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Interaction between bovine mammary epithelial cells and planktonic or biofilm *Staphylococcus aureus*: The bacterial lifestyle determines its internalization ability and the pathogen recognition

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Abstract

The main cause of mastitis, one of the most costly diseases in the dairy industry, is bacterial intramammary infection. Many of these bacteria are biofilm formers. Biofilms have been associated with resistance to antibiotics and to the host immune system. Here, we evaluated different experimental models representing bacterial biofilm lifestyle with the aim to study bacterial invasion into bovine mammary ephitelial cells and the interaction of these cells with planktonic or biofilm *Staphylococcus aureus*. *Staphylococcus aureus* V329, its nonbiofilm-forming mutant and bovine mammary alveolar cells (MAC-T) were used. Bacterial invasion was studied using the gentamicin exclusion test, cell viability by trypan blue exclusion technique, TLR2 expression by flow cytometry, IL1β/IL6 production by ELISA and IL8/TNFα gene expression by real-time polymerase chain reaction. Biofilm and planktonic *S. aureus* showed differences in their invasion ability, with the biofilm mode showing a lower ability. Planktonic *S. aureus* reduced MAC-T viability after 6 h of co-culture, while biofilms did so at 24 h. MAC-T infected with planktonic bacteria showed increased TLR2 expression. Both lifestyles increased IL8 expression and IL1β/IL6 production but did not modify TNFα expression. Our results demonstrate that the bacterial lifestyle affects the invasion behavior, suggesting that biofilms reduce the bacteria-epithelial cell interaction. Planktonic cultures seem to induce higher cellular activation than biofilms. Further knowledge about the complex host-biofilm interaction is necessary to design more efficient therapies against bovine mastitis.

Keywords: planktonic and biofilm lifestyles, innate immune response, epithelial cells, *in vitro* experimental models, *Staphylococcus aureus*, bovine mastitis

1. Introduction

Mastitis is one of the most costly diseases in the dairy industry. The main cause of mastitis is the intramammary infection produced by bacteria such as *Staphylococcus aureus, Streptococcus uberis, Escherichia coli* and coagulase negative *Staphylococcus* [1]. The severity and outcome of the intramammary infection depend on several factors, including the host innate resistance and immune status. The host immune response can vary depending on both the species and the strain of the infecting bacterium [1]. The intramammary infection caused by *S. aureus* mostly induces subclinical mastitis, which may result in a chronic disease and lifelong persistence of the pathogen [2].

In the last two decades, the observation that bacteria present in most biological systems exist in biofilms rather than in a free-living (or planktonic) state has led to a revolutionary paradigm shift in the field of microbiology [3]. Biofilm-forming bacteria are encased into extracellular polymeric substances, which allow them to be highly tolerant to antibiotics as well as to the host immune response. In addition, bacteria growing within biofilms behave differently from those growing in planktonic cultures, which suggests that biofilm communities have emerging properties that cannot be predicted from the study of free-living bacterial cells [4]. Persistent infections in plants, animals and humans, as well as in medical devices and implants, are caused by microorganisms in biofilm form [4,5]. Consequently, a considerable research effort has been made to develop novel experimental methods to replicate bacterial biofilms *in vitro* [3].

In veterinarian research, *in vitro* studies are extremely needed because animal use must be avoided as much as possible to promote animal welfare [6]. Then, *in vitro* co-cultures of prokaryotes and eukaryotes represent important tools to acquire empirical data about biofilms and to provide the basis for confirmatory *in vivo* studies [3]. Despite their great

versatility and numerous benefits, these models show some limitations, as they do not allow evaluating the interactions with other cells such as those of the host immune system or commensal flora [7].

Resident mammary epithelial cells (MECs) are proposed to be key primary actors in the initiation of a species-associated response [8–10]. Several studies have evaluated the capacity of *S. aureus* isolates from bovine mastitis to produce biofilm and its ability to adhere and invade bovine MECs [11–14]. However, few studies have addressed the role of epithelial cells in the immunity of the mammary gland against biofilm-forming mastitis pathogens.

Some of the most important etiological agents of bovine mastitis are staphylococci due to their virulence factors such as their ability to penetrate inside MECs and to form biofilm [12]. Polysaccharide intercellular adhesin, a component of *S. aureus* biofilms, has been detected in udders from *S. aureus*-infected cows [15]. Moreover, all the 209 *Staphylococcus* spp. isolated from bovine mastitis in our group have been found to produce biofilm *in vitro* [16]. Then, the biofilm lifestyle could represent an important virulence factor of bacteria to persist within the bovine mammary gland. Recently, Fang et al. studied the genome-wide expression of mRNAs and miRNAs in bovine mammary gland cells after 24 h of intramammary infection with either high or low concentrations of *S. aureus* and, in those infected with high concentrations of *S. aureus*, they identified 194 highly-confident responsive genes, predominantly involved in pathways and biological processes related to the innate immune system, such as cytokine-cytokine receptor interaction and inflammatory response [17]. Previous studies have suggested that TLRs mediate the innate immune recognition of staphylococcal species during planktonic growth, and that biofilms seem to evade host immunity by circumventing

TLR2 and TLR9 recognition [18]. TLR2 is important in the defense against *S. aureus* and recognizes lipoproteins present on the bacteria [19].

