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MICROBIOLOGICAL CHARACTERIZATION OF UNPASTEURIZED SHEEP MILK

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MICROBIOLOGICAL CHARACTERIZATION OF UNPASTEURIZED SHEEP MILK

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ABBREVIATIONS AND ACRONYMS

ABR	Antibiotic resistance
ANOVA	Analysis of variance
API	Analytical profile index
bp	Base pair
BTM	Bulk tank milk
CC	Coliform count
CDC	Centers for Disease Control and Prevention
CFC	Chives-flavoured cheese
CFSC	Cumin-flavoured-smoked cheese
CFU	Colony forming unit
cm	Centimetre
CMT	California Mastitis Test
CNS	Coagulase-negative staphylococci
CTM	Cooling tank milk
EBC	*Enterobacteriaceae* count
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FBTM	Fresh bulk tank milk
g	Gram
GFC	Garlic-flavoured cheese
GFSC	Garlic-flavoured-smoked cheese
HSGBA	Hungarian Sheep and Goat Breeders' Association
ICAR	International Committee for Animal Recording
IDF	International Dairy Federation
IRM	Individual raw milk
ISO	International Organization for Standardization
kg	Kilogram
LABC	Lactic acid bacteria count
lg	Decimal logarithm

MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
mL	Millilitre
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSP	Main spectrum profile
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
OECD	Organization for Economic Co-operation and Development
OMÉK	Országos Mezőgazdasági és Élelmiszeripari Kiállítás és Vásár (National Agricultural and Food Exhibition and Fair)
OTU	Operational Taxonomic Unit
PBC	Psychrotrophic bacteria count
PCR	Polymerase chain reaction
QIIME	Quantitative insights into microbial ecology
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SAC	*Staphylococcus aureus* count
SCC	Somatic cell count
SD	Standard deviation
SEs	Staphylococcal enterotoxins
STEC	Shiga-toxin producing *Escherichia coli*
t	Metric tones
TPC	Total plate count
US	Udder surface
USA	United States of America
WHO	World Health Organization

1. INTRODUCTION

Over the past three decades, global milk production had increased from 530 million tons in 1988 to 852 million tons in 2019 by more than 60 percent. As the population of the world rises rapidly, the consumption of milk and milk products also increases with an expected per capita consumption of 125.2 kg by 2025 [INTERNATIONAL DAIRY FEDERATION (IDF), 2016]. To improve food and nutrition security, milk products are becoming a regular requirement. Sheep milk and its products are among the functional foods with higher nutritional value and health potential (BALTHAZAR et al., 2017; MOHAPATRA et al., 2019). Milking sheep has been started since the time of domestication nearly 13,000 years ago (BALTHAZAR et al., 2017). Although sheep milk can rarely be consumed fresh, it is mainly used in yogurt and cheese making (HAENLEIN and WENDORFF, 2006; PIRISI et al., 2007).

Currently, there are approximately 1.24 billion heads of sheep in the world, and around 21.8% were anticipated for milk production (FAOSTAT, 2019). Asia shares 42.6% of this population followed by Africa (32.9%) and Europe (10.3%). Though more than 80% of world milk is from dairy cows, approximately 1.3% of milk comes from sheep (FAOSTAT, 2019). As reported by FAO (2019), Asia leads in global sheep milk production with a share of 46.9% followed by Europe (29.5%) and Africa (22.8%). In ovine milk production, Turkey, Syria, and Greece have been at the forefront of the Mediterranean Sea bordering countries (PULINA et al., 2018). Greece, Spain, Italy, and Romania are leading countries with the share of 31.8, 19.0, 16.7, and 14.3% of ovine milk production within European Union (EU) countries, respectively (FAOSTAT, 2019).

Hungary is one of the EU countries with approximately 790,000 heads of ewes out of 1.1 million heads of ovine flock size in late 2019 (HUNGARIAN CENTRAL STATISTICAL OFFICE, 2019). The available data on FAOSTAT (2019) database indicated that the ovine milk production of Hungary in 2019 was 1,580 metric tons. Even though around 80% of the sheep population in the country belongs to the Merino breed, there are other indigenous and exotic sheep breeds (TOLDI et al., 2003; KUKOVICS and NÉMETH, 2011; Nagy et al., 2011). There are currently 19 dairy sheep farms in Hungary where milking data are regularly recorded. Some of the dairy sheep breeds kept on these farms are Milking Tsigai, Lacaune, British Milksheep, and East Friesian breeds and in few farms, Awassi and Merino breed has been milked [HUNGARIAN SHEEP and GOAT BREEDERS' ASSOCIATION (HSGBA), 2020].

At commercial dairy sheep farms, the key parameters regularly inspected in milk are total plate count, chemical composition (fat and protein content), somatic cell count, and adulteration (PIRISI et al., 2007). Moreover, pH and the appearance of milk are also important (PARK et al., 2013). Understanding of public health and food safety concerns has led to more interest in milk hygiene in ovine to reduce risks and ensure optimum quality of consumption (GONZALO, 2017). However, especially in Hungary, in comparison with cow milk, less emphasis is given to ovine milk hygienic requirements, while consumer demand is increasing (KUKOVICS et al., 2009).

The microbial load is low in fresh milk drawn from a healthy animal mammary gland, which is less than 1000 CFU/mL (FOTOU et al., 2011). However, contaminant microorganisms enter into milk from several sources, predominantly from the farm environment and animal body (QUIGLEY et al., 2013; ADDIS et al., 2016). This increases the microbial load up to 100 fold or more in the milk (FOTOU et al., 2011). Microorganisms facilitate the fermentation process, cause disease, or spoils milk after entering (QUIGLEY et al., 2013). The REGULATION (EC) 853/2004 of the European Parliament and of the council for ovine milk limit value has targeted only aerobic mesophilic count. Based on this regulation, the total plant count of ovine milk to be pasteurized before processing must be ≤ 1,500,000 CFU/mL (6.2 lg CFU/mL), and to be processed without pasteurizing should be ≤ 500,000 CFU/mL (5.7 lg CFU/mL).

According to the finding of FOTOU et al. (2011) and SILVA et al. (2020) from Greece and Brazil, 6.0 and 6.9 lg CFU/mL of lactic acid bacteria count were detected in unpasteurized ovine bulk tank milk, respectively. These bacteria facilitate the fermentation process or promote health (VARGA et al., 2014). In contrast, *Listeria monocytogenes*, *Campylobacter* spp., *Salmonella* spp., *Brucella melitensis*, *Yersinia pseudotuberculosis*, Shiga toxin-producing *Escherichia coli*, and *Staphylococcus aureus* are reported as some of the pathogenic bacteria in raw ovine milk and its products **(**MUEHLHERR et al., 2003; VERRAES et al., 2014; VAN DEN BROM et al., 2020). Therefore, evaluating the hygienic status of unpasteurized ovine milk and cheese samples is necessary because of public health and economic effects (GONZALO, 2017). Another significant concern is the identification and evaluation of the characteristics of bacterial species.

Aims of the study

In Hungary, where sheep are milked, the consumption of milk and its products is so far less in terms of quantity and quality. It is therefore very important to expand dairy sheep farms with milk type ewes, with the aim of producing hygienic milk and its products. Respecting acceptable limit values for somatic cell count, total plate count, and other pathogenic microorganisms is another issue that needs consideration in ovine milk sector. Microbiological studies on ovine-associated products have so far been relatively ignored, although there is an upward worldwide interest in the consumption of ovine milk and milk products. This is also true for Hungary, where the microbiological quality of raw ovine milk and cheese samples has not been fully investigated (KUKOVICS et al., 2009).

Basic data on the microbiological quality of ovine-associated samples (e.g. udder surface, raw milk, and cheese) for further research to improve the quality of ovine-originated foods are critical. Moreover, studying the virulence factor and antibiotic resistance of pathogenic bacterial strains such as *S. aureus* is important from a public health point of view. To the best of our knowledge, this is the first report evaluating the bacterial count and characterizing bacterial strains of ovine udder surface samples in Hungary. The following specific objectives have been pursued through the duration of the research.

- Examination of the microbiological status (total plate count, *Enterobacteriaceae* count, *Escherichia coli* count, and *Staphylococcus aureus* count) of udder surface samples.
- Examination of the microbiological status (total plate count, *Enterobacteriaceae* count, *Escherichia coli* count, *Staphylococcus aureus* count, and lactic acid bacteria count) and California Mastitis Test of individual raw milk samples.
- Examination of the microbiological status (total plate count, *Enterobacteriaceae* count, *Escherichia coli* count, *Staphylococcus aureus* count, psychrotrophic bacteria count, and lactic acid bacteria count) of bulk tank milk samples.
- Examination of the microbiological status (*Enterobacteriaceae* count, *Escherichia coli* count, *Staphylococcus aureus* count, and lactic acid bacteria count) of cheese samples.

- Determination of the coefficient of correlation between the major bacterial counts; i.e. total plate count and *Enterobacteriaceae* count of corresponding udder surface and individual raw milk samples.
- Analysis of microbiota community in some raw milk samples.
- Isolation and identification of staphylococcal and lactic acid bacteria strains.
- Phenotypic (tellurite production, lecithinase activity, coagulase test, hemolysis, catalase test, oxidase test and antibiotic resistance) and genotypic (enterotoxin gene presence) characterizations of staphylococci strains.
- Phenotypic (catalase test, oxidase test, and antibiotic resistance) properties of lactic acid bacteria isolates.

2. LITERATURE REVIEW

2.1. Global ovine milk production

The continuous growth of the world population and the increasing demand for dairy products and their derivatives have resulted in a steady increase in world per capita milk consumption, reaching 111.3 kg in 2015 (IDF, 2016). World milk production has increased over the past three decades from 530 million tons in 1988 to 852 million tons in 2019 by more than 60 percent. It is expected that it will rise at 1.6% per annum over the next 9 years, (to 997 t by 2029), faster than most other big agricultural commodities (OECD/FAO, 2020). Although the world average flock growth is higher than the world average yield growth, the shifting rates are the product of rising flocks faster in relatively low yield countries. The drivers of yield growth include optimizing milk production processes, improving animal health, increasing feeding efficiencies, and improved genetics (OECD/FAO, 2020).

According to the FAOSTAT database of 2019, 81.05% of world milk production comes from cattle, followed by milk from other animals such as buffalo (15.14%), goat (2.25%), sheep (1.30%), and camel (0.35%). However, dairy sheep farms represent a significant proportion of agricultural economies in many countries, particularly on the borders of the Middle East and Mediterranean (PARK et al., 2007; PULINA et al., 2018). Sheep milk is usually not consumed directly in contrast with cow milk but it is used in the production of ethnic dairy foods, typically used for self-consumption or marketed as conventional, high-quality dairy products included in the list of main ingredients of the so-called Mediterranean diet (DENNETT, 2016; PULINA et al., 2018).

World sheep milk production increased by 25.4% during the period 2000-2019 (Table 1). Meanwhile, during the same time, in Asia, it was increased by 35.7% having a contribution of about 46.9%. In all the continents, there has been a rise in the production of sheep milk. World production of fresh sheep milk reported by FAOSTAT (2019) is estimated to be 10.6 million tons (Table 1). According to the FAOSTAT database (2019), the Asian continent is home to the first two main producing countries in the world in 2019: Turkey (1,521,455 t) and China (1,166,323 t) (Figure 1). Similarly, from Europe, Greece (944,300 t) and Spain (563,530 t) were the third and fifth largest producers of sheep milk in the world. Syria takes fourth place with 574,362 t milk production (Figure

1). There is a very small but growing production in the Americas (0.9%) and Oceania (< 0.1%) continents (Table 1).

Table 1: Sheep milk production (metric tons) in the world from 2000-2019

	Sheep milk production (metric tons)			Change (%) 2000-2019	Contribution (%) 2019
	2000	2019	Mean (2000-2019)		
Africa	1,830,070	2,410,156	2,133,503.3	31.7	22.8
Americas	79,895	91,173	90,406.7	14.1	0.9
Asia	3,655,669	4,960,306	4,299,905.2	35.7	46.9
Europe	2,876,672	3,125,385	3,051,693.4	8.6	29.5
Oceania	ND	ND	ND	ND	ND
World	8,442,305	10,587,020	9,575,508.5	25.4	100

ND: no data. [Source: FAOSTAT (2019)].

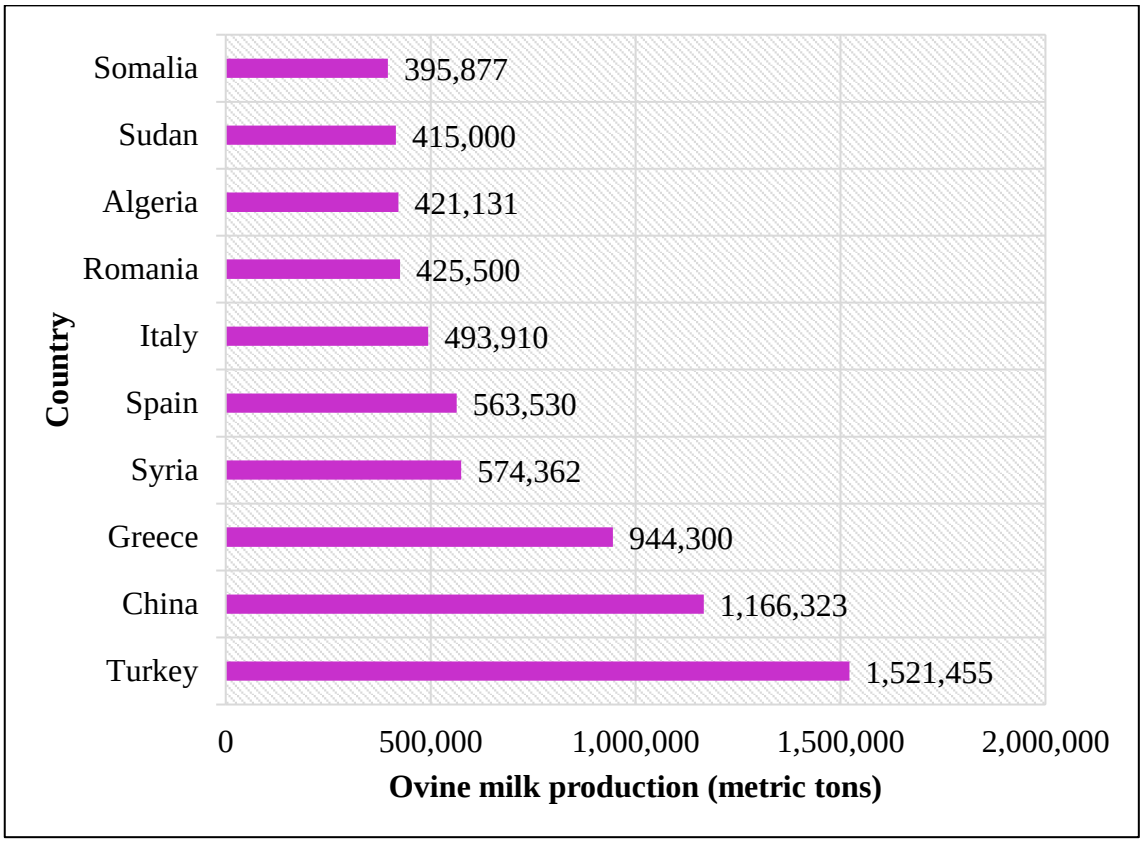

Figure 1: Ovine milk production (metric tons) of top ten producing countries during. 2019. [Source: FAOSTAT (2019)].

2.2. Ovine milk production status and breeds in Hungary

The dairy sheep industry in Hungary is one of the livestock farmings for milk and its products. According to the HUNGARIAN CENTRAL STATISTICAL OFFICE (2019), by the end of 2019, the flock size of sheep was 1.1 million and the stock of ewes was 782,000 heads. The number of the former being 48,000, and the number of the latter 16,000 less than one year earlier.

Milk production increased rapidly in the mid-1980s, and then gradually decreased until 1990 in Hungary. Prior to the 1990s, state and cooperative farms dominated the dairy sheep sector, with only a few private farms. In 1990, the sector's ownership changed, with sheep farms being privatized in the majority (95%) (KUKOVICS and NÉMETH, 2011). There has been a decrease in sheep milk production, and the majority of milk processing plants have been closed. To address this issue, the Hungarian Sheep Dairying Association was established in 1996 with the goal of increasing milk production while maintaining quality (KUKOVICS and NÉMETH, 2011). The government accepted and introduced it in 1997 with a new subsidy system. Between 1997 (1,945 t) and 2003 (2,647 t), sheep milk production increased by about 37% (FAOSTAT, 2019). However, it began to decline after 2005. FAOSTAT database of 2019 showed that Hungary ranked 14th country in the European Union by ovine milk production with an amount of 1,580 metric tons in 2019 (Figure 2).

According to Figure 2 below, in Hungary, it was observed that there was a significant decrease in the production of sheep milk from time to time. It has decreased over the past two decades from 3,200 t in 2000 to 1,580 t in 2019 by 50.6% (Figure 2). This could be due to the fact that the labour shortage in sheep farms, the lack of capital to invest in milking machines and the low profit in milking are among several factors. Furthermore, a low level of subsidy to improve milking, the dominance of lamb meat, and low milk production of Merinos (KUKOVICS and NÉMETH, 2011).

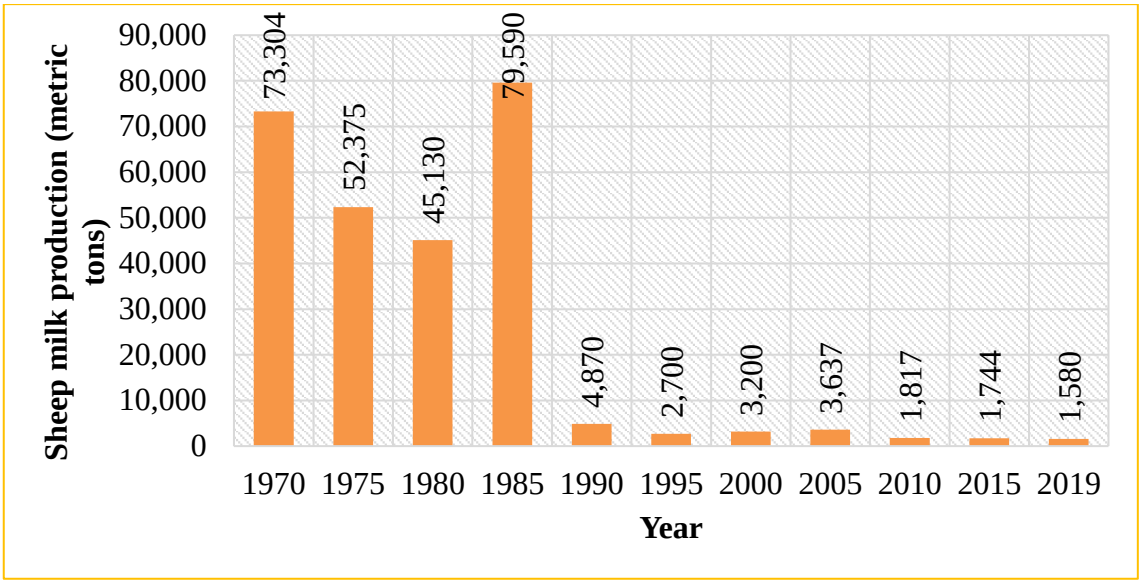

Figure 2: Sheep milk production in Hungary (1970-2019). [Source: FAOSTAT (2019)].

Data recording (flock size, breed size, quantity, and quality of milk) is one of the most important aspects of the dairy sheep sector. This works for Hungarian sheep farms as well. However, currently, only 19 dairy sheep farms in the country are recording ovine dairy farm-associated monitoring (such as flock size, type, age, the quantity of milk, and animal health) regularly. The total number of ewes kept on these farms is 4,805 heads of different flock sizes; from 16 to more than 600 heads of ewes. More than 47% (9/19) of the farms have less than 200 heads of ewes. Fourteen out of 19 farms are raising Lacaune breed, while Milking Tsigai and British Milksheep breeds are raised on the rest of the farms. Lacaune breed has a big share of 74.7% (3,590 heads) followed by British Milksheep (15.5%) and Milking Tsigai (9.8%) (Table 2).

Table 2: Summary of flock size on 19 dairy sheep farms in Hungary

	Number of sheep (heads)		
Breed	**Ewes**	**Yearling females**	**Rams**
Lacaune	3,590	1,063	453
British Milksheep	744	56	42
Milking Tsigai	471	163	104
Total	**4,805**	**1,282**	**599**

2018 data. Source: HSGBA (2020).

According to the study by TOLDI et al. (2003) and NAGY et al. (2011), in Hungary, Merino Sheep breed shares the majority of ovine population, which is around 80%. In addition to Merino, some other dairy breeds like Milking Tsigai, Lacaune, and British Milksheep are present. Merino sheep have been kept in Hungary since the mid-1700s. The predecessor of the Hungarian Merino, the Hungarian Combed Merino, was formed in the 1920s and 1930s by the crossing of the partly the Racka and the longhaired German-Swabian sheep **(**NAGY et al., 2011; HSGBA, 2020).

To improve meat production of this breed, breeding also began in the 1960s, first using French Merino and then German meat Merinos (HSGBA, 2020). From 1993, with the establishment of an independent pedigree, the development of a unified Hungarian Merino began. It has moderate maternal characteristics, lamb-raising ability and it can be bred all year round.

Another sheep breed is Tsigai. Tsigai is an indigenous breed found in Southeast and Central Europe, especially in Hungary, Slovakia, Serbia, and Romania (NASTIĆ et al., 2020). The number of this sheep breed is declining because of its relatively low milk and meat production (KUKOVICS and NÉMETH, 2011; NASTIĆ et al., 2020). However, preserving the genetic resources of Tsigai sheep was strongly needed due to the possibility of increased demand for genetic resources in the future due to climate change (AUGUSTIN et al., 2015). The genetic variability among various Tsigai sheep populations to maintain and determine their genetic diversity was studied in Hungary by ANNUS et al. (2015) and KUSZA et al. (2010). Although this breed is indigenous with low productivity, there have been some occasions where it has been successfully used, for example in Ukraine, to enhance the characteristics of other local sheep breeds (SEDILO et al., 2016).

In Hungary, as reported by KUKOVICS and NÉMETH (2011), although Tsigai is a multi-purpose (milk, wool, and meat) breed, priority was given for the production of meat. The same trends were experienced, which means the raising of this breed was oriented toward meat production in Croatia (VRDOLJAK et al., 2007), Romania (ILIŞIU et al., 2013), and Serbia (PETROVIĆ et al., 2011). Milking Tsigai was introduced to Hungary from Balkan at the end of the 18th century. The data from HSGBA (2020) website indicates that milk production of this breed is between 100-200 kg within 120-160 days (Figure 3c).

Furthermore, Dorper is also another sheep breed raised in Hungary. Dorper sheep was developed in South Africa in the 1930s by crossing the Dorset Horn and Blackhead Persian breeds. Although it is originated from South Africa, now found in various part of the world (Figure 3b). It is primarily raised for mutton with a long breeding season (BUDAI et al., 2013). Dorper ewe has docile temperament and very good maternal quality. The daily weight gain of lambs is high. This breed's early maturity, hardiness, and low shearing requirements are also advantages (HSGBA, 2020). On the other hand, poor wool and milk production are drawbacks of this breed. Dorper can certainly be raised for milk production as well, but it is not the best choice because milk production is better with milk type breeds such as East Friesians and Lacaunes.

Hungary is one of few countries where most of British Milksheep breed can be found now. British Milksheep was bred in the United Kingdom in the 1970s by crossing East Friesian, Bluefaced Leicester, Dorset-Horn, Finnish Landrace, and Welsh Llyn breeds. In addition to being available in its native area, this breed was exported to Hungary, France, Canada, and Greece. It has a very good milk-producing ability and milk production is 200-400 kg during 160-200 days (HSGBA, 2020) (Figure 3d). It has a very good milk-producing ability but is mainly recommended as a maternal line of crosses as a breed with excellent reproductive and lamb-raising ability (HSGBA, 2020). According to the data on the BRITISH MILKSHEEP SOCIETY (2021) homepage, this breed is sturdy and active sheep, easy to manage, and highly prolific. The quality of its wool and carcass has additional economic benefits. Except for lambing ewes, all types of stock are capable of wintering outside in the most challenging weather **(**EKARIUS, 2008).

Moreover, Lacaune is a dual-purpose (meat and milk) breed but mainly raised for milk in Hungary. Lacaune breed is the best-known French Dairy breed with milk production of up to 400 kg during 150-180 days (HSGBA, 2020) (Figure 3a). Data from the INTERNATIONAL COMMITTEE for ANIMAL RECORDING (ICAR) (2021) homepage showed that approximately 1 million heads of Lacaune ewe have been raised in France in 2,500 flocks, in which 373 flocks milk is recorded. In the 1960s and 1970s, the income from meat and milk was the same (BARILLET et al., 2001), but at present, the income from milk is about three times higher than the income from meat, revealing the fact that this breed is currently one of the high milk yield breeds (ROBLES-JIMENEZ et al., 2020).

Lacaune milk is nutritious with high protein and milk fat content and good flavour. This high milk fat content of milk makes it ideal for cheese production. “Roquefort”

cheese is made primarily from the milk of Lacaune sheep. It tends to excellent seasonality and breeding of this sheep can be at any time of the year, especially during the April-May period (HSGBA, 2020).

Figure 3: Sheep breeds: Figure 3(**a**) was captured on 28 May 2018, 3(**b**) and 3(**c**) on 25 April 2018, and 3(**d**) was from https://i1.wp.com/www.britishmilksheep.com/wp-content/uploads/2013/11/Triplets.jpg?fit=4320%2C3240&ssl=1.

2.3. Chemical composition of ovine milk

Ovine milk value depends on the chemical and physical properties; such as total solids, protein, fat content, density, conductivity, and pH (GONZALO et al., 2006). Depending on the stage of lactation, parity, season, animal age and nutrition, breed, and udder diseases, the chemical composition of ovine milk varies among animals (TAMIME et al., 2011; CLAEYS et al., 2014). Because of the changes in the forage composition, seasonal variations greatly influence the composition of fatty acids in ovine milk (REVILLA et al., 2017). The findings of different studies, presented in Table 3, revealed that sheep milk has a higher level of fat content than bovine, caprine, and camel milk but a lower level than buffalo milk. The highest amount of total solid and solids-not-fat was confirmed in ovine milk by different studies (Table 3).

The protein content of ovine milk has a significant impact on nutritional and technological values. According to SELVAGGI et al. (2014), ovine milk protein content is composed of casein and whey proteins. As indicated in Table 3 below, the lactose content in sheep milk is similar to dairy cow and buffalo milk. The level of calcium is higher in ovine milk than in bovine, camel, and caprine milk and lower level than buffalo milk (Table 3). Except for lower concentrations of folate, pantothenic acid, and vitamin D3, at comparable amounts to those found in cow milk, the vitamin content in ovine milk is higher than that in cow milk (WIJESINHA-BETTONI and BURLINGAME, 2013).

Table 3: Comparison of the composition of ovine milk with other animal species milk

Parameters	**Bovine milk**	**Ovine milk**	**Caprine milk**	**Camel milk**	**Buffalo milk**
Moisture (g/100g)	87.90	82.90	87.60	87.50	81.10
Ash (%)	0.72	0.85	0.82	0.79	0.82
Lactose (%)	4.82	5.10	4.51	4.30	4.90
Fat (%)	3.34	7.00	4.14	3.11	7.73
Solid-not-fat (%)	9.13	11.24	8.95	8.15	10.09
Total solid (%)	13.32	18.05	13.56	11.26	16.67
Protein (%)	3.29	5.98	3.56	3.70	4.38
Calcium (mg/100g)	119	193	134	117	195
Calories (Kcal/100g)	66	95	60	-	110

[Source: WIJESINHA-BETTONI and BURLINGAME (2013); SELVAGGI et al. (2014); MANCA et al. (2016)].

2.4. Somatic cell count and mastitis in ovine milk

2.4.1. Somatic cell count

Somatic cell count (SCC) in ewe milk indicates the udder health status of animals, to distinguish infected and uninfected mammary glands. It is also considered as one of the hygienic indicators in ewe milk quality. Somatic cell counts in milk vary depending on the genetic and environmental factors (PARK et al., 2013). The SCC is higher in healthy ewe mammary glands than in cow mammary glands, which was tested during mid-lactation (LEITNER et al., 2011). To define the safety and hygienic quality of ewe

milk, it would be important to set the exact and reasonable cut-off values of SCC in ewe milk (ALBENZIO et al., 2019).

In the case of ovine bulk tank milk, SCC must meet the regulatory standard of PASTEURIZED MILK ORDINANCE (2007) in United States, which is 1 million cells/mL. However, including the European Union, many countries have no defined limit value for SCC in sheep milk. The limit value of SCC in ewe milk is still a debatable topic. Some authors suggested that the threshold of SCC must not exceed 250,000 cells/mL (EL-KHABAZ et al., 2015). ŚWIDEREK et al. (2016) indicated that SCC in ewe milk above 200,000 cells/mL would result in mammary glands health problems and the value can be used for subclinical mastitis diagnosis. Table 4 shows the somatic cell count of ovine bulk tank milk (BTM) reported from different countries.

Table 4: Somatic cell count in ovine BTM samples examined in different countries

Country	**No.**	**SCC (lg cells/mL)**	**References**
Brazil	22	6.2	MERLIN-JUNIOR et al. (2015)
Czech Republic	25	5.7	BOGDANOVIČOVÁ et al. (2016)
Greece	155	6.1	ALEXOPOULOS et al. (2011)
Spain	751	6	DE GARNICA et al. (2013)
Spain	68,781	5.1	GONZALO et al. (2010)
United States	15	5.8	D'AMICO and DONNELLY (2010)

2.4.2. Ovine mastitis and its effect on public health

Mastitis in ovine can be classified as clinical and subclinical (CONTRERAS et al., 2007). Subclinical mastitis in ovine is characterized by changes in the quantity and quality of milk, mainly an increased number of somatic cells (ZAFALON et al., 2016). It might be difficult to identify subclinical mastitis in ovine. California Mastitis Test (HAWARI et al. 2014) and the SCC (SPANU et al., 2011) are the most frequently used methods to diagnose subclinical mastitis in the milk of infected animals. Studies on ovine milk have shown that the annual incidence of clinical mastitis is generally < 5 percentage (CONTRERAS et al., 2007), however, subclinical mastitis is between 5 and 30% or even higher (CONTRERAS et al., 2007; VASILEIOU et al., 2018).

