Research Article

Functionally Effective Immune Responses of Dairy Cows Vaccinated with *Streptococcus uberis* Adhesion Molecule during the Transition Period

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Abstract

Streptococcus uberis causes a significant proportion of mastitis in dairy cows. It is very important to induce protective immunity during the perparturient (transition) period when dairy cows are highly susceptible to mastitis. The aim of this study was to optimize immune responses of dairy cows during the periparturient period through three vaccinations at 28 days before drying off (D-28), at drying off (D0), and at 28 days after drying off (D+28) with recombinant *Streptococcus uberis* adhesion molecule (rSUAM). Forty dairy cows in the 1st or 2nd lactation were divided into two groups of 20 cows each. Cows in Group 2 were vaccinated with 200 µg of rSUAM with Montanide® ISA70VG adjuvant whereas cows in Group 1 (control) were injected with Montanide® ISA70VG mixed with PBS. Blood and milk samples were collected before each vaccination and at calving and were analyzed for anti-rSUAM IgG, IgG1, IgG2, IgA and IgM antibodies. Serum from rSUAM vaccinated cows was used in phagocytosis, as well as attachment and internalization inhibition assays. Results showed that serum and milk anti-rSUAM IgG, IgG1, IgG2, IgA and IgM titers were significantly increased after each vaccination achieving highest titers after 3rd vaccination ($P< 0.01$). Anti-rSUAM antibodies were effective in reducing adherence to and internalization of *S. uberis* into primary bovine mammary epithelial cells as well as increasing phagocytic killing of *S. uberis* by bovine macrophages. Results showed that this vaccination schedule induced robust serum and milk anti-rSUAM immune responses during periparturient period that can be protective against *S. uberis* mastitis.

INTRODUCTION

Bovine mastitis is an economically important disease of dairy herds throughout the world. Studies have clearly shown that the prevalence of environmental mastitis pathogens have steadily increased, thus becoming a major problem in well-managed dairy farms with low prevalence of contagious mastitis pathogens [1-4]. In these herds, *S. uberis* accounts for the majority of both clinical and subclinical mastitis in lactating and non-lactating cows [1,4] and heifers [5] suggesting that reduction in the incidence of contagious mastitis pathogens resulted in increased intramammary infections (IMI) caused by environmental pathogens particularly *S. uberis.* These observations also suggest that *S. uberis* have developed mechanisms by which it effectively overcome defenses of the mammary glands. An important consequence of the adaptation of *S. uberis* to the mammary gland is development of persistent infections, where the same clonal type was isolated from the same infected mammary quarter of the same cow over extended periods of time [6].

Lack of knowledge on *S. uberis* virulence factors and pathogenic mechanisms allowing *S. uberis* to overcome mammary gland defense mechanisms, have hampered the development of effective control strategies. The absence of adequate control and prevention measures for *S. uberis* mastitis plus the difficulty of controlling *S. uberis* in the dairy cow environment, calls for the development of alternative control tools such as protective vaccines. However, a critical and practical problem is how to induce protective immune responsesduring the early non-lactating (dry) and periparturient periods, when cows are highly susceptible to mastitis.

Neutrophils, dendritic cells, monocytes, and macrophages are professional phagocytes that control bacterial infections through different mechanisms [7]. For efficient phagocytosis and killing, bacteria need to be covered with opsonins such as immunoglobulins and proteins of the complement system (C3b, C3bi) [7]. The opsonization of a bacterium by immunoglobulins leads to the phagocytosis of the pathogen by phagocytes which ultimately results in the killing of the microorganism. Alternatively, the binding of antibodies to the bacterial surface mediated the activation of the complement through the classical pathway, which leads to the killing of the pathogen, but in the latter the binding of specific antibodies confers specificity to lytic activity of the complement. Therefore, the inductionof strong

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adaptive immune response through vaccination with rSUAM is of paramount importance for the efficient killing and clearance of *S. uberis* from the infected mammary gland. The combination of the complement and specific immunoglobulins are believed to be efficient in clearing bacterial infection by phagocytic killing [8].

