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Effect of commission implementing regulation (EU) 2020/1319 on the bacterial composition of PDO Provola dei Nebrodi cheese

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ABSTRACT

In this study, PDO Provola dei Nebrodi cheese was deeply characterized for its bacterial community and chemical composition. Four dairy factories (A–D) were monitored from milk to ripened cheese. Wooden vat biofilms were dominated by thermophilic rod LAB (4.6–6.5 log CFU/cm^2). Bulk milk showed consistent levels of total mesophilic microorganisms (TMM) (5.0–6.0 log CFU/mL) and, after curdling, a general increase was recorded. The identification of the dominant LAB in wooden vat biofilms and ripened cheeses showed that the majority of wooden vat LAB were lactococci and *Streptococcus thermophilus*, while cheese LAB mainly belonged to *Lacticaseibacillus paracasei* and *Enterococcus*. Illumina sequencing identified 22 taxonomic groups; streptococci, lactococci, lactobacilli and other LAB constituted the majority of the total relative abundance % of the wooden vat (69.01–97.58 %) and cheese (81.57–99.87 %) bacterial communities. Regarding chemical composition, the effect of dairy factories was significant only for protein content. Inside cheese color was lighter and yellower than surface. Differences in fatty acids regarded only myristic acid and total amount of monounsaturated fatty acids. The sensory evaluation indicated some differences among cheeses produced in the four dairies regarding color, homogeneity of structure, overall intensity, salty, spicy, and hardness. The integrated approach applied in this study showed that PDO Provola dei Nebrodi cheese characteristics are quite stable among the dairy factories analyzed and this has to be unavoidably imputed to the application of the same cheese making protocol among different dairies.

1. Introduction

Sicily is a southern Italian region where 33 traditional cheeses are produced, but only five of them enjoy a protected denomination of origin (PDO) status: Vastedda della valle del Belìce, Pecorino Siciliano, Piacentinu Ennese, Provola dei Nebrodi, and Ragusano. Among them, Provola dei Nebrodi cheese obtained the PDO recognition very recently, in light of commission implementing regulation (EU) 2020/1319 of 22 September 2020, (G.U.R.I., 2020).

Provola dei Nebrodi PDO is a stretched cheese produced with raw whole cows' milk, coming from farms located in the Nebrodi area (Sicily), according to the product specifications described in the Production Regulations. The product shows a typical pear-shaped form tended to the oval, with a weight ranging from 1 to 10 kg and, depending on the aging duration, a straw yellow to intense yellow color. This cheese is produced in five different varieties (Busetta et al., 2021).

The entire cheese production process is characterized by the use of traditional wooden tools, namely "tina" (wooden vat), "ruotula" (wooden paddle used to break the curd), "piddiaturi" (wooden container), "manuvedda" (flattened wooden staff used for cutting) and mastredda (a large wooden open-topped board on which curd is traditionally left to acidify at room temperature for about 24 h) which, due to the porous structure of wood, facilitate absorption and trapping of the native dairy microbiota (Cruciata et al., 2019).

In general, when no starter culture is added, curd acidification and maturation of traditional Sicilian cheeses depend exclusively on the

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adventitious microorganisms present in raw milk (Guarcello et al., 2016), in the animal rennet paste added for coagulation (Cruciata et al., 2014), onto the surface of the wooden equipment used during transformation (Di Grigoli et al., 2015; Licitra et al., 2007), and in the dairy factory environment (Cruciata et al., 2019). In order to produce a given ripened cheese, the microbial community has to include lactic acid bacteria (LAB) because they perform the acidification of the curd (starter LAB) and contribute to the development of the typical sensory traits (non-starter LAB) (Grujović et al., 2022). Thus, the wooden equipment used to transform milk into traditional Sicilian cheeses are defining to transfer desired starter and non-starter LAB (Carpino et al., 2017; Di Grigoli et al., 2015). These bacteria produce extracellular polymeric substances (EPS) and form an aggregate known as "biofilm" (Azeredo et al., 2017).

