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Physicochemical and microbiological characterisation of a typical Italian raw ewe's milk cheese: Pecorino bagnolese

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ABSTRACT

Pecorino Bagnolese is a typical Italian cheese made from raw ewe's milk in province of Avellino (southern Italy). The aim of this study was to evaluate the physicochemical and microbiological parameters during cheese-making and ripening (30, 90 and 180 d). Results showed that the cheese-making and ripening influenced the pH, water activity, moisture, fat content and levels of *Enterobacteriaceae*. Changes in the fatty acids profile of ripened cheeses compared to milk were observed, suggesting the influence of ripening on the fatty acids profile. Nutritional indices demonstrated positive health attributes. The concentration of malondialdehyde remained consistently low, while free fatty acids showed an increasing trend during ripening. Coagulase-positive staphylococci complied with the legislative threshold value, but colonies of *Staphylococcus aureus* were found. Pathogens were not detected. In conclusion, the data collected provided a better characterisation of this traditional cheese and can be used for broadening knowledge of raw ewe's milk cheeses.

1. Introduction

In recent years, the interest in sheep's dairy products has increased in many areas (van den Brom et al., 2020), specifically, in countries bordering the Mediterranean, where dairy sheep are concentrated. The production sector of small ruminants, generally characterised by semi-extensive systems using natural pastures (Pulina et al., 2018), allows the environment and cultural patrimony to be safeguarded through the manufacturing of traditional and high-quality dairy products. The popularity of these products among consumers is related to their familiarity and link with cultural and culinary traditions, as well as the association with the "healthy food" concept, based on possible nutritional benefits, as sources of proteins, fatty acids, and vitamins (Vargas-Bello-Pérez et al., 2022). Italy is the first ewe cheese producer among Mediterranean countries (ISMEA, 2021). The Italian hard cheeses obtained exclusively from ewes' milk, referred to as "Pecorino" cheeses, are usually produced with different ancient traditional procedures, depending on geographical areas (Settanni & Moschetti, 2014). The quality of these cheeses is strongly correlated with the territory of production and its pedoclimatic conditions, native genetic variations, and anthropic factors, which determine such a distinctive environment that is difficult to reproduce in other places (Caridi et al., 2003). The interactions between all these factors affect the composition of milk substrates (carbohydrates, fats and proteins) and enzymes, which are involved in the phenomena reliable for the characteristics of cheeses (F. Toldrá & L.M.L. Nollet, 2013).

Pecorino cheese manufacturing generally takes place in small local dairy units usually with the use of raw milk (Gonzales-Barron et al., 2017), which allows cheese to preserve the biodiversity of indigenous microbial consortia (Montel et al., 2014). The uniqueness of raw milk cheeses, generally considered "biologically active foods", is mainly based on the activity of autochthonous lactic acid bacteria (LAB), in particular their catabolic reactions (proteolysis and lipolysis), which operate during ripening and influence the development of the various sensory features. Nowadays, the consumption of raw milk product is increasing due to the growing demand for minimally processed food (Fusco et al., 2020) by certain groups in society, who recognize them as healthier and more tasteful compared to heat-treated products (van den Brom et al., 2020). Heat treatment can affect microorganism and non-

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microbial inhibitors normally present in milk, such as some enzymes (lactoperoxidase, lysozyme, and lactoferrin) and the production of free fatty acids, with antibacterial properties (Claeys et al., 2013). On the other hand, the use of raw milk may represent a food safety concern, because if it is obtained from unhealthy animals or without following the proper hygiene practices the final products could be contaminated by the pathogens survived during cheese manufacturing. Many authors have analysed the risks related to the consumption of raw milk and its derived products (Claeys et al., 2013; van den Brom et al., 2020). Several studies have shown the low prevalence of pathogens in cow's raw milk ripened cheese (Brooks et al., 2012; Little et al., 2008), but only limited attention was given to sheep's raw milk cheese.

Pecorino Bagnolese (recognized by the Italian Ministry of Agriculture as a Traditional Agri-Food Product and a Slow Food Presidium) is manufactured according to traditional technologies in a limited area of the province of Avellino (southern Italy). This territory is the most important breeding area of Bagnolese sheep, an autochthonous breed of the Campania region, which is raised in small groups in a wild or semi-wild state on herbal natural pastures, with integration only in the winter months. Following local tradition protocol, manufacturing is made from raw milk, without any starter cultures and cheese is ripened in a cave. Maturation in natural environmental conditions, such as a cave, is characterized by greater variability in thermoshygrometric parameters (relative humidity, temperature, and ventilation) compared to controlled and standardized conditions in cold rooms. This variability complicates the cheese ripening process and can affect the characteristics of cheeses (Di Paolo et al., 2023; Torracca et al., 2016). The time and type of the ripening process play a role in the total accumulation of biogenic amines in cheeses and can affect aroma intensity, that can result higher in cave-ripened cheeses (Torracca et al., 2016). Moreover, the ripening method can affect the texture characteristics of cheese, such as hardness, chewiness, and gumminess, resulting in higher levels in cheeses ripened using traditional methods under non-standardized conditions and without pH monitoring (Di Paolo et al., 2023).

Despite the widespread use of natural caves in the world, equally large scientific documentation on their specificity, nor on the biological processes that take place in cheeses ripened in these conditions. Each biodiversity factor involved in the traditional production systems influences the quality of the end products (Licitra et al., 2018) and for this reason, manufacturing procedures of Pecorino Bagnolese may represent critical points.

Typical cheeses, especially those made from raw milk, are frequently the subject of debate regarding their risks and benefits (Montel et al., 2014). In general, the evaluation of physicochemical and microbiological parameters of cheeses is important in ensuring high-quality and safe products and preserving, at the same time, their unique ecosystems. Therefore, the aim of this research was to study the physicochemical and the microbiological characteristics of Pecorino Bagnolese cheese during cheese-making and ripening in cave.

