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Reduction of PDO Pecorino Siciliano cheese making duration: Microbial dynamics and quality attributes deriving from replacing whey permeate with hot water during cooking

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ABSTRACT

This work was carried out with the aim to reduce the transformation duration of Protected Designation of Origin (PDO) Pecorino Siciliano cheese. To this purpose, the cooking in hot water (experimental production, EXP) was compared to the traditional cheese cooking under whey permeate (control production, CTR). The microbiological composition of under rind (UR) and core (Co) section of CTR and EXP cheeses was determined by a combined culture-dependent and -independent approach. Total mesophilic microorganisms and lactic acid bacteria (LAB) present in raw ewes' milk (5.0 log CFU/mL) increased during cheese making and reached values of about 8.0 log CFU/g in both sections (UR and Co) of 5-month ripened cheeses of both productions (CTR and EXP) monitored. The identification of the viable LAB populations in ripened cheeses showed that *Enterococcus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Limosilactobacillus* and *Streptococcus* dominated UR and Co sections of all cheeses. MiSeq Illumina analysis demonstrated that LAB populations (lactobacilli, lactococci and streptococci) dominated the bacterial community of cheeses at 95.63–98.41 % of relative abundance. The two different cooking operations did not influence the physicochemical characteristics of PDO Pecorino Siciliano cheeses. Sensory evaluation performed by artificial senses analysis and trained panelists confirmed that the modification of PDO Pecorino Siciliano cheese production protocol did not significantly affect product characteristics and overall acceptance. Thus, data of this work confirmed that cooking under hot water allowed to reduce transformation duration and safeguard typicality of PDO Pecorino Siciliano cheese.

1. Introduction

Pecorino Siciliano is a traditional semi-hard Italian raw ewes' milk cheese that gained the Protected Designation of Origin (PDO) status by the European Community (Commission Regulation 1107, 1996). PDO Pecorino Siciliano cheese is produced throughout Sicily only in small enterprises gathered into a protection consortium applying a century-old production protocol (GUCE C 170 EUR-Lex - 52020XC0518(03)). Recently, the production and consumption of Pecorino cheese type is on the increase (Centorotola et al., 2021), and this phenomenon is strictly linked to the re-discovery of natural and historical cheeses by the postmodern consumer (Braghieri et al., 2014). The use of wooden tools, lamb rennet paste, raw ewes' milk and the absence of lactic acid bacteria (LAB) starter cultures added characterize typical PDO Pecorino Siciliano cheese making (Gaglio et al., 2021). As a matter of fact, the production of this cheese relies on the presence of indigenous LAB present in raw materials, equipment and transformation environment (Ruta et al., 2023).

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Microbiota naturally associated with the production of PDO Pecorino Siciliano cheese include starter LAB (SLAB), able to generate a high amount of lactic acid during the very first steps of cheese productions through the fermentation of lactose, and nonstarter LAB (NSLAB), involved in the development of the typical sensory traits of cheeses (Gaglio et al., 2021). Even though some undesirable microorganisms have been found in PDO Pecorino Siciliano cheese during ripening (Todaro et al., 2011), typical dairy pathogens associated with the microbiological food safety criteria (Commission Regulation 2073, 2005), such as *Listeria monocytogenes* and *Salmonella* spp., have never been detected along the entire production process (Guarcello et al., 2016; Settanni et al., 2013).

Traditionally, PDO Pecorino Siciliano cheese is molded into rattan baskets which are kept under hot whey permeate at about 75 °C for 3–4 h; this production step is known as the cooking phase. This phase is intimately related to the production of Ricotta cheese obtained by thermal precipitation of whey proteins (Mangione et al., 2023). The whey permeate resulting from Ricotta cheese production, namely "scotta" (Settanni et al., 2020a), is used to perform PDO Pecorino Siciliano cheese cooking. Thus, PDO Pecorino Siciliano cheese production depends on the availability of scotta which takes approximately 2 h. During this time interval, Pecorino Siciliano pressed curd in the rattan baskets remains at room temperature and undesired microorganisms might negatively influence the quality of the final product.

The cooking phase represents a crucial step in cheese-making; it affects physicochemical and sensory aspects of semi-hard cooked cheeses (Güler et al., 2021). In particular, cheese cooking influences the syneresis of the curd, protein, fat and volatile organic compound losses, yield, color, texture and microstructure (Hayaloglu et al., 2008; Lucey and Kelly, 1994). Up to date, the studies available in literature on the use of whey permeate for cheese cooking only focused on the effects of the temperature applied on physicochemical and sensory traits of semi-hard cooked cheeses (Hayaloglu and Brechany, 2007; Hayaloglu et al., 2010; Sulejmani et al., 2014). To our knowledge, no studies have been conducted on the evaluation of the microbiological, chemical, textural and sensory characteristics of cheeses cooked under hot water.

In this study, the replacement of whey permeate with hot water was tested in order to accelerate PDO Pecorino Siciliano cheese production by reducing the resting time before cooking. This study is part of a research project aimed to valorize the natural historic cheeses and was performed to provide in-depth insights on the effect of the different cooking procedures on PDO Pecorino Siciliano cheese characteristics. The final cheeses were subjected to the evaluation of microbiological and chemical composition as well as sensory traits by artificial sense and panel evaluation.

2. Materials and methods

2.1. Cheese production

Cheese making trials were performed at an artisanal dairy factory, located in Santa Margherita di Belìce (Agrigento, Italy), belonging to the Consortium for the protection of PDO Pecorino Siciliano cheese. Raw ewe's milk of the autochthonous Sicilian sheep breed "Valle del Belìce" was transformed applying the traditional "PDO Pecorino Siciliano" cheese technology (Fig. 1). The experimental plan included two different trials: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water. Briefly, bulk milk (200 L), heated at 38 °C was transferred into a 12 years old Douglas wooden vat and was kept under gentle manual agitation for 5 min before addition of lamb rennet paste (36 g, Rennet Regional Consortium, Poggioreale, Italy). Curdling occurred in approximately 40–50 min. The unbroken coagulum was added with hot water (20 L) at 74 °C to facilitate syneresis and was cut with a wooden paddle called "rotula" until small rice-size grains (3–7 mm diameter) were reached. After whey draining, curd

