***Short communication*: Recombinant mammary serum amyloid A3 as a potential strategy to prevent intramammary infections in dairy cows at dry-off**. *By Parés et al.* Dairy cows are most susceptible to incur intramammary infections during the dry period. Preventive strategies other than antibiotics may be extremely useful to decrease the infection risk without increasing antibiotic resistances. The use of recombinant mammary acute phase protein M-SAA3 is a promising alternative because it is a key modulator of the mammary gland innate immunity. This study demonstrates the effects of M-SAA3 on mammary epithelia reducing the infections of four relevant mastitis pathogens in dairy cattle.

Short communication: Recombinant mammary serum amyloid A3 as a potential strategy to prevent intramammary infections in dairy cows at dry-off

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ABSTRACT

Mammary serum amyloid A3 (M-SAA3) has shown potential stimulating innate immunity during intramammary infections at calving and dry-off. Herein, we have produced recombinant caprine M-SAA3 to test its potential to reduce intramammary infections against *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*, which are common mastitis-producing pathogens. The recombinant production of M-SAA3 followed by LPS-removal to avoid LPS-unspecific stimulation of the immune system was successfully achieved. Mammary serum amyloid A3 stimulated the expression of IL-8 in a dose-dependent manner in primary mammary cultures. Although a direct killing effect on *S. aureus* by M-SAA3 was not detected, the acute phase protein was able to reduce up to 50% *S. aureus, S. uberis,* and *S. dysgalactiae* infections and induced a reduction of 67% in *E. coli* counts. In general, the best concentration of caprine M-SAA3 at inhibiting infections was the lowest concentration tested (10 μg/ml), although greater concentrations of M-SAA3 (up to 160 μg/ml) increased the antimicrobial potential against some pathogens.

Key words: dry period, intra-mammary infections, immune response, M-SAA3

Short Communication

Mastitis is the inflammatory response of the mammary tissue to bacterial infections, and it represents one of the most economically costly diseases affecting dairy animals. The udder of the dairy cow is most susceptible to incur intra-mammary infections (IMI) during the dry period, which can persist throughout the next lactation, causing important economic losses (Oliver and Murinda, 2012) and discomfort to the animal (Bertulat et al., 2013). The reasons behind this prominent risk is that a relatively large volume of milk remains in the udder at dry-off, which may lead to milk leakage (Tucker et al., 2009) and favor the colonization of the mammary gland by microorganisms (Schukken et al., 1993). Also, at dry-off, the immune

system is not fully active against pathogens because a reduced functional capability of mammary leukocyte population (Oliver and Sordillo, 1989) coupled with the fact that an important part of phagocytic cells arriving at mammary gland is engaged in removing fat and protein debris from the milk accumulated in the udder at dry-off (Burvenich et al., 2007). At present, the most common strategy to prevent IMIs at dry-off include: a) inducing a reduction of milk yield (through either reducing milking frequency (Tucker et al., 2009), nutrient supply (Ollier et al., 2014), or both, or the use of prolactin release inhibitors such cabergoline that also hasten mammary gland involution and enhance its immunity (Bach et al., 2015, Boutinaud et al., 2016, 2017)), 2) administering broad-spectrum antibiotics (Scherpenzeel et al., 2014), and 3) applying teat sealants at drying (McParland et al., 2019). However, a concern exists that use of antibiotics could be associated with an increase in antibiotic-resistant bacteria, and some governments are implementing regulations to restrict the use of prophylactic intramammary antibiotics in non-infected cows at dry-off. However, Scherpenzeel et al. (2014) showed that quarters that did not receive antimicrobial treatment at dry-off had a 1.7 times greater incidence rate of clinical mastitis during the dry period and the first 100 d in the subsequent lactation, as compared with quarters that did receive antibiotic treatment at dry-off.

