

Research Article

*These authors have contributed equally and their names are listed alphabetically

Cite this article: Chatzopoulos DC *et al* (2020). *Streptococcus* spp. from bulk-tank milk and milking machine teatcups on small ruminant farms, and factors potentially associated with their isolation. *Journal of Dairy Research* **87**, 277–281. <https://doi.org/10.1017/S0022029920000734>

Received: 29 June 2020

Revised: 29 June 2020

Accepted: 9 July 2020

First published online: 4 September 2020

Keywords:

Goat; intramammary infection; mastitis; sheep; somatic cell counts; *Streptococcus*; subclinical mastitis

Author for correspondence:

George C. Fthenakis, Email: gcf@vet.uth.gr

Streptococcus spp. from bulk-tank milk and milking machine teatcups on small ruminant farms, and factors potentially associated with their isolation

Dimitris C. Chatzopoulos^{1,*}, Daphne T. Lianou^{1,*}, Charalambia K. Michael^{1,*}, Dimitris A. Gougoulis¹, Vasia S. Mavrogianni¹, Natalia G. C. Vasileiou¹, Angeliki I. Katsafadou¹, Antonis P. Politis¹, Nikos G. Kordalis¹, Eleni I. Katsarou¹, Katerina S. Ioannidi¹, Efthimia Petinaki² and George C. Fthenakis¹

¹Veterinary Faculty, University of Thessaly, Karditsa, Greece and ²University Hospital of Larissa, Larissa, Greece

Abstract

The objectives of this work were (a) to determine the presence of streptococci in samples from small ruminant dairy farms (bulk-tank milk and, where possible, teatcup swabs), (b) to investigate the potential adverse effects of streptococci on milk quality and (c) to investigate the importance of some husbandry factors for the isolation of streptococci. Bulk-tank milk samples and teatcups swab samples were examined bacteriologically for the presence of streptococci. Somatic cell counting and milk composition measurements were also performed. The husbandry factors present in each farm were assessed for potential associations with the isolation of streptococci. Streptococci were isolated from milk samples from 31.4% of sheep and 17.4% of goat farms and from 4.8% of sheep and 5.9% of goat teatcups. Streptococci were isolated more frequently from the upper part than the lower part of teatcups: 5.0% vs. 1.9%. Most isolates (57.9%) were identified as *Streptococcus uberis*. Most isolates (68.4%) were slime-producing; slime-production was more frequent among isolates from teatcups (83.3%) than from bulk-tank milk (55.0%). Somatic cell counts and milk composition did not differ between farms in which streptococci were or were not isolated. Machine-milking was associated with the isolation of streptococci from bulk-tank milk samples. The initial stage of the milking period (first two months) was found to be associated with the isolation of streptococci from milking machine teatcups in sheep farms only.

The importance of streptococci in ovine and caprine mastitis has not been studied as extensively as that of staphylococci, probably because staphylococci cause more than 70% of the cases of mastitis in small ruminants (Gelasakis *et al.*, 2015). Various studies (mainly in Italy) have indicated that streptococci could be of greater importance for small ruminant mastitis than previously believed (Addis *et al.*, 2013; Pisanu *et al.*, 2015; Albenzio *et al.*, 2016). The objectives of this work were (a) to determine the presence of streptococci in samples from small ruminant dairy farms, specifically, in bulk-tank milk and on milking machine teatcups, (b) to investigate the potential adverse effects of streptococci on milk quality and (c) to evaluate the importance of some management practices on the presence of streptococci.

Materials and methods

Farms

In total, 51 sheep and 23 goat dairy farms were studied. Dairy farms (milking was performed by machine or by hand) were selected by collaborating veterinarians on willingness of farmers to accept a visit by University personnel for sample collection and visited once each. Three investigators (DTL, CKM and GCF) accompanied by an assisting investigator (NGCV, APP, NGK or KSI) visited all farms for sample collection. At the start of each visit, the farmer was interviewed to obtain information on udder health management practices in use (online Supplementary Material 1).

All farms visited were for dairy production. Lambs or kids born were weaned at 45 to 50 or 60 to 90 d of age, respectively. After weaning of their offspring, ewes or nannies were milked twice or thrice daily for up to 8 months.

© The Author(s), 2020. Published by Cambridge University Press on behalf of Hannah Dairy Research Foundation.



