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Capacity of two *Staphylococcus aureus* strains with different adaptation genotypes to persist and induce damage in bovine mammary epithelial cells and to activate macrophages

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ABSTRACT

The aim of this study was to evaluate and compare the ability to adhere/internalize, persist, and induce damage in mammary epithelial cells (MAC-T) of two *Staphylococcus aureus* strains with different adaptation genotypes (low and high) to the bovine mammary gland (MG). Also, the phagocytic and bactericidal capacity induced after the interaction between macrophages, isolated from mammary secretion, of both *S. aureus* strains was evaluated. Two isolates (designated 806 and 5011) from bovine intramammary infection (IMI) 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). Strains 806 and 5011 were associated with low (nonpersistent-NP) and high (persistent-P) adaptation to the MG, respectively. Strain 5011 (P), *agr* group I, *cap8* positive and strong biofilm producer showed higher capacity to adhere/internalize in MAC-T compared with strain 806 (NP), characterized as *agr* group II, *cap5* positive and weak biofilm producer. Strain 5011(P) could be recovered from MAC-T lysates up to 72 h pi; while strain 806 (NP) could be recovered only at 4 h pi. Strain 5011 (P) showed greater capacity to induce apoptosis compared with strain 806 (NP) at 4, 24 and 48 h pi. Macrophages infected with strain 5011 (P) showed a greater phagocytic capacity and higher percentage of intracellular reactive oxygen species (ROS) production than strain 806 (NP). No viable bacteria were isolated from macrophages lysates stimulated with any of the *S. aureus* strains at 2, 4, 8 and 24 h pi. The knowledge of the molecular profile of the *S. aureus* strains causing bovine mastitis in a herd could become a tool to expose the most prevalent virulence gene patterns and advance in the elucidation of the pathogenesis of chronic mastitis.

1. Introduction

Staphylococcus aureus is a major pathogen distributed worldwide, causing bovine intramammary infections (IMIs) often leading to chronic mastitis [1]. One of the most important factors during development of bovine *S. aureus* IMI is the bacterium capability to evade clearing by antibiotics and by the host immune system resulting in long-lasting persistent infections [2]. Both early interactions between *S. aureus* and host cells, as well as the events that lead to establishment of chronic mastitis are not fully understood. Several phenotypic and genotypic strain characteristics have been suggested to be linked to *S. aureus* long-term persistence in the bovine mammary gland (MG), including the capacity to form biofilms and to invade and/or survive intracellularly, the capsular polysaccharides (CP) production and the accessory gene regulator (*agr*) type of the strain [3,4].

Biofilm formation, a highly organized multicellular complex, is not only associated with epithelial adhesion but also with evasion of host immune defense [5]. Furthermore, bacterial communities in biofilm are

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less susceptible to antibiotics [6]. Biofilm production requires the presence of the gene cluster *icaADBC* (intracellular adhesion locus). Both the high prevalence of *S. aureus* isolated from bovine IMI harboring the *ica* locus [7] and the proportion of biofilm producers among isolates belonging to pulsotypes (PT) associated with milk rather than bovine extramammary sites [8], suggest its potential role as a virulence factor in the pathogenesis of mastitis.

Staphylococcus aureus is commonly recognized as an extracellular pathogen, but it can survive and even replicate inside non-professional as well as professional phagocytes [9,10]. The survival within the intracellular niche protects the bacteria from the antibiotics commonly used in mastitis treatment and enables them to persist in the host for a long time without causing apparent sings of inflammation [11]. In a previous study, using selected *S. aureus* isolates from bovine IMI categorized as persistent-P and non-persistent-NP having different genetic profiles, we demonstrated that *S. aureus* internalization into bovine mammary epithelial cells (MAC-T) was strain-dependent and internalized bacteria overexpressed adherence and biofilm-forming genes compared with those that remained in cocultures supernatants; particularly those genes encoding FnBPs and IcaD [12].

Capsular polysaccharide (CP) production by *S. aureus* isolated from bovine IMI has been shown to increase resistance to phagocytosis. Capsular polysaccharides from serotypes 5 and 8 are prevalent both in human and animal infections [13,14]. In addition, the role of CP in *S. aureus* internalization in cells has been described. The absence of CP expression was shown to enhance adherence [15] and invasion [16] of endothelial and epithelial cells. Furthermore, CP-negative *S. aureus* were shown to induce chronic experimental mastitis in mice, suggesting that the loss of CP expression may enhance the persistence of *S. aureus* in the MG [16]. More recently, Grunert et al. [4] demonstrated that lack of CP expression was associated with high within-herd *S. aureus* prevalence.

The expression of most *S. aureus* virulence genes is controlled by an *agr* locus, which encodes a two-component signal transduction system that leads to down-regulation of surface proteins and up-regulation of secreted proteins during growth [17]. The *agr* system is polymorphic and permits classification of *S. aureus* strains in four groups *Agr* group I is associated with persistence or with features that facilitate bacterial persistence in the udder: strains belonging to *agr* group I are more likely to be internalized in epithelial cells, to persist in murine MG [18] and to be associated with penicillin resistance [19] than strains belonging to the other *agr* groups.

Previous studies indicate that high within-herd prevalence and persistence of *S. aureus* is linked to specific phenotypes and genotypes [4,20,21]. Therefore, the identification of the staphylococcal phenotypic and genotypic characteristics associated with persistence of IMI is of utmost importance to understand the pathogenesis of the disease. The aim of this study was to evaluate and compare the ability to adhere/internalize, persist, and induce damage in MAC-T cells of two *S. aureus* strains with different adaptation genotypes (low and high) to the bovine MG. Also, the phagocytic and bactericidal capacity induced after the interaction between mammary macrophages with the two *S. aureus* strains was evaluated.

2. Materials and methods

2.1. Strains selection

Two *S. aureus* strains (designated 806 and 5011) isolated from milk samples taken from Holstein cows with subclinical mastitis belonging to different dairy farms located in Santa Fe province (Argentina) were selected based on differential features. Mastitis was characterized as subclinical when milk somatic cell counts were $> 250 \times 10^3$/mL without macroscopic changes in milk. Strain 806 was isolated only once from a mammary quarter and not re-isolated in three consecutive milk samplings after standard treatment with beta lactam antibiotics for 3 days was carried out. It was considered to have low adaptation to bovine MG and designated as nonpersistent (NP). Strain 5011 was isolated from the same mammary quarter in consecutive monthly milk samplings over a period of six months and was considered highly adapted to bovine MG and designated as persistent (P). To confirm persistency, in a previous study by Pereyra et al. [12], the genotypic profiles of *S. aureus* isolates obtained from the same quarters were compared using pulsed-field gel electrophoresis (PFGE).

Quantification of biofilm production was assessed by a microtiter plate assay (MPA) described in a previous study by Pereyra et al. [12]. The accessory gene regulator (*agr*) groups were determined by a multiplex PCR described by Pereyra et al. [12]. The presence of genes that code for capsular polysaccharide (CP), adhesion, biofilm formation and resistance to penicillin was evaluated by polymerase chain reaction (PCR) according to Pereyra et al. [12]. The clonal relationships of the strains were evaluated by PFGE according to the methodology employed in a previous work [12]. Strains were further characterized by a microarray-based *S. aureus* genotyping system (Alere GmbH, Jena, Germany). Genomic DNA was obtained as previously described [22]. Probe sequences used in this system were derived from published genome sequences of *S. aureus* strains and have been published previously [22].

2.2. Bacterial growth conditions

Before each experiment, a fresh bacterial suspension was activated from frozen stocks (−80 °C) by culture on Columbia agar base (CAB) (Britania, Buenos Aires, Argentina) and incubated at 37 °C for 24 h under aerobic conditions. Three colonies of each strain were inoculated into 5 mL of trypticase soy broth (TSB) (Britania) and incubated for 16 h at 37 °C. Culture was vortexed and diluted 1:100 in TSB (Britania) and incubated to mid-log phase for 2 h at 37 °C. Bacteria were collected from the culture by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) or RPMI Medium 1640 (1X GIBCO® by Life Technologies™).

