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Experimental study for evaluation of the efficacy of a biofilm-embedded bacteria-based vaccine against *Staphylococcus chromogenes*-associated mastitis in sheep

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ABSTRACT

Although coagulase-negative staphylococci are the primary aetiological agents of subclinical mastitis in ewes, there is little information regarding vaccination against that infection. The objective of this study was to evaluate the efficacy of a vaccine against staphylococcal mastitis in ewes under experimental conditions. The antigen in the vaccine is based on a bacterin of *Staphylococcus aureus* strain, expressing the exopolysaccharide poly-N-acetylglucosamine (PNAG), which is involved in biofilm formation by these bacteria. Ewes in groups A (n = 17) or B (n = 6) were given an initial vaccination 5 weeks before expected lambing, followed by a repeat administration 21 days later. Ewes in groups C (n = 8) or D (n = 6) were unvaccinated controls. Ewes in group A (n = 17) or C (n = 8) were challenged with a biofilm-forming *S. chromogenes*; animals in subgroups A1 or C1 were challenged on the 10th and those in A2 or C2 on the 50th day after lambing. Ewes in groups B or D were uninoculated controls. Clinical examinations of ewes, ultrasonographic examinations of udder, milk yield measurements, blood sampling for detection of anti-PNAG specific antibodies and milk sample collection for bacteriological and cytological examinations were performed up to 52nd day post-challenge. Finally, biopsies were performed for mammary tissue collection for histopathological examination. Among group A ewes, 29% developed systemic signs and 59% signs in the inoculated gland; the respective figures for group C were 50% and 100% ($P = 0.040$ for mammary signs). The median total clinical score was 2.0 for A and 5.5 for C ewes ($P = 0.025$). For A, but not for C, clinical scores decreased progressively during the study ($P = 0.018$ and $P = 0.47$, respectively). The duration of mastitis was shorter in A (4 days) than in C (17.5 days) ewes ($P = 0.022$). Bacterial counts were lower in milk samples from A than from C ewes, for samples collected from the inoculated and the uninoculated ($P < 0.01$) mammary glands of these ewes. Somatic cell counts in samples from inoculated and uninoculated mammary glands of A ewes were higher than in samples of C ewes ($P < 0.02$). There were differences for gray-scale evaluations during ultrasonographic examination and for milk yield measurements between groups ($P < 0.01$). Median bacterial counts in tissue samples from A ewes (0 cfu g^{-1}) were lower than in ones from C (6.5 cfu g^{-1}) ewes ($P = 0.041$). The median score for histopathological findings in tissue samples from inoculated glands of A was lower than that for C ewes: 1 *versus* 2 ($P = 0.014$). It is concluded that mastitis was less severe in vaccinated animals, as indicated by a wide array of measures.

1. Introduction

In ewes, bacterial mastitis is a financially significant problem, especially in dairy-type production systems (Gelasakis et al., 2015). It is also a predominant cause of decreased welfare in sheep farms (European Food Safety Authority, 2014). For mastitis control, a variety

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of approaches, including correct management in the milking parlour (Gelasakis et al., 2015; Vasileiou et al., 2018b) or administration of antibiotics at the end of a lactation period (Petridis and Fthenakis, 2014), can be applied; vaccination may also be implemented (Lacasta et al., 2015).

Staphylococci are the principal causes of mastitis in dairy sheep, accounting for over 65% of cases of mastitis (Gelasakis et al., 2015). Various factors have been implicated to contribute in the virulence of these organisms and to participate in the pathogenesis of mastitis. Slime production is an important virulence factor, contributing to biofilm formation by causal staphylococci, which thus survive and disseminate or adhere on epithelial cells in the mammary glands (Clarke and Foster, 2006; Melchior et al., 2006; Otto, 2008).

Coagulase-negative staphylococci (mainly *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*) are the primary aetiological agents of subclinical mastitis (Gelasakis et al., 2015; Vasileiou et al., 2018b). There is little information regarding protection of ewes against coagulase-negative staphylococci. Relevant vaccine trials have investigated efficacy against *Staphylococcus aureus*; indeed, anti-mastitis vaccines are licenced against this organism, but do not report possible effect against coagulase-negative isolates. Therefore, there is a scope for investigating the potential effect of vaccination to limit mastitis by coagulase-negative staphylococci.

The objective of this study was to evaluate the efficacy of a vaccine for protection of ewes against staphylococcal mastitis in an experimental setting. The staphylococcal antigen in the vaccine is based on a bacterin of *S. aureus* strain, expressing the exopolysaccharide poly-N-acetylglucosamine (PNAG), which is involved in biofilm formation by these bacteria (Perez et al., 2009; Prenafeta et al., 2010).

2. Materials and methods

2.1. Experimental design

2.1.1. Animals

Thirty-seven (37) Chios-cross ewe-lambs were used in the study. Animals were allocated into four groups (with two of the groups further subdivided into two subgroups each) as shown in Table 1. Allocation was made by using a random number generator to achieve complete randomisation.

Animals in each group (B, n = 6; D, n = 6) or subgroup (A1, n = 8; A2, n = 9; C1, n = 4; C2, n = 4) were penned together and separately from animals in other groups / subgroups. Reproductive control was applied. Animals were mated by rams of known fertility and repeatedly examined ultrasonographically to confirm pregnancy and its normal progress.

2.1.2. Vaccination schedule and administration

A vaccine licensed in European Union countries for protection of ewes against clinical staphylococcal mastitis (Vimco®; Hipra, Girona, Spain) was used (batches: 69VW-1, 77XW-1). Vaccination was carried out during the last stage of gestation of ewes, following the licensed schedule of the product. The initial administration was performed on the 112th day after ram introduction (*i.e.*, 5 weeks before expected lambing) and was followed by a repeat administration 21 days later.