CAMBRIDGE
UNIVERSITY PRESS

Table 1. Results of isolation of streptococci from bulk-tank milk samples and milking machine teatcups in sheep or goat farms

Origin	Sheep			Goats			Total
	Total	Slime +ve	Slime -ve	Total	Slime +ve	Slime -ve	
Bulk-tank milk							
<i>S. uberis</i>	8	5	3	2	1	1	10
<i>S. acidominimus</i>	2	0	2	0	0	0	2
<i>Streptococcus</i> spp.	6	4	2	2	1	1	8
Total	16	9	7	4	2	2	20
Milking machine							
<i>S. uberis</i>	10	7	3	2	2	0	12
<i>Streptococcus</i> spp.	4	4	0	2	2	0	6
Total	14	11	3	4	4	0	18
Grand total	30	20	10	8	6	2	38

Sampling

At each farm, sampling took place after milking and cleaning of the parlour by following the usual farm routine. Initially, two 20 mL samples were collected using aseptic techniques from the milk tank.

If machine milking was used, three teatcups were swabbed in parlours with up to 12 milking units ($n = 28$ sheep and 6 goat farms), six teatcups in parlours with 13 to 24 units ($n = 16$ and 4) and nine teatcups in parlours with 25 to 36 units ($n = 3$ and 1). In total, 207 teatcups were sampled on sheep farms and 51 on goat farms. The specific teatcups to be sampled were predetermined using an electronic random number generator. Two swab samples were taken from each teatcup, one from the upper (approx. 1–1.5 cm deep) and one from the lower (approx. 10–12 cm deep) part of the teatcup; swabbing included the entire circumference of the inner wall of the teatcup in a circular manner.

Transportation of samples to the laboratory in Karditsa was performed by car, by the investigators. Samples were stored at 0.0 to 4.0 °C using ice packs in portable refrigerators.

Laboratory examination

Bacteriological examinations started within 12 h of collection of samples. Milk samples (10 µl) and swabs were cultured in duplicate on streptococcus selective media (*Streptococcus* selective agar; BioPrepare Microbiology, Athens, Greece). Plates were incubated aerobically at 37 °C for 48 h. If there was no growth, plates were re-incubated for another 24 h. Bacterial isolation and initial identification were performed using standards methods (Barrow and Feltham, 1993; Euzebey, 1997). Detection of at least three confirmed streptococcal colonies on at least one agar plate of those inoculated with milk samples or swabs from each farm, was considered to indicate presence of the organism in the bulk-tank milk or the teatcup, respectively. Identification to species level was performed using the Microgen® Strep-ID system (Microgen Strep-ID; Microgen Bioproducts Ltd, Camberley, United Kingdom).

After identification, all streptococcal isolates were cultured on Congo Red agar plates (BioPrepare Microbiology, Athens, Greece) for evaluation of biofilm formation. The plates were incubated aerobically at 37 °C for 24 h (Freeman *et al.*, 1989). Slime-producing strains ('+') were indicated by black colonies with

dry crystalline consistency. Dark-coloured colonies with no dry crystalline culture appearance were assigned an intermediate result ('±'), whilst colonies that remained pink, were considered to reflect non slime-producing strains ('-') (Freeman *et al.*, 1989; Schönborn *et al.*, 2017).

Somatic cell counting (Lactoscan SCC; Milkotronic Ltd, Nova Zagora, Bulgaria) and milk composition measurement (Lactoscan Farm Eco; Milkotronic Ltd) were performed in duplicate; the two results were averaged, then the two means again averaged for the final result from each bulk-tank milk.

Data management and analysis

Data were entered into Microsoft Excel. Descriptive analysis was performed initially. The following outcomes were considered: 'isolation of streptococci from bulk-tank milk' and 'isolation of streptococci from the milking machine teatcups'. The importance of predictors was assessed by using cross-tabulation with the Fisher exact test or the one-way analysis of variance, as appropriate, and with simple logistic regression. Moreover, the frequency of identification of *Streptococcus uberis* and the frequency of slime-producing streptococci among relevant isolates were also calculated. Statistical significance was defined at $P < 0.05$.

Results

Isolation of streptococci

Data are shown in Table 1. Streptococci were isolated from bulk-tank milk of 16/51 sheep (31.4%) and 4/23 goat (17.4%) farms (the difference between farm type was not significant, $P > 0.05$).