Mammary serum amyloid A3 (M-SAA3) is a naturally occurring acute phase protein produced locally and involved in the regulation of the local immunity and the regeneration of the mammary gland. The concentrations of M-SAA3 are greatest in colostrum, in milk during IMI, and at dry-off (Molenaar et al., 2009). A previous study (Domènech et al., 2014) has shown that intra-mammary infusion of recombinant M-SAA3 in the cow fostered the involution process, mainly through the activation of endogenous mammary matrix metalloproteinase -9 (MMP-9) activity, which is involved in extracellular matrix remodeling. It has also been reported that M-SAA3 modulates the innate immunity by increasing mediators of the immune response, such as IL-8, to ultimately enhance neutrophil recruitment, maturation of dendritic

cells, and phagocytic activity by opsonization (Domènech et al., 2012, 2014). However, the role of recombinant M-SAA3 in the inhibition of infections caused by mastitic pathogens has not yet been studied in depth. Thus, the main objective of the present study was to explore the potential of M-SAA3 in the inhibition of the infection of primary bovine mammary epithelial cultures by common mastitis-producing pathogens including *Escherichia coli*, *Streptococcus uberis*, *Staphylococcus aureus*, and *Streptococcus dysgalactiae*.

Herein, M-SAA3 was produced recombinantly without traces of endotoxins that could interfere with the immune function using either *E. coli* BL21 Star (DE3) or ClearColi® (Lucigen, Middleton, WI, USA) transformed with pET101/D-TOPO vector codifying for the His tagged-goat M-SAA3 sequence isolated in a previous study (Domènech et al., 2012). Microbial cells were grown in 400 mL of LB-Amp media at an initial OD_{600} of 0.05 until log phase was achieved. Recombinant expression was induced by 0.1 mM IPTG at OD_{600} of 0.9-1 for 1 h and 20 min in BL21 strain and at 0.6-0.7 for 2 h in ClearColi®. The cell pellet was obtained by centrifugation at 6,000 x *g* for 10 min, and frozen at -80°C until use, and then lysed in 36 mL Tris-buffered saline lysis buffer containing 1x Bugbuster® (Merck-Millipore, Billerica, MA, USA), 0.1% v/v benzonuclease (Sigma-Aldrich, Saint Louis, MO, USA), and 1x cOmplete® EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). The lysed product was centrifuged for 15 min at 20,000 x *g* at 4 ºC. Supernatants containing protein were then used to obtain purified protein with the Amicon Pro System from Merck-Millipore (Billerica, MA, USA) and the Clontech (Mountain View, CA, USA) His60 agarose bead slurry (60 mg/mL binding capacity). Samples were eluted twice to ensure there was no protein left in the resin. The purified protein was dialyzed overnight at 4°C against PBS and quantified using spectrophotometry ((A280×Molecular weight)/Extinction coefficient; (mg/mL)). The removal of LPS from M-SAA3 produced in *E. coli* BL21 was carried out with the Mustang Q Acrodisc membrane (Pall Corporation, New York, USA) following manufacturer's instructions. Briefly,

all samples and buffers were filtered through a 0.22 μm filter before pushed through the membrane. The membrane was then washed with 5 mL 1 M NaOH and equilibrated with 1 mL 1 M NaCl and 5 mL PBS before running the sample. The presence of possible endotoxin traces in purified M-SAA3 (LPS or Lipid IV_A) was quantified by Microcoat Company using LAL Kinetic chromogenic assay. Protein production was assessed by SDS-PAGE in 12% polyacrylamide gels either with Coomassie or western blot as previously described (Gifre-Renom et al., 2018). Samples were analyzed using a lineal method in an UltrafleXtreme MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) with ion acceleration of 25 kV. In order to test M-SAA3 activity, mammary epithelial primary cultures were obtained from mammary tissue as described elsewhere (Domenech et al., 2014). Mammary epithelial cells were seeded in 24-well plates at 44,000 cells/well. After 24 h incubation at 37°C and 5% CO_2, wells were washed twice with warm PBS to remove antibiotics, and treatments were applied in a final volume of 100 μL of PBS + 400 μL of medium (DMEM/F-12 medium with 8 μg/mL bovine insulin and 50 μg/mL hydrocortisone) in the case of LPS and Lipid IV_A or in 600 μL PBS + 400 μL of medium in the case of recombinant M-SAA3. After a 3 h incubation at 37°C, 5% CO_2, cells were gently washed with PBS, and 500 μL of Trizol reagent (Thermo Fisher Scientific, Madrid, Spain) were added to each well to collect and lysate the cells. Cell lysates were kept frozen at -80°C until RNA extraction. The extraction of RNA was performed using the TRizol reagent (Thermo Fisher Scientific, Madrid, Spain). Then, RNA was retrotranscribed to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc, Shiga, Japan) following manufacturer's instructions. The RNA purity was assessed using a Nanodrop instrument at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios between 1.9-2.0 and 2.0-2.2, respectively. Quantitative PCR was performed for genes encoding for IL-8 (Primer sequences: Fw: 5'-TTGAGAGTGGGCCACACTGTG-3', Rv: 5'-TGCACCCACTTTTCCTTGG-3') and ACTB (Primer sequences: Fw: 5'-