Streptococcus uberis Adhesion Molecule (SUAM) is a novel *S. uberis* surface protein which favors adherence of *S. uberis* to host cells, plays a crucial role in the establishment, spread and persistence of infection (Almeida et al., 2006; Luther et al., 2008; Patel et al., 2009). The SUAM bound to lactoferrin (LF), a whey protein found in milk (Fang & Oliver, 1999) and binding of LF through SUAM enhanced adherence of *S. uberis* to bovine mammary epithelial cells (Fang et al., 2000). Antibodies against SUAM cross-reacted with homologous proteins present in other strains of *S. uberis* demonstrating the ubiquity of SUAM across all strains of *S. uberis* evaluated (Almeida et al., 2006).We hypothesized that a series of immunizations with recombinant *Streptococcus uberis* adhesion molecule (rSUAM) before and during the early dry period will induce enhanced intramammary immune responses suitable for preventing *S. Uberis* IMI during early dry and periparturient periods.

MATERIALS AND METHODS

Experimental animals and vaccination protocol

Pregnant Holstein cows (n=40) near the end of their 1st or 2nd lactation were randomly dividedinto two groups (rSUAM and control groups) of 20 cows each. Cows in Group 1 (rSUAM) were vaccinated with 200 µg rSUAM with adjuvant (Montanide® ISA70VG) and cows in the control group were injected with PBS mixed with adjuvant. All cows were injected 28 d prior to drying off (D-28), at drying off (D0) and 28 d after drying off (D+28). Prior to enrolment in the study, cows were tested and confirmed to be free of intramammary infection (IMI) and proven free of Johne's, brucellosis, tuberculosis, bovine leukemia virus (BLV). In addition, cows were tested and selected based on low serum anti-rSUAM titer. Additionally, cows were confirmed to have calf-hood vaccinations; minimum projected milk production of 22,000 pounds; four functional mammary quarters; pregnancy confirmation; expected calving dates; and mammary quarter somatic cell counts(SCC) <250,000 cells/ml of milk (Table 1).

Antigen Preparation and Administration

The rSUAM was prepared as described by Prado et al. [9]. Each cow of the Group 2 was vaccinated with 2 ml of the vaccine preparation containing 200 µg of rSUAM emulsified in Montanide® ISA70VG (Seppic, Paris, France) adjuvant at 30/70(vol/vol) antigen to adjuvant ratio. Group 1 (Control)

cows were injected with 2ml of a mixture of PBS (pH 7.4) and Montanide ISA 70VG at 70/30adjuvant to PBS ratio.

All cows were injected subcutaneously (SQ) 3 times onalternate sides of the neck area, approximately midway between the bases of the ear and the point of the shoulder. Experimental and control cows were under the same herd management and housed at the East Tennessee Research and Education Center-Little River Animal Experimental Unit (ETREC-LRAEU).

Sample Collection

Blood (n=4/cow) and milk (n=3/cow) samples for evaluation of humoral and cellular immune responses were collected immediately before (D-28) first vaccination, at each subsequent vaccination (D0 & D+28), and at calving (C). To avoid physiological disturbances and risk of IMI, mammary secretion samples were not collected at D+28. Blood and composite mammary secretions (pooled in equal volume from each quarter) were processed into serum and whey, respectively. For whey preparation, milk samples were ultra centrifuged at 20,000 x g for 30 min, and the supernatant (whey) was used for analysis ofimmune responses.

Enzyme-linked immuno sorbent asssay (ELISA)

ELISA was carried out as described elsewhere [10,11]. Briefly, 96-well plates were coated with 1µg/ml of rSUAM and incubated at 4°C overnight. After incubation, the coating solution was removed and plates were washed 5X with tris-buffered saline and blocked with tris-buffered saline (TBS)-0.5% Tween 20 (v/v) containing 1% gelatin (TBS-TG). Serum and whey samples were serially diluted horizontally (A1-A12) on 96-well plates in four-fold increments from 1:100 to 1: 1,638,400 and 1:40 to 1:655,360 respectively. Alkaline phosphatase-conjugated polyclonal sheep anti-bovine IgG (H+L) and monoclonal sheep anti-bovine IgG1, IgG2, IgA and IgM (Bethyl laboratories Inc, Montgomery, TX) were diluted 1:5000 and added to each well at 100 µL/well and incubated for 1h as per the manufacturer recommendation. After washing, 100 µL/well freshly prepared BCIP/NBT phosphatase substrate solutions (KPL-**Sera Care Life Sciences**, Milford, MA) was added onto each well, and incubated for 30 min at room temperature. After incubation, the absorbance was read at 405 nm with reference at 490 nm wave lengths. Serum and milk titers were calculated by the intersection of least-square regression of A405 versus logarithm of the dilution. The cutoff point in the serial dilution for titer calculation was determined using average plus two standard deviation (avg + 2st.dev.) of reading from blank (control) wells (B1- B12). The titer of each sample was evaluated in triplicates on same plates to avoid plate to plate variation.