Streptococci were isolated from 10/207 teatcups in 5/44 sheep farms (4.8% and 11.4%, respectively) and 3/51 teatcups in 2/11 goat farms (5.9% and 18.2%) (once again, the difference between farm type was not significant, $P > 0.05$); in total, 18 isolates were obtained, 14 (10 from the upper and 4 from the lower part of the teatcups) on sheep and 4 (3 and 1) on goat farms. The more frequent isolation of streptococci from the upper part of the teatcups, 5.0% (13/258) vs. 1.9% (5/258), was significantly different ($P < 0.05$), whilst in five teatcups (4 in sheep farms and 1 in a goat farm) isolations from both sites were recorded.

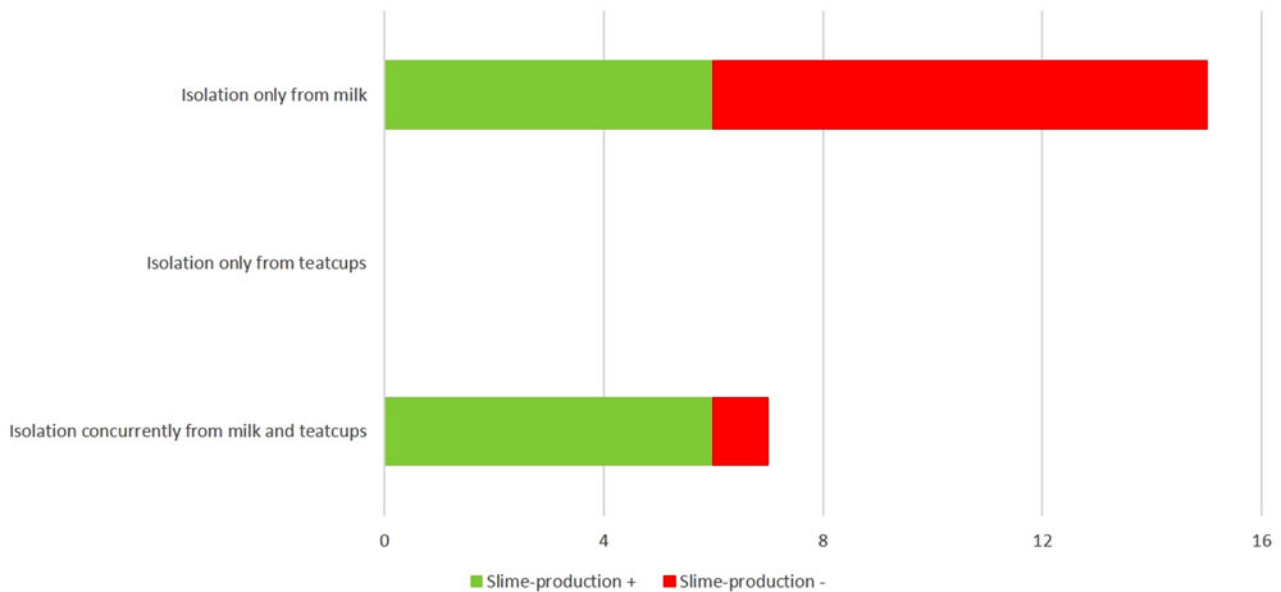


Fig. 1. Cases of isolation of slime- or non-slime-producing streptococci from the milk or the milking machine teatcups alone or concurrently, from dairy sheep or goat farms in Greece.

Most isolates (57.9%) were identified as *S. uberis*; *Streptococcus acidominimus* was also identified (5.3% of all isolates), whilst 36.8% of the isolates could not be speciated. There was no difference in the frequency of isolation of *S. uberis* from sheep (18/30) or goat (4/8) farms ($P > 0.05$) and from swabs (12/18) or milk samples (10/20) ($P > 0.05$; Table 1).

Most isolates (26/38, 68.4%) were slime-producing. Slime-production was numerically more frequent among isolates obtained from swabs from teatcups (15/18, 83.3%) than among isolates from milk (11/20, 55.0%), although this difference was not statistically significant ($P = 0.065$). There was no difference in the frequency of slime-production according to species: 68.2% of *S. uberis* isolates and 68.8% of the other isolates were slime-producing ($P > 0.05$; Table 1). As shown in Fig. 1, the concurrent isolation of slime-producing streptococci from milk and teatcups within the same farm occurred more frequently than that of non-slime producing streptococci ($n = 6$ vs. $n = 1$ respectively), although this difference was not statistically significant. This was not seen for isolation of such strains only from milk or teatcups within the same farm ($n = 6$, vs. $n = 9$ for non-slime-producing isolates; Fig. 1).