CTGGACTTCGAGCAGGAGAT-3', Rv: 5'-CCCGTCAGGAAGCTCGTAG-3') using a MyiQ Single Color Real-Time PCR Detection System Thermocycler (Bio-Rad, Madrid, Spain) with the SYBR Premix Ex Taq (Takara Bio Inc, Japan). Quantitative PCR conditions for each set of primers were individually optimized (ACTB: Annealing temperature = 57 ºC, primer concentration = 0.124 μM; IL-8: Annealing temperature = 55 ºC, primer concentration = 0.5 μM). The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in 0.8% DNA agarose gel, using each respective gene control, and a single peak in the qPCR melting curves. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which was calculated using the formula $10^{-1/slope}$, with an acceptable range of 1.8-2.2 (Chow et al., 2010). Relative gene expression was calculated using the $2^{\Delta Ct}$ method with ACTB as reference gene.

Pathogenic *E. coli*, *S. uberis*, *S. aureus*, and *S. dysgalactiae* were isolated from mastitic milk kindly provided by ALLIC (Laboratori interprofessional lleter de Catalunya, Cabrils, Spain). All strains were grown in brain-heart infusion (BHI) media and plated onto Tripticasein Soy agar plates (*S. dysgalactiae, S. uberis*) or BHI agar plates (*E. coli, S. aureus*). Single colonies were grown in 10 mL of BHI media overnight at 37°C in static conditions. A cell pellet was obtained by centrifugation at 6,000 x *g* for 10 min at 4°C and kept frozen in 10% glycerol upon use. Before infection experiments, bacterial pellets were thawed at room temperature and grown overnight in 10 mL of BHI at 37 ºC in static conditions. Bacterial concentrations were determined by spectrophotometric quantifications according to previously established colony-forming unit (CFU) counts (data not shown). Equivalence between OD_{600} and CFU/mL was the following: *E. coli* 5.00 = $5x10^9$ CFU/mL, *S. uberis* 0.50 = $8.5x10^8$ CFU/mL, *S. aureus* 5.00 = $5.1x10^9$ CFU/mL, and *S. dysgalactiae* 0.50 = $6.2x10^7$ CFU/mL. Infective bacterial doses were obtained by resuspending and diluting the cell pellet in infection medium. Mammary epithelial cells were seeded in 24-well plates at a density of 44,000 cells/well in cell culture

medium (concentrations described above). After 24 h, cells were incubated with recombinant M-SAA3 (at a concentration of 10-160 μg/ mL) for 1 h in a volume of 600 μL PBS + 400 μL infection medium. Negative control consisted of 600 μL PBS + 400 μL medium. After 1 h incubation at 37°C and 5% CO_2 mammary cells were infected with 10^6 CFU/well and incubated in the same conditions for two more hours. Then, mammary cells were gently washed with 0.5mL PBS twice and 0.5 mL of 0.9% NaCl was added until lysed. Mammary cell lysate obtained from each replicate was serially diluted and seeded in agar plates. Colonies were counted after 24-48 h of growth at 37°C. The experiment was repeated 3 times with 6 replicates per treatment. Also, direct bacterial killing activity of recombinant M-SAA3 was tested against *S. aureus*. A suspension containing $1x10^6$ CFU was incubated with PBS with or without 160 μg/mL of recombinant M-SAA3 for 3.5 h and 5.5 h at 37°C. After incubation, viable bacteria were quantified by plating the suspension in agar plates. Data were transformed to achieve a normal distribution when necessary prior to statistical analyses. All data were analyzed using an analysis of variance that accounted for the effect of treatment (i.e., M-SAA3, LPS, or Lipid IV_A).