2.3. Mammary epithelial cells culture

The established bovine mammary epithelial cell line (MAC-T) [23] was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), insulin (5 mg/mL), hydrocortisone (1 mg/mL), penicillin (100 U/mL), and streptomycin sulfate (100 mg/mL) (Sigma Chemical Co., St. Louis, MO).

2.4. Adherence and internalization assays in MAC-T

For adherence and internalization assays, approximately 10^5 MAC-T cells were seeded onto 24-well tissue culture plates and incubated at 37 °C in a 5% CO_2 (v/v) for 24 h. Strains of *S. aureus* at mid-log phase were co-cultured with confluent monolayer of MAC-T cells (2×10^5 cells/mL) in DMEM without penicillin and streptomycin sulfate at a multiplicity of infection (MOI, ratio of *S. aureus* organisms to cells) of 100:1 at 37 °C in 5% CO_2 for 2 h. To select the MOI, appropriate dilutions were performed to obtain varying numbers of organisms per epithelial cell (data not shown). The number of epithelial cells per well was estimated by counting in a hemocytometer to determine the bacteria: epithelial cell ratio used during invasion experiments. After incubation, wells were washed 3 times with 500 μL of PBS 1X (pH 7.4) and treated with medium containing gentamicin (100 mg/mL, Sigma Chemical Co., St. Louis, MO) in DMEM at 37 °C in 5% CO_2 for 2 h to kill extracellular bacteria. Supernatants were then collected and plated on TSA added with 5% calf blood to verify killing by gentamicin. Finally, MAC-T monolayers were washed three times with PBS, treated with 0.25% trypsin 0.1% EDTA (Gibco, BRL) and further lysed with Triton X-100 (Amersham, Arlington Heights, IL, USA) at a final

concentration of 0.025% (v/v) in sterile distilled water to release intracellular staphylococci. MAC-T lysates were serially diluted tenfold, plated on TSA added with 5% calf blood and incubated overnight at 37 °C. Colony forming units per mL (CFU/mL) of *S. aureus* internalized in MAC-T cells were determined by standard colony counting techniques. Adherence assays were run in parallel with the exception that antibiotic treatment was omitted. Numbers of adherent bacteria were determined by subtracting the number of internalized bacteria from the number of MAC-T cell-associated bacteria. Each strain and condition was tested in triplicate and assays were repeated independently at least three times.

2.5. Intracellular persistence assays in MAC-T cells

To determine whether *S. aureus* strains could survive or multiply within MAC-T cells, the standard invasion assay was modified by further incubation of infected monolayers for up to 72 h. Persistence assays were based on previously described methodology by Atalla et al. [24] with modifications. Briefly, cultures of MAC-T cells were grown to confluence in 24-well tissue culture plates as described above. Monolayer cells were infected with the *S. aureus* strains at a MOI of 30 for 2 h at 37 °C in 5% CO_2 followed by exposure to 100 mg/mL gentamicin (Sigma) treatment for 2 h to eliminate extracellular bacteria. Cultures were washed 3 times with PBS and incubation was continued in fresh growth medium supplemented with 100 mg/mL gentamicin for 10, 24, 48 and 72 h to prevent monolayer infections by released bacteria from infected dead cells. Cells were monitored daily by light microscopy and the growth medium was changed every other day. The numbers of intracellular CFU were determined at 24–72 h time points and compared with the numbers recovered after 4 h.

2.6. MAC-T cell death assay

Apoptotic and necrotic MAC-T cells after *S. aureus* infection were detected by flow cytometry using Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, MA) according to manufacturer's instructions. Apoptosis in *S. aureus*-infected MAC-T cells was evaluated at different time post-infection (4, 10, 48, and 72 h) using a MOI of 30. Persistence assays were performed as described above. Noninfected and *S. aureus* infected MAC-T cells were incubated for 4, 10, 24, 48 and 72 h and then were washed two times with PBS. To detach MAC-T monolayers, plates were treated with 0.25% trypsin 0.1% EDTA (Gibco, BRL) for 7 min. Suspension of MAC-T cells were centrifuged at 800 × *g* for 5 min at 4 °C and resuspended in 500 μL of 1X Annexin V binding buffer. Finally, 5 μL of Annexin V-FITC and 5 μL propidium iodide (PI) were added and cells were incubated at room temperature for 5 min in the dark. Three independent experiments were performed in triplicate. As positive control of apoptosis MAC-T cells were treated with hydrogen peroxide (5%) during 7 h. Data were collected using an Attune, NxT Acoustic Focusing Cytometer (A24860, Life Technology) and analyzed using FlowJo software (TreeStar Inc., Ashland, USA). Flow cytometry was performed on MAC-T gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. Cells in early phases of apoptosis bind annexin V but exclude PI (Anexin V + PI-), while those in the late apoptosis process or necrotic cells bind simultaneously with annexin V and PI (Anexin V + PI+). Necrotic cells were only PI positive (Anexin V– PI+). Cells in early phase of apoptosis were analyzed.

2.7. MAC-T cell viability assay

MAC-T cell viability after *S. aureus* infection were detected by Cell Proliferation Kit II (XTT) assay (Roche Life Science, Basilea, Switzerland) according to Beccaria et al. [25] with slight modifications. Briefly, MAC-T cells (2×10^5 cells/mL) were seeded in 24-well plates at 37 °C, 5% CO_2 and incubated with the *S. aureus* strains as in persistence assays described above. After each incubation time (4, 24, 48 and 72 h), MAC-T cells were treated with 500 μL/well of 0.3 mg/mL of XTT labeling mixture (XTT labeling reagent: sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate and electron-coupling reagent: PMS (*N*-methyldibenzopyrazine methyl sulfate) and incubated in a humidified atmosphere for 2 h. Uninfected cells were used as a negative control (100% viability), and cells treated with 150 μL of ethyl alchohol 99.5% (Cicarelli, Santa Fe, Argentina) served as a positive control of mortality (0% viability). Three independent experiments were performed in triplicate. Finally, the absorbance was measured at 450 nm on a microplate spectrophotometer SPECTROstar Nano (Life Technologies) and the results were expressed as optical density (OD).

2.8. Bacterial viability inside MAC-T cells

To investigate the survival of internalized *S. aureus* into MAC-T cells, 806 (NP) and 5011 (P) strains were stained with a mixture of Syto 9 and PI using Live/Dead® BacLightTM Bacterial Viability Kit (Molecular Probes, Invitrogen). Persistence assays were performed as described above with some modifications. Briefly, MAC-T cells were seeded on sterile coverslips placed in 6-well culture plates (Costar, Fisher Scientific) and incubated with *S. aureus* strains. After each incubation time (4, 24, 48 and 72 h), MAC-T cells were washed three times with PBS, fixed in PBS containing 4% paraformaldehyde for 30 min and permeabilized with PBS containing 0,1% saponin (Sigma) for 10 min. Finally, MAC-T cells were stained with the dye mixture containing 0.5 μL of Syto 9 (3.34 mM) and 1 μL of PI (20 mM) in 100 μL PBS containing 0.1% saponin for 15 min. Stain was performed in darkness and at room temperature. Uninfected cells were used as a negative control. Live and death intracellular bacteria were observed with a fluorescence microscope (Eclipse Ci, Nikon, Tokyo, Japan), and representative images were taken with a digital camera (DS-Fi3, Nikon).

2.9. Isolation of macrophages from mammary gland secretion

For phagocytosis assays, mammary secretions samples were collected from nonlactating healthy cows 10–15 days after cessation of milking and macrophages were isolated as described previously [25]. Approximately 25 mL of mammary secretion was diluted with PBS and centrifuged at 400 × *g* for 10 min at 4 °C using a high capacity centrifuge (RC6 plus, Sorvall, Thermo Fisher Scientific). Then, fat was carefully removed; the pellet was washed twice with PBS and resuspended in 40 mL of PBS. Then 5 mL of Histopaque 1083 (Sigma) were placed in the bottom of the tube and centrifuged at 400 × *g* for 30 min at room temperature. The mononuclear layer was collected and the cells were washed three times (400 × *g*, 10 min at 4 °C) in PBS. Using the trypan blue (Sigma) exclusion test, viable cells were counted in a Neubauer haemocytometer chamber and resuspended to the desired concentration in RPMI 1640 medium supplemented with 10% FBS.