In most of the studies conducted to date, bacteria-host interactions involve bacteria in their planktonic state. However, considering that epithelial cells actively contribute to the innate immune system, virulence factors such as the biofilm lifestyle are relevant to assess the outcome of staphylococcal infection. Therefore, knowledge about the host-biofilm interaction might lead to the design of new and more efficient therapies. Thus, the aim of this study was to compare different *in vitro* experimental models to represent bacterial biofilm lifestyle (planktonic growth, dislodged biofilms and established biofilms) that allow us to study bacterial invasion into bovine MECs. Additionally, we studied the interaction of bovine MECs with planktonic or biofilm *S. aureus.*

2. Materials and methods

2.1. Bacterial strains, cell line and growth conditions

The well-characterized *S. aureus* V329 strain isolated from cows with subclinical mastitis and its nonbiofilm-forming double mutant *S. aureus* V329 *ΔbapΔica* were used in this study [13,20]. *Staphylococcus aureus* Newbould 305 (ATCC 29740) was included as an invasive strain for invasion assays [21]. Bacteria were routinely grown in trypticase soy broth (TSB) containing 0.25 % glucose and agar (TSA) (Britania. Buenos Aires, Argentina).

The established bovine cell line produced from mammary alveolar cells (MAC-T) [22] was cultured as detailed in Isaac et al. [23].

2.2. Bacterial invasion in different co-culture models

To evaluate the response of eukaryotic cells to planktonic or biofilm bacterial infection, different experimental models to represent bacterial biofilm lifestyle were selected:

123 planktonic, dislodged biofilms and established biofilms [13,24–26]. These models are
124 described below and schematically represented in Figure 1.

125 <**Figure 1**>

126 For the bacterial invasion assay, MAC-T cells were seeded in 96-well plates at 5×10^4
127 cells/well and allowed to attach for 24 h. Bacteria representing planktonic or biofilm
128 lifestyles were added, and internalized bacteria were counted. Planktonic growth was
129 compared to biofilm lifestyle represented by three variants: *S. aureus* V329 biofilm-
130 forming in planktonic mode, dislodged biofilm and established biofilm-.

131 **Model 1: Planktonic mode (P).** Bovine cells were infected with *S. aureus* V329 or its
132 nonbiofilm-forming mutant, *S. aureus* V329 *ΔbapΔica*, in planktonic mode [13,26].
133 Bacteria were inoculated into TSB and incubated overnight at 37°C. Cultures were then
134 diluted to 0.5 in the McFarland scale and colony forming units (CFU) were determined
135 by plate count on TSA. A final dilution was made to reach a multiplicity of infection
136 (MOI) of 100 bacteria per cell. The bacterial invasion by *S. aureus* V329 *ΔbapΔica* was
137 compared whit that *S. aureus* V329, both in planktonic mode, at the same MOI.

138 **Model 2: Dislodged Biofilm mode (DB).** Dislodged biofilm was obtained according to
139 Daw et al. [24]. Briefly, *S. aureus* V329 was grown overnight in TSB at 37°C. The
140 culture was diluted in TSB to 0.5 in the McFarland scale and 10 mL of this bacterial
141 suspension was dispensed into a 100 mm x 20 mm TC-treated culture polystyrene dish
142 (Corning®, Corning, NY, USA) to allow biofilm formation. After incubation (37°C, 24
143 h), the medium with non-adhered bacteria was discarded and the plate was washed
144 twice with sterile phosphate buffered saline (PBS). Adhered bacterial cells were
145 resuspended in 5 mL of TSB, dislodged with a cell scraper and vortexed to disrupt
146 clumps. The CFU of the dislodged biofilm were determined by plating serial dilutions
147 on TSA. An aliquot of broth corresponding to a MOI of 100 bacteria per cell was used

as appropriate to infect the eukaryote cells. The bacterial invasion by the dislodged biofilm was compared with that by planktonic *S. aureus* V329 at the same MOI.

Model 3: Established Biofilm mode (B). Bacterial biofilms were obtained using the MBEC^{TM} Biofilm Inoculator (Innovotech. Edmonton, AB, Canada), a 96-peg lid device used to grow biofilms in a multi-well microplate as previously described [25]. The culture was diluted in TSB to 0.5 in the McFarland scale and 150 µL of this aliquot was dispensed into each well to allow biofilm formation. The bacterial load on the pegs was calculated according to Bowler et al. [25]. Briefly, after 24 h of incubation with *S. aureus*, the pegs were removed and washed with PBS, placed into 200 µL of PBS and sonicated for 5 min. Then, the bacterial suspension was vortexed and subjected to sequential 10-fold dilutions that were spot plated and counted to know the number of CFU per peg. In this way, the MOI of 100 bacteria per cell was known in biofilm lifestyle. The lid with the biofilms on the pegs was placed on a 96-well microplate with MAC-T cells and co-cultures were incubated. The bacterial invasion by the established biofilm was compared with that by planktonic *S. aureus* V329 at the same MOI.

After 2 h at 37°C of co-culture, the bacterial invasion was determined in the different experimental models using the gentamicin exclusion assay according to Valle et al. with modifications [13]. Then, the medium of the co-cultures was replaced with Dulbecco's Modified Eagle Medium (DMEM; Gibco. Grand Island, NY, USA) containing 100 µg/mL of gentamicin for 1 h to kill extracellular or adhered bacteria. Then, bacterial cell count was evaluated after eukaryotic cell lysing with 1 % Triton X-100 (Sigma-Aldrich. St. Louis, MO, USA). Intracellular bacterial count was determined by plate count in TSA for 24 h at 37ºC and expressed as Log CFU/mL.

2.3. Cell viability

Viability of uninfected MAC-T cells and MAC-T cells infected with planktonic or biofilm *S. aureus* V329 was studied by the trypan blue exclusion test of cell viability, using a Neubauer chamber [27] at different co-culture times, using experimental model 3.