2. Materials and methods

2.1. Cheese manufacturing and sampling procedure

The experimental study was conducted in a dairy farm located in Avellino's province (Campania, Italy). The flow diagram of the traditional manufacturing technology used is shown in Fig. 1. Briefly, bulk raw ewe's milk was filtered and slightly heated in a multi-purpose circular steel vat to 33 °C and the coagulation was performed by adding lamb rennet paste (3 g L^{-1} of milk) in 25 min. The coagulum was cut by a traditional wooden tool into pieces, close to a rice grain size (length 3–6 mm) and after 5 min the whey was poured through a screen strainer (1 mm holes), and the curd was allowed to drain. The obtained curd was transferred into special moulds called “fuscelle” (perforated cylindric plastic molds) and pressed by hand to increase the whey drainage. Moulded curd was steamed at 30 °C for about 2 h and dry salted by hand on the surface with coarse-grained salt. Cheeses were ripened in natural cave at 15–18 °C and around 84–85 % relative humidity (RH) with frequent rotations and cleaning of the surface during ripening. During the study period (2021–2022) four batches of Pecorino Bagnolese produced in November, April, May, and June were monitored from milk curdling until six months of ripening (180 d). In particular, the sampling plan provided samples of bulk milk, curd, cheese on the day of production (0 d) and cheeses at 30, 90 and 180 d of ripening, which were collected and sent to the laboratory in refrigerated boxes (4 ± 1 °C) for the microbiological and physicochemical analyses.

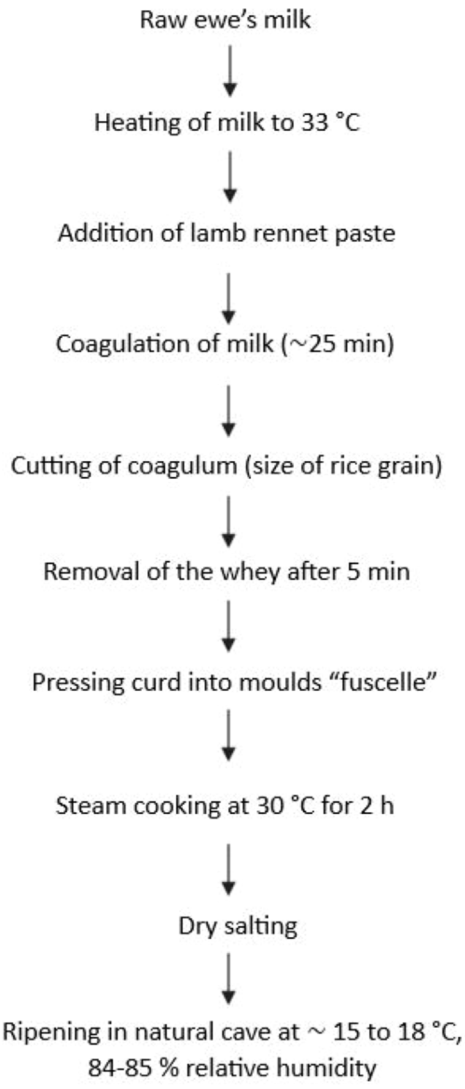

Fig. 1. Flow diagram of Pecorino Bagnolese manufacturing.

2.2. Physicochemical and fatty acids analysis

The chemical composition and the fatty acid (FA_s) profile of cheeses were determined from grated cheese samples taken 2 cm

from the rind. Protein, fat, and moisture contents were determined in accordance with the methods outlined by Association of Official Analytical Chemists (AOAC, 2002). pH and water activity (a_w) were monitored during cheese-making and ripening using a digital pH meter (Crison-Micro TT 2022, Crison Instruments, Barcelona, Spain) and Aqualab 4 TE (Decagon Devices Inc., Pullman, WA, USA) instrument, respectively. The extraction of fat for the subsequent gas chromatographic (GC) analysis was carried out according to the Official Method of Cheese Analysis (D.M., 1986.), based on the Schimith–Bondzynski–Ratzlaff traditional method of extracting lipids. Derivatization to determine the composition of fatty acids (FA_s) was performed on milk and cheese lipids extract following the method described by Di Paolo et al., 2023. To assesses the lipid quality indices in ripened cheeses, the index of atherogenicity (AI), thrombogenicity index (TI) were calculated as proposed by Ulbricht & Southgate, 1991:

$$AI = \frac{[C12:0 + (4 \times C14:0) + C16:0]}{(n3\ PUFA + n6\ PUFA + \sum MUFA)} \quad (1)$$

$$TI = \frac{(C14:0 + C16:0 + C18:0)}{[(0.5 \times \sum MUFA + 0.5 \times PUFA\ (n6) + (n3)/(n6)]} \quad (2)$$

Furthermore, the hypocholesterolaemic/hypercholesterolaemic ratio (HH) was calculated according to Santos-Silva et al., 2002:

$$h/H = \frac{(C18:1n9cis + \sum PUFA)}{(C12:0 + C14:0 + C16:0)} \quad (3)$$

More recently, S. Chen et al., 2004 developed the health-promoting index (HPI), which is the inverse of the AI, and this index was calculated as follow:

$$HPI = \frac{PUFA\ (n6) + PUFA\ (n3) + \sum MUFA}{[(12:0 + (4 \times C14:0) + C16:0]} \quad (4)$$

2.3. Lipolysis and oxidative stability in cheeses

Lipolysis and oxidative stability during cheese-making and ripening of cheeses were assessed by measuring the concentrations of free fatty acids (FFA) and thiobarbituric acid reactive substances ($TBAR_s$) according to the methods adopted by Di Paolo et al., 2023. The results for FFA_s and $TBAR_s$ were expressed as a percentage of oleic acid and as mg malondialdehyde (MDA)/kg samples, respectively.

2.4. Microbiological analyses

Samples of milk (10 mL) and of curd/cheese (10 g) were transferred to sterile stomacher bags, for the preparation of the first dilution, in 90 mL (1:10 w/v or v/v) of sterile Peptone Water (PW, Oxoid) and subsequently homogenized for 2 min at 230 rpm using a peristaltic homogenizer (BagMixer®400 P, Inter science, Saint Nom, France). Serial decimal dilutions per homogenate sample were prepared with a sterile PW solution. The following specific agar media and incubation conditions were used to enumerate microbial groups of samples: total aerobic bacteria (TAB 30 °C) on Plate Count Agar (PCA, Oxoid) incubated at 30 °C for 72 h (ISO 4833-1:2013); *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA, Oxoid) incubatedat 37 °C for 24/48 h (ISO 21528-2:2017); coagulase-positive staphylococci on Baird Parker Agar (BPA, Biolife) supplemented with Egg Yolk Tellurite Emulsion incubated at 37 °C for 24/48 h (ISO 6888-1:2021); mesophilic lactococci on Medium 17 Agar (M17, Oxoid) supplemented with 1% lactose incubated aerobically at 30 °C for 48 h (Mormile et al., 2016); mesophilic lactobacilli on De man, Rogosa and Sharpe agar (MRS CM0361, Oxoid) incubated aerobically at 30 °C for 72 h (ISO 15214:1998). Each sample was examined for the detection of *Salmonella* spp. and *Listeria monocytogenes* by using the respective reference analytical microbiological methods (ISO 6579-1:2017 and ISO 11290-1:2017).