2.10. Phagocytosis assay and intracellular reactive oxygen species production

The phagocytosis assay was performed by flow cytometry using fluorescein isothiocyanate (FITC)-labelled *S. aureus* strains according to Beccaria et al. [25]. One millilitre of PBS containing FITC (10 mg/mL in dimethylsulfoxide) and 1×10^9 CFU/mL of 806 (NP) or 5011 (P) *S. aureus* strains were incubated at room temperature for 1 h with slight shaking. Both labelled bacteria were washed four times and resuspended to initial volume with PBS. Macrophages (1×10^6 cells/mL) were seeded in tubes and then cells were incubated with both FITC-labelled bacteria in a MOI of 100:1 at 37 °C for 30 min. To stop phagocytosis cells were incubated with cool NaCl 0.85%/EDTA 0.04%, centrifuged at 400 × *g* for 5 min and incubated with 1 μM hidroethidine (HE, Molecular Probes, Invitrogen) in PBS for 15 min at 37 °C in

Table 1
Summary of phenotypic and genotypic characteristics of *S. aureus* strains used in this study.

S. aureus strains	Biofilm grading (MPA)	*agr* type	Capsule type	Adhesion genes	Biofilm producing genes	Resistance penicillinase	PFGE	Hybridization profiles
Strain NP (806)	Weak	*agrII*	*cap5*	*clfA, clfB, fnbpA, fnbpB (−), fib, cna*	*icaA, icaC, icaD, bap (−)*	*blaZ (−)*	D	ST350
Strain P (5011)	Strong	*agrI*	*cap8*	*clfA, clfB, fnbpA, fnbpB (−), fib, cna*	*icaA, icaC, icaD, bap (−)*	*blaZ*	O	ST188 (CC188)

NP: nonpersistent. P: persistent. MPA: microtiter plate assay. PFGE: pulsed-field gel electrophoresis. 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*).

darkness for intracellular reactive oxygen species (ROS) production [25]. HE, which is a cell-permeable, nonfluorescent probe, is converted to ethidium by ROS (mainly by superoxide anion) in a dose-dependent manner, resulting in fluorescence emission. Finally, the cells were centrifuged at 400 × *g* for 5 min, resuspended in focusing fluid and analyzed by flow cytometry (Attune, NxT Acoustic Focusing Cytometer A24860, Life Technology). The macrophage population was gated based on forward and side light scatter parameters (Region 1, R1). Data were collected and analyzed using FlowJo software (TreeStar Inc., Ashland, USA). The percentage of macrophages with associated bacteria (adhered and/or internalized) was assessed (Region 2) and the mean fluorescence intensity (MFI) was used to estimate the number of bacteria associated per positive cell [25]. Conversion of HE to ethidium by superoxide anion in macrophage population (R1) or in FITC + macrophage population (R2) was analyzed. The MFI was used to estimate the amount of ROS produced per cell [25].

2.11. Nitric oxide production

Macrophage supernatants from the phagocytosis assay were recovered for all conditions: uninfected macrophages (basal cells) and infected macrophages with the *S. aureus* strains; to determine nitrite concentration (NO2-) using the Griess Reagent Kit (Thermo Fisher Scientific) according to Beccaria et al. [25]. Briefly, the Griess reagent was prepared by mixing equal volumes of stock solution A (10% sulfanilamide, 40% phosphoric acid) and stock solution B [1% *N*-(1-naphthyl) ethylenediamine dihydrochloride] and then incubated with the filtered supernatant for 30 min at room temperature before the measurement of nitrite. Parallel to this, a standard curve with a nitrite standard solution (provided by the kit) was performed. Total nitrite levels, indicative of the amount of nitric oxide (NO) production, were evaluated by reading the OD of each sample at 550 nm and calculated by reference to the standard calibration curve of sodium nitrite (0.78–100 μM). Cells incubated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) and 1 mg/mL ionomycin (Io, Sigma-Aldrich) were used as positive control of NO production.

2.12. Intracellular persistence assays in bovine macrophages

Persistence assays were performed as previously described above with modifications. The *S. aureus* strains were co-cultured with bovine macrophages (2×10^6 cells/mL) in RPMI medium 1640 (Life Technologies) at a MOI of 100:1 for 1 h at 37 °C in 5% CO_2. After incubation, macrophages were washed with PBS and treated with gentamicin (100 mg/mL, Sigma) in RPMI Medium 1640 (Life Technologies) at 37 °C in 5% CO_2 for 1 h to kill extracellular bacteria. Cultures were washed 3 times with PBS and incubation was continued in fresh growth medium supplemented with 100 mg/mL gentamicin for 4, 8 and 24 h to prevent monolayer infections by released bacteria from infected dead cells. In each corresponding time (2, 4, 8, and 24 h), macrophages were washed with PBS and lysed with Triton X-100 at a final concentration of 0.025% (v/v) in sterile distilled water to release intracellular staphylococci. Macrophages lysates were serially diluted tenfold, plated on TSA added with 5% calf blood and incubated overnight at 37 °C. Colony forming units per mL of *S. aureus* internalized in macrophages were determined by standard colony counting technique. The assay was performed in triplicate and experiments were repeated five times.

2.13. Bacterial viability inside bovine macrophages

To investigate the survival of phagocytosed *S. aureus* into bovine macrophages, persistence assays were performed and both *S. aureus* strains were stained as previously described above in 2.8 with modifications. Bovine macrophages were infected with the *S. aureus* strains and after each incubation time (2 and 4 h), macrophages were washed,

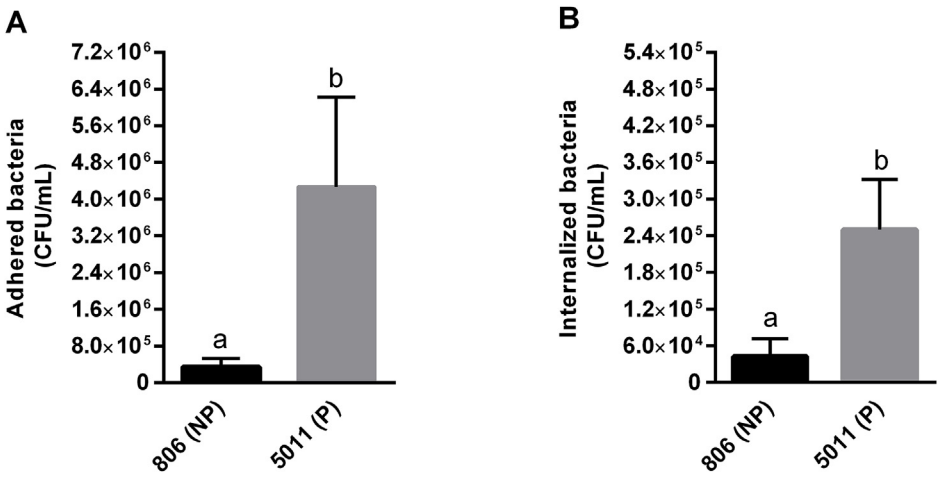

Fig. 1. Adherence (A) and internalization (B) to MAC-T cell by 806 (NP) and 5011 (P) *S. aureus* strains. Data are presented as colony forming units per millilitre (CFU/mL) of adherent and internalized bacteria and are the mean of three experiments run in triplicate. Bars represent the mean ± standard deviation (SD). Differences between groups in adherence and internalization assays were assessed by Student *t*-tests. Different letters correspond to statistically significant differences ($P < 0.05$).

