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Original article

The bacterial flora in the teat duct of ewes can protect against and can cause mastitis

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Abstract – We studied the possible effects of bacterial populations within the teat duct, in the pathogenesis of ovine mastitis. In experiment I, 32 ewes were allocated into group A (ewes from which we isolated (+++ growth) coagulase-negative staphylococci), B (ewes from whose duct we isolated (+ growth) coagulase-negative staphylococci) or C (ewes from which we isolated *Bacillus* spp.) and subdivided into A1, B1, C1 ($n = 4$; challenged by deposition of 1.250 cfu of *Mannheimia haemolytica* into the teat duct) or A2, B2, C2 ($n = 4$; used as uninoculated controls); group D ($n = 8$) contained ewes with no bacteria in their teat ducts and were challenged as above. There were less bacteriological isolations of flora ($P = 0.018$) and challenge ($P < 0.05$) organisms from A1 than from A2 and D ewes; the severity of pathological findings in A1 (summed up score: 27) ewes was smaller than in D (summed up score: 36) ewes ($P = 0.038$). No such findings were evident with B1 or C1 ewes ($P > 0.4$). In experiment II, ewes (groups E and F, $n = 6$) from whose duct we isolated coagulase-negative staphylococci (+ growth) were used; in group G ($n = 6$) ewes with no bacteria in their teat ducts were included. Teat chapping was applied in E and G ewes. All E ewes developed acute clinical mastitis within 24 h after teat chapping, although we had carried out no challenge; there were more bacteriological isolations of flora organisms from E than from F and G ewes ($P < 0.001$); the severity of pathological findings in E (score: 28) was greater than in F (score: 3) or G (score: 14) ewes. In experiment III, eight ewes with no bacteria in their teat ducts were allocated into group H or I ($n = 4$) and challenged into the teat (group H) or into the gland (group I) with 10^6 cfu of a *Staphylococcus simulans* recovered from the teat duct of a group E ewe. Group H ewes developed transiently clinical followed by subclinical mastitis (based on bacteriological and cytological evidence), whilst group I ewes developed severe clinical disease. We conclude that staphylococcal flora present in high numbers within the teat duct of ewes can afford some protection against invading microorganisms. However with impeded defence mechanisms of the teat, the same flora may invade the mammary parenchyma and cause clinical mastitis.

mastitis / sheep / teat / predisposing factor / bacterial flora

1. INTRODUCTION

The significance of the ovine teat as a defence mechanism against intramammary infections has been established [36]. Clinically healthy teats provide a substantial protection against *Mannheimia haemolytica* intramammary infection. It was found that deposition of either of two isolates of *M. haemolytica* into the teat duct did not result in clinical mastitis, although an inflammatory reaction had been elicited; direct inoculation of the same strains into the gland cistern always resulted in clinical

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mastitis. During this study, we observed lymphoid nodules at the border between the teat duct-teat cistern; we postulated that these structures might play a protective role, as in histological sections from teats inoculated with the bacteria, they were hyperplastic with germinal activity. Apart from that, the bacterial flora residing in the teat duct of healthy ewes may also contribute to the protective role of the teat. In field studies, isolation of bacteria from clinically healthy sheep teats was associated with observations of lymphoid nodules with germinal activity [40].

Coagulase-negative staphylococci are the principal organisms present as bacterial flora in sheep teats [18]. Since staphylococci are confirmed aetiological agents of ovine mastitis [16], one may suggest that perhaps and under certain circumstances, flora organisms can also cause mastitis.

However, the above hypotheses have not been tested experimentally. The objective of the work presented in this paper was to investigate the role of the bacterial flora in the teat duct of ewes. Initially, we explored possible interactions between teat duct bacterial flora and invading microorganisms; subsequently, we studied whether teat lesions may predispose ewes to clinical mastitis caused by teat duct bacterial flora. Finally, we investigated whether teat duct staphylococcal flora may cause clinical mastitis if inoculated directly into the mammary gland cistern. For the purposes of this paper, the term "bacterial flora" refers to the *bacterial populations* within the teat duct of the ewes, which appear to be inert and are considered to be innocuous for the mammary parenchyma.

2. MATERIALS AND METHODS

2.1. Experimental design

2.1.1. Overview

Three experiments were performed during this study. They were carried out under a license for experimental procedures obtained from the Greek Ministry of Agriculture.

In the first experiment, clinically healthy sheep teats with bacterial flora (coagulase-negative staphylococci or *Bacillus* spp.) in the duct were inoculated with an isolate of *M. haemolytica* (strain VSM08L). This strain had been isolated in Greece and was found to cause mastitis in ewes when inoculated directly into the gland cistern, whilst deposition into the duct or the cistern of clinically healthy teats resulted in subclinical mastitis [36].

The identity of the organism was initially established by means of conventional bacteriological techniques [2, 12]. It was then confirmed by using molecular techniques. DNA was isolated from a blood-agar colony of the organism using a commercial kit (Gentra Systems, Minneapolis, USA) according to the manufacturer's instructions. PCR amplification was carried out according to the guidelines described by Kwok and Higuchi [28]. The sequence of the primers and the PCR conditions to amplify a part of 350 bp of 16S r-RNA gene, were the same as previously described [1]. Following amplification, 10 μL of each PCR product were analysed by electrophoresis on 2% agarose gel and stained with ethidium bromide (0.5 mg/mL). A 100 bp DNA ladder was analysed on the same gel to serve as a size marker. As a negative control, DEPC treated H_2O (RNA free) was used instead of DNA in a PCR assay to exclude any contamination. As a positive control, we used strain ES26L, *M. haemolytica* serotype A9, which has been isolated and typed in England [11]. The specificity of the PCR products was verified after direct PCR product sequencing (MWG Biotech AG, Ebersberg, Germany).

In the second experiment, clinically healthy sheep teats with bacterial flora (coagulase-negative staphylococci) into the duct were subjected to skin chapping

lesions; no challenge was performed. In the third experiment, clinically healthy sheep teats or mammary glands with no bacterial flora, were inoculated with a *Staphylococcus simulans* from the teat duct of one of the ewes of the second experiment. The experimental design is summarised in Table I and presented in detail below.

2.1.2. *Deposition of* M. haemolytica *into bacteriologically positive sheep teat ducts: Experiment I*

Twenty-four, 3- to 5-year-old, Karagouniko-breed lactating ewes, which had not received antibiotic treatment throughout their pregnancy and lactation, were included in the experiment. For selection of the ewes three, 2-day interval, examinations and samplings were carried out. Initially, a thorough clinical examination was carried out; special attention was paid to their mammary glands and teats, which were examined as described before [17, 36]. A sterile plastic fine catheter 2 mm long was inserted into the teat and moved from the left to the right, in order to sample the mucosa [37]. Then, mammary secretion samples (10 to 15 mL) were obtained.