To understand subclinical mastitis pathogens in the ewe milk, a study on 1,500 milk samples of the Comisana ewe breed in Italy explored the association between SCC and

pathogenic bacteria (ALBENZIO et al., 2012). From tested milk samples, about 700 were bacteriologically positive. Coagulase-negative staphylococci (CNS) (21%), *Streptococcus* spp. (19%), *E. coli* (18%), *Pseudomonas aeruginosa* (21%) were among the detected bacteria (ALBENZIO et al., 2012).

The finding of VASILEIOU et al. (2018) revealed that subclinical mastitis was detected in 26% of 2,198 individual raw milk samples on 111 ovine farms in Greece (Table 5). According to the study of VASILEIOU et al. (2018), CNS was the most frequently isolated both in milk samples from ewes with subclinical mastitis and mammary carriage (Table 5). Besides, *S. aureus*, *Streptococcus* spp., *Corynebacterium* spp., *E. coli*, *Micrococcus* spp., *Bacillus* spp., and *Mannheimia haemolytica* were among the detected bacteria (Table 5). The same prevalence of subclinical mastitis (26% of 907 IRM) was reported by ZAFALON et al. (2016) from Brazil. Based on their study, CNS spp. (14.8%), *Enterobacter* (3.8%), *Streptococcus* spp. (2.9%), and *S. aureus* (2.1%) were dominant etiological agents of subclinical mastitis in ovine milk.

Another study by MØRK et al. (2007), in Norway, indicated that *S. aureus* was found to be the predominant bacteria detected in 63.5% of 547 ovine individual milk with clinical mastitis. In addition, *E. coli* (6.4%), CNS spp. (2.9%), *Mannheimia haemolytica* (1.8%), *Streptococcus* spp. (1.5%), and *Clostridium perfringens* (1.3%) were detected in clinical mastitis ovine milk (MØRK et al., 2007). *Staphylococcus aureus* and CNS species are pivotal agents of clinical and subclinical mastitis among staphylococci species in ovine, respectively (VASILEIOU et al., 2019). However, the aforementioned findings (MØRK et al., 2007; VASILEIOU et al., 2018) confirmed that *S. aureus* can cause subclinical and CNS species can cause clinical mastitis in ovine. Table 5 below, summarizes the frequency of bacteria from the countrywide study in Greece on the investigation of ovine subclinical mastitis.

Table 5: Bacterial isolates originated from ovine milk samples with mammary carriage and subclinical mastitis in Greece

Bacteria	**Frequency of isolates from subclinical mastitis n (%)**	**Frequency of isolates from the mammary carriage n (%)**
Coagulase-negative staphylococci spp.	454 (59.7)	166 (59.7)
Staphylococcus aureus	77 (10.1)	11 (4.0)
Streptococcus spp.	36 (4.7)	1 (0.4)
Corynebacterium spp.	27 (3.6)	40 (14.4)
E. coli	26 (3.4)	10 (3.6)
Micrococcus spp.	20 (2.6)	15 (5.4)
Mannheimia haemolytica	19 (2.5)	2 (0.7)
Trueperella pyogenes	19 (2.5)	2 (0.7)
Acinetobacter spp.	16 (2.1)	5 (1.8)
Bacillus spp.	12 (1.6)	17 (6.1)
Enterococcus spp.	9 (1.2)	0 (0.0)
Others*	45 (5.9)	9 (3.2)
Total	**760**	**278**

**Burkholderia* spp., *Klebsiella* spp., *Kokuria* spp., *Peptococcus* spp., *Proteus* spp., *Pseudomonas* spp., and *Rhodococcus* spp. [Source: VASILEIOU et al. (2018)].

Among the bacterial pathogens allied with the ingestion of mastitic sheep raw milk and its products, the most common are Shiga-toxin producing *Escherichia coli* (STEC), *Campylobacter* spp., *Salmonella* spp., *Listeria monocytogenes*, *Brucella melitensis*, and *S. aureus* (VAN DEN BROM et al., 2020). Ruminants are known to be the main STEC's reservoirs, in particular cattle and sheep. Ovine milk can be contaminated with pathogenic *E. coli* via faecal on the udder and teat surfaces, or from the environment (VAN DEN BROM et al., 2020). Of the six classical pathotypes of enteric *E. coli*, strains that belong to STEC are important in the food safety context (CLEMENTS et al., 2012; MENG et al., 2013). There have been > 100 serotype groups of STEC associated with public health issues. Out of them, *E. coli* O157 was the most frequently involved in outbreaks and others such as O26, O103, O111, and O145 (VAN DEN BROM et al., 2020).

According to the European Union overview report on trends and sources of zoonosis, in 2017, the most commonly identified cause of haemolytic-uraemic syndrome cases was Serotype group O157 (EFSA-ECDC, 2018). Findings reported by CARO et al. (2011) from Spain, showed the prevalence of *E. coli* O157 (18%), *E. coli* O111 (8%), and *E. coli* O26 (6%). A meta-analysis by GONZALES-BARRON et al. (2017) revealed that an average prevalence of STEC in ovine milk was 4.8% based on eight studies. There are few studies regarding the existence of possible pathogenic *E. coli* in ovine milk. The studies mostly concentrated on the incidence of *E. coli* O157: H7, particularly in countries from the Mediterranean region (CARO et al., 2011). However, MUEHLHERR et al. (2003) from Switzerland reported the incidence of positive non-O157 STEC with a prevalence of 12.7% from bulk tank ovine milk samples.

L. monocytogenes is one of the most important species of *Listeria* in the case of public health or food safety (ROCHA et al., 2017). For this bacterium, animal feed and contaminated equipment are the main sources of milk contamination on the farm (BUCHANAN et al., 2017). Also, milk can be directly contaminated with this bacterium by shedding animals (GONZALES-BARRON et al., 2017). This bacterium grows well at low temperatures, and particularly soft and semi-soft cheeses are the perfect medium for the growth of it (VAN ASSELT et al., 2017). In the Czech Republic, the examination of ovine BTM samples showed that *L. monocytogenes* was 4.4% prevalent (BOGDANOVIČOVA et al., 2016).

A meta-analysis by GONZALES-BARRON et al. (2017) demonstrated that an average *L. monocytogenes* prevalence in raw ovine milk and cheeses (made from ovine milk) was 3.56, and 3.61% based on nine and four studies included in the review, respectively. Even though, *L. monocytogenes* is found in raw milk, it is not considered as significant a hazard as in dairy products such as cheese (EFSA-ECDC, 2018). The prevalence of some pathogenic organisms in ovine milk and cheese from different countries were summarized in Table 6.

Table 6: Summary of studies on the prevalence of pathogenic bacteria associate with mastitic ovine milk and products in different countries

Country	Sample type	N	Summary of the findings	References
Czech Republic	BTM	23	Ovine milk samples were tested for *S. aureus*, *L. monocytogenes*, *Campylobacter*, *Salmonella*, and *E. coli*. *L. monocytogenes* in 1 (4.4%), and STEC in 1 (4.4%) samples. However, *Campylobacter* and *Salmonella* were not detected in any of the tested milk samples.	BOGDANOVIČOVA et al. (2016)
Greece	BTM	240	*Salmonella* was found to be positive in 5% of samples. *Bacillus* species was positive in 29% and *L. monocytogenes* and *Campylobacter* were not found.	FOTOU et al. (2011)
Greece	BTM	595	Out of 595 ovine BTM samples examined, 0.8% (n = 5) were *E. coli* positive. Also, of this (0.84%), 0.5% (n = 3) were STEC positive.	SOLOMAKOS et al. (2009)
Spain	BTM, fresh cheese curd, and cheese	407	In total 407 (287 BTM, 91 fresh cheese curd, and 29 cheese) samples were evaluated for the prevalence, serotype, and virulence gene of STEC in Spain. The prevalence was 10% (n = 29). Four isolates were tested for O157: H7 serotype and none of them were positive.	REY et al. (2006)
Switzerland	BTM	63	In 63 ovine BTM samples, the prevalence of *Salmonella*, *Campylobacter*, STEC, and *Mycobacterium avium* spp. *Paratuberculosis* was studied. STEC was detected in 8 (13%), *Mycobacterium avium* spp. *Paratuberculosis* in 15 (24%), and others were not detected in any of the samples.	MUEHLHERR et al. (2003)

BTM: bulk tank milk; N: number of samples; STEC: Shiga-toxin producing *Escherichia coli*.

2.5. Hygienic indicators in ovine milk

Hygienic, sensory, nutritional, and technical criteria can be used to determine the quality of milk in ovine. On the ovine dairy farm, among the key quality parameters, regularly monitored are SCC and total plate count (TPC). Moreover, taste, flavour, temperature, and appearance of milk are also important (PARK et al., 2013). In recent years, understanding public health and food safety concerns have led to more interest in milk hygiene in ovine to reduce risks and ensure optimum quality of consumption (GONZALO, 2017). However, in comparison with cow milk, less emphasis is given to ovine milk hygienic requirements.

The presence of bacteria in ovine milk is from several sources, predominantly from the farm environment and animal body to the milk (HOLM et al., 2004; QUIGLEY et al., 2013). The exterior surface of the udder and teat are the primary sources of bacterial contamination of milk (GRIFFITHS, 2010). From these sources, bacteria enter into milk and play several roles (facilitating fermentation, causing disease, and spoilage). When animals lay down, bedding materials, soil, feces, and other materials may easily adhere to the udder surface (GRIFFITHS, 2010). Indicator organisms in milk can be defined as a marker that indicates the overall microbial condition of milk or provides evidence for poor hygiene (MARTIN et al., 2016).

According to the review by METZ et al. (2020), exploring the indicator bacteria in milk was an indication for fecal contamination, which indirectly shows pathogens might be present. On the other hand, studies revealed that there was no correlation between the presence of indicator bacteria and pathogens in the milk (MARTIN et al., 2016). Moreover, the bacterial diversity originating from the environment and the initial natural diversity of bacteria present in raw milk play a crucial role in the fermentation process and are significant in the final synthesis of conventional dairy products (SANDRA et al., 2013).

2.5.1. Total plate count

Total plate count is among the main hygienic parameters in ovine milk (PARK et al., 2013). Besides, *Enterobacteriaceae*, coliform, *E. coli*, and psychrotrophic bacteria are some of the hygienic indicator organisms. Some of these bacteria resist pasteurization or can grow at low temperature and indicate faecal contamination. Despite this,

REGULATION (EC) 853/2004 of the European Parliament and of the council for ovine milk limit value has targeted only total plate count. Based on this regulation, the total plate count of ovine milk to be pasteurized before processing must be $\leq 1.5 \times 10^6$ CFU/mL (6.2 lg CFU/mL), and to be processed without pasteurizing should be $\leq 5 \times 10^5$ CFU/mL ($\leq$ 5.7 lg CFU/mL).

A recent review study of existing literature on small ruminant milk hygiene by GONZALO (2017) indicated that preservation strategies and storage time change total plate count in BTM. According to the data in Table 7, the average TPC of BTM samples was found between 2.3 and 7.2 lg CFU/mL. The TPC value from Czech Republic (BOGDANOVIČOVÁ et al., 2016), Switzerland (MUEHLHERR et al., 2003), Spain (GONZALO et al., 2010), and Brazil (MERLIN-JUNIOR et al., 2015) failed to meet the limit value of Regulation (EC) 853/2004, which was $\leq$ 6.2 and $\leq$ 5.7 lg CFU/mL for TPC of ovine milk to be processed with and without heat, respectively (Table 7).

2.5.2. *Enterobacteriaceae*, coliform, and *Escherichia coli* bacteria

Enterobacteriaceae family is Gram-negative which has > 30 genera and > 100 species (DSMZ, 2021). Along with harmless symbionts, this family includes pathogenic ones such as *Enterobacter*, *Klebsiella* spp., *Salmonella* spp., and *Shigella*. Regarding these bacteria, little information was available on ovine milk. These bacteria are also among the other hygienic indicator bacterial groups. Studies suggest that *Enterobacteriaceae* reflects the hygienic status of milk more accurately than coliforms. Although coliforms have been used as milk hygienic indicators for a long time in the dairy sector, a recent study indicates that the *Enterobacteriaceae* family is widely used as hygienic indicators throughout Europe (HERVERT et al., 2016). However, in the dairy production chain, both coliform and *Escherichia coli* are regularly used as indicators of hygienic/sanitary conditions (KADYAN et al. 2020). *E. coli* are an exact indicator of faecal contamination in raw and processed milk (KUMAR and PRASAD, 2010).

Based on the data presented in Table 7, the average *Enterobacteriaceae* count (EBC) of ovine BTM samples was found between 3.9 and 5.2 lg CFU/mL. The findings in Spain (REY et al., 2006), Czech Republic (BOGDANOVIČOVA et al., 2016), and Greece (SOLOMAKOS et al., 2009), indicated that *E. coli* was detected in 10.1, 4.4, and 0.8% of raw ovine BTM samples, respectively.

Table 7: Summary of ovine bulk tank milk hygienic indicators examined in different countries

Country	N	**Hygienic indicators tested (lg CFU/mL)**			**References**
		TPC	EBC	CC	
Brazil	22	7.2	5.4	5.5	MERLIN-JUNIOR et al. (2015)
Czech Republic	25	5.8	-	-	BOGDANOVIČOVÁ et al. (2016)
Egypt	35	5.3	5.2	5.2	OMBARAK and ELBAGORY (2017)
Greece	155	5.5	-	4.5	ALEXOPOULOS et al. (2011)
Slovakia	1256	2.3	-	-	KLIMEŠOVÁ et al. (2017)
Spain	751	5.1	-	3.8	DE GARNICA et al. (2013)
Spain	68,781	5.9	-	-	GONZALO et al. (2010)
Switzerland	63	5.9	3.9	-	MUEHLHERR et al. (2003)
United States	15	4.6	-	1.6	D'AMICO AND DONNELLY (2010)

All milk samples were originated from the bulk tank. TPC: total plate count, EBC: *Enterobacteriaceae* count; CC: coliform count; N: number of samples.

2.5.3. Psychrotrophic bacteria

Psychrotrophic bacteria that grow at seven degrees Celsius has usually a positive correlation with total bacterial load in ovine milk (DE GARNICA et al., 2013). Psychrotrophic bacteria occurring in unpasteurized milk are studied worldwide because of the difficulties associated with controlling their growth during storage (DE OLIVEIRA et al., 2015). The occurrence of higher psychrotrophic bacterial count (PBC) may lead to the spoilage of milk, predominantly by the genus *Pseudomonas* (MCPHEE and GRIFFITHS, 2011).

In Brazil, between April 2012 and March 2013, MERLIN-JUNIOR et al. (2015) examined 22 ovine BTM samples for PBC, with a mean value of 5.8×10^6 CFU/mL (6.8 lg CFU/mL). A further finding by DE GARNICA et al. (2013) reported that 5.7 lg CFU/mL of PBC was recorded in 751 BTM samples examined between January and December 2011 in Spain.

2.6. Staphylococci in ovine milk

Taxonomically, staphylococci are classified in the *Staphylococcus* bacterial genera of the *Staphylococcaceae* family. It is Gram-positive non-spore-forming bacteria. Currently, the genera include > 55 species and about 25 subspecies (DSMZ, 2021). Coagulase test, the reaction of staphylococcal strains with coagulase enzymes, identifies staphylococcal strains that can produce coagulase (coagulase-positive staphylococci) and can not produce coagulase (coagulase-negative staphylococci). Another approach refers to 16S rRNA sequencing, clustered staphylococci into 11 groups (Figure 4).

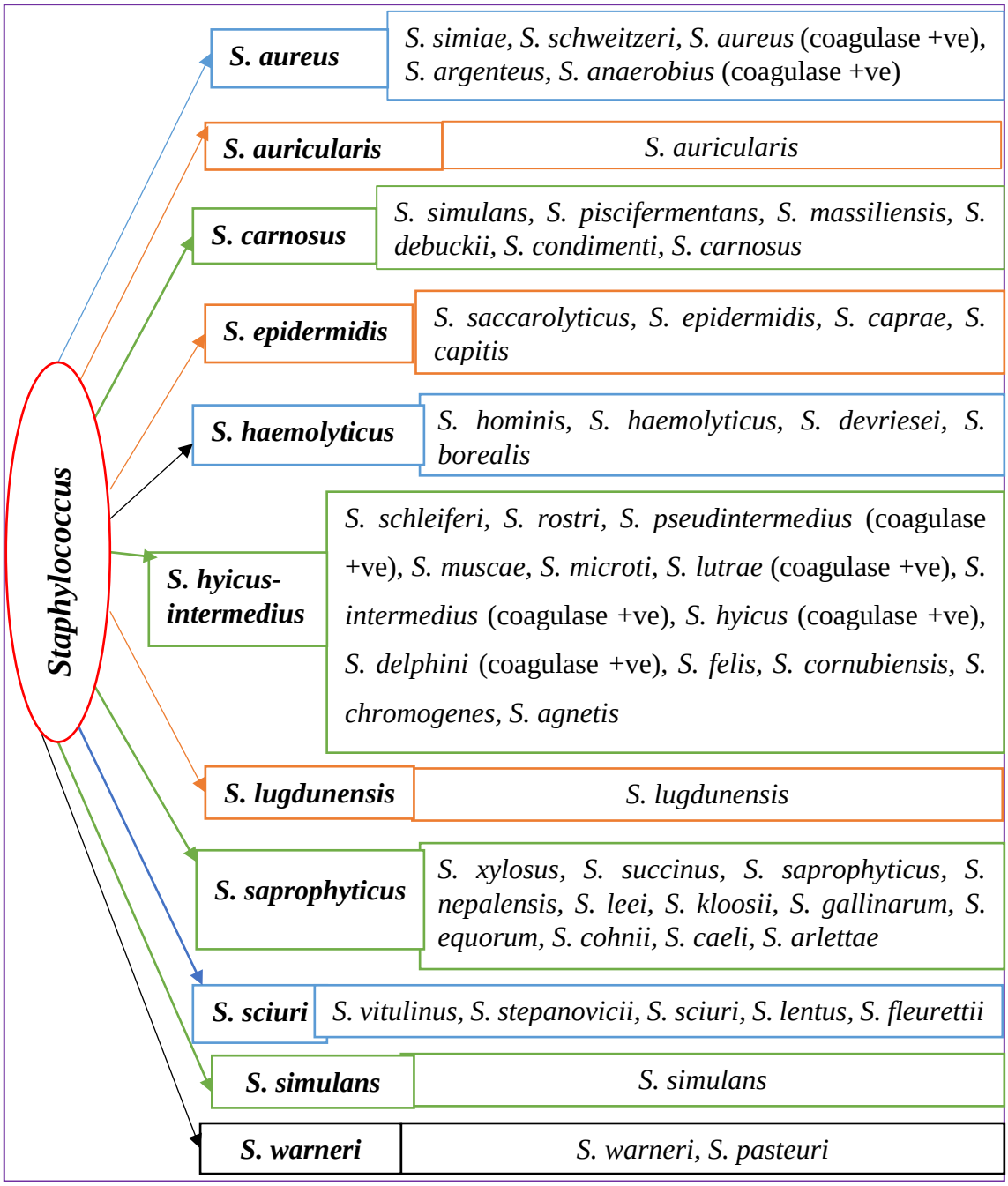

Figure 4: Staphylococci clustered into 11 groups based on 16S rRNA sequencing. Other species of staphylococci were also reported, but their valid taxonomic status was not confirmed. [Source: TAKAHASHI et al. (1999); DSMZ (2021)].

2.6.1. *Staphylococcus aureus*

S. aureus is coagulase-positive facultative anaerobic bacteria (MASALHA et al., 2001). It is non-motile catalase-positive and it forms grape-like clusters when viewed by a microscope. It grows in milk at 7 ^{o}C and may cause food poisoning, which is by

staphylococcal enterotoxins (EFSA BIOHAZ PANEL, 2015). Therefore, making bulk tank milk temperature < 6 ^{o}C is among the prevention methods of foodborne problems by *S. aureus*. In the USA, about 50,000 deaths per year are associated with *S. aureus* infection (SCHLECHT et al., 2015). This bacterium is considered as the main cause of clinical mastitis in ovine (MACORI et al., 2017).

Dairy ovine farms experienced a significant economic loss due to *S. aureus* infections of intramammary glands **(**BERGONIER et al., 2003). Contact with animals during milking, contaminated milking equipment, and bedding spread the infection within a sheep flock (CLAEYS et al., 2013; VAN ASSELT et al., 2017). According to the report of EFSA BIOHAZ PANEL (2015), *S. aureus* in ovine raw milk is not a key hazard, but in products made from it. In ovine BTM tested in different countries, *S. aureus* has been detected in 39% of the samples (BOGDANOVIČOVA et al., 2016) in the Czech Republic, 33% (MUEHLHERR et al., 2003) in Switzerland, and 24% (FOTOU et al., 2011) in Greece.

2.6.2. Coagulase-negative staphylococci

Coagulase-negative staphylococci (CNS) are heterogeneous groups of species of bacteria that commonly cause subclinical mastitis in dairy flocks **(**BERGONIER et al., 2003). Coagulase-negative staphylococci colonize the teat apex, the teat canal, and skin. It enters the mammary gland via the teat orifice resulting in the occurrence of intramammary gland infection (MARTINS et al., 2017). Subclinical mastitis in ovine caused by CNS is still poorly understood because of the heterogeneity of these bacterial groups and is not usually identified at the species level, making it difficult to control the disease (PYÖRÄLÄ and TAPONEN, 2009; MARTINS et al., 2017).

VASILEIOU et al. (2018) identified CNS species from the milk of subclinically infected ewe udder halves (Table 8). Accordingly, *S. chromogenes* (25.0%) was the most frequently identified CNS species followed by *S. epidermidis* (21.4%) and *S. simulans* (19.1%). Similarly, in another study, from Brazil, *S. simulans* (23.7%), *S. warneri* (15.2%), and *S. devriesei* (13.6%) are among the dominantly identified CNS species from noninfected ewe milk (MARTINS et al., 2017). Some of the CNS species and their frequency reported in two countries from both healthy and subclinical ovine milk were shown in Table 8.

Table 8: Frequency of coagulase-negative staphylococci isolates recovered from healthy and subclinical mastitis ovine milk

CNS species	Healthy ovine milk [n (%)] (MARTINS et al., 2017)	Subclinical mastitis ovine milk [n (%)] (VASILEIOU et al., 2018)*
S. simulans	14 (23.7)	16 (19.1)
S. warneri	9 (15.2)	1 (1.2)
S. devriesei	8 (13.6)	0 (0.0)
S. xylosus	8 (13.6)	7 (8.3)
S. epidermidis	7 (11.9)	18 (21.4)
S. haemolyticus	5 (8.4)	3 (3.6)
S. auriculares	3 (5.1)	0 (0.0)
S. chromogenes	3 (5.1)	21 (25.0)
S. caprae	1 (1.7)	3 (3.6)
S. hominis	1 (1.7)	8 (9.5)
S. capitis	0 (0.0)	2 (2.4)
S. lentus	0 (0.0)	5 (5.9)
Total	**59 (100)**	**84 (100)**

*In the original article, the percentage of isolates was calculated based on 91 isolates, but in this table, it was determined based on 84 isolates, because seven isolates belonging to other CNS species were not included.

2.6.3. Antibiotic resistance and enterotoxins profile of staphylococci species

Antibiotic resistance (ABR) is related to mutation and rearrangements of the genetic background of microbial isolates within a staphylococci genome (VASILEIOU et al., 2019). In recent years, ABR is a major public health issue, in particular the resistance of staphylococci strains (EFSA, 2009; YILMAZ and ASLANTAŞ, 2017). By 2050, 10 million people are estimated to die from antimicrobial resistance (O'NEILL, 2016). In the USA more than 35,000 (CDC, 2019) and in the EU more than 33,000 (CASSINI et al., 2019) deaths in a year are due to antibiotic resistance.

The finding by TAPONEN and PYÖRÄLÄ (2009) from Finland demonstrated that coagulase-negative staphylococci strains are more resistant to drugs than coagulase-positive strains in bovine BTM. Currently, staphylococci resistance to methicillin is a major public health concern (POVEDA et al., 2020). Prone to methicillin-resistant

Staphylococcus aureus (MRSA) can cause staphylococcal infections that are difficult to treat due to resistance to certain antibiotics (CDC, 2019). The cases due to MRSA in USA from 2012 to 2017 were demonstrated in Figure 5. The report indicates that MRSA cases in the USA decreased from time to time (Figure 5).

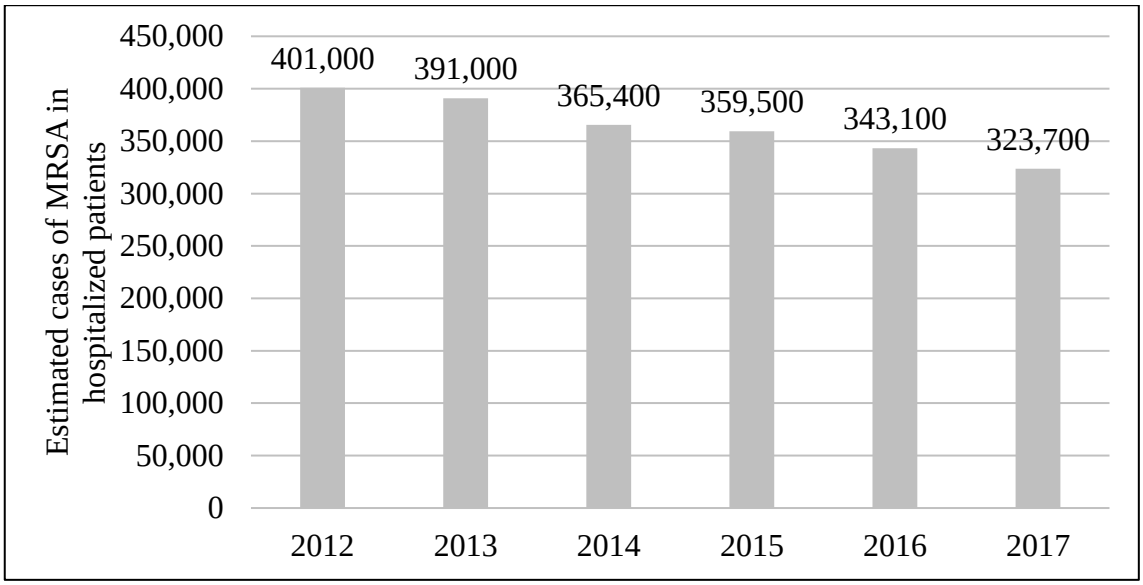

Figure 5: Cases of methicillin-resistant *S. aureus* in hospitalized patients in USA during 2012 to 2017. [Source: CDC (2019)].

Staphylococcal enterotoxin (SE) is a bacterial protein, which is pyrogenic and linked to human disease by food poisoning (PINCHUK et al., 2010). There are 24 SEs and SE-like (SE*l*s) reported so far (FISHER et al., 2018). The genetic elements, molecular weight, emetic activity, and super antigenic activity of these SEs and SE*l*s were summarized in Table 9. The molecular weight of SEs and SE*l*s falls between 19.3 and 28.6 kDa (Table 9). Staphylococcal enterotoxins were named according to the date of investigation in alphabetical order (JIN and YAMADA, 2016). The ingestion of contaminated food with SEs and SE*l*s leads to foodborne intoxication, which is another global public health problem (PEXARA et al., 2016). SEs are heat tolerant, they can survive at high temperatures (MARTINS et al., 2017).

According to the COMMISSION REGULATION (EC) No. 2073/2005 on microbiological criteria for foodstuffs, any dairy products verified positive for SEs were unacceptable. SEs concentration in food likely vary and different strains may have different dose response relationships. According to ASAO et al. (2003), less than 1 μg of SE can cause food poisoning symptoms in sensitive individuals. Estimated doses as low

as 0.017 μg of SEA can cause symptoms in children and estimated doses of 0.05 μg can cause symptoms in all age groups (ASAO et al., 2003). However, no clear limit value for SEs concentration has been established. The outbreaks due to staphylococcal food poisoning caused by the consumption of milk and milk products in different countries between 2000 and 2017 were summarized in Table 10.

Table 9: Characteristics of and SEs and SE*l*s toxins

SEs and SE*l*s	**Genetic elements**	**Molecular weight (kDa)**	**Emetic activity**	**Super antigenic activity**	**Type**
SEA	prophage	27.1	Yes	Yes	Classical
SEB	chromosome, SaPI, plasmid	28.4	Yes	Yes	Classical
SEC1-C3	SaPI	27.5-27.6	Yes	Yes	Classical
SED	plasmid (pIB485)	26.9	Yes	Yes	Classical
SEE	prophage	26.4	Yes	Yes	Classical
SEG	*egc*, chromosome	27.0	Yes	Yes	New
SEH	transposon	25.1	Yes	Yes	New
SEI	*egc*, chromosome	24.9	Yes	Yes	New
SE*l*J	plasmid (pIB485, pF5)	28.6	nd	Yes	New
SE*l*K	SaPI	25.3	Yes	Yes	New
SE*l*L	SaPI	24.7	Yes	Yes	New
SE*l*M	*egc*, chromosome	24.8	Yes	Yes	New
SE*l*N	*egc*, chromosome	26.1	Yes	Yes	New
SE*l*O	*egc*, chromosome	26.8	Yes	Yes	New
SE*l*P	prophage (Sa3n)	26.7	Yes	Yes	New
SE*l*Q	SaPI	25.2	Yes	Yes	New
SER	plasmid (pIB485, pF5)	27.0	< 100 μg/kg	Yes	New
SES	plasmid (pF5)	26.2	< 100 μg/kg	Yes	New
SET	plasmid (pF5)	22.6	< 100 μg/kg	Yes	New
SE*l*U	*egc*, chromosome	27.2	nd	Yes	New

SEs and SE*l*s	**Genetic elements**	**Molecular weight (kDa)**	**Emetic activity**	**Super antigenic activity**	**Type**
SE*l*V	*egc*, chromosome	27.6	nd	Yes	New
SE*l*W	chromosome	23.2	nd	Yes	New
SE*l*X	chromosome	19.3	nd	Yes	New

nd: not demonstrated. [Source: OMOE et al. (2005); ONO et al. (2008); ARGUDÍN et al. (2010); HU and NAKANE (2014); JIN and YAMADA (2016); HU et al. (2017); ONO et al. (2017); FISHER et al. (2018)].

