Short communication: Recombinant mammary serum amyloid A3 as a potential strategy for preventing intramammary infections in dairy cows at dryoff

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ABSTRACT

Mammary serum amyloid A3 (M-SAA3) has shown potential in stimulating innate immunity during intramammary infections, at calving and at dryoff. In this study, we produced recombinant caprine M-SAA3 to test its ability to reduce intramammary infections with *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*, which are all common mastitis-producing pathogens. Recombinant production of M-SAA3 (followed by lipopolysaccharide removal to avoid lipopolysaccharide-nonspecific 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 *Staph. aureus* by M-SAA3 was not detected, this acute phase protein was able to reduce *Staph. aureus*, *Strep. uberis*, and *Strep. dysgalactiae* infections by up to 50% and induced a reduction in *E. coli* counts of 67%. In general, the best concentration of caprine M-SAA3 for inhibiting infections was the lowest concentration tested (10 µg/mL), although higher concentrations (up to 160 µg/mL) increased its antimicrobial potential against some pathogens.

Key words: dry period, intramammary infection, immune response, mammary serum amyloid A3 (M-SAA3)

Short Communication

Mastitis is the inflammatory response of the mammary tissue to bacterial infections, and it is one of the most economically costly diseases affecting dairy animals. The udder of the dairy cow is most susceptible to IMI during the dry period, which can persist throughout

the next lactation, causing important economic losses (Oliver and Murinda, 2012), as well as discomfort for 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 dryoff, which may lead to milk leakage (Tucker et al., 2009) and favor colonization of the mammary gland by microorganisms (Schukken et al., 1993). As well, the immune system is not fully active against pathogens at dryoff because the mammary leukocyte population has reduced functional capability (Oliver and Sordillo, 1989) and because an important proportion of the phagocytic cells arriving at the mammary gland is engaged in removing fat and protein debris from the milk accumulated in the udder at dryoff (Burvenich et al., 2007). At present, the most common strategies for preventing IMI at dryoff include the following: (a) inducing a reduction of milk yield by decreasing milking frequency (Tucker et al., 2009), nutrient supply (Ollier et al., 2014), or both, or by using prolactin release inhibitors such cabergoline that also hasten mammary gland involution and enhance immunity (Bach et al., 2015; Boutinaud et al., 2016, 2017); (b) administering broad-spectrum antibiotics (Scherpenzeel et al., 2014); and (c) applying teat sealants at dryoff (McParland et al., 2019). However, the 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 noninfected cows at dryoff. Still, Scherpenzeel et al. (2014) have shown that quarters without antimicrobial treatment at dryoff had an incidence of clinical mastitis during the dry period and in the first 100 d of the subsequent lactation that was 1.7 times greater than quarters with antibiotic treatment at dryoff.

Mammary serum amyloid A3 (M-SAA3) is a naturally occurring acute phase protein that is produced locally and is involved in the regulation of local immunity and regeneration of the mammary gland. Concentrations of M-SAA3 are highest in colostrum, in milk

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during IMI, and at dryoff (Molenaar et al., 2009). A previous study (Domènech et al., 2014) has shown that intramammary infusion of recombinant M-SAA3 in cows fostered the involution process, mainly by activating endogenous mammary matrix metalloproteinase 9, which is involved in extracellular matrix remodeling. It has also been reported that M-SAA3 modulates 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. The main objective of the present study was to explore the potential of M-SAA3 to inhibit the infection of primary bovine mammary epithelial cultures with common mastitis-producing pathogens, including *Escherichia coli*, *Streptococcus uberis*, *Staphylococcus aureus*, and *Streptococcus dysgalactiae*.