The wooden vats used for milk curdling have been thoroughly characterized for their dairy LAB biofilms (Licitra et al., 2007; Lortal et al., 2009; Scatassa et al., 2015). Furthermore, those investigations did not reveal the presence of pathogenic bacteria. Regarding PDO Provola dei Nebrodi cheese production, so far, only "mastredda" has been microbiologically investigated and found to host several viable LAB belonging to *Enterococcus*, *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* genera (Busetta et al., 2021). In general, the whole production process of traditional Sicilian cheeses relies on wooden tools, from milking until ripening and even the wooden shelves used for ripening have been found to host harmless species belonging to cheese-surface-ripening groups, halophilic and moderately halophilic bacteria and LAB, confirming that all wooden equipment used to produce these cheeses are safe systems (Settanni et al., 2021).

In order to provide further insights on the microbiological significance of the wooden tools used to produce PDO Provola dei Nebrodi cheese, in this study the wooden vats used during production were characterized by a combined culture-dependent and –independent approach and their influence was evaluated until ripened cheeses which were investigated for the total microbial diversity and for the dominant strains. The final cheeses were also investigated for their chemical composition as well as sensory traits.

2. Materials and methods

2.1. Cheese production and sample collection

Four dairy factories (A–D) located in the countryside of Randazzo and Maniace (Catania province), producing exclusively PDO Provola dei Nebrodi cheeses, were monitored during the entire production cycle from milking until the third month of ripening. Cheese production occurred in all four factories applying strictly the protocol established for the protection of this traditional product from raw cows' milk processed in wooden vats without the addition of starter cultures and curdled with animal rennet paste (G.U.R.I., 2020). The flowsheet of PDO Provola dei Nebrodi cheese is reported in Fig. 1. The characteristics of the wooden vats of the four factories were identical: made from douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] wood, activated contemporarily following the common protocol with hot water, coarse salt brushing and deproteinized whey contact (Cruciata et al., 2018) and used for three years.

In each factory, cheese production was followed twice, the first was carried out in February and the second in March 2021. The wooden vat biofilms were sampled before milk contact following the brushing recovery method described by Didienne et al. (2012) using 100 cm^2 sterile plastic squares (Biogenetics s.r.l., Padua, Italy). Delimited areas were brushed with a sterile toothbrush and microorganisms recovered by a sterile gauze as reported by Settanni et al. (2021). Bulk milk (500 L in factories A–C and 350 L in factory D) was sampled before being transferred into the wooden vat and after 5 min from transfer. During the contact with the vat surfaces, milk was kept under gentle manual agitation performed with the typical wooden stick used for curd breaking. Milk coagulation occurred with 30 g of lamb rennet paste per 100 L of milk, in order to obtain a firm coagulum in about 60 min. Curd acidification, molding and ripening were carried out as reported by PDO disciplinary for production (G.U.R.I., 2020).

