

Bacteriotherapy for Staphylococcal Mastitis: Evaluating the Efficacy of Intramammary Infusion of Live *Lactococcus lactis* in Ewes

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Abstract

Purpose. Alternatives to antibiotic therapy for mastitis in ruminants are needed. We present an evaluation, in two trials, of the efficacy of an intramammary infusion of a live culture of *Lactococcus lactis* for the treatment of subclinical and clinical mastitis in ewes.

Methodology. In total, 67 animals were enrolled: 19 lactating ewes (study 1), including healthy (N=6) and coagulase-negative staphylococci (CNS)-infected ewes (N=13); and 48 lactating ewes (study 2) with either CNS mastitis (N=32), or *Staphylococcus aureus* mastitis (N=16), for a total of 123 mammary glands. Intramammary infusions were performed with either *L. lactis* or PBS for 3 (study 1) or 7 (study 2) consecutive days. Antibiotic-treated and untreated control glands were included. Milk samples for microbiology, somatic cell analysis and milk production were collected before and after treatment.

Results/Key findings. *L. lactis* rapidly activated the mammary glands' innate immune response and initiated an inflammatory response as evidenced by the recruitment of polymorphonuclear neutrophils and increased somatic cell counts. But while leading to a transient clearance of CNS in the gland, this response caused mild to moderate clinical cases of mastitis characterized by abnormal milk secretions and udder inflammation. Moreover, *S. aureus* infections did not improve, and CNS infections tended to relapse.

Conclusion. Under our experimental conditions, the *L. lactis* treatment led to a transient clearance of the pathogen in the gland, but also caused mild to moderate clinical cases of mastitis. We believe it is still early to implement bacterial formulations as alternatives in treating mastitis in ruminants and further experimentation is needed.

INTRODUCTION

Mastitis is estimated to be the most expensive disease on dairy farms worldwide due to reduced milk yield and quality, animal replacement costs, veterinary services and labour expenses. The annual incidence of clinical mastitis in small ruminants – sheep and goats – is generally lower than 5 %, but can, in rare cases, exceed 30–50 %, causing mortality or culling of up to 70 % of the flock [1].

Mastitis is an inflammation of the mammary gland, often caused by bacterial infection. *Staphylococcus* spp. are the most frequently diagnosed causal micro-organisms of ovine mastitis: the most prevalent isolates are *S. aureus* in clinical cases and coagulase-negative staphylococci (CNS) in subclinical mastitis [1, 2]. *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus simulans* and *Staphylococcus xylosus* are the most frequently isolated CNS in ewes [1, 3, 4]. Detailed protocols for the treatment of mastitis, currently available for cows, have not

yet been published for small ruminants, and in many countries, few drugs are licensed for use in sheep [5] studies are needed to perfect this strategy [2]. The improvement of general hygiene standards in flocks remains the best strategy to control mastitis.

Due to concerns regarding the presence of antibiotic residues in milk and dairy products, and the risk of transmission of antibiotic resistance to both indigenous microbiota and potential pathogens, the widespread use of antibiotics is advised against, and the development of alternatives is strongly advocated.

Several new treatments have been recently proposed as alternatives to antibiotics in preventing and treating bovine mastitis [6, 7]. Examples are the use of lactic acid bacteria (LAB), generally recognized as safe, and their antimicrobial peptides (bacteriocins) – both of which are able to inhibit the growth of Gram-positive bacteria. Previous *in vivo* studies in lactating dairy cows have shown intramammary

infusions of the bacteriocin nisin to be effective in the treatment of clinical and subclinical mastitis caused by several different pathogens [8, 9]. The intramammary infusion of another bacteriocin – lacticin 3147 – showed the potential to prevent streptococcal [10] and staphylococcal [11] infections in cattle. Encouraging results in treating bovine chronic subclinical and clinical mastitis were also obtained with the inoculation of a live culture of *Lactococcus lactis* DPC 3147, a treatment which was as safe and effective as common antibiotic therapies [12]. All the above studies opened up new and promising avenues for research on the potential use of LAB and their products as novel, natural approaches to the prevention and treatment of mastitis in ruminants.

We report the results of two field studies assessing, for the first time, the efficacy of intramammary infusion of a live culture of a nisin-producing *L. lactis* strain in the treatment of clinical and subclinical staphylococcal mastitis in naturally infected sheep.

METHODS

Selection of animals and study design

In each of the two studies, both healthy (i.e. having two healthy mammary glands) and mastitis-infected ewes (i.e. having at least one infected mammary gland) were enrolled. All animals were in late lactation. Before each trial, the health status of each animal's udder was monitored. Both infected and uninfected glands (or udder halves), were included. Diagnosis of clinical or subclinical mastitis for individual animals was made according to Fragkou et al. [13], although the somatic cell count (SCC) cutoff values for diagnosis of mastitis or healthy glands were slightly reduced on the basis of our experience on the Italian sheep breeds considered here. Briefly, SCC values $>0.510^6$ cells ml^{-1} indicate a mammary gland with clinical – mammary inflammation accompanied by clinically detectable changes in the mammary parenchyma or in milk – or subclinical mastitis – mammary inflammation in the absence of any clinically detectable changes. Diagnosis of subclinical mastitis was based on detection of infection (i.e. isolation of staphylococcal species other than *S. aureus* from the milk at least 10^3 c.f.u. ml^{-1}) and/or inflammatory reaction in the mammary gland. A gland was considered healthy in the absence of clinical signs of infection, with an SCC $<0.310^6$ cells ml^{-1} and a negative milk culture. A gland was considered 'suspected' with an SCC between 0.310^6 and 0.510^6 cells ml^{-1} and classified as 'infected' or 'healthy' if the simultaneous bacteriological result was positive or negative, respectively.

All animals were routinely milked twice a day. Pre-milking udder preparations were regularly made, consisting of forestripping and drying of teats with individual paper towels. A sanitizing product containing chlorhexidine was

used after milking all animals. Clinical examinations of the glands, as well as milk sampling for laboratory analysis, were performed in both trials.

Study 1

In 2014, 19 lactating Sarda breed ewes, including healthy (N=6) and mastitis-affected ewes (N=13), were selected from a single flock located in the area of Sassari, Sardinia, and housed at the facility of the Istituto Zooprofilattico Sperimentale of Sardinia under the same management conditions. The Sarda breed is indigenous to the island of Sardinia. The infected animals all presented subclinical mastitis by CNS, and in most of them infection was bilateral. A total of 38 mammary glands, including both infected and healthy glands, were included in the study. CNS-infected glands (n=21) were randomly divided into three groups: CNS-LL (treated with *L. lactis*), CNS-PBS (treated with PBS) and CNS-UNT (infected control glands left untreated). Healthy glands (n=17) were randomly divided into two groups: HLT-LL (healthy glands treated with *L. lactis*) and HLTUNT (healthy control glands left untreated). All groups are listed in Table 1. For further details, see Treatment protocols below.