All samples were cultured onto Columbia blood agar; the media were incubated aerobically at 37 °C for up to 72 h. The California Mastitis Test (CMT) was carried out in secretion samples; secretion films were made and stained by the Giemsa method.

Selection of animals was based on the concurrent presence of the following criteria at all three samplings: (i) clinically healthy mammary glands and teats; (ii) no bacterial isolation from mammary secretion; (iii) secretion CMT negative with a minimal number of leucocytes in Giemsa-stained secretion films; (iv) bacterial isolation from teat catheter of one teat (left or right) in pure culture; (v) no bacterial isolation from the teat catheter of the other teat. Allocation of animals into groups was carried out as follows: group A (n = 8): isolation of coagulase-negative *Staphylococcus* sp. (4 *S. epidermidis*, 4 *S. simulans*) in heavy growth (+++); group B (n = 8): isolation of coagulase-negative *Staphylococcus* sp. (4 *S. epidermidis*, 4 *S. simulans*) in mild growth (+); group C (n = 8): isolation of *Bacillus* spp. in heavy growth (+++). Amongst the 8 ewes allocated into each group, 4 (subgroups A1, B1, C1) were challenged and 4 (subgroups A2, B2, C2) were used as uninfected positive controls.

Additionally, a group D (n = 8) was also included in the experiment. Lambs of these ewes were weaned 18 days after lambing. No bacteria were isolated from any teat catheter or mammary secretion samples obtained. These were used as inoculated negative controls.

After selection, all animals were hand-milked thrice daily. Ewes were examined again and samples were collected as above, on the day of inoculation (D0), which was carried out as described by Mavrogianni et al. [36]. The ewes in A1, B1, C1 and D were challenged 2 mm deep into the teat by means of a sterile plastic fine catheter (Abbocath; Abbott Laboratories Inc., Abbott Park, IL, USA) 20 G. In the other teat of these ewes, 0.2 mL of sterile PBS was injected 2 mm deep into the teat. In ewes of subgroups A2, B2 and C2, 0.2 mL of sterile PBS was injected 2 mm deep into both teats. Ultimately, the teats of the ewes into each group were naturally infected (NI) and/or challenged (CH) as follows: subgroup A1, B1, C1: one teat NI+/CH+, the other teat NI−/CH−; subgroup A2, B2, C2: one teat NI+/CH−, the other teat NI−/CH−; group D: one teat NI−/CH+, the other teat NI−/CH−.

2.1.3. *Artificial skin chapping on sheep teats with bacteriologically positive duct: Experiment II*

Twelve, 3- to 5-year-old, Karagouniko-breed lactating ewes, which had not

Table I. Summary of experimental design.

Group – Subgroup	Procedure	Time after challenge or chapping (D0) when ewes were euthanised
Experiment I		
A1 (n = 4); coagulase-negative *Staphylococcus* sp. (growth +++) into the duct of one teat	Teat with flora: inoculation of 1250 c.f.u. *M. haemolytica* 2 mm-deep (NI+/CH+); other teat: PBS 2 mm-deep (NI–/CH–)	D1, D3, D5, D7
A2 (n = 4); coagulase-negative *Staphylococcus* sp. (growth +++) into the duct of one teat	Teat with flora: PBS 2 mm-deep (NI+/CH–); other teat: PBS 2 mm-deep (NI–/CH–)	D1, D3, D5, D7
B1 (n = 4); coagulase-negative *Staphylococcus* sp. (growth +) into the duct of one teat	Teat with flora: inoculation of 1250 c.f.u. *M. haemolytica* 2 mm-deep (NI+/CH+); other teat: PBS 2 mm-deep (NI–/CH–)	D1, D3, D5, D7
B2 (n = 4); coagulase-negative *Staphylococcus* sp. (growth +) into the duct of one teat	Teat with flora: PBS 2 mm-deep (NI+/CH–); other teat: PBS 2 mm-deep (NI–/CH–)	D1, D3, D5, D7
C1 (n = 4); *Bacillus* sp. (growth +++) into the duct of one teat	Teat with flora: inoculation of 1250 c.f.u. *M. haemolytica* 2 mm-deep (NI+/CH+); other teat: PBS 2 mm-deep (NI–/CH–)	D1, D3, D5, D7
C2 (n = 4); *Bacillus* sp. (growth +++) into the duct of one teat	Teat with flora: PBS 2 mm-deep (NI+/CH–); other teat: PBS 2 mm-deep (NI–/CH–)	D1, D3, D5, D7
D (n = 8); no bacteria into the duct of either teat	One teat: inoculation of 1250 c.f.u. *M. haemolytica* 2 mm-deep (NI–/CH+); other teat: PBS 2 mm-deep (NI–/CH–)	D1 (X2), D3 (X2), D5 (X2), D7 (X2)
Experiment II		
E (n = 6); coagulase-negative *Staphylococcus* sp. into the duct of one teat (growth +)	Both teats: chapping lesions by immersion into 1 N NaOH solution (one teat: NI+/CP+; other teat: NI–/CP+)	D5 (X3), D7 (X3)
F (n = 6); coagulase-negative *Staphylococcus* sp. into the duct of one teat (growth +)	No chapping in either teat (one teat: NI+/CP–; other teat: NI–/CP–)	D5 (X3), D7 (X3)
G (n = 6); no bacteria into the duct of either teat	One teat: chapping lesions by immersion into 1 N NaOH solution (NI–/CP+); other teat: no chapping (NI–/CP–)	D5 (X3), D7 (X3)
Experiment III		
H (n = 4); no bacteria into the duct of either teat	One side: inoculation of 10^6 c.f.u. *S. simulans* 2 mm-deep (teat: NI–/CH+, gland NI–/CH–); other side: PBS 2 mm-deep (teat: NI–/CH–, gland NI–/CH–)	D5 (X2), D7 (X2)
I (n = 4); no bacteria into the duct of either teat	One side: inoculation of 10^6 c.f.u. *S. simulans* into the gland cistern (teat: NI–/CH–, gland: NI–/CH+); other side: PBS into the gland cistern (teat: NI–/CH–, gland: NI–/CH–)	D5 (X2), D7 (X2)

In all animals, no bacteria in the mammary secretion; NI: naturally infected, CH: challenged, CP: chapped.

received antibiotic treatment throughout their pregnancy and lactation, were included in the experiment. For selection, the same procedures and criteria as in Experiment I were applied. Allocation of animals into groups was carried out as follows: group E ($n = 6$): isolation of coagulase-negative staphylococci (3 *S. simulans*, 3 *S. epidermidis*) in mild growth; group F ($n = 6$): isolation of coagulase-negative staphylococci (3 *S. simulans*, 3 *S. epidermidis*) in mild growth. Additionally, group G ($n = 6$) containing ewes with no bacteria in the teat duct was included in the experiment; their selection was carried out as above (2.1.2.) and they were used as negative controls.