Table 10: Outbreaks due to staphylococcal food poisoning caused by the consumption of milk and milk products in different countries between 2000 and 2017

Country	**Type of food poisoned**	**SEs and SE*l*s genes**	**No. of cases**	**Year**	**References**
Austria	Products of pasteurized milk	*sea*, *sed*	166	2007	SCHMID et al. (2009)
France	Sliced soft cheese	*sea*	2	2001	KÉROUANTON et al. (2007)
France	Cream	*sea*	10	2001	
France	Cheese made from raw ewe milk	*sea*	43	2002	
France	Soft cheese	*sed*	5	2008	ROUSSEL et al. (2015)
France	Unpasteurized milk-based soft cheese	*see*	23	2009	OSTYN et al. (2010)
Germany	Ice cream	*sea*	13	2013	FETSCH et al. (2014)
Italy	Ovine cheese (semi-ripened)	*sec*	2	2015	VITALE et al. (2015)
Japan	Dairy products of skim milk powder	*sea*	13,420	2000	ASAO et al. (2003)
Japan	Reconstituted milk	*sea*, *seh*	> 10,000	2000	IKEDA et al. (2005)
Norway	Mashed potatoes prepared with raw milk	*seh*	43	2003	JØRGENSEN et al. (2005)
Paraguay	Ultra-pasteurized milk	*sec*, *sed*	400	2007	WEILER et al. (2011)
Romania	Curd cheese of ovine	*sed*, *sej*, *ser*	8	2013-2017	CIUPESCU et al. (2018)
Romania	Appetizer made with a soft cheese of bovine	*sed*, *seg*, *sei*, *sej*, *ser*	52	2013-2017	
Romania	Ripened cheese made from raw bovine milk	*seh*	36	2013-2017	
Switzerland	Caprine cheese (Robiola)	*seg*, *sei*, *selm*, *seln*, *selo*	5	2007	JOHLER et al. (2015a)
Switzerland	Soft cheese made from raw milk	*sea*, *sed*	14	2014	JOHLER et al. (2015b)

2.7. Lactic acid bacteria

Lactic acid bacteria are comprised of many genera such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus,* and *Enterococcus*. Among these genera, *Lactobacillus* is diverse and complex, which is composed of more than 180 species **(**GOLDSTEIN et al., 2015). Lactic acid bacteria dominate raw ovine milk (FOTOU et al., 2011). As a major fermentation product of carbohydrate metabolism, lactic acid bacteria produce lactic acid (HAYEK and IBRAHIM, 2013). In milk and milk products, they are naturally present. It has been used since ancient times for fermenting food and feed materials, and today its main applications remain as starter cultures in the feed and food industries (HAYEK and IBRAHIM, 2013). In comparison with bovine milk, the production of acidity appears to be faster, especially during the first 8 to 12 hours of fermentation (KEHAGIAS et al., 2008). This is particularly important from a technological point of view since the probiotic strains have reduced proteolysis and the difficulty in milk to grow (TRIPATHI and GIRI, 2014).

Ovine milk is ideal for the production of fermented products with *S. thermophilus*, *L. acidophilus*, and *Bifidobacterium bifidus*, which has beneficial effects on consumers' health even after 6 weeks of refrigerated storage (VARGA et al., 2014). Lactic acid bacteria count (LABC) in raw ovine milk and milk products was examined by SILVA et al. (2020) in Brazil. In this study, LABC was examined in raw ovine milk (n = 10), pasteurized milk (n = 3), pasteurized milk cream (n = 8) and butter samples (n = 4). A higher mean value of LABC was recorded in raw milk samples (6.93 ± 0.84 lg CFU/mL) than pasteurized milk (5.19 ± 0.01 lg CFU/mL) (Figure 6). This might be due to the destruction of some bacteria during pasteurization. Pasteurized milk cream (3.58 ± 0.60 lg CFU/mL) has a lower mean value of LABC than butter (5.85 ± 0.01 lg CFU/mL), which is a raw material for butter production (SILVA et al., 2020).

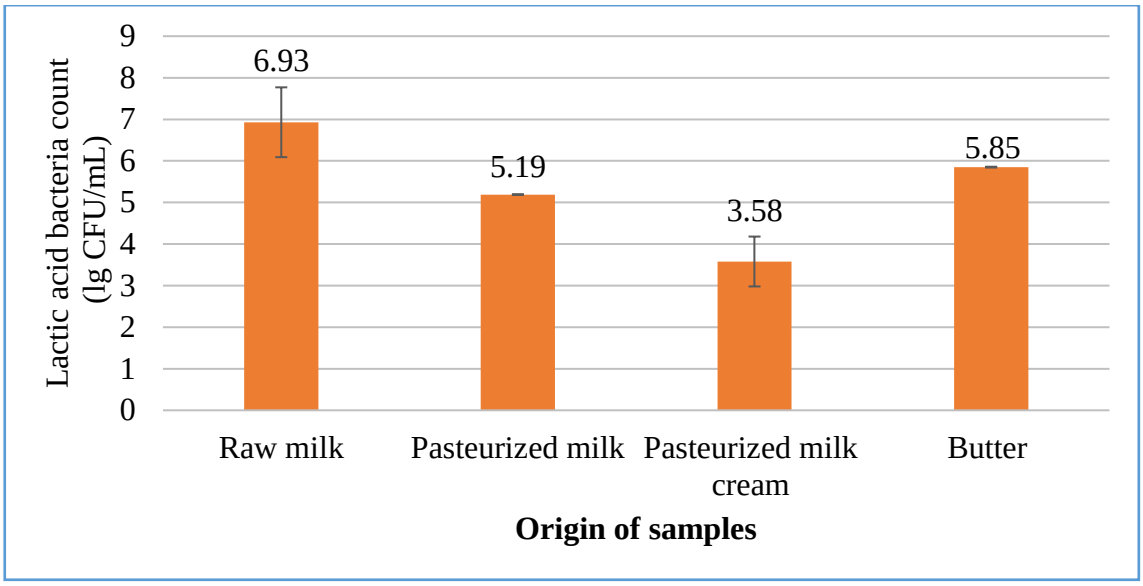

Figure 6: Lactic acid bacteria count from ovine raw milk and milk products. The bar chart was created from the data presented in tabular form in the original article. [Source: SILVA et al. (2020)].

2.8. Microbiota community profiling of ovine milk

The microbiota profile of ovine raw milk from either the bulk tank or individual animal at the farm level or processing factories is currently not well studied. However, many studies have been conducted on the microbiota of raw bovine milk using 16S rRNA (OIKONOMOU et al., 2014; KABLE et al., 2016; KIM et al., 2017; CREMONESI et al., 2018). In their studies, the core microbiotas were addressed according to the health (healthy, subclinical, and clinical mastitis cows) (OIKONOMOU et al., 2014) in USA, season (spring, summer, and fall) (KABLE et al., 2016) in USA, different regions (KIM et al., 2017) in South Korea, breed (Holstein Friesian and Rendena breed) (CREMONESI et al., 2018) in Italy of dairy cows.

The abundance of microbiota was different in bovine milk samples with different health statuses; however, *Streptococcus* and *Staphylococcus* were the most abundant genera among others in all groups (OIKONOMOU et al., 2014). According to the finding of KABLE et al. (2016), there was a change in the microbiota composition of bovine milk in different seasons. In contrast, in the bovine raw milk samples from two regions, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phyla were the most frequently detected (KIM et al., 2017). In Holstein Friesian and Rendena cow breeds milk, the microbiota

population was very different with low microbial diversity in Rendena breed (CREMONESI et al., 2018).

Microbiota profile in goat (MCINNIS et al., 2015) and water buffalo (CATOZZI et al., 2017) milk samples has also been studied with 16S rRNA gene sequencing in USA and Italy, respectively. Goat milk microbiota was analysed by considering the lactation stage (early, mid, and late lactation). *Actinobacteria* phylum was highly frequent during late lactation and *Proteobacteria* was the most frequent phylum during early and late lactation (MCINNIS et al., 2015). CATOZZI et al. (2017) from Italy reported the finding concerning water buffalo milk to define the microbiota in healthy, subclinical, and clinical mastitis. According to their study, microbiota from healthy water buffalo quarters were more diverse than subclinical and clinical mastitis quarters. In three groups of water buffalo milk samples, *Actinobacteria*, *Bacteroidetes, Cyanobacteria*, *Firmicutes*, *Fusobacteria*, *Proteobacteria,* and *Spirochaetes* main phyla were detected (CATOZZI et al., 2017).

In the case of ovine milk microbiota, only two studies by using 16S rRNA gene sequencing have been published from Spain until the 1st of November 2020 (ESTEBAN-BLANCO et al., 2020a, 2020b). The first study was about the microbiota profile of Assaf ewe raw milk concerning SCC (ESTEBAN-BLANCO et al., 2020a). Fifty raw milk samples were collected from healthy Assaf sheep ewes and classified into two groups based on SCC values. These were healthy (n = 13) with SCC of < 400, 000 cells/mL and subclinical (n = 37) with SCC of > 400, 000 cells/mL. Overall, the most abundant microbiotas at the phylum level were *Firmicutes* (64.4%), *Actinobacteria* (14.3%), and *Proteobacteria* (9.1%).

Negative correlation was observed for the bacterial diversity with somatic cell count. This indicates that small group of bacterial taxa were found in higher SCC milk samples (ESTEBAN-BLANCO et al., 2020a). In the other study (ESTEBAN-BLANCO et al., 2020b), the microbiota of Churra sheep raw milk was characterized. In their study, they compared the finding with a previous study that was on the Assaf breed (Table 11). The microbiota diversity in Assaf sheep was higher than the diversity in two Churra sheep flocks (ESTEBAN-BLANCO et al., 2020b).

Table 11: Relative abundance of two sheep breeds' (Assaf and Churra) milk microbiota for 5 topmost abundant genera analysed by 16S rRNA genome sequencing in Spain

Breed	**Milk sample of the healthy ewe (n = 37)**	**Milk sample of ewe with subclinical mastitis (n = 13)**
Assaf	*Staphylococcus* (12.4%)	*Staphylococcus* (29.8%)
	Lactobacillus (12.2%)	*Lactobacillus* (19.3%)
	Corynebacterium (9.7%)	*Corynebacterium* (6.4%)
	Streptococcus (3.4%)	*Streptococcus* (5.8%)
	Escherichia/*Shigella* (2%)	*Alloiococcus* (2.04%)
Churra	**Milk sample of healthy ewe (n = 166)**	**Milk sample of ewe with subclinical mastitis (n = 46)**
	Staphylococcus (14.1%)	*Staphylococcus* (42.6%)
	Cutibacterium (7.1%)	*Corynebecterium* (4.5%)
	Corynebacterium (4.3%)	*Streptococcus* (3.5%)
	Streptococcus (4.3%)	*Cutibacterium* (3.4%)
	Bacillus (3.7%)	*Massilia* (3.1%)

The total milk sample of Assaf ewe was 50 and Churra ewe was 212. [Source: ESTEBAN-BLANCO et al. (2020a), (2020b)].

2.9. Isolation and identification of ovine-associated bacteria

Ovine-associated bacterial isolation starts with the culturing bacteria from the milk or its products or environmental samples, especially from udder skin or teat duct of ewes (FRAGKOU et al., 2014). The inoculation of samples can be performed on selective or conventional media. After incubation at the appropriate temperature for a specific time, identification of bacteria starts with evaluating the appearance of the colonies. Gram-positive bacteria can be distinguished by Gram-staining from Gram-negative bacteria microscopically.

Besides, catalase testing is useful to differentiate colonies of staphylococci from other Gram-negative bacteria such as enterococci and streptococci (VASILEIOU et al., 2019). Subsequently, physical or biochemical, or molecular techniques can be used in the identification of bacterial species. The physical, biochemical, and genetic methods are by characterizing the proteome, metabolic pathways, and specific genes of the bacteria (DONAY et al., 2004).

Phenotypical and biochemical bacterial identification tools are commercially available to facilitate the identification of bacteria, however, many of these systems have not been found to produce accurate results when testing isolates (SAMPIMON et al., 2009). The limited number of bacterial strains evaluated and included in the database for reference could be the reason for this (ZADOKS and WATTS, 2009).

API Staph test is one of biochemical reactions based phenotypic identification methods that is simple to use and provides rapid results (KIM et al., 2018). ONNI et al. (2010) compared the performance of API Staph test method with 16S rRNA and gap genes by PCR assays for the identifications of CNS strains from ovine milk. In their study, it was confirmed that the API Staph test is poorly performed in the identification of CNS isolates that originated from ovine milk. Other researchers explain this as the system is might be designed for human-originated strains (SANTOS et al., 2008).

In recent times, Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) is widely in use for the identifications of bacterial species. This method is fast, technically easy to perform, and cost-effective that can identify a great number of isolates (KLIEM and SAUER, 2012; CAMERON et al., 2018). According to BÖHME et al. (2012), the MALDI-TOF method is approximately 67% less expensive than conventional bacteriological methods. MALDI-TOF method has so far been used by few researchers to identify bacterial strains associated with ovine (SMITH et al., 2015; KAČÁNIOVÁ et al., 2019). Figure 7 (a) shows the procedures and steps of MALDI-TOF techniques during bacterial strain identification. It has to be done carefully by minimizing the occurrence of liquid smear between spots, which raises the risk of cross-contamination (KOSTRZEWA and NAGY, 2016).

Another method is polymerase chain reaction (PCR), which is based on DNA amplification, which is more accurate, but time-consuming and requires expertise (SHELL et al., 2017). Molecular-based identification has been used for the bacterial isolates related to ovine (ONNI et al., 2010; ROVAI et al., 2014; MARTINS et al., 2017). Figure 7 (b) shows a thermal cycler, reaction mixture components, and PCR steps.

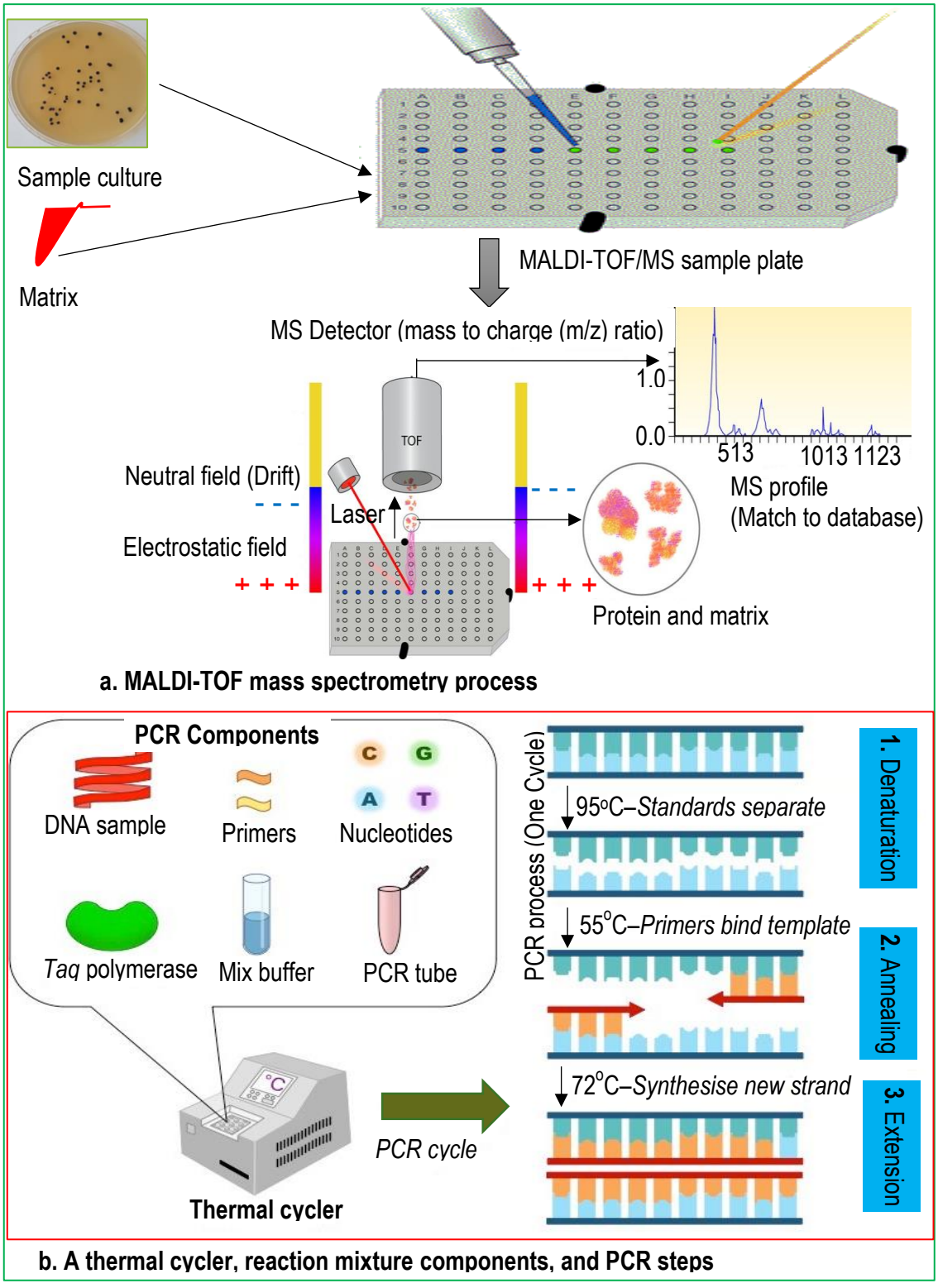

Figure 7: MALDI-TOF mass spectrometry process (a) [Source: CLARK et al. (2013)]. A thermal cycler, reaction mixture components, and PCR steps (b) [Source; https://longroadtoinnovation.files.wordpress.com/2018/11/pcr-components_med.jpeg?crop]. The figure has been modified from the original.

Furthermore, for microbiota evaluation or to identify types of bacteria in milk samples of different livestock species, 16S rRNA gene sequencing, high-throughput sequencing, is more suitable (ADDIS et al., 2016; CATOZZI et al., 2017; ESTEBAN-BLANCO et al., 2020a). In the case of ovine milk samples, few researches were conducted using 16S rRNA gene sequencing to understand milk microbiota composition (ESTEBAN-BLANCO et al., 2020a, 2020b). Figure 8 reveals the systematic overview of the 16S rRNA gene sequencing protocol.

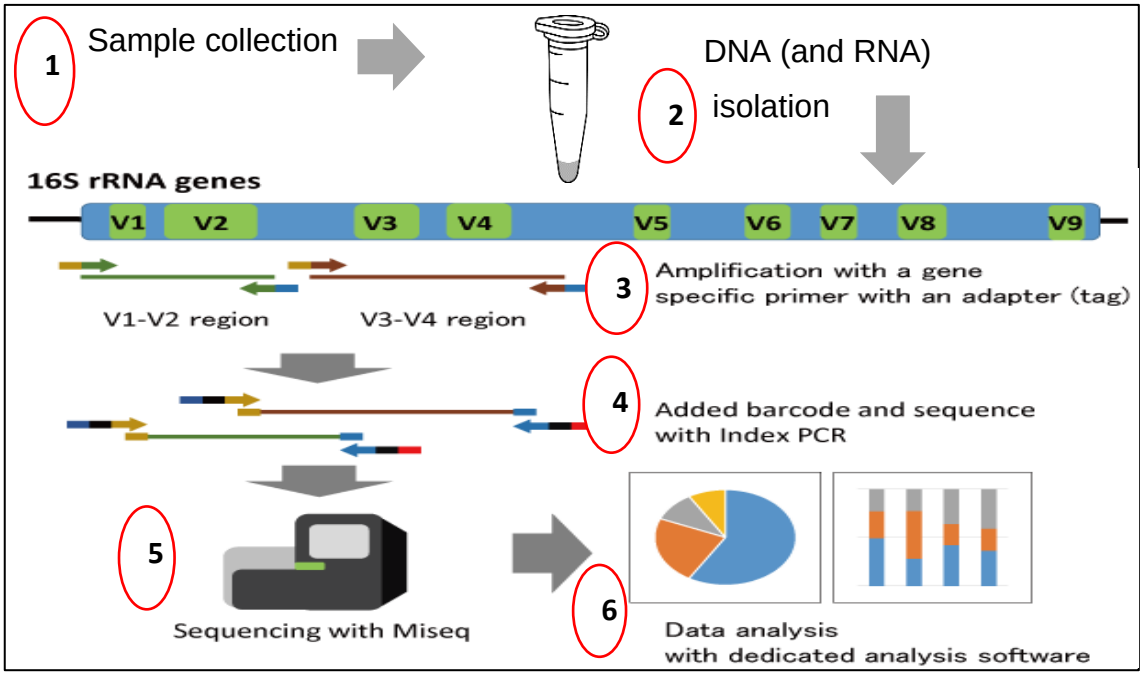

Figure 8: Overview of 16S rRNA gene sequencing protocol. [Source: https://www.repertoire.co.jp/en/asset/imgs/research/technology/16srrnainfo/thumb_01.png]. The figure has been modified from the original (Step 1 and 2 were added to the original image).

2.10. How to prevent bacterial growth in ovine milk

During different stages of production, processing, and storage, milk and its product contamination with bacteria can occurs in the dairy sheep sector (VAN DEN BROM et al., 2020). In the above-mentioned stages, the remarkable composition of ovine milk makes it an apt product for the growth and survival of pathogens (CLAEYS et al., 2013). In line with the consumption of raw ovine milk and ovine milk products, several pathogenic microorganisms pose a risk to public health. Although hygiene alone is not

sufficient to prevent pathogens, hygiene is essential to prevent contamination during ovine milk processing (WILLIS et al., 2018).

Milk and dairy products contamination with bacteria can be minimized by milking mastitis-infected animals separately, using pre and post-disinfectants before and after milking, clean bedding, and attention to the hygiene of utensils for milking, processing, and storage (VAN DEN BROM et al., 2020). Cooling lessens the multiplication rate of microorganisms but some of them, such as *Listerias* spp., are still able to proliferate even at refrigerator conditions. Therefore, the control of temperature after milking should be deliberated to retard the growth of pathogens and to reduce toxin production (PORCELLATO et al., 2018). In addition, heat treatment is suggested to minimize a load of unfavourable and pathogenic microorganisms in ovine milk. Heat treatment of milk should be according to the standard by the International Dairy Federation (JUFFS and DEETH, 2007).

3. MATERIALS AND METHODS

3.1. Description of sheep farms and animal management

Four sheep farms were enrolled in this study. One of the farms was the experimental sheep farm (Farm I) of the University of Debrecen, where Merino, Dorper, and Tsigai sheep breeds were raised. The other three dairy sheep farms (Farm II, III, and IV) were commercial farms. Farms were located in Hajdú-Bihar County (Farm I, II, and III) and Jász-Nagykun-Szolnok County (Farm IV) of eastern Hungary. The flocks in Farm II and III were Lacaune breeds. The British Milksheep were kept on Farm IV. The size of the flock ranged from 51 to 292 animals. On Farm I, 56 Merino, 74 Tsigai, and 82 Dorper ewes were kept. On Farm II, III, and IV the flock size was 51, 292, and 265 ewes respectively. Regular milking and recording activities on farms where animals are milked were performed following the HUNGARIAN SHEEP and GOAT BREEDERS' ASSOCIATION'S SHEEP PERFORMANCE TESTING CODEX (2013) in agreement with the ICAR guidelines (2018).

Animals were kept indoors on Farm I, while those on Farm II, III, and IV were graze during the daytime. Grazing occurs between mid-April and November of the year, depending on the weather. On farms where animals graze (Farm II, III and IV), animals are released to graze around 6 a.m. in the morning. As a result, milking begins early in the morning, around 4 a.m., especially in Farm III and IV. Animals were milked on Farm II and IV by a milking parlour and on Farm III by hand milking. During the period of this experiment, milking was not started on Farm I; though a milking parlour has since been installed, and milking is expected to begin in the coming years. The animals were milked twice per day on the above-mentioned farms, which are early morning and late afternoon. Only on Farm II, before milking, ewe’s teats were disinfected. Post-milking disinfection was not practiced on any of the farms where animals are milked. Before being delivered to the processing units, milk is stored in a bulk tank at 4 degrees Celsius on the farm. Milk was delivered from Farm II, III, and IV to the milk-processing plant between 24 and 72 hours. Table 12 shows the main features of the four sheep farms.

Table 12: The main characteristics of the sheep farms

Farms	Sheep breeds	Type of housing	Grazing	Pre- or post-milking disinfection	Milking method
Farm I	Merino, Tsigai and Dorper	Deep litter	No	No	No milking
Farm II	Lacaune	Deep litter	Yes	Pre-milking disinfection	Milking parlour
Farm III	Lacaune	Deep litter	Yes	No	Hand milking
Farm IV	British Milksheep	Deep litter	Yes	No	Milking parlour

3.2. Sampling and sample preparation

Data on flock size, breed, feeding, housing, milking frequency, and time of milking were gathered during farm visit for sampling. Furthermore, the condition of the farms, milking type, and milk storing, equipment used and hygienic practices applied at the farms were observed. Ewes that had a detectable mammary gland abnormality and clinical mastitis were excluded. Among ewes with clinically healthy udder, five to twenty-one animals with mixed-parity were randomly selected for the udder surface and individual raw milk sampling (Table 13). Sample collection took place after two to six weeks of weaning on all farms except on Farm I, where sampling was performed before weaning.

A preliminary study was conducted to determine the origin of the environmental sample (udder surface). As such, samples were taken from the teat and udder surface, the mouth of the lamb, and barn wall at the very beginning of this research, and examined for the bacteriological count. The count values of different sample types (teat surface, udder surface, lamb mouth, and barn walls) were compared. The results showed no significant ($P > 0.05$) differences between environmental samples for the bacterial count (Appendix 1). As a result, based on these findings, only udder surface samples were considered for the bacterial count test. For this study, udder surface and milk samples were obtained between March and July in 2018 and 2019 at different times. During these sampling periods, two to three times of sampling was performed from the same animal (Table 13). Besides these, only individual raw milk samples were obtained from Farm I once in

February 2020. During the entire study period, 77 udder surface and 96 (86 + 10) raw milk samples were examined for the bacteriological count (Table 13).

Table 13: Distribution of sample size on a farm basis (between 2018 and 2020)

Farms	Year	Ewes sampled	Sampling frequency	US	IRM	BTM	Udder halves milk examined for CMT
FI*	2018	8	3	24	24	No milking	0
	2019	6	3	18	18	No milking	36
	2020	7 (+2)	1	0	9	No milking	18
FII	2018	5	3	15	15	6	30
FIII	2019	5	2	10	10	3	20
FIV	2019	5	2	10	10	1	20
	Total	**36**	**14**	**77**	**86**	**10**	**124**

*8 ewes (2 Merino, 4 Tsigai, and 2 Dorper) in 2018, 6 ewes (2 Merino, 2 Tsigai, and 2 Dorper) in 2019, and 7 ewes (2 Merino, 3 Tsigai, and 2 Dorper) and 1 Dorper and 1 Merino from 2019 sampling in 2020. During the study, animals were not commercially milked on Farm I, so only individual raw milk sample was obtained. FI: Farm I; FII: Farm II; FIII: Farm III; FIV: Farm IV; US: udder surface; IRM: individual raw milk; BTM: bulk tank milk; CMT: California Mastitis Test.

Animals were handled gently during sampling as described by the HUNGARIAN SHEEP AND GOAT BREEDERS' ASSOCIATION'S SHEEP PERFORMANCE TESTING CODEX (2013). Samples were obtained from ewes during scheduled milking sessions immediately before milking in the early morning. Initially, the research personnel wore sterile gloves to prevent the contamination of the sample from the hand. Thereafter, swabs moistened with 0.9% NaCI (VWR International Kft., Debrecen, Hungary) and 0.1% peptone (Merck Kft., Budapest, Hungary) solution were used for the collection of udder surface samples. The udder surface samples were taken from an udder surface of 4 cm × 5 cm using a standard metal frame.

After the udder surface sampling was completed, sterile cotton balls moistened with seventy percent ethanol (Molar Chemicals, Halásztelek, Hungary) were used to clean the ewe teat ends. The teat ends were gently scrubbed before the individual raw milk sample

was obtained. The research personnel once again cleaned the hand with the same concentration of ethanol used for teat cleaning. Then the first three to four milk streams were discarded before the individual raw milk sampling.

Two to three mL of udder halves milk was collected for the California Mastitis Test (CMT). California Mastitis Test was performed at the farms by mixing 2-3 mL of udder half milk samples with the same amount of reagents. The paddle was moved gently in a circular motion. Based on a change in viscosity, the CMT reaction being visually scored as negative (no gel or slime form), weak positive (mixture becomes slimy or gel like), positive (mixture clearly forms a gel), and strongly positive (mixture thickens immediately, tends to form jelly). The recorded result was interpreted as described by ZIGO et al. (2018). Accordingly, the CMT score values were recorded as -, +, ++, and +++ for negative, weak positive, positive, and strongly positive, respectively. In total, 124 udder halves ovine milk samples were tested for California CMT. This is the first study that evaluated the CMT score value of udder halves milk sample in the studied sheep farms.

Approximately 40 mL of milk was obtained for bacteriological culturing in sterile 50 mL Falcon tubes. No contact was made with the inside part of the Falcon tube and the lid with the hand and teat ends. To avoid dirt or hair from dropping into it, the milk sample was collected by keeping the tube at an angle. A clean dipper was used to take samples of milk from a bulk tank. Milk sampling was performed in compliance with the guidelines of the International Dairy Federation for milk and milk products sampling (ISO 707:2008). Individual raw milk samples from two udder halves were collected in one Falcon tube. Individual raw milk samples with one udder half positive for the CMT score were considered as positive samples. A permanent marker was used to label the sample tubes.

3.3. Ovine cheese samples

Fifteen samples of ovine cheese were purchased from three sources. Nine from the 79th OMÉK on 26 September 2019, three from the market in Debrecen, and the other three from the farmer near to Debrecen. Based on their flavour, four types of sheep cheeses were evaluated in this study. Chives-flavoured cheese (n = 3), garlic-flavoured cheese (n = 3), garlic-flavoured-smoked cheese (n = 6) and cumin-flavoured-smoked cheese (n = 3). The cheese samples were evaluated for *Enterobacteriaceae* count,

Escherichia coli count, *Staphylococcus aureus* count, and lactic acid bacteria count (6/15 samples) in parallel. The procedure of cheese sample preparation such as cutting of cheese, homogenizing and decimal dilution was followed as described by PETRÓCZKI et al. (2018). Comparing the results from three different sources proved difficult due to differences in cheese flavour and uneven sample size distribution. Thus, instead of considering the source of origin, the bacterial count was compared based on the flavour of the cheese samples.