Study 2

In 2015, 48 mastitis-affected lactating ewes, mainly of the Valle del Belice and Comisana breeds – both breeds are indigenous to the island of Sicily, were selected from two neighbouring flocks from the area of Catania, Sicily. The Valle del Belice breed has originated from cross-breeding between Sarda, Cosimana and Pinzirita sheep breeds which are all autochthonous Italian sheep breeds. The trial was carried out in the animals' native farms. All ewes were subject to the same management conditions. The ewes suffered either from subclinical mastitis by CNS (N=32), or from clinical mastitis by *S. aureus* (N=16). Infections were either unilateral or bilateral, and bilateral infections could be caused by one or both of the pathogens (with one gland infected by CNS and the other by *S. aureus*). Glands whose microbiology or SCC results were unavailable during the selection period, were excluded from the study. A total of 85 mammary glands, including both infected (n=72) and healthy (n=13) glands, were considered. The infected glands were culture-positive either for CNS (n=56) or *S. aureus*

Table 1. Mammary glands: groups and administered treatments in the two studies

Health status of ewes (N)	Health status of glands (n)	Treatment*	Group
Study 1			
Unilateral or bilateral subclinical CNS mastitis (13)	CNS-infected glands (21)	L. lactis	CNS-LL (13)
		PBS	CNS-PBS (4)
		Untreated	CNS-UNT (4)
Healthy (6)	Healthy glands (17)	L. lactis	HLT-LL (7)
		Untreated	HLT-UNT (10)
Total ewes (19)	Total glands (38)	–	–
Study 2			
Unilateral or bilateral subclinical CNS mastitis (32)	CNS-infected glands (56)	L. lactis	CNS-LL (25)
		PBS	CNS-PBS (8)
		Oxytetracycline	CNS-DR (14)
		Untreated	CNS-UNT (9)
Unilateral clinical <i>S. aureus</i> mastitis (8)	<i>S. aureus</i> -infected glands (16)	L. lactis	S.AU-LL (10)
		Oxytetracycline	S.AU-DR (6)
Bilateral CNS and <i>S. aureus</i> clinical mastitis (8)	Healthy glands (13)	Untreated	HLT-UNT (13)
Total ewes (48)	Total glands (85)	–	–

N, number of ewes; n, number of mammary glands; PBS, sterile saline solution.

*L. lactis and PBS were administered daily into the teat canal for 3 consecutive days, with a 24 h interval between treatments. Oxytetracycline was administered following the manufacturer's instructions.

(n=16). CNS-infected glands were randomly divided into four groups: CNS-LL, CNS-PBS, CNS-DR (treated with oxytetracycline) and CNS-UNT (infected control group left untreated). *S. aureus*-infected glands were randomly divided into two groups: S.AU-LL (treated with *L. lactis*) and S.AU-DR (treated with oxytetracycline). Healthy glands were all included in a single, untreated control group: HLT-UNT. All groups are listed in Table 1. For further details, see Treatment protocols below.

Treatment protocols

During the course of experimental procedure ewes were housed in establishments approved by the Italian Ministry of Health (study 1) or in the animals' native farms (study 2), according to their biological characteristics, husbandry and feed requirements to ensure animal welfare. All experimental procedures were designed to minimize discomfort distress and pain to the animals and were approved by the Italian Ministry of Health (permit numbers 17757-31/12/ 2013 and I/0002003/14-31/01/2014). Veterinarians and animal care staff assured that the

approved procedures were followed. Animal care and treatment were conducted in accordance with both institutional guidelines and international laws and policies. At the end of the studies, animals returned to the herd of origin according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.

With the exception of oxytetracycline in study 2, which was injected intramuscularly, all treatments were infused directly into the teat sinus via the streak canal. Syringes with blunt, smooth tips (Bovivet Patteansats Cat. N 170260, Kuuse, UK) were used in order to ensure minimum pain, suffering or distress and prevent injury to the teat. Infusions were performed after the morning milking. In study 1, all treatments were administered for 3 consecutive days. In study 2, *L. lactis* and PBS treatments were extended to 7 consecutive days, while oxytetracycline treatments were administered following the manufacturer's instructions.

L. lactis

A live culture of the nisin-producing *L. lactis* subsp. *lactis* LMG 7930 (BCCM/LMG Bacteria Collection, Belgium) was prepared as follows: the bacterium was grown at 37 C for 24 h in Trypticase Soy Broth (TSB, BD, Italy). Altogether, 3 ml of this overnight culture (approximately 10^8 c.f.u.ml⁻¹; pH range of 5.0–5.30) were used for intramammary injections (groups CNS-LL, HLT-LL of study 1; and groups CNS-LL and S.AU-LL of study 2). In both studies, a 24 h interval between *L. lactis* treatments was observed.

PBS

Sterile pyrogen-free PBS solution (3 ml) was used for intramammary injections (groups CNS-PBS of study 1 and CNSPBS of study 2). In both studies, a 24 h interval between PBS treatments was observed.

Drug

Oxytetracycline (30 mg kg⁻¹ BW) was administered twice, by deep intramuscular injection (groups CNS-DR and S. AU-DR, study 2), with a 6 day interval between inoculations, as per the manufacturer's instructions (ALAMYCIN LA 300; Norbrook Laboratories, Ireland).

Clinical observations

Clinical examinations were performed daily throughout both studies, but recorded only in study 2. The udders were palpated and examined for soreness, swelling, hardness, redness and heat, and milk was inspected for alterations in colour and consistency (flakes, clots or watery appearance).

For each half udder, clinical mastitis was classified according to the severity of clinical signs as either mild (abnormal appearance of milk being the only sign), moderate (abnormal appearance of milk accompanied by swelling or redness of mammary gland) or severe (ewe exhibiting systemic signs of illness) [14].

Milk sampling and analysis

In both studies, milk samples for microbiology, somatic cell analysis and milk yield measurements were collected from each gland before the morning milking. In study 1, milk samples were collected just before the first treatment (day 0), and subsequently 24 h after the first dose (day 1), as well as on days 3, 7, 15, 30 and 45. In study 2, milk samples were collected on days 0, 3, 7, 15 and 30.

Milk yield

The production of milk from each gland during morning milking was measured by collecting milk into single-use sterile plastic containers.