Milk somatic cell counts and composition

The data are in Table 2. There were no significant differences in somatic cell counts between farms from the bulk-tank milk samples of which streptococci were or were not isolated ($P > 0.05$ for both sheep and goats). Also, there were no significant differences in milk composition between farms from the bulk-tank milk samples of which streptococci were or were not isolated ($P > 0.05$ for sheep and goats; Table 2).

Potential risk factors for isolation of streptococci

Data are in Table 3. Only machine-milking was associated with the isolation of streptococci from bulk-tank milk samples. This effect was significant for goat farms ($P < 0.05$) but not for sheep

farms ($P = 0.058$). For the other variables evaluated, no such associations were evident ($P > 0.21$). On farms with sheep during the initial stage of the milking period (first two months), isolation of streptococci from teatcups was more frequent than from other farms ($P < 0.01$). For the other variables, no such associations were evident ($P > 0.2$; Table 3, online Supplementary Material 2).

Discussion

Streptococci are little-studied mastitis pathogens in sheep and goats, with most mastitis-related literature in these species referring to staphylococcal infections (Vasileiou *et al.*, 2019). Nevertheless, the importance of streptococci as potential pathogens for mastitis in small ruminants has recently been highlighted. Various species, including *S. acidominimus*, *S. agalactiae*, *S. bovis*, *S. dysgalactiae*, *S. equi*, *S. pluranimalium*, *S. mitis*, *S. porcinus*, *S. suis*, *S. uberis*, have been implicated as aetiological agents of the infection in both sheep and goats (Devriese *et al.*, 1999; Fernandez *et al.*, 2004; Marogna *et al.*, 2012; Queiroga, 2017).

Previously, we reported that the prevalence of subclinical streptococcal mastitis in ewes in Greece was approximately 5% (Vasileiou *et al.*, 2018). Here, we took a different approach and examined samples from the bulk-tank and the milking machine teatcups, to obtain information from the entire flock or herd, which might be a potential indicator of the presence of streptococci in the animals. The results indicate a high frequency of streptococci in the bulk-tank milk. The two species identified, *S. acidominimus* and *S. uberis*, are both mammary gland pathogens, hence, this is likely to indicate intramammary infections.

It is also noteworthy that *S. uberis* is an environmental pathogen, present in the bedding in animal houses and on various sites of the animals (eg intestine), as described by Bramley (1982) and Kruze and Bramley (1982). These authors isolated *S. uberis* in high numbers from straw samples in cattle farms, as well as from body sites (rectum, vulva). The present results did not indicate an association of *S. uberis* recovery with the use of straw as bedding in the farms studied.

Table 2. Milk somatic cell counts and composition in relation to isolation of streptococci from the bulk-tank milk in sheep or goat farms

Farms	Isolation of streptococci	Somatic cell counts ($\times 1000$ cells/ml)	Fat (%)	Protein (%)	Lactose (%)
Sheep	Yes	530 \pm 59	5.97 \pm 0.14	4.49 \pm 0.06	4.31 \pm 0.04
	No	668 \pm 126	5.95 \pm 0.25	4.51 \pm 0.06	4.38 \pm 0.10
Goats	Yes	754 \pm 91	5.17 \pm 0.30	3.32 \pm 0.10	4.74 \pm 0.09
	No	783 \pm 28	4.86 \pm 0.97	3.64 \pm 0.28	4.87 \pm 0.33

$P > 0.22$ between samples from farms in milk samples from which streptococci were or were not isolated.

Table 3. Findings regarding factors associated with isolation of streptococci from the bulk-tank milk or the milking machine teatcups in sheep or goat farms

Factor	Farms	Proportion of farms, in which streptococci were isolated	Odds ratio (95% confidence intervals)	P
<i>Isolation of streptococci from bulk-tank milk samples</i>				
Milking method applied	Sheep			0.058
Machine-milking ($n = 44$)		16/44 (0.364)	8.684 (0.465–162.029)	0.148
Hand-milking ($n = 7$)		0/7 (0.000)	reference	
Milking method applied	Goats			0.024
Machine-milking ($n = 10$)		4/10 (0.400)	18.692 (0.870–401.853)	0.061
Hand-milking ($n = 13$)		0/13 (0.000)	reference	
<i>Isolation of streptococci from milking machine teatcups</i>				
Month of milking period	Sheep			0.005
1st–2nd		3/5	43.400 (1.682–1119.780)	0.062
3rd–4th		1/12	4.044 (0.151–108.573)	0.405
5th–6th		0/15	reference	
7th–8th		1/12	4.044 (0.151–108.573)	0.405

Only factors with $P \leq 0.2$ are presented.