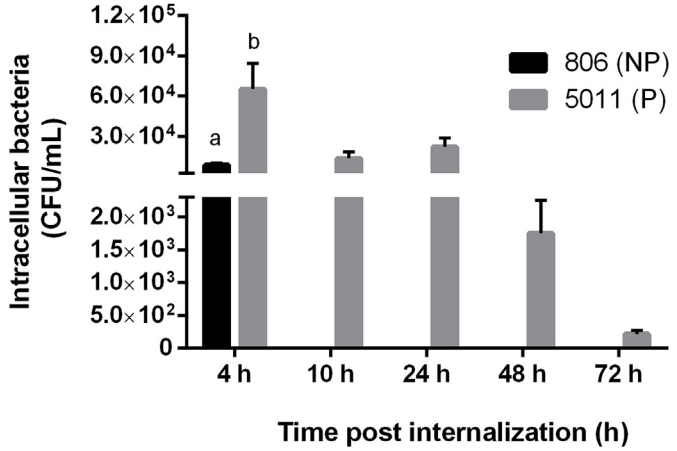

Fig. 2. Persistence of 806 (NP) and 5011 (P) *S. aureus* strains within MAC-T cells over time. All values indicate the mean of three independent experiments, each performed in triplicate. Bars represent the mean ± standard deviation (SD). Differences between groups in *S. aureus* persistence assays were assessed by Mann-Whitney non-parametric test. Different letters correspond to statistically significant differences ($P < 0.05$).

fixed in PBS containing 4% paraformaldehyde for 30 min and permeabilized with PBS containing 0,1% saponin for 10 min. Finally, macrophages were stained with 100 μL of PBS-0.1% saponin containing the dye mixture (Syto 9/PI) for 15 min. Bovine macrophages without *S. aureus* treatment were used as the negative control. Three independent experiments were performed in triplicate. Live and death intracellular bacteria were observed with a fluorescence microscope and representative images were taken with a digital camera.

2.14. Statistical analysis

All statistical analyses were performed using a software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois). Results were expressed as mean ± standard deviation (SD). Differences between groups in adherence and internalization assays were assessed by Student *t*-tests. Differences between groups in cell death and viability assays in MAC-T and NO production from phagocytosis assays were assessed by one-way analysis of variance (ANOVA), followed by a multiple comparision Duncan's tests. Differences between groups in *S. aureus* persistence assays in MAC-T, macrophage phagocytosis assays and ROS production were assessed by Mann-Whitney non-parametric test. P values of < 0.05 in all tests employed were considered significant.

3. Results

3.1. Diferential characteristics of S. aureus strains

Table 1 summarizes the differential characteristics of the two *S. aureus* strains. Strain 806 (NP) was susceptible to penicillin and weak biofilm producer; while strain 5011 (P) was resistant to penicillin and strong biofilm producer. Strain 806 (NP) was characterized as *agr* group II and *cap*5 gene positive and strain 5011 (P) as *agr* group I and *cap8* positive. Presence of clumping factor A and B genes (*clfA*, *clfB*); fibronectin binding proteins A gene (*fnbpA*); fibrinogen binding protein gene (*fib*); collagen adhesion gene (*cna*); and intercellular adhesion genes A, C and D (*icaA*, *icaC*, *icaD*) were detected in both *S. aureus* strains. Both strains were methicillin-susceptible and *bap* (biofilm-associated protein gene) negatives. Presence of beta-lactamase gene (*blaZ*) was detected in 5011 (P) strain. Both isolates were not clonally related and belonged to different PFGE types. The hybridization profiles allowed the assignment of isolates to different sequence type (ST) and clonal complex (CC).

3.2. Adherence and internalization in MAC-T cells

The ability to adhere and internalize in host cells of *S. aureus* belonging to different clonal types causing subclinical NP and P bovine IMI was evaluated by the epithelial MAC-T cell adhesion and invasion assays. Results indicated that the *S. aureus* strains presented different adhesion and internalization capacities. The number of CFU/mL from strain 5011 (P) that attached and internalized to MAC-T cells with a MOI of 100:1 were higher ($P < 0.01$) than that of strain 806 (NP) (Fig. 1).

3.3. Intracellular S. aureus persistence in MAC-T cells

The persistence capacity of both *S. aureus* strains in MAC-T cells was evaluated up to 72 h post-infection (pi) with a MOI of 30:1. Results showed that the two *S. aureus* strains presented different capacity to persist in MAC-T cells over time. Strain 806 (NP) was recovered from MAC-T cells lysates only after 4 h of co-culture and the number was significantly lower than the one obtained for 5011 (P) strain ($P = 0.002$; Fig. 2). Strain 5011 (P) was recovered from MAC-T cells lysates from 4 to 72 h pi. At 4 h of co-culture, the highest number of bacteria was recovered ($6.5 \times 10^4 \pm 5.2 \times 10^4$ CFU/mL); after that a decrease was observed, detecting at 72 h a low number of bacteria ($2.1 \times 10^2 \pm 5.7 \times 10^1$ CFU/mL) (Fig. 2).

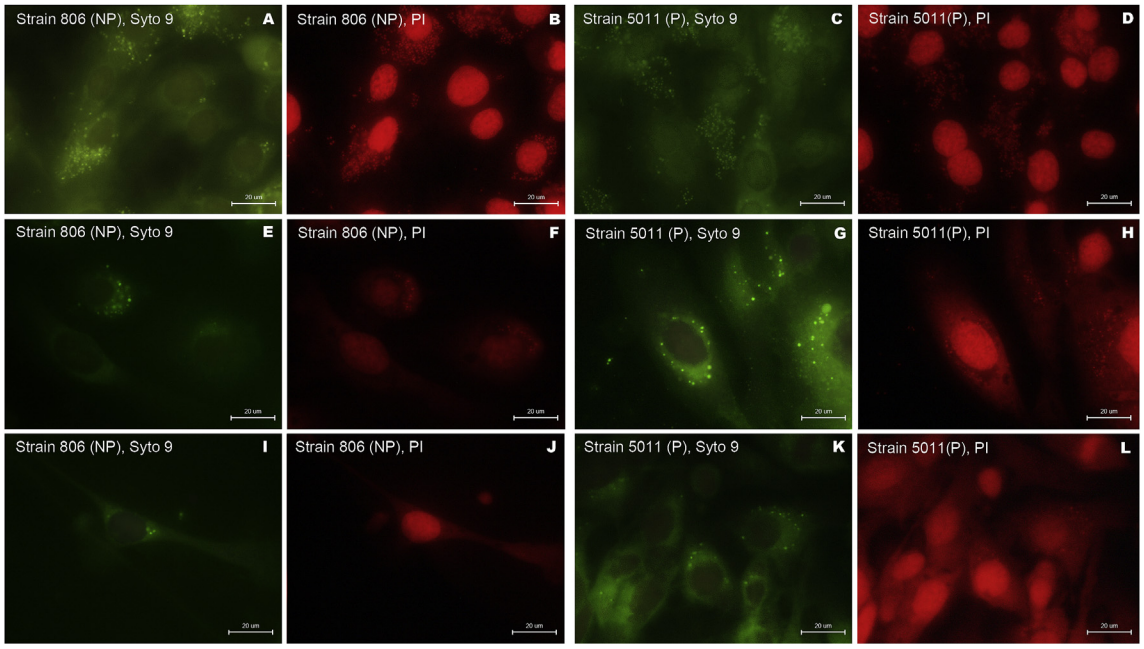

Fig. 3. Fluorescence imaging of Syto 9/PI stained samples from MAC-T cells infected by 806 (NP) and 5011 (P) *S. aureus* strains at 4, (A–D), 24 (E–H) and 48 (I–L) h pi. Live bacteria in the cytoplasm of infected MAC-T cells labelled with Syto 9 are shown in green. At 72 h pi, Syto 9 stained bacteria were observed in MAC-T cells infected by 5011 (P) *S. aureus* strain (data not shown). Dead bacteria in the cytoplasm of infected MAC-T cells labelled with PI are shown in red. Each panel is from a representative experiment. Magnification, ×1000.