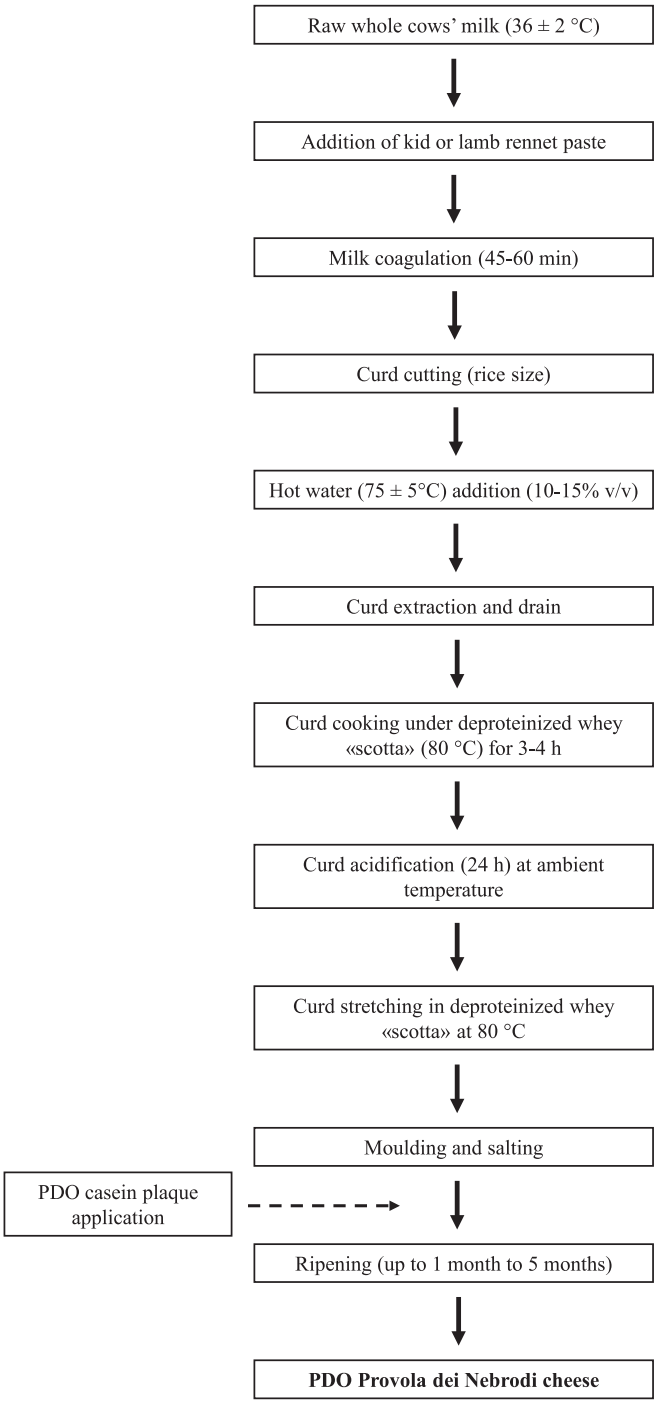

Fig. 1. Flowsheet of PDO Provola dei Nebrodi cheese production.

The samples of wooden vat biofilms, bulk milk before and after wood contact, curd, acidified curd, stretched curd and 3-month ripened cheeses were transported under refrigeration in insulated boxes containing reusable ice packs to the laboratories of Agricultural Microbiology of University of Palermo.

2.2. Microbiological analysis and LAB isolation

All samples collected during PDO Provola dei Nebrodi cheese production, including vat biofilms, as well as 3-month ripened cheeses were subjected to the decimal serial dilution. In particular, the wooden vat biofilms were diluted in Ringer's solution; the first dilution of these samples was obtained from 1 mL of the cell suspensions deriving from toothbrush and gauze. Milk samples (1 mL) were also diluted in Ringer's solution, while curd and cheese samples (15 g) were first homogenized in 2 % (*w/v*) sodium citrate solution (135 mL) using a stomacher (Bag-Mixer 400, Interscience, Saint Nom, France) at the maximum speed for 2 min and then serially diluted as reported above in Ringer's solution.

Cell suspensions were plated on agar media to allow the development of total mesophilic microorganisms (TMM), total psychrotrophic microorganisms (TPM), members of the Enterobacteriaceae family, total coliforms, *Escherichia coli*, *Salmonella* spp., coagulase-positive staphylococci (CPS), *Listeria monocytogenes*, pseudomonads, thermophilic and mesophilic LAB cocci and rods, enterococci, yeasts, molds as reported by Gaglio et al. (2021a). LAB incubation occurred in anaerobiosis in hermetically sealed jars equipped with the AnaeroGen AN25 sachets. All media, supplements and the anaerobic gas generating sachets were purchased from Oxoid (Basingstoke, England). All microbiological counts were carried out in duplicates for all samples at each collection time.

2.3. Phenotypic and genetic characterization of LAB

Presumptive LAB colonies developed from cell suspensions of wooden vat biofilm and ripened cheese samples were isolated at the highest dilutions plated. Colonies (at least five) sharing the same morphological characteristics (width, thickness, shape, color and opaqueness, uniformity of structure and margin) were picked up from MRS, M17 and WBAM, considering all different morphologies, and transferred into the corresponding broth media. After growth at the optimal incubation conditions, the isolates were streaked repeatedly onto agar media until reaching the uniformity of colonies. Purified bacteria were further phenotypically characterized following the procedure described by Gaglio et al. (2014).

All putative LAB were further processed by genetic tools. Overnight grown cultures were used to extract genomic DNAs by means of the DNA-SORB-B kit (Sacace Biotechnologies Srl, Como, Italy) applying manufacturer's guidelines. Strain differentiation was obtained through random amplification of polymorphic DNA (RAPD)-PCR analysis that is used as a fingerprinting for bacteria; to this purpose, each DNA was subjected to the amplification by single primers M13, AB111, and AB106 (Gaglio et al., 2017). The resulting polymorphic profiles were analyzed by GelCompar II software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). This program allows to generate a dendrogram to evaluate the similarity among the LAB isolates. The isolates sharing a high similarity levels of their RAPD patterns were considered to be the same strain.

