

Coagulase-negative staphylococci in ewe udder surface and raw milk samples

Andualem Tonamo^{1,2} – István Komlósi² – Flóra Mária Petróczki^{1,3} – Ferenc Peles³

¹Doctoral School of Animal Husbandry, University of Debrecen, Böszörményi Street 138, 4032 Debrecen, Hungary

²Institute of Animal Science, Biotechnology and Nature Conservation, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, Böszörményi Street 138, 4032 Debrecen, Hungary

³Institute of Food Science, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, Böszörményi Street 138, 4032 Debrecen, Hungary
andytonamo@agr.unideb.hu

SUMMARY

Coagulase-negative staphylococci (CNS) are among the major causes of subclinical mastitis in dairy ewe flocks. This has a financially significant impact on the ewe dairy sector and consumer health. The determination of the bacterial count, particularly CNS, is critical in terms of the quantity and quality of ovine milk. Thus, the purpose of this study was to quantify the CNS count in the udder surface and raw milk samples of the ewe, in addition, to identify CNS strains ($n=8$) collected from udder surface and individual raw milk samples by 16S rRNA gene sequencing. A total of 164 samples of udder surface and raw milk originated from four sheep farms were tested. The obtained values were compared between the different farms. Furthermore, values during 2018 and 2019 in the case of Farm I were compared. Significantly higher ($p<0.05$) average count was observed in udder surface samples taken from Farm I (2.8 ± 1.0 lg CFU/cm²) than that of Farm III samples (2.3 ± 0.6 lg CFU/cm²). In the case of individual raw milk, the higher value was observed in samples derived from Farm III (3.5 ± 0.9 lg CFU/mL), while the obtained value was significantly lower ($p<0.05$) in samples originated from Farm IV (1.8 ± 0.4 lg CFU/mL). In the bulk tank milk samples, the highest mean value was 5.3 ± 0.4 CFU/mL, and there was no significant ($p>0.05$) variation between farms. Coagulase-negative staphylococci counts were decreased to a certain extent in both sample types tested during 2019 except for individual raw milk derived from the Tsigai breed. The correlation between the mean CNS counts of udder surface and individual raw milk was very weak ($r=0.048$). *Staphylococcus simulans*, *Staphylococcus auricularis*, and *Staphylococcus equorum* were identified by molecular sequencing and *Staphylococcus simulans* were the most frequently identified CNS species. A higher CNS count of bulk tank milk than individual raw milk indicates possible contamination during milking and storage. Therefore, further studies are required to investigate the other sources of bulk tank milk contamination to improve the hygienic quality of milk.

Keywords: Coagulase-negative staphylococci; individual raw milk; udder surface

INTRODUCTION

Based on their ability to produce coagulase enzymes, staphylococcal bacteria are categorized as coagulase-positive (e.g. *S. aureus*, *S. hyicus*, and *S. delphini*) and coagulase-negative (e.g. *S. haemolyticus*, *S. simulans*, and *S. epidermidis*) (Vasileiou et al., 2019). Consumption of ewe milk contaminated with coagulase-negative staphylococci (CNS) can cause health problems in humans, particularly milk products made from unpasteurized raw milk (Zell et al., 2008; Kadariya et al., 2014). Some CNS strains produce heat resistant enterotoxins that cause food poisoning. However, CNS strains had a lower level of enterotoxigenicity and pathogenicity in comparison to *Staphylococcus aureus*, which is coagulase-positive (Ünal and Çinar, 2012; Podkowik et al., 2013). Like *S. aureus*, enterotoxins were detected in CNS strains such as *S. epidermidis*, *S. cohnii*, *S. haemolyticus*, and *S. xylosum* from ovine milk and cheese samples (Podkowik et al., 2013). Many studies have shown that CNS can enter into milk from the udder and teat surfaces of animals as well as from the milker's hand (Bergonier and Berthelot, 2003; Mavrogianni et al., 2007; Vasileiou et al., 2019). These bacteria are associated with subclinical mastitis in dairy ewe flocks (Spanu et al., 2011; Gelasakis et al., 2015). The prevalence of subclinical mastitis in dairy ewe flocks is

usually between 5 and 30% or higher (Contreras et al., 2007; Vasileiou et al., 2018).

