1 *Short communication*: **Recombinant mammary serum amyloid A3 as a potential strategy**

2 **to prevent intramammary infections in dairy cows at dry-off**. *By Parés et al.* Dairy cows

3 are most susceptible to incur intramammary infections during the dry period. Preventive

4 strategies other than antibiotics may be extremely useful to decrease the infection risk without

5 increasing antibiotic resistances. The use of recombinant mammary acute phase protein M-

6 SAA3 is a promising alternative because it is a key modulator of the mammary gland innate

7 immunity. This study demonstrates the effects of M-SAA3 on mammary epithelia reducing the

8 infections of four relevant mastitis pathogens in dairy cattle.

9

10 *Short communication*: **Recombinant mammary serum amyloid A3**

11 **as a potential strategy to prevent intramammary infections in dairy**

12 **cows at dry-off**

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23

24 ABSTRACT

25 Mammary serum amyloid A3 (M-SAA3) has shown potential stimulating innate immunity

26 during intramammary infections at calving and dry-off. Herein, we have produced recombinant

27 caprine M-SAA3 to test its potential to reduce intramammary infections against

28 *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia*

29 *coli*, which are common mastitis-producing pathogens. The recombinant production of M-

30 SAA3 followed by LPS-removal to avoid LPS-unspecific stimulation of the immune system

31 was successfully achieved. Mammary serum amyloid A3 stimulated the expression of IL-8 in

32 a dose-dependent manner in primary mammary cultures. Although a direct killing effect on *S.*

33 *aureus* by M-SAA3 was not detected, the acute phase protein was able to reduce up to 50% *S.*

34 *aureus, S. uberis*, and *S. dysgalactiae* infections and induced a reduction of 67% in *E. coli*

35 counts. In general, the best concentration of caprine M-SAA3 at inhibiting infections was the

36 lowest concentration tested (10 μg/ml), although greater concentrations of M-SAA3 (up to 160

37 μg/ml) increased the antimicrobial potential against some pathogens.

38

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

40

41 **Short Communication**

42 Mastitis is the inflammatory response of the mammary tissue to bacterial infections, and it

43 represents one of the most economically costly diseases affecting dairy animals. The udder of

44 the dairy cow is most susceptible to incur intra-mammary infections (IMI) during the dry

45 period, which can persist throughout the next lactation, causing important economic losses

46 (Oliver and Murinda, 2012) and discomfort to the animal (Bertulat et al., 2013). The reasons

47 behind this prominent risk is that a relatively large volume of milk remains in the udder at dry-

48 off, which may lead to milk leakage (Tucker et al., 2009) and favor the colonization of the

49 mammary gland by microorganisms (Schukken et al., 1993). Also, at dry-off, the immune

50 system is not fully active against pathogens because a reduced functional capability of

51 mammary leukocyte population (Oliver and Sordillo, 1989) coupled with the fact that an

52 important part of phagocytic cells arriving at mammary gland is engaged in removing fat and

53 protein debris from the milk accumulated in the udder at dry-off (Burvenich et al., 2007). At

54 present, the most common strategy to prevent IMIs at dry-off include: a) inducing a reduction

55 of milk yield (through either reducing milking frequency (Tucker et al., 2009), nutrient supply

56 (Ollier et al., 2014), or both, or the use of prolactin release inhibitors such cabergoline that also

57 hasten mammary gland involution and enhance its immunity (Bach et al., 2015, Boutinaud et

58 al., 2016, 2017)), 2) administering broad-spectrum antibiotics (Scherpenzeel et al., 2014), and

59 3) applying teat sealants at drying (McParland et al., 2019). However, a concern exists that use

60 of antibiotics could be associated with an increase in antibiotic-resistant bacteria, and some

61 governments are implementing regulations to restrict the use of prophylactic intramammary

62 antibiotics in non-infected cows at dry-off. However, Scherpenzeel et al. (2014) showed that

63 quarters that did not receive antimicrobial treatment at dry-off had a 1.7 times greater incidence

64 rate of clinical mastitis during the dry period and the first 100 d in the subsequent lactation, as

65 compared with quarters that did receive antibiotic treatment at dry-off.