3.4. Microbiological examination

Tightly closed sample tubes were placed in a box with ice packs and were instantly refrigerated (4 ^{o}C) upon arrival. On the same day as the collection of the samples, bacteriological analyses (US, IRM, BTM, and cheese samples) were carried out immediately in the microbiological laboratory of the Institute of Food Sciences at the University of Debrecen. The decimal dilution was performed on peptone water (9 mL) prepared in test tubes with NaCl (8.5 g) (VWR International Ltd., Hungary) and peptone (1.0 g) (Merck Kft., Hungary). According to the available data, this is the first study in Hungary that examined the microbiological quality of udder surface and individual raw milk of sheep. Table 14 presents the procedure details for the bacterial count.

Table 14: Procedure detail for the bacterial count

Bacterial count	**Culture media**	**Incubation**	**Standard**
Total plate count	Plate count agar	30 ^{o}C, 72 hrs	ISO 4833-1:2013
Enterobacteriaceae	Violet Red Bile Glucose agar	37 ^{o}C, 24 hrs	ISO 21528-2:2017
Escherichia coli	Tryptone Bile X-Glucoronide agar	37 ^{o}C, 24 hrs	ISO 16649-2:2001
Staphylococcus aureus	Baird-Parker agar	37 ^{o}C, 48 hrs	ISO 6888-1:2008
Lactic acid bacteria	de Man, Rogosa and Sharpe agar	30 ^{o}C, 72 hrs	ISO 15214:2005
Psychrotrophic bacteria	Plate count agar	7 ^{o}C, ten days	ISO 17410:2005

During the procedures for the total plate count, *Enterobacteriaceae* count, psychrotrophic bacteria count, and lactic acid bacteria count, the bacteria were cultured

in duplicate with 1 mL of the diluted sample by the pour plate technique. However, *Staphylococcus aureus* count and *Escherichia coli* count were inoculated in duplicate with 0.1 mL of the diluted sample by surface inoculation technique. All agar media were purchased from Biolab Ltd., Hungary. Furthermore, sterile egg yolk tellurite emulsion (LAB-KA Ltd., Hungary) was used on the plate during the procedure for *Staphylococcus aureus* culturing. The bacterial plates were evaluated manually after incubation at the required temperature for the relevant time. *Staphylococcus aureus* was differentiated from the other bacteria that grown on Baird-Parker agar through the latex agglutination test (Biolab Ltd., Hungary).

3.5. Sequencing and microbiota community analysis

Five ovine raw milk samples were collected from three dairy sheep farms for analysing microbiota composition. Three milk samples; one individual raw milk, one fresh bulk tank, and one cooling tank milk (24 hrs old) samples were taken from Farm II for microbiota analysis. These three raw milk samples were analysed in the Eurofins Genomics GmbH, Germany for microbiota profiling. After a year, one cooling tank and one individual raw milk, originated from Farm III and IV respectively were analysed for microbiota diversity at Biomi Ltd. Company, Hungary. The former one (Eurofins Genomics) was by 16S rRNA gene sequencing method by targeting the V3-V4 gene region and the latter one (Biomi Ltd.) was by LoopSeq™ 16S-18S Microbiome SSC 24-Plex Kit (Version 1.7, July 2019) method (https://www.loopgenomics.com/16s) with single sample calibration.

In the case of three milk samples analysed in Eurofins Genomics Center, the microbiota community analysis was performed on 27 August 2018. Bacterial universal primer sequencing was used for amplification of the 16S rRNA gene by targeting the V3-V4 gene region. Sequencing was done on Illumina MiSeq with 270 bp of a total read length to remove low-quality bases. The reads with undefined bases were removed at the first step of microbiota analysis. Based on the de-novo algorithm of UCHIME (EDGAR et al., 2011), chimeric reads were detected and removed as applied in the VSEARCH kit (ROGNES et al., 2016). The FLASH software version 2.2.00 (MAGOČ and SALZBERG, 2011) was used to merge paired-end reads. To minimize wrong positive merges, merging of pairs was performed with a minimum overlap size of 10 bp. For bacterial species, a copy-number correction was made according to ANGLY et al. (2014).

Minimum Entropy Decomposition, a strategy for picking Operational Taxonomic Unit, was used to process the high-quality reads (EREN et al., 2013, 2015). Sequences with a very low abundance (less than ≈ 0.02 percent of the average sample size) were detected and filtered by the Minimum Entropy Decomposition method.

Discontiguous Mega the Basic Local Alignment Search Tool of cluster representative sequences to the sequence database (NCBI_nt-release 2018-07-07) were performed to assign taxonomic details to each Operational Taxonomic Unit. For each Operational Taxonomic Unit, from the set of best-matched reference sequences, the most precise taxonomic units were transferred. A 70 percent identity across at least 80 percent of the representative sequence was the minimum criterion for a reference sequence. Using the QIIME software package version 1.9.1 (CAPORASO et al., 2010), further processing of Operational Taxonomic Unit and taxonomic assignments was done. To enhance estimates the bacterial taxonomic unit abundances were normalized by lineage-specific copy numbers of the related marker genes (ANGLY et al., 2014).

In the case of two milk samples analysed in Biomi Ltd. Company, the analysis was in July 2019. The workflow schematic described in the Loop genomics version 1.7, July 2019 was used for LoopSeq^{TM} based milk microbiota analysis. The DNA extraction to the taxonomic assignment of each OTU was done based on this manual (https://www.loopgenomics.com/start). The result of the taxonomic classification of the complete 16S molecules formed from the sample was based on the SILVA_16S_18S database (https://www.arb-silva.de/). The difference in microbiological composition of milk samples was not compared due to differences in analysis methods used. In the figure (13-17), the microbial community of milk samples was depicted by leaving a space between the results of two methods on the bar chart.

3.6. Isolation and identification of staphylococcal and lactic acid bacteria strains

3.6.1. Bacterial isolates

Seventy-one presumptive staphylococci colonies, 29 *Staphylococcus aureus,* and 42 coagulase-negative staphylococci colonies were picked from Baird-Parker agar for characterization. In addition to staphylococcal colonies, presumptive lactic acid bacteria (n = 35) colonies were also picked from de Man, Rogosa and Sharpe (MRS) agar. Both types of bacterial colonies were morphologically characterized by colony shape, size,

colour, appearance, and texture. Then sub-culturing of individual isolates was performed on total plate agar (Biolab Ltd., Hungary), which was incubated at 37 ^{o}C for 24 hours to clean the contamination. By using a plastic inoculating loop (Starstedt, Germany), a single pure colony from a total plate was transferred into 7 mL Tryptone Soya Broth (Biolab Ltd., Hungary) and incubated at 37 ^{o}C for 24 hours. The Cryo Tube (Thermo Fisher Scientific, China) was then suspended with 1 mL of bacterial broth and 0.5 mL of glycerol in duplicate. Until further examination, the colonies were stored at -80 ^{o}C.

3.6.2. Identification of bacterial strains

Staphylococcal isolates identification was done either by API Staph and/or by MALDI-TOF and/or PCR method. Three identification methods were not performed on each of the isolates because of the cost and time. Therefore, bacterial strains that had a reliable identification by one of the methods were not considered to be subjected to additional identification methods. Lactic acid bacteria isolates were identified by MALDI-TOF. The detailed procedures and number of bacterial strains subjected to the three identification methods were stated below.

3.6.2.1. API-Staph kit identification for *Staphylococcus*

API-staph kit (BioMerieux, Marcyl'Étoile, France) was used to further confirm eight *S. aureus* and to identify 13 suspected coagulase-negative staphylococci isolates at different times between July 2019 and April 2020 in the microbiological laboratory of the Institute of Food Sciences at the University of Debrecen. Firstly, the sub-culturing of the staphylococcal colonies was performed on Colombia agar media with 5% sheep blood for 24 hr at 37 ^{o}C. Then to create a humid environment, the honeycombed wells of the tray were filled with 5 mL of distilled water and uniformly distributed. Twenty microtubes with dehydrated substrates were included in an API-Staph strip. Inoculation of these microtubes was performed in API-Staph medium, using a bacterial suspension, to reconstitute the samples.

In the case of ADH and URE tests, to ensure anaerobic condition, mineral oil was filled into the microtubes. The metabolism created changes in colour during incubation, which were either spontaneous or seen by adding reagents. The reagents used were for the NIT test (NIT 1 and 2 reagents), PAL test (ZYM A and B reagents), and VP test (VP

1 and 2 reagents). The reading table was used to read the reactions and the identification was obtained by reference to the index of the analytical profile https://apiweb.biomerieux.com. Content of the kit (kit for 25 tests) were 25 API-Staph strips, 25 incubation boxes (tray and lid), 25 ampules of API-Staph medium, and 25 result sheets.

3.6.2.2. *Staphylococcus* and lactic acid bacteria strains identification by MALDI-TOF MS Biotyper

MALDI-TOF MS Biotyper identification method was employed to 21 *S. aureus* and 21 presumptive coagulase-negative staphylococci for confirmation and identification respectively. Furthermore, 35 suspected lactic acid bacteria colonies were subjected to MALDI-TOF MS Biotyper for identification. A 24 hr incubation treatment at 37 ^{o}C was applied on total plate agar (Biolab Ltd., Hungary) to individually sub-culture the isolates, which were then used for identification. Bacterial colonies were suspended in distilled water (300 μL) in a Cryo Tube through plastic inoculating loops, which were homogenized with a vortex mixer after adding 900 μL of 96% ethanol (Scharlab Ltd., Hungary). The samples were then transported to the AgroBioTech Research Centre at the Slovak University of Agriculture for the experiments, where they were refrigerated at -20 ^{o}C until the examination was completed.

The samples and plates were prepared accordingly for the MALDI-TOF test as defined by KAČÁNIOVÁ et al (2020). The generating of spectra and analysing were done on 29 October 2019 as defined by KAČÁNIOVÁ et al. (2019). MALDI-TOF identification with a score value of ≥ 2.300 is considered to be high-probability species identification, whereas the results were classified as secure and uncertain genus level when it is 2.00-2.299 and 1.700-1.999 respectively. When a score value is below 1.700, the identification is considered as not reliable (TOMAZI et al., 2014; SMITH et al., 2015). Additionally, the figure, which shows the meaning of the MALDI-TOF score value, was found in Appendix 2 of this study.

3.6.2.3. Identification of staphylococcal strains by a partial 16S rRNA gene sequencing

The identification of 15 suspected coagulase-negative staphylococcal strains into genera and species level was performed between the 5th and 23rd of April 2019 at Biomi Ltd. Company, Hungary. Following DNA extraction from the bacteria isolates, a partial 16S rRNA gene sequence method was used for bacterial identifications. With 519R and 27F primers (LANE, 1991), the sequencing of the partial 16S rRNA gene was used in the amplification. For reference, the partial double-stranded 16S rRNA gene fragment has been mapped into two databases. The TrueBac^{TM} ID database (DB ver. 20190409) (YOON et al., 2017) and the NCBI (National Center for Biotechnology Information) database. The Basic Local Alignment Search Tool algorithm was used to carry out alignment and the alignment is limited to cultivable strains.

3.7. Characterization of the identified strains

3.7.1. Coagulase-, hemolysis-, catalase-, and oxidase test

Forty-five staphylococcal (29 *S. aureus* and 16 coagulase-negative staphylococci) and 11 lactic acid bacteria strains that were identified and belonging to respective genera were further examined. Characterizations were carried out on all colonies, which were catalase and oxidase tests, while coagulase and hemolysis test were carried out for only staphylococcal isolates. For catalase test, a small amount of colony from a total plate agar was placed onto sterile slide. A droplet of 3% H_2O_2 (Biolab Ltd., Hungary) was added to the smear and mixed with a plastic loop. Based on the formation of bubbles, the result was categorized as positive (bubbles presence) and negative (no bubbles). Oxidase test was performed by scraping a colony-using loop on filter paper (Biolab Ltd., Hungary). Appearance of purple-blue colour and no colour change considered as positive and negative reactions respectively. Coagulase test was a carried out as LINAGE et al. (2012) on rabbit plasma. Hemolysis test was performed as described by VASIĽ et al. (2020) on 5% sheep blood Colombia agar (Biolab Ltd., Hungary).

3.7.2. Antibiotic resistance

Staphylococci and lactic acid bacterial strains were tested against 8 and 10 antibiotics (Biolab Ltd., Hungary) (Table 15) for antibiotic resistance respectively in the microbiological laboratory of the Institute of Food Sciences at the University of Debrecen. Antibiotic resistance was tested at different times between the second week of February and the first week of August 2020. The disk diffusion method has been applied to Mueller-Hinton (staphylococci isolates) and MRS (lactic acid bacteria isolates) agar (Biolab Ltd., Hungary) in compliance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017). In a normal saline tube, the bacterial suspension was calibrated to 0.5 McFarland turbidity. After 24 hrs of incubation at 37 ^{o}C, the inhibition was measured. *S. aureus* ATCC25923 (susceptible) was used as a control in every test. Isolates that are resistant to at least three antibiotics are known to be multidrug-resistant strains.

Table 15: Antibiotics used for antibiotic resistance test

Antibiotics[*]	**Dosages**
Penicillin G (P)	10 U/disk
Cefoxitin (FOX)	30 μg/disk
Chloramphenicol (C)	30 μg/disk
Clindamycin (DA)	2 μg/disk
Erythromycin (E)	15 μg/disk
Gentamicin (CN)	10 μg/disk
Tetracycline (TE)	30 μg/disk
Trimethoprim/Sulfamethoxazole (SXT)	1.25 + 23.75 μg/disk
Streptomycin (S)	10 μg/disk
Vancomycin (VA)	30 μg/disk

[*]*Staphylococcus* strains were evaluated against the first eight antibiotics (penicillin G to trimethoprim/sulfamethoxazole), and lactic acid bacteria strains were against all ten antibiotics (penicillin G to vancomycin).

3.8. Enterotoxin gene profiles of staphylococcal strains

For PCR reactions, DNA was extracted from staphylococci strains using the PrepManTM Ultra Sample Preparation Reagent (Biocenter Kft., Hungary) as urged by the manufacturer. There were one multiplex, three duplex, and two uniplex PCR reactions to detect 13 staphylococcal enterotoxins and SE-like toxins. To detect the well-characterized classical five SEs (SEA, SEB, SEC, SED and SEE), the multiplex PCR reaction was carried out. Furthermore, three duplex (SEG and SEI, SEH and SER, SE*l*M and SE*l*O) and two uniplex (SEJ, SE*l*N) PCR reactions were to detect 5 new forms of SEs (SEG, SEH, SEI, SEJ, and SER) and 3 SE*l*s (SE*l*M, SE*l*N, and SE*l*O). As Figure 9 shows, T100™ Thermal Cycler (Bio-Rad Magyarország Kft., Hungary) was used to perform DNA amplification.

One percent gel was prepared with 80 mL of 1 × TBE buffer containing ethidium bromide and 0.1 g agarose (Lab Mark Ltd., Czech Republic). Four µL of MidoriGreen Advance stain (Nucleotest Bio Kft., Hungary) was dissolved in the liquid gel before pouring into a gel tray. After the gel was dried for 30 minutes, it was placed into a gel box. Electrophoresis was carried out at 120 V for 40 minutes for all PCR products. For molecular weight marker, 100 bp GeneRuler™ Plus DNA Ladder (Biocenter Kft., Hungary) was used. The ethidium bromide was used to stain the gels and photographed under FluorChem M system (Bio-Science Kft., Hungary).

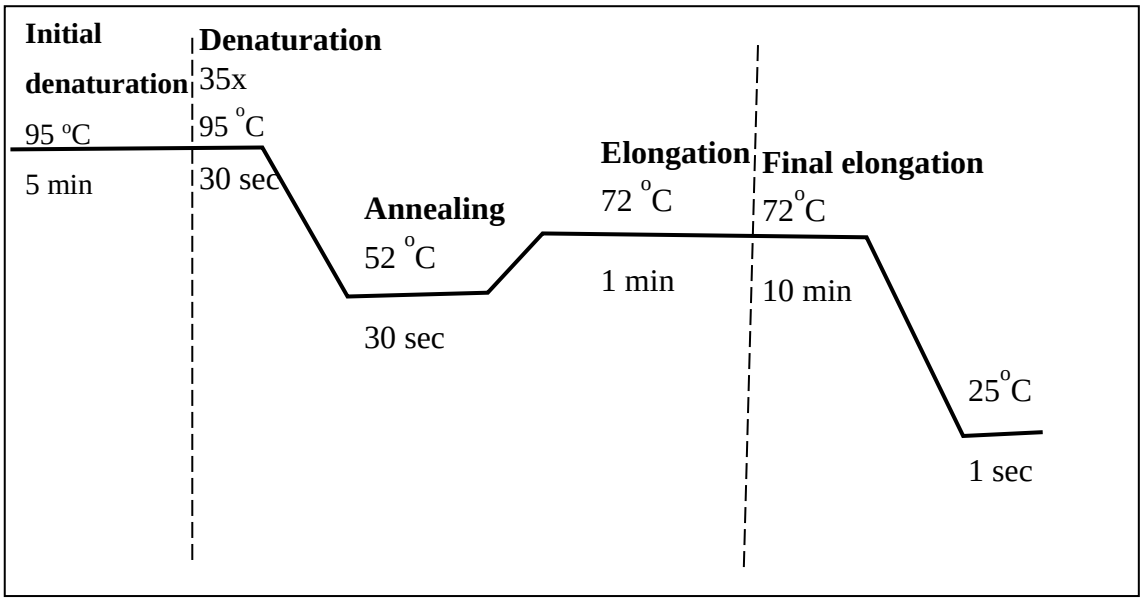

Figure 9: Thermal Cycler cycling conditions of PCR reaction.

Positive reference staphylococcal strains used for the PRC reaction were shown in Table 16. Using the primers shown in Table 17, the detection was carried out in the microbiological laboratory of the Institute of Food Sciences at the University of Debrecen. For PCR, the procedures were followed as suggested by MEHROTRA et al. (2000), SHARMA et al. (2000), BANIA et al. (2006), and CHIANG et al. (2008) with a minor change.

Table 16: The positive reference staphylococcal strains used for the PCR reaction

Strains ID	**Staphylococcal enterotoxin produced**
S. aureus ATCC29213	SEA, SEG, SEI
ATCC14458	SEB
ATCC19095	SEC, SEG, SEH, SEI
ATCC23235	SED, SEG, SEI, SEJ, SER
ATCC27664	SEE
SA54A	SE*l*M, SE*l*O
SA54B	SE*l*N

Table 17: PCR primers used for staphylococcal enterotoxins detection

Genes	Primers	Sequence (5´- 3´)	Size	Amplicon size (bp)	References
sea	GSEAR-F	GGTTATCAATGTGCGGGTGG	20	102	MEHROTRA et al. (2000)
	GSEAR-R	CGGCACTTTTTTCTCTTCGG	20		
seb	GSEBR-F	GTATGGTGGTGTAACTGAGC	20	164	
	GSEBR-R	CCAAATAGTGACGAGTTAGG	20		
sec	GSECR-F	AGATGAAGTAGTTGATGTGTATGG	24	451	
	GSECR-R	CACACTTTTAGAATCAACCG	20		
sed	GSEDR-F	CCAATAATAGGAGAAAATAAAAG	23	278	
	GSEDR-R	ATTGGTATTTTTTTTCGTTC	20		
see	SA-U	TGTATGTATGGAGGTGTAAC	20	213	SHARMA et al. (2000)
	SA-E rev	GCCAAAGCTGTCTGAG	16		
seg	SEG-F	GTTAGAGGAGGTTTTATG	18	198	BANIA et al. (2006)
	SEG-R	TTCCTTCAACAGGTGGAGA	19		
seh	SEH-F	CAACTGCTGATTTAGCTCAG	20	173	
	SEH-R	CCCAAACATTAGCACCA	17		
sei	SEI-F	GGCCACTTTATCAGGACA	18	328	
	SEI-R	AACTTACAGGCAGTCCA	17		
sej	SEJ-F	GTTCTGGTGGTAAACCA	17	131	
	SEJ-R	GCGGAACAACAGTTCTGA	18		

Genes	**Primers**	**Sequence (5´- 3´)**	**Size**	**Amplicon size (bp)**	**References**
selm	SEM-F	CATATCGCAACCGCTGA	17	148	
	SEM-R	TCAGCTGTTACTGTCGA	17		
seln	SEN-F	GGCAATTAGACGAGTCA	17	237	
	SEN-R	ATCGTAACTCCTCCGTA	17		
selo	SEO-F	GTCAAGTGTAGACCCTA	17	288	
	SEO-R	TGTACAGGCAGTATCCA	17		
selp	SEP-F	TCAAAAGACACCGCCAA	17	396	
	SEP-R	ATTGTCCTTGAGCACCA	17		
ser	SER1-F	AGATGTGTTTGGAATACCCTAT	22	123	CHIANG et al. (2008)
	SER2-R	CTATCAGCTGTGGAGTGCAT	20		

3.9. Data analysis

Data were stored in Microsoft Excel version 2016 (16.0.5110.1000). Before calculating the mean and standard deviation (SD) of the colony counts, the values of the bacterial count were converted to the decimal logarithm (lg) to normalize the distributions. The mean values were expressed by CFU/cm^2, CFU/mL, and CFU/g for udder surface, milk and cheese samples respectively. Bacterial count (TPC, EBC and SA) of individual raw milk samples were compared based on CMT scores. Individual raw milk samples with one udder half positive for the CMT score were considered as positive samples during analysis. The effects on bacterial counts by farms (Farm I-IV), breed (Farm I), and year (2018 and 2019) were evaluated by one-way ANOVA, t-test (for two levels of factors), and the non-parametric Kruskal-Wallis test using GraphPad Prism 3.02 (San Diego, California, USA) and SPSS (2019) version 26.0 (IBM SPSS Corp., Armonk, NY, USA). In the case of cheese samples, the mean values were compared between cheese types.

Using the Pearson correlation, the coefficient of correlation (r) was determined between the major bacterial counts; i.e. total plate count and *Enterobacteriaceae* count of 77-77 US and IRM samples. Furthermore, the correlation between total plate count and psychrotrophic bacterial count for BTM samples was evaluated. The frequency of resistance or antibiotic susceptibility between staphylococci isolates (i) from different farms (Farm I, II, III, and IV) or (ii) from different sources (US, IRM, BTM, and cheese) or (iii) of different identities (*S. aureus* and coagulase-negative isolates) was evaluated using the Pearson chi-square test. The level of significance was considered at $P < 0.05$.

4. RESULTS AND DISCUSSION

4.1. California Mastitis Test of udder halves milk

In this study, 124 udder halves ovine milk samples were tested for California Mastitis Test (CMT). Of 124 udder halves milk samples, 87.1% (n = 108) were negative for CMT. The remaining 16 (12.7%) were found to be weak positive-to-positive CMT. Eleven (68.8%) and five (31.2%) were recorded as weak positive (+) and positive (++) respectively (Table 18). Within farms studied, the overall positive CMT results varied from 10 to 25%. In Farm III samples, we observed an increased prevalence of positive CMT (25%) as well as a lower number of negative CMT (Table 18). This is the first study to evaluate the CMT score value of milk samples from udder halves in the sheep farms studied.

California Mastitis Test is one of the most frequently used methods to diagnose subclinical mastitis in the milk of infected animals (HAWARI et al., 2014). Similarly, ZIGO et al. (2018) reported that 16.6 to 23.2% of the udder halves ovine milk showed either weak positive, positive or strong positive CMT score in two Slovakian sheep flocks. In addition, our results concurs with those reported by FOTOU et al. (2011), conducted in Greece, only 42 (17.5%) had positive CMT scores out of 240 individual ewe milk samples tested.

Table 18: Distribution of California Mastitis Test scores

Farm	**No. of udder halves milk sample examined**	**California Mastitis Test score**			
		Negative (-)	Weak positive (+)	Positive (++)	Overall (+ and ++)
Farm I	54	48 (88.9%)	5 (9.3%)	1 (1.8%)	6 (11.1%)
Farm II	30	27 (90.0%)	1 (3.3%)	2 (6.7%)	3 (10.0%)
Farm III	20	15 (75.0%)	3 (15.0%)	2 (10.0%)	5 (25.0%)
Farm IV	20	18 (90.0%)	2 (10.0%)	0 (0.0%)	2 (10.0%)
Overall	**124**	**108 (87.1%)**	**11 (8.9%)**	**5 (4.0%)**	**16 (12.9%)**

As our results show (Table 19) as an average, total plate count (TPC) was 2.9 ± 1.0 lg CFU/mL, *Enterobacteriaceae* count (EBC): 1.5 ± 0.5 lg CFU/mL and *Staphylococcus aureus* count (SAC): 2.6 ± 0.7 lg CFU/mL in overall (n = 108) CMT negative udder halves milk samples. Overall, individual raw milk samples with weak positive (n = 11) and positive (n = 5) CMT scores had TPC mean value of 3.6 ± 1.1 and 3.6 ± 0.8 lg CFU/mL, EBC: 1.8 ± 0.6 and 3.0 ± 0.9 lg CFU/mL, SAC: 2.8 ± 0.3 and 3.4 lg CFU/mL, respectively (Table 19). Except for Farm III milk samples, there was no growth of *S. aureus* in individual raw milk samples with negative CMT scores. Surprisingly, *S. aureus* was detected in Farm III individual raw milk samples with negative CMT scores (2.6 ± 0.7 lg CFU/mL) rather than individual raw milk samples with weak positive and positive CMT scores.

All individual raw milk samples (n = 124) with negative to positive CMT scores had TPC mean value of 3.0 ± 1.1 lg CFU/mL, EB: 1.9 ± 0.9 lg CFU/mL and SAC: 2.9 ± 0.6 lg CFU/mL. For TPC, EBC and SAC, there was no significant difference between individual raw milk samples with negative CMT scores (n = 108) and individual raw milk samples with negative to positive CMT scores (n = 124) (Table 19). Almost all individual raw milk samples with weak positive or positive CMT scores had a slightly higher TPC value than milk samples with negative CMT scores in all farms.

Table 19: Bacterial count of individual milk samples (lg CFU/mL) according to the California Mastitis Test score

Farm	CMT score	No. of udder halves milk sample	TPC	EBC	SAC
Farm I	Negative	48	2.6 ± 0.9	1.1 ± 0.3	ND
	CMT +	5	4.2 ± 0.6	ND	2.9
	CMT ++	1	4.1	ND	3.4
	Overall	54	2.9 ± 1.1	1.1 ± 0.3	3.2 ± 0.3
Farm II	Negative	27	3.3 ± 0.9	1.8 ± 0.4	ND
	CMT +	1	4.7	1.1	ND
	CMT ++	2	3.5 ± 1.0	3.0 ± 0.8	ND
	Overall	30	3.3 ± 1.0	2.3 ± 0.9	ND
Farm III	Negative	15	3.4 ± 1.1	ND	2.6 ± 0.7
	CMT +	3	3.7 ± 0.1	2.2 ± 0.0	ND
	CMT ++	2	3.6 ± 0.2	ND	ND
	Overall	20	3.5 ± 0.9	2.2 ± 0.0	2.6 ± 0.7
Farm IV	Negative	18	1.8 ± 0.5	1.4 ± 0.0	ND
	CMT +	2	1.6 ± 0.2	ND	2.8 ± 0.3
	CMT ++	0	-	-	-
	Overall	20	1.8 ± 0.4	1.4 ± 0.0	2.8 ± 0.3
Overall	**Negative**	**108**	**2.9 ± 1.0[a]**	**1.5 ± 0.5[a]**	**2.6 ± 0.7[a]**
	CMT +	11	3.6 ± 1.1	1.8 ± 0.6	2.8 ± 0.3
	CMT ++	5	3.6 ± 0.8	3.0 ± 0.9	3.4
	Overall	**124**	**3.0 ± 1.1[a]**	**1.9 ± 0.9[a]**	**2.9 ± 0.6[a]**

Individual raw milk samples from two udder halves were collected in one Falcon tube, thus, milk samples with one udder half positive for the CMT score were considered as positive samples during analysis. CMT: California Mastitis Test; EBC: *Enterobacteriaceae* count; ND: not detected; SAC: *Staphylococcus aureus* count; TPC: total plate count. Mean values in the same column with the same letters are not significantly ($P > 0.05$) different.

4.2. Total plate count of udder surface and milk samples

In our study, a total of 77 udder surface (US), 86 individual raw milk (IRM) and 10 bulk tank milk (BTM) samples from four sheep farms were analysed for total plate count (TPC) between 2018 and 2020. Total plate counts of udder surface (US) samples were ranged from 1.0 to 5.1 lg CFU/cm^2 with overall mean value of 2.5 ± 0.8 lg CFU/cm^2 (Table 20). With regard to individual raw milk (IRM) samples, TPC was between 1.0 and 5.5 lg CFU/mL with overall average value of 3.1 ± 1.1 lg CFU/mL. Bulk tank milk samples had TPC values ranged from 5.2 to 8.9 lg CFU/mL with overall mean value of 7.0 ± 0.9 lg CFU/mL (Table 20).

The TPC counts of US, IRM, and BTM samples of each ovine farm were presented below in Table 20. In this regard, the mean of TPC was 2.7 ± 1.0, 2.5 ± 0.7, 2.2 ± 0.5, and 2.4 ± 0.4 lg CFU/cm^2 on ewes' udder surfaces kept on Farm I, II, III, and IV, respectively (Table 20). Results indicated the highest value on Farm I and the lowest value on Farm III, the differences between the values in these two farms were statistically significant ($P < 0.05$).

Due to the same breed (Lacaune) of sampled animals on Farm II and III, there was no significant ($P > 0.05$) variation in the US samples TPC value (Table 20). This is the first study that dealt with the bacteriological quality of ewe udder surface samples in the studied sheep farms. In addition, similar findings on the bacteriological status of US ovine samples were scarce in other countries as well. It is therefore not easy to compare our findings with others.

The mean TPC of IRM was found between 1.8 ± 0.4 and 3.5 ± 0.9 lg CFU/mL. The result of IRM samples originated from animals kept on Farm IV was significantly lower ($P < 0.05$) than that of IRM samples from Farm I, II, and III based on total plate counts. In the same way, the finding of BYTYQI et al. (2013) from Kosovo revealed that a significant ($P < 0.0001$) difference of bacterial content was observed in individual ewe raw milk samples of different farms. Remarkably lower TPC of IRM samples obtained from British Milksheep on Farm IV was may be partially related to the adaptability and vigorousity of this hardy and strong sheep breed. The highest value of TPC (3.5 ± 0.9 lg CFU/mL) was recorded in the IRM samples of ewes on Farm III.