Furthermore, the different strains were subjected to the rRNA gene sequencing analysis using the 16S rRNA fragment as target sequence. The analysis was performed using the primers rD1/fD1 as described by Weisburg et al. (1991). The final sequences were loaded in the database available at http://www.ncbi.nlm.nih.gov (GenBank/EMBL/DDBJ) and http://eztaxon-e.ezbiocloud.net/ (EzTaxon-e). The doubtful identity of *Enterococcus* was resolved by the *sodA* gene-based multiplex PCR assay of Jackson et al. (2004).

2.4. Culture-independent analysis of total bacterial community

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, USA) were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy) as described 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 2018.2). 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 bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013; Price et al., 2009). For bacteria, taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99 % Operational Taxonomic Units (OTUs) database which had been previously trimmed to the V3–V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. Number PRJNA893874.

2.5. Physicochemical characterization, antioxidant properties and oxidative stability of final cheeses

Cheese samples were freeze-dried and analyzed for dry matter (DM), fat, protein (N × 6.38), and ash content as reported by Bonanno et al. (2019). 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 core and under rind color, measured in duplicate by a Minolta Chroma Meter CR300 (Minolta, Osaka, Japan) and for hardness by an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy) as reported by Gaglio et al. (2021b).

The antioxidant activity of PDO Provola dei Nebrodi cheeses followed in this study was evaluated by TEAC assay, according to Re et al. (1999) and Bonanno et al. (2019). The method applied is based on a decolorization assay that measures the radical scavenging ability of samples using the ABTS radical cation (ABTS•+), and Trolox as standard. A Trolox solution in phosphate-buffered saline, ranging from 0 to 2.5 mM, was used to develop a calibration curve ($R^2 = 0.99$), and the results were expressed as mmol Trolox/kg DM of cheese.

Regarding the oxidative stability of PDO Provola dei Nebrodi cheeses, the thiobarbituric acid–reactive substances (TBARS) as a measure of secondary lipid oxidation was evaluated according to the methods proposed by Tarladgis et al. (1960) and modified by Mele et al. (2011). To quantify TBARS, 1,1,3,3-tetramethoxypropane solutions at concentrations ranging from 0.016 to 0.165 μg/mL were used for the calibration curve ($R^2 = 0.99$). Results were expressed as μg malonylaldehyde (MDA)/kg DM of cheese.

2.6. Cheese fatty acid profile determination

Fatty acids (FA) in lyophilized cheese samples (100 mg) were directly methylated in 1 mL hexane with 2 mL 0.5 M $NaOCH_3$ at 50 °C for 15 min, followed by 1 mL 5 % (*v*/v) HCl in methanol at 50 °C for 15 min, based on the bimethylation procedure described by Lee and Tweed (2008). Fatty acid methyl esters (FAME) were recovered in 1.5 mL hexane. One microliter of each sample was injected by means of an autosampler, into an HP 6890 gas chromatography system equipped with a flame-ionisation detector (Agilent Technologies, Santa Clara, CA, USA). FAME from each sample were separated using a CP-Sil 88 capillary column (100 m long, 0.25 mm internal diameter, 0.25 μm film thickness) (Chrompack, Middelburg, The Netherlands). The injector temperature was kept at 255 °C and the detector temperature was kept at 250 °C, with hydrogen flow of 40 mL/min, air flow of 400 mL/min, and a constant helium flow of 45 mL/min. The initial oven temperature

was held at 70 °C for 1 min, increased by 5 °C/min to 100 °C, held for 2 min, increased by 10 °C/min to 175 °C, held for 40 min, then finally increased by 5 °C/min to a final temperature of 225 °C held for 45 min. Helium, with a pressure of 158.6 kPa and a flow rate of 0.7 mL/min (linear velocity 14 cm/s), was used as carrier gas. A FAME hexane mix solution (Nu-Check-Prep, Elysian, MN, USA) was used to identify each FA. Individual standards (Larodan Fine Chemicals AB, Malmö, Sweden) were used to identify some branched FA, as C15:0 iso, C15:0 anteiso, C17:0 iso, and C17:0 anteiso. To quantify total FA, C23:0 (Sigma-Aldrich, Milan, Italy) was used as internal standard (4 mg/g lyophilized cheese).