The M-SAA3 yields obtained after protein expression in *E. coli* BL21 and ClearColi® were 1.8 and 1.33 mg/L culture with a purity of 98 and 95%, respectively. The two main bands of 15 kDa and 10 kDa observed by Coomassie staining were identified as M-SAA3 by western blot using anti-His antibodies and MALDI-TOF analyses (data not shown). Also, a band corresponding to lysine decarboxylase CadA from *E. coli* was identified in M-SAA3 samples purified from ClearColi®. The inclusion of a second purification step based on a cation-exchange chromatography for M-SAA3 obtained from ClearColi® allowed the removal of this contaminating protein. However, although the addition of this extra purification step increased the purity of M-SAA3, it also resulted in a loss of 70% of M-SAA3 yield (data not shown). The results indicated that the amount of Lipid IV_A associated to 1 μg of M-SAA3 produced in

ClearColi® was much greater than the amount of LPS traces in 1 μg of M-SAA3 produced in BL21 strain (76 ng or 54 pmols of Lipid IV_A compared with 0.08 ng or 0.016 pmols of LPS). Although it has been described that Lipid IV_A does not trigger the TLR4-based responses in human cells (Planesse et al., 2015), an IL-8 stimulation when using high doses of Lipid IV_A was observed herein. Since the amount of Lipid IV_A traces was high in the purified M-SAA3 protein, the use of high M-SAA3 concentrations (around 100 μg) in bovine cell culture experiments and further *in vivo* assays would include a sufficient amount of Lipid IV_A to stimulate IL-8 expression itself, which would interfere with the analysis of M-SAA3 activity. The inconsistent production rates and presence of high amounts of Lipid IV_A in the recombinant product from Clearcoli were the main reason to select the protein produced in *E. coli* BL21 followed by a 800-fold LPS removal with Mustang filters. This final amount of LPS present should not interfere in the study of M-SAA3 functionality (< 0.001 EU (0.0001 ng LPS) /μg of M-SAA3).

The expression of IL-8 increased ($P < 0.0001$) up to 13-fold in M-SAA3 treatments compared with control in a dose-dependent manner, with 10 μg/mL representing the lowest dose with a significant effect (Figure 1B). We were not able to demonstrate direct killing activity of *S. aureus* (Figure 3), which is one of the most important pathogens involved in IMI in dairy cows (Rainard, 2005). Molenaar et al. (2009) reported a moderate activity at 18 μg/mL and a complete killing activity of *E. coli* at concentrations ≥63 μg/mL of bovine M-SAA3.2. The greater effect on *E. coli* could be linked to a greater efficacy of M-SAA3 against Gram-negative microorganisms, due its binding affinity to OmpA (Hari-Dass et al., 2005; Molenaar et al., 2009). Alternatively, differences observed between studies could be due to the sequence of the M-SAA3 protein used. Bovine and caprine M-SAA3 forms share the 91.3% of their nucleotide and 86.3% of their amino acid sequence, but they are not identical molecules. Moreover, the recombinant M-SAA3 produced herein lost a 9 amino acids at the N terminus of the native

mature form, which might be involved in this direct killing activity. However, MSAA-treated mammary epithelial cells had reduced bacterial counts of *S aureus , S uberis and E coli* with all the concentrations tested (Figure 2A, 2C, 2D). Specifically, a reduction of 50% of *S. aureus* infection was achieved at 160 μg/mL. From a general point of view, the best concentration of caprine M-SAA3 at inhibiting infections was the lowest concentration tested (10 μg/mL). In the case of *S. dysgalactiae* and *E. coli* (Figure 2A, 2B) this concentration elicited the best efficiency of inhibition, being 67 and 51%, respectively. In the case of *S. aureus* and *S. uberis* (Figure 2C, 2D), although 10 μg/mL of SAA3 inhibited infection by 23 and 29%, respectively, greater concentrations of M-SAA3 (up to 160 μg) increased the antimicrobial potential to 50%. Intriguingly, high M-SAA3 concentrations were not as efficient as low M-SAA3 concentrations in *E. coli* and *S. dysgalactiae*. In contrast, the greatest concentrations of M-SAA3 performed better with *S. aureus* and *S. uberis* than with *E. coli*. Regarding the direct bacterial killing of *S. aureus*, there was no effect of caprine M-SAA3 on the viability of *S. aureus* (Figure 3). Thus, it was decided to explore the potential effect of four different concentrations of M-SAA3 on the reduction of bacterial infection by *S. aureus*, *S. dysgalactiae*, *S. uberis*, and *E. coli* in mammary cultures. A decrease in bacterial infection with all four strains was found and, interestingly, the response to the different doses varied among the pathogens, which could be explained by the different inflammatory pathways elicited by different bacteria. It has been previously described that M-SAA3 binds to MD-2 to activate p38 and NF-κB pathways in a MyD88-dependent manner (Deguchi et al., 2013). This regulates not only the stimulation of inflammatory components such as several cytokines, but also the synthesis of antimicrobial peptides, tight junctions, and inflammation components that allow epithelial cells control the infection (Tak and Firestein, 2001; Zarubin and Han, 2005). Moreover, M-SAA3 increases the expression of mucins in several tissues (Tashiro et al., 2017) and the mammary