Of the four farms included in this study, animals were milked in three farms from which BTM samples were obtained and tested for bacterial counts. As this study reveals, based on the specific bacterial group examined, milk samples of the bulk tank had up to

1.0 to 4.1 lg CFU/mL more bacterial counts than their corresponding IRM samples. The concentration of TPC in BTM indicates the effect of the hygienic condition during milking, milking equipment and extended time storage of raw milk on bacterial content. The duration and technique of storage contribute significantly to the change in the bacteriological content of ewe milk (DE GARNICA et al., 2011). The value of TPC in the BTM derived Farm II (7.4 ± 0.6 lg CFU/mL) and Farm III (6.3 ± 0.4 lg CFU/mL) did not meet the limit of REGULATION (EC) 853/2004, which was ≤ 6.2 lg CFU/mL for TBC of ovine milk to be processed with heat treatment. A Significant ($P < 0.05$) difference was observed between TPC of BTM samples examined from Farm II and III, where the machine and hand milking was practiced respectively.

Table 20: Total plate count in udder surface and milk samples (between 2018 and 2020)

Type of samples	**Selected statistical value**	**Farm I (n = 42)**	**Farm II (n = 15)**	**Farm III (n = 10)**	**Farm IV (n = 10)**	**Overall (n = 77)**
Udder surface (lg CFU/cm^2)	Minimum value	1.1	1.0	1.2	1.6	1.0
	Maximum value	5.1	4.1	3.2	3.6	5.1
	Mean ± SD	2.7 ± 1.0^{a}	2.5 ± 0.7^{ab}	2.2 ± 0.5^{b}	2.4 ± 0.4^{ab}	2.5 ± 0.8
		Farm I (n = 51)	**Farm II (n = 15)**	**Farm III (n = 10)**	**Farm IV (n = 10)**	**Overall (n = 86)**
Individual raw milk (lg CFU/mL)*	Minimum value	1.0	1.3	1.5	1.0	1.0
	Maximum value	5.4	5.5	5.0	2.5	5.5
	Mean ± SD	3.1 ± 1.1^{a}	3.3 ± 1.0^{a}	3.5 ± 0.9^{a}	1.8 ± 0.4^{b}	3.1 ± 1.1
		Farm I (n = 0)	**Farm II (n = 6)**	**Farm III (n = 3)**	**Farm IV (n = 1)**	**Overall (n = 10)**
Bulk tank milk (lg CFU/mL)	Minimum value	No milking	6.4	5.5	5.2	5.2
	Maximum value	No milking	8.9	6.9	5.2	8.9
	Mean ± SD	No milking	7.4 ± 0.6^{a}	6.3 ± 0.4^{b}	5.2	7.0 ± 0.9

*Total plate count of individual milk samples with positive CMT scores was included in the analysis due to a non-significance between individual raw milk samples with negative CMT (n = 108) scores and negative to positive CMT scores (n = 124). During the sampling period, milking was started on Farm I. ^{ab}Mean values in the same row with different letters are significantly ($P < 0.05$) different.

Total plate count in US and IRM samples obtained from Farm I was analysed by year of investigation and on breed basis (Table 21). The overall mean of TPC, the two years was 2.5 ± 1.0, 3.2 ± 1.0, and 2.5 ± 0.8 lg CFU/cm^2 on the udder surfaces of Merino, Tsigai, and Dorper ewes at Farm I, respectively (Table 21). There was significantly higher count ($P < 0.05$) on US samples of Tsigai breed than the other two breeds. This was might be due to denser and longer hair on the udder surface of Tsigai ewes than others to which dust particles and bedding materials can be easily attached. In the case of individual raw milk samples, a similar effect of breed on TPC was observed. Tsigai ewes had a substantially higher mean count ($P < 0.05$) for TPC than Merino ewes. As shown in Table 21, the overall mean TPC determined in the IRM samples of Merino, Tsigai, and Dorper ewes on Farm I was 2.8 ± 1.2, 3.4 ± 1.3, and 3.2 ± 0.8 lg CFU/mL, respectively (Table 21). In terms of TPC, the IRM samples from Tsigai were higher than ($P < 0.05$) those from Merino.

Udder surface samples examined in 2018 seemed to contain higher ($P < 0.05$) TPC than those examined in 2019. A similar pattern was found concerning IRM samples, in which the number of TPC decreased over the year. This reduction of the TPC from 2018 to 2019 may be due to an awareness of hygienic steps by sheep farm workers during milk production. At the end of each sampling year, our results were shared with the farm manager, which helps to enhance the management practices of the farm. Over this, the hygienic status of the farm may have improved over time. Likewise, GONZALO et al. (2010) reported that the number of total bacteria in ewe BTM in Spain statically varies ($P <0.0001$) from year to year.

Table 21: The effect of breed and year of investigation on total plate count of udder surface and individual raw milk samples on Farm I

Breed	Year	No.	Udder surface (lg CFU/cm^2)	Individual raw milk (lg CFU/mL)
Merino	2018	6	3.8 ± 0.5^a	3.6 ± 1.3^a
	2019	6	1.9 ± 0.4^b	2.3 ± 0.8^b
	Overall	**12**	**2.5 ± 1.0^a**	**2.8 ± 1.2^a**
Tsigai	2018	12	4.0 ± 0.7^a	3.8 ± 1.1^a
	2019	6	2.1 ± 0.5^b	3.2 ± 1.2^{ab}
	Overall	**18**	**3.2 ± 1.0^b**	**3.5 ± 1.2^b**
Dorper	2018	6	3.5 ± 0.7^a	3.3 ± 0.9^a
	2019	6	2.0 ± 0.3^b	3.3 ± 0.5^a
	Overall	**12**	**2.5 ± 0.8^a**	**3.3 ± 0.7^{ab}**

^{ab}Mean values in the same column with different letters are significantly ($P < 0.05$) different.

4.3. *Enterobacteriaceae* count of udder surface, milk and cheese samples

In this study, a total of 77 US, 86 IRM, 10 BTM and 15 ovine cheese samples were analysed for *Enterobacteriaceae* count (EBC) between 2018 and 2020. *Enterobacteriaceae* counts of US samples were ranged from 0.1 to 2.6 lg CFU/cm^2 with an average value of 1.2 ± 0.6 lg CFU/cm^2 (Table 22). With regard to IRM samples, EBC was between 0.0 and 3.9 lg CFU/mL with overall mean value of 1.9 ± 0.8 lg CFU/mL. Bulk tank milk samples had EBC values ranged from 3.9 to 7.0 lg CFU/mL with overall mean value of 5.1 ± 0.9 lg CFU/mL (Table 22).

As our results show, the average EBC in US samples of ewe in Farm I, II, III, and IV was 1.5 ± 0.8, 1.0 ± 0.5, 1.0 ± 0.1, and 0.5 ± 0.3 lg CFU/cm^2, respectively (Table 22). Udder surface samples originated from Farm IV had a significantly lower ($P < 0.05$) EBC than Farm I. The difference in husbandry practices, which involves feeding, housing, breeds, etc. in two farms, might partially explain the significant difference of EBC in US samples.

The EBC of IRM samples collected from ewes on Farm I (1.1 ± 0.3 lg CFU/mL) and Farm II (2.3 ± 0.8 lg CFU/mL) was significantly varied ($P < 0.05$). Individual raw milk samples in Farm III and IV had 2.2 ± 0.0 and 1.4 ± 0.0 lg CFU/mL of EBC,

respectively ($P > 0.05$). The average value of EBC in BTM samples of Farm II, III and IV was 5.1 ± 0.9, 5.6 ± 0.9 and 3.9 lg CFU/mL respectively. Even if the milking methods practiced on Farm II and III were different, which is parlour machine with pre-milking disinfection and hand milking without disinfection, respectively, the mean value of EBC of BTM samples was not significantly different ($P > 0.05$). In our findings, EBC values in BTM of three farms were similar to those reported from Spain (5.4 lg CFU/mL) by MERLIN-JUNIOR et al. (2015), from Egypt (5.2 lg CFU/mL) by OMBARAK and ELBAGORY (2017) and from Switzerland (3.9 lg CFU/mL) by MUEHLHERR et al. (2003).

Table 22: *Enterobacteriaceae* count in udder surface and milk samples (between 2018 and 2020)

Type of samples	**Selected statistical value**	**Farm I (n = 42)**	**Farm II (n = 15)**	**Farm III (n = 10)**	**Farm IV (n = 10)**	**Overall (n = 77)**
Udder surface (lg CFU/cm^2)	Minimum value	0.3	0.1	0.9	0.2	0.1
	Maximum value	2.6	1.9	1.1	0.7	2.6
	Mean ± SD	1.4 ± 0.7^{a}	1.0 ± 0.5^{ab}	1.1 ± 0.1^{ab}	0.5 ± 0.3^{b}	1.2 ± 0.6
		Farm I (n = 51)	**Farm II (n = 15)**	**Farm III (n = 10)**	**Farm IV (n = 10)**	**Overall (n = 86)**
Individual raw milk (lg CFU/mL)*	Minimum value	0.7	1.1	0.0	0.0	0.0
	Maximum value	1.4	3.9	2.2	1.4	3.9
	Mean ± SD	1.1 ± 0.3^{a}	2.3 ± 0.9^{b}	2.2 ± 0.0^{ab}	1.4 ± 0.0^{ab}	1.9 ± 0.8
		Farm I (n = 0)	**Farm II (n = 6)**	**Farm III (n = 3)**	**Farm IV (n = 1)**	**Overall (n = 10)**
Bulk tank milk (lg CFU/mL)	Minimum value	No milking	3.9	4.3	3.9	3.9
	Maximum value	No milking	7.0	6.8	3.9	7.0
	Mean ± SD	No milking	5.1 ± 0.9^{a}	5.6 ± 0.9^{a}	3.9	5.1 ± 0.9

**Enterobacteriaceae* count of individual milk samples with positive CMT scores was included in the analysis due to a non-significance between individual raw milk samples with negative CMT (n = 108) scores and negative to positive CMT scores (n = 124). During the sampling period, animals were not milked on Farm I. ^{ab}Mean values in the same row with different letters are significantly ($P < 0.05$) different.

The overall mean EBC on the udder surfaces of Merino, Tsigai, and Dorper ewes of Farm I was 0.9 ± 0.3, 2.4 ± 0.7, and 1.7 ± 0.2 lg CFU/cm^2, respectively, with Tsigai showing significantly increased EBC ($P < 0.05$) compared to Merino (Table 23). The EBC of IRM samples from Merino and Dorper individuals did not differ significantly ($P > 0.05$), showing means of 1.0 ± 0.3 and 1.4 ± 0.0 lg CFU/mL, respectively (Table 23). Udder surface samples examined in 2019 seemed to contain lower ($P < 0.05$) EBC than those examined in 2018. This reduction of the EBC from 2018 to 2019 may be due to an awareness of hygienic steps by sheep farm workers during milk production. Over this, the hygienic status of the farm may have improved over time.

Table 23: The effect of breed and year of investigation on *Enterobacteriaceae* count of udder surface and individual raw milk samples on Farm I

Breed	Year	No.	Udder surface (lg CFU/cm^2)	Individual raw milk (lg CFU/mL)
Merino	2018	6	1.1 ± 0.2^a	0.0 ± 0.0^a
	2019	6	0.9 ± 0.3^a	1.0 ± 0.3^b
	Overall	**12**	**0.9 ± 0.3^a**	**1.0 ± 0.3^a**
Tsigai	2018	12	2.2 ± 0.9^a	0.0 ± 0.0^a
	2019	6	0.2 ± 0.1^b	0.0 ± 0.0^a
	Overall	**18**	**2.4 ± 0.7^b**	**0.0 ± 0.0^b**
Dorper	2018	6	1.7 ± 0.2^a	0.0 ± 0.0^a
	2019	6	0.0 ± 0.0^b	1.4 ± 0.0^b
	Overall	**12**	**1.7 ± 0.2^{ab}**	**1.4 ± 0.0^a**

^{ab}Mean values in the same column with different letters are significantly ($P < 0.05$) different.

Enterobacteriaceae counts in chives-flavoured cheese (CFC), cumin-flavoured-smoked cheese (CFSC), garlic-flavoured cheese (GFC) and garlic-flavoured-smoked cheese (GFSC) samples were presented in Figure 10 below. The mean of EBC was 5.5 ± 0.1, 5.5 ± 0.2, 4.3 ± 0.6 and 0.0 ± 0.0 lg CFU/g in CFC, GFSC, CFSC and GFC, respectively (Figure 10). There was significant difference ($P < 0.05$) between cheese samples in the case of EBC. EBC mean value in CFSC was significantly lower ($P < 0.05$) compared to CFC and GFSC. Relatively lower value was reported by FRECE et al. (2016)

from Croatia, for cheese from ewe milk (2.5 lg CFU/g), while EBC reported by SANDRA et al. (2013) was higher than that obtained in this study for fresh cheese from ewe milk (7.2 lg CFU/g). The existence of *Enterobacteriaceae* in cheese samples usually suggests direct or indirect faecal contamination of the product during processing, and thus the likelihood of pathogenic faecal origin bacteria in the cheese.

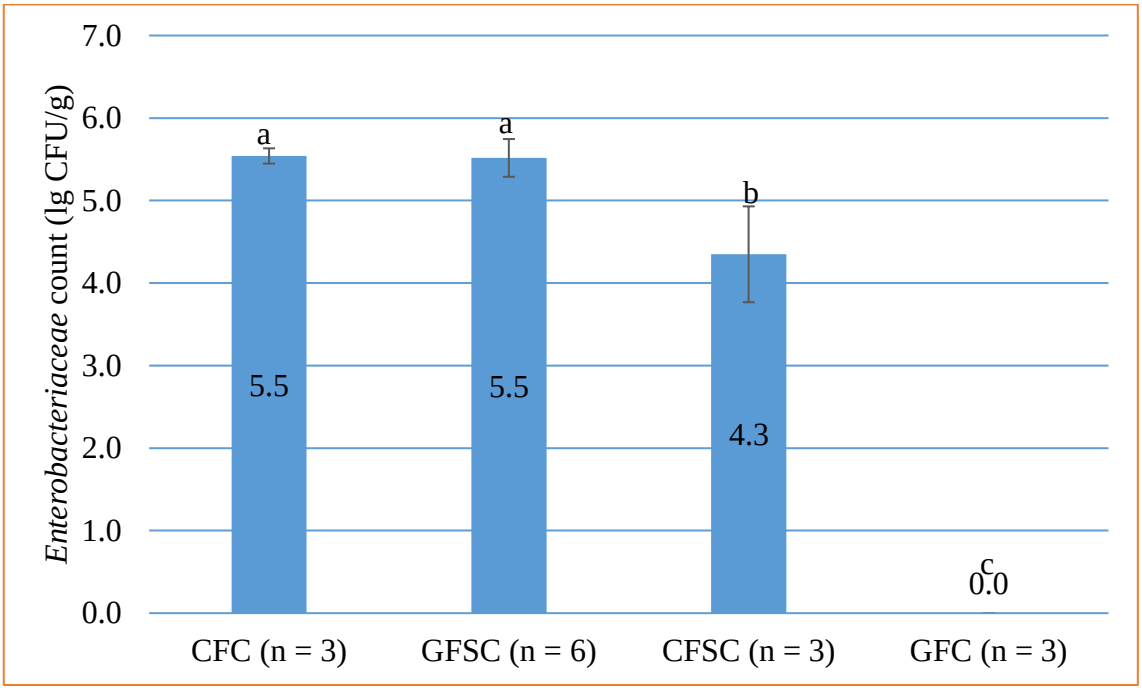

Figure 10: *Enterobacteriaceae* count in ovine cheese samples. CFC: chives-flavoured cheese; CFSC: cumin-flavoured-smoked cheese; GFC: garlic-flavoured cheese; GFSC: garlic-flavoured-smoked cheese.

4.4. *Escherichia coli* count of udder surface, milk and cheese samples

In our study, 47 udder surface, 56 individual raw milk, 4 bulk tank milk and 15 cheese samples were analysed for *Escherichia coli* count (ECC) between 2018 and 2020. *Escherichia coli* was not detected in all US samples except for Farm II US samples, where *Escherichia coli* was not tested. *Escherichia coli* was not tested for Farm II individual animal milk samples and interestingly, it was not present in IRM samples obtained from three other farms. In the case of BTM samples from Farm III and IV, the mean count of *E. coli* was 2.9 ± 0.5 and 3.8 lg CFU/mL, respectively. It was diagnosed in all (100%) four (three BTM Farm III and one BTM Farm IV) BTM samples tested. Similarly, in studies that had investigated the occurrence of *E. coli* in ovine BTM, *E. coli* was detected

in 10.1% of the sample in Spain (REY et al., 2006), 4.4% of the sample in Czech Republic (BOGDANOVIČOVA et al., 2016), and 0.8% of the sample in Greece (SOLOMAKOS et al., 2009).

The colonies of *E. coli* were detected in CFC and GFSC cheese samples. The mean value of ECC was 5.1 ± 0.4 and 4.4 ± 0.3 lg CFU/g in GFSC and CFC respectively, while not detected in CFSC and GFC samples (Figure 11). Higher count of *E. coli* was found in GFSC. These values exceeded the *E. coli* limit value of 2.0 lg CFU/g set by Regulation (EC) No. 2073/2005 for cheese made from heat-treated milk. Comparatively higher *E. coli* (8.7 lg CFU/g) count was reported by SANDRA et al. (2013) from Macedonia, for ewe-milk cheese at the beginning of ripening due to the use of raw milk and handmade rennet, as well as high humidity and low salt content. On the other hand, FRECE et al. (2016) reported a lower count (0.3 lg CFU/g) from Croatia for cheese from ewe milk. Based on the ECC results in this study, CFC and GFSC cheese samples failed to meet regulatory standards in DECREE NO. 4/1998. (XI. 11.) EÜM, which defines mandatory criteria for this bacterial group in the milk products and curd, should be less than one CFU/g.

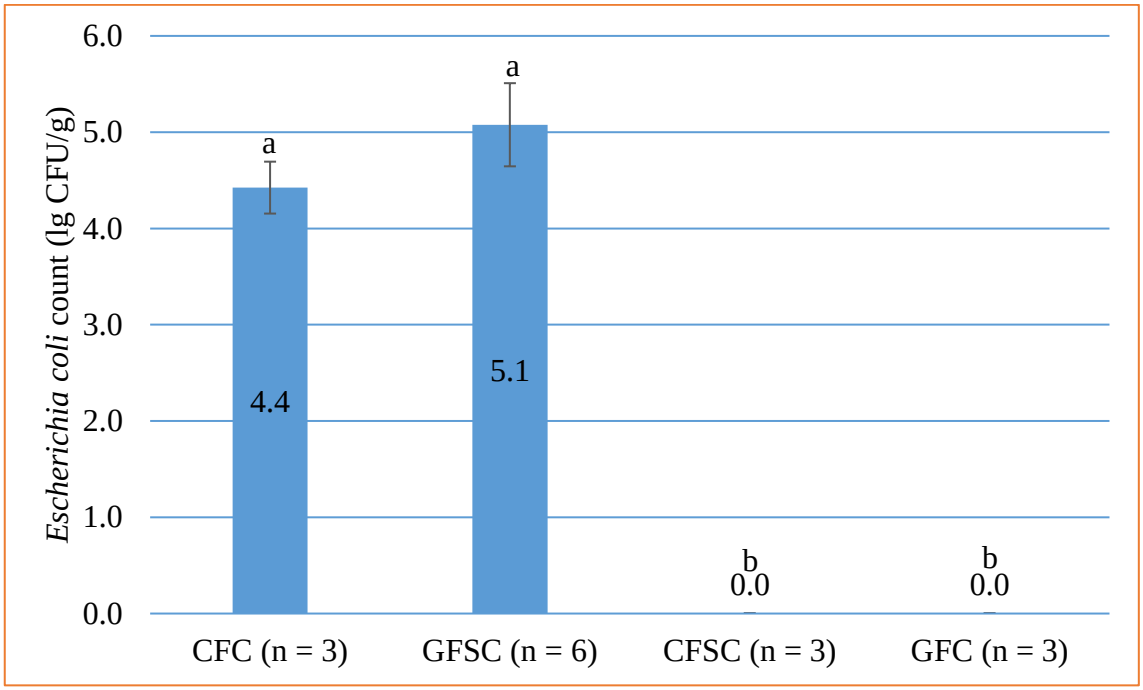

Figure 11: *Escherichia coli* count in ovine cheese samples. CFC: chives-flavoured cheese; CFSC: cumin-flavoured-smoked cheese; GFC: garlic-flavoured cheese; GFSC: garlic-flavoured-smoked cheese.

4.5. *Staphylococcus aureus* count in udder surface, milk and cheese samples

A total of 77 US, 86 IRM, 10 BTM and 15 ovine cheese samples were analysed for *Staphylococcus aureus* count (SAC) between 2018 and 2020. Out of 77 udder surfaces examined from four farms, *Staphylococcus aureus* was detected in only two samples (2.6%) (1 US of Farm I and 1 US of Farm IV) with SAC of < 1 lg CFU/mL. It was not detected in US samples belonging to other farms (Farm II and III). Out of 51 IRM samples obtained from Farm I during 2018 and 2020, *S. aureus* was detected in three samples (5.9%) with mean value of 3.2 ± 0.3 lg CFU/mL. None of the IRM samples in Farm II was detected for *S. aureus*.

In the case of IRM samples originated from Farm III and IV, out of 10-10 samples, it was diagnosed in two (20%) and one (10%) samples, respectively. The average value of SAC was 2.6 ± 0.7 and 2.8 lg CFU/mL in Farm III and IV IRM samples, respectively. Overall, out of 86 IRM samples examined, *S. aureus* was detected in six (7.0%) samples with overall mean value of 2.9 ± 0.6 lg CFU/mL. Out of 10 BTM samples, *S. aureus* was detected in only two (20%) samples of Farm III with a mean of 3.4 ± 0.6 lg CFU/mL. These SAC values were lower than 4.1 lg CFU/mL, which was reported by OMBARAK and ELBAGORY (2017).

The *Staphylococcus aureus* count of ovine cheese samples was summarized below based on the types. Coagulase-positive staphylococci mean value was 5.7 ± 0.2 lg CFU/g in CFC and not detected in the other three cheese types. This result exceeded the Regulation (EC) No. 2073/2005 limit value in which cheese made from raw milk should not exceed *S. aureus* of 4.0 lg CFU/g. Significantly, a higher value ($P < 0.05$) was recorded in CFC samples compared to others. The count of *S. aureus* CFC in this study agrees to some extent with that recorded by FRECE et al. (2016) from Croatia, for cheese from ewe milk (5.5 lg CFU/g).

The high count of coagulase-positive staphylococci in CFC samples should be considered as a potential safety hazard. This is because of the poor hygiene conditions during production (as indicated also by the high numbers of EBC and ECC). The growth of pathogenic microorganisms in cheese could be influenced by the addition of leaves or plants for organoleptic, decorative, or preservation purposes. However, it is difficult to conclude that higher *S. aureus* count in cheese samples was because of the chives. Probably unpasteurized milk was used for cheese production, ripening duration, utilities used in cheese processing/production were not sterilized to avoid contamination by

staphylococci. Furthermore, smoking may have an effect on the growth of this bacteria and the cheese in which *S. aureus* detected was not smoked. The absence of coagulase-positive staphylococci in CFSC, GFSC and GFC and indicates good safety of the cheese.

4.6. Psychrotrophic bacteria count in bulk tank milk samples

Psychrotrophic bacteria count (PBC) was examined in four BTM samples. Three samples were originated from Farm III and one sample from IV. According to this study, PBCs were 4.3 ± 1.0 and 3.6 CFU/mL in BTM samples of Farm III and IV respectively. Compared to our results, higher PBCs were determined from Brazilian and Spanish ovine BTM samples by MERLIN-JUNIOR et al. (2015) and DE GARNICA et al. (2013), which were 6.8 and 5.7 lg CFU/mL respectively. The results of several researchers indicate that the psychrotrophic bacterial counts in ovine BTM samples are positively correlated with the total plate count (DE GARNICA et al., 2013). This was also shown by our results in which a non-significant strong positive correlation ($r = 0.693$; $P = 0.307$) was observed between total plate count and psychrotrophic bacterial counts evaluated in four BTM samples.

4.7. Lactic acid bacteria in milk and cheese samples

A total of 29 IRM, 4 BTM and 6 ovine cheese samples were tested for Lactic acid bacteria count (LABC). It was tested in only nine individual animal raw milk samples originated from Farm I in 2020 with a mean value of 5.0 ± 0.6 lg CFU/mL. Milk samples from Farm II were not tested. The result of LABC in the IRM samples from Farm III ($n = 10$) and IV ($n = 10$) was the same (3.3 lg CFU/mL). LABC in BTM obtained from Farm III ($n = 3$) and IV ($n = 1$) was 6.7 ± 0.4 and 4.2 lg CFU/mL respectively. A similar mean value of LABC was reported from Brazil in raw ovine milk samples ($n = 10$), which was 6.9 ± 0.8 lg CFU/mL (SILVA et al., 2020).

Lactic acid bacteria count was enumerated for six cheese samples out of 15. The mean of LABC was 7.5 ± 0.1 and 6.5 ± 0.3 lg CFU/g in GFSC and GFC, respectively (Figure 12). There was significant difference ($P < 0.05$) between two types of cheese samples. LABC value in GFC was significantly lower ($P < 0.05$) compared to GFSC.

Higher value of LABC, up to 9.7 lg CFU/g according to the different regions and the medium was reported from Turkey in fresh sheep cheese samples (KIRMACI et al.,

2016). Lactic acid bacteria are used as starter culture, which have a crucial role to play in the process of cheese making and ripening. Apart from this, it plays major role in inhibiting the growth of pathogenic bacteria. Thus, one of the advantages of a higher count of LAB in cheese samples examined in our study is that it can impede the spoilage causing and pathogenic bacterial growth. These can be by producing lactic acid and other antimicrobial metabolites such as hydrogen peroxide, organic acid, etc. Similarly, others also stated that higher LABC in milk and milk products has importance (SANDRA et al., 2013; FRECE et al., 2016).

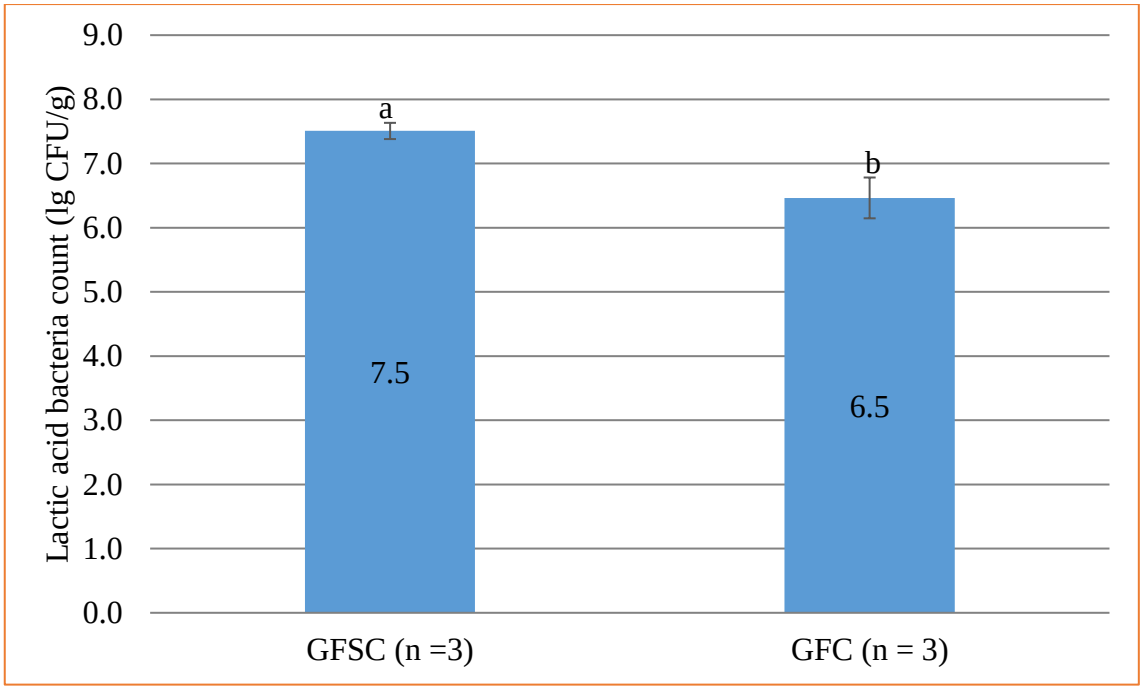

Figure 12: Lactic acid bacteria count in ovine cheese samples (n = 6). GFC: garlic-flavoured cheese; GFSC: garlic-flavoured-smoked cheese.

4.8. Correlation between total plate count and *Enterobacteriaceae* count

The correlation coefficients (r) between major bacterial counts (TPC and EBC) of udder surface and corresponding individual raw milk samples were calculated (Table 24). The r value was interpreted according to the explanation by SCHOBER et al. (2018). In this regard, in our study, weak to moderate positive correlations were observed between the bacterial count pairs evaluated. TPC^{IRM} had a significantly ($P < 0.01$) weak-positive correlation with EBC^{IRM} ($r = 0.295$). The same is true for TPC^{US} and EBC^{US} ($r = 0.323$, $P < 0.01$). As it was expected, EBC^{US} revealed a significantly ($P < 0.01$) moderate-

positive correlation with EBC^{IRM} (r = 0.565). There was also a weak positive correlation between TPC^{US} and EBC^{IRM} (r = 0.271, P < 0.05).

Significantly (P < 0.01) moderate correlation between US and IRM samples for EBC (r = 0.565) indicates that unhygienic farm environments, such as dirty bedding, are prejudicing factors for the occurrence of unwanted microorganisms in unpasteurized ovine milk. So far, no study has assessed the correlation between environmental and milk samples for ovine-associated bacterial parameters. However, the correlation among microbiological parameters of ovine and bovine BTM samples was evaluated in Spain (DE GARNICA et al., 2013) and USA (PANTOJA et al., 2009) respectively.

Table 24: Pearson correlation coefficients (r) between major bacterial counts of udder surface samples (n = 77) and individual raw milk samples (n = 77)

	TPC^{IRM}	TPC^{US}	EBC^{IRM}	EBC^{US}
TPC^{IRM}		0.185	0.295^{**}	0.025
TPC^{US}			0.271^{*}	0.323^{**}
EBC^{IRM}				0.565^{**}
EBC^{US}				

$^{*}P < 0.05$; $^{**}P < 0.01$. TPC: total plate count; EBC: *Enterobacteriaceae* count; ^{IRM}: individual raw milk; ^{US}: udder surface.