Vaccine (groups A, n = 17, and B, n = 6) or placebo (groups C, n = 8, and D, n = 6) administration was performed after clipping the wool in the neck region of the animal. A dose of 2 mL of the product (A and B) or normal saline (C and D) was injected intramuscularly. The first injection to each animal was performed on the left side of the neck, with the repeat injection performed on the right side. All vaccine and placebo administrations were carried out by the same person (NGCV).

The other vaccines that had been administered to the animals during the study period were a vaccine against bacterial respiratory infections (on the 6th week of pregnancy) and an anti-clostridial vaccine (on the 98th day of pregnancy, *i.e.* 14 days prior to the initial administration of the vaccine under evaluation).

Table 1
Design of an experimental study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.

Group or subgroup	n	Vaccination	Intramammary inoculation with *S. chromogenes*
A1	8	+	10th day post-partum
A2	9	+	50th day post-partum
B	6	+	–
C1	4	–	10th day post-partum
C2	4	–	50th day post-partum
D	6	–	–

+: performed, -: not performed.

2.1.3. Inoculations

After lambing, animals in group A (n = 17) or C (n = 8) were challenged (day D0) with the *S. chromogenes* biofilm-forming (Vasileiou et al., 2018a) strain 6684, which was isolated from a case of mastitis in a ewe. Animals in subgroups A1 or C1 were challenged on the 10th day after lambing (= D0), whilst animals in subgroups A2 or C2 were challenged on the 50th day after lambing (D0).

For inoculation, the challenge isolate was grown on Columbia blood agar and checked for purity; then it was inoculated into Soy broth (BioMerieux) and incubated aerobically at 37 °C for 5 h. The broth culture was serially diluted in phosphate-buffer-saline (PBS), pH 7.3; finally, 0.2 mL of the desired dilution was drawn into a syringe. The inoculum contained 4.88×10^5 to 5.22×10^5 c.f.u., as estimated by the method of Miles and Misra (1938).

The ewes were inoculated directly into the *sinus lactiferous*. The mammary gland to be challenged was chosen randomly, at the toss of a coin. Inoculation was performed under strict aseptic conditions. A sterile fine plastic catheter 20 G (Abbocath, Abbot) was inserted into the teat; a syringe containing the inoculum was attached to the catheter and the bacterial suspension was injected. The same technique was used to inject 0.2 mL of PBS into the other mammary cistern of each ewe, as a control.

2.1.4. Licence for experimental procedures

The study was performed under a licence for experimental procedures obtained from the local offices of the Hellenic Ministry of Agricultural Development and Food, at the Department of Obstetrics and Reproduction of the Veterinary Faculty of University of Thessaly, according to EU regulations (project reference no.: 2566).

2.2. Examination of animals and collection of samples

2.2.1. Clinical examinations

After each vaccine administration, animals were observed for any systemic or local reactions.

A general clinical examination of animals was routinely performed 2 days after lambing and then at 5-day intervals. Before inoculation, on D-4 and D-1, a standardised detailed clinical examination of the udder (observation, palpation, comparison between glands) was performed (NGCV).

After challenge, detailed clinical examinations were performed daily on D1 to D7, D10, D14, D17 and thereafter weekly until D52 for groups A and C. For groups B and D (10th day after lambing = D0), examinations were performed on the same days as above, as well on D36, daily on D39 to D47, D50, D54, D57 and thereafter weekly until D92. Lambs were with their dams throughout the study.

2.2.2. Ultrasonographic examination of udder

An ultrasound scanner (MyLab® 30; ESAOTE SpA, Genova, Italy),

fitted with a linear transducer with 7.5–12.0 MHz imaging frequency, was used. Coupling gel was applied. The probe was placed on the caudal surface of the udder and moved around it. The method of examination was as described in detail by Barbagianni et al. (2017). For this examination, a 40–60 mm scanning depth and 10.0 or 12.0 MHz frequency were used. B-mode images were frozen and saved on the equipment's hard-disk for performing subsequently appropriate measurements and data analysis.

2.2.3. Collection of milk samples

On each of the above occasions, milk samples were collected from all animals, under strict aseptic conditions. Then, 10 to 15 mL of secretion were collected into a sterile container; separate samples were collected from each mammary gland.

2.2.4. Milk yield measurements

On eight occasions for groups A (n = 17) and C (n = 8) (D-1, D10, D17 and thereafter weekly until D52) and on 16 occasions for groups B (n = 6) and D (n = 6) (D-1, D10, D17 and thereafter weekly until D38, and then on D39, D45, D50, D52, D57 and thereafter weekly until D92), the milk yield of all ewes was measured.

Measurements were carried out in the morning of each evaluation day. Ewes were injected intramuscularly with 5 i.u. oxytocin and were milked out by hand. The lambs were separated from their dams. Four hours later, they were injected again with 5 i.u. oxytocin and once more were milked out. The milk was collected into a plastic container. To avoid misreading because of foam and for accuracy of results, the milk was slowly poured into a volumetric glass container with 2 mL graduations and allowed to sit for 5 min before reading.

2.2.5. Collection of blood samples

Blood samples (for serum extraction) were collected from all animals on seven occasions during the study. Samples were collected as follows: (i) before initial vaccination, (ii) before booster vaccination, (iii) three weeks after booster vaccination (5th – 7th day post-partum), (iv) on D-1, (v) on D14, (vi) on D31 and (vii) on D52.

2.2.6. Mammary tissue biopsy

In ewes in groups A (4 of each of A1 and A2) and C (2 of each of C1 and C2), mammary tissue biopsy samples were collected after the end of the study monitoring period, on D53. The operation was carried out under sedation with local anaesthesia using lidocaine. All surgical procedures were performed under strict aseptic conditions. A biopsy of the parenchyma of both mammary glands was performed; two cubes of tissue, approximately 1 × 1 × 1 cm, were removed from the caudal part of each mammary gland. After obtaining the tissue samples, the wounds were repaired.