After selection, the animals were hand-milked thrice daily. Then, the lower 3.0 to 3.5 cm of both teats of group E ewes or one teat of group G ewes were immersed into a 1 N solution of NaOH for 1 min; the procedure was repeated on the following day (D−1 and D0). The resulting chapping was scored according to the standards described by Fox et al. [15] and Mavrogianni et al. [38].

Ultimately, the teats of the ewes into each group were naturally infected (NI) and/or chapped (CP) as follows: group E: one teat NI+/CP+, the other teat NI−/CP+; group F: one teat NI+/CP−, the other teat NI−/CP−; group G: one teat NI−/CP+, the other teat NI−/CP−.

2.1.4. *Experimental inoculation of* S. simulans *from the teat duct of a ewe, into sheep mammary gland cisterns or teat ducts: Experiment III*

Eight lactating primiparous Karagouniko-breed ewes were used in the experiment. Lambs of these ewes were weaned 18 days after lambing and subsequently, the animals were hand-milked thrice daily. No bacteria were isolated from any teat catheter or mammary secretion samples obtained. They were allocated into one of two groups (H or I, $n = 4$) and challenged with a *S. simulans*, recovered from the teat duct of one of group E ewes (Experiment II). Ewes in group H were challenged 2 mm deep into the teat duct and ewes in group I were challenged directly into the gland cistern.

For inoculation (D0), the same procedure as in Experiment I was carried out. The inoculum contained 10^6 c.f.u., as estimated by the method of Miles and Misra [43]. The left teat duct (group F) or mammary gland cistern (group G) of the ewes was challenged. By using the same technique, 0.2 mL of PBS was injected into the right teat duct (group F) or mammary gland cistern (group G) of the ewes.

Ultimately, the teats and the mammary glands of the ewes into each group were naturally infected (NI) and/or challenged (CH) as follows; group H: one teat NI−/CH+, respective gland NI−/CH−, the other teat NI−/CH−, respective gland NI−/CH−; group I: one teat NI−/CH−, respective gland NI−/CH+, the other teat NI−/CH−, respective gland NI−/CH−.

2.2. Post-inoculation/chapping examinations

After challenge or chapping, detailed clinical examination of the mammary glands and teats was carried out daily (unless of course, a ewe had been euthanised before that). Teat catheter samples and mammary secretion samples were collected. All samples were cultured onto Columbia blood agar; the media were incubated aerobically at 37 °C for up to 72 h.

The CMT was carried out in secretion samples, as described before [19]. Secretion films were stained by the Giemsa method; the percentage of leucocyte (macrophages, neutrophils, lymphocytes) subpopulations was determined.

The ewes were euthanised on the time-points detailed in Table I. Dissection of the mammary glands and the teats started immediately; it was carried out as described before [11, 36]. In A1, B1, C1, D and H group ewes, an electronic cutimeter was used to measure 2 mm from the teat orifice, in order to determine the precise site within the teat, where the inoculum had been deposited. Scrapings from each of the two sites sampled in each teat, as well as parenchyma samples were plated onto Columbia blood agar for bacteriological examination; the media were incubated aerobically at 37 °C for up to 72 h.

Throughout this study, conventional bacteriological techniques were used [2, 12]. Identification of staphylococci was carried by means of API-Staph SYSTEM quick identification strips (BioMerieux, Marcy-l'Étoile, France) and a complete series of biochemical tests. The API-Staph SYSTEM was carried out in all staphylococcal isolates recovered during this study; profiles of isolates were compared among them.

Longitudinal sections, involving all the structures of the teat, were taken out for histological examination. Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax, using conventional techniques. Haematoxylin and eosin (HE) standard staining procedures were performed for histopathological examination.

2.3. Data management and analysis

Quantitative information on the cellular content of the mammary secretion of the ewes was obtained by using two sets of data. First, the CMT results were assigned numerical values as follows; value 0 to score "negative", value 1 to score "trace", value 2 to score "1", value 3 to score "2" and value 4 to score "3". Second, the results of the Microscopic cell counting method (Mccm, IDF reference method) [8,24,49], which had been applied as part of the departmental quality assurance procedure, were taken into account. Although it is generally well established that CMT results are reliable proxy measurements for somatic cell counts (SCC) [17, 20], we confirmed this as part of the quality control in this study. Following log-transformation, the correlation between Mccm SCC and CMT was $r = 0.93$ with a corrected R^2 of 87%.

A scoring system previously developed and described [36] was used and numerical values were assigned for the pathological findings in the experimental animals. A separate score (0–4 scale) was given for macroscopic and for histological findings in the teat and the mammary gland; these were then added to a 0–16 scale to produce a pathology score for the findings in each ewe. The system is detailed in Table II.

Statistical analyses were performed in Minitab 14 (Minitab Inc., State College, PA, USA) and Epi-Info 6 (CDC, Atlanta, GA, USA). For analysis, the proportion of positive bacteriological and CMT results between the different groups/subgroups were compared by using the Chi-square test or the Fisher Exact Test, as appropriate. Total pathology scores were compared using the Friedman Test using each day's total score as the unit and with group as "Treatment" and day number as "Block". Exact binomial Confidence Intervals (CI) for proportions were calculated. For the comparison of Mccm SCC the readings were first logged. For each experimental group a "baseline" value was obtained as the mean pre-treatment value (i.e., before treatment or chapping) and this baseline value was subtracted from all subsequent readings. Comparisons between treatments used one-way Analysis of Variance on all baseline-corrected readings obtained after Day 0. Statistical tests were 2-Sided.

Table II. Description of scores given for pathological findings in teats and mammary parenchyma of experimental ewes.

Score	Description
Teat – Macroscopic pathological findings	
0	Normal
1	Presence of folds on the mucosa of the teat
2	Hyperaemia of the mucosa of the teat
3	Thickening of the mucosa of the teat, with increased number of folds and presence of petechiae
4	Extreme thickening of the mucosa of the teat, with loss of the separation of the compartments of the teat
Teat – Histopathological findings	
0	Normal
1	Presence of a few, scattered leucocytes
2	Presence of increased numbers of leucocytes clustered under the epithelium of the teat
3	Presence of high numbers of leucocytes evenly distributed under the epithelium of the teat
4	Presence of high numbers of leucocytes, plus hyperplasia of lymphoid nodules in the teat duct–teat cistern border
Parenchyma – Macroscopic pathological findings	
0	Normal
1	Enlarged and swollen glands, with presence of subcutaneous oedema
2	Presence of fibrin and clots of milk, with focally reddened parenchyma
3	Mutlifocally reddened parenchyma, with distension of veins
4	Extensive haemorrhagic appearance, with masses of exudate and demarcation from the adjacent tissue
Parenchyma – Histopathological findings	
0	Normal
1	Presence of a few, scattered leucocytes
2	Presence of increased numbers of leucocytes clustered in the intra- and inter-alveolar area
3	Diffuse presence of leucocytes, extravasation and destruction of epithelial cells
4	Haemorrhages, destruction of alveoli and loss of the internal architecture of the parenchyma

3. RESULTS

3.1. Pre-inoculation/pre-chapping examinations

The mammary glands and the teats of all ewes were clinically healthy before challenge. The teats were soft with no external abnormalities. All selection criteria were fulfilled in the animals used. No bacteria were isolated from the mammary secretion. In Experiment I and Experiment II, bacteria recovered from teat duct catheter samples met the allocation criteria. In

Experiment III, no bacteria were isolated from the mammary secretion or the teat duct catheter samples obtained. The CMT was always negative; in Giemsa-stained secretion films, no leucocytes were observed.