66 Mammary serum amyloid A3 (M-SAA3) is a naturally occurring acute phase protein produced

67 locally and involved in the regulation of the local immunity and the regeneration of the

68 mammary gland. The concentrations of M-SAA3 are greatest in colostrum, in milk during IMI,

69 and at dry-off (Molenaar et al., 2009). A previous study (Domènech et al., 2014) has shown

70 that intra-mammary infusion of recombinant M-SAA3 in the cow fostered the involution

71 process, mainly through the activation of endogenous mammary matrix metalloproteinase -9

72 (MMP-9) activity, which is involved in extracellular matrix remodeling. It has also been

73 reported that M-SAA3 modulates the innate immunity by increasing mediators of the immune

74 response, such as IL-8, to ultimately enhance neutrophil recruitment, maturation of dendritic

75   cells, and phagocytic activity by opsonization (Domènech et al., 2012, 2014). However, the

76   role of recombinant M-SAA3 in the inhibition of infections caused by mastitic pathogens has

77   not yet been studied in depth. Thus, the main objective of the present study was to explore the

78   potential of M-SAA3 in the inhibition of the infection of primary bovine mammary epithelial

79   cultures by common mastitis-producing pathogens including *Escherichia coli*, *Streptococcus*

80   *uberis*, *Staphylococcus aureus*, and *Streptococcus dysgalactiae*.

81   Herein, M-SAA3 was produced recombinantly without traces of endotoxins that could interfere

82   with the immune function using either *E. coli* BL21 Star (DE3) or ClearColi® (Lucigen,

83   Middleton, WI, USA) transformed with pET101/D-TOPO vector codifying for the His tagged-

84   goat M-SAA3 sequence isolated in a previous study (Domènech et al., 2012). Microbial cells

85   were grown in 400 mL of LB-Amp media at an initial OD_{600} of 0.05 until log phase was

86   achieved. Recombinant expression was induced by 0.1 mM IPTG at OD_{600} of 0.9-1 for 1 h and

87   20 min in BL21 strain and at 0.6-0.7 for 2 h in ClearColi®. The cell pellet was obtained by

88   centrifugation at 6,000 x *g* for 10 min, and frozen at -80°C until use, and then lysed in 36 mL

89   Tris-buffered saline lysis buffer containing 1x Bugbuster® (Merck-Millipore, Billerica, MA,

90   USA), 0.1% v/v benzonuclease (Sigma-Aldrich, Saint Louis, MO, USA), and 1x cOmplete®

91   EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). The lysed product was

92   centrifuged for 15 min at 20,000 x *g* at 4 °C. Supernatants containing protein were then used to

93   obtain purified protein with the Amicon Pro System from Merck-Millipore (Billerica, MA,

94   USA) and the Clontech (Mountain View, CA, USA) His60 agarose bead slurry (60 mg/mL

95   binding capacity). Samples were eluted twice to ensure there was no protein left in the resin.

96   The purified protein was dialyzed overnight at 4°C against PBS and quantified using

97   spectrophotometry ((A280×Molecular weight)/Extinction coefficient; (mg/mL)). The removal

98   of LPS from M-SAA3 produced in *E. coli* BL21 was carried out with the Mustang Q Acrodisc

99   membrane (Pall Corporation, New York, USA) following manufacturer's instructions. Briefly,

100   all samples and buffers were filtered through a 0.22 μm filter before pushed through the

101   membrane. The membrane was then washed with 5 mL 1 M NaOH and equilibrated with 1 mL

102   1 M NaCl and 5 mL PBS before running the sample. The presence of possible endotoxin traces

103   in purified M-SAA3 (LPS or Lipid IV$_A$) was quantified by Microcoat Company using LAL

104   Kinetic chromogenic assay. Protein production was assessed by SDS-PAGE in 12%

105   polyacrylamide gels either with Coomassie or western blot as previously described (Gifre-