In this study, M-SAA3 was produced recombinantly without traces of endotoxins that could interfere with immune function using either *E. coli* BL21 Star (DE3) or ClearColi (Lucigen, Middleton, WI) transformed with pET101/D-TOPO vector encoding the His-tagged goat M-SAA3 sequence isolated in a previous study (Domènech et al., 2012). Microbial cells were grown in 400 mL of lysogeny broth–ampicillin medium at an initial optical density at 600 nm (OD_{600}) of 0.05 until log phase was achieved. We induced recombinant expression by 0.1 mM isopropyl β -D-1-thiogalactopyranoside at an OD_{600} of 0.9 to 1 for 1 h 20 min in the BL21 strain and 0.6 to 0.7 for 2 h in ClearColi. The cell pellet was obtained by centrifugation at $6,000 \times g$ for 10 min, frozen at -80°C until use, and then lysed in 36 mL Tris-buffered saline lysis buffer containing 1 $\times$ Bugbuster (Merck-Millipore, Billerica, MA), 0.1% (vol/vol) benzonuclease (Sigma-Aldrich, Saint Louis, MO), and 1 $\times$ complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). The lysed product was centrifuged for 15 min at $20,000 \times g$ at 4°C . Supernatants containing protein were then used to obtain purified protein using the Amicon Pro System (Merck-Millipore) and His60 agarose bead slurry (60 mg/mL binding capacity; Clontech, Mountain View, CA). Samples were eluted twice to ensure that no protein was left in the resin. The purified protein was dialyzed overnight at 4°C against PBS and quantified using spectrophotometry $[(A_{280} \times \text{molecular weight})/\text{extinction coefficient; mg/mL}]$. We removed LPS from the M-SAA3 produced in *E. coli* BL21 using the Mustang Q Acrodisc membrane (Pall Corporation, New York) following the manufacturer's instructions ([https://laboratory.pall.com/content/dam/pall/laboratory/literature-library/non-gated/syringefilters/Acrodisc®%20Units%20with%20Mustang®%20Q%20and%20S%20Membranes%20.pdf](https://laboratory.pall.com/content/dam/pall/laboratory/literature-library/non-gated/syringefilters/Acrodisc%20Units%20with%20Mustang%20Q%20and%20S%20Membranes%20.pdf)). Briefly, all samples and buffers were passed through a 0.22- μm filter before being pushed through the membrane. The membrane was then washed with 5 mL of 1 M NaOH and equilibrated with 1 mL of 1 M NaCl and 5 mL of PBS before the sample was run. The presence of possible endotoxin traces in purified M-SAA3, either LPS or Lipid IVA (LPS modified molecule), was quantified in BL21 and ClearColi, respectively. This was performed by the MicroCoat Company (Bernried am Starnberger See, Germany) using a kinetic chromogenic LAL assay. Protein production was assessed by SDS-PAGE in 12% polyacrylamide gels with either Coomassie or Western blot as previously described (Gifre-Renom et al., 2018). Samples were analyzed using a linear method in an UltrafleXtreme MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) with ion acceleration of 25 kV.

To test M-SAA3 activity, we obtained mammary epithelial primary cultures from mammary tissue as described elsewhere (Domènech et al., 2014). Mammary epithelial cells were seeded in 24-well plates at 44,000 cells per 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 at a final volume of 100 μL PBS + 400 μL medium (Dulbecco's modified Eagle medium/Nutrient Mixture F-12 with 8 $\mu\text{g/mL}$ bovine insulin and 50 $\mu\text{g/mL}$ hydrocortisone) for LPS and Lipid IV_A or in 600 μL of PBS + 400 μL of medium for recombinant M-SAA3. After a 3 h incubation at 37°C and 5% CO_2 , cells were gently washed with PBS, and then 500 μL of Trizol reagent (Thermo Fisher Scientific, Madrid, Spain) was added to each well to collect and lyse the cells. Cell lysates were kept frozen at -80°C until RNA extraction. The extraction of RNA was performed using Trizol reagent (Thermo Fisher Scientific). Then, RNA was retrotranscribed to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan) following the manufacturer's instructions (https://www.takarabio.com/assets/documents/User%20Manual/RR037A_e.v1705Da.pdf). The RNA purity was assessed using a Nanodrop instrument at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios of 1.9 to 2.0 and 2.0 to 2.2, respectively. We performed quantitative PCR for genes encoding for *IL8* (primer sequences: forward 5'-TTGAGAGTGGGCCACTGTG-3'; reverse 5'-TGCACCCACTTTTCCTTGG-3') and *ACTB* (primer sequences: forward 5'-CTG-GACTTCGAGCAGGAGAT-3'; reverse 5'-CCCGT-CAGGAAGCTCGTAG-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.). Quantitative PCR conditions for each set of primers were individually optimized (*ACTB*: annealing temperature 57°C, primer concentration 0.124 μ M; IL-8: annealing temperature 55°C, primer concentration 0.5 μ M). We evaluated the specificity of the amplification by 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 quantitative PCR melting curves. We plotted a standard curve of Ct (cycle threshold) versus log concentration to obtain the efficiency, which we calculated using the formula $10^{-1/\text{slope}}$, with an acceptable range of 1.8 to 2.2 (Chow et al., 2010). We calculated relative gene expression using the $2^{\Delta\text{Ct}}$ method with *ACTB* as the reference gene.