Table 1: Vaccination Protocol.

Group	Antigen	Adjuvant	Route	Dose &Regimen	No. of Cows[a]
1	None	Montanide® ISA70VG	SQ[b]	2 ml, 3 times at D-28[c], D0[d], D+28[e] (28 d apart)	20
2	rSUAM[f] (200 µg)	Montanide® ISA70VG	SQ	2 ml, 3 times at D-28, D0, D+28 (28 d apart)	20

Abbreviations: [a]Number of cows, [b]Subcutaneous, [c]28 days before drying off, [d]at drying off, [e]28 days after drying off, [f]recombinant *Streptococcus uberis* adhesion molecule.

Bovine mammary epithelial cells and bacterial growth conditions

Primary bovine mammary epithelial cells (PBMEC) were grown in 24 well (Corning Inc., Corning, NY, USA) tissue culture plates at 37°C in 5% CO_2/95% air until 90-100% confluent [12] mono layers was reached. *Streptococcus uberis* strain UT888, originally isolated from a cow with chronic mastitis, was grown in Todd Hewitt broth (THB, Becton Dickinson Company, Sparks, MD, USA) overnight and sub-cultured to mid-log phase at 37°C in THB. After incubation, the bacterial suspension was washed 3X and re-suspended in PBS (pH 7.4) at a density of ~10^7 colony forming units (CFU)/ml.

Bacterial adherence and Internalization Inhibition Assay

The adherence assay protocol as well as bacterial growth and pre-incubation with hyper immune sera were conducted as described with modifications [12,13]. Briefly, after incubation with anti-rSUAM antibodies, or fetal calf serum (control), bacterial suspensions were washed with PBS (pH7.4) to remove unbound antibodies, and incubated with PBMEC monolayers for 1 h at 37°C in a 5% CO2: 95% air balance incubator. For internalization assay, monolayers were washed 3X with PBS (pH7.4) and incubated with cell growth media containing gentamicin (100 µg/ml; Sigma) and penicillin G (100 IU/ml) for 2 h at 37°C in 5% CO2:95% air (vol/vol). The number of CFU/ml in supernatants was determined by standard plate dilution techniques to monitor effectiveness of gentamicin/penicillin G in killing extracellular *S. uberis.* After removal of cell growth media containing antibiotics, PBMEC monolayers were washed three times with PBS (pH 7.4), trypsinized and lysed. Number of internalized bacteria was determined by standard plate dilution technique. For adherence, monolayers were washed 3X with PBS (pH7.4) to remove non-attached bacteria, and monolayers were detached and lysed by treating with 0.25% trypsin and Triton X-100 (Amersham Arlington Heights, IL) at a final concentration of 0.025% (vol/vol) in sterile distilled water. Cell associated bacteria (bacteria attached to or internalized into the cells) from lysates were determined by standard plate dilution techniques. The numbers of adherent bacteria were determined by subtracting the number of internalized bacteria from the corresponding number of cell-associated bacteria. Both, internalization and adherence inhibition assays were run in parallel, in triplicate, and the assays were repeated three independent times.