106   Renom et al., 2018). Samples were analyzed using a lineal method in an UltrafleXtreme

107   MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) with ion acceleration of 25

108   kV. In order to test M-SAA3 activity, mammary epithelial primary cultures were obtained from

109   mammary tissue as described elsewhere (Domenech et al., 2014). Mammary epithelial cells

110   were seeded in 24-well plates at 44,000 cells/well. After 24 h incubation at 37°C and 5% CO_2,

111   wells were washed twice with warm PBS to remove antibiotics, and treatments were applied

112   in a final volume of 100 μL of PBS + 400 μL of medium (DMEM/F-12 medium with 8 μg/mL

113   bovine insulin and 50 μg/mL hydrocortisone) in the case of LPS and Lipid IV$_A$ or in 600 μL

114   PBS + 400 μL of medium in the case of recombinant M-SAA3. After a 3 h incubation at 37°C,

115   5% CO_2, cells were gently washed with PBS, and 500 μL of Trizol reagent (Thermo Fisher

116   Scientific, Madrid, Spain) were added to each well to collect and lysate the cells. Cell lysates

117   were kept frozen at -80°C until RNA extraction. The extraction of RNA was performed using

118   the TRizol reagent (Thermo Fisher Scientific, Madrid, Spain). Then, RNA was retrotranscribed

119   to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc, Shiga, Japan) following

120   manufacturer's instructions. The RNA purity was assessed using a Nanodrop

121   instrument at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios between 1.9-2.0 and

122   2.0-2.2, respectively. Quantitative PCR was performed for genes encoding for IL-8 (Primer

123   sequences: Fw: 5'-TTGAGAGTGGGCCACACTGTG-3',   Rv: 5'-

124   TGCACCCACTTTTCCTTGG-3')   and   ACTB   (Primer   sequences: Fw: 5'-

125 CTGGACTTCGAGCAGGAGAT-3', Rv: 5'-CCCGTCAGGAAGCTCGTAG-3') using a

126 MyiQ Single Color Real-Time PCR Detection System Thermocycler (Bio-Rad, Madrid, Spain)

127 with the SYBR Premix Ex Taq (Takara Bio Inc, Japan). Quantitative PCR conditions for each

128 set of primers were individually optimized (ACTB: Annealing temperature = 57 ℃, primer

129 concentration = 0.124 μM; IL-8: Annealing temperature = 55 ℃, primer concentration = 0.5

130 μM). The specificity of the amplification was evaluated by the single band identification at the

131 expected molecular weight in 0.8% DNA agarose gel, using each respective gene control, and

132 a single peak in the qPCR melting curves. A standard curve of Ct versus log concentration was

133 plotted to obtain the efficiency, which was calculated using the formula $10^{-1/slope}$, with an

134 acceptable range of 1.8-2.2 (Chow et al., 2010). Relative gene expression was calculated using

135 the $2^{\Delta Ct}$ method with ACTB as reference gene.

136 Pathogenic *E. coli*, *S. uberis*, *S. aureus*, and *S. dysgalactiae* were isolated from mastitic milk

137 kindly provided by ALLIC (Laboratori interprofessional lleter de Catalunya, Cabrils, Spain).

138 All strains were grown in brain-heart infusion (BHI) media and plated onto Tripticasein Soy

139 agar plates (*S. dysgalactiae, S. uberis*) or BHI agar plates (*E. coli, S. aureus*). Single colonies

140 were grown in 10 mL of BHI media overnight at 37°C in static conditions. A cell pellet was

141 obtained by centrifugation at 6,000 x *g* for 10 min at 4°C and kept frozen in 10% glycerol upon

142 use. Before infection experiments, bacterial pellets were thawed at room temperature and

143 grown overnight in 10 mL of BHI at 37 ℃ in static conditions. Bacterial concentrations were

144 determined by spectrophotometric quantifications according to previously established colony-

145 forming unit (CFU) counts (data not shown). Equivalence between OD_{600} and CFU/mL was