3.2. Post-inoculation/post-chapping clinical, bacteriological and cytological findings

3.2.1. Deposition of M. haemolytica *into bacteriologically positive sheep teat ducts: Experiment I*

None of the ewes in subgroup A1, B1 or C1 developed clinical mastitis. From the NI+/CH+ side, *M. haemolytica* was isolated: in total, from 16/32, 24/32, 25/32 samples from A1, B1, C1 ewes, respectively; additionally, the initial bacterial flora was also isolated (confirmed by matching profiles in the API-Staph SYSTEM) from duct, but not from secretion, samples: in total, from 10/32, 14/32, 15/32 samples. The CMT increased (>"1"). Somatic cell counts in mammary secretion also increased significantly. Leucocytes (up to D2 mostly neutrophils with fewer macrophages and lymphocytes, subsequently lesser neutrophils with more macrophages and lymphocytes increased) were seen in Giemsa-stained secretion films.

None of the ewes in subgroup A2, B2 or C2 developed clinical or subclinical mastitis. From the NI+/CH- side, only the initial bacterial flora was isolated from the duct, but not from secretion, samples: in total, from 16/32 samples from ewes of each subgroup. The CMT remained negative (<"1"). Somatic cell counts in mammary secretion did not change.

None of the ewes in group D developed clinical mastitis. From the NI−/CH+ side, *M. haemolytica* was isolated: in total, from 49/64 samples. The CMT increased (>"1"). Somatic cell counts in mammary secretion also increased significantly. Leucocytes (proportions as above) were seen in Giemsa-stained secretion films.

No clinical signs were observed in any of the NI−/CH− sides (A, B, C, D ewes). No bacteria were recovered there. The CMT was always negative. Somatic cell counts in mammary secretion did not change. Details are given in Tables III, IV and V.

3.2.2. Artificial skin chapping on sheep teats with bacteriologically positive duct: Experiment II

All ewes in group E developed systemic and mammary signs. The teats became chapped to score "2" to "3". *Staphylococcus* spp., the same species as originally (before chapping) recovered from the teat duct catheter sample, were isolated in pure culture from the duct and secretion samples obtained after chapping: in total, from 71/72 samples. The CMT increased (≥ "2"). Somatic cell counts in mammary secretion also increased significantly. In Giemsa-stained secretion films, cocci were seen; leucocytes (initially neutrophils and macrophages, subsequently neutrophils with fewer lymphocytes and macrophages) were also observed. Control teats (NI−/CP+) of ewes of group E remained chapped to a score "2" to "3"; no clinical findings characteristic of mastitis were observed. No bacteria were recovered. The CMT was mildly positive (score "1"). Somatic cell counts in mammary secretion also increased. In Giemsa-stained secretion films, neutrophils were seen almost exclusively.

None of the ewes in group F developed clinical or subclinical mastitis. From the NI+/CP- side, only the initial bacterial flora was isolated from the duct, but not from secretion, samples: in total, from 36/72 samples. The CMT remained negative (<"1"). Somatic cell counts in

Table III. Cumulative bacteriological findings and CMT results in samples after challenge of ewes during the three experiments.

	Experiment I: subgroups						
	A1	A2	B1	B2	C1	C2	D
Bacterial isolation							
D-F[a]	10/16[b]	16/16	14/16	16/16	15/16	16/16	0/32
D-Mh[a]	10/16	0/16	14/16	0/16	15/16	0/16	29/32
S-F[a]	0/16	0/16	0/16	0/16	0/16	0/16	0/32
S-Mh[a]	6/16	0/16	10/16	0/16	10/16	0/16	20/32
CMT results							
Positive	14/16	0/16	14/16	0/16	14/16	0/16	28/32

	Experiment II: groups		
	E	F	G
Bacterial isolation			
D-F[a]	36/36	36/36	0/36
S-F[a]	35/36	0/36	0/36
CMT results			
Positive	36/36	0/36	32/36

	Experiment III: groups	
	H	I
Bacterial isolation		
D-F[a]	0/24	0/24
D-Ss[a]	22/24	4/24
S-F[a]	0/24	0/24
S-Ss[a]	16/24	24/24
CMT results		
Positive	19/24	24/24

[a] D-F = teat duct–flora, D-Mh = teat duct–*M. haemolytica*, S-F = secretion–flora, S-Mh = secretion–*M. haemolytica*, D-Ss = teat duct–*S. simulans*, S-Ss = secretion–*S. simulans.*
[b] n/m = positive results out of total animals sampled.

mammary secretion did not change. The chapped teat of ewes of group G were scored "2" to "3". No mastitis was observed. From the NI−/CP+ side, no bacteria were recovered from any duct or secretion samples: from 0/72 samples. The CMT was positive (score "1"). Somatic cell counts in mammary secretion increased significantly. Neutrophils were mostly seen in Giemsa-stained secretion films. No clinical signs were observed in any of the NI−/CP− sides (F, G ewes). No bacteria were recovered. The CMT was negative. Somatic cell counts in mammary secretion did not change. Details are in Tables III, IV and V.

Table IV. Results (median values) of cellular content in secretion samples from experimental animals during the study.

	Experiment I/Subgroups/Treatments													
	A1		A2		B1		B2		C1		C2		D	
	NI+/CH+	NI–/CH–	NI+/CH–	NI–/CH–	NI+/CH+	NI–/CH–	NI+/CH–	NI–/CH–	NI+/CH+	NI–/CH–	NI+/CH–	NI–/CH–	NI–/CH+	NI–/CH–
Somatic cell counts ($\times 10^6$ cells per mL) – Microscopic cell counting method														
Period 1	0.249	0.173	0.225	0.198	0.194	0.149	0.226	0.245	0.198	0.174	0.153	0.200	0.205	0.212
Period 2	2.183	0.308	0.204	0.254	1.709	0.209	0.179	0.265	1.595	0.307	0.212	0.307	1.632	0.241
CMT results (min. 0, max. 4)														
Period 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Period 2	4	0	0	0	4	0	0	0	4	0	0	0	4	0

	Experiment II/Groups/Treatments					
	E		F		G	
	NI+/CP+	NI–/CP+	NI–/CP+	NI–/CP–	NI–/CP+	NI–/CP–
Somatic cell counts ($\times 10^6$ cells per mL) – Microscopic cell counting method						
Period 1	0.182	0.293	0.165	0.343	0.182	0.215
Period 2	Abnormal samples	0.740	0.258	0.222	0.786	0.149
CMT results (min. 0, max. 4)						
Period 1	0	0	0	0	0	0
Period 2	4	3	0	0	2	0

	Experiment III/Groups/Treatments			
	H		I	
	t NI–/CH+ // g NI–/CH–	t NI–/CH– // g NI–/CH–	t NI–/CH– // g NI–/CH+	t NI–/CH– // g NI–/CH–
Somatic cell counts ($\times 10^6$ cells per mL) – Microscopic cell counting method				
Period 1	0.219	0.223	0.166	0.232
Period 2	0.883	0.138	Abnormal samples	0.181
CMT results (min. 0, max. 4)				
Period 1	0	0	0	0
Period 2	3	0	4	0

Period 1: D–6 to D–2, Period 2: D1 to D7 (after treatment: challenge or chapping).