Milk cultures and bacterial identification

Appropriate milk aliquots were used for microbiological investigations and somatic cell analyses. Microbiological investigations were performed on the milk samples according to the National Mastitis Council protocols. In brief, 10 µl of each milk sample were plated in duplicate on

blood agar and mannitol salt agar plates for pathogen isolation. Plates were incubated at 37 C for 24–48 h and bacteria were identified according to Holt and colleagues [15] and API Staph kit (BioMerieux, France). Counts from duplicate plates were averaged. In study 1, milk cultures in MRS agar (de Man, Rogosa, Sharpe; Thermo Fisher Scientific, UK) for *L. lactis* isolation were also performed. Cultures were considered pathogen-free when the average number of colonies were below the detection limits recommended by the National Mastitis Council.

Somatic cell analysis and polymorphonuclear neutrophils

In both studies, somatic cells were measured in each sample using a Fossomatic FC cell counter (Foss Electric, Hillerød, Denmark) according to UNI EN ISO 13366-2 : 2007 (IDF 148-2 : 2006).

In addition, in study 2, 20 glands were randomly selected from the CNS-LL (n=5), CNS-PBS (n=5), CNS-UNT (n=5) and HLT-UNT (n=5) groups to study the polymorphonuclear neutrophil (PMN) response. Briefly, milk samples collected from each gland on days 0, 3, 7 and 15 were used to make smears. Ten fields for each slide were examined at high magnification by light microscopy following MayGrunwald Giemsa staining, for PMN identification and quantification. The mean PMN count across the ten fields (mPMN) of each slide, corresponding to a milk sample taken from one gland at a single point in time, was then calculated and used for statistical analyses.

Histological investigations

Five ewes from study 2 were slaughtered on day 7 after milk samples were collected. A total of eight infected udder tissue samples (three glands from the CNS-LL group, three from the CNS-PBS and two from the S.AU-LL group) underwent histopathological examinations. Udder tissue fragments were fixed in 10 % buffered formalin at room temperature, then embedded in paraffin, cut into 5 µm sections and finally stained with hematoxylin and eosin.

Statistical analysis

Independence between udder halves within ewe was assumed. Median and interquartile ranges were used to graphically describe trends in SCC, milk production and mPMN (see Milk sampling and analysis above) data sets.

Intra- and inter-group analyses were performed within each study. Intra-group differences in SCC, milk production and PMN values were determined by the Friedman test, followed by Dunn's pairwise multiple comparisons test where applicable. Data sets recorded on day 0, before any treatment was administered, were considered as the control and compared with those obtained on all other treatment and follow-up days. Glands whose milk samples

presented clots preventing an accurate enumeration of somatic cells were excluded from the SCC intra-group analysis. Glands belonging to slaughtered animals were also excluded from the SCC, milk production and PMN within-group analyses.

Inter-group analyses of differences between the control group and treated groups at different time points were performed by the Mann–Whitney test for two groups, while for three or more groups the Kruskal–Wallis test was used, followed by Dunn’s pairwise multiple comparisons test where applicable. CNS-infected and healthy glands left untreated (CNS-UNT and HLT-UNT) were considered as controls. For the inter-group analysis of SCC and milk production, the following comparisons were considered: CNS-LL vs CNS-UNT, CNS-PBS vs CNS-UNT, HLT-LL vs HLT-UNT in study 1; and CNS-LL vs CNS-UNT, CNS-PBS vs CNS-UNT, CNS-DR vs CNS-UNT and S.AU-LL vs S.AU-DR in study 2. For PMN inter-group analysis, CNS-LL vs CNS-UNT and CNS-PBS vs CNS-UNT were evaluated (study 2).

GraphPad Prism 6 version 6.07 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com, was used for both intra- and inter-group analyses. Differences were considered significant at $P < 0.05$.

RESULTS

Study 1

In study 1, 17 healthy glands and 21 glands with subclinical mastitis caused by CNS, for a total of 38 glands, were enrolled. Glands were divided into five groups and underwent different treatments, as shown in Table 1.

Microbiology

The results of milk cultures are shown in Table 2. On day 0, most of the subclinical infections were caused by *S. epidermidis* (17 out of 21 infected glands). In the CNS-LL group, after the first dose of *L. lactis* (day 1), eight glands were CNS-free (61.5 %), of which three were culture-negative and five shed *L. lactis*. After two additional *L. lactis* doses (day 3), the number of CNS-free glands increased to 12 (92 %) – all of which shed *L. lactis*. Glands resumed shedding CNS in milk as of the fourth day following the last *L. lactis* dose, going from 9 out of 13 (69 %) on day 7, to 10 out of 11 (91 %) on day 45. *L. lactis* was no longer isolated from milk after the third and last dose. *S. epidermidis* was the most common pathogen isolated after the treatment. In the CNS-PBS group, only one of the four glands was culture-negative after the three PBS doses. In the CNS-UNT group, three out of four glands showed spontaneous clearance of CNS on day 7 and were still culture-negative on day 15, but CNS reappeared in the cultures on day 30. The healthy glands of the HLT-LL group shed *L. lactis* until a few days after the last doses and almost all (6/7) remained culture-negative until day 45. Similarly, nearly all of the

HLT-UNT glands (8/10) remained pathogen-free throughout the trial.

SCC measurements

Trends and intra-group analysis

Median SCC results in milk are shown in Fig. 1. A sharp increase in median SCC values was observed in CNS-LL glands (panel A): the value rose from 3.48×10^6 cells ml^{-1} before treatment (day 0) to 16.37×10^6 cells ml^{-1} 24h after the first dose of *L. lactis* (day 1), and then further to 21.76×10^6 cells ml^{-1} 24h after the third and last dose (day 3), a >fivefold increase. However, 4 days after the last dose (day 7), the value dropped back to pre-treatment levels ($<3.01 \times 10^6$ cells

ml^{-1}) and then gradually rose, albeit not significantly, until day 45. The *L. lactis*-infused healthy glands (HLT-LL) experienced a dramatic increase in SCC values during the 3 day treatment, from a median value of 21.01×10^4 cells ml^{-1} immediately prior to the infusion (day 0) to 19.32×10^6 cells ml^{-1} 24 h after the first *L. lactis* dose (day 1) and further to 20.61×10^6 cells ml^{-1} after the third and last dose (day 3) – a >90-fold increase. Subsequently, from day 7 on, SCC levels

Table 2. Study 1: microbiological results from milk samples by group and day of follow-up

Group (n)	Bacterial isolate	Day 0 (n)	Day 1 (n)	Day 3 (n)	Day 7 (n)	Day 15 (n)	Day 30 (n)	Day 45 (n)
CNS-LL (13)*	<i>S. chromogenes</i>	2		1	1	2		1
	<i>S. epidermidis</i>	10	3		6	8	7	9
	<i>S. hyicus</i> †				1		2	
	Untyped CNS‡	1						
	<i>Streptococcus</i> spp.				1			
	Mixed flora		2					
	<i>L. lactis</i>		5	12				
	No growth		3		4	3	2	1
CNS-PBS (4)	<i>S. epidermidis</i>	4	4	3	3	3	2	4
	<i>S. hyicus</i> †						2	
	No growth			1	1	1		
CNS-UNT (4)	<i>S. epidermidis</i>	3	3	3	1	1	2	2
	<i>S. hyicus</i> †						1	
	No growth	1§	1	1	3	3	1	2
HLT-LL (7)	<i>S. epidermidis</i>				1	1	1	1
	<i>L. lactis</i>		7	1	2			
	No growth	7	0	6	4	6	6	6
HLT-UNT (10)	<i>S. epidermidis</i>				1	1		2
	<i>S. hyicus</i> †						1	
	Mixed flora			1				
	No growth	10	10	9	9	9	9	8

n, number of mammary glands.

