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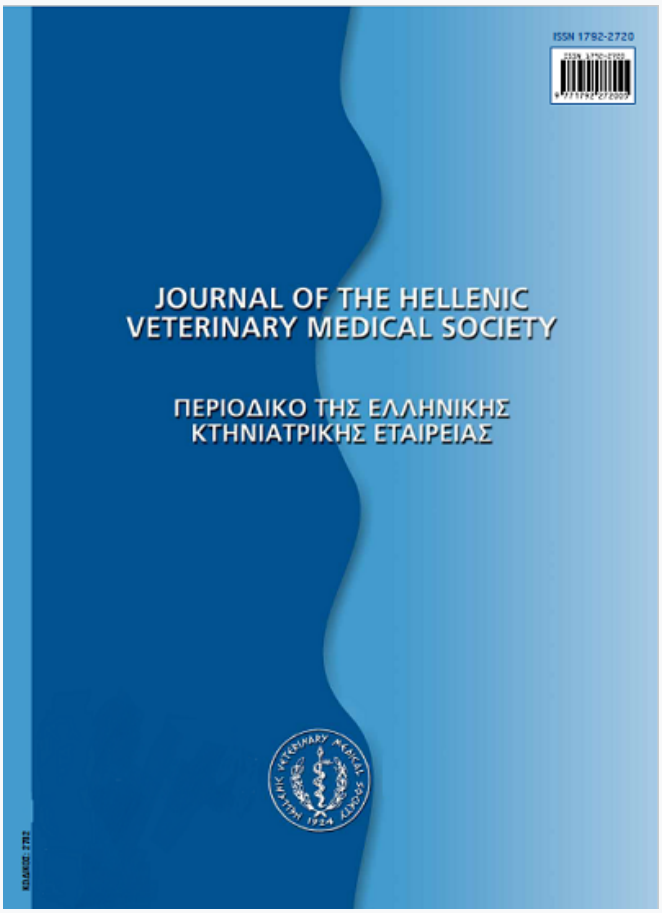

Τα αποτελέσματα του ενοφθαλμισμού αιγών με Staphylococcus aureus στη θηλή του μαστού

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Research article
Ερευνητικό άρθρο

Results of challenge of goats with Staphylococcus aureus into the teat of the udder

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Τα αποτελέσματα του ενοφθαλμισμού αιγών με Staphylococcus aureus στη θηλή του μαστού

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ABSTRACT. Objective of the present study was to study the outcome of inoculation of *Staphylococcus aureus* into the teat duct of female goats, which simulates mammary natural infections. In total, 22 lactating goats were used in the study; 8 animals were challenged with a *S. aureus* strain at a depth of 2 mm into one teat duct (group A), 8 animals were challenged with the same strain at 6 mm into one teat duct (group B) and 6 animals were challenged directly into one gland cistern (group C). Challenge dose was always 1300 cfu. Animals were examined clinically before and after challenge; milk samples were collected for bacteriological and cytological examination, and milk yield measurements were also performed. Goats in group A or B developed a significantly milder response than animals in group C. It is concluded that the evidence indicates a protective role of the normal teat of the udder of goats and that the results also underline the significance of maintaining healthy teats for prevention of mastitis in dairy herds.

Keywords: *immunity, goat, mastitis, Staphylococcus aureus*

ΠΕΡΙΛΗΨΗ. Σκοπός της μελέτης ήταν η αξιολόγηση των αποτελεσμάτων του ενοφθαλμισμού *Staphylococcus aureus* στη θηλή του μαστού αιγών, ώστε να προσομοιάζει στη φυσική μόλυνση. Στη μελέτη περιλήφθηκαν συνολικά 22 αίγες σε γαλακτική περίοδο. Σε 8 αίγες έγινε εναπόθεση *S. aureus* στη θηλή του μαστού σε βάθος 2 mm από το στόμιο αυτής (ομάδα Α), σε άλλες 8 εναπόθεση *S. aureus* στη θηλή του μαστού σε βάθος 6 mm από το στόμιο αυτής (ομάδα Β) και σε άλλες 6 έγινε ενοφθαλμισμός απευθείας στο γαλακτοφόρο κόλπο (ομάδα Γ). Η δόση ενοφθαλμισμού ήταν 1.300 cfu. Στα ζώα έγινε κλινική εξέταση πριν και μετά τον ενοφθαλμισμό. Επίσης, συλλέχθηκαν δείγματα γάλακτος για βακτηριολογική και κυτταρολογική εξέταση πριν και μετά τον ενοφθαλμισμό, επιπλέον δε πραγματοποιήθηκαν μετρήσεις του παραγόμενου γάλακτος. Οι αίγες στην ομάδα Α ή Β εκδήλωσαν σημαντικά πιο ήπια ευρήματα στον ενοφθαλμισμό από τα ζώα στην ομάδα Γ. Από το σύνολο των ευρημάτων συμπεραίνεται ότι η θηλή του μαστού ασκεί προστατευτική δράση έναντι εισβαλλόντων μικροοργανισμών, πού αποδεικνύει τη σημασία της διατήρησης της υγείας των θηλών για την πρόληψη της μαστίτιδας σε εκτροφές αιγών.