Table V. Statistical significance in comparison with bacteriological findings and CMT results in samples after challenge of ewes during the three experiments ($P =$).

Comparisons between	Bacteriological results				CMT results	SCC results
	D-F[a]	D-Mh[a]	S-F[a]	S-Mh[a]		
A1 vs. A2	0.018	< 0.001	1.00	0.018	< 0.001	< 0.001
A1 vs. D	< 0.001	0.044	1.00	0.101	1.00	0.671
B1 vs. B2	0.484	< 0.001	1.00	< 0.001	< 0.001	0.001
B1 vs. D	< 0.001	> 0.9	1. 00	> 0.9	1.00	0.935
C1 vs. C2	> 0.9	< 0.001	1.00	< 0.001	< 0.001	0.005
C1 vs. D	< 0.001	> 0.9	1.00	> 0.9	1.00	0.925
A1 vs. B1 vs. C1	0.046	0.046	1.00	> 0.25	> 0.25	0.921
Comparisons between	Bacteriological results				CMT results	SCC results
	D-F[a]		S-F[a]			
E vs. F	1.00		< 0.001		< 0.001	ND
E vs. G	< 0.001		< 0.001		0.11	0.012
F vs. G	< 0.001		1.00		< 0.001	ND
Comparisons between	Bacteriological results				CMT results	SCC results
	D-F[a]	D-Ss[a]	S-F[a]	S-Ss[a]		
H vs. I	1.00	< 0.001	1.00	< 0.004	0.049	ND

[a] D-F = teat duct–flora, D-Mh = teat duct–*M. haemolytica*, S-F = secretion–flora, S-Mh = secretion–*M. haemolytica*, D-Ss = teat duct–*S. simulans*, S-Ss = secretion–*S. simulans.*
ND: not done (abnormal samples).

3.2.3. Experimental inoculation of S. simulans *from the teat duct of a ewe, into sheep mammary gland cisterns or teat ducts: Experiment III*

Ewes in group H did not become systemically ill, nor developped abnormal mammary signs. The secretion was found to be transiently abnormal (serous with flakes) from D2 to D3. The inoculated teats became hard (D5) and expression of milk was difficult. *S. simulans* was consistently isolated in pure culture from all duct and many secretion samples: in total, from 38/48 samples. The CMT increased (≥ "2"). Somatic cell counts in mammary secretion increased significantly. In Giemsa-stained secretion films, cocci and leucocytes (up to D2 mainly neutrophils, subsequently neutrophils, lymphocytes and macrophages) were seen.

Ewes in group I became systemically ill and developed abnormal mammary signs. *S. simulans* was consistently isolated in pure culture from few duct and all secretion samples: in total, from 28/48 samples. The CMT increased (≥ "2"). Somatic cell counts in mammary secretion also increased significantly. In Giemsa-stained secretion films, cocci and leucocytes (up to D2 mainly neutrophils, subsequently neutrophils, lymphocytes and macrophages) were seen.

No clinical signs were observed in any of the teats and glands (NI–/CH–) of these ewes injected with PBS. No bacteria were recovered from any duct or secretion sample obtained from these. The CMT was negative. Somatic cell counts in mammary

secretion did not change. Details are given in Tables III, IV and V.

3.3. Pathological findings

3.3.1. Deposition of M. haemolytica *into bacteriologically positive sheep teat ducts: Experiment I*

Measurement of the length of the internal teat structures after dissection of the teats of ewes of A1, B1, C1 and D groups, showed that the inoculum had always been deposited within the teat duct.

Post-mortem bacterial isolations were as follows. *M. haemolytica* was isolated from the NI+/CH+ side of A1, B1 and C1 ewes: from 4/12, 10/12, 9/12 sites sampled, respectively ($P = 0.024$). The initial bacterial flora was also isolated: from 3/12, 8/12, 8/12 sites sampled, respectively ($P = 0.062$). Only the initial bacterial flora (confirmed by matching profiles in the API-Staph SYSTEM) was isolated from the NI+/CH- side of A2, B2 and C2 ewes: from 7/12, 8/12, 7/12 sites sampled, respectively ($P = 0.89$). *M. haemolytica* was isolated in pure culture from the NI–/CH+ side of D ewes: from 15/24 sites sampled (0.625, 95% C.I.: 0.41-0.81). Statistical comparisons revealed that for A1 versus D, $P = 0.044$, whilst for B1 or C1 versus D, $P > 0.4$. No bacteria were isolated from the contralateral side (NI–/CH–) of these ewes (Tab. VI).

Mild macroscopic changes were seen in the NI+/CH+ teats of ewes in subgroups A1, B1 and C1; some folds and mild thickening were evident. Histologically, sub-epithelial leucocytic infiltration was the salient feature; in some teats, lymphoid nodules with or without germinal activity, characteristically present at the border between teat duct-teat cistern were recorded. No macroscopic changes were seen in the respective parenchyma and the supra-mammary lymph nodes. Histologically, leucocytic infiltration (mainly neutrophils), lysis of neutrophils and extravasation were evident. The total pathology scores summed over all days were 27, 33 and 35 for A1, B1 and C1 ewes ($P = 0.041$), respectively (maximum possible: 64; Tab. VII).

No macroscopic changes were seen in the NI+/CH− teats of ewes in subgroups A2, B2 and C2. Histologically, mild subepithelial leucocytic infiltration was the salient feature; these leucocytes were observed as small aggregates of cells deep in the epithelium. Lymphocytes were the predominant cell type subjacent to the teat cistern epithelium. In the teat duct wall however, sub-epithelial lymphocytes and neutrophils were seen in almost equal proportions. Lymphoid nodules were detected in 6 (50%) ewes at the border between the teat duct-teat cistern. No changes were seen in the respective mammary parenchyma or the supra-mammary lymph nodes of these ewes. The total pathology scores summed over all days were 8, 8 and 6 ($P = 0.37$) for A2, B2 and C2 ewes, respectively (maximum possible: 64; Tab. VII).