Phagocytic killing by bovine macrophages

Milk macrophages isolation, phagocytosis and phagocytic killing assay were conducted as described by Denis et al. [14,15] with modification. Briefly, mammary gland secretions were collected fromcows with highest antibody titers at calving (C0) or medium titers at drying off (D0) or lowest titers prior to vaccination (D-28) and centrifuged at 400 xg for 10 min. The cell pellets were washed 3x times in RPMI-1640 (Gibco-Thermofisher Scientific, Waltham, MA). Cell pellets were then re-suspended in RPMI-1640 with 10% fetal calf serum (FCS) and 200mM L-glutamine (Sigma Aldrich, St. Louis, MO). To isolate macrophages, cells were incubatedat 37°C in 5% C0$_2$: 95% air incubator for 2 h in 5 ml of medium in Corning® CellBIND®

surface culture dishes (Cat. No. **CLS3294, Sigma**) pre-coated with FCS to allow adherence of macrophages. Non-adherent cells were removed by washing 3X with RPMI-1640 medium. The adherent cells were then detached by incubation with cell dissociation solution (Cat. No. C5789, Sigma).Cells were washed 3X with RPMI-1640 medium and evaluated by esterase staining and zymosan uptake as described elsewhere [14]. *Streptococcus uberis* treated with anti-rSUAM serafrom vaccinated cows (C0 & D0) or from cows prior to vaccination (D-28) for 1has described elsewhere [9,13] were co-cultured with bovine macrophages at the multiplicity of infection (M.O.I.) of 50 *S. uberis* to 1 macrophage cell (50:1) in a total volume of 200 µl of RPMI-1640 with 10% fetal bovine serum (Gibco-Thermofisher Scientific) in round-bottomed 96-well plates (Corning-sigma)at 37°C in 5% C0$_2$: 95% air incubator for 2 h. The bacteria were also inoculated into cell culture media (RPMI-1640) in a separate plate that serve as control in parallel to phagocytosis and killing assay. The percentage of phagocytosis was determined by comparing numbers of *S. uberis* in the supernatant of macrophage *S. uberis* co-cultures and control cultures of *S. uberis* in cell-culture media run in parallel. Percentage of killing was calculated by lysing macrophages with 0.1% Triton-X (Sigma) and determining the number of viable *S. uberis*. In both assays, numbers of *S. uberis* were calculated using standard plate dilution techniques. The results were expressed as percentage (%) phagocytosis by macrophages after 2 h of co-culture with macrophages in the presence of each serum (D-28, D0 & C0). Each serum (D-28 or D0 or C0) was evaluated with multiplicity of infection (M.O.I.) of 50:1 (bacteria to macrophage).

Data analysis

Data were evaluated for statistical significance by analysis of variance using graph pad Prism version 7.03.

RESULTS AND DISCUSSION

Results

Vaccination with rSUAM induced significant increase in specific antibody titers in milk and serum of vaccinated cows compared to control cows (Figures 1-5). Anti-rSUAM antibody titers were significantly increased after each vaccination in rSUAM cows compared to the control cows (Figures 1-5). The serum anti-rSUAM IgG titer at D +28 was significantly (*P*<0.05) higher than titer at D0 indicating an increased response to the booster vaccination. However, serum anti-rSUAM IgG titer after 3rd vaccination was relatively higher but not significantly different from the response observed after the 2nd vaccination (Figure 1). Milk anti-rSUAM IgG titers at D0 and the correspondingtiterafter the 3rd vaccination at calving (C0), were significantly higher in rSUAM vaccinated cows, as compared to the control cows. Milk anti-rSUAM IgG titer at calving (C0) was significantly (P< 0.001) higher than the response observed at D0, indicating an increment in milk antibody titer (Figure 1). Serum anti-rSUAM IgG1 and IgG2 titers at calving (C0) were significantly (P<0.001) higher in rSUAM vaccinated cows as compared to control cows. Similarly, milk anti-rSUAM IgG1 and IgG2 titers at calving (C0) were significantly (P<0.001) higher in rSUAM cows compared to control cows (Figures 2,3). Serum IgG1/IgG2 ratios were not changed, thus indicating a similar increase in both isotypes. In

contrast, milk IgG1/IgG2 ratios were decreased; indicating a bias toward cell mediated immune responses (Figure 6). At calving (C0) both anti-rSUAM IgA and -IgM titers in milk and serum were increased significantly in rSUAMvaccinated cows compared to the control group (Figure 4,5). Anti-rSUAM antibodies reduced adherence and internalization of *S. uberis* to bovine mammary epithelial cells, and increased phagocytosis of *S. uberis* by bovine macrophages (Figure 7).