According to the study from Greece, the overall prevalence of CNS in subclinical mastitis ewe milk was 59.7% with the *S. chromogenes* (23%) predominant species followed by *S. epidermidis* (19.8%), *S. simulans* (17.6%), and *S. hominis* (8.8%) (Vasileiou et al., 2018). European Union legislation No. 2073/2005 on foods of animal origin (European Commission, 2005) sets the limit value for *S. aureus* (coagulase-positive staphylococci). On the other hand, the limit value for CNS has not been established. To improve milk hygiene in dairy ewe flocks, it is crucial to know the milk's CNS count and the source of contamination. However, in the studied area, the data on CNS count in the milk and udder surface of the ewe were very scarce. Therefore, this study aimed to examine coagulase-negative staphylococci in the udder surface and raw milk samples from the ovine. Furthermore, to identify CNS strains ($n=8$) collected from udder surface and individual raw milk samples by 16S rRNA gene sequencing.

MATERIALS AND METHODS

One hundred fifty-four samples were obtained from four sheep farms. Of these, 77–77 samples were taken from the udder surface (US) and individual raw milk (IRM). Moreover, 10 bulk tank milk (BTM) samples



were collected from farms where ewes were milked. Animals were not milked on Farm I during this study, an experimental sheep farm. However, the installation of a milking parlour was done, and milking is expected in the coming years. The flock on the Farm I consists of Merino, Tsigai, and Dorper breeds, while Lacaune in Farm II and Farm III, and British Milkshopeep in Farm IV. During March–July 2018 and 2019, the samples were collected from Hajdú-Bihar (Farm I–Farm III) and Jász-Nagykun-Szolnok (Farm IV) Counties of eastern Hungary.

Farm characteristics, date of sample collection, sample collection procedures, and sample handling have been described in our previous publication (Tonamo et al., 2020). Bacteria were cultured on Baird-Parker agar (Biolab Ltd., Hungary) enriched with egg yolk and tellurite by spreading 0.1 mL of dilution. After 48 hours of incubation at 37 °C, the colonies were counted.

Eight CNS strains collected from 12 samples (6 US and 6 IRM) obtained from Farm I were identified by partial 16S rRNA gene sequencing. The sequencing was done in the Biomi Ltd. Company, Hungary. The sequencing of the partial 16S rRNA gene was with 519R and 27F primers (Lane, 1991), used in the amplification. Two databases with a partial double-stranded 16S rRNA gene fragment were used as a reference. The National Center for Biotechnology Information and the TrueBac™ ID (DB ver.

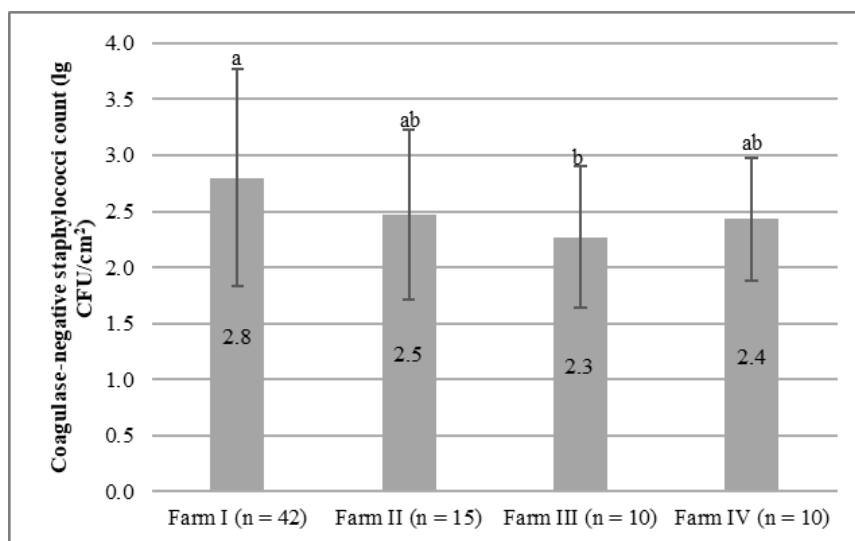
20190409) (Yoon et al., 2017) databases. The Basic Local Alignment Search Tool algorithm was used to carry out alignment and the alignment is limited to cultivable strains.

GraphPad Prism 3.02 and Microsoft Excel 2016 were used to analyze data. The US sample's CNS count was divided by 20, as the sample was obtained from a surface area of 20 cm². Then the results were transformed into a logarithm. Farm (Farm I–Farm IV) and year (Farm I) effect on CNS count were evaluated. The mean difference was compared using a one-way analysis of variance and the Kruskal-Wallis test. The correlation between the US and the corresponding individual result of the raw milk sample was determined by Pearson correlation. The significance level was considered at $p < 0.05$.

RESULTS AND DISCUSSION

The mean values of coagulase-negative staphylococci (CNS) in udder surface (US) samples originated from four sheep farms were shown in *Figure 1*. The average count in udder surface samples obtained from Farm I, II, III, and IV was 2.8 ± 1.0 , 2.5 ± 0.8 , 2.3 ± 0.6 , and 2.4 ± 0.6 lg CFU/cm², respectively (*Figure 1*). The mean value of US samples derived from Farm I was significantly ($p < 0.05$) higher than that of Farm III samples (*Figure 1*).