Λέξεις ευρετηρίασης: *αίγες, αμυντικοί μηχανισμοί, μαστίτιδα, Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus is the most important mammary pathogen in goats, although other organisms (e.g. coagulase-negative *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli*) are also implicated in the aetiology of the disease. The organism causes clinical or subclinical mastitis, which adversely affects the welfare of animals and causes significant financial losses (e.g., death of affected does, veterinary expenses, reduced milk production, changes in quality of milk) (Marogna et al., 2012). Infection takes place through the teat, which, in cows and ewes, acts defensively to limit invading bacteria (Mavrogianni et al., 2006b). Potential defense role of the teat of goats had not been adequately described, although evaluation would be helpful in formulating strategies for control of mastitis in goats. Objective of the present study was to study the outcome of deposition of *S. aureus* into the teat duct of female goats and to evaluate the results of infection and production parametres; this model simulates natural mammary infections.

MATERIALS AND METHODS

Experimental design

In total, 22 lactating goats of the Greek indigenous *Capra prisca* goat breed were allocated into one of three groups (A, n=8; B, n=8; C, n=6) and challenged with a strain of *S. aureus* that had been isolated from a case of clinical caprine mastitis. The animals were purchased directly from small-scale farmers. Selection at the first stage was by visual appraisal. Only animals that were structurally sound, free from obvious physical defects and appeared healthy were purchased.

The breeding animals were all between 38 and 40 months old when they are kidded. Kids of these does were taken away from their mothers at the age of 18 days and subsequently, the does were hand-milked thrice daily. Does were also monitored at weekly intervals with clinical examination and bacteriological and cytological examination of milk samples, to confirm that they remained free of mammary infections.

Challenge (the day of inoculation) was performed on the 35th day after lambing. For inoculation, the strain was grown on Columbia blood agar and checked for purity; then it was inoculated into Soy-broth (BioMerieux) and incubated aerobically at 37^{o}C for 5 h.

Does in group A were challenged by deposition of 1,300 cfu of the organism into the left teat duct (2 mm deep) (Mavrogianni et al., 2006b). Does in group B were challenged by deposition of the same dose into the left teat cistern (6 mm deep). Does in group C (received as «positive controls») were challenged by introduction of the same dose directly into the gland cistern. The contralateral side of the udder was used for control purposes and a small amount of sterile PBS (1 mL) was inoculated in the respective sites.

Pre- and post-inoculation examinations and sample collections

Three days (D-3) and one day (D-1) before challenge, which took place on D0, a thorough clinical examination of the experimental animals was performed. Special attention was paid to their mammary glands and teats, which were palpated to evaluate their shape, size and consistency. Teat duct material was collected for bacteriological examination by using a previously described technique; in brief, an Abbocath catheter cut at a length of 2 mm was inserted into the teat duct, mildly swirled to collect material and then taken out (Mavrogianni et al., 2006a). Finally, milk samples were withdrawn and subjected to bacteriological and cytological examinations; the first two squirts of secretion were discarded and, then, 10 to 15 mL of secretion were carefully collected into a sterile container (Fthenakis, 1994). Subsequently, similar examinations and sample collections were performed 12 hours, as well as 1, 2, 4, 7 and 10 days after challenge and every 7 days thereafter.