*One ewe injured one leg and was sent to the slaughterhouse on day 15. Microbiological data on two infected glands are therefore missing on days 30 and 45.

†Coagulase variable species. <https://doi.org/10.5281/zenodo.19096969>

‡The species-level identification of some CNS could not be obtained by biochemical testing.

§Classified as infected based on milk SCC >0.5 (10^6 cells ml^{-1}) on day 0, also in light of positive CNS cultures during the pre-trial monitoring period.

declined again and remained low through day 45 (panel D). On the other hand, the median SCC values in untreated healthy glands (HLT-UNT) remained below the breakpoint value for healthy glands ($<0.310^6$ cells ml^{-1}) on days 0, 1, 3, 15 and 30, rising slightly over that limit on days 7

and 45 (panel E). Intra-group analysis by the Friedman test was significant in the CNS-LL, HLT-LL and HLT-UNT groups ($P=0.0022$, $P=0.0020$ and $P=0.0038$, respectively). Pairwise multiple comparisons as compared to day 0 showed significant differences in SCC values on days 1 and 3 in CNSLL ($P=0.0119$ and $P=0.0012$, respectively) and HLT-LL ($P=0.0046$ and $P=0.0027$, respectively), and on days 7 and 45 in HLT-UNT ($P=0.0466$ and $P=0.0005$, respectively), as shown with asterisks (Fig. 1). No statistically significant changes in SCC values from day 0 to day 45 were observed in either the CNS-PBS (panel B), or the CNS-UNT control group (panel C).

Inter-group analysis

Both treatment groups – CNS-LL and CNS-PBS – were compared with the CNS-UNT group, here considered as the control. The Kruskal–Wallis test was positive only on day 3 ($P=0.0148$). Pairwise multiple comparisons were significant only between CNS-LL and CNS-UNT ($P=0.0383$). When comparing the HLT-LL with the HLT-UNT group, significant differences in SCC levels were found on days 1, 3 and 15 ($P=0.0001$, $P=0.0007$ and $P=0.002$, respectively). Results are shown in Table S1 (available in the online version of this article).

Milk production

Trends and intra-group analysis

Results regarding milk yield medians from CNS-infected and healthy glands are shown in Fig. 2. Throughout the study, the median milk production values of Sarda ewes remained below the average milk yield for this breed (175 ml/gland/milking). The Friedman test was positive in the CNS-LL, HLT-LL and HLT-UNT groups ($P<0.0001$, $P=0.0002$ and $P=0.0001$, respectively). Pairwise multiple comparisons as compared to day 0 showed significant reductions in milk yields only on day 45 in CNS-LL, HLTLL and HLT-UNT ($P=0.0003$, $P=0.0002$ and $P=0.0161$ in panels A, D and E, respectively), and on day 3 in HLT-LL, after the last *L. lactis* dose ($P=0.0119$, panel D).

Inter-group analysis

No differences were observed in milk yield between CNS-infected groups (CNS-LL, CNS-PBS and CNS-UNT). Conversely, a comparison between the milk yields of *L. lactis*-treated and untreated healthy groups (HLT-LL and HLT-UNT, respectively), showed significant reductions on days 3 and 45 ($P=0.0003$ and $P=0.0075$, respectively). Results are shown in Table S1.

Study 2

In study 2, 56 glands with subclinical mastitis caused by CNS, 16 glands with clinical mastitis by *S. aureus* and 13 healthy glands, for a total of 85 glands, were enrolled. Glands were divided into seven groups and given different treatments as shown in Table 1.

Macroscopic changes in udders and milk The health status of each gland before any treatment (day 0) was used as the control for intra-group comparisons.

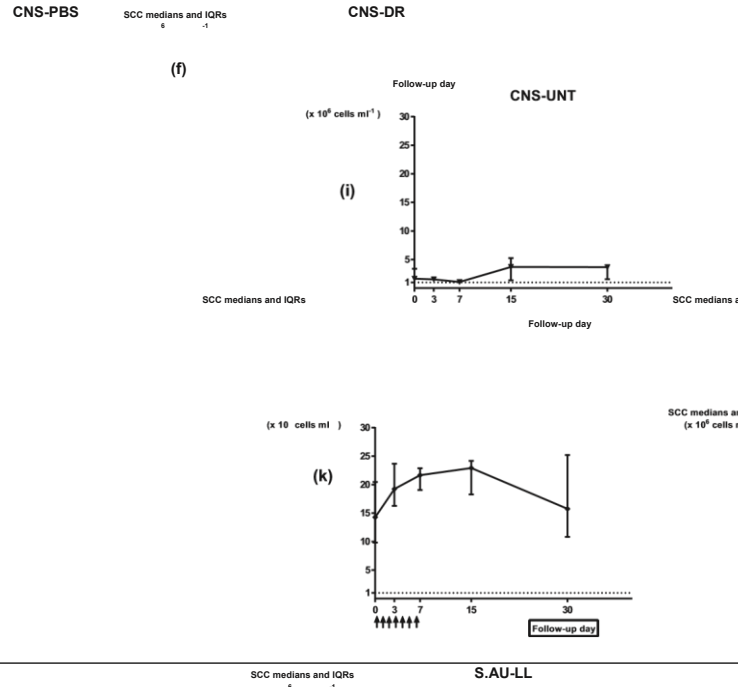
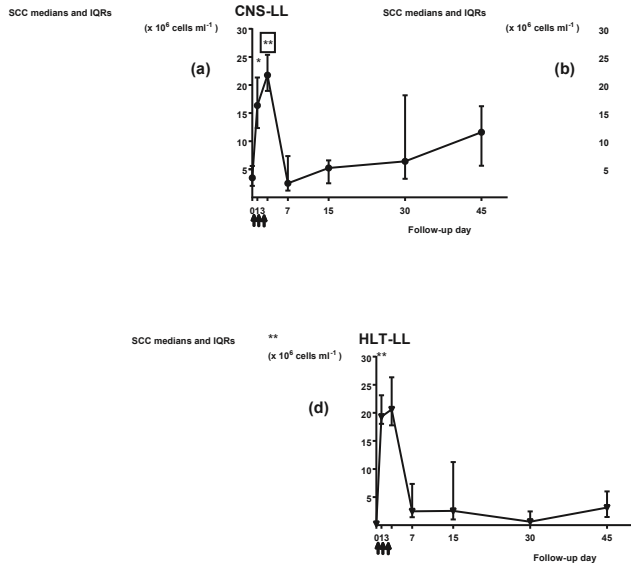
In the CNS-LL group, where infections had initially been subclinical, after three *L. lactis* doses (day 3) clinical mastitis was recorded in 40 % of glands (10/25). The clinical signs consisted of abnormal milk secretions with deviations in milk colour and consistency (7/25, 28 % mild inflammation) or acute inflammation of the mammary gland and/or the teat canal (3/25, 12 % moderate inflammation). Seven days after the *L. lactis* treatment was discontinued (day 15), the prevalence of abnormalities in milk declined to 20 % (5/25) and all gland inflammations returned to pretreatment levels. In the CNS-PBS group, three PBS doses caused macroscopic alterations in 50 % of infected glands (4/8). Of these, two glands (25 %) developed acute inflammation (moderate inflammation) and two (25 %) developed abnormal milk secretions (mild inflammation). Raised SCC values were recorded in those glands. Gland inflammation resolved on day 7 and abnormalities in milk resolved on day 15. In the CNS-DR group, one gland out of 14 showed mild inflammation on day 1, while milk abnormalities, also found in a single gland, resolved soon after the first oxytetracycline dose. In the S.AU-LL group, a worsening of clinical signs was observed on day 3 in 30 % of glands (3/10). These consisted of abnormal milk secretions (2/10) and udder inflammation (1/10). Milk abnormalities persisted until day 30, while the udder inflammation resolved 7 days after the last dose of *L. lactis* (day 15). No clinical signs of inflammation were observed in either of the two control groups – CNS-UNT and S.AU-DR.