3.4. Bacterial viability inside MAC-T cells

The viability of 806 (NT) and 5011 (P) *S. aureus* strains in MAC-T cells was evaluated by fluorescence microscopy after 4, 10, 24, 48 and 72 h of the co-culture. Fig. 3 shows representative images of MAC-T cells infected with both *S. aureus* strains stained with a mixture of Syto 9 and PI at 4, 24 and 48 h pi. In the MAC-T cells infected with 806 (NT) strain, the presence of viable intracytoplasmic bacteria (positive to Syto 9) was observed from 4 to 24 h pi, showing a decreasing density with the progress of time. At 72 h after co-culture it was not possible to identify viable bacteria in the cytoplasm of MAC-T infected with 806 (NT) strain. In MAC-T cells infected with 5011 (P) strain, the presence of viable intracytoplasmic bacteria was evidenced from 4 to 72 h pi, showing a greater density than that observed with the strain 806 (NP), which was decreasing with the progress of time. Dead bacteria (positive to PI) were observed inside the MAC-T cells infected with both *S. aureus* strains at all evaluated times except at 72 h pi. The number of positive bacteria to PI was scarce at 4 hs pi and increased until 24 h pi and then decreased until 72 h pi; in this last time no PI positive bacteria were detected for any strain.

3.5. MAC-T cells death and viability after S. aureus infection

The capacity of 806 (NP) and 5011 (P) *S. aureus* strains to induce MAC-T cell death by apoptosis and/or necrosis at different times of co-culture (4, 24, 48 and 72 h pi) was evaluated by flow cytometry (Fig. 4A). The results showed that both *S. aureus* strains induced apoptosis to MAC-T cells at all times evaluated. However, differences in the induction of apoptosis between both strains over time were observed (Fig. 4A). At 24 h pi both strains induced the highest percentages of apoptosis ($P < 0.05$) in relation to the uninfected cells (basal). Strain 5011 (P) induced higher percentages of apoptosis compared with 806 (NP) strain and basal at 4, 24 and 48 h pi ($P < 0.05$). At 72 h pi the percentages of apoptosis of both strains were similar to basal.

The viability of MAC-T cells was evaluated after co-culture with 806 (NP) and 5011 (P) *S. aureus* strains by a colorimetric kit that measures the reduction of XTT in metabolically active or viable cells (Fig. 4B). At 4 h pi no differences were observed between MAC-T cells infected with 806 (NP) and 5011 (P) strains and uninfected cells (basal). At 24 h pi the viability of MAC-T cells infected with 806 (NP) strain was similar to uninfected cells and greater than of cells infected with 5011 (P) strain ($P < 0.05$). At 48 and 72 h pi the viability of the infected MAC-T cells was similar for both strains and lower than uninfected cells ($P < 0.05$).

3.6. Phagocytic activity, ROS and NO production in vitro

Both FITC + *S. aureus* strains were co-cultured with mammary secretion macrophages for phagocytosis assays by flow cytometry. Macrophages were initially separated by FSC-A and FSC-H to obtain singlets (Region 1-R1) (Fig. 5A). Then, based on light scatter properties, we defined the Region 2 (R2) to further analyze macrophage phagocytosis and the percentage of intracellular ROS production (Fig. 5A).

The percentage of macrophages with associated bacteria (FITC + macrophages with adhered or intracellular bacteria) was higher in macrophages stimulated with 5011 (P) strain compared to 806 (NP) strain ($P = 0.041$; Fig. 5B). The average number of bacteria associated per macrophage (measured by the mean fluorescence intensity-MFI) showed significant differences between both *S. aureus* strains; being higher in macrophages stimulated with 5011 (P) strain compared with 806 (NP) strain ($P = 0.026$; Fig. 5C).

No differences were observed in the intracellular ROS production (percentage of positive HE cells) between macrophages stimulates with 806 (NP) and 5011 (P) strains ($P = 0.24$; Fig. 5D). The MFI (a measure of the amount of ROS produced per macrophage) was similar for both evaluated strains ($P = 0.86$; Fig. 5E).

The percentage of FITC + macrophages that produced ROS (HE + cells) was higher in cells stimulated with 5011 (P) strain compared with 806 (NP) strain ($P = 0.029$; Fig. 5F). However, the MFI of FITC + macrophages that produced ROS was similar for both evaluated strains ($P = 0.34$; Fig. 5G).

The NO production by phagocytic macrophages stimulated with 806 (NP) and 5011 (P) strains was similar to basal production (macrophages without bacteria). PMA/Io used as positive control stimulated the released of NO in mammary secretion macrophages (Fig. 6).

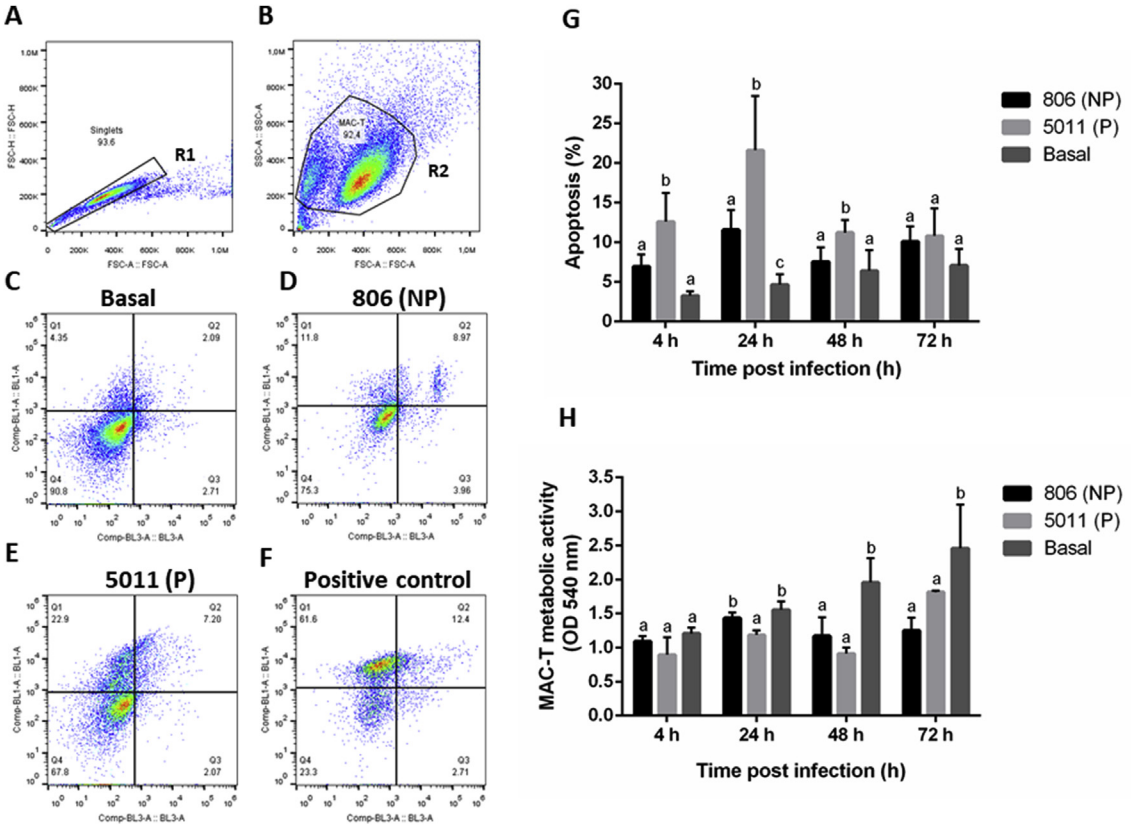

Fig. 4. Apoptosis and viability of MAC-T cells infected by 806 (NP) and 5011 (P) *S. aureus* strains over time. Apoptosis was evaluated by flow cytometry using Annexin V-FITC Apoptosis Detection Kit. (A–F) Gating strategy for apoptosis analysis in MAC-T cells. (A) The cells were separated by FSC-A and FSC-H to obtain singlets (Region 1, R1). (B) Representative forward scatter vs. side scatter dot plot showing gate in Region 2 (R2) for further MAC-T cell death analysis. (C–E) Representative dot plots showing apoptosis in basal and *S. aureus*-infected MAC-T cells evaluated at 24 h post infection. (F) Representative dot plot showing apoptosis in MAC-T treated with hydrogen peroxide as a positive control of cell death. (G) Percentage of early apoptotic cells over time. Bars represent the mean ± standard deviation (SD). Different letters correspond to statistically significant differences ($P < 0.05$). (H) Cell viability was evaluated by XTT assays. Bars represent the mean ± SD. Different letters correspond to statistically significant differences ($P < 0.05$). Differences between groups in apoptosis and viability assays were assessed by one-way analysis of variance (ANOVA), followed by a multiple comparision Duncan's tests. Basal: uninfected MAC-T cells.