Discussion

Because of previous exposure of cows to streptococcal mastitis pathogens or similar environmental bacteria there were background anti-rSUAM titer in control cows. However, significant increases in all antibody isotopes were observed after 1st, 2nd and 3rd vaccinations, as shown by comparing titers of DO, D+28 and C0, to pre-vaccination titers (D-28) (Figure 1-5). In general, these results are in line with our previous study, in which we evaluated humoral IgG, IgG1 and IgG2 response of dairy cows vaccinated with two different doses of rSUAM (Prado et al. 2011). The major differences between this and the previous study was that in the present study, cows were vaccinated 28 days before drying off, at drying off, and 28 days after drying off and also IgA and IgM were evaluated in addition to IgG, IgG1 & IgG2 antibodies during transition period. In our previous study, after the first vaccination a significant increase in anti-rSUAM antibodies was detected at D+28 in cows vaccinated with 200 µg and 400 µg rSUAM, as compared to the control group. Such increment in anti-rSUAM antibodies continued following the second vaccination at D+28 reaching the highest levels at calving (C0) after which, antibodies titers remain constant. Similarly, in the current study significant rise in antibody titers were detected at D0 (28 days after first vaccination) in rSUAM cows compared to control cows and the increment continues after 2nd (D0) vaccination reaching the highest level after 3rd vaccination at 28 days after drying off (D+28). These observations clearly showed that there were increments in antibody production during transition period reaching highest titer after 3rd vaccination around calving. At calving milk titer remain constant and start decreasing as large

volume of milk is being removed daily through milking. It seems that increase in volume of milk produced and continuous removal of milk through milking diluted out the amount of antibodies in milk during early lactation. Therefore, it is very important to maintain high antibody titer in milk during early lactation through optimizing adjuvant-antigen formulation, routes of vaccination, and time of vaccination to prevent IMI during the periparturient period. The adjuvant Montanide® ISA70VG renders water-in-oil emulsions which allow the enclosed antigen to remain stable for a long period when in contact with components of the emulsion at 37°C, thus creating a stable depot capable of inducing long-term immunity. The Montanide® ISA70VG was already marketed worldwide in vaccines against bovine viral diarrhea and bacterial diseases.Use of this adjuvant with rSUAM antigen optimizes antibody titers in milk during early lactation.

Measuring antibody isotype increase or decrease by evaluating the IgG1/IgG2 ratio over time provides an indication of the type of immune response elicited and it can relate to resistance or susceptibility to the infection [16]. In cattle, IgG1 has been shown to be a type 2 isotype directed by production of cytokines such as IL-4, whereas IgG2 is a type 1 isotype influenced largely by IFN-g [17]. An increase in the IgG1/IgG2 antibody ratio indicates humoral immune responses, whereas the opposite indicates cell mediated immune responses. Results from this study showed that serum IgG1/IgG2 ratios were not changed, thus suggesting a similar increase in both isotypes and similar dominance of the both arms of the immune response. However, the milk IgG1/IgG2 ratios were decreased; indicating an increase toward cell mediated immune responses (Figure 6).

In vitro assays showed that anti-rSUAM antibodies blocked adherence to and internalization of *S. uberis* into bovine mammary epithelial cells and enhanced phagocytosis by bovine macrophages (Figure 7). Moreover, further work from our lab using a passive protection model showed that anti-rSUAM antibodies were partially protective against experimentally induced *S. uberis* IMI [13]. So, it seems that the major problem is not specificity or protective function but rather concentration
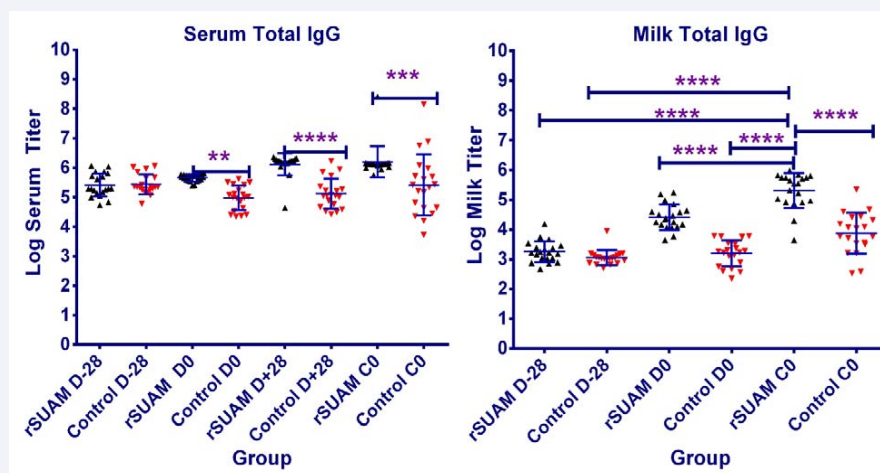