Microbiology

The results of milk sample cultures are shown in Table 3. A wide variety of CNS species were identified in study 2. In CNS-LL glands, *S. epidermidis* was the most commonly identified species on day 0, followed by *S. xylosus*. After three *L. lactis* doses (day 3), 11 out of 25 glands (44 %) were CNS-free. The number of CNS-free glands increased to 15 (60 %) after four additional *L. lactis* doses (day 7). No data are available on the shedding of *L. lactis* during treatment, as *L. lactis* cultures were not performed in this study. As observed in study 1, once treatment was discontinued, glands resumed the shedding of CNS, although the pathogen species identified on day 15 were different from those found on day 0. In the CNS-PBS group, the seven PBS doses were unable to resolve CNS infections: two glands out of eight were culture-negative on day 7 and none on day 15. Similarly, in the CNS-DR group, oxytetracycline did not resolve CNS infections: only 2 out of 14 glands were culture-negative on day 7, after the second and last dose of oxytetracycline, and none on day 15. In the CNS-UNT group, the glands showed no substantial spontaneous recovery from CNS infections. Changes in CNS species were observed here as well. Neither *L. lactis* nor oxytetracycline were

effective against *S. aureus* infection in the groups S.AU-LL and S.AU-DR, respectively. As in study 1, almost all of the untreated glands in the HLT-UNT group remained pathogen-free throughout the trial.

Study 1



Study 2

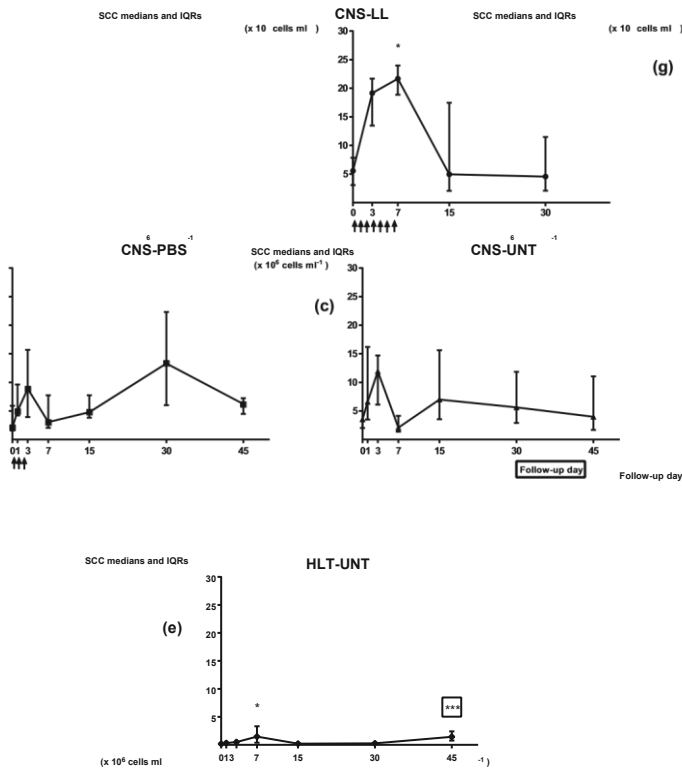


Fig. 1. Trends and intra-group differences in SCCs in study 1 and study 2. Median values are shown for samples collected before (day 0) and after treatment. Arrows indicate the administration of oxytetracycline. Intra-group differences were calculated as compared to day 0. ***P<0.001.

SCC measurements

Trends and intra-group analysis

Median SCC results are shown in Fig. 1. An increase was observed among CNS-LL glands, from 5.5810^6 cells ml⁻¹ before treatment (day 0) to 21.6710^6 cells ml⁻¹ 24 h following the last *L. lactis* dose (day 7), a >threefold increase (panel F). The Friedman test was positive only in CNS-LL ($P=0.0005$). Pairwise multiple comparisons from day 0 showed significant differences in SCC values only on day 7 ($P=0.012$). One week after the last *L. lactis* dose (day 15), the median value of SCC dropped to a level slightly lower than the pre-treatment value ($<5.010^6$ cells ml⁻¹) and remained essentially unchanged until day 30. No statistically significant changes in SCC values from day 0 to day 30 were observed in the CNS-PBS, CNS-DR or the CNS-UNT control group

(panels G, H and I, respectively). Similarly, SCC values of *S. aureus*-infected glands did not significantly change following treatment, neither with the live culture of *L. lactis* in S.AU-LL glands nor with oxytetracycline in S.AU-DR glands (panels K and L, respectively). Untreated healthy glands (HLT-UNT) remained below the breakpoint value for healthy glands throughout the trial (panel J).