2.4.1. MALDI-TOF mass spectrometry (MS) identification

All presumptive colonies of pathogens investigated (*L. monocytogenes* and *Salmonella* spp.) and presumptive colonies of *Staphylococcus aureus* were analysed by MALDI-TOF MS (MALDI Biotyper® Sirius) using "direct sample spotting". In brief, single colonies from the agar plate were smeared directly in duplicate onto a 96-spotsteel plate (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature (Topić Popović et al., 2021). Subsequently, colonies were covered with a 1 μL matrix solution containing 10 mg mL^{-1} of α-cyano-4-hydroxycinnamic acid in acetonitrile (Sigma–Aldrich, Berlin, Germany), deionized water and trifluoracetic acid (50:47.5:2.5, [v/v/v]). Bruker's Bacterial Test Standard (BTS Bruker Daltonics) was used for the mass calibration and Flex Control 3.4 software (Bruker Daltonics, Bremen, Germany) was set in a linear positive ion detection mode (Bruker Daltonics). Identification was obtained by comparing the collected spectra to those contained in the Bruker MSP database (MBT Compass Library) using the Bruker Compass software at default settings (Peruzy et al., 2021). According to the Brukerrecommendations, the identification score criteria were classified as follows: a log (score) value ≥ 2 indicates highly probable species identification; a score between 1.7 and 1.99 indicates secure genus identification and probable species; a score <1.7 indicates non-reliable identification.

2.5. Statistical analysis

Statistical analyses were performed on data from this study using IBM SPSS Statistics, version 28 (IBM Analytics, Armonk, NY, USA). Microbiological parameters (TAB 30 °C, *Enterobacteriaceae*, coagulase-positive staphylococci, mesophilic lactobacilli and mesophilic lactococci) as well as chemical parameters (chemical composition, fatty acid profile and lipolysis and oxidation index) were statistically analysed with a one-way analysis of variance (ANOVA). This analysis considered milk, curd, and cheeses collected on different days of cheese ripening (0, 30, 90, and 180). A probability value of less than 0.05 ($P < 0.05$) was considered statistically significant. All data were presented as mean ± standard deviation.

3. Results and discussion

3.1. Physicochemical parameters of Pecorino Bagnolese

The physicochemical parameters of Pecorino Bagnolese during cheese-making and ripening period are summarised in Table 1. The results showed a significant decrease in moisture content and activity water (a_w) during cheese-making and ripening period. As expected, most of the water (approximately 10 % of initial water content in the curd) was lost during the draining prosses which involves manual pressing and steaming. This process allowed the curd to reach a moisture content of around 50 % in the cheese on the day of production. During the first 30 d of the ripening period, a decrease in the moisture content and the water activity (a_w) values was observed as a result of environmental conditions that influenced the surface evaporation of the water (Di Paolo et al., 2023). Subsequently, the moisture content of cheese significantly decreased ($P < 0.01$) until 90 d of about 20 % (from 50.75 % at 0 d to 31.83 % at 90 d). This decrease could be attributed by the acid development produced by lactic acid bacteria (LAB) that decrease

the pH value creating an environment that promotes the activity of proteolytic enzymes present in cheese, the solubility of casein and consequently its hydrolysis (Nazari et al., 2020). These factors may have contributed to cheese syneresis and consequently a reduction of moisture content and a_w value of cheese during ripening due to the changes in water binding with the new carboxylic acid and amino groups formed during hydrolysis (Nazari et al., 2020). However, it was worth noting that there is no significant change in the moisture content of cheese between 90 d and the end of ripening period (31.83 % at 90 d *vs.* 29.04 % at 180 d; $P > 0.05$). This result may be attributed to the natural ventilation and the relative humidity in the ripening cave, that create an equilibrium between water absorption by amino groups formed through secondary proteolysis and water release due to osmotic pressure, as observed by Rani & Jagtap, 2019. In agreement with our results, Rani & Jagtap, 2019 noted a similar behavior of moisture in Swiss cheese ripened for 90 d. The total fat content increased during cheese-making and ripening, but no significant differences in fat content were found between cheeses ripened for 30, 90, and 180 d, probably due to the variability associated with the sampling scheme. Protein content showed a significant increase ($P < 0.05$) during cheese-making, from curd to cheese on the day of production. Subsequently, the protein content remained relatively constant, increasing ($P < 0.05$) at the end of ripening period due to the moisture decrease and the concentration of nutritional components according to García et al., 2016.

The pH value decreased significantly ($P < 0.05$) during production and the first 30 d of ripening. The intense enzyme activity of the autochthonous lactic acid bacteria probably led to acid production and subsequent decrease in the pH value (Caridi et al., 2003). The pH values reached their lowest point at 30 d, and then gradually increased until the end of ripening. This increase is likely attributed to secondary proteolysis and the subsequent production of basic compounds resulting from the deamination of amino acids (Sousa et al., 2001), which influenced the pH value reaching a final value of 5.75 in cheese at 180 d. A similar behavior of pH was observed in goat cheese ripened for 90 d (Gonzales-Barron et al., 2017).

3.2. Fatty acid profile of milk and Pecorino Bagnolese

Fatty acids (FA_s) composition of milk and Pecorino Bagnolese during cheese-making and ripening period (Table 2) revealed that the most abundant fatty acids were palmitic acid (C16:0), oleic acid (C18:1 *n*-9 *cis*), myristic acid (C14:0) and stearic acid (C18:0). These findings align with those Serrapica et al., 2020 who studied the fatty acid profile of traditional Pecorino cheese. During cheese-making (from milk to cheese at 0 d), no significant changes were observed in the fatty acid concentrations. However, differences emerged in the FA_s composition of ripened cheeses (30 d, 90 d and 180 d) compared to milk, suggesting the influence of ripening on the FA_s profile. According to Di Paolo et al., 2023, the most notable changes were related to the profile of C18 fatty acids. Oleic acid (C18:1 *n*-9 *cis*) increased by the end of ripening, rising from 19.16 % in milk to 21.73 % in cheese at 180 d. Stearic acid (C18:0) also increased at the end of ripening, with values changing from 13.54 % in milk to 15.49 % in cheese at 180 d. During cheese ripening, the content of butyric acid (C4:0) decreased, probably due to the lipolytic activity of the autochthonous microflora (Collins et al., 2003). This decrease could suggest the liberation of C4:0 as free fatty acid within cheeses, a phenomenon recognized for enhancing the flavor and taste of the cheese (Akin et al., 2003). Saturated fatty acids (SFA_s) were the most abundant class of FAs, followed by monounsaturated ($MUFA_s$), and finally, polyunsaturated ($PUFA_s$) fatty acids. These classes, along with individual fatty acids, were influenced by ripening period. The levels of SFA_s remained stable during the initial cheese production process (until 30 d). Subsequently, they increased at 90 d of ripening, and, finally, by the end of the ripening period (180 d), they significantly ($P < 0.01$) decreased. Instead, $MUFA_s$ and $PUFA_s$ exhibited the opposite trend compared to SFA_s according to Laskaridis et al., 2013.