Figure 1 Anti-rSUAM total IgG titers in serum and milk of vaccinated (▲) and control (▼) cows at D-28, DO, D+28 and C0. D-28= 28 days before drying off, D0= at drying off, D+28= 28 days after drying off, C0= at calving. The bars show the mean ± S.D. of the mean. The asterisks represent statistically different (**: P<0.01; ***: P<0.001) groups.
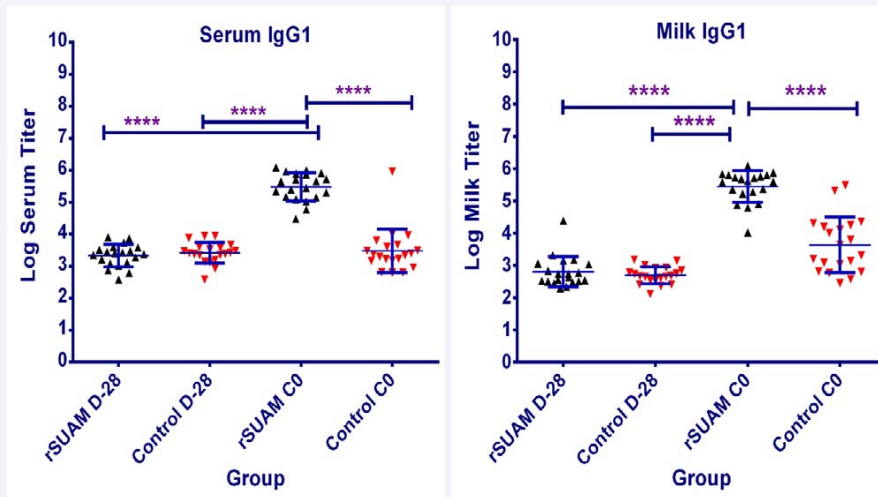
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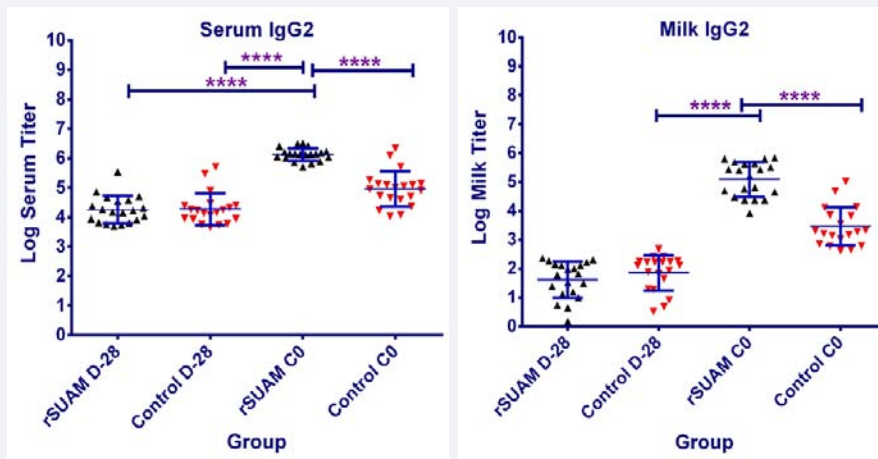*Email: okerrode@utk.edu*

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Figure 2 Anti-rSUAM IgG1 titers in serum and milk of vaccinated (▲) and control (▼) cows at D-28 and C0. D-28= 28 days before drying off, C0= at calving. The bars show the mean ± S.D. of the mean. The asterisks represent statistically different (***: P<0.001) groups.