2.4. Cellular immune mediators released in response to planktonic or biofilm *S. aureus*

The key mediators of the immune response of MAC-T cells to *S. aureus* V329 in planktonic or biofilm lifestyle was studied according to model 3, using the MBEC™ Biofilm Inoculator.

2.4.1. Toll-like receptor (TLR) 2 expression

TLR2 surface expression in MAC-T cells was evaluated by flow cytometry. MAC-T cells were grown in DMEM medium (Control condition) or co-cultured with *S. aureus* in planktonic or biofilm mode. Bacterial infection was performed at a MOI of 100 bacteria per cell, at 37 °C for 2 and 4 h. TLR2 (CD282) expression was assessed by staining with human anti-bovine CD282:Alexa Fluor® 647 (BIO-RAD, Hercules, CA, USA) for 30 min at 4 °C in the dark. Cell suspensions were acquired on a BD Accuri™ C6 flow cytometer (BD Biosciences, San José, CA, USA) and the data were analyzed using FlowJo software V 7.6.2 (Tree Star Inc., Ashland, OR, USA).

2.4.2. Expression and production of cytokines

2.4.2.1. Tumor Necrosis Factor alpha (TNFα) and Interleukins (IL) 8 expression

TNFα and IL8 mRNA levels were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Total RNA was isolated from MAC-T cells after 4 h of co-culture and DNase treated using the EasyPure RNA kit (TransGen Biotech Co., Beijing, China) according to the manufacturer's instructions. The quantity and quality of purified RNA was determined

using a microliter spectrophotometer (Picodrop, Hinxton, UK) and by visualization on a denaturing agarose gel. The RNA was stored at -80 ◦C until use.

Total RNA (100 ng) was used for RT-qPCR, which was carried out using the iTaq Universal SYBR® Green One-Step Kit (BIO-RAD, Hercules, CA, USA) on a CFX96 Touch Time™ PCR-Real Time (BIO-RAD, Hercules, CA, USA) according to the manufacturer's instructions with modifications. The reaction mixture consisted of 5 µL of iTaq Universal SYBR® Green reaction mix (2X), 0.094 µL iScript reverse transcriptase, 0.3 µL of 10 µM forward and reverse primers, 2 µL of template RNA and 2.31 µL of nuclease-free water. The RT-qPCR reactions were initiated with a reverse transcription reaction at 50 °C for 10 min, polymerase activation and DNA denaturation at 95 °C for 1 min, followed by 40 cycles of amplification at 95 °C for 10 sec (denaturation), and 55-60 °C for 30 sec (annealing/extension plus plate read). A melting curve was performed at the end of the run according to the instrument user guide. No template controls were included for each primer pair reaction and each RT-qPCR reaction was carried out in duplicate. Amplification plots and dissociation curves were obtained with the CFX Manager Software version 2.1 (BIO-RAD, Hercules, CA, USA). The baseline and Cq were automatically determined by the software. Gene-specific amplification was confirmed by a single peak in the melting-curve analysis. Two reference genes, β-actin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were evaluated. The sequences of the oligonucleotides used in this study and the amplicon lengths are shown in Table 1. The relative changes in gene expression data of TNFα and IL8 were calculated using the threshold cycle method ($2^{-\Delta\Delta CT}$) with untreated samples as controls and GAPDH as the reference gene [28]. The transcript quantities were expressed as changes (n-fold) relative to the values of the control.

<Table 1>

2.4.2.2. IL1β and IL6 secretion

IL1β and IL6 concentrations in culture supernatants were measured by ELISA after 2 and 4 h of infection using commercial kits (Thermo Scientific-Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's instructions.

2.5. Statistical analyses

All experiments were performed three times in triplicate. The results are reported as the mean ± standard error. The data were analyzed with the independent t-test, one-way ANOVA and Bonferroni's post-test or Kruskal-Wallis test, as appropriate. Mean values were considered significantly different at $p < 0.05$. The Infostat software version 2017 was used for all statistical analyses [29].

3. Results

3.1. Invasion of MAC-T by *S. aureus* on different experimental models to represent bacterial biofilm lifestyle

In a first step, we compared the invasion of *S. aureus* using the different experimental models mentioned above. The intracellular bacterial counts in MAC-T cells infected by the wild type form of *S. aureus* V329 (Planktonic or Dislodged Biofilm modes) and its nonbiofilm-forming mutant were similar (Fig. 2A), suggesting that the bacterial invasion was not affected by the inability to grow in biofilm or loss of biofilm structure. In contrast, the bacterial load found in MAC-T cells co-cultured with the established biofilm was significantly lower than that found in cells co-cultured with planktonic bacteria (-22 %) (Fig. 2B). This may be because the structure of the biofilm in that model may have been preserved. Taking these results into account, for the following assays, we selected the biofilm mode (obtained by the inoculator methodology) as representative of biofilm to evaluate the interactions between bacteria and host cells.

<Figure 2>

3.2. Analysis of MAC-T cell viability after infection with planktonic or biofilm *S. aureus* at different co-culture times

MAC-T cell viability was assessed by trypan blue exclusion test. MAC-T cells were infected with *S. aureus* in planktonic or biofilm mode, and uninfected MAC-T cells were used as controls. Cells were observed at 2, 4, 6 and 24 h at 37 °C. The left column in Figure 3A shows the aspect of uninfected MAC-T cells. The middle column shows a large number of free-living *S. aureus* V329 contacting the MAC-T cells. The right column shows fragments of *S. aureus* biofilms detached from the pins of the MBEC™ biofilm inoculator used to obtain the established biofilm; these fragments were larger at longer infection times.