2.7. Sensory analysis

The sensory characteristics of PDO Provola dei Nebrodi cheese were evaluated after three months of ripening by a panel test carried out by eight trained panelists following the ISO 13299:2016 protocol. The sensory analysis was performed for both cheese productions (February and March). Each panellist was asked to score a set of 14 descriptive attributes grouped into appearance, aroma, taste, and texture categories. All sensory attributes were evaluated on an increasing line-scale from 1 to 15 (from left to right) in steps of 0.1 and recorded by each panellist in individual computerized booths (UNI EN ISO 8589:2010). Data were gathered using the Compusense five V 4.6 software (Compusense, 2003).

Cheese samples, at room temperature at the time of testing, were portioned (approx. 1 cm of thickness) and presented on white plates to the panelists. An entire slice of each cheese was also shown to the panelists for the evaluation of appearance attributes. The samples were identified using random 3-digit codes.

2.8. Statistical analysis

Microbiological data were subjected to One-Way Variance Analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, USA). Chemical, physical and fatty acid composition of PDO Provola dei Nebrodi cheese were analyzed with the following ANOVA linear model: $Y_{ik} = \mu + DF_i + \varepsilon_{ik}$ where DF_i is the Dairy Factory (A.D), ε_{ik} is the error using the software SAS 9.1.2. Data on cheese sensory characteristics were analyzed with the JMP 16 software (SAS Institute 2022) using a GLM mixed model to test the effect of the farm, while the variable panellist was considered as random effect. The Tukey's test was used for means comparisons. Statistical significance was attributed to p values of $p < 0.05$.

3. Results

3.1. Microbiological analysis by culture-dependent approach

The levels of the viable microbial groups investigated on wooden vat biofilms and all samples collected during cheese making and after ripening of PDO Provola dei Nebrodi cheeses are reported in Table 1. Regarding wooden vat biofilms, their levels in terms of TMM ranged between 5.1 and 6.7 log CFU/cm^2 and LAB dominated the wooden vat microbial community of the four factories investigated. The wooden vats were actually characterized by different microbiological traits among the dairy factories; although *E. coli*, *Salmonella* spp., *Listeria monocytogenes* and CPS were not detected in any vat, WV-A was characterized by better hygienic conditions than the other three equipment used to collect milk, since members of Enterobacteriaceae, but also that of pseudomonads, enterococci and molds were below the detection limit. Also WV-C showed undetectable levels of pseudomonads. The levels of molds were particularly high (4.3 log CFU/cm^2) on WV-D surface. Within LAB community, a clear dominance of thermophilic cocci was registered in the vats WV-A and WV-C, while the vat WV-D showed a massive presence of mesophilic rods.

Bulk milk showed consistent levels of TMM (5.0–6.0 log CFU/mL) and, although no big differences were registered after contact with the wooden surfaces (5.2–6.6 log CFU/mL), the levels of LAB increased, especially for the thermophilic coccus group. The milk after contact with the wooden vats was still characterized by the absence of *Salmonella* spp., *L. monocytogenes* and CPS, but the levels of members of Enterobacteriaceae, coliforms and *E. coli* were almost around 10^3 CFU/mL for all dairy factories.

After curdling, a general increase of the majority of microbial groups investigated was recorded. In particular, TMM reached 8.0 log CFU/g in factory C. This factory showed the highest increase of all LAB groups while *Salmonella* spp., *L. monocytogenes* and CPS were not registered. On the contrary, the curds of the factories A and B showed detectable levels of CPS (2.8 and 3.4 log CFU/g, respectively). After overnight acidification, plate counts increased showing levels above 8.0 log CFU/g of TMM for all four dairy factories. In all cases, the highest increase was registered in correspondence of thermophilic and mesophilic LAB cocci. In particular, thermophilic LAB cocci in acidified curds from factories A and B reached 9.0 and 9.1 log CFU/g, respectively. The acidification step decreased below the detection levels the group of pseudomonads in factories A, B and D, and that of mold in factories B and C. Furthermore, CPS compared during curdling in factories A and B were reduced until undetectable levels with the acidification.