Figure 3 Anti-r SUAM IgG2 titers in serum and milk of vaccinated (▲) and control (▼) cows at D-28 and C0. D-28= 28 days before drying off, C0= at calving. The bars show the mean ± S.D. of the mean. The asterisks represent statistically different (***: P<0.001) groups.
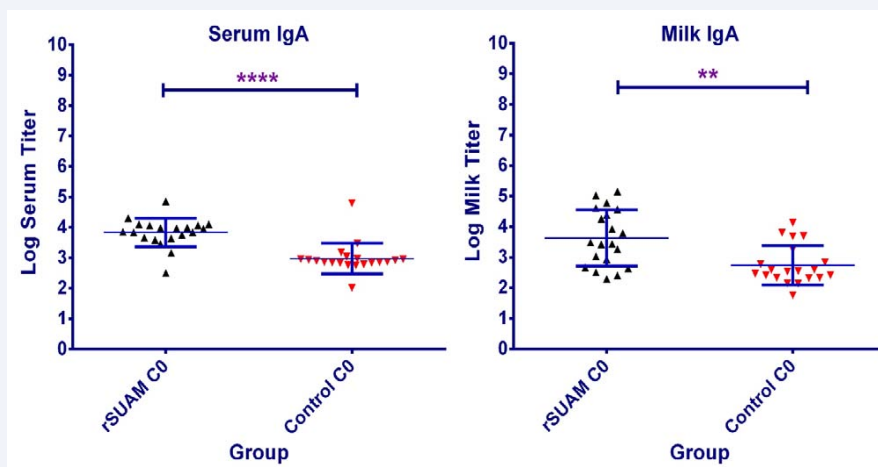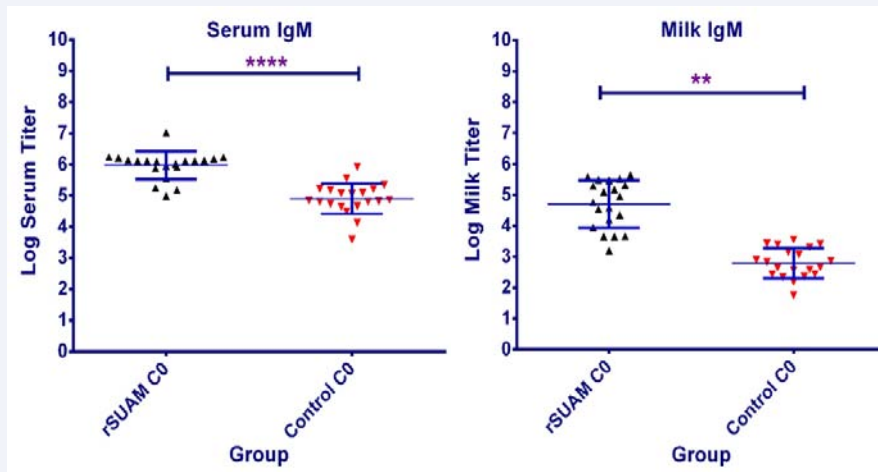

Figure 4 Anti-rSUAM IgA titers in serum and milk of vaccinated (▲) and control (▼) cows at C0. C0= at calving. The bars show the mean ± S.D. of the mean. The asterisks represent statistically different (**: P<0.01; ***: P<0.001) groups.

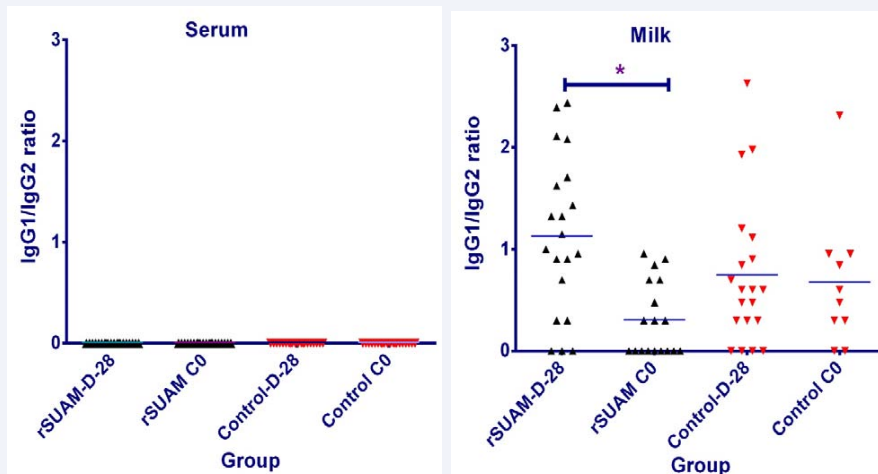*Oudessa Kerro Dego et al. (2017)*
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Figure 5 Anti-rSUAM IgM titers in serum and milk of vaccinated (▲) and control (▼) cows at C0. C0= at calving. The bars show the mean ± S.D. of the mean. The asterisks represent statistically different (**: P<0.01; ***: P<0.001) groups.