2.3. Laboratory examinations

2.3.1. Bacteriological examination of milk and tissue samples

Milk samples (10 μL) were cultured using Columbia blood agar plates incubated aerobically at 37 °C for 48 h. If nothing had grown, the plates were incubated for another 24 h. Bacterial colonies morphologically similar to those of *S. chromogenes* (smooth, entire, glistening, with yellow or yellow-orange pigment) in pure culture were considered to be those of the challenge organism. Moreover, counts of the organism in milk samples were performed following the method of Miles and Misra (1938); starting milk volume for the counting was 20 μL

Tissue samples were washed with PBS, to remove mammary secretion, and were then homogenised (10 g of tissue sample with 50 mL of sterile PBS blended for 3 min) in a tissue blender (Mixwel; Alliance Bio Expertise, Guipry, France). The mixture was cultured. Also, bacterial counts were performed in the mixture, following the method of Miles and Misra (1938).

A proportion of the bacterial isolates recovered from challenged animals, which were considered as the challenge organism, were subjected to detailed identification using the Vitek® 2 automated system (BioMerieux, Marcy-l'-Étoile, France). All 11 (100%) isolates recovered from tissue samples were thus identified, whilst 25% (68 / 272) of the isolates from milk samples, selected at random using an electronic random number generator (www.randomresult.com), were also subjected to detailed identification

2.3.2. Cytological examination of milk samples

All milk samples were assessed using the Microscopic cell counting method (Mccm) (IDF reference method) (International Dairy Federation, 1984). Smears were also produced from milk samples and dried; these were stained using the Giemsa method for estimation of leucocyte subpopulations. The proportion of leucocyte types therein was calculated by examining at least 10 fields of each milk film using the 40 × objective lens of a Zeiss-Axiostar Microscope (Carl Zeiss, Göttingen, Germany) with a 10× eyepiece lens and counting at ≥ 100 leucocytes.

2.3.3. Detection of anti PNAG-specific antibodies in blood serum samples

Blood samples were centrifuged for serum collection in preparation for measurement of PNAG-specific IgG. Serum samples were diluted 1:100. Diluted samples were assayed in an indirect ELISA in plates coated with purified PNAG (Perez et al., 2010). In brief, 96-well plates were coated with the purified PNAG and blocked with StabilCoat Immunoassay Stabilizer (SurModics, Eden Prairie, USA). The diluted samples were added to the wells and bound IgG was then detected withprotein G conjugated to peroxidase (Pierce; Thermo Fisher Scientific, Waltham, USA) (Prenafeta et al., 2010). The persons who processed the samples were not aware of their origin (group).

Known positive and negative control serum samples were included on each assay plate. Finally, wells were incubated with a chromogenic substrate for the peroxidase; absorbance was measured in a microplate reader at 405 nm (Sunrise equipped with Magellan 3.11 software; Tecan, Männedorf, Switzerland). Each sample was assayed twice.

2.3.4. Histopathological examination of tissue samples

Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin for histopathological evaluation.

2.4. Data management and analysis

2.4.1. Clinical scores

During the clinical examination, clinical scores were determined using a scheme described previously that took into account systemic (attitude, appetite, temperature) and local mammary (secretion, temperature, pain, oedema, size, colour) signs. For each sign, a score from 0 to 4 was employed; in the udder, separate scores were given to the inoculated and the uninoculated mammary gland. Hence, possible scores ranged from 0 to 12 for systemic signs and from 0 to 24 for mammary signs. At the end, a total score with possible values ranging from 0 to 60 was produced by adding together scores given for systemic signs and for mammary signs in each gland. Details are provided in Supplementary material 1.

2.4.2. Definitions of mastitis

Mastitis was classified as clinical or subclinical mastitis. Mastitis was defined as clinical in ewes with abnormal gross findings in a mammary gland (including changes in secretion) (Fragkou et al., 2014); mastitis was defined as subclinical in ewes in which a bacteriologically positive (with the challenge organism) milk sample with concurrently increased somatic cell counts (≥ 0.5×10^6 cells mL^{-1}) plus a high proportion of neutrophils and lymphocytes (≥ 65% of all leucocytes) was detected (Fragkou et al., 2014; Vasileiou et al., 2018b). Mastitis in a ewe was defined as recurrent when a ewe with mastitis was later

found not to have mastitis and then again was found to have it. Mastitis definitions referred to ewes. Mastitis definitions referred to ewes; therefore if one or both mammary glands were affected one cases of mastitis was recorded.

An incident of mammary infection (IMI) was defined, when the above findings referred to individual mammary glands. Therefore, on a sampling point, if both mammary glands of a ewe were affected, one case of mastitis and two IMIs were recorded.

For analysis, the post-challenge experimental period was divided in three stages: S1 (acute post-inoculation period, sampling points D1, D2, D3), S2 (subacute post-inoculation period, sampling points D4 to D10) and S3 (chronic post-inoculation period, sampling points D14 to D52).

2.4.3. Evaluation of ultrasonographic images

During the ultrasonographic examination, images of mammary parenchyma were recorded. Stored images of mammary parenchyma were processed using of ImageJ (National Institutes of Health, Rockville Pike, MD, USA), which can edit, process and analyse grey-scale images by calculating area and pixel value statistics to yield intensity values (National Institutes of Health, 2013). In an image processing context, grey-scale analysis referred to the image's overall pixel grey intensity values (Ojala et al., 2002). For analysis of grey-scale intensity, intensity values of each of the three images stored from each mammary gland on each occasion were assessed conjointly. Areas with vessels or ducts were not taken into account for the grey-scale analysis. Results were expressed on a 0 (black) to 255 (white) scale.

For analysis of the results of the grey-scale measurements, data were normalised by calculating the ratio $[GS_i / GS_c]_n / [GS_i / GS_c]_{D-1}$, where GS_i was the grey-scale intensity of the inoculated mammary gland, GS_C was the grey-scale intensity of the uninoculated mammary gland, n was day of measurement after challenge (*i.e.*, D1, D2, D3 *etc.*) and D-1 was the day before challenge.