The evolution of short-chain ($SCFA_s$), medium-chain ($MCFA_s$) and long-chain ($LCFA_s$) fatty acids is showed in Fig. 2. Short-chain fatty acids increased during the first stages of cheese-making (milk, curd and 0 d) and exhibited a slight decrease during the later stages of ripening (30, 90, and 180 d). In particular, the concentrations of C4:0, C6:0, C8:0 and C10:0 decreased during the ripening process, according to Correddu et al., 2021 who studied the fatty acid profile of Pecorino Sardo cheese during the ripening period. Medium-chain fatty acids remained relatively stable during the initial phases of cheese-making and throughout the ripening process until 30 d. However, they increased at 90 d and were lower at the end of the ripening period. Long-chain fatty acids increased during cheese-making and the initial stages of ripening until 30 d, then decreased at 90 d, and subsequently showed a significant increase ($P < 0.01$) at the end of the ripening period (180 d). During the cheese ripening, a multitude of intricate microbial and enzymatic processes occur, giving rise to various compounds that contribute to the cheese's aroma and texture characteristics (Fox et al., 2017). Among these compounds are the free fatty acids (FFA_s), that result from the enzymatic breakdown of fatty acids found in triglycerides. These FFA_s are further metabolized into highly flavorful compounds, such as methyl ketones and lactones (Collins et al., 2003). Therefore, the decrease in the proportion of $SCFA_s$ observed during the ripening process, along with the behavior of $MCFA_s$ and $LCFA_s$, might be considered an indication of the hydrolysis of FA_s from triglycerides, primarily those of the $SCFA_s$ (Chamba & Perreard, 2002).

The content of conjugated linoleic (CLA_s), omega 3 (*n*-3) and omega 6 (*n*-6) fatty acids, atherogenicity index (IA), thrombogenicity index (IT), the hypocholesterolemic/Hypercholesterolemic fatty acids ratio ($h\ H^{-1}$) and the health-promoting index (HPI) were determined (Table 3). The CLA_s content remained relatively stable during the cheese-making (milk, curd and 0 d), followed by an

Table 1
Chemical composition of Pecorino Bagnolese during the cheese-making and ripening.[a]

	Curd	Ripening time (d)			
		0	30	90	180
Moisture, %	63.4 ± 2.19^{A}	50.75 ± 2.80^{B}	43.97 ± 2.01^{C}	31.83 ± 1.66^{D}	29.04 ± 0.66^{D}
Fat, %	19.55 ± 1.71^{aA}	22.85 ± 0.70^{bA}	29.71 ± 1.21^{B}	30.19 ± 1.60^{B}	31.76 ± 2.37^{B}
Protein, %	13.75 ± 0.96^{A}	18.40 ± 1.08^{B}	19.10 ± 2.15^{B}	20.35 ± 1.39^{BC}	22.89 ± 0.26^{C}
pH	6.85 ± 0.06^{A}	6.61 ± 0.05^{B}	5.26 ± 0.05^{C}	5.60 ± 0.05^{aD}	$5.75 \pm 0.05b^{D}$
a_w	0.986 ± 0.001^{aA}	0.980 ± 0.002^{bA}	0.958 ± 0.005^{B}	0.935 ± 0.002^{C}	0.914 ± 0.002^{D}

[a] Values were expressed as mean ± standard deviation. $^{A–D}$ Different superscript uppercase letters indicate a significant difference at $P < 0.01$. $^{a-b}$ Different superscript lowercase letters indicate a significant difference at $P < 0.05$.

Table 2
Fatty acid profile of Bagnolese ewe milk and Pecorino Bagnolese during cheese-making and ripening.[a]