The stretching operation exerted a sanitizing effect of the curds. In fact, even though very high levels of TMM and thermophilic and mesophilic LAB cocci were still enumerated in all factories, psychrotrophic microorganisms and pseudomonads, together with *Salmonella* spp., *Listeria monocytogenes* and CPS disappeared from the four stretched curds and, in case of samples from factories A and B it was also registered the decrease below detectability of *E. coli*, coliforms and members of Enterobacteriaceae. Stretching also determined a reduction of molds until being undetectable from the curds A, C and D.

The characteristics of the ripened cheeses were quite comparable among the four dairy factories. The only groups detected were TMM and all LAB, while all other groups object of investigation were below the detection limit. However, within LAB community, enterococci ranged between 4.3 and 6.2 log CFU/g in ripened cheeses.

3.2. LAB differentiation and identification

The preliminary characterization of the 728 isolates for general LAB traits determined the recognition of 678 Gram-positive and catalase-negative bacteria which were distinguished into 559 cocci and 119 rods. Considering cell arrangement, growth in different conditions and use of different substrates, 14 phenotypic groups (Table S1) were obtained with the LAB isolated from wooden vats and final cheeses.

Strain typing was performed exclusively on the isolates within each phenotypic group that developed from the highest dilutions of the cell suspensions of the wooden vat biofilms and ripened cheeses. The comparison of the polymorphic profiles indicated that the isolates from the wooden vat biofilms represented 14 strains (Fig. 2A), while those from ripened cheeses showed a similar biodiversity as being 18 strains (Fig. 2B).

All strains were identified by 16S rRNA gene sequencing and were confirmed to belong to the group of LAB (Ac. No. OP714481 – OP714512). Furthermore, the application of the species-specific *sodA* gene based PCR confirmed that enterococci were *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* (results not shown). The majority of LAB from the wooden vats were identified as lactococci (groups VI and VII) and *Streptococcus thermophilus* (group XIII), while those isolated from ripened cheeses were mainly allotted into *Lacticaseibacillus paracasei* (groups II and III) species and *Enterococcus* (groups IX and X) genus. In particular, the species *Enterococcus faecalis* was also found as member of the wooden vat biofilm community together with *Lactobacillus delbrueckii* (group I) and leuconostocs (groups VIII and XI), while the other species found in ripened cheeses were *Lactiplantibacillus*

Table 1
Microbial loads of samples collected through experimental cheese production.