2.4.4. Milk yield ratios

For analysis of results of milk yield measurements, data were normalised by calculating the ratio $[MY_i / MY_c]_n / [MY_i / MY_c]_{D-1}$, where MY_i was the milk yield of the inoculated mammary gland, MY_C was the milk yield of the contralateral mammary gland, n was the day of the measurement after challenge (*i.e.*, D10, D17, D24 *etc.*) and D-1 was the day before challenge.

2.4.5. Measurement of anti-PNAG specific antibodies in serum samples

The results of antibody measurements on serum samples were expressed as optical density (OD) values. The mean OD value of the two duplicate assays was converted into a RIPC (relative index percent) using the formula:

RIPC = [OD value sample - OD value negative control] / [OD value positive control - OD value negative control] × 100.

A cut-off point of 6.0 RIPC for the anti-PNAG-specific total IgG in serum had been established during validation of the assay (Prenafeta et al., 2010), as follows:

cut-off = (mean RIPC from negative serum samples + 2sd) + [(mean RIPC from negative serum samples + 2sd) × (variation coefficient of the technique)].

Values above the cut-off point were regarded as 'positive', while values below that were regarded as 'negative'.

2.4.6. Histopathological scores

A scoring system previously developed and described was used and numerical values were assigned for the histopahtological findings in the tissue samples. A 0 to 4 scale was used, as detailed in Supplementary material 2. Separate scores were assigned for the tissue samples collected from each mammary gland.

2.4.7. Statistical analysis

Standard basic descriptive statistics were performed and for further analyses the following methods were employed as appropriate: Analysis of Correlation (CORR), Analysis of Variance (ANOVA), Fisher's exact test (FET), Kruskal-Wallis test (KWT), Linear Mixed models (LMM), Mann-Whitney test (MWT), Pearson's chi-square test (PCST), Wilcoxon sign-rank test (WSRT). More details of these analyses are provided with the results and in Supplementary material 3.

Initially, comparisons were performed between subgroups A1 and A2 and between C1 and C2; evaluations did not reveal any significant differences between subgroups. Hence, subgroups were taken together and their results combined for assessment of outcomes. In all analyses, statistical significance was defined at $P < 0.05$.

3. Results

3.1. Post-vaccination adverse reactions

After vaccination or placebo administration, no adverse reactions were recorded in any ewe (total vaccinated: n = 23). Hence, the incidence rate of adverse reactions was 0.0% (95% confidence interval [CI]: 0.0%–14.3%).

3.2. Development of mastitis and clinical findings

All animals in groups A (n = 17) and C (n = 8) developed mastitis after the intramammary challenge. Among group A ewes, 5 (29.4%) developed systemic clinical signs and 10 (58.8%) developed clinical signs in the inoculated gland. The respective figures for group C were 4 (50%) and 8 (100%) ewes (for comparisons between groups A and C: $P = 0.28$ for presence of systemic signs, $P = 0.040$ for presence of mammary signs, FET). No mastitis was developed by in any ewe in group B (n = 6) or D (n = 6) ($P < 0.01$ compared with groups A or C, FET). Details are shown in Table 2.

Table 2
Development of mastitis during a study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.

Group or subgroup	Ewes with mammary infection in		Ewes with clinical signs		Duration of mastitis (d)	Ewes with recurrent mastitis
	inoculated side	uninoculated side	systemic	mammary		
A1 (n = 8)	8	0	4	4	4.5 (1 – 52)	3
A2 (n = 9)	9	0	1	6	4.0 (1 – 52)	8
A (n = 17)	$17^{a,b}$	0^{a}	5	$10^{a,b}$	4.0^{a} (1 – 52)	11
B (n = 6)	$0^{a,c}$	0^{b}	0	$0^{a,c}$	0 (0 – 0)	0
C1 (n = 4)	4	3	3	4	17.5 (11.5 – 52)	3
C2 (n = 4)	4	2	1	4	17.5 (2 – 52)	2
C (n = 8)	$8^{c,d}$	$5^{a,b,c}$	4	$8^{c,d}$	17.5^{a} (2 – 52)	5
D (n = 6)	$0^{b,d}$	0^{c}	0	$0^{b,c}$	0 (0 – 0)	0

$^{a-d}$: $P < 0.05$ for differences between values with similar letters within the same column (for frequencies: FET, for duration of mastitis: MWT).
Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.

Table 3
Median (min. – max.) clinical scores (systemic plus mammary signs in inoculated and uninoculated mammary glands) of ewes in a study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.

Group or subgroup	All study	S1 of study	S2 of study	S3 of study
A1 (n = 8)	5 (0 – 7)	2.5 (0 – 5)	1 (0 – 2)	0.5 (0 – 3)
A2 (n = 9)	1 (0 – 5)	1 (0 – 5)	0 (0 – 0)	0 (0 – 0)
A (n = 17)	2^{a} (0 – 7)	2 (0 – 5)	0 (0 – 2)	0 (0 – 3)
B (n = 6)	0^{b} (0 – 0)	0^{a} (0 – 0)	0 (0 – 0)	0 (0 – 0)
C1 (n = 4)	5.5 (4 – 10)	3.5 (0 – 10)	0 (0 – 2)	2 (0 – 2)
C2 (n = 4)	4.5 (1 – 14)	2.5 (0 – 14)	2 (0 – 2)	0 (0 – 1)
C (n = 8)	$5.5^{a,b,c}$ (1 – 14)	$3.5^{a,b}$ (0 – 14)	0 (0 – 2)	1 (0 – 2)
D (n = 6)	0^{c} (0 – 0)	0^{b} (0 – 0)	0 (0 – 0)	0 (0 – 0)

$^{a-c}$. $P \leq 0.05$ between respective values within the same column (MWT).
S1: D1 - D3, S2: D4 - D10, S3: D14 - D52.
Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.