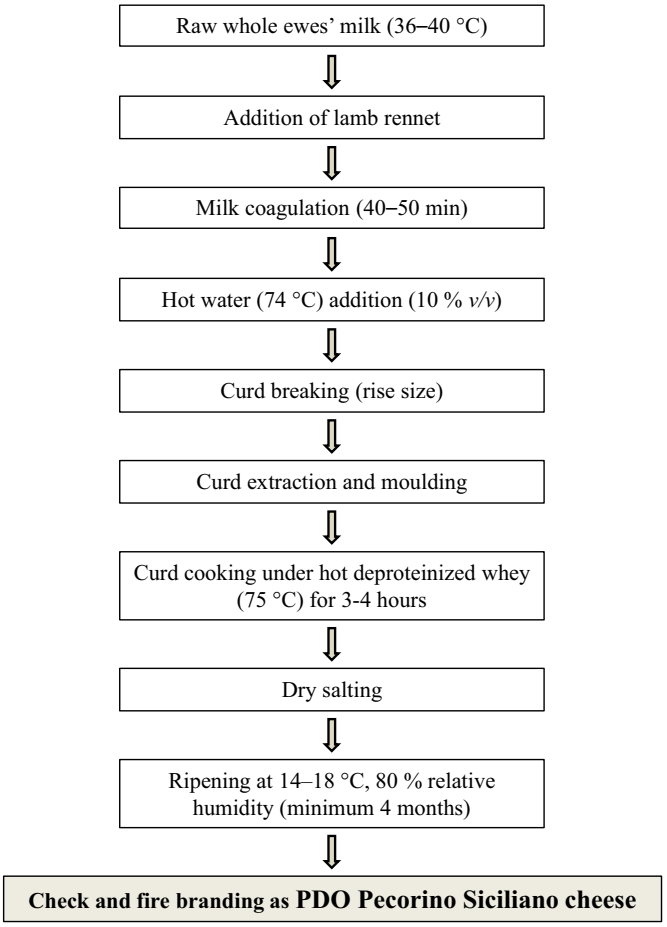


Fig. 1. Flowsheet of PDO Pecorino Siciliano cheese production.

was hand pressed into 7 kg rattan baskets, forming a total of four cheeses. Two cheeses representing EXP trial were immediately cooked under hot water at 72 °C for 3 h, while two cheeses of CTR trial remained at room temperature for 2 h, miming the average time generally occurring to obtain whey permeate from Ricotta cheese production. Also for CTR trial cheeses the cooking phase occurred at 72 °C for 3 h. Twenty-four hours after cooking, all cheeses were salted in saturated brine for 24 h, and ripened for 5 months in a storage chamber at 16 °C and 85 % of relative humidity (RH). Cheese productions was carried out in triplicate (one production per month) during February, March and April 2020 which represent the months with the higher green forage availability and traditionally suited for high quality production of PDO Pecorino Siciliano cheese.

2.2. Sample collection, temperature and pH monitoring

Control and experimental cheese making trials were thoroughly monitored; samples were collected from bulk milk before being transferred into the wooden vat and after 5 min from the transfer, curds just after curdling, cheese soon after cooking, and 5-month ripened cheeses. In order to analyze the entire cheese profile, the under rind (UR) section, located at 3 cm from the upper face, and core (Co), taken at a distance of 11 cm from both faces of each cheese, were collected. The wooden vat surface before milk contact was sampled following the brushing method described by Didienne et al. (2012).

The monitoring of pH during the production processes (from raw ewe's milk to final cheeses) was performed with a portable pH-meter (waterproof pHTestr 30, Eutech Instruments, Nijkerk, The Netherlands). The temperature of cheeses during the cooking process was

registered with two Thermo Button 22 T 8 K data loggers (VWR International Srl, Milano, Italy) inserted at molding in cheese Co and UR sections.

2.3. Microbiological analysis

One milliliter of liquid (wooden vat biofilms and milk) samples was directly serially decimally diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy) (Health Canada, 2015), while 25 g of solid (curd and cheese) samples were first homogenized in 225 mL of sodium citrate (2 % w/v) solution by a stomacher (Solís et al., 2009) and then serially diluted in Ringer's solution.

Appropriate dilutions from each sample were plated on agar media to allow the development of: total mesophilic microorganisms (TMM) spread on Skim Milk Agar (SMA) and incubated for 72 h at 30 °C; thermophilic and mesophilic coccus LAB poured in Medium 17 (M17) agar containing 5 g/L of lactose, incubated for 48 h at 44 °C and 30 °C, respectively; thermophilic rod LAB poured in whey-based agar medium (WBAM) prepared as described by Settanni et al. (2012) and incubated for 48 h at 44 °C; mesophilic rod LAB poured in de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 M), incubated for 48 h at 30 °C; total coliforms poured in violet red bile agar (VRBA), incubated for 24 h at 37 °C. In addition, all samples were also analyzed for the presence of the main pathogenic microorganisms: coagulase-positive staphylococci (CPS) applying the UNI EN ISO 6888-2 (ISO, 2021); *Escherichia coli* the ISO 4832 (ISO, 2006); sulfite-reducing anaerobes the ISO 15213 (ISO, 2003); *Listeria monocytogenes* and *Salmonella* spp. by the Enzyme Linked Fluorescent Assay (ELFA) method as reported by Cruciata et al. (2018). All media and supplements were purchased from Biokar Diagnostics (Allonne, French), except SMA provided by Microbiol Diagnostici (Uta, Italy). All microbiological counts were carried out in duplicates.

2.4. LAB isolation, differentiation and identification

Presumptive LAB colonies developed from the highest cell suspensions of CTR and EXP ripened cheese samples were isolated and purified by successive sub-culturing following the approach reported by Busetta et al. (2023a). Pure cultures were tested for the preliminary LAB characteristics: Gram-positive and catalase-negative. Gram type was determined after treatment with 3 % (w/v) KOH (Gregersen, 1978), while catalase test was carried out by 3 % (v/v) H_2O_2 contact (Koneman et al., 1997). All pure cultures preliminary belonging to the LAB group were microscopically investigated to evaluate cell morphology and arrangement (Barbaccia et al., 2021). Physiological and biochemical characteristics were analyzed following the procedure described by Gaglio et al. (2014). All cultures with a coccus shape were tested for growth at pH 9.2 and with 6.5 g/L NaCl to identify enterococci able to grow in both conditions.

All LAB were subjected to genomic DNA extraction and strain differentiation following the approach reported by Gaglio et al. (2017). Briefly, crude cell extracts were analyzed by randomly amplified polymorphic DNA (RAPD)-PCR and the resulting polymorphic profiles were elaborated by the program GelCompar II version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). The isolates showing different RAPD patterns were subjected to 16S rRNA gene sequencing following the procedures applied by Weisburg et al. (1991). The resulting DNA fragments of about 1600 bp were purified by the ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced at BMR Genomics (Padova, Italy). The unequivocal identities of the sequences were determined by comparison with those available in two distinct databases, NCBI and EZ-Taxon (Gaglio et al., 2016).