All samples were cultured onto Columbia blood agar; the media were incubated aerobically at 37 oC for up to 72 h. Throughout this study, all bacteria isolated were identified by using conventional microbiological techniques (Barrow et al., 1993).

The Microscopic cell counting method (Mccm, IDF reference method) was applied for measuring cell numbers in milk samples (International-Dairy-Federation, 1984; Raynal-Ljutovac et al., 2007). Further, after stain of milk films by the Giemsa technique, the type of 200 cells was also identified, in order to estimate proportion of the various leucocyte numbers in the samples (Mavrogianni et al., 2006b).

Finally, actual milk yield measurements were performed before challenge, as well as on D10 and every 14 days thereafter. Measurements were performed on the day of estimation of milk yield 12 h after complete emptying of the mammary gland. Actual milk yield measurements at day: D-3, D10, D24, D38, D52, D66 and D79, were calculated using individual milk yields recorded at each a.m. and p.m.

milking session by electronic milk meters and flock management software.

Data management and analysis

The number of bacteriologically positive results obtained by each of the three different procedures of challenging the animals, were compared between them.

For somatic cell counts and milk yields, repeated measures mixed effect linear regression models were used to determine significance of differences between groups throughout the course of the study period. Effect of experimental subjects (animals) was included as random effect in the model. Models were adjusted for repeated measures within animals. Independent variables (fixed effects) included experimental group, time of the study and a time of the study by experimental group interaction.

Separate analyses were performed for results from the challenged and the contralateral side of the udder. Statistical significance was defined as $P < 0.05$.

RESULTS

Findings before challenge

Both mammary glands and teats of all goats (groups A, B and C) were clinically healthy during the period from kidding to challenge. No bacteria were isolated from teat duct material or milk samples. Cell counts in milk samples were always $<0.4\times10^6$ cells mL^{-1}; difference between the three groups were not significant ($P>0.65$). Most (80-85%) cells present in milk films were macrophages, with some (15-20%) neutrophils and scarce (<5%) lymphocytes also present.

Clinical findings after challenge

In group A, no clinical findings were observed in any goat after bacterial deposition into the teat. In group B, clinical signs were transiently (1 day) seen in only one goat after challenge. In contrast, in group C, all ewes developed clinical mastitis in the inoculated side (abnormal secretion with flakes and clots, enlarged mammary gland) for up to 38 days after challenge. The difference in development of clinical disease after challenge between groups A/B and C was statistically significant ($P<0.001$). Duration of clinical signs was also longer in group C goats (median value: 27.25 days) than in group A or B animals (median value: 0 days) ($P<0.001$).

The contralateral side of the udder of all experimental animals remained healthy throughout the study.

Bacteriological findings after challenge

In group A animals, *S. aureus* were isolated from 50/210 samples (34/105 teat duct material samples, 16/105 milk samples) from the challenged side of the udder. In group B animals, *S. aureus* were isolated from 93/210 samples (47/105, 46/105, respectively) from the challenged side of the udder ($P<0.035$ compared to group A). In group C animals, S. aureus were isolated from 95/168 samples (45/84, 50/84, respectively) from the challenged glands ($P<0.003$ compared to group A, $P=0.016$ for milk samples compared to group B).

No bacteria were isolated from milk samples collected from the contralateral side of the udder from any goat at any sampling.

Cytological findings after challenge

In all cases, after challenge, somatic cell counts increased in milk from the inoculated side of the udder. Mean (±standard error of the mean) cumulative somatic cell counts were $0.625\times10^6\pm0.06\times10^6$ cells mL^{-1} for group A goats, $0.962\times106\pm0.11\times106$ cells mL-1 for group B goats and $1.409\times106\pm0.22\times106$ cells mL-1 for group C goats ($P<0.04$ between groups).

Neutrophils predominated (>75% of total leucocytes) in milk films up to D7. From D10 to D17, neutrophils (35-55%) and lymphocytes (35-45%) were seen in milk films, whilst thereafter lymphocytes predominated (>50%) in milk films. In groups A and B, progressively (after D38), macrophages again became the predominant cell type in the animals.