Throughout the study, the median total clinical score was 2.0 for group A ewes (n = 17) and 5.5 for group C ewes (n = 8) ($P = 0.025$, MWT). For group A, but not for group C, clinical scores decreased progressively during the study ($P = 0.018$ and $P = 0.47$, respectively, between the three stages, KWT). One group C ewe died on D2; the post-mortem examination revealed that the inoculated mammary gland was enlarged, oedematous and hyperaemic, the ipsilateral lymph node was enlarged and there was also evidence of septicaemia (petechiae on kidneys and heart, presence of serohaemorrhagic exudate in thoracic and peritoneal cavities) with peritonitis (fibrin deposition on the peritoneum coupled with presence of serohaemorrhagic exudate therein). Details are shown in Table 3.

The duration of mastitis was shorter in group A (n = 17): 4 days, than in group C (n = 8): 17.5 days ewes ($P = 0.022$, MWT). There was no difference in recurrence of mastitis between ewes in group A (11 ewes) or C (5 ewes) ($P = 0.63$, FET).

In group A ewes (n = 17), the proportion of occasions with IMIs was smaller than in group C ewes (n = 8) ($P < 0.001$, PCST). For the inoculated glands, no significant difference was evident between the two groups during stages 1 and 3 ($P > 0.07$, PCST), but only during stage 2 ($P < 0.001$, PCST); for the uninoculated glands, lower relative frequencies were seen in group A ewes in the three stages ($P < 0.03$ for all comparisons, PCST). In group C (n = 8), IMIs were also recorded in 5 uninoculated mammary glands ($P = 0.001$ *versus* group A, FET). In group A, inoculated mammary glands were more likely to develop IMI than their own within-ewe uninoculated glands ($P < 0.001$, FET); in group C, no such difference was found ($P = 0.10$, FET). Relative frequencies of IMIs decreased during the study; in stage 3, they were lower than in the other two stages ($P < 0.025$ for all comparisons). Details are shown in Table 4.

3.3. Bacterial counts in milk

Bacterial counts were lower in samples from vaccinated (group A, n = 17) ewes than control (group C, n = 8) ewes for samples collected from the inoculated ($P < 0.001$, ANOVA) and the uninoculated ($P < 0.01$, ANOVA) mammary glands (Fig. 1). There were also differences in bacterial counts in the milk of inoculated mammary glands (groups A, n = 17, and C, n = 8) compared to counts in the milk of uninoculated animals (groups B, n = 6, and D, n = 6), in which counts were always 0 ($P < 0.01$ for all comparisons, ANOVA). Comparisons of differences in bacterial counts between the inoculated and the uninoculated gland revealed that these were greater in group C than in group A during stages S1 and S2 ($P = 0.01$, WSRT), but not during stage S3 ($P > 0.05$, WSRT). Details are shown in Table 5.

Within the inoculated groups (A, n = 17, and C, n = 8), bacterial counts in samples from the inoculated glands were higher than counts in samples from the uninoculated glands ($P < 0.025$ for all comparisons, ANOVA). Within these groups, no differences were seen in bacterial counts between the three stages ($P > 0.22$ for all comparisons, ANOVA).

Table 4
Proportion (%) of occasions of mammary infection in ewes recorded during a study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.

(a) inoculated mammary glands

Group or subgroup	All study	Stage 1	Stage 2	Stage 3
A1 (n = 8)	67.3	87.5	75.7	51.0
A2 (n = 9)	63.0	88.9	57.8	55.6
A (n = 17)	$64.9^{a,b,c}$	$88.2^{a,b,p,q}$	$65.0^{a,b,c,p}$	$53.6^{a,b,q}$
B (n = 6)	$0.0^{a,d}$	$0.0^{a,c}$	$0.0^{a,d}$	$0.0^{a,c}$
C1 (n = 4)	81.7	100.0	100.0	60.7
C2 (n = 4)	87.2	100.0	93.3	76.2
C (n = 8)	$84.1^{b,d,e}$	$100.0^{c,d,p}$	$97.1^{b,d,e,q}$	$67.3^{c,d,p,q}$
D (n = 6)	$0.0^{c,e}$	$0.0^{b,d}$	$0.0^{c,e}$	$0.0^{b,d}$

(b) uninoculated mammary glands

Group or subgroup	All study	Stage 1	Stage 2	Stage 3
A1 (n = 8)	0.0	0.0	0.0	0.0
A2 (n = 9)	0.0	0.0	0.0	0.0
A (n = 17)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
B (n = 6)	0.0^{b}	0.0	0.0^{b}	0.0^{b}
C1 (n = 4)	21.6	8.3	25.0	25.0
C2 (n = 4)	21.3	18.2	6.7	33.3
C (n = 8)	$21.5^{a,b,c}$	13.0^{a}	$17.1^{a,b,c}$	$28.0^{a,b,c}$
D (n = 6)	0.0^{c}	0.0	0.0^{c}	0.0^{c}

$^{a-g}$. $P \leq 0.05$ between respective values with similar letters within the same column in each table (PCST).
p,q. $P \leq 0.05$ between respective values with similar letters within the same row in each table (PCST).
S1: D1 - D3, S2: D4 - D10, S3: D14 - D52.
Groups A and B: vaccinated, groups C and D: unvaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.

All bacteria recovered from the inoculated ewes were identified as coagulase-negative staphylococci, with colonial morphology similar to that of *S. chromogenes*: large (5–7 mm in diametre), yellow to orange, glistening and butyrous, non-haemolytic colonies with entire margins. All isolates submitted for full identification were confirmed as *S. chromogenes*.

3.4. Somatic cell counts in milk

In inoculated mammary glands, milk somatic cell counts increased after challenge. Cell counts in inoculated and uninoculated mammary glands of A ewes (n = 17) were significantly higher than counts in respective mammary glands of C ewes (n = 8) ($P < 0.02$ for both comparisons, ANOVA). Also, milk somatic cell counts in inoculated mammary glands of A and C ewes were significantly higher than counts in samples from mammary glands of B (n = 6) and D (n = 6) ewes ($P < 0.001$ for all comparisons, ANOVA). Cell counts in samples from uninoculated mammary glands of C ewes were higher than respective counts in samples from mammary glands of B and D ewes ($P < 0.001$ for both comparisons, ANOVA), whilst no significant difference was seen in cell counts of samples from uninoculated mammary between A ewes and B and D ewes ($P > 0.08$ for both comparisons, ANOVA). Details are in Supplementary material 4 and 5.