2.5. DNA extraction, MiSeq library preparation, Illumina sequencing, data analysis and identification of sequences

Total genomic DNA was extracted with the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNAs were quantified by the Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Wilmintog, DE, USA).

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system were carried out at Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy) sequencing platform. The methodology was reported in detail by Gaglio et al. (2020).

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology, Qiime2, version 2020.11 (Bolyen et al., 2019). The sequences were quality-filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013). Taxonomic and compositional analysis were carried by using the plugins feature classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained, accessed Naive Bayes classifier based on the Greengenes gg_13_5_otus. tar.tgz Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/?prefix=downloads/greengenes_database/gg_13_5/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to the generate taxonomy tables. Data generated by Illumina sequencing were uploaded in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA997145.

2.6. Physicochemical analysis

Curd and cheese samples were analyzed for dry matter (DM), fat, protein (N × 6.38), and ash content according to the International Dairy Federation (IDF) standards [4A (IDF, 1982), 5B (IDF, 1986), 25 (IDF, 1964a), and 27 (IDF, 1964b), respectively]. Cheese samples soluble nitrogen (N) was determined on an aqueous filtrate using the Kjeldahl method (MAF, 1986). Water activity was determined at 23 °C at the surface of each sample slice by using an activity-meter instrument (Rotronic Int., USA). Cheese samples were assessed for Co and UR color, measured in duplicate by a Minolta Chroma Meter CR300 (Minolta, Osaka, Japan) using the illuminant C; results are expressed as lightness (L*, from 0 = black, to 100 = white), redness (a*, from red = +a, to green = −a), and yellowness (b*, from yellow = +b, to blue = −b), according to the CIE L* a* b* system. Cheese hardness was evaluated with an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy) measuring the maximum resistance to compression (compressive stress, N/mm^2) of samples (2 cm × 2 cm × 2 cm) kept at room temperature (22 °C).

2.7. Electronic nose and tongue

Odor and taste profiles of CTR and EXP cheeses were evaluated using an E-nose (FOX 4000, Alpha M.O.S., Toulouse, France) with 18 MOS sensors and a potentiometric E-tongue (αAstree, Alpha M.O.S., Toulouse, France) with 7 chemical sensors. The electronic senses were developed to mimic the function of the human senses and, in particular, the electronic nose and electronic tongue to perceive smells and tastes, respectively. This system consists of an array of non-specific, slightly selective electrochemical sensors with high stability and cross-selectivity towards volatile compounds or groups of substances present in complex liquid systems (Di Rosa and Leone, 2018). The array of sensors is combined with an appropriate pattern recognition system that can interpret complex signals from those sensors producing the

fingerprint of the product as human senses produce in brain. Each sample was tested five times with the electronic nose and 10 times with the electronic tongue.

2.8. Sensory analyses

Five-month ripened CTR and EXP cheeses were also evaluated for their sensory traits by a panel of judges. All cheeses were judged by 12 assessor members including six men and six women (aged between 21 and 65 years old) familiar with the sensory analysis of cheeses. The analysis was carried out in single chambers and the panelists were specifically trained following the ISO 8589 (ISO, 2007) indications. The cheeses were acclimated at about 20 °C for 1 h, cut into cubes (3 cm × 3 cm × 3 cm) and then coded and served in a random order. Sixteen descriptive attributes grouped into aspect, aroma, taste, and texture categories were judged and scored using a line scale from 1 to 9 (cm) as reported by Gaglio et al. (2019a).

2.9. Statistical analyses

Microbiological, chemical and physical data of cheeses were statistically analyzed with the following ANOVA linear model: $Y_{ijk} = \mu + (\text{cooking} \times \text{cheese})_{ij} + \varepsilon_{ijk}$ where cooking is the liquid utilized to cook the cheeses (CTR, EXP); cheese is the fixed factor cheese type (cheese soon after cooking, PDO Pecorino Siciliano); Chemical and physical cheese data were analyzed with the following ANOVA linear model: $Y_{ijk} = \mu + (\text{cooking} \times \text{sampling})_{ij} + \varepsilon_{ijk}$ where cooking is the different liquid utilized to cook the cheeses (CTR, EXP); sampling is the zone of cheese analyzed (UR, Co). Sensorial parameters were analyzed with the following ANOVA linear model: $Y_{ijk} = \mu + \text{Panellist}_i + \text{cooking}_j + \varepsilon_{ijk}$ were Panellist_i is the fixed factor "expert judges" (1.12) and cooking is the liquid utilized to cook the cheeses (CTR, EXP); least square means were reported as spider graph. The Student "t" test was used for means comparisons at $p < 0.05$ and $p < 0.01$ significance level, while the statistical software used was SAS 9.1.2 (Procedure General Linear Model procedure). Results from E-nose and E-tongue sensors were subjected to exploratory Principal Component Analysis (PCA) and expressed based upon the Discrimination Index (DI).

3. Results and discussion

3.1. Monitoring of temperatures during cheese making

Table 1 shows the temperatures of the curd detected during the entire cheese-making process. During molding, the temperature of the curds was highly similar, independently on the cooking system (CTR vs EXP) and cheese section (UR vs Co). After molding, EXP cheeses exhibited higher temperatures than CTR cheeses at the beginning of the cooking operation ($p < 0.01$). This observation is undoubtedly the result of the resting of CTR cheeses before being covered by the whey permeate residual from Ricotta cheese production. During cooking the temperatures increased for both trials, but, after 3 h, the differences between CTR and EXP cheeses were still significant ($p < 0.05$). After 30 and 120 min from the end of cooking, the temperatures were not statistically significant between the two production trials. Regarding cheese sections, core temperature was characterized by almost 2 °C higher than the under rind. The slow increase of core temperature is due to the slow heat penetration into the solid matrix of the cheese depending on its low thermal conductivity that delays heat transfer. The low thermal conductivity of dairy products depends on their composition; it is directly related to water content and inversely to fat and protein content (Tavman and Tavman, 1999).