3.7. Intracellular S. aureus persistence in bovine macrophages

The intracellular persistence of 806 (NP) and 5011 (P) strains in bovine macrophages purified from mammary secretion was evaluated. No bacteria were isolated from macrophages lysates stimulated with any of the *S. aureus* strains at any evaluated time (2, 4, 8 and 24 h pi).

3.8. Bacterial viability inside bovine macrophages

The viability of 806 (NP) and 5011 (P) *S. aureus* strains in macrophages was evaluated by fluorescence microscopy after 2 and 4 h of the co-culture. Fig. 7 shows representative images of macrophages infected with both *S. aureus* strains stained with a mixture of Syto 9 and PI. At 2 h pi, the presence of live bacteria (positive to Syto9) was identified in the cytoplasm of macrophages stimulated with both *S. aureus* strains; showing a higher density of phagocytosed bacteria the macrophages stimulated with 5011 (P) strain compared with 806 (NP) strain (Fig. 7 A,C). In macrophages challenged with 5011 (P) strain was possible to identify the presence of live bacteria in the cytoplasm of phagocytes until 4 h pi. At 2 and 4 h pi, PI positive bacteria were observed in macrophages stimulated with both *S. aureus* strains (Fig. 7 B,D).

4. Discussion

To gain insights into the mechanism allowing *S. aureus* to successfully persist in the MG, this study focused on two isolates collected from bovine IMI with different genotypic profiles harboring genes involved in adherence and biofilm production, belonging to different CP type, *agr* group, PT and ST/CC. Strain 806 belonged to ST350-MSSA and strain 5011 to ST188 (CC188)-MSSA and were associated with low (NP) and high (P) adaptation to the MG, respectively. CC188-MSSA is considered mostly a human lineage although it has also been found in association with bovines [26,27]; while, ST350-MSSA is a rare ST that has been isolated from humans, dogs, horses, a wild deer and cows with mastitis (Monecke, S; data not published). In this study, we compared the ability of the two selected strain to adhere/internalize, persist and induce damage in MAC-T cells. Also, we evaluated the phagocytic and bactericidal capacity induced after the interaction between mammary macrophages with both *S. aureus* strains.

The binding capacity is closely related to the pathogenicity of staphylococci since their adherence to mammary epithelial cells is a crucial step in the invasion of host cells and in the biofilm formation [28,29]. Internalization might protect bacteria from clearance by the immune system and allow for long-term persistence in chronically infected hosts [30]. In a recent study, Gruner et al. [4], observed that lack of CP expression and high cellular invasiveness were associated with high within-herd prevalence of *S. aureus* persistent subtypes. In this context, Bardiau et al. [31] demonstrated that isolates belonging to *agr* group II, *cap8* positive and expressing CP8, were less likely to survive intracellularly than isolates belonging to *agr* group I which do not express any CP. Moreover, Buzzola et al. [18] showed that strains of *agr* group I (capsulated or noncapsulated) were internalized in MAC-T cells at significantly higher rates than isolates of *agr* groups II, III and IV. In the present study, strain 5011 (P), characterized as *agr* group I and *cap8*

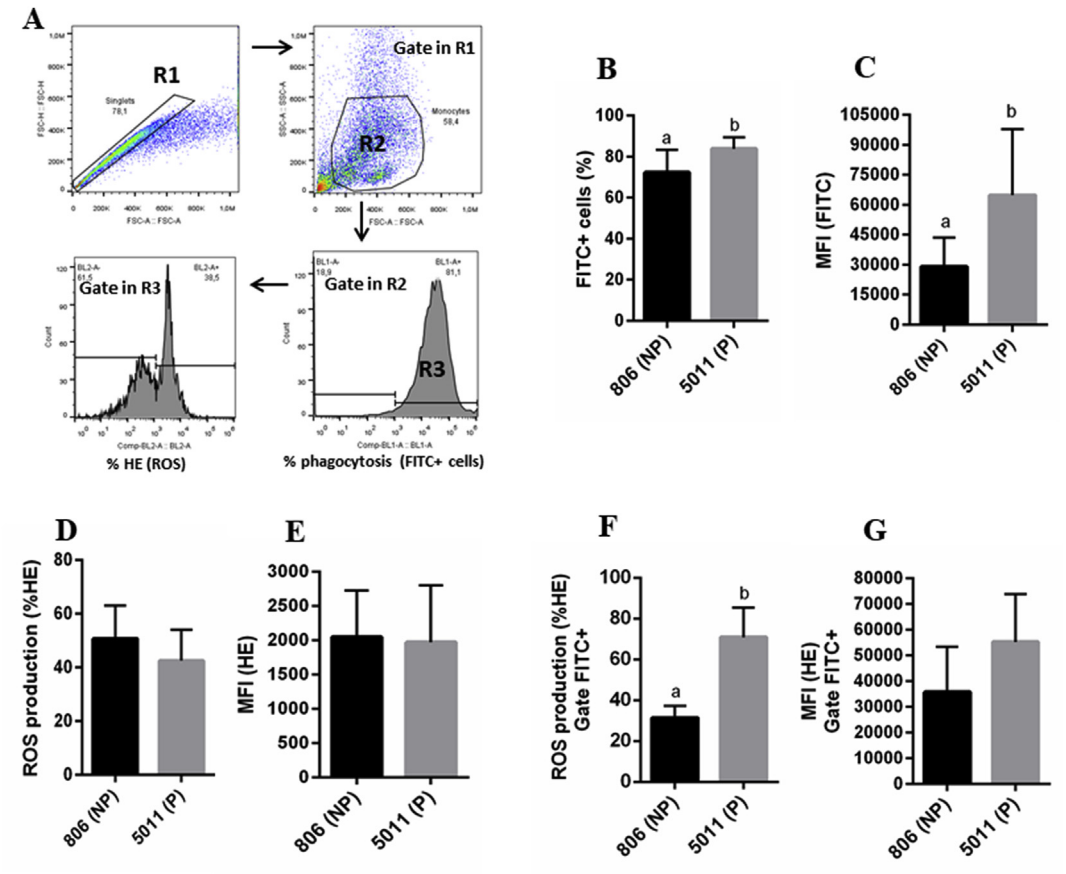

Fig. 5. (A) Gating strategy for analysing phagocytosis and ROS production in the population of total macrophages with at least one associated bacteria (attached and/or internalized). The cells were separated by FSC-A and FSC-H to obtain singlets (Region 1, R1). Representative forward scatter vs. side scatter dot plot showing gate in Region 2 (R2) for further macrophages phagocytosis and ROS production analysis. Region 3 (R3), representative histogram of the percentage of macrophages that are associated with at least one bacterium (FITC + macrophages). Representative histogram of total macrophages (Gate in R2) or FITC + macrophages (Gate in R3) that produce ROS. (B) Percentages of FITC + macrophages after co-culture with strains 806 (NP) and 5011 (P) of *S. aureus*. (C) Mean fluorescence intensity (MFI) of FITC + macrhophages. (D) Percentages of intracellular production of ROS in total macrophages after co-culture with strains 806 (NP) and 5011 (P) of *S. aureus*. (E) MFI of ROS production in total macrophages. (F) Percentages of the intracellular production of ROS in FITC + macrophages after co-culture with strains 806 (NP) and 5011 (P) of *S. aureus* (G) MFI of ROS production in FITC + macrophages. All values indicate the mean of three independent experiments, each performed in triplicate. Bars represent the mean ± standard deviation (SD). Differences between groups in macrophage phagocytosis assays and ROS production were assessed by Mann-Whitney non-parametric test. Different letters correspond to statistically significant differences ($P < 0.05$).

positive showed a significantly higher capacity to adhere and internalize in MAC-T cells compared with strain 806 (NP), characterized as *agr* group II and *cap5* positive. In our study, although both *S. aureus* strains carried *cap* genes, the expression of CP5 or CP8 *in vitro* was not determined, therefore direct comparisons with previous research [18,31] cannot be carried out.