3.5. Ultrasonographic examination of udder

There were differences in the grey-scale ratios of the ultrasonographic images of the mammary glands between the four groups ($P < 0.001$, ANOVA). Between group differences were significant for A1, B

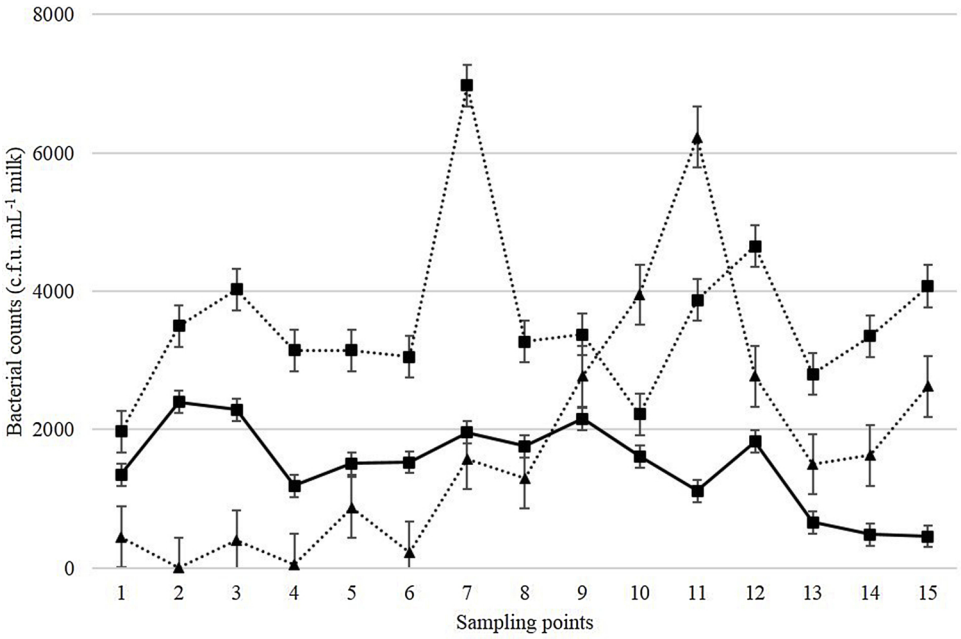

Fig. 1. Mean (± standard error of the mean) bacterial counts in milk from ewes throughout a study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.
Solid line: inoculated glands of group A (vaccinated ewes), dotted line with square points: inoculated glands of group C (unvaccinated ewes), dotted line with triangular points: uninoculated glands of group C (unvaccinated ewes); from uninoculated glands of group A and both glands of groups B and D no bacteria were recovered.

and D *versus* C1 ($P < 0.01$, ANOVA) and for A2, B and D *versus* C2 ($P < 0.01$, ANOVA). Details are shown in Supplementary material 6.

3.6. Milk yield measurements

There were differences in the milk yield ratios of the ewes between the four groups ($P < 0.001$, ANOVA). Between group differences were significant for A1, B and D *versus* C1 ($P < 0.01$, ANOVA) and for A2, B and D *versus* C2 ($P < 0.01$, ANOVA). Details are shown in Fig. 2 and in Supplementary material 7.

3.7. Anti PNAG-specific antibodies in serum samples

After vaccination, there was an increase in anti-PNAG antibody titres in ewes of groups A (n = 17) and B (n = 6) ($P < 0.001$, LMM) compared to pre-vaccination titres. In contrast, no changes in antibody titres were seen throughout the study in ewes in groups C (n = 8) and D (n = 6) ($P > 0.17$, LMM). Antibody titres in ewes in group A (vaccinated) were higher than those in group C (non-vaccinated) ($P < 0.001$). There were no differences between groups A and B and between groups C and D ($P > 0.28$). Details are shown in Supplementary material 8.

Table 5
Mean (± standard error of the mean) bacterial counts (× 10^2 c.f.u.) in milk from ewes during a study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.

(a) inoculated mammary glands

Group or subgroup	All study	Stage 1	Stage 2	Stage 3
A1 (n = 8)	12.0 ± 1.5	11.4 ± 2.5	14.3 ± 3.1	8.9 ± 1.8
A2 (n = 9)	15.8 ± 0.9	18.1 ± 2.6	18.2 ± 0.9	13.0 ± 1.3
A (n = 17)	14.1 ± 0.8^{a,b,c}	14.8 ± 1.9^{a,b,c}	17.4 ± 1.4^{a,b,c}	11.2 ± 1.1^{a,b,c}
B (n = 6)	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{a,d}
C1 (n = 4)	36.0 ± 4.7	33.5 ± 9.0	41.8 ± 10.3	32.9 ± 5.9
C2 (n = 4)	35.3 ± 4.3	29.8 ± 8.3	36.6 ± 9.6	36.7 ± 5.4
C (n = 8)	35.6 ± 3.1^{b,d,e}	31.7 ± 5.9^{b,d,e}	39.2 ± 6.9^{b,d,e}	34.8 ± 4.0^{b,d,e}
D (n = 6)	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{c,e}

(b) contralateral mammary glands

Group or subgroup	All study	Stage 1	Stage 2	Stage 3
A1 (n = 8)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A2 (n = 9)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A (n = 17)	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
B (n = 6)	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
C1 (n = 4)	24.2 ± 4.5	5.7 ± 3.6	14.6 ± 5.6	39.1 ± 6.8
C2 (n = 4)	10.9 ± 3.7	0.0 ± 0.0	1.5 ± 1.2	22.3 ± 6.9
C (n = 8)	17.6 ± 3.0^{a,b,c}	2.8 ± 1.9^{a,b,c}	8.1 ± 3.2^{a,b,c}	30.7 ± 5.0^{a,b,c}
D (n = 6)	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}

$^{a-e}$. $P \leq 0.05$ between respective values with similar letters within the same column in each table (ANOVA).
S1: D1 - D3, S2: D4 - D10, S3: D14 - D52.
Groups A and B: vaccinated, groups C and D: unvaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.