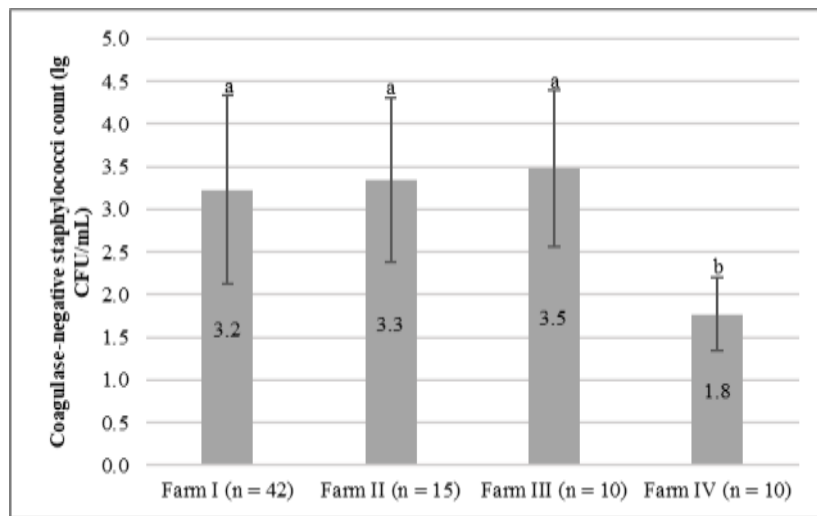
Figure 1. Coagulase-negative staphylococci count in ovine udder surface



The mean of individual raw milk (IRM) samples derived from Farm I, II, III, and IV was 3.2 ± 1.1 , 3.3 ± 1.0 , 3.5 ± 0.9 , and 1.8 ± 0.4 lg CFU/mL, respectively (*Figure 2*). The value of IRM samples from Farm IV was substantially ($p < 0.05$) lower value than that originating from other farm samples (*Figure 2*). This may be attributed to the British Milkshopeep breed on

Farm IV, which is one of the strong and hardy sheep breeds. This ewe breed can withstand bacteria invading the mammary gland via the teat canal. Similar to our results, another study from Kosovo revealed that the farm had a substantial impact on the total bacterial count of individual raw ewe milk (Bytyqi et al., 2013).

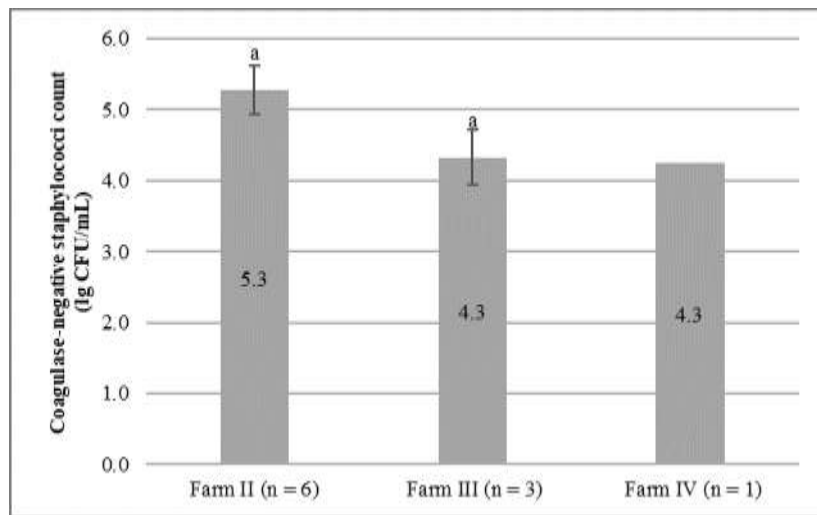
Figure 2. Coagulase-negative staphylococci count in ovine individual raw milk



The average value of BTM samples in Farm II, III, and IV was 5.3 ± 0.4 , 4.3 ± 0.4 , and 4.3 lg CFU/mL, respectively (Figure 3). Only one BTM sample in Farm IV was examined for the bacterial count. Therefore, the result of Farm IV was not statistically compared to the results of other farms. Indeed, there is no significant ($p > 0.05$) difference between Farm II and III. However,

the average value of BTM from Farm II is slightly higher (Figure 3). A higher CNS count of BTM than IRM indicates possible contamination during milking and storage. Comparatively similar staphylococci mean count (4.4 ± 0.5 lg CFU/mL) was reported from Spain for a bulk tank ewe's milk samples (Poveda et al., 2020).