Biofilm formation is an important mechanism for bacterial attachment to cells. In certain bacterial genera, the ability to form biofilm appears to be associated with invasiveness [32,33]. However, in *S. aureus*, this relationship has not yet been clarified; although it has been shown that capsule and surface proteins may inhibit internalization [34], no association was observed between biofilm formation and intracellular invasion to mammary epithelial cells [3,35]. Biofilm production requires the presence of the gene cluster *icaADBC*. Most isolates of *S. aureus* from bovine mastitis harbor the *ica* genes, but not all are capable of producing biofilm [36]. In the present study, *icaADC* genes were identified in both *S. aureus* strains that showed different capacity to produce biofilm *in vitro*. Strain 806 (NP) a weak biofilm producer showed lower adhesion/internalization capacity to MAC-T compared with strain 5011 (P) characterized as strong biofilm producer and high adhesion/internalization capacity to MAC-T. This is in line with previous findings showing that strains with an intracellular survival rate higher than 2% were biofilm producers whereas strains with an intracellular survival rate lower than 2% were not biofilm producers [31]. Although *in vitro* studies contribute to understand these host–pathogen interactions, it has to be taken in account that during a natural IMI it is not known whether the bacteria that have a first contact with the epithelial cells are in the planktonic or biofilm mode and at what stage of the establishment of the IMI the biofilm is formed promoting bacterial survival within the MG. The strong capacity to produce biofilm of the 5011 (P) strain could be a characteristic of its lineage, since in a study by Li et al. [27] CC188 strains showed higher biofilm formation ability than other lineages.

Bacterial factors that contribute to intracellular persistence, and host factors leading to *S. aureus* clearance or survival during IMI are poorly documented. Most of the studies on persistence in non-phagocytic cells, such as epithelial cells, have focused on an altered bacterial phenotype called small-colony variants (SCV) [24,28,37]. In this study, to investigate whether the isolate from P IMI could have a higher capacity to adapt and persist in an intracellular location than the isolate from NP IMI, we performed long-term infection experiments in MAC-T cells. Strain 5011(P) showed superior adaptation to the intracellular niche and could be recovered up to 72 h pi with a gradual decrease in the amount of intracellular bacteria recovered over time; while strain 806 (NP) could only be recovered at 4 h pi. These results coincided with those of Atalla et al. [24], who observed a higher recovery of viable *S.*

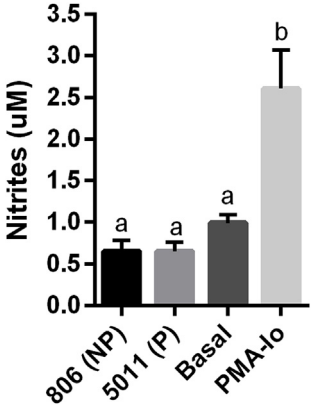

Fig. 6. Concentration of nitrites (generated by the spontaneous oxidation of NO) produced by macrophages after co-culture with strains 806 (T) and 5011 (P) of *S. aureus*. Basal: uninfected macrophages. PMA/Io (Phorbol 12-Myristate 13-Acetate/Ionomicine): Macrophages stimulated with PMA and Ionomycin, positive control for NO production. All values indicate the mean of three independent experiments, each performed in triplicate. Bars represent the mean ± standard deviation (SD). Differences between groups were assessed by one-way analysis of variance (ANOVA), followed by a multiple comparision Duncan's tests. Different letters correspond to statistically significant differences ($P < 0.05$).

aureus SCV Heba3231 from the MAC-T after 3.8 h with MOI of 100 compared with recovery of parent strain *S. aureus* 3231 and prototype strain Newbould 305. Also, they detected viable SCV up to 96 h while 3231 and Newbould 305 were not isolated at 24 h or later. In our study, the ability to survive intracellularly from strain 5011 (P) was similar to that observed for SCV, indicating that *S. aureus* can display several strategies to achieve intracellular persistence and this ability is strain-dependent. Although our findings partially coincide with those of Attalla et al. [24] caution must be exercised when comparing results from both studies since strains used in our study were not from SCV phenotype.

Fluorescent assays confirmed the results obtained from classical internalization assays and showed the ability of both *S. aureus* strains to survive intracellularly. In MAC-T infected with strain 806 (NP) the presence of viable intracytoplasmic bacteria was evidenced from 4 to 48 h pi, showing a decreasing density as time progressed; whereas, in the persistence assays the bacteria could only be recovered up to 4 h pi. This discrepancy could be based on differences in the ability of the methods used to detect viable organisms present in small numbers. The culture method is highly dependent on the inoculum volume, which may not have been high enough to detect bacterial growth. In addition, a viable non-culturable state has been described for several bacteria, including *Staphylococci* under certain conditions [39], which could make viable bacteria undetectable by standard microbiological assays. In MAC-T cells infected with strain 5011 (P), in agreement with the persistence assays, the presence of viable intracytoplasmic bacteria was evidenced from 4 to 72 h pi, demonstrating greater adaptation to the intracellular milieu. Similar results were observed by Tamilselvam et al. [38] using similar methodology to evaluate intracellular survival of two *Streptococcus uberis* strains (UT366 and UT888) and *S. aureus* UT955 strain in MAC-T cells, demonstrating Syto 9-labelled *S. uberis* (UT888 and UT366) at 96 h of co-culture, and *S. aureus* UT955 at 72 h of co-culture. Results of our study not only confirmed the previously described ability of *S. aureus* strains to persist within bovine mammary epithelial cells, but also the capability of both *S. aureus* strains tested to survive at low numbers in the cells.

Several studies have demonstrated that *S. aureus* can induce apoptosis in neutrophils [40], lymphocytes [41], macrophages [42] and bovine mammary epithelial cells [43]. Induction of epithelial cells apoptosis upon infection with *S. aureus* may result in the break-down of the epithelial barrier that favour bacterial access to deep tissues without stimulating bactericidal activities, while simultaneously being provided with a protective barrier against exogenous host immune defences and/or antibiotics [43]. In agreement with previous research, in this study the main mechanism of death of *S. aureus* infected cells was apoptosis, with the highest percentages at 24 h pi. In addition, strain 5011 (P) showed higher capacity to induce apoptosis than strain 806 (NP) at 4, 24 and 48 h pi, suggesting a greater induction of mammary epithelial cell damages. Our results are in accord with those of Haslinger-Löffler et al. [44] who observed that apoptosis induction in human endothelial cells was strongly strain-dependent and determined by multiple