4.9. Microbiota and taxonomic profile of five ovine milk samples

To our knowledge, little is known about the ovine milk microbiota so far. Five samples of raw ovine milk obtained from Farm II (n = 3), III (n = 1), and IV (n = 1) were tested for the taxonomic profile of microbiota. One fresh bulk tank milk (FBTM), two cooling tank milk (CTM1 and 2) stored at > 6 ^{o}C for 24 hours in the farms, and two individual raw milk (IRM1 and 2) samples were sampled. As explained in the materials and methods chapter of the dissertation, three out of five raw milk samples were analysed at Eurofins Genomics GmbH, Germany by 16S rRNA gene sequencing by targeting V3 and V4 gene regions. The other two raw milk samples were analysed for microbiota diversity at Biomi Ltd. Company, Hungary by LoopSeq™ 16S-18S Microbiome SSC 24-Plex Kit (Version 1.7, July 2019) method.

Regarding milk microbiota analysis by 16S rRNA gene sequencing that targets V3 and V4 gene regions, with in-house scripts, based on primer sequences, processing of

sequencing reads has been carried out. For further analysis, only read pairs, in which both expected forward and reverse primers were found, were considered. During the identification of primer sequences, mismatches were not allowed. Illumina Miseq produced 305,647 readings of microbiota sequencing, of which 73,357, 103,505, and 128,785 readings belonging to FBTM, CTM1, and IRM1 sample, respectively. Short and low-quality sequences and chimeras were removed and the lineage-specific copy-number correction count was 122, 316. A total of 623 OTUs were produced from the clustering of all sequences.

In the case of LoopSeq^{TM} 16S and 18S microbiome sequencing, the total number of reads produced by short Illumina was 3,505,517, of which 1,552,044 and 1,953,473 reads belonged to the IRM2 and CTM2 respectively. The total number of reads that mapped to a reference database after the removal of overlaps was 11825. In this pilot study, microbiota richness and diversity indexes based on sample types were not calculated due to insufficient sample size. Furthermore, the use of two different analysis methods made comparison impossible.

Four bacterial phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria)* were present in all milk samples analysed for microbiota taxonomic profile. The dominating microbiota at the phylum level was *Proteobacteria* in the three milk samples with a relative abundance of 98.8, 52.5, and 45.0% in IRM2, FBTM, and CTM1 respectively. *Firmicutes* and ‘’others’’ (all taxonomic units with less than 0.0% of reads at phylum level are collapsed in the category) were abundant in the CTM2 (82.4%) and IRM1 (47.5%) sample, respectively (Figure 13). *Bacteroidetes* was the second abundant microbiota in CTM1 (37.1%) and FBTM (29.4%) at the phylum level. However, the milk sample of IRM1 was dominated by *Proteobacteria* phylum (41.1%) next to others.

Firmicutes was another phylum, the third dominant microbiota in FBTM (17.4%) and CTM1 (15.9%) samples whereas *Actinobacteria* (14.8%) in the IRM1 sample (Figure 13). In contrast to our finding, ESTEBAN-BLANCO et al. (2020a) reported that *Firmicutes* (64.4%) was dominating phylum followed by *Actinobacteria* (14.3%) and *Proteobacteria* (9.1%) in the overall individual milk samples of the Assaf breed reported from Spain. In another study, from the same country, the microbiota phylum level was also dominated by *Firmicutes* (50.3%) followed by *Proteobacteria* (25.5%), and *Actinobacteria* (18.9%) in the Churra sheep breed individual milk samples (ESTEBAN-BLANCO et al., 2020b).

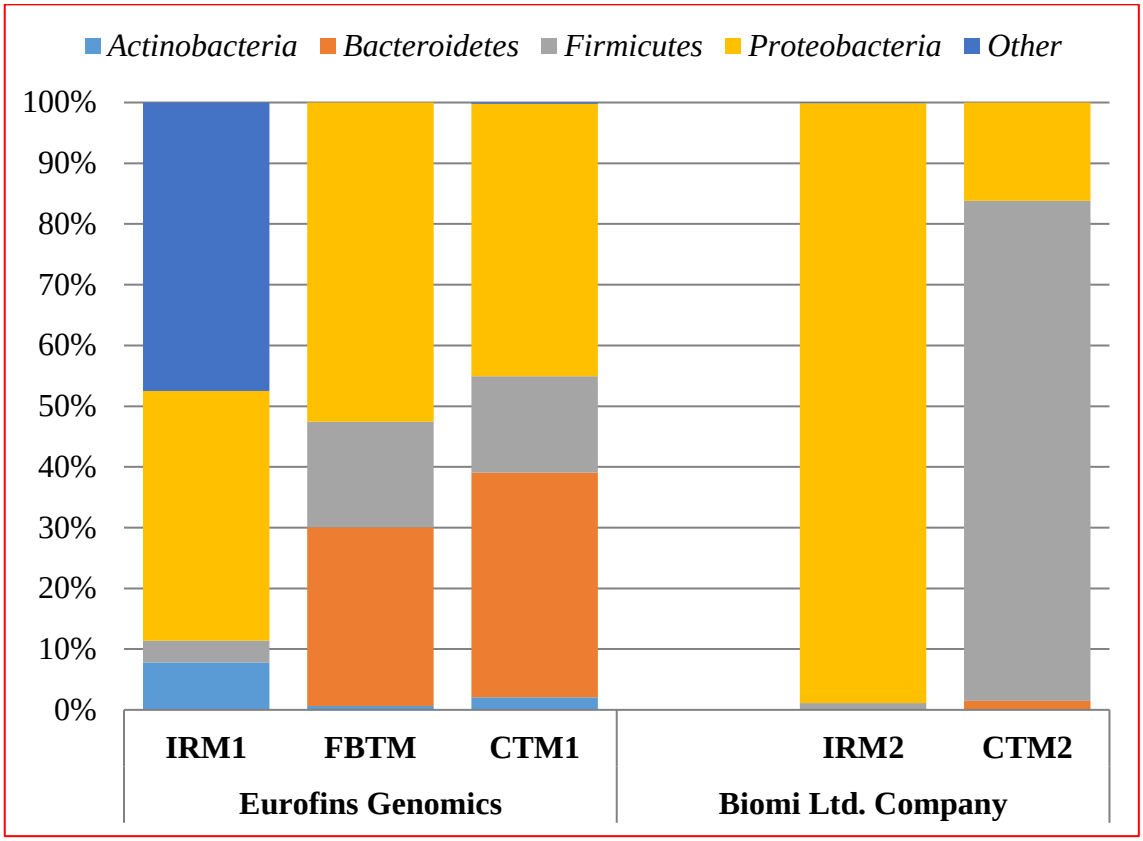

Figure 13: Taxonomic distribution of microbiota community in ovine milk across sample types at the phylum level. IRM1: individual raw milk (Farm II), IRM2: individual raw milk (Farm IV), FBTM: fresh bulk tank milk (Farm II), CTM1: cooling tank milk (Farm II), CTM2: cooling tank milk (Farm III).

At the class level, CTM1 sample of ewe milk microbiota was dominated by *Flavobacteriia* (40.7%), whereas *Gammaproteobacteria* dominated in IRM2 (98.8%), IRM1 (74.9%), and FBTM (46.0%) samples (Figure 14). *Bacilli* was the first and second dominant microbiota in the case of CTM2 (82.3%) and CTM1 (19.4%). Bacilli was third in the case of FBTM (19.1%) and IRM1 (7.3%) samples (Figure 14).

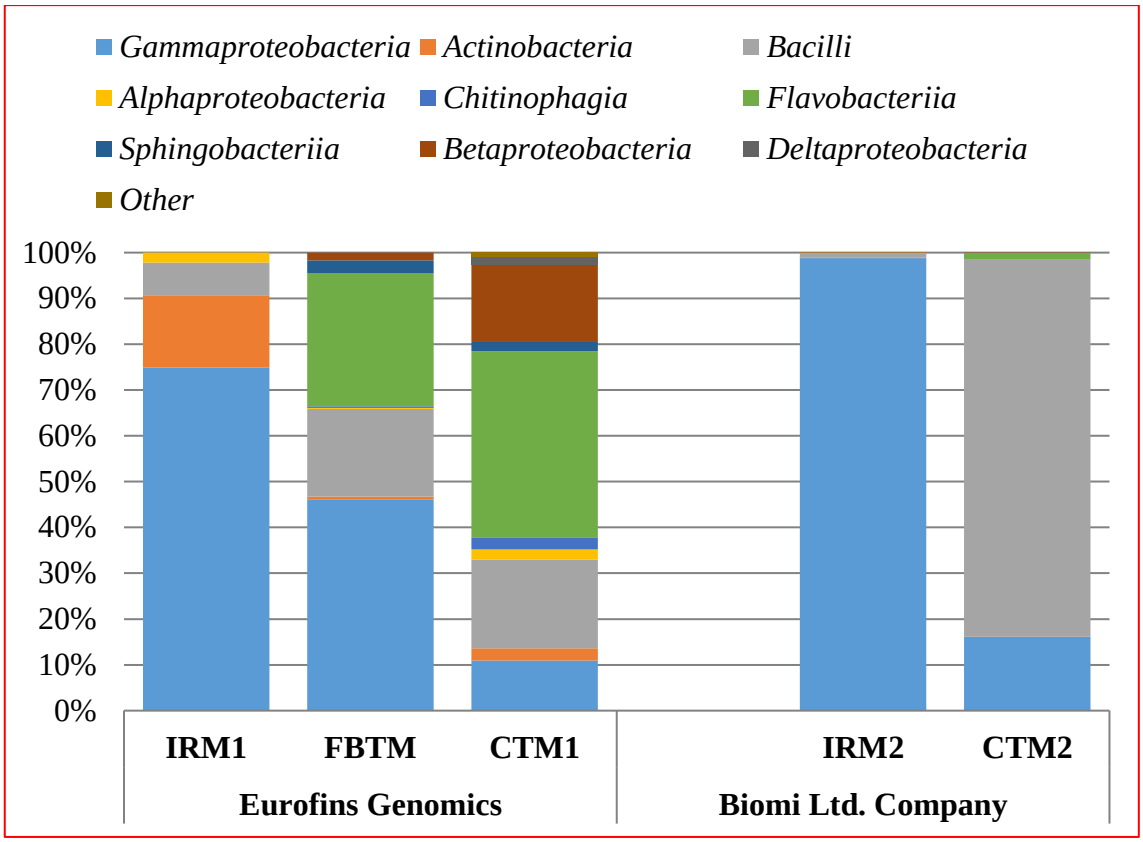

Figure 14: Taxonomic distribution of top nine-microbiota community in ovine milk across sample types at the class level. IRM1: individual raw milk (Farm II), IRM2: individual raw milk (Farm IV), FBTM: fresh bulk tank milk (Farm II), CTM1: cooling tank milk (Farm II), CTM2: cooling tank milk (Farm III). Microbiota taxa with a relative abundance of less than 0.5% were clustered under the ‘‘others’’ label.

At order level, *Flavobacteriales* was the dominant microbiota in FBTM (36.8%) and CTM1 (41.8%) samples whereas *Enterobacterales* (86.7%), *Lactobacillales* (79.4%) and *Campylobacterales* (52.8%) in IRM2, CTM2 and IRM1 sample respectively (Figure 15).

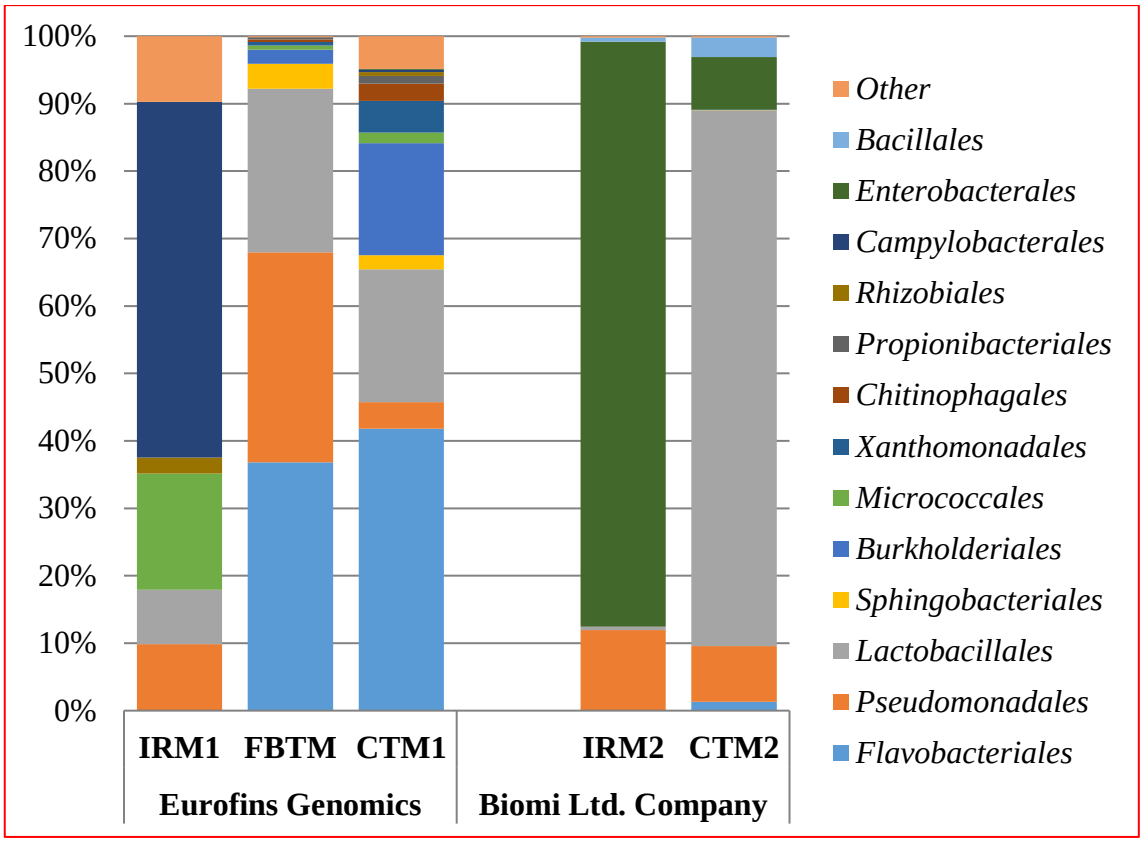

Figure 15: Taxonomic distribution of top thirteen-microbiota community in ovine milk across sample types at the order level. IRM1: individual raw milk (Farm II), IRM2: individual raw milk (Farm IV), FBTM: fresh bulk tank milk (Farm II), CTM1: cooling tank milk (Farm II), CTM2: cooling tank milk (Farm III). Microbiota taxa with a relative abundance of less than 0.5% were clustered under the ‘‘others’’ label.

At the family level, the first and second abundant microbiotas were *Flavobacteriaceae* (38. 9%) and *Pseudomonadaceae* (32.0%), *Flavobacteriaceae* (47.1%) and *Burkholderiaceae* (10.8%), and ‘‘others’’ (75.1%) and *Microbacteriaceae* (9.2%) in the case of FBTM, CTM1 and IRM1 samples respectively (Figure 16). In the case of IRM2 and CTM2, *Pseudomonadaceae* (49.3%) and *Moraxellaceae* (38.1%), and *Streptococcaceae* (71.0%) and *Leuconostacaceae* (10.6%) were first and second abundant at the family level respectively (Figure 16).

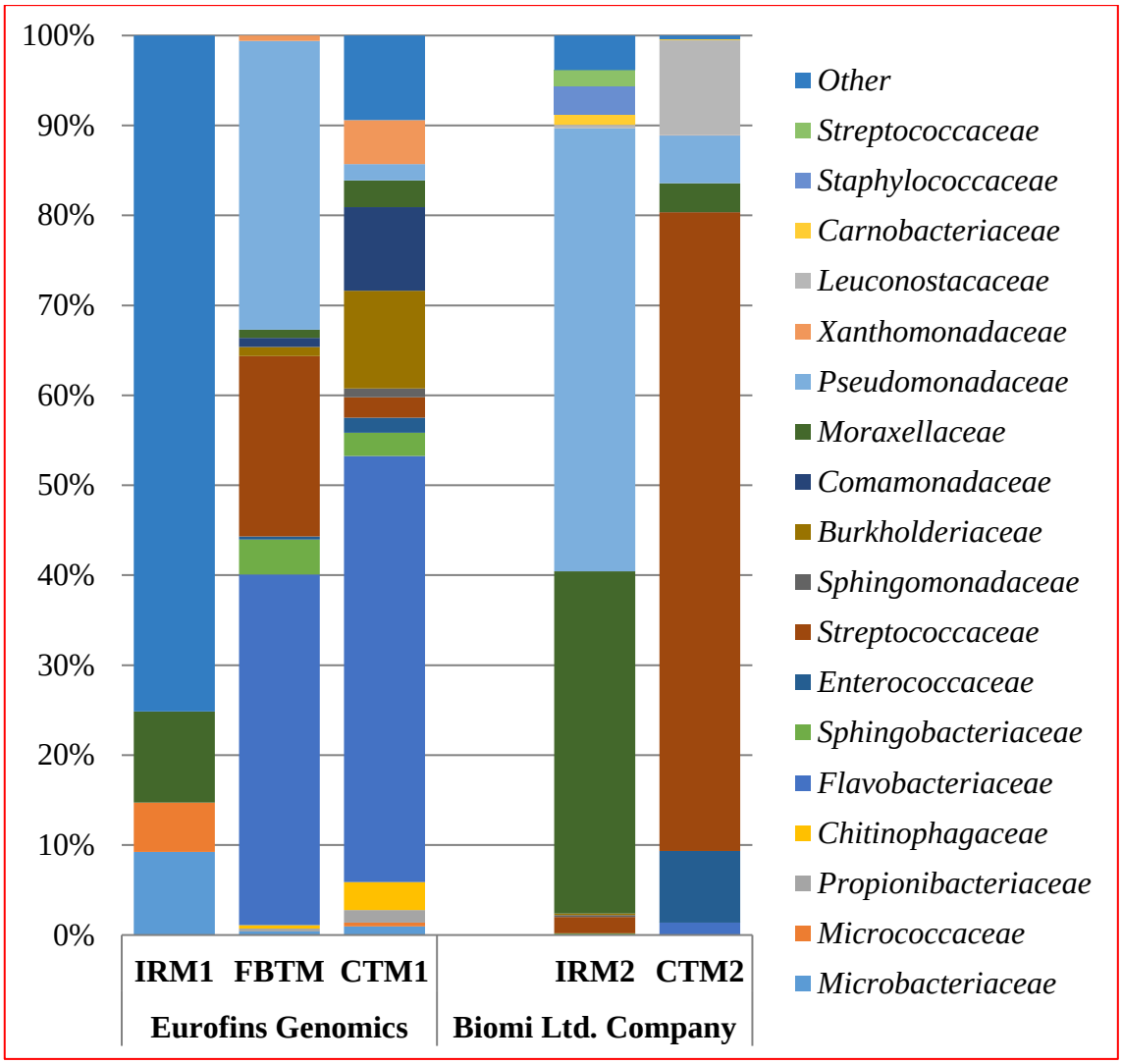

Figure 16: Taxonomic distribution of top eighteen-microbiota community in ovine milk across sample types at the family level. IRM1: individual raw milk (Farm II), IRM2: individual raw milk (Farm IV), FBTM: fresh bulk tank milk (Farm II), CTM1: cooling tank milk (Farm II), CTM2: cooling tank milk (Farm III). Microbiota taxa with a relative abundance of less than 0.5% were clustered under the ‘‘others’’ label.

Taxonomic distribution of top eighteen-microbiota community in ovine milk across sample types at genus level was displayed in Figure 17. According to the analyses, the IRM2 and FBTM samples were dominated by the genera *Pseudomonas*, accounting for 40.8 and 35.5%, followed by *Chryseobacterium* (32.2%) and *Acinetobacter* (31.5%) respectively (Figure 17). *Chryseobacterium* (43.0%), *Ralstonia* (12.8%), and *Ottowia* (9.6%) genera dominated the sample of milk from the cooling tank (CTM1). Next to ‘‘others’’ groups of bacterial genera, *Lonsdalea* (22.5%) was the second abundant genus in IRM1 sample followed by *Acinetobacter* (19.0%) and *Microbacterium* (17.4%). In

opposite to the findings of this study, *Staphylococcus*, *Lactobacillus*, *Cutibacterium*, *Streptococcus*, and *Corynebacterium* genera were the abundant ones in two ovine milk microbiota analysis studies from Spain (ESTEBAN-BLANCO et al., 2020a, 2020b).

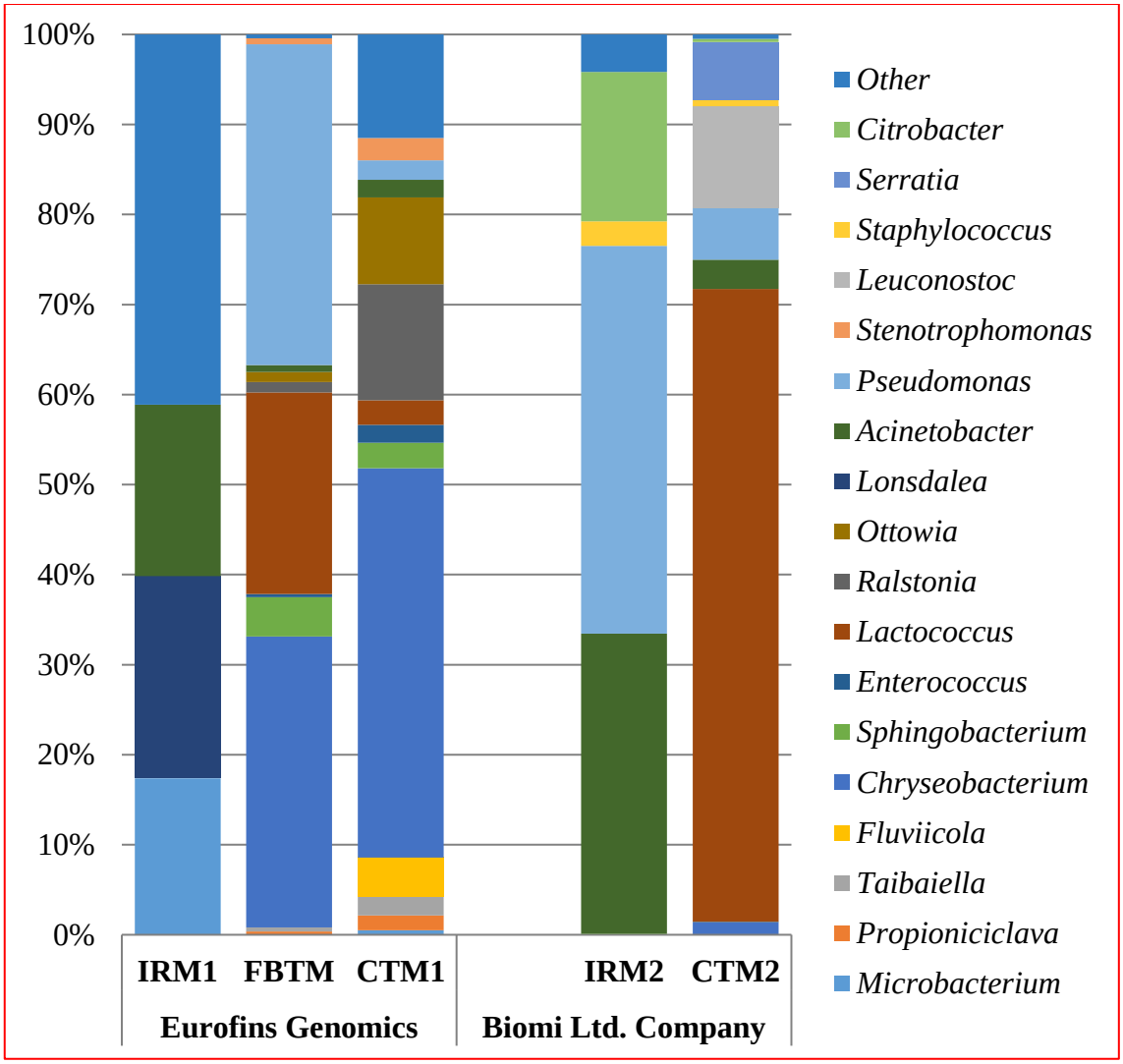

Figure 17: Taxonomic distribution of top eighteen-microbiota community in ovine milk across sample types at the genus level. IRM1: individual raw milk (Farm II), IRM2: individual raw milk (Farm IV), FBTM: fresh bulk tank milk (Farm II), CTM1: cooling tank milk (Farm II), CTM2: cooling tank milk (Farm III). Microbiota taxa with a relative abundance of less than 0.5% were clustered under the ‘‘others’’ label.

4.10. Identification and characterization of *Staphylococcus* strains

4.10.1. Identification of *Staphylococcus aureus*

Coagulase and latex agglutination tests were performed for presumptive 29 *S. aureus* colonies. Accordingly, the coagulase test revealed that all presumably *S. aureus* isolates had positive reactions. The latex agglutination test appropriately distinguished (positive) all *S. aureus* isolates. Furthermore, conformation was done either by API Staph or by MALDI-TOF. Out of 29 *S. aureus* isolates, eight isolates were subjected to API Staph kit (BioMerieux, Marcyl'Étoile, France) for confirmation. The homology values of the eight isolates were between 66.80 and 97.70%. API software suggests that identification with a homology value of > 80 percentage was considered to be high-probability species identification, whereas the results were classified as low-probability when the homology value was < 80% (PARK et al., 2011; KIM et al., 2018).

In this regard, six of the *S. aureus* isolates that were confirmed with the API test had the homology value above the suggested level (Table 25). The result of the API Staph of two *S. aureus* isolates (SAA2 and SAA21), below the recommended level, was approved by coagulase testing. *Staphylococcus chromogenes* (27.9%) was the second-best match for both strains. We used coagulase test to confirm the species in which positive reaction was observed for SAA2 and SAA21.

The other 21 *S. aureus* isolates were confirmed by MALDI-TOF. The MALDI-TOF score values of the best-matched organisms were presented in Table 25. The meaning of the MALDI-TOF score value was defined as suggested by the MALDI-TOF Biotyper software (Appendix 2) and other researchers (TOMAZI et al., 2014; SMITH et al., 2015). Accordingly, out of 21 *S. aureus* isolates, 15 *S. aureus* isolates had MALDI-TOF score values in the range of 2.000-2.299, which was secure genus or probable species identification (Table 25).

The highest score value was 2.385, which was highly probable species identification. Six *S. aureus* isolates had score values between 1.700-1.999 that was probable genus identification. The isolate with the lowest score value was 1.810 (Table 25). Regarding the origin of *S. aureus*, 13 isolates were originated from the samples of Farm III (n = 13/29), followed by Farm I (n = 10/29), OMÉK (n = 4/29), and Farm IV (n = 2/29). *S. aureus* was not detected in any of Farm II samples. 55.2% of *S. aureus* isolates

were recovered from IRM (n = 16), 24.1% from BTM (n = 7), 13.8% from cheese (n = 4) and 6.9% from US (n = 2) (Table 25).

Table 25: *Staphylococcus aureus* strains identification

No.	Origin	Sample type	Analyte ID	Coagulase test	Latex agglutination test	Identification method	Score value or HV (%)
1	FI	US	SAA22	+ve	+ve	MALDI-TOF	2.136
2	FI	US	SAA23	+ve	+ve	MALDI-TOF	2.005
3	FI	IRM	SAA1	+ve	+ve	MALDI-TOF	1.810
4	FI	IRM	SAA2	+ve	+ve	API Staph	66.80*
5	FI	IRM	SAA17	+ve	+ve	API Staph	86.60
6	FI	IRM	SAA18	+ve	+ve	API Staph	86.60
7	FI	IRM	SAA19	+ve	+ve	API Staph	86.60
8	FI	IRM	SAA20	+ve	+ve	API Staph	86.60
9	FI	IRM	SAA21	+ve	+ve	API Staph	66.80*
10	FI	IRM	SAA24	+ve	+ve	MALDI-TOF	2.018
11	FIII	IRM	SAA7	+ve	+ve	MALDI-TOF	1.814
12	FIII	IRM	SAA8	+ve	+ve	MALDI-TOF	2.049
13	FIII	IRM	SAA9	+ve	+ve	MALDI-TOF	2.245
14	FIII	IRM	SAA10	+ve	+ve	MALDI-TOF	2.132
15	FIII	IRM	SAA26	+ve	+ve	MALDI-TOF	2.244
16	FIII	IRM	SAA27	+ve	+ve	API Staph	97.70
17	FIII	BTM	SAA3	+ve	+ve	MALDI-TOF	2.385
18	FIII	BTM	SAA4	+ve	+ve	MALDI-TOF	2.240

No.	Origin	Sample type	Analyte ID	Coagulase test	Latex agglutination test	Identification method	Score value or HV (%)
19	FIII	BTM	SAA5	+ve	+ve	MALDI-TOF	2.186
20	FIII	BTM	SAA6	+ve	+ve	MALDI-TOF	2.074
21	FIII	BTM	SAA13	+ve	+ve	MALDI-TOF	2.214
22	FIII	BTM	SAA14	+ve	+ve	MALDI-TOF	1.846
23	FIII	BTM	SAA25	+ve	+ve	MALDI-TOF	2.123
24	FIV	IRM	SAA11	+ve	+ve	MALDI-TOF	1.893
25	FIV	IRM	SAA12	+ve	+ve	MALDI-TOF	1.919
26	OMÉK	Cheese	SAA28	+ve	+ve	MALDI-TOF	2.050
27	OMÉK	Cheese	SAA29	+ve	+ve	MALDI-TOF	2.011
28	OMÉK	Cheese	SAA15	+ve	+ve	API Staph	86.70
29	OMÉK	Cheese	SAA16	+ve	+ve	MALDI-TOF	1.857

FI: Farm I; FIII: Farm III; FIV: Farm IV; US: udder surface; IRM: individual raw milk; BTM: bulk tank milk; HV: homology value; OMÉK: 79th National Agriculture and Food Exhibition and Fair held in Budapest, Hungary, in September 2019. MALDI-TOF identification with a score value of ≥ 2.300 is considered to be high-probability species identification, whereas the results were classified as secure and uncertain genus level when it is 2.00-2.299 and 1.700-1.999 respectively. When a score value is below 1.700, the identification is considered as not reliable (TOMAZI et al., 2014; SMITH et al., 2015). *According to the API software suggestion, *S. aureus* was differentiated from the second-best match (*S. chromogenes*: 27.9% HV) for SAA2 and SAA21 by coagulase testing.