Milk yield measurement

In all cases, after challenge, milk yield decreased from the inoculated side of the udder. Mean (±standard error of the mean) cumulative milk yields were 232±19 mL for group A, 186±13 mL for group B and 91±3 mL for group C ($P<0.04$ between groups). On average, milk yield from the inoculated side of the udder was smaller from the contralateral side by 14% to 36% for group A, by 22.5% to 39.5% for group B and by 64.5% to 68% for group C animals ($P=0.07$ for group A *versus* group B, $P<0.001$ for group A or B *versus* group C).

Detailed results are in Table 1.

DISCUSSION

In previous experimental studies of caprine mastitis, the bacteria were deposited directly into the mammary gland (Herwijnen, 2010). In our work, we inoculated the staphylococci into the teat of goats, which simulates natural conditions of infection, as the teat is the

portal of entry of mammary pathogens. The results provide interesting evidence or facts regarding a potential protective role of the teat against mammary infections.

Animals in group C were «positive controls». Mastitis was consistently induced in these does after inoculation of *S. aureus* directly into the gland cistern. This strain had been isolated from a case of clinical mastitis in a goat, hence induction of the disease after intramammary challenge and re-isolation of the strain confirmed its pathogenicity for the caprine mammary gland. The inoculated animals showed increased somatic cell counts (>2.0×10^6 cells mL^{-1}) and dramatic reduction (>60%) of milk yield, which is likely the result of destruction of mammary epithelial cells caused by the virulent factors of the staphylococci (Fthenakis and Jones, 1990).

In contrast to the above, deposition of the same dose into the teat of the udder did not result in clinical mastitis. Goats in group A showed only a transient excretion of the organism in the milk, whilst isolation of the organism from the teat duct (i.e., the exact site of deposition) also ceased some days after challenge. Milk somatic cell counts were significantly smaller than in goats of group C, which indicates a milder inflammatory reaction mounted by goats in group A. Further, reduction of milk yield was smaller (<35%), which suggests a smaller effect of bacterial factors to mammary cells and likely less severe damage to the mammary cells, as the degree of mammary destruction is related with reduction in milk yield. Similar findings were recorded in animals of group B, in which bacteria were deposited into the teat cistern, although the reaction of the animals in that group was more severe (e.g., longer time required to clean bacteria from the udder, higher somatic cell counts).

These findings support a hypothesis regarding a protective role of the teat of ewes. Bacteria placed into the teat duct ascended to the mammary gland (isolation from milk), but did not cause clinical mastitis. In cows and ewes, the protective role of the teat has been confirmed, but similar studies have never been reported in goats. Knowledge from ewes cannot be applied directly to goats, as these are two different animal species with distinct physiological differences (Agrawal et al., 2014). The present results indicate that the potential defence mechanisms act at the lower end of the teat, at a distance of up to 6 mm from the teat orifice.

Various mechanisms can be responsible and may play a defensive role at the teat of the udder (Paape and Capuco, 1997; Fragkou et al., 2007). These include the keratin of the teat duct, which inhibits proximal progression of bacteria, whilst subsequent cellular and humoral defence mechanisms occur in the teat cistern, e.g., leucocytes and non-specific antibacterial proteins (Ezzat Alnakip et al., 2014). Also, possibly, under field conditions, bacterial flora into the teat duct (e.g., coagulase-negative staphylococci) can play a protective role against invading bacteria, for example by acting in competition with invading organisms and ultimately leading to reduction in the number of the latter bacteria (Fragkou et al., 2007).

An interesting question is why the bacteria, although they reached the mammary gland, did not cause clinical mastitis in groups A or B. One may postulate that, perhaps, the elicited influx of neutrophils (as confirmed by results of cytological examination) after deposition of bacteria into the teat has prepared the mammary gland to respond better to the bacteria which arrived thereafter to the parenchyma. Direct inoculation of bacteria into the gland cistern elicits an inflammatory response within 6 to 12 hours, by which time the bacteria will have multiplied massively and will have already damaged the mammary tissue. Deposition of bacteria into the teat gives the opportunity for an initial, mild leucocytic response, which would prepare the mammary gland to counter-act efficiently the bacteria if they would reach the parenchyma.

The above evidence confirms the protective role of the normal teat of the udder of goats. The results also indicate that maintenance of healthy teats will contribute to effective control of mastitis.