The only factor associated with isolation of streptococci from bulk-tank milk was the application of milking machine in the farm. It is interesting that in the extensive countrywide study of Vasileiou *et al.* (2018), machine-milking was also found to be the factor associated with subclinical mastitis of streptococcal aetiology. Machine-milking encompasses a variety of factors (e.g. milking conditions, cleaning practices), which, to a varying extent, may potentially contribute to either the development of mastitis or its effective control. Although on their own these factors were not associated with the isolation of streptococci, their combination can possibly contribute to the development of streptococcal mastitis, as indicated by the present results allied to those of Vasileiou *et al.* (2018). One may thus postulate that with the expansion of mechanical milking of sheep and goats, the significance of streptococci as aetiological agents of mastitis might also increase.

Slime-production and biofilm formation has been shown to occur frequently in streptococcal isolates (including *S. uberis*) from mastitis in cows, with a frequency as high as 85% among evaluated collections of strains (Kaczorek *et al.*, 2017; Schönborn *et al.*, 2017; Dieser *et al.*, 2019). The current results present for the first time the slime-production and biofilm-formation from streptococcal isolates from small ruminants and are comparable to those reported in strains from cattle.

The current study also reports for the first time the isolation of streptococci from milking machine teatcups in small ruminant

farms. In cattle, Santos *et al.* (2013) reported the identification of *S. agalactiae* from teatcups and suggested their recovery as an indicator of incorrect cleaning procedures in the farms. In this context, it is noteworthy that *S. uberis* is an environmental organism and can be found in farm bedding. One may postulate that the organism contaminates the skin of the udder and the teats possibly when animals would be lying on straw, particularly in wet and muddy conditions. When the animals are brought into the milking parlour, *S. uberis* present on the teat-end may possibly colonise the milking machine teatcups, especially slime-producing strains, which attach easily onto these. The bacteria may then possibly colonise the teat-end(s) of the animals milked subsequently, which can potentially lead to an increase in intramammary infections. In a recent challenge study, Hillerton (2020) reported that streptococci could cause clinical mastitis more frequently if infused immediately after milking than at other times in relation to milking. This may possibly be allied to a potential transmission of the bacteria from the teatcups to ewes occurring during milking.

The increased frequency of slime-producing isolates recovered from the teatcups, especially from their upper part, indicates that these bacteria could attach and multiply on these, thus increasing potential of intramammary infections. Moreover, presence of slime-production in streptococcal isolates also raises concerns regarding treatment of these pathogens in cases of intramammary infections, as it increases the antimicrobial resistance of biofilm-forming bacteria (Hickey *et al.*, 2018; Dieser *et al.*, 2019).

In conclusion, the study found frequent recoveries of streptococci from bulk-tank and milking teatcups in sheep and goat dairy farms. *S. uberis* predominated among isolates. Machine-milking was associated with the isolation of streptococci from bulk-tank milk samples; on sheep farms, isolation of streptococci from teatcups was more frequent during the initial stage of the milking period. Whilst the results do not prove origin of these bacteria from intramammary infections, they nevertheless indicate a potential to create streptococcal intramammary infections during milking of the animals.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0022029920000734>.