Planktonic *S. aureus* did not affect MAC-T cell viability at 2 or 4 h of infection, but significantly reduced it at 6 h (- 69 %) (Fig. 3B). In contrast, bacterial biofilms affected cell viability significantly only after longer times, as shown at 24 h of co-culture (- 97 %) (Fig. 3B). For that reason, in subsequent tests, we used co-culture times of 2 and 4 h.

<Figure 3>

3.3. Effect of *S. aureus* lifestyle on TLR2 expression in MAC-T cells

We evaluated TLR2 expression in MAC-T cells co-cultured with planktonic or biofilm *S. aureus* V329 for 2 and 4 h by flow cytometry (Fig. 4A). We found that TLR2 expression was significantly higher (4-fold) in MAC-T cells infected with planktonic *S. aureus* V329 for 4 h than in uninfected cells. In contrast, after the co-culture with biofilm *S. aureus* V329, TLR2 expression was intermediate between those conditions (Fig. 4B). Similar results were obtained at 2 h of co-culture (data not shown).

<Figure 4>

3.4. Effect of *S. aureus* lifestyle on the induction of immune mediators by bovine cells

Then, we evaluated transcripts of IL8 and TNFα in MAC-T cells and the production of IL6 and IL1β in supernatants after infection with planktonic or biofilm *S. aureus* V329. Both the planktonic cultures and the bacterial biofilm significantly increased twice IL-8 mRNA levels at 4 h co-culture than those of uninfected condition (Fig. 5A). However, TNFα expression was not modified by the infection after 4 h (Fig. 5A). The planktonic cultures increased IL1β concentration in the supernatant at 2 and 4 h of co-culture (145 and 84-fold respectively) and IL6 secretion at 2 h co-culture (3-fold) as compared to the uninfected condition (Fig. 5B). On the other hand, the bacterial biofilm significantly increased both IL1β and IL6 secretion at 2 h (86 and 5-fold respectively) and 4 h (150 and 6-fold respectively) of co-culture (Fig. 5B).

<Figure 5>

4. Discussion

Chronic biofilm infections have great economic impact on the dairy industry. Therefore, understanding the mechanisms whereby staphylococcal biofilms alter immune recognition pathways requires new *in vitro* models. In this work, we compared different experimental models (planktonic, dislodged biofilms and established biofilms) used indistinctly in research to represent bacterial biofilm lifestyle, and selected bacterial invasion as a representative parameter of biofilm-host cell interactions. The results obtained showed that the biofilm model affected bacterial invasion, suggesting that bacterial biofilms reduce the interaction with epithelial cells *in vitro*.

It has been shown that *S. aureus, S. uberis*, and *E. coli* are able to invade and persist within bovine MECs, representing a niche where they may evade phagocytosis and

immune system recognition [1]. In the present study, epithelial cells showed a reduction of bacterial load when cultured with the bacterial biofilm mode. In agreement, by using *Pseudomonas aeruginosa* and human epithelial cells A549 as pathogen-host model, Bowler et al. found that planktonic bacteria are significantly more internalized than biofilms [25]. Similarly, other researchers have reported lower bacterial invasion of bovine MECs after co-culture with a biofilm-producing *S. aureus* strain [11,13]. In contrast, Kunyanee et al. found that the biofilm phenotype, unlike a nonbiofilm-forming mutant strain, promoted internalization of *Burkholderia pseudomallei* into human epithelial A549 cells (MOI of 10, 2 h) [26]. Differences with our findings could be attributed to the different biofilm-producing phenotype, strong for *S. aureus* V329 and moderate for *B. pseudomallei* H777. On the other hand, RAW 264.7 macrophages and JAWS II dendritic cells phagocyte intact and dislodged *Enterococcus faecalis* biofilms at levels similar to or higher than the planktonic mode [24]. The differences observed could be related to the experimental model used as well as to the way in which the biofilm is represented. The dislodged biofilms studied consisted of fragments that may retain some features of the intact biofilm [30]. However, the invasion assay showed that dislodged biofilms presented no differences with planktonic bacteria, suggesting that free bacteria from dislodged biofilms can retain the characteristics of planktonic invasion. In fact, different outcomes can be expected with young newly forming biofilms as opposed to mature (24 h growth) biofilms [25], which was the model chosen in this work to represent the established biofilm. Probably, intracellular or biofilm lifestyles constitute strategies to avoid detection by professional phagocytes used by planktonic bacteria and biofilms, respectively.

Planktonic *S. aureus* V329 bacteria affected the viability of MAC-T cells after 6 h of co-culture, while biofilms did so at 24 h. Similarly, Bowler et al. found that planktonic

bacteria cause more death of A549 cells than biofilm bacteria, although the 4 h exposure to biofilm also produced cell death [25]. In contrast, in our study, biofilms did not affect MAC-T viability in short co-cultures. Differences could be explained because cell death induction by *S. aureus* depends on the specific cell types and strains investigated, as well as on the MOI used [31].

One mechanism used by biofilms to evade host immunity is to circumvent TLR2 and TLR9 recognition [32]. For *S. aureus* biofilms, the mechanism(s) responsible for TLR2/TLR9 evasion is/are not known but could also be explained by ligand inaccessibility. Biofilms consist of a complex three-dimensional structure with few bacteria exposed at the outer surface, which allows them to avoid detection by the pattern recognition receptors expressed on the surface of phagocytes [33]. The lower induction of TLR2 expression by *S. aureus* V329 biofilms observed in the present study supports the above. In agreement, it has been shown that *S. aureus* D30 isolated from the anterior nares of a healthy human donor promotes a delay in the up-regulation of TLR2 receptor in nasal epithelial cells, a strategy that may enable a significant window to evade the host's innate immunity [34].