3.2. Evolution of microbial populations during cheese making

The results of the plate counts carried out throughout cheese production from wooden vat surface to CTR and EXP cheeses after 5-month of ripening are reported in Table 2. The specific search for *L. monocytogenes* and *Salmonella* spp., responsible for food-borne outbreaks (Nguyen et al., 2016), did not show any growth from either of the samples analyzed (for this reason, these results are not included in Table 2). The surfaces of the wooden vat, hosted levels of TMM, mesophilic rod and coccus, as well as thermophilic rod and coccus LAB of about 10^6 CFU/cm^2. High levels of these microorganisms are often detected in wooden equipment used to process PDO traditional Sicilian cheeses (Busetta et al., 2021; Gaglio et al., 2016; Settanni et al., 2021), and this is imputable to the ability of LAB to colonize the inner surfaces of the wooden vats used to process these cheeses (Cruciata et al., 2018). Within the undesired bacteria, CPS were undetectable (<1 log CFU/mL), while total coliforms and *E. coli* were around 10^2 CFU/cm^2. Similar levels of these bacteria have been previously observed onto the surfaces of the wooden vats used for the production of Caciocavallo Palermitano and PDO Vastedda della Valle del Belìce (Scatassa et al., 2015). Bulk milk hosted TMM at almost 10^5 CFU/mL and it was dominated by mesophilic LAB cocci. An increase of about 1 log cycle was registered for TMM and all LAB groups, after contact with the wooden vat. No differences were found for the levels of total coliforms, *E. coli* and CPS before and after contact with the wooden vat surface. A similar trend was previously reported by Didienne et al. (2012). After curdling, an almost 1 log increase was registered for the densities of the majority of the microbial groups investigated. This increase is an expected phenomenon after whey draining (Settanni et al., 2013). As reported in Table 2, no significant differences ($p > 0.05$) were found for the levels of all microbial groups object of investigation between the different portions (UR and Co) of both EXP and CTR cheese soon after cooking and even after 5-month of ripening. Regardless of the cooking treatment applied (water or whey permeate) and the section analyzed, all cheeses soon after production showed values of TMM and all LAB groups of about 10^8 CFU/g. These levels remained almost constant in both CTR and EXP cheeses after 5-month of ripening as previously reported for this type of cheese by different authors (De Pasquale et al., 2016; Guarcello et al., 2016; Randazzo et al., 2006). In general, higher differences are registered in terms of LAB populations characterizing the different sections of high-volume cheeses, such as Trentingrana cheese, during ripening (Monfredini et al., 2012). Concerning safety aspects, after 5-

Table 1
Temperatures detected during experimental cheese productions.

Items	Cooking (C)	Sampling (S)		SEM	*p* value "C × S"
		Co	UR		
Molding	EXP	38.6	38.2	0.49	0.327
	CTR	37.6	37.4		
Start of cooking	EXP	38.5	37.3 x	0.57	0.008
	CTR	37.1 A	34.7 B Y		
End of cooking	EXP	43.6 a	40.9 b	0.97	0.029
	CTR	41.3 a	38.9 b		
After 30 min from end of cooking	EXP	44.9 A	41.3 B	0.80	0.008
	CTR	43.0 a	40.4 b		
After 120 min from end of cooking	EXP	45.3 a	37.3 b	1.80	0.126
	CTR	42.6	39.3		
Brine	EXP	17.5	16.5	1.03	0.884
	CTR	17.4	16.9		

On the row, values with different superscript letters are significant A, B: $p \leq 0.01$; a, b: $p \leq 0.05$. On the column, values with different underscript letters are significant X, Y: $p \leq 0.01$. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; SEM = standard error of mean.

Table 2
Microbial evolution during experimental cheese productions.

Samples	Growth media							
	SMA	M17 30 °C	M17 44 °C	MRS	WBAM	VRBA	BP	TBX
Wooden vat surface	6.49 ± 0.31	6.42 ± 0.19	6.21 ± 0.22	5.88 ± 0.56	5.49 ± 0.48	1.87 ± 0.40	<1	1.69 ± 0.35
Bulk milk	4.91 ± 0.35	4.94 ± 0.42	3.89 ± 0.18	3.99 ± 0.52	1.94 ± 0.59	2.85 ± 0.59	2.26 ± 0.18	1.38 ± 0.37
Bulk milk in wooden vat	5.87 ± 0.25	5.80 ± 0.48	4.54 ± 0.18	4.40 ± 0.52	3.38 ± 0.37	3.09 ± 0.34	2.64 ± 0.24	1.64 ± 0.37
Curd	6.61 ± 0.15	6.30 ± 0.27	5.68 ± 0.19	5.68 ± 0.29	4.08 ± 0.24	3.76 ± 0.26	3.58 ± 0.16	3.24 ± 0.18
Cheese after cooking "UR"								
CTR	7.95 ± 0.21	8.20 ± 0.34	7.79 ± 0.28	7.57 ± 0.32	7.31 ± 0.53	4.41 ± 0.27	4.71 ± 0.24	4.25 ± 0.24
EXP	7.75 ± 0.17	7.88 ± 0.30	7.75 ± 0.50	7.22 ± 0.24	7.56 ± 0.57	4.29 ± 0.31	4.66 ± 0.28	3.99 ± 0.12
p value	0.269	0.289	0.910	0.215	0.608	0.640	0.826	0.169
Cheese after cooking "Co"								
CTR	8.13 ± 0.34	8.20 ± 0.34	7.79 ± 0.28	7.57 ± 0.32	7.31 ± 0.53	4.41 ± 0.27 b	4.71 ± 0.24	4.25 ± 0.24 b
EXP	8.21 ± 0.22	8.13 ± 0.36	8.08 ± 0.24	7.71 ± 0.32	7.75 ± 0.36	5.09 ± 0.27 a	5.34 ± 0.45	4.81 ± 0.24 a
p value	0.749	0.819	0.245	0.620	0.300	0.037	0.099	0.046
Ripened cheese "UR"								
CTR	7.83 ± 0.21	8.00 ± 0.40	7.87 ± 0.33	7.51 ± 0.17	7.30 ± 0.33	<2	<2	<2
EXP	7.98 ± 0.31	7.95 ± 0.33	8.19 ± 0.29	7.88 ± 0.33	7.28 ± 0.35	<2	<2	<2
p value	0.795	0.857	0.276	0.159	0.946	n.e.	n.e.	n.e.
Ripened cheese "Co"								
CTR	7.91 ± 0.23	7.73 ± 0.22	8.09 ± 0.37	7.79 ± 0.32	7.53 ± 0.35	<2	<2	<2
EXP	7.74 ± 0.21	7.79 ± 0.30	7.87 ± 0.39	7.93 ± 0.43	7.70 ± 0.30	<2	<2	<2
p value	0.398	0.794	0.518	0.674	0.654	n.e.	n.e.	n.e.