References

- Addis MF, Pisanu S, Marogna G, Cubeddu T, Pagnozzi D, Cacciotto C, Campesi F, Schianchi G, Rocca S and Uzzau S (2013) Production and release of antimicrobial and immune defense proteins by mammary epithelial cells following *Streptococcus uberis* infection of sheep. *Infection and Immunity* **81**, 3182–3197.
- Albenzio M, Santillo A, Caroprese M, Ciliberti MG, Marino R and Sevi A (2016) Effect of stage of lactation on the immune competence of goat mammary gland. *Journal of Dairy Science* **99**, 3889–3895.
- Barrow GI and Feltham RKA (1993) *Manual for the Identification of Medical Bacteria*, 3rd Edn. Cambridge, UK: Cambridge University Press.
- Bramley AJ (1982) Sources of *Streptococcus uberis* in the dairy herd: I. Isolation from bovine faeces and from straw bedding of cattle. *Journal of Dairy Research* **49**, 369–373.
- Devriese LA, Vandamme P, Collins MD, Alvarez N, Pot B, Hommez J, Butaye P and Haesebrouck F (1999) *Streptococcus pluranimalium* sp. nov., from cattle and other animals. *International Journal of Systematic Bacteriology* **49**, 1221–1226.
- Dieser SA, Fessia AS, Zanotti AR, Raspanti CG and Odierno LM (2019) Fibronectin and laminin induce biofilm formation by *Streptococcus uberis* and decrease its penicillin susceptibility. *Microbial Pathogenesis* **136**, 103652.
- Euzeby JP (1997) List of bacterial names with standing in nomenclature: a folder available on the Internet. *International Journal of Systematic Bacteriology* **47**, 590–592. (list of prokaryotic names with standing in nomenclature, <http://www.bacterio.net>).
- Fernandez E, Blume V, Garrido P, Collins MD, Mateos A, Dominguez L and Fernandez-Garayzabal JF (2004) *Streptococcus equi* subsp. *ruminatorum* Subsp. nov., isolated from mastitis in small ruminants. *International Journal of Systematic and Evolutionary Microbiology* **54**, 2291–2296.
- Freeman DJ, Falkiner FR and Keane CT (1989) New method of detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology* **42**, 872–874.
- Gelasakis AI, Mavrogianni VS, Petridis IG, Vasileiou NGC and Fthenakis GC (2015) Mastitis in sheep – The last 10 years and the future of research. *Veterinary Microbiology* **185**, 136–146.
- Hickey EE, Wong HS, Khazandi M, Ogunniyi AD, Petrovski KR, Garg S, Page SW, O’Handley R and Trott DJ (2018) Repurposing ionophores as novel antimicrobial agents for the treatment of bovine mastitis caused by Gram-positive pathogens. *Journal of Veterinary Pharmacology and Therapeutics* **41**, 746–754.
- Hillerton JE (2020) Timing of entry of *Streptococcus uberis* into the mammary gland of the dairy cow. *Journal of Dairy Research* **87**. doi: 10.1017/S0022029920000357.
- Kaczorek E, Małaczewska J, Wójcik R and Siwicki AK (2017) Biofilm production and other virulence factors in *Streptococcus* Spp. isolated from clinical cases of bovine mastitis in Poland. *BMC Veterinary Research* **13**, 398.
- Kruze J and Bramley AJ (1982) Sources of *Streptococcus uberis* in the dairy herd: I. Evidence of colonization of the bovine intestine by *Str. uberis*. *Journal of Dairy Research* **49**, 375–379.
- Marogna G, Pilo C, Vidili A, Tola S, Schianchi G and Leori SG (2012) Comparison of clinical findings, microbiological results, and farming parameters in goat herds affected by recurrent infectious mastitis. *Small Ruminant Research* **102**, 74–83.
- Pisanu S, Cubeddu T, Pagnozzi D, Rocca S, Cacciotto C, Alberti A, Marogna G, Uzzau S and Addis MF (2015) Neutrophil extracellular traps in sheep mastitis. *Veterinary Research* **46**, 59.
- Queiroga MC (2017) Prevalence and aetiology of sheep mastitis in Alentejo region of Portugal. *Small Ruminant Research* **153**, 123–130.
- Santos AI, Pires ACS, Behaine JJS, Araújo EA, de Andrade NJ and de Carvalho AF (2013) Effect of cleaning treatment on adhesion of *Streptococcus agalactiae* to milking machine surfaces. *Food and Bioprocess Technology* **6**, 1868–1872.
- Schönborn S, Wente N, Paduch JH and Krömker V (2017) In vitro ability of mastitis causing pathogens to form biofilms. *Journal of Dairy Research* **84**, 198–201.
- Vasileiou NGC, Cripps PJ, Ioannidi KS, Chatzopoulos DC, Gougoulis DA, Sarrou S, Orfanou DC, Politis A, Calvo Gonzalez-Valerio T, Argyros S, Mavrogianni VS, Petinaki E and Fthenakis GC (2018) Extensive country-wide field investigation of subclinical mastitis in sheep in Greece. *Journal of Dairy Science* **101**, 7297–7310.
- Vasileiou NGC, Chatzopoulos DC, Sarrou S, Fragkou IA, Katsafadou AI, Mavrogianni VS, Petinaki E and Fthenakis GC (2019) Role of staphylococci in mastitis in sheep. *Journal of Dairy Research* **86**, 254–266.