Pro-inflammatory cytokines like IL1, IL6 and TNFα are major cytokines that arbitrate the inflammatory response during bovine mastitis [35]. IL6 is a key pro-inflammatory cytokine, which also has anti-inflammatory properties [35]. The chemokine IL8 promotes the recruitment of neutrophils, which are potent phagocytic leukocytes, to the udder, and protection from infection is dependent on the rapid recruitment of neutrophils and subsequent phagocytosis of invading microorganisms [36]. It has been reported that staphylococcal biofilms divert the innate immune response of the host from a proinflammatory phenotype to an anti-inflammatory phenotype to promote bacterial persistence [18]. *S. aureus* culture supernatant is sensed by TLR2 in mammary

346 epithelial cells, that activate the NF-κB pathway [37]. Here, *S. aureus* V329 in both
347 lifestyles (planktonic and biofilm) induced IL8 expression and promoted IL1β and IL6
348 secretion (2 h of co-culture) in levels similar in comparison whith to those of uninfected
349 cells. TNFα expression was similar in uninfected MAC-T cells and MAC-T cells
350 infected with *S. aureus* V329 in both lifestyles. Despite the differences in the ability to
351 invade, effects on cell viability and stimulation of TLR2, biofilms were able to induce
352 the expression of certain pro-inflammatory cytokines. Together, these results suggest
353 that innate host defense epithelial cells recognize staphylococcal biofilms and are mildly
354 stimulated.
355 Staphylococcal biofilms escape immune recognition thanks to their chronic and indolent
356 nature and may shift the host immune response from a proinflammatory bactericidal
357 phenotype toward an anti-inflammatory response that favors bacterial persistence [18].
358 It is possible that the IL6 induction here observed in MAC-T cells co-cultured with *S.*
359 *aureus* on biofilm mode (4 h of co-culture) is related to the anti-inflammatory response.
360 In fact, IL6 is a pleiotropic cytokine with pro- and anti-inflammatory properties and, in
361 several mouse models, IL6 classical signaling is essential to induce the regeneration of
362 epithelial cells after damage [38].
363 As mentioned in the introduction of the work, it has been postulated that the bacterial
364 biofilm lifestyle would be an escape route for pathogens, which would allow bacteria
365 not to be recognized by the host's immune system and not to be affected by
366 antimicrobial therapies. However, finding *in vivo* evidence of *Staphylococcus* biofilm
367 formation in the bovine mammary gland and studying its implication in the
368 development of mastitis is very complex. Our group has reported that *Staphylococcus*
369 spp. isolates from bovine mastitis have the ability to form biofilms *in vitro* [16]. In this
370 work, the aim was to study the interaction between biofilms and bovine mammary

epithelial cells and the induced immune response. Given the difficulties of evaluating this interaction, we used three experimental models and demonstrated that bacteria released from biofilms were able to internalize in bovine mammary epithelial cells and stimulate mediators of the innate immune response. Although we expected to observe greater differences between the lifestyles of these pathogens, biofilms presented certain differences in their interaction with host cells, which must be considered to combat their resistance to current therapies, being a challenge to study them *in vivo*. Finally, the results of this work highlight the importance of developing models that allow studying the interactions between bacterial biofilms and host cells to find more clues that can be used in the development of anti-biofilm strategies.

5. Conclusion

The results of this study corroborate that the recognition and initiation of the innate immune response depend on the bacterial lifestyle. Our results showed differences in how the biofilms invaded and affected the viability of bovine MECs. In addition, TLR2 expression in MAC-T cells was less stimulated by biofilms than by planktonic bacteria. Nevertheless, biofilms were able to stimulate key mediators of the immune response in levels comparable to planktonic cultures, suggesting that biofilms could stimulate a differential delayed response. Additional studies are needed to investigate the mechanisms that lead to the impairment of the host response upon contact with *Staphylococcus* spp. biofilms. Taking into account that staphylococcal biofilms are considered a significant virulence factor in persistent and chronic infections, biofilm-host interactions should be better understood to develop new strategies to treat them. In summary, our findings try to compare the different experimental models and shed new data about the behavior of mature staphylococcal biofilms in comparison with

396 planktonic cultures. These data can lay the basis to better understand the behavior of
397 bacterial biofilms and their interaction with MECs.
398
399 **Conflict of interest statement:** The authors report no conflicts of interest in this work.
400
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566 **Table 1. Oligonucleotides used in this study**

Gene	Primers sequences (5´ → 3´)	Amplicon length (bp)	Tº annealing (°C)	Reference
TNFα	F: CCCCTGGAGATAACCTCCCA R: CAGACGGGAGACAGGAGAGC	101	60	Mookherjee et al. [39]
IL8	F: ACCAATGGAAACGAGGTCTGCCTA R: ACACCAGACCCACACAGAACATGA	217	55	Primers were derived from the published sequence in the Genbank database under the accession number NM_173925
GAPDH	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	120	55/60	Herath et al. [40]
β-actin	F: CAAGGAGAAGCTCTGCTACG R: GATGTCGACGTCACACTTCA	231	55/60	Ibeagha-Awemu et al. [41]

567 F: forward primer, R: reverse primer

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Figure legends

Figure 1. Schematic representation of the different experimental models used to represent bacterial biofilm lifestyle to study bacterial invasion.