Macroscopically, a rough internal lining, folds and petechiae were seen in the teat duct of ewes in group D (NI–/CH+ side). Histologically, there was subepithelial leucocytic infiltration at the border between the teat duct-teat cistern; hyperplastic lymphoid nodules with germinal activity were observed. No macroscopic changes were seen in the respective mammary parenchyma or the supra-mammary lymph nodes; leucocytic infiltration, lysis of neutrophils, extravasation and destruction of epithelial cells were histologically evident. The median total pathology score summed over all days was 36 (maximum possible: 64; Tab. VII). Statistical comparisons revealed that for A1 versus D, $P = 0.038$, whilst for B1 or C1 versus D, $P > 0.6$. No macroscopic or histological lesions were recorded on the contralateral side of these ewes (NI–/CH–). The median total pathology score summed

Table VI. Post-mortem bacteriological findings in samples from ewes during Experiment I.

	Days after challenge / Groups													
	D1							D3						
	A1	A2	B1	B2	C1	C2	D	A1	A2	B1	B2	C1	C2	D
D-F[a]	1/1[b]	1/1	1/1	1/1	1/1	1/1	1/2	0/1	1/1	1/1	1/1	1/1	1/1	0/2
D-Mh[a]	1/1	0/1	1/1	0/1	1/1	0/1	2/2	0/1	0/1	1/1	0/1	1/1	0/1	2/2
C-F[a]	1/1	1/1	1/1	1/1	1/1	1/1	0/2	0/1	1/1	1/1	1/1	1/1	1/1	0/2
C-Mh[a]	1/1	0/1	1/1	0/1	1/1	0/1	1/2	0/1	0/1	1/1	0/1	1/1	0/1	2/2
P-F[a]	0/1	0/1	0/1	0/1	0/1	0/1	0/2	0/1	0/1	0/1	0/1	0/1	0/1	0/2
P-Mh[a]	1/1	0/1	1/1	0/1	1/1	0/1	1/2	0/1	0/1	0/1	0/1	0/1	1/1	1/2
	D5							D7						
	A1	A2	B1	B2	C1	C2	D	A1	A2	B1	B2	C1	C2	D
D-F[a]	1/1	1/1	1/1	1/1	1/1	1/1	0/2	0/1	1/1	1/1	1/1	1/1	1/1	0/2
D-Mh[a]	1/1	0/1	1/1	0/1	1/1	0/1	2/2	0/1	0/1	1/1	0/1	1/1	0/1	1/2
C-F[a]	0/1	0/1	1/1	1/1	1/1	1/1	0/2	0/1	1/1	1/1	1/1	1/1	0/1	0/2
C-Mh[a]	0/1	0/1	1/1	0/1	1/1	0/1	1/2	0/1	0/1	1/1	0/1	1/1	0/1	1/2
P-F[a]	0/1	0/1	0/1	0/1	0/1	0/1	0/2	0/1	0/1	0/1	0/1	0/1	0/1	0/2
P-Mh[a]	0/1	0/1	1/1	0/1	0/1	0/1	1/2	0/1	0/1	0/1	0/1	0/1	1/1	0/2

[a] D-F = teat duct–flora, D-Mh = teat duct–*M. haemolytica*, C-F = teat cistern–flora, C-Mh = teat cistern–*M. haemolytica*; P-F = parenchyma–flora, P-Mh = parenchyma–*M. haemolytica.*
[b] n/m = positive results out of total animals sampled.

over all days was 0 (maximum possible: 64).

3.3.2. Artificial skin chapping on sheep teats with bacteriologically positive duct: Experiment II

Post-mortem bacterial isolations were as follows. From the NI+/CP+ side of E ewes, *Staphylococcus* sp. same species as originally recovered from the teat duct catheter sample were consistently isolated in pure culture from the teat duct, teat cistern and mammary parenchyma: from 18/18 sites sampled; no bacteria were isolated from the contralateral side (NI−/CP−). *Staphylococcus* sp. same species as originally recovered from the teat duct catheter, were consistently isolated in pure culture from the teat duct from the NI+/CP− side of F ewes: from 6/18 sites sampled. No bacteria were isolated from the other side (NI−/CP−). No bacteria were recovered either from the NI−/CP+ side of group G ewes: from 0/18 sites sampled, or from their other side (NI−/CP−) (Tab. VIII).

The principal macroscopic lesions inside the teats of group E ewes (NI+/CP+ side) were folds, hyperaemia and thickness of the mucosa. Histologically, we recorded principally extensive subepithelial neutrophilic infiltration, lysis of neutrophils and destruction of epithelial cells; a conspicuous lymphoid area with germinal center was also observed. In the skin of all teats studied, erosion and ulceration with the presence of sero-cellular crusting and leucocytic accumulation were evident. Macroscopic lesions in the respective mammary parenchyma included subcutaneous oedema and sanguineous fluid exuding from sections of the reddened

Table VII. Total scores for pathology findings in ewes during Experiment I.

Subgroup	Day after challenge												Total sum
	D1			D3			D5			D7			
	Teat	Gland	Total	Teat	Gland	Total	Teat	Gland	Total	Teat	Gland	Total	
A1	7	1	8	6	1	7	6	2	8	3	1	4	27
A2	2	0	2	2	0	2	1	1	2	1	1	2	8
B1	7	2	9	7	2	9	7	2	9	5	1	6	33
B2	1	0	1	2	0	2	2	1	3	1	1	2	8
C1	7	3	10	6	3	9	6	2	8	6	2	8	35
C2	1	0	1	2	1	3	1	0	1	1	0	1	6
D[a]	7	1	8	6	2	8	6	4	10	7	3	10	36

[a] Scores in group D are presented as median score of the two animals euthanised at each time-point.

Table VIII. Post-mortem bacteriological findings in samples from ewes during Experiment II.

	Days after challenge / Groups					
	D5			D7		
	E	F	G	E	F	G
D-F[a]	3/3[b]	3/3	0/3	3/3	3/3	0/3
C-F[a]	3/3	0/3	0/3	3/3	0/3	0/3
P-F[a]	3/3	0/3	0/3	3/3	0/3	0/3

[a] D-F = teat duct–flora, C-F = teat cistern–flora, P-F = parenchyma–flora.
[b] n/m = positive results out of total samples.

parenchyma; the supramammary lymph nodes were enlarged. Histologically, neutrophilic infiltration, extravasation, intraalveolar live and exhausted neutrophils, destruction of epithelial cells, alveolar destruction, lymphocytic infiltration and haemorrhages were evident. The median total pathology score summed over all days was 28 (maximum possible: 32; Tab. IX).

In ewes of group F (NI+/CP− side), macroscopic and histological changes in the teat and the respective mammary parenchyma were in general similar to those described for ewes in subgroups A2, B2, C2. The median total pathology score summed over all days was 3 (maximum possible: 32; Tab. IX).