CONCLUDING REMARKS

Deposition of a *S. aureus* strain with confirmed pathogenicity into the teat of the udder of goats leads to a milder effects compared to the ones recorded after direct inoculation of the strain into mammary parenchyma. The evidence indicates a protective role of the normal teat of the udder of goats. Furthermore, the results also underline the significance of maintaining healthy teats for prevention of mastitis in dairy herds.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

Table 1. Detailed results of clinical, bacteriological and cytological findings after deposition of *Staphylococcus aureus* into the teat duct of goats.

Group A (bacterial deposition into the teat duct)											
		D -3	**D -1**	**D0+12 h**	**D1**	**D2**	**D4**	**D7**	**D10**	**D17**	**D24**
Disease development											
Clinical findings		0/8	0/8	0/8	0/8	0/8	0/8	0/7	0/6	0/6	0/6
Sub-clinical mastitis		0/8	0/8	0/8	4/8	3/8	3/8	2/7	1/6	1/6	1/6
Bacterial isolations											
Teat duct material		0/8	0/8	8/8	8/8	6/8	5/8	3/7	2/6	1/6	1/6
Mammary secretion		0/8	0/8	1/8	4/8	3/8	3/8	2/7	1/6	1/6	1/6
Cytological results											
Milk cell content (cells mL^{-1})		0.236×10^6 $\pm0.05\times10^6$	0.228×10^6 $\pm0.04\times10^6$	0.325×10^6 $\pm0.32\times10^6$	0.764×10^6 $\pm0.13\times10^6$	0.942×10^6 $\pm0.16\times10^6$	1.003×10^6 $\pm0.18\times10^6$	0.864×10^6 $\pm0.14\times10^6$	0.896×10^6 $\pm0.13\times10^6$	0.808×10^6 $\pm0.11\times10^6$	0.690×10^6 $\pm0.09\times10^6$
Milk yield measurement results											
Milk yield (mL)	inoculated gland	251±7							177±7		190±3
	control gland	269±8							275±8		289±8
		D31	**D38**	**D45**	**D52**	**D59**	**D66**	**D73**	**D79**	**Cumulative results**	
Disease development											
Clinical findings		0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/8^a	
Sub-clinical mastitis		0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	8/8	
Bacterial isolations											
Teat duct material		0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	34/105^{c,d}	
Mammary secretion		0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	16/105^a	
Cytological results											
Milk cell content (cells mL^{-1})		0.567×10^6 $\pm0.06\times10^6$	0.529×10^6 $\pm0.07\times10^6$	0.510×10^6 $\pm0.07\times10^6$	0.426×10^6 $\pm0.05\times10^6$	0.447×10^6 $\pm0.06\times10^6$	0.375×10^6 $\pm0.04\times10^6$	0.422×10^6 $\pm0.06\times10^6$	0.424×10^6 $\pm0.06\times10^6$	$0.625\times10^6\pm0.06\times10^{6a,a}$	
Milk yield measurement results											
Milk yield (mL)	inoculated gland		218±19		259±21		262±21		285±15	232±18^{c,a}	
	control gland		296±9		308±8		326±16		333±17	305±9	