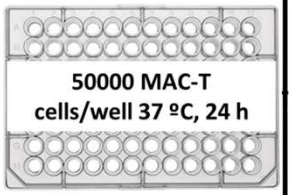
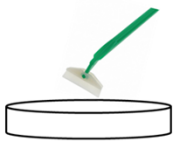
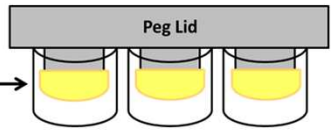
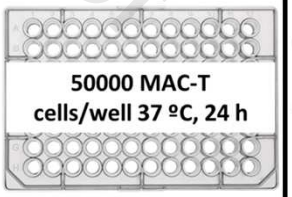
Figure 2. Bacterial invasion of bovine epithelial cells under different experimental models to represent bacterial biofilm lifestyle. *Staphylococcus aureus* invasion was compared after 2 h of co-culture with MAC-T cells at a MOI of 100 bacteria per cell. A) Planktonic (P) and dislodged biofilm (DB), *Staphylococcus aureus* Newbould 305 strain (ATCC 29740) was included as control of invasion. B) Planktonic (P) and Established Biofilm (B). Different letters show significant differences ($p < 0.05$).

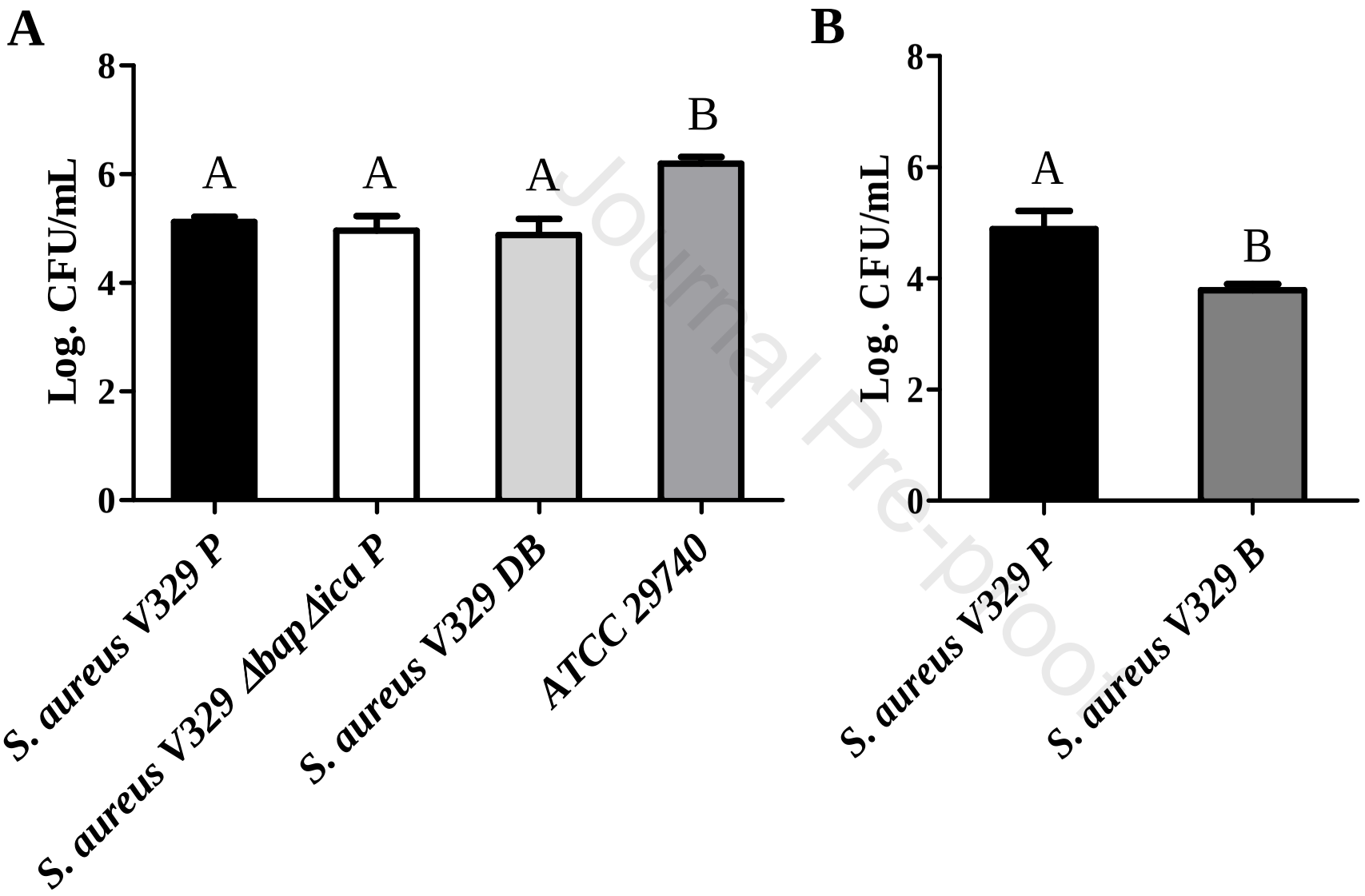
Figure 3. Viability of MAC-T cells uninfected or infected with planktonic or biofilm *S. aureus* V329 (MOI of 100) at different co-culture times. (A) Photomicrographs of MAC-T cells. 10x magnification, scale bar: 160 µm. (B) The trypan blue exclusion test was used to study MAC-T cell viability. Different letters show significant differences ($p < 0.05$). P: Planktonic, B: Established Biofilm.