In the teats of ewes G (NI−/CP+ side), skin lesions as above were evident. Mild mucosal thickening, leucocytic infiltration and presence of a hyperplastic lymphoid area were found. No macroscopic changes were seen in the mammary parenchyma; histologically, some leucocytes were seen. The median total pathology score summed over all days was 14 (maximum possible: 32; Tab. IX).

Macroscopic or histological lesions recorded on the contralateral side of group E ewes (NI-/CP+) were limited to skin lesions similar to those described above; on the inside, mild mucosal thickening, leucocytic infiltration and presence of a hyperplastic lymphoid area were found. No macroscopic changes were in the parenchyma; histologically, a few leucocytes were observed. The median total pathology score summed over all days was 14 (maximum possible: 32). No lesions were recorded on the contralateral teat and mammary gland of group F and G ewes (NI-/CP+ side). The median total pathology score summed over all days was 0 for ewes of both groups (maximum possible: 32).

Table IX. Total scores for pathology findings in ewes during Experiment II.

Group	Days after challenge						Total sum
	D5			D7			
	Teat	Gland	Total	Teat	Gland	Total	
E[a]	7	7	14	6	8	14	28
F[a]	1	1	2	1	0	1	3
G[a]	6	1	7	6	1	7	14

[a] Scores are presented as median score of the three animals euthanised at each time-point.

3.3.3. Experimental inoculation of **S. simulans** *from the teat duct of a ewe, into sheep gland cisterns or teat ducts: Experiment III*

Measurement of the length of the internal teat structures after dissection of the teats of ewes of group H showed that the inoculum had always been deposited within the teat duct.

Post-mortem bacterial isolations were as follows. *S. simulans* was isolated in pure culture from group H ewes: from 7/12 sites sampled (CH+ side); no bacteria were isolated from the other teat and mammary gland of these ewes (CH− side). *S. simulans* was isolated in pure culture from group I ewes: from 9/12 sites sampled (CH+ side); no bacteria were isolated from the other side (CH− side) (Tab. X).

Macroscopically, a rough internal lining, folds and petechiae were seen in the teat duct of group H ewes (CH+ side). Histologically, there was subepithelial leucocytic infiltration; the presence of hyperplastic lymphoid nodules with germinal activity was recorded. No gross pathological findings were evident in the respective mammary parenchyma or the supra-mammary lymph nodes; leucocytic infiltration (neutrophils and lymphocytes), lysis of neutrophils, extravasation and destruction of epithelial cells were histologically evident. The median total pathology score summed over all days was 22 (maximum possible: 32; Tab. XI).

Table X. Post-mortem bacteriological findings in samples from ewes during Experiment III.

	Days after challenge / Groups			
	D5		D7	
	H	I	H	I
D-Ss[a]	2/2[b]	1/2	1/2	0/2
C-Ss[a]	2/2	2/2	1/2	2/2
P-Ss[a]	1/2	2/2	0/2	2/2

[a] D-F = teat duct–*S. simulans*, C-F = teat cistern–*S. simulans*, P-F = parenchyma–*S. simulans.*

[b] n/m = positive results out of total samples.

Macroscopically, no gross pathological changes were seen in the teat ducts of group I ewes (CH+ side); histologically, there was mild subepithelial leucocytic infiltration in some of these. Macroscopic lesions in the respective mammary parenchyma included grossly swollen gland with subcutaneous oedema and sanguineous fluid exuding from sections of the reddened parenchyma; threads of fibrin were recorded; the supramammary lymph nodes were enlarged. Histologically, the salient feature was conspicuous neutrophilic infiltration and elimination of structural elements of the gland. The total pathology score summed over all days was 16 (maximum possible:32; Tab. XI).

No macroscopic or histological lesions were recorded on the contralateral

Table XI. Total scores for pathology findings in ewes during Experiment III.

Group	Days after challenge						Total sum
	D5			D7			
	Teat	Gland	Total	Teat	Gland	Total	
H[a]	7	3.5	10.5	7.5	4	11.5	22
I[a]	0.5	7.5	8	0.5	7.5	8	16

[a] Scores are presented as median score of the two animals euthanised at each time-point.

side (NI−/CH− side). The median total pathology score summed over all days was 0 for ewes of both groups (maximum possible: 32).

4. DISCUSSION

Previous experimental studies on ovine mastitis have established the protective role of the teat against intramammary infections; Mavrogianni et al. [36] studied the effects of the inoculation of *M. haemolytica* in different sites of healthy, bacteriologically negative teats. The results of that study showed that the ovine teat acts as a barrier against bacteria. During that study, we also suggested that the bacterial flora present in the teat duct of healthy ewes[1] [18] might act competitively against invading bacteria and thus provide one of the defence mechanisms active in the teat.

Most authors who studied the possible role of bacterial flora in the mammary gland of cows have indeed reported a protective effect: the presence of coagulase-negative staphylococci within the mammary gland was associated with reduced incidence risk of experimentally induced mastitis [29, 33, 35, 46]. However, Hogan et al. [23] did not find any effect under field conditions and did not support the hypothesis. There are marked differences between cows and ewes: *M. haemolytica* is a common mastitis agent in sheep, whilst *Escherichia coli* or *Streptococcus uberis* are not; ewes suckle their lambs for a period of up to three months, subsequently being hand-milked [3, 4]. Therefore, findings from one species should not be directly extrapolated for the other. The role of bacterial flora residing apically at the teat duct in the pathogenesis of ovine mastitis has not been studied.

In the present work, we inoculated bacteriologically positive teats with a *M. haemolytica* isolate, in order to study possible interactions between the bacterial flora and a confirmed mastitis causal agent [3, 4]. Ewes in subgroup A1 and D developed subclinical mastitis. However, recoveries of the challenge organism from the former animals were significantly fewer than from controls, thus suggesting an effect on the challenge strain; furthermore, the severity of the mammary lesions was significantly smaller in A1 than in D ewes. Adherence of *M. haemolytica* on mammary epithelial cells is required for its multiplication and leucotoxin production [55]; based on the present findings, one may postulate that the bacterial flora inhibited that process. No such bacteriological and pathological differences were seen in B1 and C1 ewes; this suggests that the protective effect of bacterial flora was exercised preferentially and only by staphylococcal

[1] Mavrogianni V.S., Protective role of the teat and importance of its disorders in ovine mastitis associated with *Mannheimia haemolytica*, Ph.D. thesis, University of Thessaly, Greece, 2006.

species present in large numbers within the teat duct.

Bacterial populations interact among themselves and constitute a "community" where each species contributes to its stability. Many mechanisms by which bacteria and their interactions prevent the invasion and colonisation of pathogenic microorganisms have been proposed, but are not all fully understood. Occupation of the host's epithelial surfaces by bacterial flora and thus, prevention of pathogen adherence on these cells [5, 6], can be particularly important, given that adherence on mammary epithelial cells by *M. haemolytica* is necessary for leucotoxin production [55]. Since staphylococci adhere to mammary epithelial cells forming biofilms [13, 42], one may postulate this as the primary mechanism by which the protective effects took place in the present experiments.