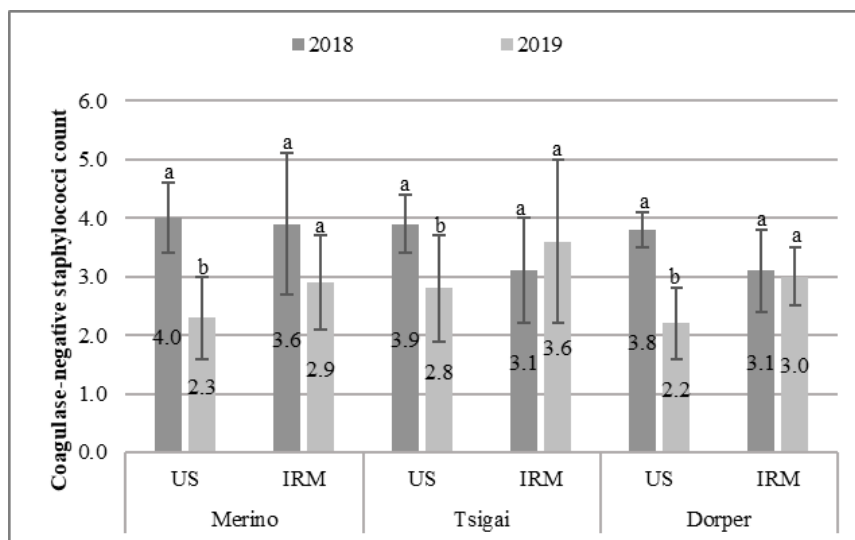
Figure 3. Coagulase-negative staphylococci count in ovine bulk tank milk



The mean was statistically compared between the year of investigation (2018 and 2019) for Merino, Tsigai, and Dorper ewe breeds on Farm I (Figure 4). The CNS count of all breeds was significantly ($p < 0.05$) lower in US samples tested during 2019 than in 2018. Unlike US samples, a non-significant ($p > 0.05$) difference was observed in IRM samples taken from three ewe breeds between 2018 and 2019. The variation of mean value between years could be explained as a difference in management practices every year. There

might be an improvement in the hygienic conditions of sheep farms over time. Similarly, differences in the total bacterial count in ewe BTM have been observed between years in Spain (Gonzalo et al., 2010). The mean of the US and the corresponding IRM results were used to measure the coefficient of correlation. There was none significant ($p > 0.05$) positive correlation coefficient ($r = 0.048$) between CNS counts of US and IRM. This shows that the correlation between CNS counts of US and IRM is very weak.

Figure 4. The effect of year of investigation on CNS count of udder surface (lg CFU/cm²) and individual raw milk (lg CFU/mL) samples of three ewe breeds on Farm I



During 2018 and 2019, 6 udder surface and 6 individual raw milk samples were examined for all breeds, but in the case of Tsigai breed in 2018, 12–12 udder surface and individual raw milk samples were examined. US: udder surface, IRM: individual raw milk.

Eight CNS isolates were successfully identified using sequencing analysis (Table 1). In this study, three CNS species were identified from US and IRM samples. The most frequently identified species was *S. simulans* (37.5%) followed by *S. auricularis* (25%), and *S. equorum* (25%) (Table 1). Similarly, from

Brazil, Martins et al. (2017) reported *S. simulans* was the most frequent isolate in both ewes with subclinical mastitis and in healthy ewes. On the contrary, the finding by Onni et al. (2010) from Italy revealed that *S. epidermidis* was the most isolated CNS species in ovine milk by molecular analysis.

Table 1. Frequency of coagulase-negative staphylococci isolates identified by 16S rRNA sequencing

Sources	Species	Frequency n (%)
Individual raw milk	<i>Staphylococcus simulans</i>	3 (37.5)
Individual raw milk	<i>Staphylococcus auricularis</i>	2 (25)
Individual raw milk	<i>Staphylococcus equorum</i>	1 (12.5)
Udder surface	<i>Staphylococcus equorum</i>	2 (25)
Total		8 (100)

CONCLUSIONS

The mean of coagulase-negative staphylococci count was low in the udder surface and individual raw milk samples. There was a very weak association between the mean values of udder surface samples and the parallel individual raw milk samples. *Staphylococcus simulans* was mostly identified species among other coagulase-negative staphylococcal species identified by molecular sequencing. A higher coagulase-negative staphylococcal count of bulk tank milk than individual raw milk shows possible contamination during milking and storage. Therefore,

further studies are required to investigate the other sources of bulk tank milk contamination to improve the hygienic quality of milk in the studied farms.

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