) **(1)**	(-) **(1)**	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
806 (NP) (Animal 2 RF)	- (0)	- (0)	**300 CFU/ml (1)**	(-) (1)	(-) (1)	(-) (1)	(-) (1)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
806 (NP) (Animal 2 LH)	(-) (0)	(-) (0)	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
806 (NP) (Animal 3 RF)	(-) (0)	(-) (0)	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
806 (NP) (Animal 3 LH)	(-) (0)	(-) (0)	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
806 (NP) (Animal 4 RF)	(-) (0)	(-) (0)	**300 CFU/ml (1)**	**1500 CFU/ml (1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
806 (NP) (Animal 4 LH)	(-) (0)	(-) (0)	(-) **(1)**	**(-)** **(1)**	(-) **(1)**	(-) **(1)**	(-) **(1)**	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
5011 (P) (Animal 5 RF)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	**250 CFU/ml** (0)	**380 CFU/ml** (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
5011 (P) (Animal 5 LH)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	**200 CFU/ml** (0)	**320 CFU/ml** (0)	(-) (0)	**250 CFU/ml** (0)	(-) (0)	100 CFU/ml (0)	(-) (0)	(-) (0)
5011 (P) (Animal 6 RF)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	**400 CFU/ml** (0)	(-) (0)	**400 CFU/ml** (0)	(-) (0)	**250 CFU/ml** (0)	**100 CFU/ml** (0)	(-) (0)
5011 (P) (Animal 6 LH)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	**500 CFU/ml (1)**	**200 CFU/ml** (0)	**150 CFU/ml** (0)	**200 CFU/ml** (0)	(-) (0)	(-) (0)	**150 CFU/ml** (0)
5011 (P) (Animal 7 RF)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	**200 CFU/ml**	(-)	(-)	(-)	(-)	(-)	(-)

	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
5011 (P) (Animal 7 LH)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	**360 CFU/ml (1)**	**100 CFU/ml** (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
5011 (P) (Animal 8 RF)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	**340 CFU/ml** (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)
5011 (P) (Animal 8 LH)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	**380 CFU/ml** (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)	(-) (0)

RF: Right front quarter. LH: Left hind quarter. (-) Negative microbiological culture to *S. aureus.*): Score (0) = no overt changes in gland or milk; Score (1) = overt changes in milk with no observed MG inflammation (Middleton et al., 2004).







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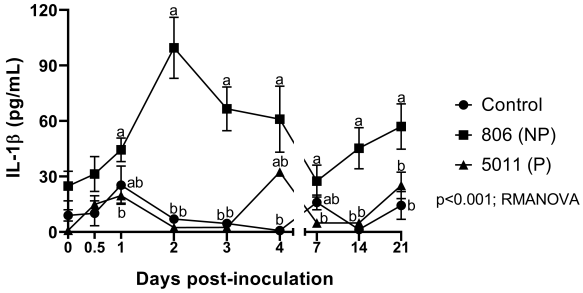

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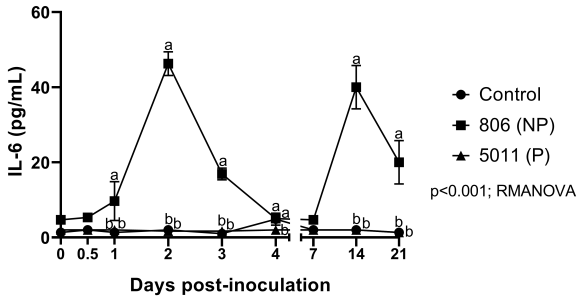

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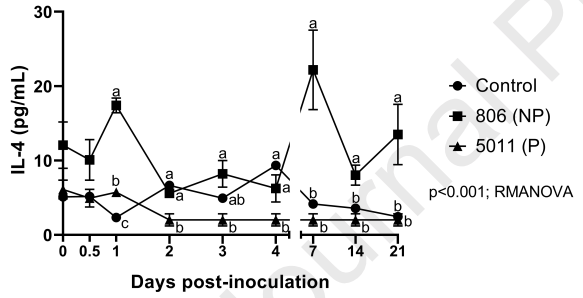