Loads are reported as log CFU/cm^2 for vat surface, log CFU/mL for milk samples, and log CFU/g for curd. Results indicate the mean values ± standard deviation (S.D.) of six plate counts (carried out in duplicate for three independent productions). Data within a row followed by different letters are significantly different according to Tukey's test. Abbreviations: SMA, Skim Milk Agar for detection of total mesophilic microorganisms; M17 30 °C, medium 17 agar incubated at 30 °C for detection of mesophilic coccus LAB; M17 44 °C, medium 17 agar incubated at 44 °C for detection of thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for detection of mesophilic rod LAB; WBAM, whey-based agar medium for detection of thermophilic rod LAB; VRBA, violet red bile agar for detection of total coliforms; BP, baird-parker agar for detection of coagulase-positive staphylococci; TBX, Tryptone Bile X-Gluc agar for detection of *E. coli*; CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; UR, under rind; Co, core; n.e., not evaluated.

month ripening, all potentially harmful microorganisms (total coliforms, *E. coli* and CPS) were below the detection limits and were in compliance with the Commission Regulation 2073 (2005) on microbiological criteria for foodstuffs (Commission Regulation 2073, 2005).

3.3. Phenotypic differentiation and genotypic identification of LAB

Four hundred and forty-two colonies of LAB (Gram-positive and catalase-negative) distinguished into 220 cocci and 222 rods were isolated from the 5-month ripened PDO Pecorino Siciliano cheeses processed in this study by cooking in water or whey permeate. Considering morphological, physiological and biochemical features, the 442 presumptive LAB cultures were separated into eight groups (Table 3). The community of coccus isolates included three groups with cells organized in long chains (group I) and short chains (groups II and III). LAB rods constituted five groups (IV to VIII), all forming short chains of cells. Among these, only one group (VII) showed a hetero-fermentative metabolism. Two hundred and nine presumptive LAB cultures (approximately 50 % of the isolates from eight phenotypic groups) were selected from both sections (UR and Co) of CTR and EXP cheeses and processed by RAPD analysis. This PCR-based technique is commonly applied for the intra- and inter-specific differentiation of dairy LAB (Rossetti and Giraffa, 2005). The dendrogram reported in Fig. 2 shows the presence of 29 distinct strains representing the viable populations dominating all sections of the final cheeses.

The identification by 16S rRNA gene sequencing indicated that all 29 strains belonged to the LAB group and they were allotted into nine species: *Enterococcus durans* (Ac. No. OR226616-OR226617); *Enterococcus faecalis* (Ac. No. OR226618-OR226619); *Enterococcus lactis* (Ac. No. OR226620); *Lacticaseibacillus paracasei* (Ac. No. OR226621-OR226631); *Lacticaseibacillus rhamnosus* (Ac. No. OR226632); *Lactiplantibacillus pentosus* (Ac. No. OR226633); *Levilactobacillus brevis* (Ac. No. OR226634-R226635); *Limosilactobacillus fermentum* (Ac. No. OR226636); *Streptococcus gallolyticus* subsp. *macedonicus* (Ac. No. OR226637-OR226644). These species are part of the typical NSLAB cultures involved in traditional cheese productions (Settanni and Moschetti, 2010). In particular, enterococci are generally isolated from PDO Pecorino Siciliano cheeses (Randazzo et al., 2006; Vernile et al., 2008; Todaro et al., 2011) and, thanks to their biochemical traits, such

Table 3
Phenotypic grouping of the LAB isolated from ripened cheeses.

Characters	Clusters							
	I (n = 143)	II (n = 61)	III (n = 16)	IV (n = 157)	V (n = 10)	VI (n = 14)	VII (n = 9)	VIII (n = 32)
Morphology	C	C	C	R	R	R	R	R
Cell disposition	lc	sc	sc	sc	sc	sc	sc	sc
Growth:								
15 °C	−	+	+	+	−	+	−	+
45 °C	+	+	+	−	+	+	+	+
pH 9.6	−	+	+	n.d.	n.d.	n.d.	n.d.	n.d.
6.5 % NaCl	−	+	+	n.d.	n.d.	n.d.	n.d.	n.d.
Resistance to 60 °C	−	−	−	−	−	−	−	+
Hydrolysis of:								
Arginine	−	+	+	−	−	−	+	+
Aesculin	−	+	+	+	−	−	−	+
Acid production from:								
Arabinose	−	+	+	−	−	+	+	+
Ribose	−	+	+	+	+	+	+	+
Xylose	−	+	+	−	−	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Lactose	+	+	+	−	+	+	+	+
Sucrose	+	+	−	+	+	+	+	+
Glycerol	+	+	+	−	−	+	+	+
CO_2 from glucose	−	−	−	−	−	−	+	−

Abbreviations: C, coccus; R, rods; lc, long chain; sc, short chain; n.d., not determined.

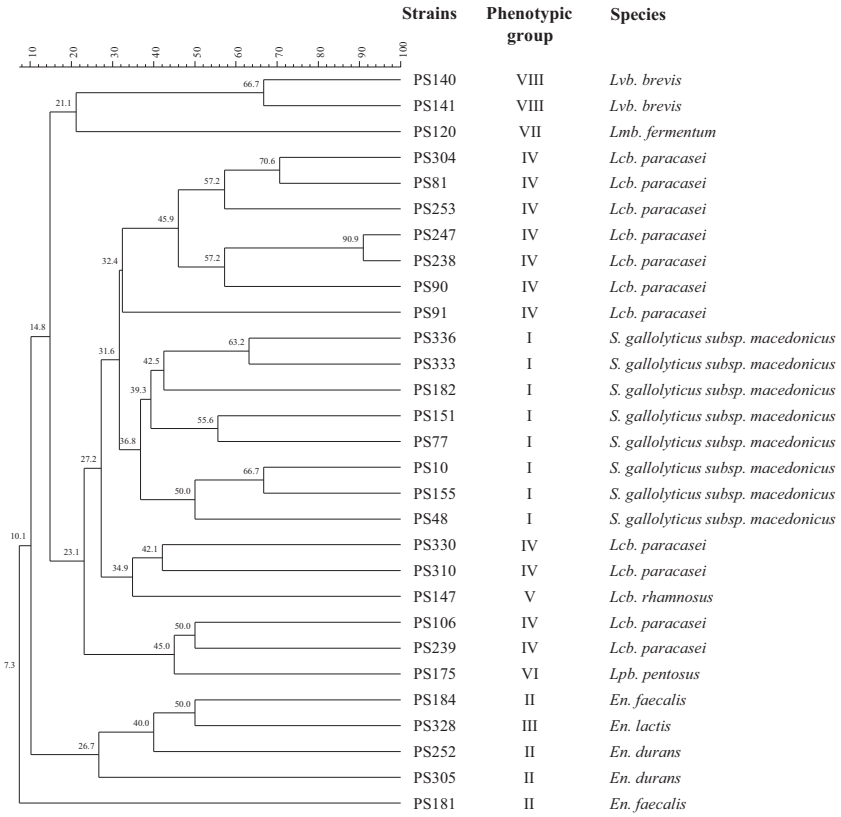


Fig. 2. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains isolated from 5-month ripened cheeses. Abbreviations: *En.*, *Enterococcus*; *Lcb.*, *Lacticaseibacillus*; *Lactiplantibacillus*; *Lvb.*, *Levilactobacillus*; *Lmb.*, *Limosilactobacillus*; *Lpb.*, *S.*, *Streptococcus*.