gland expresses Mucin1 and Lingual Antimicrobial Peptide (LAP) as protective molecules (Isobe et al., 2009; Patton et al., 1995; Pallesen et al., 2001).

As a Gram-negative bacterium, *E. coli* stimulates, the inflammatory response of epithelia via TLR4, which is also activated by M-SAA3. However, some authors have suggested that *S. aureus* does not activate this receptor at the same extent than in *E. coli* but mainly activates TLR2 instead (Fu et al., 2013). On the other hand, Yang et al. 2008 suggested that although *S. aureus* actives both TLR2 and TLR4, the pathogen impairs the final NF-kB activation (Yang et al., 2008). Thus, these observations suggest that there is a greater immune stimulation in *E. coli* than in *S. aureus*, which combined with M-SAA3 effects on the mammary cells could lead to an over-stimulation causing negative effects on the tissue (Blach-Olszewska et al., 2007). Alternatively, we could not discard a possible competitive effect for TLR4 between some bacteria strains used (*E. coli* / *S. dysgalactiae*) and M-SAA3. Although further research is needed to elucidate the exact mechanisms, it can be inferred that the recombinant M-SAA3 used herein represents a plausible strategy to prevent IMI in dairy cows.

Overall, these results show potential for the use of recombinant M-SAA3 in preventing IMI in dairy cows. We have demonstrated effects of recombinant M-SAA3 on mammary epithelia and bacteria resulting in the reduction of infection by four relevant mastitic pathogens, which coupled with the potential to recruit immune cells into the mammary gland, increased phagocytic activity and dendritic cells maturation (Domènech et al., 2014, 2012) make M-SAA3 a plausible alternative to antimicrobial agents to control IMI in cattle.

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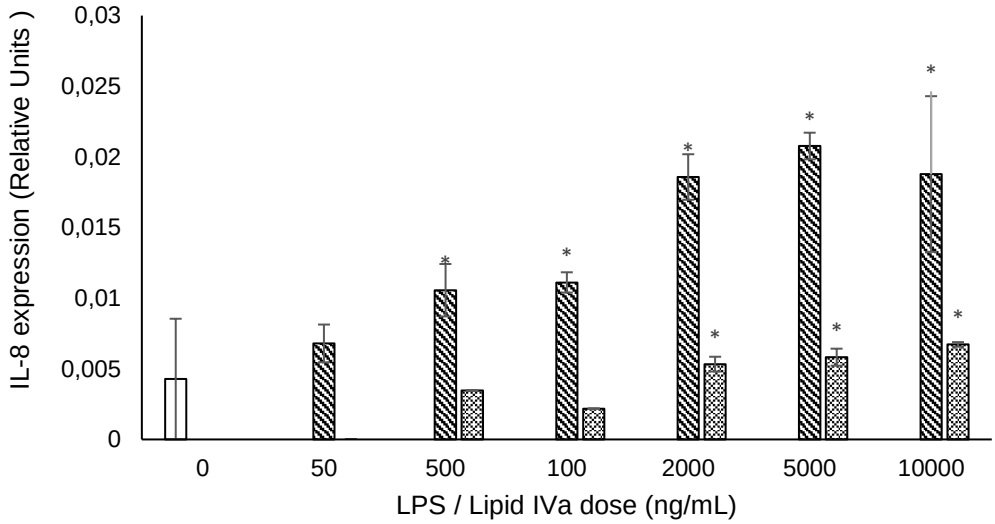
Figure 1

A.


IL-8 expression (Relative Units)
0,03
0,025
0,02
0,015
0,01
0,005
0
0
50
500
100
2000
5000
10000
LPS / Lipid IVa dose (ng/mL)

B.