146 the following: *E. coli* $5.00 = 5 \times 10^9$ CFU/mL, *S. uberis* $0.50 = 8.5 \times 10^8$ CFU/mL, *S. aureus* 5.00

147 $= 5.1 \times 10^9$ CFU/mL, and *S. dysgalactiae* $0.50 = 6.2 \times 10^7$ CFU/mL. Infective bacterial doses

148 were obtained by resuspending and diluting the cell pellet in infection medium. Mammary

149 epithelial cells were seeded in 24-well plates at a density of 44,000 cells/well in cell culture

150   medium (concentrations described above). After 24 h, cells were incubated with recombinant

151   M-SAA3 (at a concentration of 10-160 μg/ mL) for 1 h in a volume of 600 μL PBS + 400 μL

152   infection medium. Negative control consisted of 600 μL PBS + 400 μL medium. After 1 h

153   incubation at 37°C and 5% CO_2 mammary cells were infected with 10^6 CFU/well and incubated

154   in the same conditions for two more hours. Then, mammary cells were gently washed with

155   0.5mL PBS twice and 0.5 mL of 0.9% NaCl was added until lysed. Mammary cell lysate

156   obtained from each replicate was serially diluted and seeded in agar plates. Colonies were

157   counted after 24-48 h of growth at 37°C. The experiment was repeated 3 times with 6 replicates

158   per treatment. Also, direct bacterial killing activity of recombinant M-SAA3 was tested against

159   *S. aureus*. A suspension containing 1×10^6 CFU was incubated with PBS with or without 160

160   μg/mL of recombinant M-SAA3 for 3.5 h and 5.5 h at 37°C. After incubation, viable bacteria

161   were quantified by plating the suspension in agar plates. Data were transformed to achieve a

162   normal distribution when necessary prior to statistical analyses. All data were analyzed using

163   an analysis of variance that accounted for the effect of treatment (i.e., M-SAA3, LPS, or Lipid

164   IV_A).

165   The M-SAA3 yields obtained after protein expression in *E. coli* BL21 and ClearColi® were 1.8

166   and 1.33 mg/L culture with a purity of 98 and 95%, respectively. The two main bands of 15

167   kDa and 10 kDa observed by Coomassie staining were identified as M-SAA3 by western blot

168   using anti-His antibodies and MALDI-TOF analyses (data not shown). Also, a band

169   corresponding to lysine decarboxylase CadA from *E. coli* was identified in M-SAA3 samples

170   purified from ClearColi®. The inclusion of a second purification step based on a cation-

171   exchange chromatography for M-SAA3 obtained from ClearColi® allowed the removal of this

172   contaminating protein. However, although the addition of this extra purification step increased

173   the purity of M-SAA3, it also resulted in a loss of 70% of M-SAA3 yield (data not shown).

174   The results indicated that the amount of Lipid IV_A associated to 1 μg of M-SAA3 produced in

175   ClearColi® was much greater than the amount of LPS traces in 1 µg of M-SAA3 produced in

176   BL21 strain (76 ng or 54 pmols of Lipid IV$_A$ compared with 0.08 ng or 0.016 pmols of LPS).

177   Although it has been described that Lipid IV$_A$ does not trigger the TLR4-based responses in

178   human cells (Planesse et al., 2015), an IL-8 stimulation when using high doses of Lipid IV$_A$

179   was observed herein. Since the amount of Lipid IV$_A$ traces was high in the purified M-SAA3

180   protein, the use of high M-SAA3 concentrations (around 100 µg) in bovine cell culture

181   experiments and further *in vivo* assays would include a sufficient amount of Lipid IV$_A$ to

182   stimulate IL-8 expression itself, which would interfere with the analysis of M-SAA3 activity.