Bacterial competition is the situation where two bacterial populations compete for multiplication and survival, usually resulting in cell population reduction or impeded growth rate than if the two populations were separated [25]. This was evident in subgroup A1, where a distinct protective effect of the flora was recorded. Both the flora populations and the challenge, invading organism were subsequently recovered from a reduced number of samples than from the respective controls. This type of relationship between bacteria occurs when two species compete to occupy a particular site [32, 54]. Rainard and Poutrel [46] have also reported that new infections were less frequent in glands already harbouring a pathogen. All these findings further support the above hypothesis.

Production of antagonistic substances by bacterial flora and competition for necessary nutritional substances between flora and invading organisms [52] are also contributing mechanisms. The direct toxic effects of certain bacterial species against other ones invading the host have also been considered, since flora populations can secure their domination over invading pathogens by producing antibacterial substances [9, 26]. Staphylococcal strains isolated from the cows' teat orifice or mammary secretion have been found to produce bacteriocins and reduce in vitro growth of other pathogens [10, 44].

Previous attempts to explain protective roles of the bacterial flora in the teat and the mammary gland have often been based on bacterial interactions. One may also suggest that cellular defence mechanisms already elicited by the bacterial flora, could contribute to the efficient defensive process exhibited by the teat. For example, inflammation mediators may be present in the teat from the previous (flora) infection; these may facilitate the local cellular response and contribute to efficient host defence. Mavrogianni et al. [36] reported the presence of lymphoid nodules with germinal activity in the teat in cases of challenge with *M. haemolytica*; in subsequent field studies, these nodules were also described in clinically healthy teats and associated with bacterial isolation from these [40]. Detection of these nodules in the current work, with evidence of germinal activity indicates that they were active and that they participated in the efficient defence process against the challenge strain. Ramney [48] proposed that bacteria within the buccal cavity provoke an immune response resulting in the release of cytokines by lymphocytes and macrophages at the oral mucosa; these activated host's defence pathways in cases of new bacterial invaders. Similar mechanisms may be available at the teat.

However, when the microbial equilibrium is disrupted for any reason, it is possible that pathogenicity of the flora strains would increase, leading to disease. Mayrand and Grenier [41] studied bacterial interactions and found that once the intra-bacterial balance was broken, pathological changes were initiated. Under those circumstances, the flora would contribute to development of disease either

by facilitating an invader to fully expressing its pathogenicity or even by participating in the infectious process itself in order to establish the pathological findings. The findings of Experiments II and III clearly indicate that under certain circumstances, the resident bacterial flora can become pathogenic. Ewes in group E rapidly developed acute clinical mastitis without bacterial challenge; the disease was caused by the bacterial flora organisms, which multiplied and ascended to the mammary parenchyma. Lesions observed during this study were typical of staphylococcal mastitis[2] [16]. In this case, the "trigger factor" that led to the equilibrium shift was the teat chapping.

The strain used in Experiment III had been isolated from the teat duct of a healthy ewe, but was found to be pathogenic for the mammary gland when inoculated directly into the gland cistern (group I). This finding confirms that there was an equilibrium between host defences and bacterial virulence factors in the ewe, from which this strain had been originally isolated; this was changed in the experimental animals, where we inoculated the organism directly into the gland cistern, resulting in mastitis. In contrast to that, inoculation of the same isolate into healthy teats (group H) did not result in clinical mastitis, further confirming the protective role of healthy teats.

In a recent paper, Mavrogianni et al. [38] provided evidence that teat chapping predisposed ewes to mastitis in cases of new bacterial infections. Chapped teats are considered an increased risk for mastitis [45, 47]. During cold weather, increased incidence of chapped teats has been reported [14]. In ewes, Leyshon [31] and Clark[3] have reported that mastitis was more prevalent in cold weather; this could have been the consequence of chapped teats.

In damaged tissues there is reduced responsiveness and defective chemotaxis of neutrophils [7], which cannot withstand the low pH and high temperature in chapped tissues [21, 27]. Additionally, the reduced hydration of chapped skin alters skin microflora, consequently decreasing resistance to bacterial colonisation. We thus believe that in these circumstances, depletion of cellular defences consequently to chapping, resulted in shifting of the balance and allowed bacterial invasion and mastitis. One may also suggest that exposure to trauma may cause degranulation and lysis of mast cells, which are active during acute stages of inflammation [22], consequently reducing the defence abilities of the teat.

Perhaps under field conditions and on a longer-term basis, any factors affecting the immune status of the animals, would affect the equilibrium of flora organisms within the teat, thus resulting in mastitis. A characteristic example of another pathological condition, where bacterial flora can become pathogenic after "trigger factors" act on the animals, is enterotoxaemia [30]. Although intestinal bacterial flora is more abundant and more varied than that in the teat duct, one may suggest some similarities between enterotoxaemia and mastitis caused by teat flora. For example, the causative organisms are present in clinically unaffected animals. Predisposing factors range from changes in management to traumatic damage, causative organisms exhibit rapid multiplication and toxin production, the course of the disease is rather rapid [30, 53].

[2] Fthenakis G.C., Ovine mastitis with special reference to subclinical mastitis associated with coagulase-negative staphylococci, Ph.D. thesis, The Royal Veterinary College, University of London, Great Britain, 1988.

[3] Clark R.G., Field observations on ovine mastitis, Proc. 2nd Semin. N. Z. Sheep Vet. Assoc., Palmerston North, New Zealand, 1972, pp. 47-54.

It is also interesting that Mavrogianni et al. [39] have described cases of *M. haemolytica* isolation from the teat duct of hand-milked ewes; they attributed this to colonisation by the organism during the preceding suckling period. In such cases mastitis may be caused if the equilibrium within the teat changed. This hypothesis would explain cases of *M. haemolytica*-associated mastitis after weaning of lambs, for which no reasonable explanation had been available so far [51]. Alternatively, one may also consider this as the result of persistent subclinical infection, periodically causing exacerbation of the disease.

In the past, the presence of bacterial flora within a mammary gland has been advocated as a means of preventing mastitis in cows [34]. From that viewpoint, preservation of a protective teat duct flora would be useful for prevention of the disease. Nevertheless, an intramammary infection with a microorganism, even in small doses, might result in increased somatic cell counts, tissue damage and adverse production effects [50]. On the contrary, teats harbouring bacteria can be a source of infection for the mammary gland; in fact, long-term colonisation of the teat duct has been shown and confirms that bacteria can adapt to living in that area [47]. In a recent paper [39], it was shown that hand milking results in increased rate of bacterial colonisation within the teat duct. Therefore, if post-milking teat disinfection was omitted, the bacteria might become pathogenic for the mammary gland. Any impediment of the defence mechanisms (local or systemic) may shift the balance and allow the bacteria to multiply, invade the mammary gland and cause mastitis.

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