Fatty acids (% of total fatty acids)	Milk	Curd	Ripening time (d)			
			0	30	90	180
C4:0	2.67 ± 0.38	2.53 ± 0.57	3.75 ± 1.56[a]	1.59 ± 0.81	1.55 ± 0.46	1.26 ± 0.87[b]
C6:0	3.20 ± 0.38	2.50 ± 0.75	3.63 ± 1.68	2.45 ± 0.82	2.53 ± 0.98	1.01 ± 0.48
C8:0	1.55 ± 0.05	1.32 ± 0.43	1.69 ± 0.68	1.41 ± 0.26	1.56 ± 0.85	0.95 ± 0.47
C10:0	2.18 ± 0.14	1.64 ± 0.45	2.10 ± 0.91	2.16 ± 0.64	2.15 ± 1.04	1.54 ± 0.57
C11:0	0.08 ± 0.01	0.06 ± 0.01	0.08 ± 0.04	0.08 ± 0.02	0.08 ± 0.04	0.07 ± 0.05
C12:0	1.51 ± 0.32	1.01 ± 0.06	1.18 ± 0.26	1.41 ± 0.49	1.52 ± 0.76	1.12 ± 0.37
C13:0	0.07 ± 0.02	0.05 ± 0.002	0.06 ± 0.007	0.06 ± 0.02	0.07 ± 0.02	0.05 ± 0.01
C14:0	6.92 ± 1.49	6.55 ± 0.88	5.69 ± 0.37	6.68 ± 1.11	8.10 ± 0.78[a]	5.45 ± 0.66[b]
C14:1	0.13 ± 0.03	0.13 ± 0.02	0.12 ± 0.008	0.13 ± 0.02	0.14 ± 0.03	0.10 ± 0.02
C15:0	1.16 ± 0.22	1.34 ± 0.22	1.14 ± 0.20	1.24 ± 0.07	1.30 ± 0.24	1.13 ± 0.15
C16:0	23.86 ± 1.23	26.29 ± 3.29	23.02 ± 3.39	24.24 ± 1.24	26.22 ± 2.48	23.67 ± 2.73
C16:1	1.47 ± 0.07[A]	0.54 ± 0.14[B]	0.49 ± 0.12[B]	0.42 ± 0.02[B]	0.41 ± 0.11[B]	0.37 ± 0.03[B]
C17:0	1.10 ± 0.015	1.27 ± 0.19	1.18 ± 0.25	1.16 ± 0.14	1.13 ± 0.08	1.23 ± 0.19
C17:1	0.29 ± 0.01	0.33 ± 0.06	0.30 ± 0.08	0.29 ± 0.03	0.30 ± 0.04	0.30 ± 0.03
C18:0	13.68 ± 2.28	14.07 ± 1.95	14.48 ± 1.43	14.56 ± 1.11	14.97 ± 1.72	15.65 ± 1.53
C18:1*n*-9 *trans*	7.94 ± 0.38	7.02 ± 2.59	7.16 ± 2.15	8.39 ± 0.68	6.45 ± 2.67	9.45 ± 0.15
C18:1*n*-9 *cis*	19.35 ± 0.84	21.17 ± 1.71	21.22 ± 2.64	20.48 ± 0.89	20.55 ± 0.69	21.95 ± 1.51
C18:2*n*-6 *trans*	0.23 ± 0.025	0.19 ± 0.05	0.19 ± 0.04	0.21 ± 0.004	0.18 ± 0.08	0.23 ± 0.01
C18:2*n*-6 *cis*	2.85 ± 0.03	3.01 ± 0.13	3.00 ± 0.33	2.75 ± 0.16	2.72 ± 0.12	2.89 ± 0.11
C20:0	0.86 ± 0.15	0.75 ± 0.20	0.77 ± 0.15	0.78 ± 0.03	0.72 ± 0.33	0.93 ± 0.17
C18:3*n*-6	0.08 ± 0.01	0.08 ± 0.00	0.07 ± 0.006	0.07 ± 0.01	0.07 ± 0.004	0.07 ± 0.01
C20:1	0.12 ± 0.02	0.08 ± 0.02	0.08 ± 0.03	0.09 ± 0.01	0.11 ± 0.10	0.09 ± 0.02
C18:3*n*-3	2.72 ± 0.11	3.08 ± 0.05	3.06 ± 0.27	2.90 ± 0.09	2.46 ± 0.76	3.51 ± 0.44
CLA *cis*-9, *trans*-11	3.21 ± 0.05	2.76 ± 1.49	3.02 ± 1.47	3.65 ± 0.42	2.35 ± 1.33	4.12 ± 0.55
CLA *trans*-10, *cis*-12	0.13 ± 0.03[a]	0.15 ± 0.05	0.15 ± 0.05	0.19 ± 0.007	0.14 ± 0.02	0.23 ± 0.03[b]
Others isomers	0.13 ± 0.02[a]	0.15 ± 0.05	0.16 ± 0.04	0.23 ± 0.05	0.13 ± 0.03[a]	0.25 ± 0.03[b]
C22:0	0.46 ± 0.09	0.36 ± 0.02	0.42 ± 0.08	0.46 ± 0.05	0.42 ± 0.21	0.46 ± 0.12
C20:3n6	0.07 ± 0.009	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.005	0.07 ± 0.05	0.06 ± 0.01
C22:1n9	0.11 ± 0.08	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.32 ± 0.50	0.04 ± 0.02
C20:3*n*-3	0.03 ± 0.005	0.04 ± 0.01	0.05 ± 0.04	0.04 ± 0.004	0.02 ± 0.005	0.05 ± 0.01
C20:4*n*-6	0.41 ± 0.11	0.33 ± 0.03	0.40 ± 0.06	0.40 ± 0.05	0.23 ± 0.04	0.38 ± 0.09
C23:0	0.47 ± 0.10	0.35 ± 0.10	0.38 ± 0.09	0.41 ± 0.03	0.33 ± 0.08	0.41 ± 0.08
C22:2*n*-6	0.25 ± 0.12	0.21 ± 0.13	0.24 ± 0.16	0.30 ± 0.07	0.21 ± 0.03	0.31 ± 0.13
C24:0	0.48 ± 0.13	0.35 ± 0.02	0.42 ± 0.09	0.44 ± 0.06	0.34 ± 0.08	0.43 ± 0.11
C20:5*n*-3	0.22 ± 0.01[A]	0.18 ± 0.04	0.19 ± 0.03	0.21 ± 0.01	0.13 ± 0.01[B]	0.22 ± 0.03[A]
$\sum SFA_s$	60.25 ± 0.40	60.47 ± 2.91	59.98 ± 0.85	59.14 ± 1.34	62.98 ± 3.64[A]	55.36 ± 1.76[B]
$\sum MUFA_s$	29.40 ± 0.34	29.30 ± 1.33	29.40 ± 0.72	29.84 ± 1.09	28.29 ± 1.96[A]	32.30 ± 1.41[B]
$\sum PUFA_s$	10.35 ± 0.23	10.23 ± 1.58	10.61 ± 1.19	11.02 ± 0.48	8.73 ± 2.13[a]	12.34 ± 0.84[b]

[a] Abbreviations: $\sum SFA_s$, sum of saturated fatty acids; $\sum MUFA_s$, sum of monounsaturated fatty acids; $\sum PUFA_s$, polyunsaturated fatty acids. Values were expressed as mean ± standard deviation. [A–B] Different superscript uppercase letters indicate a significant difference at $P < 0.01$. [a-b] Different superscript lowercase letters indicate a significant difference at $P < 0.05$.

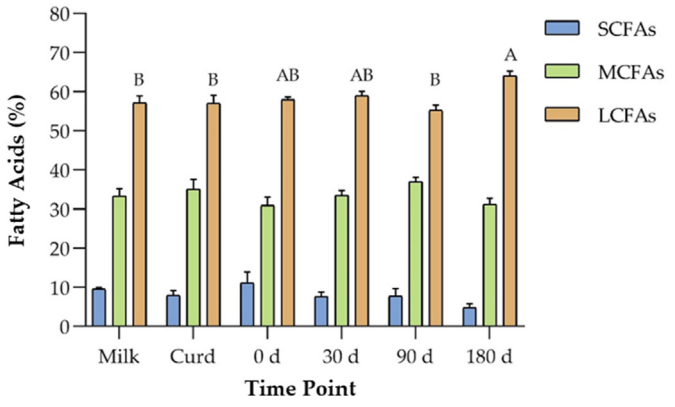

Fig. 2. Changes in content of short-chain fatty acids, $SCFA_s$ (blue bars); medium-chain fatty acids, $MCFA_s$ (green bars) and long-chain fatty acids, $LCFA_s$ (orange bars) at each time point during cheese-making and ripening of Pecorino Bagnolese. $SCFA_s$, short-chain fatty acids (C4–C10); $MCFA_s$, medium-chain fatty acids (C12–C16); $LCFA_s$, long-chain fatty acids (C18:0 - C20:5). No letters are provided above the bars where there were no statistically significant differences observed between the FA groups at any time point ($P > 0.05$). [A–B] Different uppercase letters above the bars indicate a significant difference at $P < 0.01$.