183   The inconsistent production rates and presence of high amounts of Lipid IV$_A$ in the

184   recombinant product from Clearcoli were the main reason to select the protein produced in *E.*

185   *coli* BL21 followed by a 800-fold LPS removal with Mustang filters. This final amount of LPS

186   present should not interfere in the study of M-SAA3 functionality (< 0.001 EU (0.0001 ng

187   LPS) /µg of M-SAA3).

188   The expression of IL-8 increased (P < 0.0001) up to 13-fold in M-SAA3 treatments compared

189   with control in a dose-dependent manner, with 10 µg/mL representing the lowest dose with a

190   significant effect (Figure 1B). We were not able to demonstrate direct killing activity of *S.*

191   *aureus* (Figure 3), which is one of the most important pathogens involved in IMI in dairy cows

192   (Rainard, 2005). Molenaar et al. (2009) reported a moderate activity at 18 µg/mL and a

193   complete killing activity of *E. coli* at concentrations ≥63 µg/mL of bovine M-SAA3.2. The

194   greater effect on *E. coli* could be linked to a greater efficacy of M-SAA3 against Gram-negative

195   microorganisms, due its binding affinity to OmpA (Hari-Dass et al., 2005; Molenaar et al.,

196   2009). Alternatively, differences observed between studies could be due to the sequence of the

197   M-SAA3 protein used. Bovine and caprine M-SAA3 forms share the 91.3% of their nucleotide

198   and 86.3% of their amino acid sequence, but they are not identical molecules. Moreover, the

199   recombinant M-SAA3 produced herein lost a 9 amino acids at the N terminus of the native

200 mature form, which might be involved in this direct killing activity. However, MSAA-treated

201 mammary epithelial cells had reduced bacterial counts of *S aureus , S uberis and E coli* with

202 all the concentrations tested (Figure 2A, 2C, 2D). Specifically, a reduction of 50% of *S. aureus*

203 infection was achieved at 160 μg/mL. From a general point of view, the best concentration of

204 caprine M-SAA3 at inhibiting infections was the lowest concentration tested (10 μg/mL). In

205 the case of *S. dysgalactiae* and *E. coli* (Figure 2A, 2B) this concentration elicited the best

206 efficiency of inhibition, being 67 and 51%, respectively. In the case of *S. aureus* and *S. uberis*

207 (Figure 2C, 2D), although 10 μg/mL of SAA3 inhibited infection by 23 and 29%, respectively,

208 greater concentrations of M-SAA3 (up to 160 μg) increased the antimicrobial potential to 50%.

209 Intriguingly, high M-SAA3 concentrations were not as efficient as low M-SAA3

210 concentrations in *E. coli* and *S. dysgalactiae*. In contrast, the greatest concentrations of M-

211 SAA3 performed better with *S. aureus* and *S. uberis* than with *E. coli*. Regarding the direct

212 bacterial killing of *S. aureus*, there was no effect of caprine M-SAA3 on the viability of *S.*

213 *aureus* (Figure 3). Thus, it was decided to explore the potential effect of four different

214 concentrations of M-SAA3 on the reduction of bacterial infection by *S. aureus*, *S. dysgalactiae*,

215 *S. uberis*, and *E. coli* in mammary cultures. A decrease in bacterial infection with all four strains

216 was found and, interestingly, the response to the different doses varied among the pathogens,

217 which could be explained by the different inflammatory pathways elicited by different bacteria.

218 It has been previously described that M-SAA3 binds to MD-2 to activate p38 and NF-κB

219 pathways in a MyD88-dependent manner (Deguchi et al., 2013). This regulates not only the

220 stimulation of inflammatory components such as several cytokines, but also the synthesis of

221 antimicrobial peptides, tight junctions, and inflammation components that allow epithelial cells

222 control the infection (Tak and Firestein, 2001; Zarubin and Han, 2005). Moreover, M-SAA3

223 increases the expression of mucins in several tissues (Tashiro et al., 2017) and the mammary

224 gland expresses Mucin1 and Lingual Antimicrobial Peptide (LAP) as protective molecules

225 (Isobe et al., 2009; Patton et al., 1995; Pallesen et al., 2001).