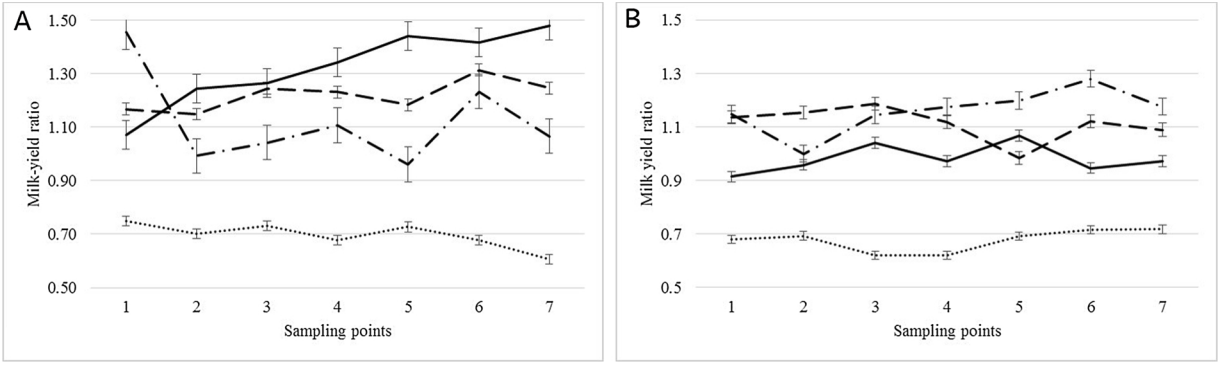

Fig. 2. Milk yield ratios in groups of ewes throughout a study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.
(a) Groups A1, B*, C1, D*
(b) Groups A2, B^{+}, C2, D^{+}
Solid line: group A (vaccinated, inoculated ewes), dashed line: group B (vaccinated, uninoculated ewes), dotted line: group C (unvaccinated, inoculated ewes), dashed-dotted line: group D (unvaccinated, uninoculated ewes).
* period from day 9 to day 62 post-partum, + period from day 49 to day 102 post-partum.

Among vaccinated ewes (groups A, n = 17 and B, n = 6), values varied from -6.24 to 69.64; values below 6.0 were obtained for 7 samples (6.1% of all samples) ($P < 0.001$ compared to groups C and D, PCST). Among challenged unvaccinated ewes (group C), values varied from -4.94 to 23.12; values above 6.0 were obtained for 10 samples (47.6% of all samples). During the same period, among unchallenged unvaccinated ewes (group D) values varied from -4.17 to 51.95; values above 6.0 were obtained for 6 samples (33.3% of all samples) ($P = 0.28$ compared to group C, PCST).

3.8. Findings in tissue samples

Bacteria were isolated from tissue samples of the inoculated mammary gland of two group A ewes (of 8 sampled, 25.0%) and four group C ewes (of 5 sampled, 80.0%) ewes ($P = 0.086$, FET). Bacteria were also isolated from tissue samples of the uninoculated mammary gland only of three group C ewes (of 5 sampled, 60.0%) ewes ($P = 0.035$ *versus* group A, FET). Median bacterial counts in tissue samples from group A ewes (n = 8) (0 cfu g^{-1}, 0–4) were lower than median bacterial counts in tissue samples from group C ewes (n = 5) (6.5 cfu g^{-1}, 0–75) ($P = 0.041$).

The median score for histopathological findings in tissue samples from inoculated glands of group A (n = 4) was lower than that for group C (n = 5) ewes [1 (0–2) *versus* 2 (2–3) ($P = 0.014$, MWT)].; The findings were similar for tissue samples from uninoculated glands [0 (0 – 0) *versus* 1 (0–2) ($P = 0.012$, MWT)], as well as when results for all tissue samples (*i.e.*, from both glands) were considered [0.5 (0–2) *versus* 2 (0–3) ($P = 0.005$, MWT)].

In tissue samples, lesions included lymphocytic infiltration, alveolar destruction (which was particularly marked in ewes of group C) collapsed alveoli and fibrous tissue proliferation; in group C ewes, the glandular elements were replaced to a large extent by fibrous tissue. In the tissue samples from the udder of the ewe that died 2 days post-challenge (group C2), there was extensive neutrophilic infiltration with degeneration of alveolar epithelial cells, destruction of alveoli and extensive extravasation throughout the mammary parenchyma.

3.9. Correlations

There was an inverse correlation between antibody titres in serum and bacterial counts in milk ($P < 0.001$, CORR). Bacterial counts in milk were positively correlated with clinical scores, IMIs and histopathological scores and inversely correlated with milk yield ratios ($P \leq 0.001$, CORR). Details of the significance of the correlations between the various parameters assessed are shown in Table 6.

4. Discussion

The results indicated that the vaccine under evaluation contributed to a reduction in the severity of mastitis in vaccinated ewes after challenge. Ewes were inoculated directly into the mammary gland with a pathogenic *S. chromogenes* isolate. This organism caused mastitis in all challenged ewes; moreover, it also caused bilateral mammary infection in control ewes and even death in one animal, confirming its virulence and invasiveness in the mammary gland. Coagulase-negative staphylococci have traditionally been considered of 'milder' pathogenicity than *S. aureus*. However, the death of ewes inoculated with *S. chromogenes* has been reported before (Fthenakis, 1988).