increase ($P > 0.05$) during the first month of ripening (30 d). The same behavior of CLA_s content is described by Buccioni et al., 2012; Govari et al., 2020, who reported higher values of CLA *cis*-9, *trans*-11 in Pecorino Toscano ovine cheese and Kefalotyri cheese compared to their respective curds after the first month of ripening. However, with further ripening (30–90 d), the CLA_s content decreased ($P > 0.05$), only to increase again at the end of the ripening period (180 d). Some authors that have studied the effect of various ripening stages in different varieties of Swedish hard cheeses (Jiang et al., 1997) and Spanish Protected Designation of Origin (PDO) cheeses (Luna et al., 2007), have concluded that the ripening process has minimal impact on the CLA_s content of cheese. However, the effect of processing and ripening on CLA_s content in cheeses remains uncertain because of the various cheese types documented in the literature and their apparent dissimilarities, making data comparisons challenging.

As for the nutritional indices, AI and TI, they remained stable throughout the cheese-making and ripening period, although at the end of ripening, AI showed a slight but significant ($P < 0.01$) decrease. Overall, both indices demonstrated positive health attributes, as reported by Chen & Liu, 2020 and Beata Paszczyk et al., 2022. At the same time, both HPI and h/H increased ($P < 0.05$) at the

Table 3
Nutritional index of Bagnolese ewe milk and Pecorino Bagnolese during cheese-making and ripening.[a]

	Milk	Curd	Ripening time (d)			
			0	30	90	180
CLA_s	4.44 ± 0.09	4.02 ± 1.58	4.31 ± 1.57	5.03 ± 0.41	3.60 ± 1.37	5.57 ± 0.54
n-3	2.95 ± 0.12	3.26 ± 0.09	3.27 ± 0.24	3.13 ± 0.10	2.57 ± 0.76[a]	3.74 ± 0.46[b]
n-6	3.84 ± 0.07	3.83 ± 0.02	3.92 ± 0.17[A]	3.75 ± 0.22	3.46 ± 0.07[B]	3.91 ± 0.28
AI	1.46 ± 0.22	1.47 ± 0.23	1.28 ± 0.09	1.43 ± 0.18	1.75 ± 0.15[A]	1.16 ± 0.13[B]
TI	2.50 ± 0.06	2.69 ± 0.34	2.46 ± 0.23	2.58 ± 0.18	2.97 ± 0.18	2.35 ± 0.27
h/H	0.96 ± 0.12	0.97 ± 0.16	1.10 ± 0.09	1.01 ± 0.08	0.85 ± 0.04[a]	1.17 ± 0.16[b]
HPI	0.69 ± 0.12	0.69 ± 0.12	0.78 ± 0.05	0.70 ± 0.08	0.57 ± 0.05[a]	0.86 ± 0.09[b]

[a] Abbreviations: CLA_s, conjugated linoleic acids; *n*-3, omega 3 PUFA; *n*-6, omega 6 PUFA; IA, Index of Atherogenicity; IT, Index of Thrombogenicity; h/H, Hypocholesterolemic/Hypercholesterolemic ratio and HPI, Health-Promoting Index. Values were expressed as mean ± standard deviation. [A–B] Different superscript uppercase letters indicate a significant difference at $P < 0.01$. [a-b] Different superscript lowercase letters indicate a significant difference at $P < 0.05$.

end of the ripening process. From a nutritional standpoint, elevated HPI and h/H values are deemed advantageous for human health, as indicated by Chen & Liu, 2020.

3.3. Lipolysis and oxidative stability in Pecorino Bagnolese

The lipolysis and oxidative changes during production and ripening period of Pecorino Bagnolese are summarised in Table 4. The assessment of $TBAR_s$ and FFA_s levels is crucial in ripened cheese, as their progression is affected by the chemical attributes of raw milk and cheeses, including the fatty acids profile and the presence of antioxidant substance (Boutoial et al., 2013). The traditional production of cheese ripened in caves and the environmental conditions of ripening, contributes to increasing the variability in cheese quality, impacting the $TBAR_s$ and FFA levels. During the production and ripening period, the concentration of oxidation products ($TBAR_s$) remained consistently low. However, lipid oxidation was not a significant process in this cheese, likely due to the presence of natural milk antioxidants and a low redox potential as suggested by Moreira et al., 2020; Balthazar et al., 2017; Boutoial et al., 2013 observed similar trends in fresh goat milk cheese, whereas Delgado et al., 2011 documented an increase in malondialdehyde (MDA) levels over the course of 30 d during the ripening of raw goat milk cheese. Fat hydrolysis (FFA) showed an increasing trend during the ripening period, highlighting an initial intense enzymatic activity. During the last step of ripening (90 and 180 d), the rate of lipolysis in the cheese decreased. This decline may be attributed to reduced enzymatic activity resulting from micro-environmental changes (moisture or pH value) within the cheese (Delgado et al., 2011).

3.4. Microbiological profile

In the present study, the populations of different microbial groups during the cheese-making and ripening was monitored (Table 5). Since the safety and quality of the raw matrix may impact on the composition and quality of the derived products (Fusco et al., 2020) the microbiological profile in raw milk was also evaluated. The microbiological counts revealed no significant differences, except between total aerobic bacteria 30 °C (TAB 30 °C) and mesophilic lactobacilli at 180 d of ripening ($P < 0.05$).

The mean level of TAB 30 °C (5.16 log cfu ml^{-1}) in raw milk (Table 5) is slightly lower than in other studies in raw ewe's milk (Pisano et al., 2006 = 5.72 log cfu ml^{-1}; Schirone et al., 2012 = about 6–7 log cfu ml^{-1}; Centi et al., 2017 = 5.40 log cfu ml^{-1}) and complies with the legislative threshold value. According to European Regulation (EC) No. 853/2004 raw milk, from species other than cow, intended for the manufacturing of raw milk products should not contain more than 500 000 cfu mL^{-1}. This parameter represents an important indicator of sanitary quality because it reflects the extent of the microbiological contaminations of the raw material. In the present study, the count reached the maximum value of 8.92 log cfu g^{-1} after 30 d of ripening, which was in accordance with Blaiotta et al., 2021, who showed a load of 8–9 log cfu g^{-1} after 25 d of ripening of Conciato Romano cheese. After that, the count suffered a decrease of about two log units until the end of ripening (180 d). The decrease in microbial counts during maturation was presumably due to the decrease in a_w and moisture, as shown in Table 1. In fact, it is known that microbial dynamics are strongly influenced by parameters such as pH, a_w, salt concentration and redox potential, which may vary widely during ripening (Schirone et al., 2012).