Inter-group analysis

The CNS-LL, CNS-PBS and CNS-DR groups were compared with the CNS-UNT control group. Significant SCC inter-group differences were found only on days 3 ($P=0.0002$) and 7 ($P<0.0001$) and only between CNS-LL and CNS-UNT glands ($P=0.0037$ and $P<0.0001$, respectively). The S.AU-LL group was compared with S.AU-DR udder halves. A significant difference was found only on day 3 ($P=0.0040$). Results are shown in Table S2.

PMN response

Trends and intra-group analysis

Median mPMN results are shown in Fig. S1. A significant rise in mPMN values was observed only in the CNS-LL group ($P=0.0067$ in the Friedman test), and only after the third *L. lactis* dose ($P=0.0212$) by the pairwise multiple comparisons. No significant changes in mPMN levels were documented in CNS-PBS, CNS-UNT and HLT-UNT udder halves.

Inter-group analysis

The CNS-LL and CNS-PBS groups were compared with the CNS-UNT control group. Significant differences in mPMN levels were found only on days 3 ($P<0.0001$) and 7 ($P=0.0114$) and only between CNS-LL and CNS-UNT glands ($P=0.0014$ and $P=0.0104$, respectively). Results are shown in Table S3.

Milk production

Trends and intra-group analysis

Median milk yields from CNS-infected, *S. aureus*-infected and healthy glands are shown in Fig. 2. Median milk production values of Valle del Belice and Comisana ewes remained below the average milk yield for these breeds (230 ml/gland/milking) throughout the study in all infected groups. Among HLT-UNT glands (panel J), on the other hand, median milk production levels approached and then exceeded the average value for the breed.

The Friedman test was positive in the CNS-LL ($P=0.0006$), CNS-PBS ($P=0.0022$), CNS-DR ($P=0.0092$), CNS-UNT ($P=0.0004$) and HLT-UNT groups ($P<0.0001$). Pairwise multiple comparisons from day 0 showed significant declines in milk yield limited to day 3 in CNS-LL and day 15 in CNS-DR ($P=0.0016$ and $P=0.0062$, respectively, panels F and H). Conversely, significant rises in milk yield from day 0 were observed on days 15 and 30 in CNS-PBS ($P=0.0149$ and $P=0.0055$, respectively, panel G), day 30 in CNS-UNT ($P=0.0070$, panel I), and days 15 and 30 in HLTUNT ($P=0.0078$ and $P<0.0001$, respectively, panel J). No significant changes in milk yields were recorded in either S.AU-LL or S.AU-DR (panels K and L, respectively).

Inter-group analysis

Comparing groups significant results were recorded only on day 15 ($P=0.0010$) and only between CNS-DR and CNS-UNT ($P=0.0023$). Results are shown in Table S2.

Histological evaluation

Nearly all infected glands (7/8; $n=3$ CNS-LL, $n=2$ CNS-PBS and $n=2$ s.AU-LL) showed chronic mastitis characterized by moderate to severe fibrosis, multifocal to diffuse mononuclear cellular infiltration (macrophages, lymphocytes and plasma cells) and mild to moderate epithelial desquamation. The remaining gland – belonging to the CNS-PBS group, showed no histological changes. In addition, one CNS-LL gland and one S.AU-LL gland showed multifocal alveolar and interstitial PMN infiltration.

DISCUSSION

The use of LAB and bacteriocins has been recently proposed for the control of bovine mastitis thanks to their ability to elicit a rapid local immune response [12, 16].

Previous *in vivo* studies in cows have shown the intramammary infusion of nisin or live *L. lactis* to be effective against Gram-positive mastitis-causing pathogens [8, 9, 12]. More recent studies have demonstrated that live LAB infused into bovine mammary glands are able to activate the local immune system [16–20]. Based on the above findings, we chose *L. lactis* LMG 7930 as a potential therapeutic agent against mastitis, to be assessed *in vivo* in small ruminants. *L. lactis* LMG 7930 is a commercially available nisin-producing strain, considered safe for human consumption (it is used in the production of Swiss cheese), and able to inhibit numerous mastitis-causing pathogens in

vitro [21]. We have decided to inoculate a live overnight culture of *L. lactis* LMG 7930, as overnight cultures of LAB have been shown to elicit stronger immunomodulatory activities than heat-killed cells or cell-free supernatants [16, 18].

In study 1 we assessed the efficacy of a three-dose treatment of live *L. lactis* culture in subclinical ewes with CNS mastitis. The three doses of live culture compromised CNS survival in the glands: 92 % of the CNS-LL glands were

Study 1

milk yield medians and IQRs (ml) CNS-LL milk yield medians and IQRs (ml) and IQRs (ml) 200200

(a)

200(b)

200

150150

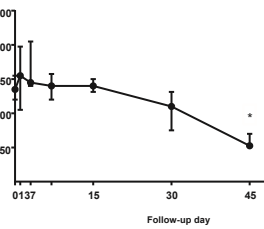
100100

5050

HLT-UNT

(e)

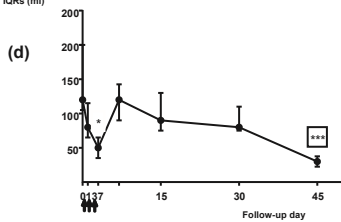
milk yield medians and IQRs (ml)



milk yield medians and IQRs (ml) HLT-LL

(d)

milk yield medians and IQRs (ml)

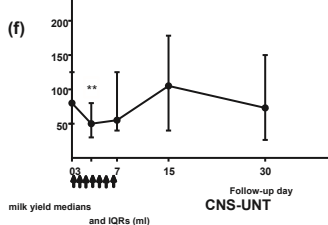


Study 2

milk yield medians and IQRs (ml) CNS-LL

(f)

milk yield medians and IQRs (ml)

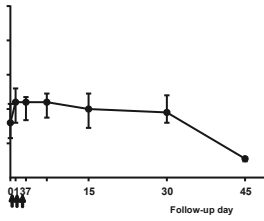


milk yield medians and IQRs (ml)

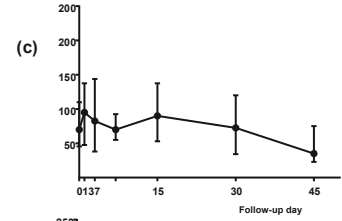
(g)

milk yield medians and IQRs (ml)