4.10.2. Identification of coagulase-negative staphylococci isolates

Based on the latex agglutination test result, 42 presumably CNS colonies that had negative reactions were considered for identification. The identification was done either by API Staph and/or by MALDI-TOF and/or molecular method. Out of 42 presumably CNS isolates, 13 suspected CNS isolates were subjected to API-Staph kit (BioMerieux, Marcyl'Étoile, France) for identification. Out of 13-suspected CNS isolates, 12 were identified to genus and species levels with the homology value between 44.8 and 99.9%. However, identifications with a homology value of > 80 percent were considered to be high-probability species identification (PARK et al., 2011; KIM et al., 2018).

On this basis, only 30.8% (n = 4) of the identifications resulted in a high-probability identification with a homology value greater than 80 percent (Table 26). Based on our result, the API Staph method had limited ability to provide reliable results for suspected CNS isolates. This might be because of phenotypic dissimilarities among staphylococcal strains of the same species. Similarly, KOOKEN et al. (2014) have experienced that phenotypic identification methods do not provide reliable results, particularly for non-*aureus* staphylococci isolates.

Among the remaining 29 presumably CNS colonies, MALDI-TOF was employed to identify 14 colonies. In addition, seven probably CNS colonies identified with API Staph that have a dubious identification profile were also subjected to MALDI-TOF for sake of clarity. In total, the identification of 21 (14 + 7) presumably CNS colonies was carried out by MALDI-TOF. The meaning of the MALDI-TOF score value was defined as suggested by the MALDI-TOF Biotyper software (Appendix 2) and other researchers (TOMAZI et al., 2014; SMITH et al., 2015). Out of 21 presumably CNS isolates, unexpectedly, only six (28.6%) were identified to at least uncertainly genus level by MALDI-TOF. The remaining 15 (71.4%) were identified as other bacterial groups or to non-reliable identification. Similar to our finding, CAMERON et al. (2018) had the same experience that 40 bovine-associated CNS isolates were not identified at the species level after the first round of duplicated MALDI-TOF.

This was might be due to some bacteria that could not be detected by MALDI-TOF MS owing to the limited bacterial database as explained by others (BARREIRO et al., 2017; CAMERON et al., 2017). Moreover, another study revealed that the variability of CNS identification was due to the conditions of the bacterial growth, sample preparation,

number of reference strains, and version of the software Biotyper (TOMAZI et al., 2014). In the same way, the insufficient number (TOMAZI et al., 2014) and the origin (WANECKA et al., 2019) of spectra incorporated into the reference databases affect the MALDI-TOF result.

The remaining 15 probably CNS isolates were subjected to the 16S rRNA gene sequencing method for identification. 16S rRNA gene-based sequencing to genus and species levels identified all 15 isolates. Of these 15 identified strains, 8 (53.3%) were identified as staphylococci, whereas 7 (46.7%) were diagnosed to be another bacterial group. These identifications were similar to those reported by ZADOKS et al. (2014) from the United Kingdom, in which *Staphylococcus* species were identified by 16S gene sequencing in clinically healthy 219 udder halves ovine milk samples.

Among the 42 probably CNS colonies subjected to either of three identification methods, 15 colonies were originated from Farm I followed by Farm IV (n = 10), OMÉK (n = 10), Farm III (n = 5), and Farm II (n = 2). For sample types, 16 probably CNS colonies were recovered from IRM, 15 from the US, 1 from BTM, and 10 from cheese samples. In comparison to ovine milk-originated CNS colonies, udder surface originated colonies had less percentage of reliable identification. Out of 15 CNS colonies, 3 were identified as *Staphylococcus* genus. This could be explained as these unidentified bacteria from US have not been previously included in the database or might have developed an extra protein layer as protection against unfavourable environmental conditions. Similarly, MAHMMOD et al. (2018) reported that the majority of unidentified CNS isolates were from cow teat skin. Reliable identification was not observed in any of the cheese-originated isolates but six colonies were identified to uncertain *Staphylococcus* genus level.

The overall identification results indicate that identification using the molecular method is necessary especially for those suspected CNS isolates of US samples. Our results revealed that the overall number of CNS isolates identified either by API Staph and/or by MALDI-TOF and/or molecular method were 18. However, two isolates with doubtful identification were not considered for further characterization. Thus, a coagulase test was performed for 16 identified CNS colonies. The coagulase test revealed that all identified CNS isolates had negative reactions (Table 26). This finding revealed that the overall number of CNS diagnosed were 6 (37.5) *S. simulans*, 3 (18.7%) *S. auricularis*, 3

(18.7%) *S. auricularis,* 2 (12.5%) *S. caprae*, 1 (6.3%) *S. haemolytic us*, and 1 (6.3%) *S. xylosus* (Table 26).

Table 26: Identified coagulase-negative staphylococci strains

No.	Origin	Sample type	Analyte ID	Coagulase test	Latex agglutination test	Organisms	Identification method	Score value or HV (%)
1	FI	US	CNS4	-ve	-ve	*S. equorum*	16S rRNA gene sequencing	100.00
2	FI	US	CNS8	-ve	-ve	*S. equorum*	16S rRNA gene sequencing	99.85
3	FI	IRM	CNS1	-ve	-ve	*S. auricularis*	16S rRNA gene sequencing	99.85
4	FI	IRM	CNS2	-ve	-ve	*S. equorum*	16S rRNA gene sequencing	100.00
5	FI	IRM	CNS3	-ve	-ve	*S. auricularis*	16S rRNA gene sequencing	99.85
6	FI	IRM	CNS5	-ve	-ve	*S. simulans*	16S rRNA gene sequencing	99.07
7	FI	IRM	CNS6	-ve	-ve	*S. simulans*	16S rRNA gene sequencing	99.07
8	FI	IRM	CNS7	-ve	-ve	*S. simulans*	16S rRNA gene sequencing	99.07
9	FII	IRM	CNS15	-ve	-ve	*S. xylosus*	API Staph	99.90
10	FII	IRM	CNS16	-ve	-ve	*S. simulans*	API Staph	99.20
11	FIII	IRM	CNS10	-ve	-ve	*S. simulans*	MALDI-TOF	1.820
12	FIII	IRM	CNS13	-ve	-ve	*S. caprae*	API Staph	82.10
13	FIII	IRM	CNS14	-ve	-ve	*S. caprae*	API Staph	97.40
14	FIII	BTM	CNS9	-ve	-ve	*S. haemolyticus*	MALDI-TOF	1.799
15	FIV	US	CNS12	-ve	-ve	*S. auricularis*	MALDI-TOF	1.713
16	FIV	IRM	CNS11	-ve	-ve	*S. simulans*	MALDI-TOF	2.093

FI: Farm I; FII: Farm II; FIII: Farm III; FIV: Farm IV; US: udder surface; IRM: individual raw milk; BTM: bulk tank milk; HV: homology value. MALDI-TOF identification with a score value of ≥ 2.300 is considered to be high-probability species identification, whereas the results were classified as secure and uncertain genus level when it is 2.00-2.299 and 1.700-1.999 respectively. When a score value is below 1.700, the identification is considered as not reliable (TOMAZI et al., 2014; SMITH et al., 2015).

4.10.3. Characteristics of *Staphylococcus aureus* strains

Out of 45 staphylococci isolates considered for characterization, 64.4% (n = 29) were *S. aureus*. All *S. aureus* had positive reactions for coagulase and latex agglutination test. Out of 29 *S. aureus* isolates, 72.4% (n = 21) and 27.6% (n = 8) of *S. aureus* strains were confirmed by MALDI-TOF and API Staph for identification, respectively (Table 27). Table 27 indicates that most of the *S. aureus* isolates were detected in samples from Farm III (n = 13/29) and Farm I (n = 10/29). In this study, more than 55% (n = 16) of the *S. aureus* isolates were from IRM (Table 27). Of the remaining 13 *S. aureus* strains, 24.1, 13.8, and 6.9% were originated from ovine BTM, cheese, and US samples respectively (Table 27).

All *S. aureus* isolates tested for hemolysis were able to produce either both α and β or one among the two-hemolysis types. In twenty *S. aureus* strains, α and β hemolysis types were shared, whereas six strains produced only β type and three strains produced α type of the hemolysis (Table 27). Twenty-seven *S. aureus* strains were black in which three were dark-black in the case of tellurite reduction. Two grey strains were also observed (Table 27). Positive lecithinase activity/formation of the zone, by the *S. aureus* colony, was detected in only five strains out of 29 strains (Table 27).

Table 27: Characteristics of *Staphylococcus aureus* isolates from ovine-associated samples

Origin	Type of sample	Strains ID	Tellurite reduction	Lecithinase activity	Hemolysis	Antibiotic test	Catalase test	Oxidase test	Enterotoxins
FI	US	SAA22	black	-ve	α-β	I (E)	+ve	-ve	SEC
FI	US	SAA23	black	-ve	α-β	S	+ve	-ve	SEC
FI	IRM	SAA1	black	-ve	α-β	S	+ve	-ve	SEC
FI	IRM	SAA2	grey	+ve	α-β	S	+ve	-ve	SEC
FI	IRM	SAA17	black	+ve	α-β	S	+ve	-ve	SEB, SEG, SEI
FI	IRM	SAA18	black	-ve	α-β	S	+ve	-ve	SEB
FI	IRM	SAA19	black	-ve	α-β	S	+ve	-ve	SEB
FI	IRM	SAA20	black	-ve	α-β	S	+ve	-ve	SEB
FI	IRM	SAA21	dark-black	-ve	α-β	S	+ve	-ve	SEB
FI	IRM	SAA24	dark-black	-ve	α-β	S	+ve	-ve	SEC
FIII	IRM	SAA7	black	-ve	α-β	I (E)	+ve	-ve	SEC
FIII	IRM	SAA8	black	-ve	α-β	S	+ve	-ve	SEC
FIII	IRM	SAA9	grey	-ve	α-β	S	+ve	-ve	SEC
FIII	IRM	SAA10	black	-ve	α-β	S	+ve	-ve	SEC
FIII	IRM	SAA26	black	-ve	weak β	R (TE)	+ve	-ve	SEC
FIII	IRM	SAA27	black	-ve	α-β	S	+ve	-ve	SEC
FIII	BTM	SAA3	black	-ve	α	I (DA), R (TE)	+ve	-ve	SEC

Origin	Type of sample	Strains ID	Tellurite reduction	Lecithinase activity	Hemolysis	Antibiotic test	Catalase test	Oxidase test	Enterotoxins
FIII	BTM	SAA4	black	-ve	α-β	R (TE)	+ve	-ve	SEC
FIII	BTM	SAA5	black	+ve	α-β	R (TE)	+ve	-ve	SEC
FIII	BTM	SAA6	black	+ve	α-β	R (TE)	+ve	-ve	SEB, SEI
FIII	BTM	SAA13	black	-ve	α	S	+ve	-ve	-
FIII	BTM	SAA14	black	-ve	α	S	+ve	-ve	-
FIII	BTM	SAA25	black	-ve	weak β	R (TE)	+ve	-ve	SEC
FIV	IRM	SAA11	black	-ve	α-β	S	+ve	-ve	SEC
FIV	IRM	SAA12	black	-ve	α-β	S	+ve	-ve	SEC
OMÉK	Cheese	SAA15	dark-black	+ve	β	I (E)	+ve	-ve	-
OMÉK	Cheese	SAA16	black	-ve	β	S	+ve	-ve	SEG, SEI
OMÉK	Cheese	SAA28	black	-ve	β	S	+ve	-ve	-
OMÉK	Cheese	SAA29	black	-ve	β	S	+ve	-ve	SEG, SEI

FI: Farm I; FIII: Farm III; FIV: Farm IV; US: udder surface; IRM: individual raw milk; BTM: bulk tank milk; OMÉK: 79th National Agriculture and Food Exhibition and Fair held in Budapest, Hungary, in September 2019; S: susceptible; I: intermediate; R: resistant; DA: clindamycin; E: erythromycin; TE: tetracycline.

4.10.4. Characteristics of coagulase-negative staphylococci strains

Out of 45 staphylococci isolates considered for characterization, 35.6% (n = 16) were coagulase-negative staphylococci. Out of 16 coagulase-negative staphylococci strains considered for characterization, 50% (n = 8), 25% (n = 4), and 25% (n = 4), were originated from strains identified by 16S rRNA gene sequencing, API Staph, and MALDI-TOF MS Biotyper, respectively (Table 28). From the 16 CNS isolates, 37.5% *S. simulans* (n = 6), followed by *S. auricularis* (n = 3) and *S. equorum* (n = 3) were identified. Furthermore, *S. caprae* (n = 2), *S. haemolyticus* (n = 1), and *S. xylosus* (n = 1) were also identified (Table 28). VASILEIOU et al. (2018) from Greece also reported *S. simulans* and *S. haemolyticus* were among the major frequent CNS species in ovine milk. Also, another study by MARTINS et al. (2017) from Brazil reported that a higher number of these two CNS species were identified in both subclinical and healthy ovine milk.

Most of the CNS isolates were diagnosed in samples from Farm I (n = 8/16) and Farm III (n = 4/16), followed by Farm II (n = 2) and Farm IV (n = 2). Twelve (75%) of the coagulase-negative staphylococci strains were originally from IRM samples (Table 28). The remaining two strains were originated from US samples and one isolate was from the BTM sample. Out of 16 CNS isolates tested for hemolysis, eight were able to produce weak α or β or γ hemolysis types. In five CNS strains weak α hemolysis was detected, whereas two strains produced a very weak β type and one strains produced weak γ type of the hemolysis (Table 28). Nine CNS strains were black in which one was black-yellow in the case of tellurite reduction. Four grey and two white CNS strains were also detected (Table 28). In all CNS strains, lecithinase activity was negative (Table 28).

Table 28: Characteristics of coagulase-negative staphylococcal isolates from ovine-associated samples

Farm	Origin of isolates	Strains ID	Organisms	Tellurite reduction	Lecithinase activity	Hemolysis	Antibiotic test	Catalase test	Oxidase test	Enterotoxins
FI	US	CNS4	*S. equorum*	black-yellow	-ve	weak α	I (DA, E)	+ve	-ve	-
FI	US	CNS8	*S. equorum*	black	-ve	-	S	+ve	-ve	-
FI	IRM	CNS1	*S. auricularis*	black	-ve	weak γ	R (SXT)	+ve	-ve	SEG, SEI
FI	IRM	CNS2	*S. equorum*	black	-ve	weak α	I (E)	+ve	-ve	-
FI	IRM	CNS3	*S. auricularis*	grey-whitish	-ve	-	R (SXT)	+ve	-ve	-
FI	IRM	CNS5	*S. simulans*	white	-ve	-	S	+ve	-ve	-
FI	IRM	CNS6	*S. simulans*	white	-ve	-	S	+ve	-ve	-
FI	IRM	CNS7	*S. simulans*	grey	-ve	-	S	+ve	-ve	-
FII	IRM	CNS15	*S. xylosus*	black	-ve	weak α	S	+ve	-ve	-
FII	IRM	CNS16	*S. simulans*	black	-ve	weak α	S	+ve	-ve	-
FIII	IRM	CNS10	*S. simulans*	grey	-ve	-	I (SXT)	+ve	-ve	-
FIII	IRM	CNS13	*S. caprae*	black	-ve	very weak β	R (P, TE)	+ve	-ve	-
FIII	IRM	CNS14	*S. caprae*	black	-ve	very weak β	R (P, E)	+ve	-ve	-
FIII	BTM	CNS9	*S. haemolyticus*	black	-ve	-	I (DA), R (P, TE)	+ve	-ve	-
FIV	US	CNS12	*S. auricularis*	black	-ve	weak α	I (SXT)	+ve	-ve	-
FIV	IRM	CNS11	*S. simulans*	grey	-ve	-	I (SXT)	+ve	-ve	-

FI: Farm I; FII: Farm II; FIII: Farm III; FIV: Farm IV; US: udder surface; IRM: individual raw milk; BTM: bulk tank milk; S: susceptible; I: intermediate; R: resistant; P: penicillin G; DA: clindamycin; E: erythromycin; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

4.10.5. Antibiotic resistance of staphylococcal strains

Twenty-nine *S. aureus* and 16 CNS strains were evaluated for ABR against eight antibiotics (cefoxitin, chloramphenicol, clindamycin, erythromycin, gentamicin, penicillin G, tetracycline, and trimethoprim/sulfamethoxazole). All 45 isolates were susceptible to chloramphenicol and cefoxitin. As in this study, in Iran, all staphylococci from caprine and ovine milk were susceptible to chloramphenicol (RAHMDEL et al., 2018). In our study, all *S. aureus* and 13 CNS isolates were susceptible to penicillin G. 20.7 and 31.2% of *S. aureus* and CNS isolates were resistant against at least one of the antibiotics respectively. This in line with the finding by VASILEIOU et al. (2019) from Greece that indicated 18.5 and 47.0% of *S. aureus* and CNS isolates from ovine milk were resistant to at least one of the exposed antibiotics, respectively.

Overall, eleven isolates (24.4%) were resistant to at least one of the tested antibiotics (Figure 18). Similarly, in Greece, 41.5% of ovine milk isolates were found to be resistant to at least one of the antibiotics assayed (VASILEIOU et al., 2019). However, higher ABR (82.9%) of staphylococcal isolates in ovine BTM was reported from Spain (POVEDA et al., 2020). In the present study, isolates were resistant to tetracycline (17.8%), trimethoprim/sulfamethoxazole (11.1%), penicillin G (6.7%), and erythromycin (2.2%). In all the resistant isolates (n = 11/45), multidrug-resistance was not detected. Contrastingly, VASILEIOU et al. (2019) reported that 13.6% of resistant staphylococcal isolates were multidrug-resistant. Studies proved that *S. aureus* is intrinsically resistant to vancomycin (VESTERGAARD et al., 2016). In this study, vancomycin was not tested in the case of staphylococcal strains. In this regard, intrinsic resistancy can not affect the overall result of staphylococcal strains resistancy.

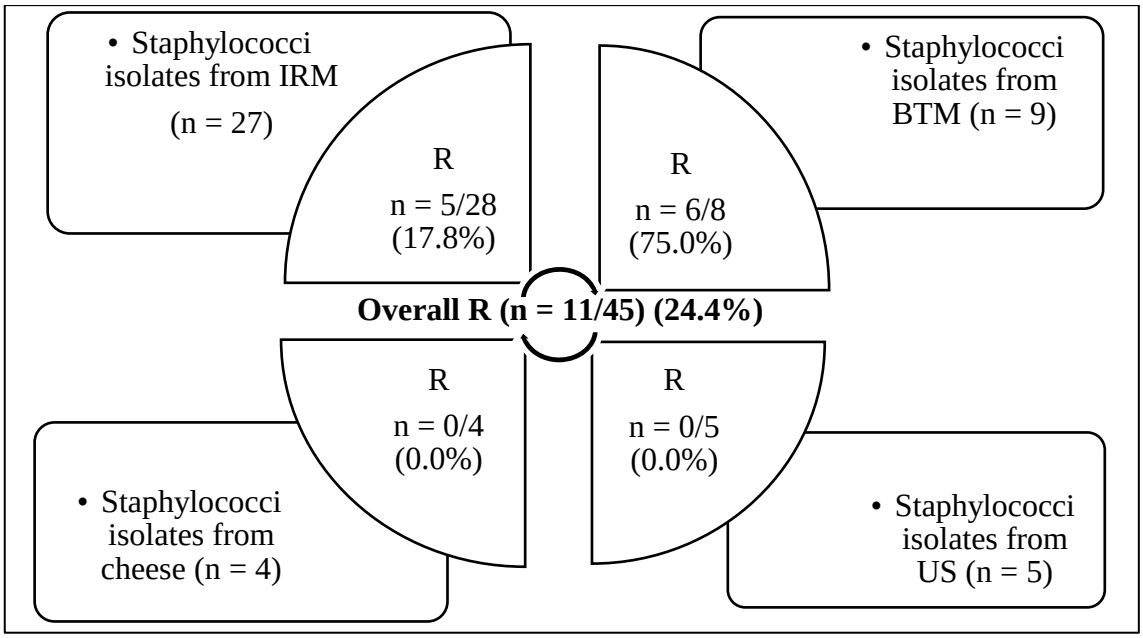

Figure 18: Number and percentage of antibiotic resistance of staphylococci isolate recovered from individual milk (IRM), bulk tank milk (BTM), cheese, and udder surface (US) of ewe. N: number of isolates; R: resistant isolates to at least one antibiotic.

4.10.5.1. Antibiotic resistance of *Staphylococcus aureus* strains

In our study, all *S. aureus* strains were sensitive to five of eight antibiotics tested. Out of 29 *S. aureus* isolates, 6 (20.7%) were resistant to tetracycline, whereas three and one *S. aureus* strains were intermediately resistant to erythromycin and clindamycin respectively (Table 27). UNAL et al. (2012) from Turkey reported a lower percentage of resistance by *S. aureus* isolates 8% to tetracycline (8%). The resistant *S. aureus* isolates were originated from Farm III. Five of them were from BTM samples and one was from the IRM sample. In all the resistant *S. aureus* isolates (n = 6/29), multidrug-resistance was not detected. Contrastingly, VASILEIOU et al. (2019) reported that 13.6% of resistant staphylococcal isolates were multidrug-resistant. *S. aureus* isolates were tested for methicillin resistance using cefoxitin according to the criteria of CLSI (2017) and none of the isolates was found to be resistant.

4.10.5.2. Antibiotic resistance of coagulase-negative staphylococci strains

In the present study, out of 16 CNS strains tested for antibiotic resistance against eight antibiotics, in five (31.2%) of CNS strains, at least one antibiotic resistance was detected (Table 28). Coagulase-negative staphylococci isolates were resistant to penicillin G (18.7%), tetracycline (12.5%), trimethoprim/sulfamethoxazole (12.5%) and erythromycin (6.2%) (Table 28). MARTINS et al. (2017) also reported a similar percentage of resistance by CNS species to a penicillin (17%) and tetracycline (10.7%). Out of five resistant CNS isolates, three were resistant to two antibiotics. In addition, the finding of MARTINS et al. (2017) indicated that seven (6.25%) isolates of the resistant isolates were resistant to more than one antimicrobial agent. Among the resistant CNS isolates, in our study, three were originated from Farm III and two were from Farm I. Four of them were from IRM samples and one was from BTM sample. Intermediately resistant/susceptible to antibiotic was detected in six CNS isolates. All CNS isolates were susceptible to cefoxitin, gentamicin, and chloramphenicol. In sharp contrast to the above findings, FRANÇA et al. (2012) determined a higher percentage of *Staphylococcus* spp. resistance to tetracycline (40.5%), followed by erythromycin (33.8%) from mastitic ovine milk.

4.10.5.3. Comparison of the frequency of antibiotic resistance in staphylococci strains between farms, origin, and identity

In this study, the difference in frequency of antibiotic resistance between farms (Farm I, II, III, and IV), sample types (US, IRM, BTM, and cheese), and identity of isolates (*S. aureus* and CNS) was compared. In this regard, in our study, the frequency of resistance was significantly different ($\chi^2 = 10.253$; $P = 0.017$) between farms (Table 29). Out of 11 resistant staphylococcal strains, 9 (81.82%) were originated from Farm III. These nine isolates were resistant to penicillin G and/or tetracycline antibiotics. Although the farm manager was not willing to tell the type of antibiotic used in the above-mentioned farm, the animals could be treated with these two antibiotics. This partly explains the high frequency of resistance strains recovered from Farm III.

In addition, there was a significant difference ($\chi^2 = 8.696$; $P = 0.034$) in the frequency of resistance between the origin of isolates (udder surface, individual raw milk, bulk tank milk, and cheese) (Table 29). None of the udder surface and cheese sample

strains was resistant to antibiotics tested. However, no significant difference ($\chi 2 = 1.853$; P = 0.173) was observed between *S. aureus* and CNS isolates for the frequency of resistance. The number of resistant isolates seemed to be equal in *S. aureus* and CNS isolates. This was in disagreement with the finding of MARTINS et al. (2017) from Brazil, where ABR was more common in CNS species than *S. aureus*. In addition, the report of POVEDA et al. (2020) from Spain showed that the frequency of resistance was varied between *S. aureus* and CNS isolates (P = 0.007).

Table 29: Frequency of resistant and susceptible isolates to antibiotic according to the farm, origin and identity of isolates

	Frequency of antimicrobial resistance and susceptibility of isolates n (%)*		
Farms	Resistant isolates (n = 11)	Susceptible isolates (n = 23)	χ^2 test
Farm I	2 (18.18)	13 (56.52)	
Farm II	0 (0.00)	2 (8.69)	$\chi^2 = 10.253$
Farm III	9 (81.82)	6 (26.09)	P = 0.017
Farm IV	0 (0.00)	2 (8.69)	
Origin of isolates	Resistant isolates (n = 11)	Susceptible isolates (n = 26)	
Udder surface	0 (0.00)	2 (7.69)	
Individual raw milk	5 (45.45)	19 (73.07)	$\chi^2 = 8.696$
Bulk tank milk	6 (54.54)	2 (7.69)	P = 0.034
Cheese	0 (0.00)	3 (11.54)	
Identity of isolates	Resistant isolates (n = 11)	Susceptible isolates (n = 26)	
S. aureus	6 (54.54)	20 (76.92)	$\chi^2 = 1.853$
Coagulase-negative staphylococci	5 (45.45)	6 (23.08)	P = 0.173

*intermediately resistant/susceptible isolates were not included in the table, but two isolates counted as resistant, which are resistant to one antibiotic and intermediate to another.

4.10.6. Enterotoxins profile of staphylococcal strains

In this study, forty-five staphylococcal strains were tested for 13 staphylococcal enterotoxins (Table 17) producing ability. Five classical SEs (SEA, SEB, SEC, SED and SEE), five new forms of SEs (SEG, SEH, SEI, SEJ, and SER,) and three SE*l*s (SE*l*M, SE*l*N, and SE*l*O) were tested. Out of 45 strains, 26 (57.8%) were able to produce at least one of the 13 tested enterotoxins (Table 28, 29). Among the enterotoxins detected, SEC was the most prevalent, being detected in 17 (37.8%) strains. However, the most frequently detected enterotoxin gene was the *sea*, in 56 ovine-associated strains in Brazil as reported by (MARTINS et al., 2017). The enterotoxin SEB present in its isolated form or concomitantly with other enterotoxins in six (13.3%) strains. Similarly, MARTINS et al. (2017) detected *seb* gene in 19 isolates from ovine milk as the second most prevalent.

SEI (n = 5) and SEG (n = 4) were the third and fourth most found enterotoxins. Concerning the SE*l*s tested by PCR, none of them was detected in our strains. In the case of farms, higher occurrence of enterotoxins was observed in strains originated from Farm I (n = 11/17) and III (n = 11/18), followed by Farm IV (n = 2/4) and Farm 2 (n = 0/2). With regard to sample types (US, IRM, BTM and cheese), enterotoxins detected more frequently in IRM (n = 17) followed by BTM (n = 5) and cheese (n = 2) and udder surface (n = 2) samples. The enterotoxins were found in only one CNS strain out of 16 (Table 28, 29).

Milk and milk products provide a suitable environment for staphylococci growth and staphylococcal enterotoxins (SEs) production (CRETENET et al., 2011; HENNEKINNE et al., 2012; KADARIYA et al., 2014; SABIKE et al., 2014). Consuming staphylococcal enterotoxins contaminated food results in foodborne intoxication, which is a worldwide public health issue (HENNEKINNE et al., 2012; LINAGE et al., 2012; PAINTER et al., 2013; PEXARA et al., 2016). The SEs are heat resistant, they can survive at high temperatures, which makes them responsible for food poising (ARGUDÍN et al., 2010; MARTINS et al., 2017).

4.11. Identification and characterization of lactic acid bacteria strains

4.11.1. MALDI-TOF MS identification of lactic acid bacteria strains

A total of 35 LAB isolates were submitted for identification by MALDI-TOF. MALDI-TOF score value was defined as suggested by the MALDI-TOF Biotyper software (Appendix 2) and other researchers (TOMAZI et al., 2014; SMITH et al., 2015). Of the 35 LAB isolates, 16 (45.7%) were identified to at least to uncertain genera level, while the remaining 19 (54.3%) were identified as other bacterial groups or with a score value below 1.700. Four LAB isolates had the MALDI score value of > 2.00 with a maximum value of 2.251. Seven LAB isolates had score values between 1.700-1.999 (Table 30). The LAB isolate with the minimum score value was 1.734 (Table 30).

All the identified isolates (16) did not belong to the genera comprising the LAB, but only 11 (68.8%) of the identifications were in the genera that consisting of the LAB. The other five (33.2%) strains were in the genera other than that of the LAB. Of the 11 isolates, which were identified as genera and species belonging to the LAB, 63.6% were isolated from cheese (n = 7), 18.2% from IRM (n = 2) and BTM (n = 2) (Table 30). The wrong/unacceptable identification of nineteen LAB isolates was might be due to the problems encountered during the preparation of the sample, the growth status of the bacteria, and the limited number of reference strains (TOMAZI et al., 2014). Furthermore, the version of the Biotyper software also affects the degree of identification. Three different LAB genera were identified namely *Lactobacillus*, *Leuconostoc* and *Enterococcus*. *Lactobacillus plantarum* was the most common species of LAB (n = 7, 63.6%), followed by *Lactobacillus paracasei* (n = 2, 18.2%), *Leuconostoc lactis* (n = 1, 9.1%) and *Enterococcus faecalis* (n = 1, 9.2%) (Table 30). Similarly, the report from Slovakia revealed that *Lactobacillus plantarum* was the most frequently identified in milk and milk products (KAČÁNIOVÁ et al., 2017).