Determination of the number of *Enterobacteriaceae* can be used as a marker of sanitary quality of the raw milk as well as the hygiene practices prevailing during the cheese-making (Giammanco et al., 2011). These bacteria are considered a negative microflora. Some of these are potential pathogens as well as responsible for cheese texture defects, blowing, and off-flavours (Nam et al., 2010). In the present study, the count of *Enterobacteriaceae* (3.48 log cfu ml^{-1}) in raw milk increased during the cheese-making reaching a level of 5.64 log cfu g^{-1} in cheese on the day of production. This result is only slightly higher than the limit described for indicator microorganisms ($\leq 10^5$ cfu g^{-1}) by the European Recommendation 2005/2073, according to which the sample of raw milk cheese is classified as having good hygienic quality. During ripening, the counts declined to <10 cfu g^{-1} at 90 d of ripening (Table 5). Results agree with those of Tabla et al., 2016; Blaiotta et al., 2021 which observed the same decreasing trend during the aging of two semi-

Table 4
Lipolysis and oxidative stability of Pecorino Bagnolese during cheese-making and ripening.[a]

	Curd	Ripening time (d)			
		0	30	90	180
TBARs, mgMDA/kg	0.00 ± 0.00[A]	0.02 ± 0.01[aA]	0.06 ± 0.01[bB]	0.09 ± 0.01[cB]	0.13 ± 0.02[dB]
FFA, % olecic acid	0.04 ± 0.00[aA]	0.06 ± 0.01[aA]	0.14 ± 0.00[A]	0.19 ± 0.02[b]	0.35 ± 0.14[aB]

[a] Values were expressed as mean ± standard deviation. [A–B] Different superscript uppercase letters indicate a significant difference at $P < 0.01$. [a-b] Different superscript lowercase letters indicate a significant difference at $P < 0.05$.

Table 5
Counts of the microbial groups (log cfu g^{-1} or mL^{-1}) in Bagnolese ewe milk and Pecorino Bagnolese during cheese-making and ripening.[a]

Microbial Groups	Milk	Curd	Ripening time (d)			
			0	30	90	180
TAB 30 °C	5.16 ± 0.25	5.61 ± 0.37	6.66 ± 0.47	8.92 ± 0.08	7.86 ± 0.14	7.02 ± 0.18
Enterobacteriaceae	3.48 ± 0.94	4.21 ± 0.96	5.64 ± 0.80	3.60 ± 0.84	<10 cfu g^{-1}	<10 cfu g^{-1}
Coagulase-positive Staphylococci	2.59 ± 0.55	3.37 ± 0.67	4.74 ± 0.67	3.58 ± 0.35	3.78 ± 0.34	2.66 ± 0.36
Mesophilic lactobacilli	3.80 ± 0.35	5.38 ± 0.54	6.30 ± 0.76	7.29 ± 0.56	6.45 ± 0.55	3.65 ± 0.50
Mesophilic lactococci	4.61 ± 0.41	5.12 ± 0.63	6.44 ± 1.65	7.96 ± 1.51	6.26 ± 1.05	7.24 ± 0.37

[a] Abbreviation: TAB 30 °C, total aerobic bacteria 30 °C. Values were expressed as mean ± standard deviation.

hard ewe cheeses; in particular in them studies *Enterobacteriaceae* were no longer detectable after 60 and 25 d of ripening, respectively. The evolution of these bacteria is closely interconnected with the long ripening and to the related harsher physicochemical conditions at the end of storage, which contribute significantly to their lower survival. It is known that the decrease in pH is due to the metabolic products (especially organic acids such as lactic acid) obtained from lactose fermentation by the lactic acid bacteria (LAB), which indirectly and directly with an antagonistic interaction, prevent the growth of *Enterobacteriaceae* (Nuraida, 2015).

The count of coagulase-positive staphylococci is recognised as process hygiene criteria by European Regulations (EC) No. 2073/2005. In the present study, their presence was detected in all the samples analysed, including the raw milk with a value of 2.59 log cfu g^{-1}. The highest value was found in cheese on the day of production. Their mean value (4.74 log cfu g^{-1}) was only slightly below the limit of 10^5 cfu g^{-1} set by the aforementioned regulation since the cheese produced in June exceeded this limit (5.61 log cfu g^{-1}), as can also be observed from the standard deviation. Exceeding this threshold requires improved hygienic conditions as well as testing of staphylococcal toxins in the final products, because they may represent a risk factor for consumers (Giammanco et al., 2011). It is worth noting that during ripening the count ranged from 2.66 to 3.78 log cfu g^{-1}, in agreement with results of Schirone et al., 2012 for Pecorino cheeses (about 2–5 log cfu g^{-1}).

Concerning *S. aureus*, in raw milk samples it was never identified (out of a total of 10 identified colonies: 8 of *Staphylococcus chromogenes,* one of *Staphylococcus pasteuri* and one of *Staphylococcus warneri*). This result is in contrast with the study of van den Brom et al., 2020 who reported a high prevalence of this pathogen in sheep milk. Instead, the presence of *S. aureus* was confirmed in curd of the batches produced in May and June (out of a total of 15 identified colonies: 10 of *S. chromogenes*, three of *S. aureus* and two of *Staphylococcus borealis*) in cheese on the day of production of the batches produced in May and June (out of a total of 35 identified colonies: 18 of *S. chromogenes*, 12 of *S. aureus*, five of *Staphylococcus epidermidis*) and in a 1-month-old cheese sample of the batch of June (three colonies of *S. aureus* were identified). This result suggests a possible contamination from handling and insufficient sanitary conditions during manufacturing. In the samples after 90 and 180 d of maturation *S. aureus* was no longer detected, in line with the disappearance of this pathogen after 60 d of maturation of Pecorino Siciliano cheese, investigated by Cardamone et al., 2020. Although the coagulase-positive staphylococci mean load was below the limit value for toxin production, the presence of *S. aureus* must not to be ignored, because, as reported by the annual report of European Food Safety Authority (EFSA)and European Centre for Disease Prevention and Control (ECDC) (EFSA and ECDC, 2021), it continues to be a threat for the safety of milk and raw milk dairy products causing food poisonings (nausea, vomiting, and diarrhoea).