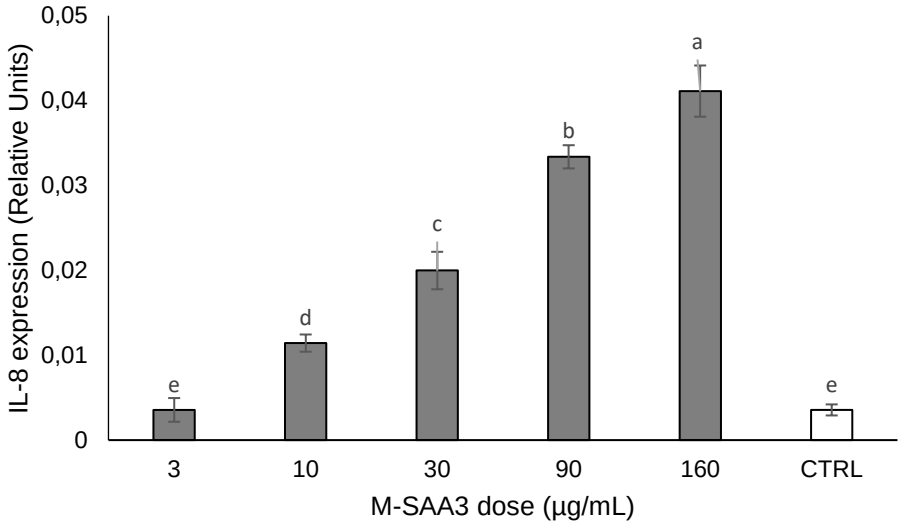
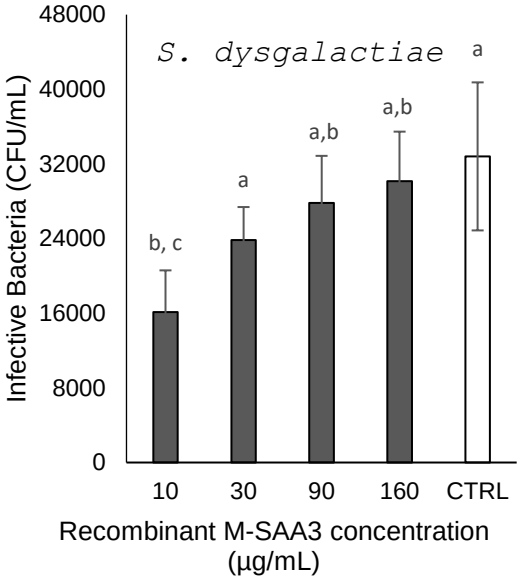

IL-8 expression (Relative Units)
0,05
0,04
0,03
0,02
0,01
0
e
d
c
b
a
e
3
10
30
90
160
CTRL
M-SAA3 dose (µg/mL)

Figure 2

A.



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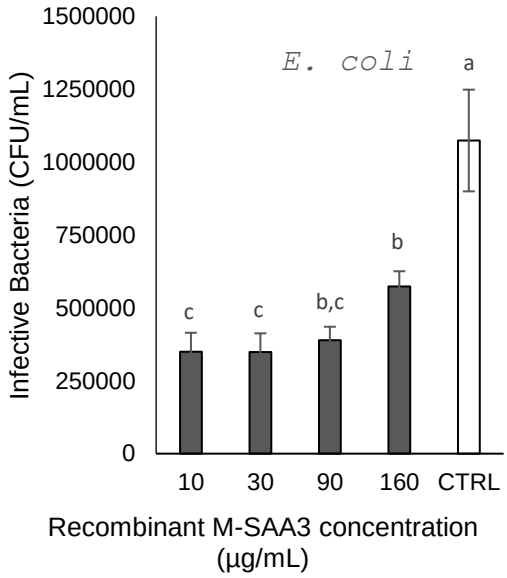