226 As a Gram-negative bacterium, *E. coli* stimulates, the inflammatory response of epithelia via

227 TLR4, which is also activated by M-SAA3. However, some authors have suggested that *S.*

228 *aureus* does not activate this receptor at the same extent than in *E. coli* but mainly activates

229 TLR2 instead (Fu et al., 2013). On the other hand, Yang et al. 2008 suggested that although *S.*

230 *aureus* actives both TLR2 and TLR4, the pathogen impairs the final NF-kB activation (Yang

231 et al., 2008). Thus, these observations suggest that there is a greater immune stimulation in *E.*

232 *coli* than in *S. aureus*, which combined with M-SAA3 effects on the mammary cells could lead

233 to an over-stimulation causing negative effects on the tissue (Blach-Olszewska et al., 2007).

234 Alternatively, we could not discard a possible competitive effect for TLR4 between some

235 bacteria strains used (*E. coli / S. dysgalactiae*) and M-SAA3. Although further research is

236 needed to elucidate the exact mechanisms, it can be inferred that the recombinant M-SAA3

237 used herein represents a plausible strategy to prevent IMI in dairy cows.

238 Overall, these results show potential for the use of recombinant M-SAA3 in preventing

239 IMI in dairy cows. We have demonstrated effects of recombinant M-SAA3 on mammary

240 epithelia and bacteria resulting in the reduction of infection by four

241 relevant mastitic pathogens, which coupled with the potential to recruit immune cells into the

242 mammary gland, increased phagocytic activity and dendritic cells maturation (Domènech et

243 al., 2014, 2012) make M-SAA3 a plausible alternative to antimicrobial agents to control IMI

244 in cattle.

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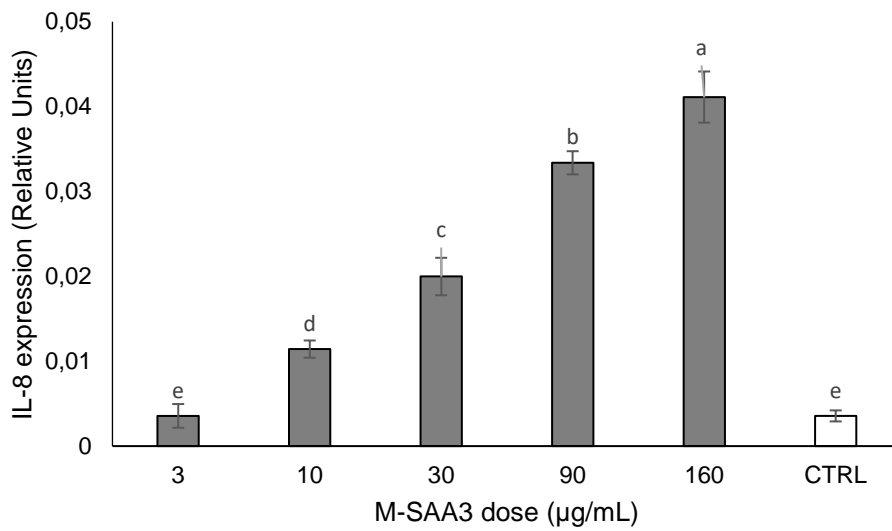
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361   **Figure 1**