Table 30: MALDI-TOF MS Biotyper identifications of lactic acid bacteria strains

No.	Origin	Sample type	Analyte ID	Organisms (best match)	MALDI score value
1	Farm III	IRM	LAB5	*Lactobacillus plantarum*	2.015
2	Farm III	IRM	LAB19	Presumably *Lactobacillus paracasei*	1.793
3	Farm III	BTM	LAB4	Presumably *Lactobacillus paracasei*	1.736
4	Farm III	BTM	LAB16	Presumably *Leuconostoc lactis*	1.736
5	OMÉK	Cheese	LAB28	Presumably *Lactobacillus plantarum*	1.877
6	OMÉK	Cheese	LAB29	*Enterococcus faecalis*	2.251
7	OMÉK	Cheese	LAB30	Presumably *Lactobacillus plantarum*	1.737
8	OMÉK	Cheese	LAB31	Presumably *Lactobacillus plantarum*	1.946
9	OMÉK	Cheese	LAB32	Presumably *Lactobacillus plantarum*	1.734
10	OMÉK	Cheese	LAB33	*Lactobacillus plantarum*	2.074
11	OMÉK	Cheese	LAB35	*Lactobacillus plantarum*	2.108

BTM: bulk tank milk; IRM: individual raw milk; OMÉK: 79th National Agriculture and Food Exhibition and Fair held in Budapest, Hungary, in September 2019. MALDI-TOF identification with a score value of ≥ 2.300 is considered to be high-probability species identification, whereas the results were classified as secure and uncertain genus level when it is 2.00-2.299 and 1.700-1.999 respectively. When a score value is below 1.700, the identification is considered as not reliable (TOMAZI et al., 2014; SMITH et al., 2015).

4.11.2. Phenotypical characteristics of lactic acid bacteria strains

Catalase and oxidase tests were performed for 11 lactic acid bacteria strains identified from ovine milk and cheese samples. As a result, all the lactic acid bacteria isolates were negative for catalase and oxidase tests (Table 31). They are therefore unable

to degrade hydrogen peroxide into water and oxygen molecules and do not have cytochrome C oxidase that oxidizes the test reagent.

Furthermore, antibiotic resistance to 10 (cefoxitin, chloramphenicol, clindamycin, erythromycin, gentamicin, penicillin G, streptomycin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin) antibiotics was carried out by disc diffusion method on MRS agar. Different studies indicated that the resistance of lactic acid bacteria against vancomycin is natural/intrinsic (ERGINKAYA et al., 2018; ZHANG et al., 2018). And, in this study, seven LAB isolates were resistant to vancomycin which is not considered a health risk, according to WHO. As a result, vancomycin (VA) resistance was not included in the overall resistance of lactic acid bacteria, despite the fact that seven LAB isolates were resistant. Eight (72.7%) of 11 lactic acid bacteria isolates were identified to be resistant to cefoxitin. Only one strain (LAB5) was resistant against tetracycline. 45.5% (n = 5/11) and 18.2% (n = 2/11) of LAB strains were intermediately resistant to penicillin G and tetracycline, respectively (Table 31). Five of the resistant lactic acid bacteria strains were originated from cheese samples and three were from ovine milk samples (Table 31).

Table 31: Characteristics of lactic acid bacteria strains

Origin	Sample type	Analyte ID	Organism	Catalase test	Oxidase test	Antibiotic resistance profile*
Farm III	IRM	LAB5	*Lactobacillus plantarum*	-ve	-ve	R (FOX, TE, VA), I (P)
Farm III	IRM	LAB19	Presumably *Lactobacillus paracasei*	-ve	-ve	R (FOX, VA)
Farm III	BTM	LAB4	Presumably *Lactobacillus paracasei*	-ve	-ve	R (FOX, VA)
Farm III	BTM	LAB16	Presumably *Leuconostoc lactis*	-ve	-ve	Susceptible
OMÉK	Cheese	LAB28	Presumably *Lactobacillus plantarum*	-ve	-ve	R (FOX, VA), I (P, TE)
OMÉK	Cheese	LAB29	*Enterococcus faecalis*	-ve	-ve	R (FOX), I (TE)
OMÉK	Cheese	LAB30	Presumably *Lactobacillus plantarum*	-ve	-ve	R (FOX, VA), I (P, S)
OMÉK	Cheese	LAB31	Presumably *Lactobacillus plantarum*	-ve	-ve	R (FOX, VA), I (P)
OMÉK	Cheese	LAB32	Presumably *Lactobacillus plantarum*	-ve	-ve	R (FOX, VA), I (P)
OMÉK	Cheese	LAB33	*Lactobacillus plantarum*	-ve	-ve	I (FOX, VA)
OMÉK	Cheese	LAB35	*Lactobacillus plantarum*	-ve	-ve	Susceptible

*Resistance to vancomycin in lactic acid bacteria is intrinsic, which is acceptable/not considered a health risk. Vancomycin resistant LAB strains were tot included in overall resistancy percentage. BTM: bulk tank milk; IRM: individual raw milk; FOX: cefoxitin; I: intermediate; P: penicillin G; R: resistance; S: streptomycin; TE: tetracycline; VA: vancomycin; OMÉK: 79th National Agriculture and Food Exhibition and Fair held in Budapest, Hungary, in September 2019.

5. CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the presence of a positive California Mastitis Test score in few udder halves milk samples might indicates the good health condition of flocks. However, disinfecting the teats of the animal before and after milking and cleaning the udder surface can prevent the occurrence of subclinical mastitis on the farms, where positive results were detected. Relatively low bacterial counts in udder surface and individual raw milk samples have reflected good housing and health conditions of sheep flocks kept on the four farms studied. However, the presence of total plate count in bulk tank milk of two farms at levels exceeding regulatory limit, which is 6.2 lg CFU/mL for milk that will undergo pasteurization, is indicative of poor hygienic standards during milking and on-farm milk handling. Therefore, measures should be taken to improve the sanitary conditions of ovine milk production on the studied farms. Besides, the present delivery practice of bulk tank milk from the farm to processing units should be revised. The length of storage of milk on the farm should be minimized by more frequent delivery of fresh milk to the dairy processing plant.

In the case of some ovine cheese samples, the high counts of *Enterobacteriaceae* and *Escherichia coli* were a sign of the existence of safety risk. The higher value of *Staphylococcus aureus* in chives-flavoured cheese samples may also indicate the unhygienic nature of ovine-milk cheese. As a result, awareness should be raised on the importance of some pre-requisites and process control points. For example, the utilization of high-quality raw materials, production in fashionable enterprises, and healthful conditions are going to be effective in minimizing contamination of bacterium.

In this study, a significant correlation between udder surface and individual raw milk samples for *Enterobacteriaceae* count indicates that unhygienic farm environments, such as dirty bedding, are prejudicing factors for the occurrence of unwanted microorganisms in unpasteurized ovine milk. Four bacterial phyla (*Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria*) were present in five milk sample types analysed for microbiota taxonomic profile. Due to the small sample size, microbiota richness and diversity indexes based on three milk sample types were not determined. This suggesting that sufficient sample size from different milk and environmental sample types should be considered in future for ovine-associated microbiota community studies.

Furthermore, the resistance against tetracycline was observed in six *S. aureus,* and penicillin G, tetracycline, trimethoprim/sulfamethoxazole, and erythromycin resistance was detected in at least one of five resistant coagulase-negative staphylococci strains. Eight lactic acid bacteria strains were resistant to at least one of the two antibiotics (cefoxitin and tetracycline). Also, SE genes were detected in 25 *Staphylococcus aureus* and one coagulase-negative *Staphylococcus* strains in which SEC was the most prevalent, being detected in 17 *Staphylococcus aureus* strains. Although the farm manager was not willing to tell the type of antibiotics used in the above-mentioned farm, the animals could be treated with antibiotics in which resistant strains were found. The presence of antibiotic resistance and SEs genes in the raw milk and cheese originated strains affects consumer's health. The interventions are needed to prevent the occurrence of pathogenic staphylococci in dairy ovine farms to ultimately prevent the milk supply from contamination with these bacteria. Moreover, careful use of antibiotics by veterinarians, avoiding raw milk consumption, and implementing a surveillance system to monitor staphylococcal enterotoxins in the ovine milk chain are of utmost crucial to minimize public health risk.

6. NEW SCIENTIFIC RESULTS

1. Total plate count was 2.9 ± 1.0 lg CFU/mL, *Enterobacteriaceae* count: 1.5 ± 0.5 lg CFU/mL and *Staphylococcus aureus* count: 2.6 ± 0.7 lg CFU/mL in overall (n = 108) California Mastitis Test negative udder halves milk samples. Individual raw milk samples with weak positive (n = 11) and positive (n = 5) CMT scores had TPC mean value of 3.6 ± 1.1 and 3.6 ± 0.8 lg CFU/mL, *Enterobacteriaceae* count: 1.8 ± 0.6 and 3.0 ± 0.9 lg CFU/mL, *Staphylococcus aureus* count: 2.8 ± 0.3 and 3.4 lg CFU/mL, respectively.

2. The overall mean value of total plate count of udder surface samples obtained from four sheep farms was 2.5 ± 0.8 lg CFU/cm^2 (ranged from 1.0 to 5.1 lg CFU/cm^2). *Enterobacteriaceae* count of udder surface samples was 1.2 ± 0.6 lg CFU/cm^2 (ranged from 0.1 to 2.6 lg CFU/cm^2). *S. aureus* was detected in only 2.6% (n = 2/77) of udder surface samples.

3. Overall average value of total plate count of individual raw milk samples originated from four sheep farms was 3.1 ± 1.1 lg CFU/mL (ranged from 1.0 to 5.5 lg CFU/mL). *Enterobacteriaceae* count of individual raw milk samples was 1.9 ± 0.8 lg CFU/mL (ranged from 0.0 to 3.9 lg CFU/mL). *S. aureus* was detected in six (7.0%) individual raw milk samples with overall mean value of 2.9 ± 0.6 lg CFU/mL.

4. Six (20.7%) *S. aureus* isolates of Farm III (five isolates of bulk tank milk and one of individual raw milk) were resistant to tetracycline. Staphylococcal enterotoxin genes were detected in 25 *S. aureus* isolates in which *sec* was the most prevalent, being detected in 17 *S. aureus* strains. Higher occurrence of enterotoxins was observed in strains originated from Farm I (n = 11/17) and III (n = 11/18), followed by Farm IV (n = 2/4) and Farm 2 (n = 0/2). Enterotoxins detected more frequently in IRM (n = 17) followed by BTM (n = 5) and cheese (n = 2) and udder surface (n = 2) samples.

5. Six coagulase-negative *Staphylococcus* (*S. auricularis, S. caprae, S. equorum, S. haemolyticus, S. simulans* and *S. xylosus*) were identified from either from udder surface and/or from raw milk samples. Penicillin G, tetracycline, trimethoprim/sulfamethoxazole, and erythromycin antibiotics resistance was detected in at least one of five resistant

coagulase-negative staphylococci strains. Among the five resistant coagulase-negative staphylococci isolates, three were originated from Farm III (2 strains of individual raw milk and 1 of bulk tank milk) and two were from Farm I individual raw milk. Enterotoxin gene (*seg* and *sei*) was diagnosed in only one coagulase-negative *Staphylococcus* strain (*S. auricularis*) of Farm I individual raw milk.

6. Three different lactic acid bacteria genera were identified namely *Lactobacillus*, *Leuconostoc* and *Enterococcus*. Eight (72.7%) lactic acid bacteria strains, five from cheese samples and three from Farm III milk samples, were resistant to cefoxitin. Out of the eight cefoxitin resistant isolates, one was also resistant to tetracycline.

7. RESULTS APPLICABLE IN PRACTICE

1. Total plate count in bulk tank milk of two farms (Farm II and III) where it exceeded regulatory limit, which is 6.2 lg CFU/mL for milk that will undergo pasteurization can be improved by improving sanitary conditions and delivering fresh milk more frequently to dairy processing plants. In the case of some ovine cheese samples, the high counts of *Staphylococcus aureus*, *Enterobacteriaceae* and *Escherichia coli* might be minimized through the utilization of high-quality raw materials (such as pasteurized milk), production in fashionable enterprises, and healthful conditions.

2. The identification and characterization of staphylococcal and lactic acid bacteria strains is crucial in dairy microbiology in order to fully understand the properties of the bacterial strains and to prevent contamination of ovine milk and cheese effectively.

3. Antibiotic-resistant staphylococcal strains were discovered in some of milk samples from Farms I and III. Moreover, resistant (except intrinsic resistance against vancomycin) lactic acid bacteria strains were found in some milk samples of Farm III and cheese samples. Disease prevention, avoiding misuse and overuse of antibiotic in accordance with WHO guideline (2017) on use of antibiotics in food producing-animals can all help to reduce the incidence.

4. Staphylococcal enterotoxin gene producing strains were found in some of udder surface, milk and cheese samples. According to COMMISSION REGULATION (EC) 2073/2005 on microbiological criteria for foodstuffs, dairy and dairy products sample should be free of enterotoxins. Even though, there is no clear staphylococcal enterotoxin concentration limit value, studies suggested that staphylococcal enterotoxin production is correlated with bacterial growth, i.e., the more bacterial counts, the more toxin production. Therefore, hygienic measures such as storing pasteurized milk and the dairy product below 6-7 ^{o}C, pasteurization of raw milk, and personal hygiene during milking can be taken to reduce *Staphylococcus* contamination.

8. SUMMARY

The consumption of ovine milk has been started a long time ago, during the beginning of sheep domestication. Ovine milk has superior nutritive value and more acceptable to the human digestive system in comparison to bovine and caprine milk. Due to its high solid content, 1 kg of cheese can be made from 5.5 litre of ovine milk while 1 kg of cow-milk cheese is needed twice as much milk. Even if ovine milk has medicinal value, in countries such as Hungary, where sheep are milked, the consumption is so far less in terms of quantity and quality of ovine milk and its products. Therefore, enough amount of hygienic ovine milk and its products is very important. Respecting acceptable limit values for somatic cell count, total plate count, and other pathogenic microorganisms is another issue that needs consideration in ovine milk value chain. The hygienic status of raw ovine milk affects the hygienic status of products made from it such as cheese and butter.

The consumption of ovine milk and its products is an actual microbiological risk to consumers due to the possible presence of undesirable food spoilage bacteria such as total plate count, *Enterobacteriaceae, E. coli, S. aureus,* and psychrotrophic bacteria. Ovine milk and its products can be contaminated during the time of milking, processing, and storage. To produce good quality milk and milk products, strict hygienic procedures must be followed. Moreover, knowing the bacteriological characteristics (hygienic indicator and pathogenic) and its sources are very crucial. Then, in this study, udder surface samples were examined for bacteriological counts to see if the bacterial load is correlated with the corresponding milk samples. This has probably a contribution to know the source of the milk contamination on the farm.

Therefore, this research was carried out with the following specific objectives;

- Examination of the microbiological status (total plate count, *Enterobacteriaceae* count, *E. coli* count, and *S. aureus* count) of udder surface samples.
- Examination of the microbiological status (total plate count, *Enterobacteriaceae* count, *E. coli* count, *S. aureus* count, and lactic acid bacteria count) and California Mastitis Test of individual raw milk samples.
- Examination of the microbiological status (total plate count, *Enterobacteriaceae* count, *E. coli* count, *S. aureus* count, psychrotrophic bacteria count, and lactic acid bacteria count) of bulk tank milk samples.

- Examination of the microbiological status (*Enterobacteriaceae* count, *E. coli* count, *S. aureus* count, and lactic acid bacteria count) of cheese samples.
- Determination of the coefficient of correlation between the major bacterial counts; i.e. total plate count and *Enterobacteriaceae* count of corresponding udder surface and individual raw milk samples.
- Analysis of microbiota community in some raw milk samples.
- Isolation and identification of staphylococcal and lactic acid bacteria strains.
- Phenotypic (tellurite production, lecithinase activity, coagulase test, hemolysis, catalase test, oxidase test, and antibiotic resistance) and genotypic (enterotoxin gene presence) characterizations of staphylococci strains.
- Phenotypic (catalase test, oxidase test, and antibiotic resistance) properties of lactic acid bacteria isolates.

In this study, four sheep farms located in Hajdú-Bihar County (Farm I, II, and III) and Jász-Nagykun-Szolnok County (Farm IV) of eastern Hungary were enrolled. In total, 124 udder halves ovine milk samples were tested for California Mastitis Test. One hundred seventy-three (77 udder surface, 86 individual raw milk, and 10 bulk tank milk) samples of five ewe breeds were examined for bacteriological quality. In addition, 15 cheese samples that were purchased from three sources (9 from the 79th OMÉK, 3 from the market in Debrecen, and 3 from the farmer near to Debrecen) were tested for bacteriological quality. Upon arrival, all the samples were kept at temperatures below 4°C before the examination. On the same day as the collection of the samples, culturing of bacteria was carried out immediately in the microbiological laboratory of the Institute of Food Sciences at the University of Debrecen. All the bacteriological examinations were carried out based on the procedures suggested by International Organization for Standardization.

Then, suspected staphylococcal (29 *S. aureus* and 42 coagulase-negative staphylococci) and lactic acid bacteria (n = 35) isolates were picked from Baird-Parker and MRS agar, respectively, for characterization. One hundred six bacterial colonies were characterized based on morphology, including size, shape, colour, appearance, and texture. The identification of 21 staphylococci strains (8 *S. aureus* and 13 coagulase-negative staphylococci) was performed by API Staph in the microbiological laboratory of the Institute of Food Sciences at the University of Debrecen. MALDI-TOF based identification was carried out for 42 staphylococci (21 *S. aureus* and 21 coagulase-

negative staphylococci) and 35 lactic acid bacteria colonies at AgroBioTech Research Centre at the Slovak University of Agriculture. Further, 15 coagulase-negative staphylococci colonies were identified by molecular method at the Biomi Ltd. Company, Hungary. Forty-five staphylococcal and 11 lactic acid bacteria strains were tested for catalase, oxidase, and antibiotic resistance tests, while coagulase, hemolysis and enterotoxin gene test was carried out for only staphylococcal isolates.

In our study, of 124 udders half milk samples evaluated, positive California Mastitis Test score was recorded only in 16 (12.9%) samples. Within farms studied, the overall positive California mastitis test results varied from 10 to 25%. Slightly higher total plate count was observed in milk samples with positive California mastitis test results.

The results revealed that the overall total plate count mean value examined between 2018 and 2020 was 2.5 ± 0.8 lg CFU/cm^2, 3.1 ± 1.1, and 7.0 ± 0.9 lg CFU/mL in udder surface, individual raw and bulk tank milk samples respectively. Udder surface and individual raw milk samples originated from animals kept on Farm I and IV had significantly lower and higher ($P < 0.05$) values than that of samples from other farms based on total plate counts. The value of total plate count in the BTM obtained from Farm II and III did not meet the limit of Regulation (EC) 853/2004, which was ≤ 6.2 lg CFU/mL for total plate count of ovine milk to be processed with heat treatment. There was a significantly higher count ($P < 0.05$) on udder surface samples of Tsigai breed than the other two breeds. Samples examined in 2018 seemed to contain a higher ($P < 0.05$) total plate count than those examined in 2019. In the case of *Enterobacteriaceae* count, the overall mean value tested between 2018 and 2020 was 1.2 ± 0.7 lg CFU/cm^2, 1.9 ± 0.8 and 5.1 ± 0.9 lg CFU/mL in udder surface, individual raw and BTM samples, respectively. Udder surface samples originated from Farm IV had a significantly lower ($P < 0.05$) *Enterobacteriaceae* count than Farm I. The *Enterobacteriaceae* count of individual raw milk samples collected from ewes on Farm I (1.1 ± 0.3 lg CFU/mL) and II (2.3 ± 0.8 lg CFU/mL) was significantly varied ($P < 0.05$). Tsigai breed shows significantly increased *Enterobacteriaceae* count ($P < 0.05$) compared to Merino in the udder surface samples. The *Enterobacteriaceae* count of individual raw milk samples from Merino and Dorper individuals did not differ significantly ($P > 0.05$). Udder surface samples examined in 2019 seemed to contain lower ($P < 0.05$) *Enterobacteriaceae* count than those examined in 2018. *Enterobacteriaceae* count mean value in cumin-flavoured-smoked cheese was

significantly lower ($P < 0.05$) compared to chives-flavoured cheese and garlic-flavoured-smoked cheese.

E. coli was not detected in any of udder surface and individual raw milk samples. *E. coli* count in chives-flavoured and garlic-flavoured-smoked cheese samples failed to meet regulatory standards in decree No. 4/1998. (XI. 11.) EÜM, which defines mandatory criteria for this bacterial group in the milk products and curd, should be less than one CFU/g. *Staphylococcus aureus* was detected in only two udder surface (2.6%) and six individual raw milk samples (7.0%). *S. aureus* was detected in only two bulk tank milk (20%) samples of Farm III with a mean of 3.4 ± 0.6 lg CFU/mL. *S. aureus* count mean value was 5.7 ± 0.2 lg CFU/g in chives-flavoured cheese and not detected in the other cheese types.

Psychrotrophic bacteria count was 4.3 ± 1.0 and 3.6 CFU/mL in bulk tank milk samples of Farm III and IV respectively. A non-significant strong positive correlation ($r = 0.693$) was observed between total plate count and psychrotrophic bacterial count. Lactic acid bacteria count in bulk tank milk obtained from Farm III and IV was 6.7 ± 0.4 and 4.2 lg CFU/mL, respectively. The mean of lactic acid bacteria count was 7.5 ± 0.1 and 6.5 ± 0.3 lg CFU/g in garlic-flavoured-smoked cheese and garlic-flavoured cheese, respectively. Lactic acid bacteria count value in garlic-flavoured cheese was significantly lower ($P < 0.05$) compared to garlic-flavoured-smoked cheese. Weak to moderate positive correlations were observed between the bacterial count pairs evaluated. Significantly ($P < 0.01$) moderate correlation ($r = 0.565$) was observed between udder surface and individual raw milk samples for *Enterobacteriaceae* count.

Four bacterial phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) were present in five milk samples analysed for microbiota taxonomic profile. Twenty-nine *S. aureus*, 16 coagulase-negative staphylococci and 11 lactic acid bacteria isolates were identified to at least genes level either by API Staph and/or by MALDI-TOF and/or molecular identification methods. Seven *Staphylococcus* species (*S. aureus*, *S. auricularis*, *S. caprae*, *S. equorum*, *S. simulans*, *S. haemolyticus*, and *S. xylosus*) were identified. Moreover, three lactic acid bacteria genera were identified namely *Lactobacillus*, *Leuconostoc* and *Enterococcus*. Thirty-two isolates (82.2%) of the staphylococci strains were able to produce at least one hemolysis type (α, β, γ). In twenty staphylococci strains, α-β hemolysis was detected. Eleven (24.4%) of staphylococci isolates were resistant to at least one of the tested antibiotics. Six *S. aureus* isolates were

resistant to tetracycline. Penicillin G, tetracycline, trimethoprim/sulfamethoxazole, and erythromycin antibiotic resistance was detected in at least one of five resistant coagulase-negative staphylococci strains. Eight (72.7%) lactic acid bacteria strains were resistant to at least one of the two antibiotics (cefoxitin and tetracycline). Out of 45 staphylococci strains, 26 (57.8%) were able to produce at least one of the 13 tested enterotoxins. Among the enterotoxins detected, SEC was the most prevalent, being detected in 17 (37.8%) strains. SEI (n = 5) and SEG (n = 4) were the third and fourth most found enterotoxins.

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10. PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

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Subject: PhD Publication List

Candidate: Andualem Tonamo
Doctoral School: Doctoral School of Animal Husbandry
MTMT ID: 10061765

List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (3)

1. **Tonamo, A.**, Komlósi, I., Szabóné Petróczki, F. M., Peles, F.: Coagulase-negative staphylococci in ewe udder surface and raw milk samples.
   Agrártud. Közl. 1, 221-225, 2021. ISSN: 1587-1282.
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2. **Tonamo, A.**, Komlósi, I., Varga, L., Kačániová, M., Peles, F.: Identification of ovine-associated staphylococci by MALDI-TOF mass spectrometry.
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   DOI: http://dx.doi.org/10.1556/066.2020.00246
   IF: 0.458 (2019)
3. **Tonamo, A.**, Komlósi, I., Peles, F.: Bacteria in the milk of sheep with or without mastitis- mini Review.
   Agrártud. Közl. 2019 (1), 47-52, 2019. ISSN: 1587-1282.
   DOI: http://dx.doi.org/10.34101/actaagrar/1/2369

Foreign language scientific articles in international journals (1)

4. **Tonamo, A.**, Komlósi, I., Varga, L., Czeglédi, L., Peles, F.: Bacteriological Quality of Raw Ovine Milk from Different Sheep Farms.
   Animals (Basel). 10 (7), 1-9, 2020. ISSN: 2076-2615.
   DOI: http://dx.doi.org/10.3390/ani10071163
   IF: 2.323 (2019)

Foreign language conference proceedings (1)

5. **Tonamo, A.**, Komlósi, I., Szabóné Petróczki, F. M., Orosz, É., Aamir, M., Peles, F.: Microbiological quality of raw milk and udder surface samples from Dorper, Merino and Cigaja sheep breeds.
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Foreign language abstracts (3)

6. **Tonamo, A.**, Komlósi, I., Peles, F.: Microbiological properties of raw ewe milk and udder surface samples in a Hungarian dairy sheep farm.
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List of other publications

Foreign language scientific articles in Hungarian journals (2)

9. Zegeye, Z. B., Vass, N., **Tonamo, A.**: Application of laparoscopic artificial insemination in conventional Lacaune sheep farm using frozen-thawed semen.
Agrártud. Közl. 2020 (2), 133-138, 2020. ISSN: 1587-1282.
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Foreign language scientific articles in international journals (1)

11. **Tonamo, A.**, Berhan, T., Gebeyehu, G., Ermias, B.: Characterization of cattle husbandry practices in Essera Woreda, Dawuro Zone, Southern Ethiopia.
Afr. J. Agric. Res. 10 (34), 3421-3435, 2015. EISSN: 1991-637X.
DOI: http://dx.doi.org/10.5897/AJAR2015.9939

Total IF of journals (all publications): 2.781
Total IF of journals (publications related to the dissertation): 2.781

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

07 June, 2021

11. DECLARATIONS

DECLARATION

I hereby declare that I have prepared this dissertation in the framework of the Doctoral School of Animal Science, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, in order to obtain the doctoral (Ph.D.) degree in Animal Husbandry.

Debrecen, 2021/……/……

Andualem Tonamo Tema
Ph.D. candidate

DECLARATION

We hereby declare that **Andualem Tonamo Tema** Ph.D. candidate has carried out his work under our supervision between 2017 and 2021 within the framework of the Doctoral School of Animal Science, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen. The findings of the dissertation represent the candidate's own ideas and independent work. We recommend accepting the dissertation.

Debrecen, 2021/……/……

----------	**----------**
Prof. Dr. István Komlósi, D.Sc.	**Dr. Ferenc Árpád Peles, Ph.D.**
professor	*assistant professor*

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The LORD is my strength and my shield; my heart trusts in him, and I am helped (Psalm 28:7).

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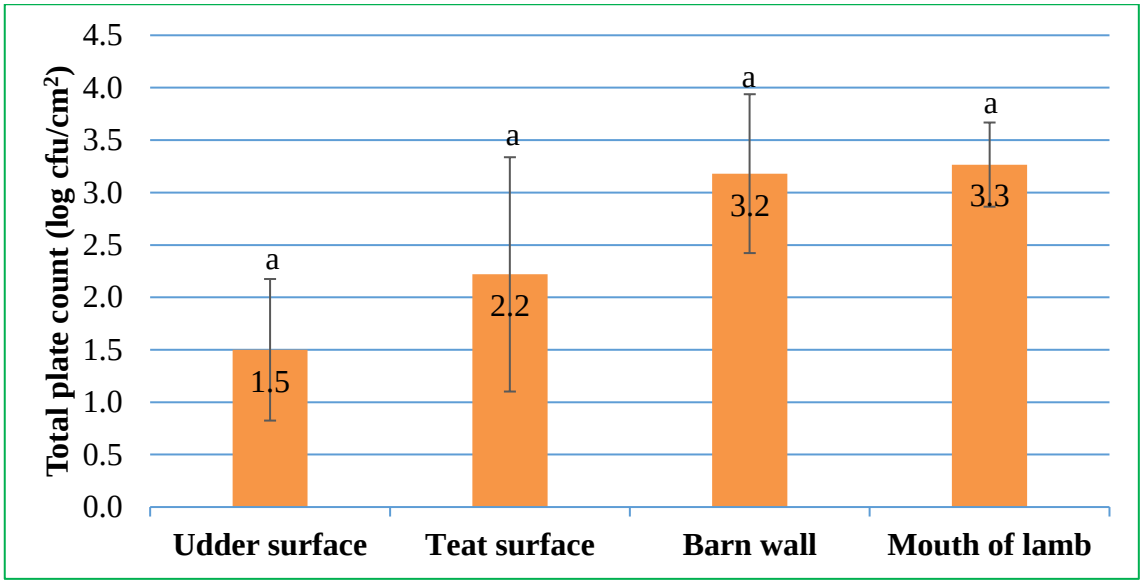
12. APPENDICES

Appendix 1: Total plate count of environmental samples obtained from Farm I



This result was from a preliminary study to determine the origin of the environmental sample (udder surface). The total plate count values of different environmental samples (teat surface, udder surface, lamb mouth, and barn walls) were compared and none-significant ($P > 0.05$) difference between samples.

Appendix 2: Meaning of MALDI-TOF score value

Range	Description	Symbols	Colour
2.300 ... 3.000	**highly probable species identification**	**(+++)**	green
2.000 ... 2.299	**secure genus identification, probable species identification**	**(++)**	green
1.700 ... 1.999	**probable genus identification**	**(+)**	yellow
0.000 ... 1.699	**not reliable identification**	**(-)**	red

Analyte Name: H2
Analyte Description:
Analyte ID: LAB35
Analyte Creation Date/Time: 2019-10-29T12:54:07.319
Applied MSP Library(ies):
Applied Taxonomy Tree: Bruker Taxonomy

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (++)	*Lactobacillus plantarum* DSM 1055 DSM	2.108	1590
2 (++)	*Lactobacillus plantarum* DSM 20246 DSM	2.049	1590
3 (++)	*Lactobacillus plantarum* DSM 2601 DSM	2.018	1590
4 (+)	*Lactobacillus plantarum* ssp *plantarum* DSM 20174T DSM	1.954	337330
5 (+)	*Lactobacillus plantarum* DSM 2648 DSM	1.803	1590
6 (-)	*Lactobacillus plantarum* DSM 13273 DSM	1.664	1590
7 (-)	*Lactobacillus plantarum* DSM 12028 DSM	1.64	1590
8 (-)	*Lactobacillus paraplantarum* DSM 10667T DSM	1.627	60520
9 (-)	*Lactobacillus pentosus* DSM 20314T DSM	1.606	1589
10 (-)	*Lactobacillus plantarum* ssp *argentoratensis* DSM 16365T DSM	1.582	271881