We isolated pathogenic *E. coli*, *Strep. uberis*, *Staph. aureus*, and *Strep. dysgalactiae* from mastitic milk provided by ALLIC (Laboratori interprofessional lleter de Catalunya, Cabriels, Spain). All strains were grown in brain-heart infusion (BHI) medium and plated onto trypticase soy agar plates (*Strep. dysgalactiae*, *Strep. uberis*) or BHI agar plates (*E. coli*, *Staph. aureus*). Single colonies were grown in 10 mL of BHI medium overnight at 37°C in static conditions. A cell pellet was obtained by centrifugation at $6,000 \times g$ for 10 min at 4°C and kept frozen in 10% glycerol until use. Before the infection experiments, bacterial pellets were thawed at room temperature and grown overnight in 10 mL of BHI at 37°C in static conditions. We determined bacterial concentrations by spectrophotometric quantification according to previously established counts (cfu; data not shown). The equivalences between OD₆₀₀ and cfu/mL were as follows: *E. coli* 5.00 = 5×10^9 cfu/mL; *Strep. uberis* 0.50 = 8.5×10^8 cfu/mL; *Staph. aureus* 5.00 = 5.1×10^9 cfu/mL; and *Strep. dysgalactiae* 0.50 = 6.2×10^7 cfu/mL. Infective bacterial doses were obtained by resuspending and diluting the cell pellet in the infection medium. Mammary epithelial cells were seeded in 24-well plates at a density of 44,000 cells per well in cell culture medium (concentrations described above). After 24 h, cells were incubated with recombinant M-SAA3 (at concentrations of 10 to 160 μ g/mL) for 1 h in a volume of 600 μ L PBS + 400 μ L infection medium. The negative control consisted of 600 μ L of PBS + 400 μ L of medium. After 1 h incubation at 37°C and 5% CO₂, the mammary cells were infected with 10^6 cfu per well and incubated in the same conditions for 2 h longer. Then, the mammary cells were gently washed twice with 0.5 mL PBS, 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 to 48 h of growth

at 37°C. The experiment was repeated 3 times, with 6 replicates per treatment.

The direct bacterial killing activity of recombinant M-SAA3 was tested against *Staph. aureus*. A suspension containing 1×10^6 cfu was incubated with PBS with or without 160 μ g/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 before statistical analyses. All data were analyzed using an ANOVA 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 purities of 98 and 95%, respectively. The 2 main bands of 15 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). A band corresponding to lysine decarboxylase CadA from *E. coli* was identified in the M-SAA3 samples purified from ClearColi. The inclusion of a second purification step based on cation-exchange chromatography for M-SAA3 obtained from ClearColi allowed for 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 with 1 μ g of M-SAA3 produced in ClearColi was much greater than the amount of LPS in 1 μ g of M-SAA3 produced in the BL21 strain (76 ng or 54 pmol of Lipid IV_A compared with 0.08 ng or 0.016 pmol of LPS). Although it has been said that Lipid IV_A does not trigger toll-like-receptor 4 (TLR4)-based responses in human cells (Planesse et al., 2015), we observed IL-8 stimulation when using high doses of Lipid IV_A. Because the amount of Lipid IV_A was high in the purified M-SAA3 protein, the use of high M-SAA3 concentrations (approximately 100 μ g) in bovine cell culture experiments and further in vivo assays should 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 reasons for selecting the protein produced in *E. coli* BL21 followed by 800-fold LPS removal with Mustang filters. The final amount of LPS should not interfere in the study of M-SAA3 functionality [< 0.001 endotoxin units (0.0001 ng of LPS)/ μ g of M-SAA3].