Group B (bacterial deposition into the teat cistern)											
		D -3	**D -1**	**D0+12 h**	**D1**	**D2**	**D4**	**D7**	**D10**	**D17**	**D24**
Disease development											
Clinical findings		0/8	0/8	0/8	1/8	0/8	0/8	0/7	0/6	0/6	0/6
Sub-clinical mastitis		0/8	0/8	2/8	6/8	7/8	7/8	5/7	4/6	3/6	3/6
Bacterial isolations											
Teat duct material		0/8	0/8	8/8	8/8	8/8	7/8	3/7	2/6	2/6	2/6
Mammary secretion		0/8	0/8	2/8	7/8	7/8	7/8	5/7	4/6	3/6	3/6
Cytological results											
Milk cell content (cells mL^{-1})		0.267×10^6 $\pm0.03\times10^6$	0.259×10^6 $\pm0.04\times10^6$	0.412×10^6 $\pm0.08\times10^6$	0.834×10^6 $\pm0.12\times10^6$	1.198×10^6 $\pm0.17\times10^6$	1.336×10^6 $\pm0.17\times10^6$	1.555×10^6 $\pm0.15\times10^6$	1.879×10^6 $\pm0.11\times10^6$	1.327×10^6 $\pm0.15\times10^6$	1.082×10^6 $\pm0.13\times10^6$
Milk yield measurement results											
Milk yield (mL)	inoculated gland	229±14							146±14		163±12
	control gland	233±16							241±14		260±15
		D31	**D38**	**D45**	**D52**	**D59**	**D66**	**D73**	**D79**	**Cumulative results**	
Disease development											
Clinical findings		0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/8^b	
Sub-clinical mastitis		3/6	3/6	2/6	1/6	1/6	0/6	0/6	0/6	7/8	
Bacterial isolations											
Teat duct material		3/6	2/6	2/6	1/6	0/6	0/6	0/6	0/6	47/105^c	
Mammary secretion		3/6	3/6	2/6	1/6	1/6	0/6	0/6	0/6	46/105^d	
Cytological results											
Milk cell content (cells mL^{-1})		0.975×10^6 $\pm0.08\times10^6$	0.999×10^6 $\pm0.09\times10^6$	0.872×10^6 $\pm0.08\times10^6$	0.825×10^6 $\pm0.05\times10^6$	0.780×10^6 $\pm0.06\times10^6$	0.643×10^6 $\pm0.08\times10^6$	0.358×10^6 $\pm0.02\times10^6$	0.317×10^6 $\pm0.02\times10^6$	$0.962\times10^6\pm0.11\times10^{6c,c}$	
Milk yield measurement results											
Milk yield (mL)	inoculated gland		182±11		185±11		209±11		234±10	186±13^{c,b}	
	control gland		267±14		274±14		285±14		302±14	271±8	

Group C (inoculation into the mammary parenchyma)											
		D -3	**D -1**	**D0+12 h**	**D1**	**D2**	**D4**	**D7**	**D10**	**D17**	**D24**
Disease development											
Clinical findings		0/6	0/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	3/6
Sub-clinical mastitis		0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	2/6	2/6
Bacterial isolations											
Teat duct material		0/6	0/6	1/6	6/6	6/6	6/6	6/6	5/6	6/6	2/6
Mammary secretion		0/6	0/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	2/6
Cytological results											
Milk cell content (cells mL^{-1})		0.245×10^6 $\pm0.06\times10^6$	0.243×10^6 $\pm0.05\times10^6$	1.581×10^6 $\pm0.12\times10^6$	2.517×10^6 $\pm0.09\times10^6$	2.612×10^6 $\pm0.06\times10^6$	2.690×10^6 $\pm0.05\times10^6$	2.366×10^6 $\pm0.14\times10^6$	2.224×10^6 $\pm0.15\times10^6$	1.878×10^6 $\pm0.17\times10^6$	1.416×10^6 $\pm0.11\times10^6$
Milk yield measurement results											
Milk yield (mL)	inoculated gland	239±10							87±9		81±7
	control gland	242±9							251±9		257±8
		D31	**D38**	**D45**	**D52**	**D59**	**D66**	**D73**	**D79**	**Cumulative results**	
Disease development											
Clinical findings		1/6	1/6	0/6	0/6	0/6	0/6	0/6	0/6	6/6^{a,b}	
Sub-clinical mastitis		3/6	1/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6	
Bacterial isolations											
Teat duct material		3/6	2/6	1/6	1/6	0/6	0/6	0/6	0/6	45/84^d	
Mammary secretion		3/6	2/6	1/6	1/6	0/6	0/6	0/6	0/6	50/84^{a,d}	
Cytological results											
Milk cell content (cells mL^{-1})		1.581×10^6 $\pm0.12\times10^6$	2.517×10^6 $\pm0.09\times10^6$	2.612×10^6 $\pm0.06\times10^6$	2.690×10^6 $\pm0.05\times10^6$	2.366×10^6 $\pm0.14\times10^6$	2.223×10^6 $\pm0.15\times10^6$	1.878×10^6 $\pm0.17\times10^6$	1.415×10^6 $\pm0.11\times10^6$	$1.409\times10^6\pm0.22\times10^{6a,c}$	
Milk yield measurement results											
Milk yield (mL)	inoculated gland		86±5		90±5		99±4		103±5	91±3^{a,b}	
	control gland		270±8		278±8		286±7		293±5	272±7	

Comparisons between same parameters of different groups: a, b: P<0.001, c: 0.05<P<0.025, d: 0.025<P<0.01, e: 0.01<P<0.001

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