as lipolytic activity and citrate utilization, are considered important for the development of typical organoleptic traits that the cheese acquire with ripening (Foulquié Moreno et al., 2006). *Lacticaseibacillus paracasei* and *Lcb. rhamnosus* isolated from PDO Pecorino Siciliano cheeses have been object of selection for NSLAB addition due to their probiotic features (Caggia et al., 2015). Regarding *Streptococcus* species detected in

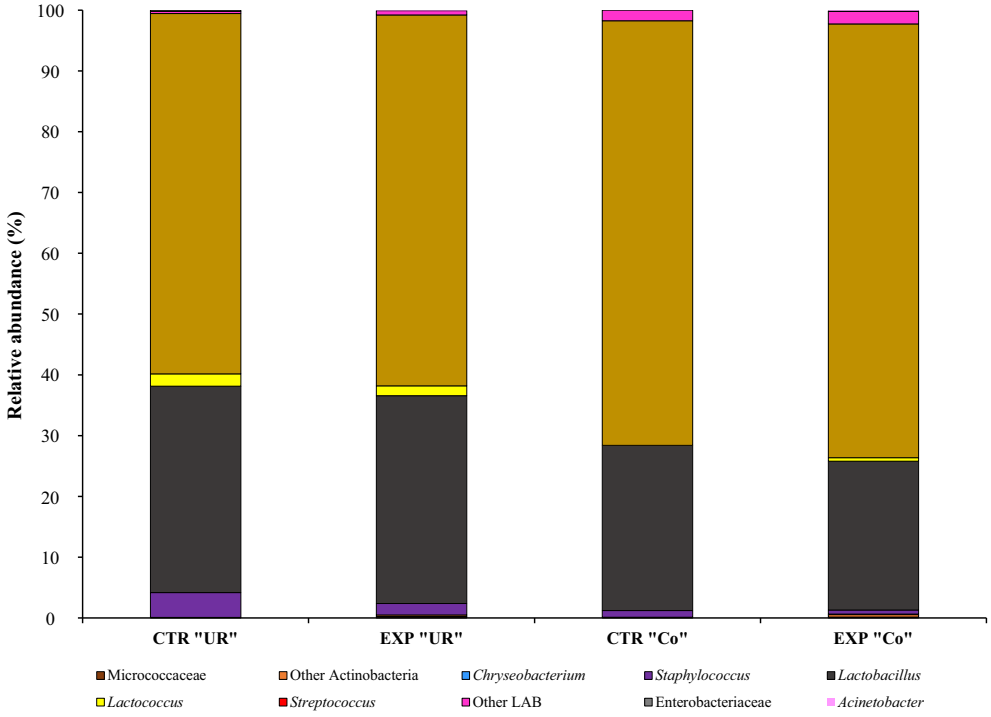


Fig. 3. Relative abundances (%) of the bacterial operational taxonomy units (OTUs) identified by MiSeq Illumina. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; UR, under rind; Co, core.

this study, *S. gallolyticus* subsp. *macedonicus* is of dairy origin and has been used as NSLAB in PDO Pecorino Siciliano cheese production (Guarcello et al., 2016; Gaglio et al., 2020; Settanni et al., 2013).

3.4. Culture-independent microbiological investigation

The microbiota associated with the different sections (UR and Co) of CTR and EXP cheeses after 5-month of ripening was also studied by DNA-based Illumina technology. This approach has been widely applied to provide a deep description of the entire bacterial composition of processed foods (Jagadeesan et al., 2019; Settanni et al., 2020b). Fig. 3 reports the bar plot of the distribution of the relative abundance (RA) % of the bacterial operational taxonomy units (OTUs) identified by MiSeq Illumina. The figure includes only the OTUs with a RA > 0.1 %, which is the minimum level generally fixed as the threshold for abundant communities (Logares et al., 2014). The taxonomy classification allowed the identification of 10 groups, the majority of which at family and genus level. LAB were detected in all samples at consistent RA % (95.63–98.41), and were classified as lactobacilli, lactococci and streptococci. The major group among cheeses, both in UR and Co section, was *Streptococcus* that ranged between 59.28 % (in UR section of CTR cheeses) and 71.31 % (in Co section of EXP cheeses) of the bacterial RA. Generally, *Streptococcus* is component of the starter LAB group (Settanni and Moschetti, 2014), but the high RA % registered in 5-month ripened cheeses could derive from the residual DNAs of no more viable cells (Gaglio et al., 2019b) or from the DNAs of *St. gallolyticus* subsp. *macedonicus* isolated at high cell densities in all sections of cheeses. However, the high percentage of these microorganisms found in the Co of both CTR and EXP cheeses is probably due to the temperatures higher to 40 °C kept in this section for at least 2 h from end of cooking, which represent the optimal growth temperatures of the species belonging to *Streptococcus* genus associated to dairy products (Gobbetti and Calasso, 2014). *Lactobacillus* was the second group most abundant in all cheese samples analyzed (27.15–33.97 % of RA). This group includes several genera in addition to *Lactobacillus*, as a consequence of the reclassification by Zheng et al. (2020). Regarding *Lactococcus*, low percentages of OTUs ranged between <0.1 % and 2.03 % were identified in the UR section of both cheese productions. The presence of lactobacilli, lactococci and streptococci at the same percentage of RA revealed in this study was previously reported for PDO Pecorino Siciliano cheese (Gaglio et al., 2020), Gran Ovino cheese (Gaglio et al., 2019a) and Grana Padano cheese (Bassi et al., 2015).

All samples object of investigation were characterized by the presence of *Staphylococcus* at very low RA % (<4 %). The presence of these bacteria at low levels is imputable to high hygiene conditions of the raw milk used for the cheese productions (Giammanco et al., 2011). Micrococcaceae, other Actinobacteria, *Chryseobacterium*, Enterobacteriaceae and *Acinetobacter* were present in all cheeses at negligible levels (<1 % of total bacterial diversity). These bacteria are commonly presents at low percentages in ripened raw milk cheeses (Busetta et al., 2023a; Busetta et al., 2023b; Gaglio et al., 2020). These results confirmed those obtained by culture-dependent approach that highlighted the dominance of LAB in both sections (UR and Co) of CTR and EXP cheeses.