Lactobacilli and lactococci usually dominate the microflora of almost all cheese varieties, whether traditional or not (Montel et al., 2014). The autochthonous LAB, thanks to their enzymatic systems which allow them to use nutrients of milk efficiently, are capable of growing in the hostile conditions of pH, a_w and moisture (Wouters et al., 2002), like those recorded in our study during ripening (Table 1). In the present research, mesophilic lactobacilli and mesophilic lactococci showed similar loads in raw milk (3.80 and 4.61 log cfu ml^{-1}, respectively) with an increase of about 1 log unit in the curd and a comparable temporal evolution, recording a peak after 30 d of ripening followed by a slight reduction at 90 d. The values of mesophilic lactobacilli achieved at 90 d (6.45 log cfu g^{-1}) are consistent with those recorded by Schirone et al., 2011 in Pecorino di Farindola and with those of Mormile et al., 2016 in Pecorino di Tramonti, but they were lower (about 2 log units) than loads in Pecorino Abruzzese in the research of Centi et al., 2017. Instead, mesophilic lactococci (6.26 log cfu g^{-1}) were lower than the results obtained in Pecorino di Tramonti (about 7 log cfu g^{-1}) and Pecorino Abruzzese (about 8 log cfu g^{-1}) by the same authors. At 180 d of maturation, a statistically significant ($P < 0.05$) reduction of mesophilic lactobacilli (3.65 log cfu mL^{-1}) was recorded, compared to TAB 30 °C (7.02 log cfu g^{-1}). This result is in agreement with the study by Pisano et al., 2006 on Fiore Sardo (2.58 log cfu g^{-1}) but strongly in disagreement with the study on Pecorino Abruzzese (about 8 log cfu g^{-1}). According to our knowledge, there is no extensive bibliography on the microbiological profile of sheep's milk cheeses at 180 d of ripening. The trend of autochthonous LAB of cheese in our study corresponded with the pH values found (Table 1). Indeed, when a high bacterial load was observed, a lower pH was recorded (from 6.85 ± 0.06 in the curd to 5.26 ± 0.06 at 30 d; $P < 0.01$). The key role of LAB in the acidification of the product is therefore confirmed, as it has been widely demonstrated that this group of bacteria produces a high amount of lactic acid, as a consequence of their intense enzymatic activity (Caridi et al., 2003).

Therefore, according to the results of the present study, a similar behaviour of all microbial groups investigated is evident during the cheese-making, with an increase of approximately 1 log from milk to curd, and equally from curd to cheese on the day of production. This trend could be attributed to the physical entrapment of microorganisms during the curd-making process and their multiplication after curdling (Metz et al., 2020).

Regarding pathogenic bacteria investigated, *L. monocytogenes* and *Salmonella* spp. were never detected (data not shown). These bacteria are among the main pathogens that can contaminate sheep dairy products (Ricci et al., 2018). In the present study cheese samples were conformed to the food safety criteria prescribed in the European Regulation (EC) No. 1441/2007 for ready-to-eat foods able to support the growth of *L. monocytogenes* as Pecorino Bagnolese had pH values higher than 5.0 (as shown in Table 1). *L. monocytogenes* is primarily an environmental pathogen, and its contamination may occur at any stage of food processing, due to its ubiquitous nature and capacity to survive in several conditions. The absence in milk samples, in this research, is consistent with the low prevalence in sheep's milk, as reported by the study of Condoleo

et al., 2020 in which *L. monoytogenes* was never detected in 372 milk samples. The possibilities of pathogen's survival in artificially contaminated fresh cheese have been already proved by Murru et al., 2018, results obtained in the present work confirm its lesser ability to grow in hard cheeses (Buchanan et al., 2017). Likewise, the absence of Salmonella spp. in milk is in accordance with the low prevalence (1.4%) in sheep raw milk reported by (Gonzales-Barron et al., 2017). Our findings demonstrate the application of correct hygiene procedures in the farm since the major cause of the presence of this bacterium into raw milk is the contamination with faecal material from the external surface of the udder during milking operations. A low prevalence (0.2 %) of *Salmonella* in cheese made from sheep milk was reported also by van den Brom et al., 2020. Hurdles such as those present during ripening inactivate *Salmonella*, whereby, outbreaks related to consumption of dairy products predominantly involve raw milk and fresh raw milk cheeses (Fusco et al., 2020).

4. Conclusions

Overall, this research represents the first study focused on the characterisation of Pecorino Bagnolese during cheese-making and ripening and provides better knowledge of its pysicochemical and microbiological characteristics connected with traditional production procedures. The data collected in this study indicate that the manufacturing technology used supported to ensure the nutritional quality and safety of the cheese. Nutritional indices (atherogenicity, thrombogenicity and health-promoting index) demonstrated positive health attributes. This aspect is supported by the decrease in $SCFA_s$ compared to the raw milk and the high content of CLA_s in the cheese, that increased during the ripening period. The oxidation of lipids was not a significant process in the ripening of Pecorino Bagnolese, thus preserving the cheese's quality attributes. The sanitary quality of raw milk used for manufacturing was of an acceptable level, respecting the legislative threshold value. In this study cheese can be considered a safe product due to the absence of the main pathogenic bacteria (*Salmonella* spp. and *L. monocytogenes*). However, the presence of *S. aureus* in samples of curd and cheese on day of production and cheese after 30 d of ripening suggests that a major attention should be paid to hygienic practices used during the cheese-making process. The study demonstrated that the long ripening of cheese decreases the number of undesired bacteria such as *Enterobacteriaceae* and coagulase-positive staphylococci. The characteristics of Pecorino Bagnolese, coupled with the biodiversity significance of Bagnolese sheep, have the potential to bolster the distinctiveness of this cheese while also supporting the livelihoods of local farmers.

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CRediT authorship contribution statement

Roberta Mazzocca: Formal analysis, Investigation, Writing – original draft. **Marika Di Paolo:** Formal analysis, Investigation, Writing – original draft. **Maria Francesca Peruzy:** Supervision, Validation, Writing – review & editing. **Antonio Rippa:** Formal analysis. **Adriano Michele Luigi Santoro:** Methodology. **Vincenzo Peretti:** Funding acquisition, Project administration, Writing – review & editing. **Raffaele Marrone:** Supervision, Validation, Writing – review & editing. **Nicoletta Murru:** Supervision, Validation, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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