362   **A.**
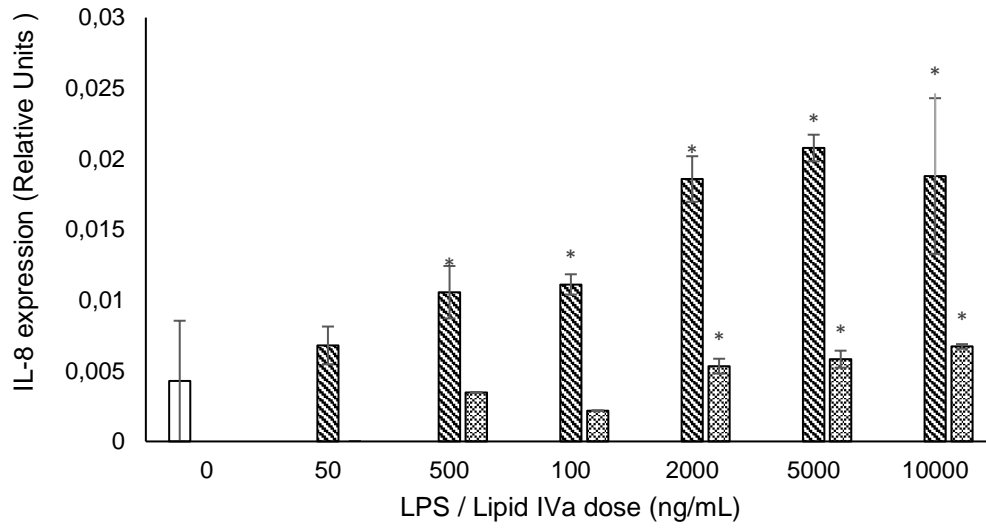
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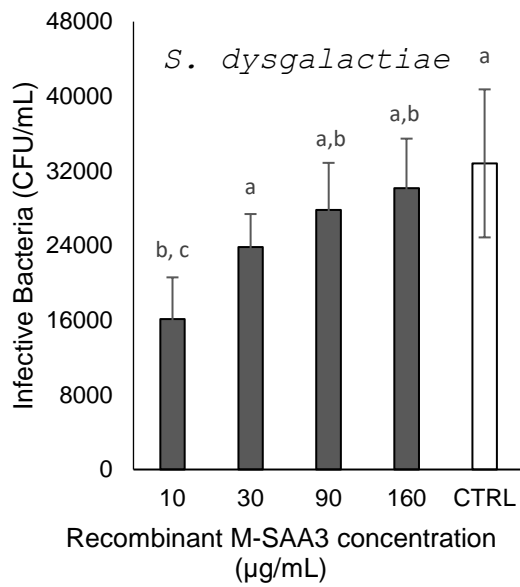