3.5. Physicochemical analyses of cheeses

Gross composition of the cheeses produced in this study is reported in Table 4. The different cooking procedures did not affect the chemical composition of the final cheeses. In particular, 5-month ripened CTR and EXP cheeses were characterized by an average DM, fat, protein, and ash content of 61.56, 43.19, 48.30, and 7.06 %, respectively. These results are comparable to those registered for PDO Pecorino Siciliano cheeses produced at a large scale level in dairy factories characterized by different pedoclimatic conditions, sheep breed, and pasture (Guarcello et al., 2016). The increase of DM and ash registered during ripening is mainly due to dehydration and to the increase of chloride content over time (Caridi et al., 2003).

Table 4
Chemical analysis of curds and cheeses.

Items	Curd just after curdling	Cooking (C)	Cheese (Ch)		*p* value "C × Ch"
			Cheese soon after cooking	Ripened cheeses	
Dry matter (DM) (%)	47.02 ± 1.69	EXP	54.07 ± 0.50 ^{A}	60.72 ± 0.70 ^{B}	0.001
		CTR	55.52 ± 0.50 ^{A}	62.39 ± 0.70 ^{B}	
Fat (g/100 g DM)	41.99 ± 0.79	EXP	40.94 ± 0.68	42.78 ± 0.97	0.147
		CTR	41.49 ± 0.68	43.60 ± 0.97	
Protein (g/100 g DM)	45.76 ± 0.73	EXP	47.08 ± 0.50	48.14 ± 0.70	0.408
		CTR	47.70 ± 0.50	48.46 ± 0.70	
Ash (g/100 g DM)	5.50 ± 0.12	EXP	5.91 ± 0.11 ^{A}	7.09 ± 0.15 ^{B}	0.001
		CTR	5.83 ± 0.11 ^{A}	7.02 ± 0.15 ^{B}	

Results indicate least square means ± e.s. On the row, values with different superscript letters are significant A, B: $p \leq 0.01$; a, b: $p \leq 0.05$. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water.

The results of the physicochemical analysis performed on the different sections (UR and Co) of CTR and EXP cheeses are reported in Table 5. No differences ($p > 0.05$) were found among the sections (UR and Co) of CTR and EXP cheeses for N soluble/N total ratio (on average, 23.88 and 23,72 %, respectively). Values of this ratio between 12 and 24 % indicate that the cheeses are characterized by high amounts of bioactive peptides (Rizzello et al., 2005). Regarding colorimetric index, significant differences ($p = 0.008$) were found only for the redness (a*) value. This parameter showed a lower value in the Co of CTR cheeses. However, similar colorimetric parameters, on the whole, were previously registered for 5-month ripened PDO Pecorino Siciliano cheeses (Todaro et al., 2011) and, in general, in Pecorino cheese typology (Bennato et al., 2023; Grasso et al., 2023).

Table 5
Physicochemical parameters of different section of cheeses.

Items	Cooking (C)	Sampling (S)		SEM	*p* value "C × S"
		Co	UR		
a_w, activity water	EXP	0.99	0.98	0.01	0.292
	CTR	0.98	0.98		
pH	EXP	5.76	5.56	0.06	0.183
	CTR	5.65	5.60		
Soluble N/total N (%)	EXP	23.72	23.72	0.49	0.991
	CTR	23.88	23.88		
Hardness (kg/cm^2)	EXP	0.47	0.60	0.13	0.538
	CTR	0.52	0.73		
L*, lightness	EXP	76.87 ^{a}	68.88 ^{b}	2.25	0.008
	CTR	77.69 ^{A}	64.39 ^{B}		
a*, redness	EXP	−3.49 $_{x}$	−3.57	0.21	0.174
	CTR	−4.16 $_{y}$	−3.81		
b*, yellowness	EXP	14.60	12.45	1.26	0.152
	CTR	15.63	11.45		

Results indicate the mean values of six determination (carried out in duplicate for three independent productions).
On the row, values with different superscript letters are significant A, B: $p \leq 0.01$; a, b: $p \leq 0.05$. On the column, values with different underscript letters are significant x, y: $p \leq 0.05$.
Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; UR, under rind; Co, core; SEM = standard error of mean.

3.6. Electronic nose and tongue response

Fig. 4 shows the separate PCA plot for E-nose and E-tongue. Results indicate that the modification of traditional protocol of production of PDO Pecorino Siciliano cheese did not affect the organoleptic attributes in terms of volatile profile and taste properties. The DI is −9 % for E-nose and −12 % for E-tongue and the first two planes (PC1 and PC2) represent 99.3 % and 95.4 % of the total variance between CTR and EXP cheeses, respectively for E-nose and E-tongue. Overall, the results showed a great potential of artificial senses to find even the most subtle differences. In fact, within the CTR and EXP cheeses, the E-senses underline the variability due to the different quality of the milk over the months and the sensory attributes of the cheeses are generally correlated with the sensory quality of milk that is the result of different components related to feed and chemical compounds. Moreover, the diet can influence the sensory perception of milk and dairy products (Chiofalo et al., 2020; Liotta et al., 2019) as well as other potential factors play a role at farm level (Kilcawley et al., 2018) or during the production phase (Di Rosa et al., 2018). The different response of the E-nose and E-tongue sensors could be explained by the distribution and abundance of volatile compounds of pasture as a consequence of the different plant species (Mariaca et al., 1997), and seasonal climatic changes (Rajeswara Rao et al., 1996). The sensors that responded mostly to the volatile profile of cheese in the various months were TA/2, P30/1 and P30/2 with a strong affinity for ethanol, hydrocarbons, ketones and hydrogen sulfide (Lo Presti et al., 2023). These findings are in agreement with the observations of some authors (Bendall and Olney, 2001; Coppa et al., 2011) which found two ketones (hept-cis-4-enal and 2,3-octanedione) as important volatile aromatic components in milk obtained from different pastures, suggesting that this was due to the oxidation of linoleic and linolenic acids mostly represented in pasture milk samples. These results confirmed that the different protocols applied for cheese cooking did not influence the sensory profile of PDO Pecorino Siciliano cheeses.

3.7. Sensory evaluation of cheeses

Fig. 5 reports the radar chart of the sensory attributes evaluated on CTR and EXP ripened cheeses during taste sessions. This analysis is mandatory before marketing of a new food product since the application of a different technology of production might influence the consumers' acceptability (Fiorentini et al., 2020). In this study, the modification of traditional protocol of production of PDO Pecorino Siciliano cheese through cooking in water did not particularly affect the sensory attributes of final products. Except for the intensity of odor and unpleasant smell, all other sensory attributes object of evaluation did not differ among CTR and EXP ripened cheeses. The absence of big differences among CTR and EXP for the main sensory attributes evaluated is undoubtedly due to the use of the same raw ewes' milk for both productions. In fact, the sensory attributes of cheeses are generally correlated with the microbiological quality of milk (Fox et al., 2004), the animal diet and farm management (Kilcawley et al., 2018).