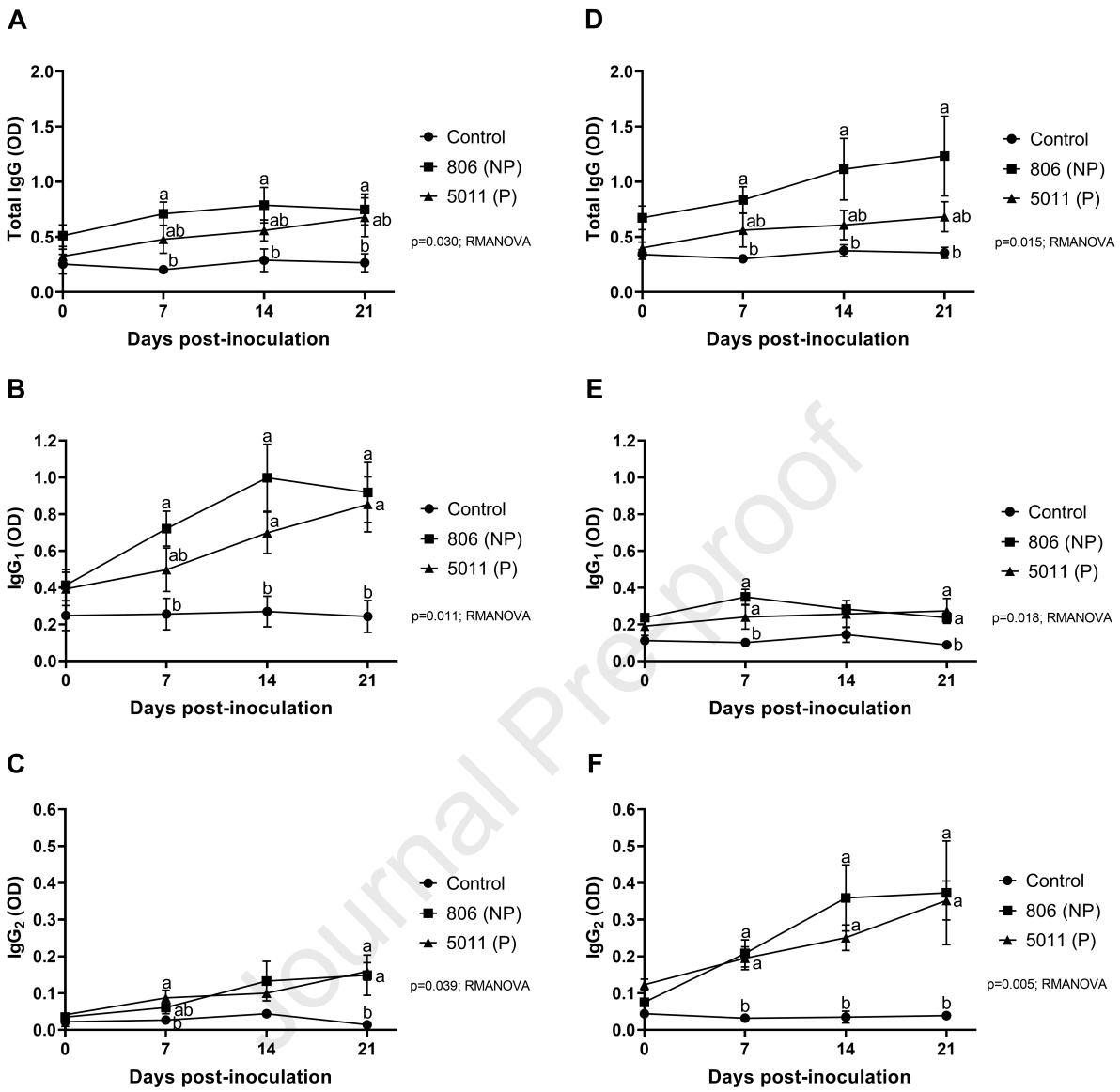

Highlights:

Mammary quarters challenged with *S. aureus* strain 806 (NP) developed mild clinical mastitis.

Mammary quarters challenged with *S. aureus* strain 5011 (P) developed persistent mastitis.

Strain 806 (NP) triggered a rapid and intense immune response that was associated with clearance of the organism from milk.

Humoral immune responses were not associated with clearance of both *S. aureus* strain.

S. aureus strains with distinct adaptation capabilities to the MG are associated with different immune response characteristics.

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Santa Fe, Argentina, May 19, 2022

Editor-in-Chief
Microbial Pathogenesis
Dear Editor,

Please find enclosed our manuscript entitled: "Differential immune response to two *Staphylococcus aureus* strains with distinct adaptation genotypes after experimental intramammary infection of dairy cows" from Carolina Engler, María S. Renna, Camila Beccaria, Paula Silvestrini, Silvana Pirola, Elizabet A.L. Pereyra, Celina Baravalle, Cecilia Camussone, Stefan Monecke, Luis F. Calvinho and Bibiana E. Dallard, for publication in Microbial Pathogenesis.

All listed authors have participated in this study and approved the final version of this manuscript. This material has not been published previously and will not be submitted for publication elsewhere. There are **no conflicts of interest** of all listed authors.

Sincerely yours,

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