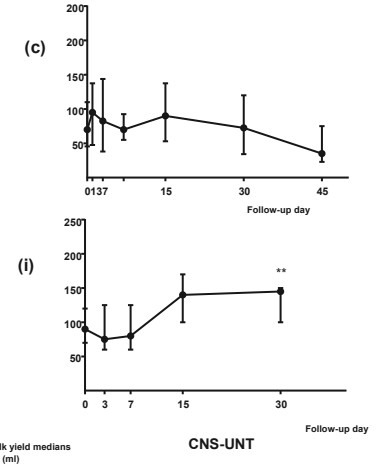
milk yield medians and IQRs (ml) CNS-UNT



(c)



(i)

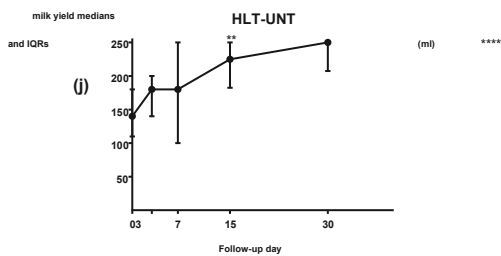
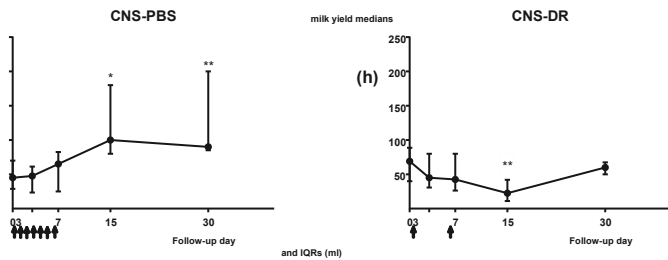


CNS-PBS

milk yield medians and IQRs (ml)

CNS-UNT

Follow-up day



CNS-free on day 3. The pathogens were not permanently eliminated, however, and CNS reappeared in milk cultures as soon as treatment was interrupted (from day 7 on).

We also observed that during *L. lactis* treatment, both infected and healthy glands temporarily shed *L. lactis*. This

A three-dose treatment of PBS (group CNS-PBS), on the other hand, was ineffective in contrasting pathogen survival in the glands, which remained CNS-positive throughout the study. The only exception was a single case of clearance – probably spontaneous rather than PBS-induced, as observed also among untreated, infected udders (CNS-UNT). Negative results of bacteriological cultures can also result from the cyclical shedding of chronic bacterial infections [22], the presence of few bacterial colonies in milk [22], or the engulfment of pathogens by professional phagocytes [23, 24].

Both infected and healthy glands infused with *L. lactis* experienced a dramatic increase in SCC during treatment suggesting a strong mammary immune stimulation by the culture. This rise in SCC was short-lived, however: SCC rapidly declined to pre-treatment levels as observed on day 7, probably due to the above-mentioned inability of *L. lactis* to colonize the treated glands. Although healthy PBStreated glands were not included in our study, on the grounds that the pyrogen-free PBS used here is a sterile isotonic saline solution with no documented toxicity to epithelial cells, our results seem to suggest that the *L. lactis* culture itself is able to activate the mammary gland immune system. Our results are in line with previous studies where mammary quarters infused with LAB experienced a transient rise in SCC [12, 25].

The fact that our ewes' milk production was constantly below the average milk yield for their breed is not surprising in light of the fact that they were in late lactation. But while a significant drop in milk yield was observed among healthy glands after the third *L. lactis* dose, the treatment did not

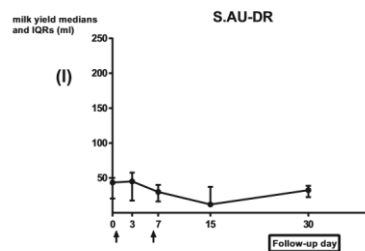
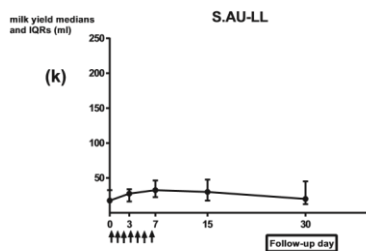


Fig. 2. Trends and intra-group differences in milk yield in study 1 and study 2. Medians and interquartile ranges of morning milk production (ml) measured before (day 0) and after treatment. Arrows indicate the administration of intramammary doses of *L. lactis*, PBS or oxytetracycline. Intra-group differences were calculated as compared to day 0, here considered as the control. *P0.05, **P0.01, ***P0.001 and ****P0.0001.

result is in agreement with a previous study on cattle [12], suggesting that *L. lactis* cultures may be unable to colonize the gland, because all inoculated glands stopped shedding lactobacilli a few days after cessation of intramammary administration.

seem to significantly alter the milk production of infected glands, probably already compromised by the infection.

On the basis of the results obtained in study 1, namely the fact that *L. lactis*-treated glands resumed pathogen shedding once treatment was discontinued, we decided to perform another study, study 2, where *L. lactis* treatment

was extended to 7 days in the hope of completely eradicating the pathogen from the gland. In CNS-LL, the extension of *L. lactis* treatment increased the proportion of CNS-free glands from 44 % on day 3 to 60 % on day 7, but did not resolve CNS infections as we had hoped. In contrast, the maximum clearance obtained in the first study was higher than that obtained in the second. This may be due to the fact that a larger proportion of CNS-LL glands in the second study was affected by chronic infection than in study 1. Here too, although different but genetically closed sheep breeds have been used, infection in the glands relapsed as soon as *L. lactis* treatment was discontinued, as in study 1.

In clinical cases of *S. aureus* mastitis, the *L. lactis* culture was ineffective in contrasting pathogen survival. These results stand in contrast to those of Klostermann and colleagues [12], where an *L. lactis* DPC3147 culture was effective in reducing *S. aureus* infection. Different experimental conditions as well as host (cows vs sheep), pathogen and LAB characteristics may account for this discrepancy.

In our study, oxytetracycline treatment was not efficacious against either of the pathogens tested. Oxytetracycline was chosen because it had previously resolved ovine mastitis on these same farms. This may have resulted in resistance to the antibiotic, however.

As in study 1, *L. lactis* infusions caused a transient rise in SCC among CNS-infected glands in study 2 (>threefold increase). Once again, following the cessation of treatment, SCC values rapidly reverted to pre-treatment levels. No significant increase in SCC values was observed among *S. aureus*-infected glands treated with *L. lactis*. This was likely due to the already high pre-treatment SCC values caused by

S. aureus infection. We also observed that, unlike PBS, the infusion of *L. lactis* into CNS-infected glands stimulated a significant recruitment of PMNs to the gland. Our results are in agreement with previous studies where the infusion of LAB cultures, such as *L. lactis* DPC 3147 [16], *Lactobacillus perolens* CRL 1724 [26] and *Weissella confusa* [18], into bovine udders activated the innate immune response, resulting in a transient recruitment of PMNs and lymphocytes to the gland, cells which in turn promoted a rapid inflammatory response. Evidence of such recruitment was obtained from the histological examination of one of the CNS-LL glands.