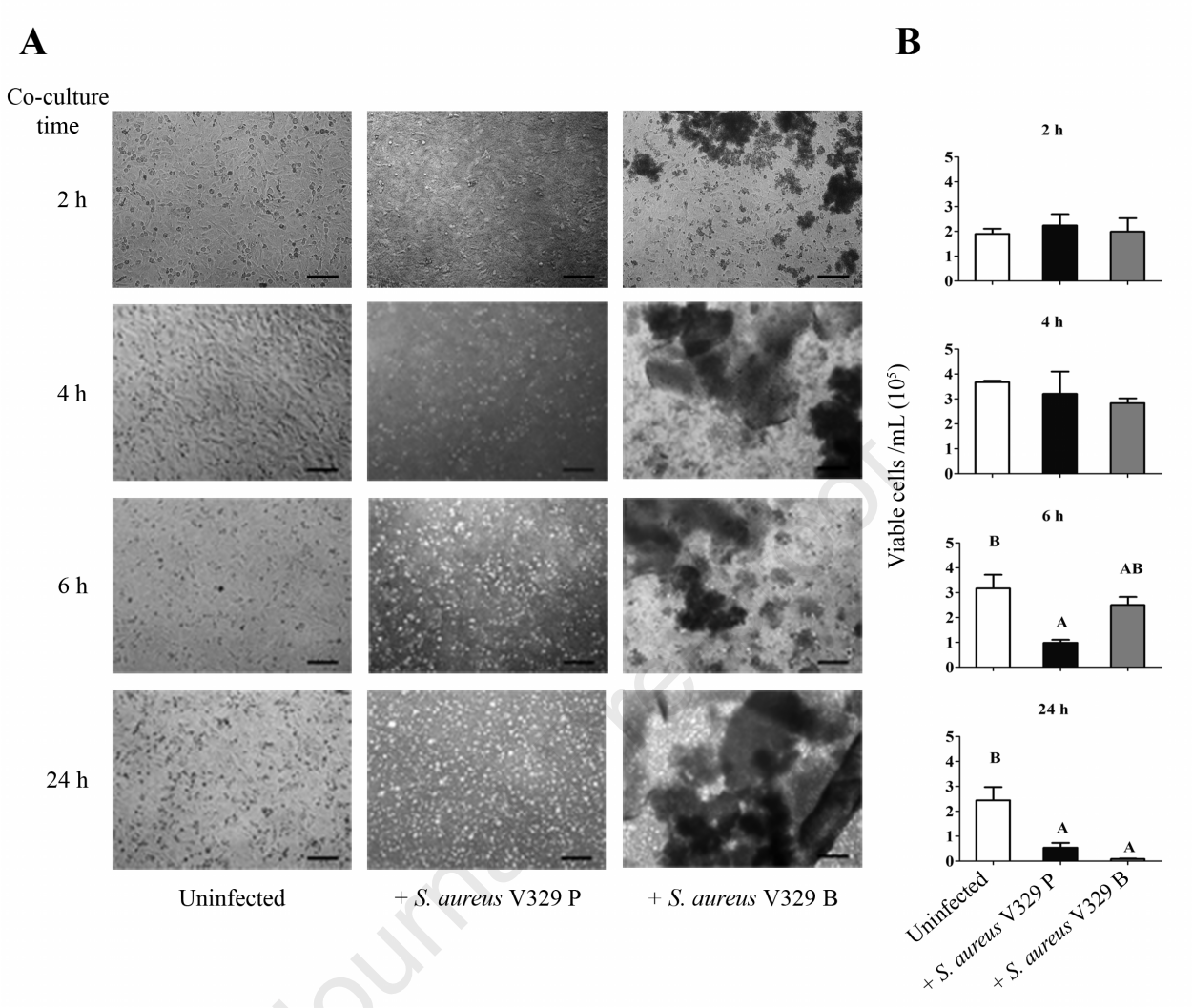
Figure 4. TLR2 expression in MAC-T cells uninfected or infected with planktonic or biofilm *S. aureus* V329 (MOI of 100, 4 h co-culture). (A) Representative dot plot and histograms of negative controls and infected MAC-T cells. (B) Percentage of TLR2-positive MAC-T cells. Different letters show significant differences ($p < 0.05$). P: Planktonic, B: Established Biofilm.