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

trol in a dose-dependent manner; 10 $\mu\text{g}/\text{mL}$ represented the lowest dose with a significant effect (Figure 1B). We were not able to demonstrate direct killing activity of *Staph. aureus*, one of the most important pathogens involved in IMI in dairy cows (Rainard, 2005). Molenaar et al. (2009) reported moderate killing activity of *E. coli* at 18 $\mu\text{g}/\text{mL}$ and complete activity at concentrations ≥ 63 $\mu\text{g}/\text{mL}$ of bovine M-SAA3. This greater effect on *E. coli* could be linked to greater efficacy of M-SAA3 against gram-negative microorganisms, because of its binding affinity to outer membrane protein A (OmpA; Hari-Dass et al., 2005; Molenaar et al., 2009). Alternatively, the differences observed between studies could have been due to the sequence of the M-SAA3 protein used. Bovine and caprine M-SAA3 forms share 91.3% of their nucleotide and 86.3% of their amino acid sequences, but they are not identical. Moreover, the recombinant M-SAA3 produced in this study lost 9 amino acids at the N terminus of the native mature form, which might have been involved in direct killing activity. However, mammary epithelial cells treated with M-SAA3 showed reduced bacterial counts of *Staph. aureus*, *Strep. uberis*, and *E. coli* at all concentrations tested (Figure 2A, 2C, 2D). Specifically, a reduction of 50% of *Staph. aureus* infection was achieved at 160 $\mu\text{g}/\text{mL}$. From a general point of view, the best concentration of caprine M-SAA3 at inhibiting infections was the lowest tested (10 $\mu\text{g}/\text{mL}$). For *Strep. dysgalactiae* and *E. coli* (Figure 2A, 2B), this concentration elicited the best efficiency of inhibition, at 67 and 51%, respectively. For *Staph. aureus* and *Strep. uberis* (Figure 2C, 2D), 10 $\mu\text{g}/\text{mL}$ of M-SAA3 inhibited infection by 23 and 29%, respectively, but higher concentrations (up to 160 μg) increased the antimicrobial potential to 50%. Intriguingly, high M-SAA3 concentrations were not as efficient as low concentrations in *E. coli* and *Strep. dysgalactiae*. In contrast, the highest concentrations of M-SAA3 performed better with *Staph. aureus* and *Strep. uberis* than with *E. coli*. Regarding direct bacterial killing of *Staph. aureus*, we found no effect of caprine M-SAA3 on the viability of *Staph. aureus* (Figure 3). Thus, we decided to explore the potential effect of 4 different concentrations of M-SAA3 on the reduction of bacterial infection by *Staph. aureus*, *Strep. dysgalactiae*, *Strep. uberis*, and *E. coli* in mammary cultures. We found a decrease in bacterial infections with all 4 strains and, interestingly, the response to the different doses varied among the pathogens, which could be explained by the different inflammatory pathways elicited by the various 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), regulating not only the stimulation of inflammatory components such as cytokines, but also the synthesis

of antimicrobial peptides, tight junctions, and inflammation components that allow epithelial cells to control 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 mucin 1 and lingual antimicrobial peptide as protective molecules (Patton et al., 1995; Pallesen et al., 2001; Isobe et al., 2009).

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 *Staph. aureus* does not activate this receptor to the same extent as *E. coli*; instead, it mainly activates TLR2 (Fu et al., 2013). In contrast, Yang et al. (2008) suggested that although *Staph. aureus* activates both TLR2 and TLR4, it impairs the final NF- κB activation. These observations suggest greater immune stimulation in *E. coli* than in *Staph. aureus*, which combined with M-SAA3 effects on the mammary

Figure 1. (A) Expression of IL-8 in primary mammary cell cultures treated with LPS (striped bars), Lipid IV_A [modified LPS present in Clearcoli (Lucigen, Middleton, WI) cell wall; crosshatched bars], or control (CTRL; white bar) expressed in relative units (RU). Asterisks indicate differences ($P < 0.05$) from control. (B) Expression of IL-8 in primary mammary cell cultures treated with different concentrations of recombinant mammary serum amyloid A3 (M-SAA3) expressed in RU. Different letters (a–e) denote differences ($P < 0.05$) among treatments. Error bars depict SEM at each time point.

Figure 2. Bacterial counts of infective bacteria (expressed as cfu/mL) associated with mammary primary cell cultures after infection with 10^6 cfu/mL of (A) *Streptococcus dysgalactiae*, (B) *Escherichia coli*, (C) *Staphylococcus aureus*, or (D) *Streptococcus uberis* and treated with different concentrations of recombinant mammary serum amyloid A3 (M-SAA3). Different letters (a–c) denote differences ($P < 0.05$) among treatments. Error bars depict SEM at each time point.

cells could lead to overstimulation, causing negative effects for the tissue (Blach-Olszewska and Leszek, 2007). Alternatively, we could not discard a possible competitive effect for TLR4 between some bacteria strains used (*E. coli*, *Strep. 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 for preventing 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 the effects of recombinant M-SAA3 on mammary epithelia and bacteria, resulting in the reduction of infection by 4 relevant mastitic pathogens. These findings, along with the potential of M-SAA3 to recruit immune cells into the mammary gland, its involvement in increased phagocytic activity, and its role in dendritic cell maturation (Domènech et

Figure 3. Bacterial counts (expressed as cfu/mL) of *Staphylococcus aureus* treated with PBS in the absence (white bars) or presence (striped bars) of 50 µg/mL recombinant mammary serum amyloid A3. Error bars depict SEM at each time point.

al., 2014, 2012), make M-SAA3 a plausible alternative to antimicrobial agents for controlling IMI in cattle.

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