All inoculated ewes developed mastitis, but it was less severe in vaccinated animals, as indicated by a wide array of measures. In vaccinated ewes, mammary infections were not seen in the uninoculated mammary glands; further, grey-scale ratios and milk yield ratios indicated milder damage to the mammary parenchyma. Finally, the histopathological scores were lower. We postulate that these milder effects were the consequences of the lower bacterial counts in milk and tissue samples collected from vaccinated ewes, as indicated by the results of the correlation analyses (*e.g.*, inverse correlation with milk yield ratios and positive correlation between bacterial counts in tissue samples and histopathological scores).

The association of bacterial numbers in milk with adverse effects in the mammary parenchyma has been reported in previous studies performed in mice, an animal species found to be as appropriate model for the study of staphylococcal mastitis of ewes (Fthenakis, 1988, 1992). Leitner et al. (2003a) showed that the pathogenicity of staphylococcal isolates recovered from cases of mastitis was related to the bacterial dose inoculated into experimental animals. Brouillette et al. (2004) found that the numbers of *S. aureus* recovered from murine mammary tissue samples after intramammary challenge were correlated with the dose inoculated and Chinchali and Kaliwal (2014) reported that histological lesions in mammary glands were correlated with the challenge dose. These studies were performed with varying challenge doses. In the present study, although a similar challenge dose was used, at the end bacterial counts in milk and tissue samples differed between vaccinated and control ewes. It may be that the anti-PNAG antibodies, which were found to increase after vaccination, led to the lower bacterial counts of the challenge isolate post-inoculation. The increase in antibody titres in vaccinated ewes confirm development of immune responses by these animals. The inverse correlation between the antibody titres and the bacterial counts in milk and in tissue samples, coupled with the shorter duration of mammary infection in vaccinated ewes, also lends support to this hypothesis.

Table 6
Significance (*P* values) of correlations (CORR) between parameters* assessed throughout a study of the efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep.

	Parameter							
Parameter	Clinical scores	Mammary infections	Bacterial counts in milk	Grey-scale ratios	Milk yield ratios	Anti-PNAG antibody titres	Bacterial counts in tissues	Histopathological scores
Clinical scores	•	< 0.001	0.001	< 0.001	0.044	0.248	0.001	0.230
Mammary infections		•	< 0.001	0.001	0.124	< 0.001	0.059	< 0.001
Bacterial counts in milk			•	0.032	< 0.001	< 0.001	0.005	0.001
Grey-scale ratios				•	0.003	0.061	0.002	0.28
Milk yield ratios					•	0.009	0.061	0.34
Anti-PNAG antibody titres						•	0.032	0.004
Bacterial counts in tissues							•	0.123
Histopathological scores								•

Shaded cells indicate the *P* value of an inverse correlation.
* Analysis of correlation in the evaluated parameters was performed among ewes in groups A (vaccinated and challenged with *S. chromogenes*) and C (non-vaccinated and challenged with *S. chromogenes*).

In the experimental design employed, the teat, which has a well-documented effect against bacterial invaders (Mavrogianni et al., 2005, 2006), was by-passed in order to guarantee development of mastitis in the inoculated ewes. Hence, it was not possible to examine its effects on development of mastitis in the study. Nevertheless, effective clearance of the challenge isolate, as postulated above, might have limited dissemination to the uninoculated gland and reduced the risk of it becoming infected.

Staphylococci express a variety of virulence factors, which may not all be targets of one vaccine; no single virulence factor is known to be necessary for infection. The variability of the various staphylococcal antigens further impedes development of vaccines (Golubchik et al., 2013). Moreover, recurrence of staphylococcal mastitis indicates that these bacteria do not induce an immunity in infected animals. This is reflected in the failure of repeated attempts to develop staphylococcal vaccines for people (Pier, 2013).

Perez et al. (2009) have pointed out that anti-PNAG antibodies can be used only to confirm induction of immunity after administration of the vaccine, but their use for detection of natural mammary infections might not be straightforward. This seems reasonable, given the nature of the infection and that staphylococci can cause a variety of infections in ewes, some of them totally unrelated to the udder, *e.g.*, osteomyelitis or vaginal infections (Kaarsemaker et al., 1997; de Paula Vasconcelos et al., 2016).

Various attempts have been reported to develop vaccines against staphylococcal mastitis. For example, a product containing inactivated whole cells of *S. aureus* and *S. simulans*, as well as *S. aureus* exopolysaccharide antigens presented within liposomes have been found to reduce the incidence risk of mastitis (Amorena et al., 1994). In another approach, a vaccine including recombinant Target of RNAIII Activating Protein, a membrane protein present in staphylococci found to be involved in the pathogenesis of staphylococcal mastitis, has been employed to protect dairy animals against staphylococcal mastitis (Leitner et al., 2003b; 2003c; 2011). More recently, a multivalent whole cell staphylococcal vaccine has been assessed by Alekish et al. (2018), who showed that in that cases it did not show an effect in reduction in new cases of mastitis in vaccinated animals, with no effects on milk production or composition.

In a field study of the vaccine under evaluation, a reduction of the incidence risk of subclinical mastitis has been seen in vaccinated ewes (Vasileiou et al., 2019). Variations in the pathogenicity of causal staphylococcal strains may account for the differences from the present study; here, a particularly invasive isolate was inoculated, one that even caused bilateral mammary infection in unvaccinated ewes. The differences could also reflect the different types of study; under field conditions, various management factors may contribute to limiting or exacerbating infection (Gelasakis et al., 2015; Fthenakis et al., 2017; Vasileiou et al., 2018b).

In cases of mastitis, the damage caused in infected mammary glands is likely to be reflected in the milk yield of the ewes (Fthenakis and Jones, 1990). Moreover, less severe tissue damage in mammary glands during the lactation period can result in a reduced culling rate at the end of the lactation period, when ewes are clinically examined and those with extensive lesions culled (Fthenakis et al., 2012; Petridis and Fthenakis, 2014). Hence, there is a benefit with financial consequences in reducing the effects of infection with a mammary pathogen in ewes.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108480.

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