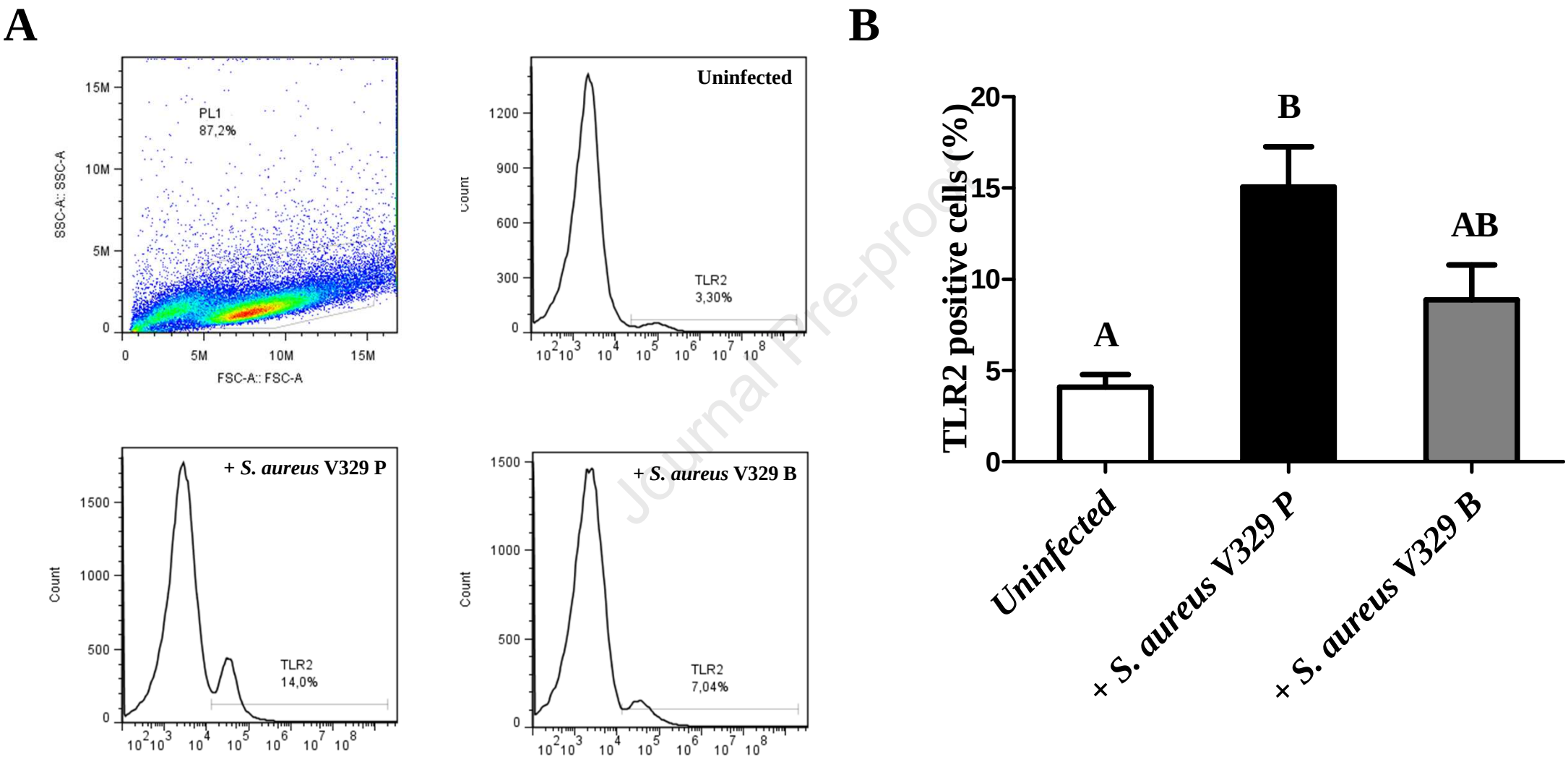
Figure 5. Expression and production of cytokines by MAC-T cells after infection with planktonic or biofilm *S. aureus* V329. (A) The RNA of MAC-T cells was isolated after 4 h of co-culture with planktonic or biofilm *S. aureus* V329. TNFα and IL8 mRNA levels were determined by reverse transcription quantitative polymerase chain reaction. (B) IL1β and IL6 concentrations were analyzed in culture supernatants of MAC-T cells after 2 or 4 h of co-cultures with planktonic or biofilm *S. aureus* V329

by ELISA. Different letters show significant differences ($p < 0.05$). P: Planktonic, B: Established Biofilm.

	Day 1	Day 2	Day 3
Model 1. Planktonic mode (P)		**Planktonic** *S. aureus* V329 *S. aureus* V329 *Δbap Δica* **37 ºC, 24 h**   **50000 MAC-T cells/well 37 ºC, 24 h**	**Co-cultures** (MOI 100, 2 h at 37°C) **and bacterial invasion**
Model 2. Dislodged Biofilm mode (DB)	 *S. aureus* V329	**Dislodged biofilm** *S. aureus* V329 **37 ºC, 24 h** 	(Model 1: P biofilm-forming strai *vs.* P nonbiofilm-forming strain, Model 2: P *vs.* DB)
Model 3. Established Biofilm mode (B)		**Planktonic**  *S. aureus* V329 **37 ºC, 24 h, 150 rpm** **Established Biofilm** →  Peg Lid *S. aureus* V329 **37 ºC, 24 h**  **50000 MAC-T cells/well 37 ºC, 24 h**	**Co-cultures** (MOI 100, 2 h at 37°C **and bacterial invasion** (Model 3: *vs.* B)







Highlights

Incidence of biofilms in bovine mastitis demands innovative research strategies

Different experimental models representing bacterial biofilm lifestyle were evaluated

Biofilms reduce the bacteria-epithelial cell interaction

Planktonic cultures seem to induce higher cellular activation than biofilms

Conflict of interest statement: The authors report no conflicts of interest in this work.

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Villa María, April 4, 2020.

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We are submitting our work entitled "Interaction between bovine mammary epithelial cells and planktonic or biofilm *Staphylococcus aureus*: the bacterial lifestyle determines its internalization ability and the pathogen recognition" by Luciana Paola Bohl, Paula Isaac, María Laura Breser, María Soledad Orellano, Agustín Conesa, Silvia Graciela Correa, Nori Tolosa de Talamoni and Carina Porporatto to be considered for publication in *Microbial Pathogenesis* as an original research paper.

Our work is an original scientific contribution on host-pathogen interactions between bacteria in different lifestyles and epithelial cells, in different experimental models of coinfection. It provides evidence on the host cell responses elicited by bacteria of veterinary importance. The topic may be of interest to journal readers who want to design their investigations taking into account the latest trends in microbiology, specifically about infections caused by bacteria growing in biofilm. The results may also be of interest the scientific and public health community in general.

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Thank you for the consideration you may give to our work. If there are any problems with this manuscript or you need more information, please do not hesitate to contact me. I look forward to your response.

Sincerely yours,

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