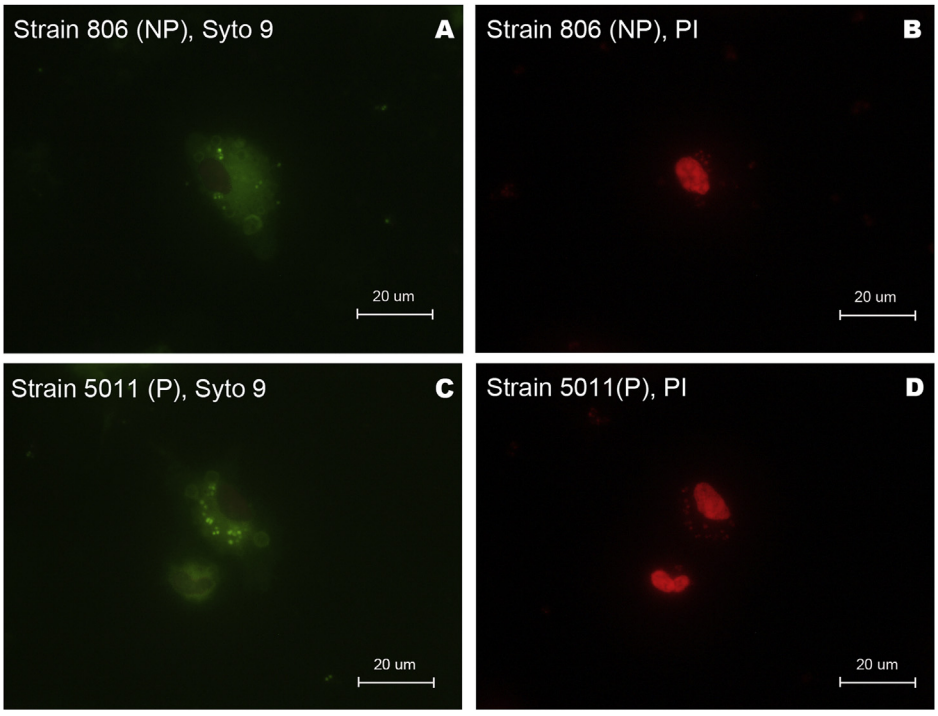

Fig. 7. Fluorescence imaging of Syto 9/PI stained samples from macrophages infected by 806 (NP) and 5011 (P) *S. aureus* strains at 2 h pi. Live bacteria in the cytoplasm of infected macrophages labelled with Syto 9 are shown in green (A, C). Dead bacteria in the cytoplasm of infected macrophages labelled with PI are shown in red (B, D). Each panel is from a representative experiment. Magnification, ×1000.

virulence factors. These researchers showed that only strongly haemolytic and invasive staphylococci, but not non-invasive strains induced caspase-dependent but Fas-independent apoptosis. In agreement with the results of apoptosis, at 24 h pi, the viability of cells infected with strain 5011 (P) was significantly lower than those infected with strain 806 (NP). Intracellular colonization could be one of the factors that leads to decreased viability of MAC-T infected with strain 5011 (P), since this strain showed high adhesion/internalization capacity and greater persistence in MAC-T compared with strain 806 (NP). From these results we could hypothesise that the *in vitro* behavior of 806 (NP) and 5011 (NP) *S. aureus* strains regarding the ability to invade, persist and induce damage in MAC-T cells, could be associated with their behavior during a natural bovine IMI. However, further research will be needed to confirm this hypothesis in experimentally induced IMI.

Macrophages are involved at multiple levels during mastitis and are indispensible for bacterial recognition and elimination [45]. The ability of macrophages to kill pathogens depends, in part, on oxygen and nitrogen-dependent mechanisms. Oxygen-dependent bactericidal mechanisms include the production of ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals [46]. In this study, macrophages infected with strain 5011 (P) showed a greater phagocytic capacity and higher percentage of intracellular ROS production than those infected with strain 806 (NP); however, the quantity of ROS produced was similar for both *S. aureus* strains. These results suggest that strain 5011 (P) of *S. aureus* was recognized and phagocytosed more efficiently than 806 (NP) and was able to induce the activation of a greater number of macrophages capable of producing ROS compared with strain 806 (NP); however, 5011 (P) strain was able to persist intracellularly inside of macrophages demonstrating greater resistance to microbicidal mechanisms. Our results are in agreement with other studies that indicate that after phagocytosis one of the main microbicidal mechanisms used by macrophages to eliminate *S. aureus* was oxygen dependent [47,48]. However, to the best of our knowledge, there is no information about the interaction of different *S. aureus* strains from bovine mastitis with macrophages isolated from mammary involution secretion. Macrophages obtained from mid dry period have shown enhanced bactericidal capacity against a virulent *Streptococcus uberis* strain compared with those from mid lactation [49].

The ability of bovine macrophages/monocytes to kill microbial pathogens has been linked to their capacity to generate NO, a highly bactericidal moiety [46]. There is scarce information about the ability of macrophages from MG involution period secretions to kill *S. aureus* [50]. Furthermore, it has not been shown if macrophages from drying off secretion produce NO in response to *S. aureus*. In this study, macrophages from mammary secretion confronted with both *S. aureus* strains, produced similar nitrites levels that uninfected macrophages. Nitric oxide is produced by all immune cells; however, it is not equally effective against all pathogens [51]. Sarantis et al. [46] observed that Gram-positive bacteria as single stimulus induction of NO synthesis by bovine monocyte-derived macrophages was four orders of magnitude less potent than Gram-negative bacteria. Since pathogen-host coevolution includes the development of mechanisms subverting host defense, and since NO synthesis is thought to contribute to antimicrobial defense, we might conceive that both *S. aureus* strains tested possessed mechanisms to subvert induction of NO synthesis in macrophages. However, in this study, macrophages isolated from mammary secretion were able to produce ROS and kill both *S. aureus* strains after 4 h pi, highlighting the importance of oxygen-dependent bactericidal mechanisms in *S. aureus* IMI.

Previous reports suggest that *S. aureus* induces macrophage apoptosis [52,53], while others have described intracellular persistence of *S. aureus* in macrophages resistant to apoptosis by microbial factors [54,55]. In chronically infected cows, the presence of viable *S. aureus* inside macrophages and alveolar cells isolated from milk has been demonstrated [10], which would favour the evasion of host immune defenses by the bacterium with the consequent persistence in the MG. In this study, in macrophages challenged with strain 5011 (P) it was possible to identify live bacteria in the cytoplasm for a longer time (4 h pi) than in those challenged with strain 806 (NP); indicating a greater capacity for resistance to microbicidal mechanisms. However, it was not possible to recover the bacteria from the lysates of the macrophages that engulfed strains 806 (NP) and 5011 (P) at any of the evaluated times, indicating a limited number of intracellular viable bacteria. In contrast with our results, Lacoma et al. [56] found that *S. aureus* Newman strain was able to persist intracellularly inside murine alveolar macrophages MH-S at least until 28 h pi. Tuchscherr et al. [30] observed that *S. aureus* strain 6850 isolated from osteomyelitis, were cleared within 3 days pi from human macrophages. In our study, macrophages from drying off mammary secretion were capable of phagocyte and trigger bactericidal mechanisms to kill both *S. aureus* strains after 4 h pi. Discrepancies between studies may rely in the host phagocytic cell types, *S. aureus* strain origin, and MOI used. Considering the results of the persistence assays on MAC-T and macrophages isolated from mammary secretion, intracellular survival of both *S. aureus* strains would be restricted to non-professional phagocytes.

5. Conclusion

Strain 806 (NP), selected for its low adaptation to bovine MG showed a low adhesion/internalization and intracellular persistence capacity in MAC-T cells, demonstrating a better adaptation to the extracellular niche. Strain 5011 (P), selected for its high adaptation to bovine MG showed high adhesion/internalization and persistence capacity in MAC-T cells. Although this strain was recognized and phagocytosed with greater efficiency by mammary secretion macrophages compared with strain 806 (NP), it showed greater resistance to microbicidal mechanisms, demonstrating a better adaptation to the intracellular niche. The knowledge of the molecular profile of the *S. aureus* strains causing bovine mastitis in a herd could become a tool to expose the most prevalent virulence gene patterns and advance in the elucidation of the pathogenesis of chronic mastitis.

CRediT authorship contribution statement

Sofía C. Sacco: Conceptualization, Methodology, Writing - original draft. **Natalia S. Velázquez:** Conceptualization, Methodology, Writing - original draft. **María S. Renna:** Methodology, Visualization, Investigation. **Camila Beccaria:** Methodology, Visualization, Investigation. **Celina Baravalle:** Methodology, Software, Validation. **Elizabet A.L. Pereyra:** Methodology, Visualization. **Stefan Monecke:** Software, Data curation. **Luis F. Calvinho:** Conceptualization, Supervision, Writing - review & editing. **Bibiana E. Dallard:** Conceptualization, Supervision, Writing - review & editing, Resources, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104017.

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