In study 2, the clinical status of glands was also recorded. After three *L. lactis* doses, 40 % of initially subclinical CNS mastitis turned into clinical mastitis, with mild (28 %) or moderate (12 %) inflammation. Likewise, 30 % of *S. aureus*-infected glands infused with *L. lactis* experienced a worsening of clinical conditions, mostly with regard to abnormalities in milk. But while the cases of udder inflammation resolved in both groups (CNS-LL and S.AU-LL) as soon as the *L. lactis* treatment was discontinued, anomalies in milk generally persisted.

Interestingly, PBS also caused macroscopic changes in 50 % of CNS-PBS glands, consisting of udder inflammation or abnormal milk secretions. Here, as opposed to CNS-LL glands, both mild and moderate inflammations resolved as soon as treatment was discontinued. Conversely, in cattle, repeated trials have shown that the infusion of sterile water into udders did not cause irritation or inflammation [19]. Our results thus seem to suggest that, in ewes, even a sterile isotonic saline solution like PBS can elicit an inflammatory response when infused into the glands, although a larger

Table 3. Study 2: microbiological results from milk samples by group and day of follow-up

Group (n)	Bacterial isolate	Day 0 (n)	Day 3 (n)	Day 7 (n)	Day 15 (n)	Day 30 (n)
CNS-LL (25)*	<i>S. capitis</i>	2				
	<i>S. caprae</i>		1			
	<i>S. caseolyticus</i>		1			2
	<i>S. chromogenes</i>	1		1	4	1
	<i>S. epidermidis</i>	5	1	1	1	6
	<i>S. haemolyticus</i>	2				
	<i>S. lentus</i>					1
	<i>S. saccharolyticus</i>			1	1	
	<i>S. sciuri</i>		1			
	<i>S. simulans</i>	1	2	1		

	S. warneri		1		1	
	S. xylosus	3				
	Untyped CNS†	11	7	6	9	4
	S. aureus				1	1
	Not tested§				3	2
	No growth		11	15	1	6
CNS-PBS (8)*	S. capitis					
	S. caseolyticus			1		
	S. chromogenes			1	1	
	S. epidermidis		3			2
	S. hyicus‡		1			
	S. lentus			2		
	S. sciuri			1		1
	S. xylosus		1			
	Untyped CNS†	8	2	1	4	2
	No growth		1	2		
	CNS-DR (14)	S. epidermidis	10			
S. xylosus		1				
Untyped CNS†		3	10	11	14	12
S. aureus			1	1		1
No growth			3	2		1
CNS-UNT (9)	S. caprae		1			
	S. capitis	1				
	S. caseolyticus		1	1		2
	S. chromogenes	2		1	3	
	S. cohnii					1
	S. epidermidis	3		2	3	1
	S. hyicus‡					1
	S. saccharolyticus	1		1		
	S. simulans			1		
S. warneri				1		
	Mixed CNS (S. cohnii/S. epidermidis/S. sciuri)		1			

S.AU-LL (10)*	Untyped CNS†	1	5	2	1	2
	S. aureus				1	
	No growth	1#	1	1		2
	S. intermedius‡		1			
	S. simulans			1	1	
	Untyped CNS†					2
S.AU-DR (6)	S. aureus	10	8	9	7	6
	No growth		1			
	Untyped CNS†				1	2
	S. aureus	6	6	6	5	4

Table 3. cont.

Group (n)	Bacterial isolate	Day 0 (n)	Day 3 (n)	Day 7 (n)	Day 15 (n)	Day 30 (n)
HLT-UNT (13)*	No growth					
	S. carnosus					1
	S. hyicus‡					1
	S. sciuri		1			
	S. warneri					1
	S. xylosum		1			
	Untyped CNS†		2	1	1	
No growth	13	9	12	11	9	

n, number of mammary glands.

*A total of five ewes were slaughtered on day 7 after milk samples were collected and mammary glands removed for histological examination. Microbiological data of nine infected glands – three from CNS-LL, three from CNS-PBS, two from S.AU-LL and one from HLT-UNT – are therefore missing on days 15 and 30.

†The species-level identification of some CNS could not be obtained by biochemical testing.

‡Coagulase-variable or coagulase-positive species.

§Cultures were not performed in cases of mastitis with poor milk flow.

||Samples were not cultured for *L. lactis*.

#Classified as infected based on milk SCC >0.5 (10⁶ cells ml⁻¹) on day 0, also in light of positive CNS cultures during the pre-trial monitoring period.

number of PBS-treated glands should be estimated to make the results more representative. We believe that the slight inflammatory response observed in CNS-PBS glands, which was transient and not coupled with significant PMN recruitment or rises in SCCs, was iatrogenic rather than the result of a direct effect of PBS on host immune system. To the best of our knowledge, only one previous paper described iatrogenic mastitis in sheep (following the injection of two different formulations) [27].

Clinical signs of inflammation and abnormalities in milk were described in the literature after the infusion of live cultures of *L. lactis* DPC 3147 [17], *W. confusa* [18] and *L. perolens* CRL 1724 [19] in mammary quarters of healthy dairy cows. The immune response to LAB infusion appears to be dose-dependent [16, 19]. Our findings, coupled with the above-mentioned literature, suggest that the intramammary infusion of overnight cultures of live LAB may produce adverse clinical signs in ruminants, such as udder inflammation or abnormal milk secretions. We think

that the dose inoculated, rather than the strain or the host, plays a decisive role in regulating the mammary gland immune response. We believe that this response might be the key to mastitis control by reducing the chance of colonization/proliferation of mastitis-causing pathogens without causing mammary gland health deterioration.

Despite encouraging evidence indicating that live LAB cultures elicit the response of the immune system in mammary glands, creating unfavourable conditions for the proliferation of pathogens, it may nevertheless be early to implement this strategy as an alternative to antibiotics in ruminants. Indeed, it may not be desirable to inoculate bacteria that increase milk SCCs and produce clinical signs of inflammation, especially in animals affected by mastitis, whose mammary gland health is already compromised. Additional studies are therefore needed to find bacterial formulations able to modulate mammary gland immune response without substantially increasing PMN mobilization that would impair udder function and milk quality.

In conclusion, under our experimental conditions, the live culture of *L. lactis* LMG 7930 rapidly activated the mammary innate immune system. This led to a transient clearance of the pathogen in the gland, but also caused mild to moderate clinical cases of mastitis. In our opinion, it is still early to implement bacterial formulations as alternatives to antibiotics in treating mastitis in ruminants, and further experimentation is needed.

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