Figure 6 Anti-rSUAM IgG1/IgG2 ratios in serum and milk of vaccinated (▲) and control (▼) cows at D-28 and C0. D-28= 28 days before drying off, C0= at calving. The bars show the mean. The asterisks represent statistically different (*: P<0.05) groups.
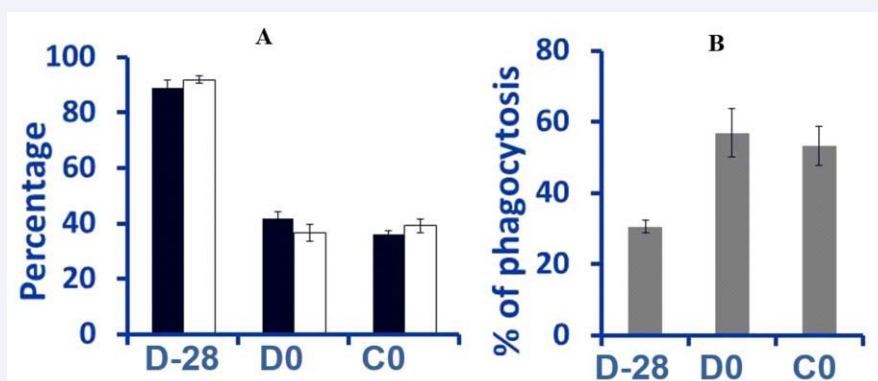

Figure 7 Protective effect of anti-rSUAM antibodies in serum at D-28, D0 and C0 through inhibition of adherence and internalization of S. uberis strain UT888 to mammary epithelial cells (BMEC) or through improving phagocytic killing by bovine macrophages. Inhibition of adherence (■) to and internalization (□) of the S. uberis strain UT888 into bovine mammary epithelial cells (BMEC) (A) or phagocytic killing (■) of S. uberis strain UT888 by bovine macrophages (B). D-28 = 28 days before drying off, D0= at drying off, C0= at calving. Data are presented as percentage of untreated controls and error bars represent the standard error of the mean (SEM) of 3 independent observations.

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of antibodies in milk during lactation decreased because of large volume of milk that dilute the antibody and also continuous removal through milking.

CONCLUSION

Immunizations with rSUAM at 28 d before drying off, at drying off, and 28 d after drying off induced high serum and milk anti-rSUAM antibody titers, which were increased after each vaccination reaching the highest titers after the 3rd vaccination. Milk IgG1/IgG2 ratios decreased, which suggest a rise in the cell mediated immune response. In contrast, serum IgG1/IgG2 ratios were not changed. These differences in serum and milk IgG1/IgG2 ratios, suggest a local cell-mediated response probably induced by the adjuvant used.

Anti-rSUAM antibodies reduced adherence to and internalization of *S. uberis* into PBMEC and increased phagocytosis of *S. uberis* by bovine macrophage as well. Taken together, findings of this study, strategic vaccination of dairy cows with rSUAM during dry period induced significant increase in immune responses that can be protective at periparturient period, when dairy cows are high susceptible to mastitis.

The periparturient period of dairy cows is a very critical period in the mammary health of dairy cows, and to achieve full protection against *S. uberis* mastitis further detailed research on optimal antigen-adjuvant formulation, route of vaccination, and realistic controlled experimental challenge models are needed.

ACKNOWLEDGEMENTS

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