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365   **Figure 2**

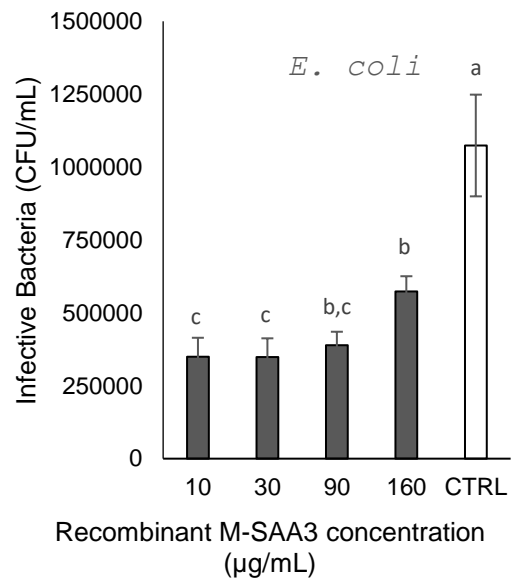366   **A.**   **B.**

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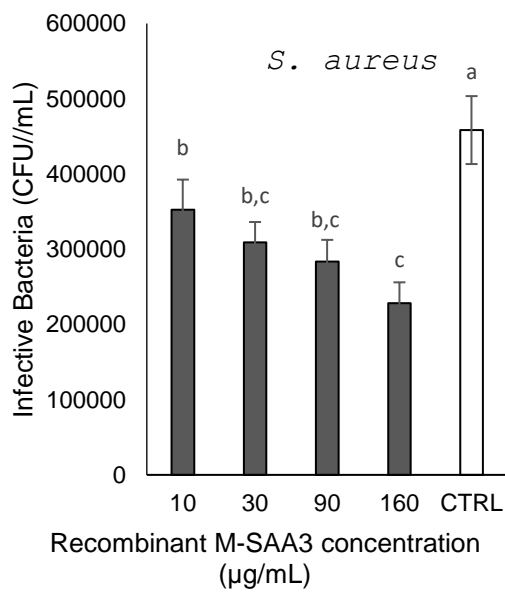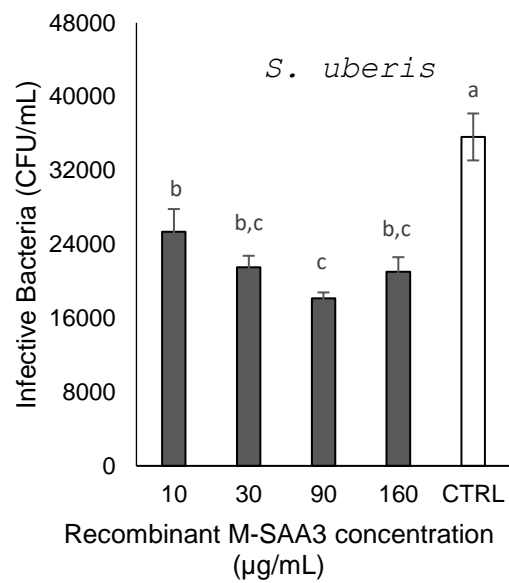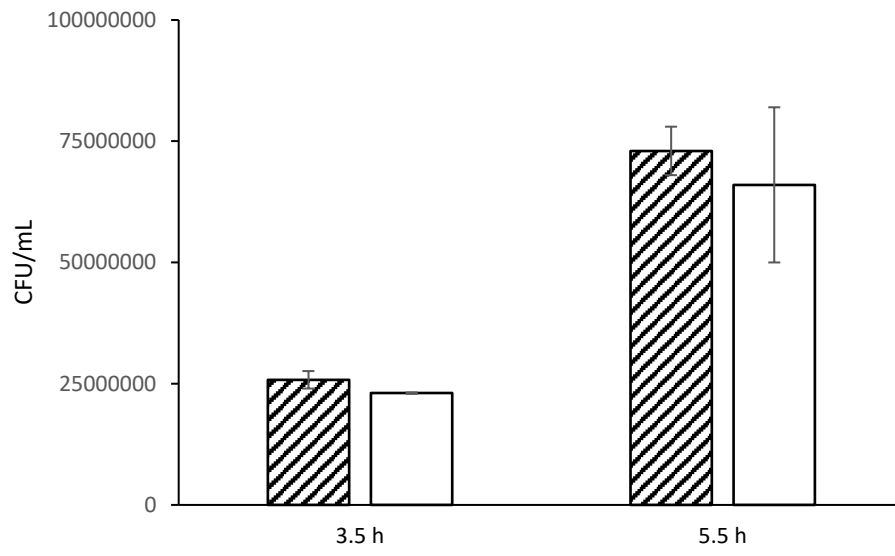368   **C.**   **D.**

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371   **Figure 3.**

372

373   **Figure captions**

374   **Figure 1**. **A.** Expression of IL-8 in primary mammary cell cultures treated with LPS (lined

375   bars), Lipid IV$_A$ (dotted bars), or control (white bar) expressed in relative units (RU). Asterisks

376   indicate differences from control. **B.** Expression of IL-8 in primary mammary cell cultures

377   treated with different concentrations of recombinant M-SAA3 expressed as relative units (RU).

378   Different letters denote differences ($P < 0.05$) among treatments. Error bars depict SEM at each

379   time point.

380

381   **Figure 2.** Bacterial counts or infective bacteria, expressed as CFU/mL, associated to mammary

382   primary cell cultures after an infection with 10^6 CFU/mL of (A) *S. dysgalactiae*, (B) *E. coli*,

383   (C) *S. aureus*, or (D) *S. uberis* and treated with different concentrations of recombinant M-

384   SAA3. Different letters denote differences ($P < 0.05$) among treatments. Error bars depict SEM

385   at each time point.

386

387   **Figure 3.** Bacterial counts expressed as CFU/mL of *S. aureus* treated with PBS in the absence

388   (white bars) or presence (dashed bars) of 50 µg/mL of recombinant M-SAA3. Error bars depict

389   SEM at each time point.

390