The cheese cooking under hot water influenced negatively intensity of odor and unpleasant smell. These results are not surprising, since whey permeate used for the cooking of CRT cheeses is rich in aromatic compounds (Saglam et al., 2019), which can affect the odor intensity of the final products. However, the scores registered in this research are similar to those reported in literature for PDO Pecorino Siciliano cheeses (Gaglio et al., 2020; Guarcello et al., 2016; Settanni et al., 2013). Overall satisfaction, intended as an overall rating determined on the basis of all sensory attributes with their scores (Qasem et al., 2017), did not differ significantly ($p > 0.05$) among CTR and EXP cheeses. These results confirmed that the modification of the production protocol of PDO Pecorino Siciliano cheeses did not affect the product characteristics and overall acceptance.

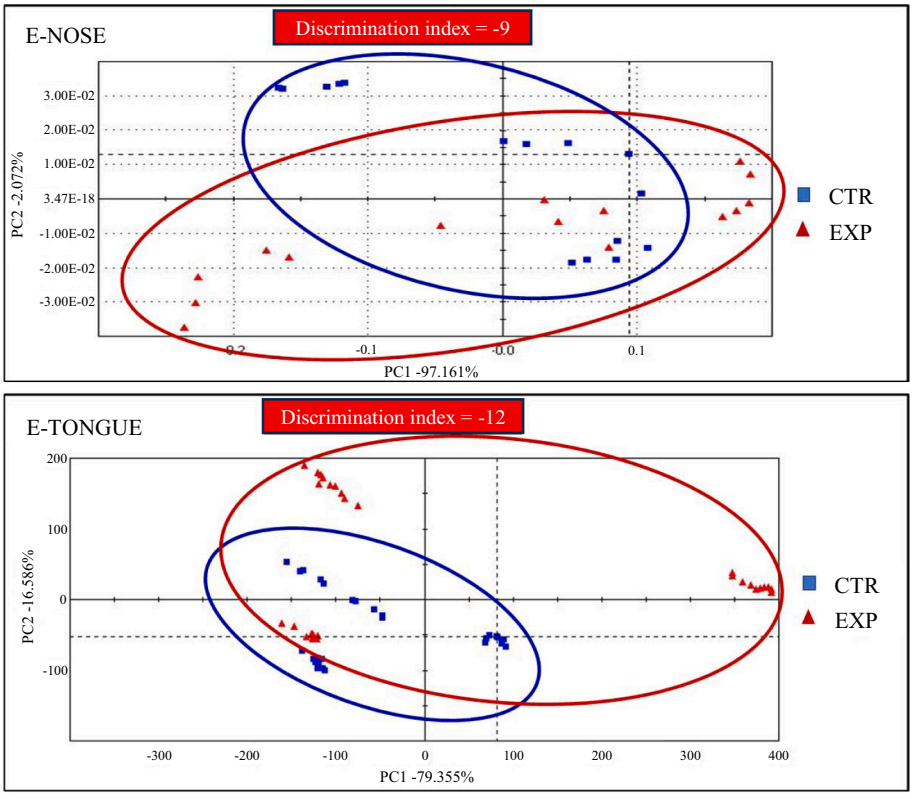


Fig. 4. Principal component analysis plot resulting from e-nose and e-tongue. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water.

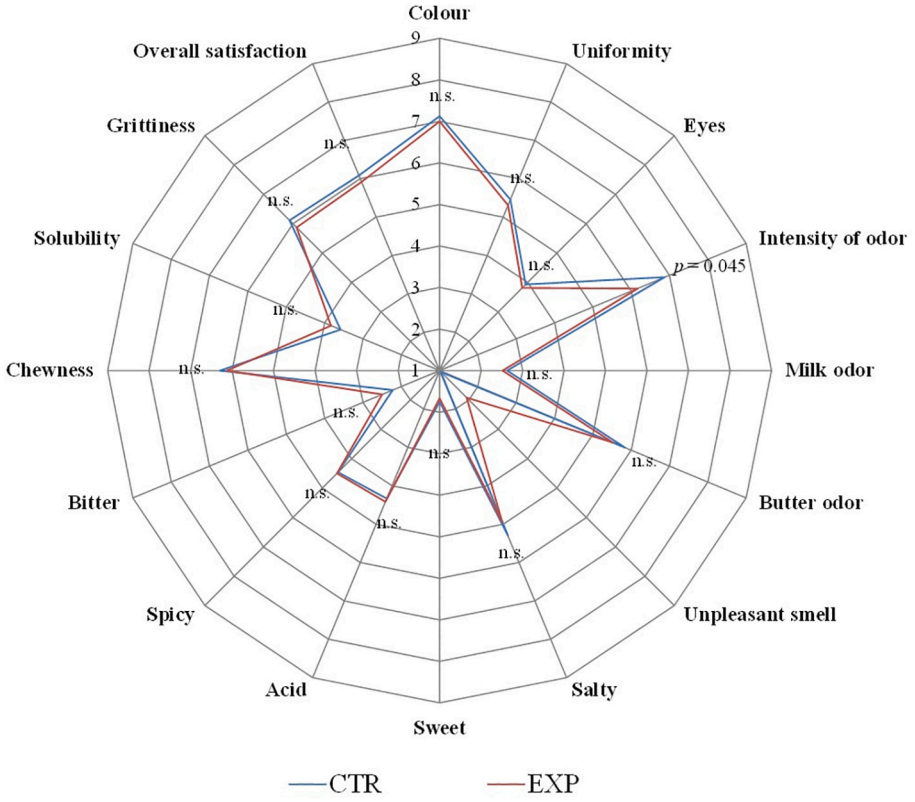


Fig. 5. Spider graph of descriptive sensory analysis of cheeses. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; n.s., not significant.

4. Conclusions

This study provides an analysis of the microbiological, chemical, textural and sensory characteristic of PDO Pecorino Siciliano cheese cooked under hot water. This technology did not affect the growth, survival and LAB species evolution in the different PDO Pecorino Siciliano cheese sections (UR and Co). Experimental PDO Pecorino Siciliano cheese was characterized by the same ash, dry matter, fat and protein content in comparison to CTR productions. Sensory traits evaluated on the basis of human and artificial senses were comparable among CTR and EXP cheeses. The results of this study clearly highlighted that the cheese cooking under hot water represent a useful innovation to reduce the transformation duration of PDO Pecorino Siciliano cheese without compromising the typical sensory profile of this traditional cheese.

Declaration of competing interest

No conflict of interest exists.

Data availability

Data will be made available on request.

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