Sample	Media[b]															
	PCA-SkM 30 °C	PCA-SkM 7 °C	VRBGA	VRBA	HEA (*E. coli*)	HEA (*Salmonella* spp.)	BP (CPS)	LSAB	PAB	M17 44 °C	M17 30 °C	WBAM	MRS	KAA	YPD	PDA
WV-A	6.4 ± 0.3 b	1.3 ± 0.5 b	<1 d	0.8 ± 0.2 d	<1	<1	<1	<1	<1 c	6.5 ± 0.4 a	3.9 ± 0.0 c	3.6 ± 0.1 c	2.0 ± 0.0 c	<1 c	2.1 ± 0.1 d	<1 d
WV-B	6.7 ± 0.1 a	3.6 ± 0.3 a	3.1 ± 0.1 b	2.1 ± 0.1 b	<1	<1	<1	<1	3.6 ± 0.0 a	4.6 ± 0.2 b	4.7 ± 0.3 b	4.6 ± 0.1 b	4.6 ± 0.4 b	3.4 ± 0.2 a	3.6 ± 0.1 a	3.0 ± 0.0 b
WV-C	5.1 ± 0.1 c	3.3 ± 0.3 a	1.9 ± 0.1 c	1.7 ± 0.1 c	<1	<1	<1	<1	<1 c	6.7 ± 0.2 a	5.0 ± 0.3 b	5.7 ± 0.3 d	2.3 ± 0.5 c	1.4 ± 0.4 b	3.1 ± 0.2 b	1.6 ± 0.1 c
WV-D	5.9 ± 0.2 b	1.0 ± 0.1 b	4.1 ± 0.1 a	5.3 ± 0.0 a	<1	<1	<1	<1	2.2 ± 0.5 b	5.4 ± 0.4 b	6.1 ± 0.1 a	5.7 ± 0.6 a	7.1 ± 0.0 a	1.8 ± 0.2 b	2.7 ± 0.1 c	4.3 ± 0.1 a
SEM	0.19	0.36	0.46	0.51	–	–	–	–	0.47	0.27	0.24	0.54	0.62	0.37	0.17	0.48
p-value	<0.0001	<0.0001	<0.0001	<0.0001	–	–	–	–	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
BM-A	6.0 ± 0.2 a	3.8 ± 0.2 a	3.4 ± 0.3 a	2.3 ± 0.0 a	3.9 ± 0.0 a	<1	<1 b	<1	4.6 ± 0.4 ab	5.9 ± 0.1 a	5.6 ± 0.1 ab	4.5 ± 0.5 a	5.0 ± 0.2 b	3.8 ± 0.0	3.9 ± 0.2 a	2.4 ± 0.0 a
BM-B	5.7 ± 0.1 a	2.5 ± 0.1 c	1.7 ± 0.1 b	1.1 ± 0.1 c	1.8 ± 0.2 b	<1	2.0 ± 0.0 a	<1	3.3 ± 0.2 c	5.2 ± 0.1 b	5.9 ± 0.3 a	3.5 ± 0.2 b	6.0 ± 0.1 a	3.8 ± 0.1	2.7 ± 0.1 c	2.0 ± 0.0 b
BM-C	5.6 ± 0.2 a	3.5 ± 0.1 a	2.1 ± 0.2 b	3.2 ± 0.1 a	2.9 ± 0.4 ab	<1	<1 b	<1	4.2 ± 0.3 b	5.2 ± 0.2 b	5.2 ± 0.3 b	4.1 ± 0.0 ab	4.2 ± 0.1 c	3.9 ± 0.3	3.4 ± 0.2 b	1.8 ± 0.2 bc
BM-D	5.0 ± 0.2 b	3.0 ± 0.2 b	3.3 ± 0.2 a	1.5 ± 0.3 c	2.1 ± 0.6 ab	<	2.1 ± 0.1 a	<1	5.1 ± 0.1 a	4.0 ± 0.1 c	4.2 ± 0.1 c	4.0 ± 0.0 ab	3.6 ± 0.0 d	3.9 ± 0.0	2.8 ± 0.0 c	1.7 ± 0.0 c
SEM	0.12	0.15	0.23	0.25	0.26	–	0.31	–	0.21	0.21	0.20	0.13	0.27	0.04	0.15	0.08
p-Value	0.001	<0.0001	<0.0001	<0.0001	0.008	–	<0.0001	–	0.0001	<0.0001	<0.0001	0.013	<0.0001	0.757	<0.0001	0.0001
MAC-A	5.9 ± 0.1 b	4.3 ± 0.0 b	3.5 ± 0.3 a	3.4 ± 0.1	3.8 ± 0.1 a	<1	<1	<1	3.5 ± 0.1 a	6.2 ± 0.0 a	5.5 ± 0.2 a	5.1 ± 0.2 a	5.0 ± 0.0 ab	3.3 ± 0.2	3.7 ± 0.1 a	2.8 ± 0.2 b
MAC-B	5.7 ± 0.1 b	3.5 ± 0.1 c	2.8 ± 0.1 b	2.5 ± 0.5	2.2 ± 0.7 b	<1	<1	<1	3.4 ± 0.1 a	5.4 ± 0.2 b	5.7 ± 0.3 a	5.1 ± 0.1 a	5.3 ± 0.2 a	3.3 ± 0.2	3.3 ± 0.0 b	3.6 ± 0.1 a