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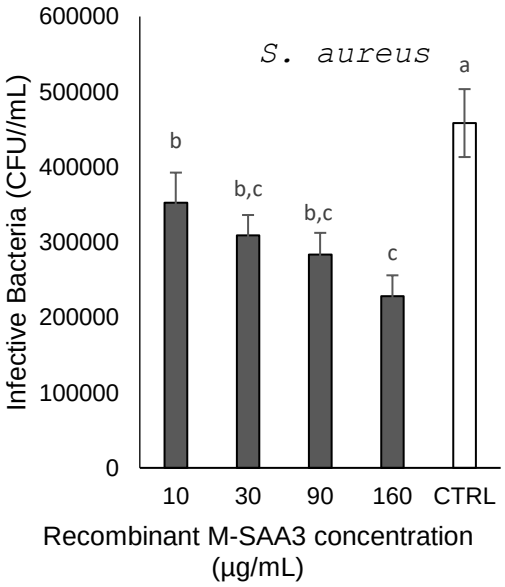
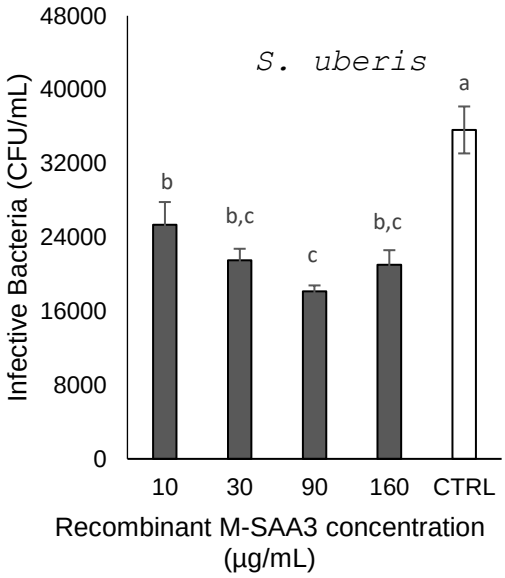


D.



371 **Figure 3.**

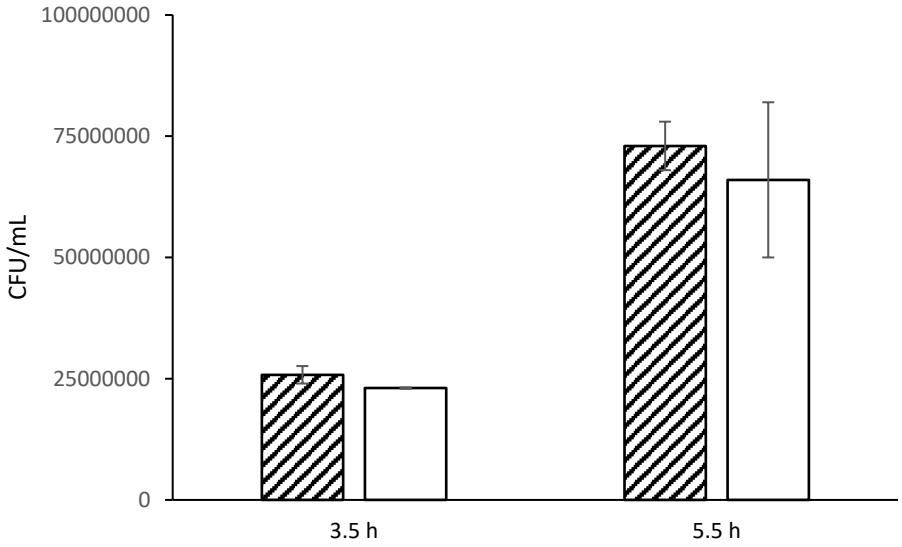


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Figure captions

Figure 1. **A.** Expression of IL-8 in primary mammary cell cultures treated with LPS (lined bars), Lipid IV_A (dotted bars), or control (white bar) expressed in relative units (RU). Asterisks indicate differences from control. **B.** Expression of IL-8 in primary mammary cell cultures treated with different concentrations of recombinant M-SAA3 expressed as relative units (RU). Different letters denote differences ($P < 0.05$) among treatments. Error bars depict SEM at each time point.

Figure 2. Bacterial counts or infective bacteria, expressed as CFU/mL, associated to mammary primary cell cultures after an infection with 10^6 CFU/mL of (A) *S. dysgalactiae*, (B) *E. coli*, (C) *S. aureus*, or (D) *S. uberis* and treated with different concentrations of recombinant M-SAA3. Different letters denote differences ($P < 0.05$) among treatments. Error bars depict SEM at each time point.

Figure 3. Bacterial counts expressed as CFU/mL of *S. aureus* treated with PBS in the absence (white bars) or presence (dashed bars) of 50 μg/mL of recombinant M-SAA3. Error bars depict SEM at each time point.