MAC-C	6.6 ± 0.2 a	5.0 ± 0.0 a	2.8 ± 0.3 b	3.4 ± 0.5	3.9 ± 0.1 a	<1	<1	<1	2.3 ± 0.1 b	6.4 ± 0.0 a	6.2 ± 0.4 a	5.0 ± 0.1 a	4.4 ± 0.5 b	3.3 ± 0.1	3.8 ± 0.0 a	2.1 ± 0.1 c
MAC-D	5.2 ± 0.1 c	3.9 ± 0.4 bc	2.4 ± 0.2 b	3.3 ± 0.2	3.2 ± 0.1 a	<1	<1	<1	2.7 ± 0.8 ab	4.8 ± 0.1 c	4.7 ± 0.0 b	3.9 ± 0.2 b	4.5 ± 0.2 b	3.1 ± 0.1	3.9 ± 0.2 a	3.9 ± 0.2 a
SEM	0.15	0.17	0.13	0.15	0.23	–	–	–	0.18	0.20	0.18	0.16	0.13	0.05	0.07	0.22
p-Value	<0.0001	0.0001	0.003	0.048	0.004	–	–	–	0.020	<0.0001	0.001	<0.0001	0.015	0.370	0.001	<0.0001
C-A	7.9 ± 0.2 a	5.1 ± 0.0 ab	5.1 ± 0.1 a	4.1 ± 0.2 b	4.2 ± 0.2	<2	2.8 ± 0.2 b	<2	2.5 ± 0.3 c	8.2 ± 0.1 a	7.2 ± 0.3 a	4.8 ± 0.2 a	4.6 ± 0.1 c	5.5 ± 0.0 a	4.2 ± 0.0 b	2.5 ± 0.2 b
C-B	6.7 ± 0.1 b	4.1 ± 0.4 c	3.2 ± 0.3 c	2.3 ± 0.5 c	3.6 ± 0.1	<2	3.4 ± 0.2 a	<2	3.1 ± 0.1 bc	6.2 ± 0.1 b	6.2 ± 0.1 b	3.8 ± 0.2 b	4.7 ± 0.1 c	5.2 ± 0.2 b	3.2 ± 0.3 c	3.6 ± 0.0 a
C-C	8.0 ± 0.3 a	5.6 ± 0.0 a	4.5 ± 0.2 b	4.9 ± 0.0 a	4.6 ± 0.3	<2	<2 c	<2	5.1 ± 0.0 a	8.3 ± 0.3 a	7.5 ± 0.4 a	4.9 ± 0.3 a	6.2 ± 0.0 a	4.9 ± 0.0 c	4.8 ± 0.2 a	3.6 ± 0.1 a
C-D	7.1 ± 0.2 b	4.9 ± 0.1 b	4.3 ± 0.1 b	4.2 ± 0.0 ab	3.3 ± 0.1	<2	<2 c	<2	3.6 ± 0.4 b	6.1 ± 0.4 b	6.2 ± 0.3 b	4.2 ± 0.1 b	5.7 ± 0.1 b	3.0 ± 0.0 d	4.1 ± 0.2 b	2.8 ± 0.3 b
SEM	0.17	0.17	0.21	0.30	0.16	–	0.47	–	0.30	0.32	0.19	0.15	0.20	0.30	0.18	0.15
p-Value	0.0001	0.0001	<0.0001	<0.0001	0.174	–	<0.0001	–	<0.0001	<0.0001	0.001	0.001	<0.0001	<0.0001	<0.0001	0.0001
AC-A	8.4 ± 0.0	2.7 ± 0.3 b	4.9 ± 0.0 a	5.0 ± 0.1	4.8 ± 0.7	<2	<2	<2	<2 b	9.0 ± 0.2	8.2 ± 0.3 ab	5.5 ± 0.1 b	5.2 ± 0.1 b	5.1 ± 0.2 b	5.3 ± 0.2 a	2.8 ± 0.2 b
AC-B	8.3 ± 0.3	2.8 ± 0.2 b	3.5 ± 0.0 c	4.1 ± 0.1	4.0 ± 0.1	<2	<2	<2	<2 b	9.1 ± 0.1	8.1 ± 0.1 b	4.7 ± 0.1 c	5.2 ± 0.2 b	5.9 ± 0.0 a	5.5 ± 0.0 a	<2 c
AC-C	8.3 ± 0.0	3.1 ± 0.5 b	4.3 ± 0.1 b	5.9 ± 1.5	4.7 ± 0.3	<2	<2	<2	2.8 ± 0.2 a	8.8 ± 0.4	8.7 ± 0.2 a	4.6 ± 0.1 c	5.1 ± 0.1 b	5.2 ± 0.1 b	4.6 ± 0.1 b	<2 c
AC-D	8.7 ± 0.4	4.0 ± 0.3 a	4.3 ± 0.1 b	5.7 ± 0.3	5.4 ± 0.1	<2	<2	<2	<2 b	8.5 ± 0.3	8.6 ± 0.1 ab	6.4 ± 0.1 a	6.9 ± 0.6 a	5.2 ± 0.0 b	5.2 ± 0.1 a	3.2 ± 0.2 a
SEM	0.08	0.18	0.15	0.28	0.15	–	–	–	0.37	0.10	0.09	0.22	0.24	0.10	0.11	0.46
p-Value	0.240	0.006	<0.0001	0.075	0.077	–	–	–	<0.0001	0.109	0.013	<0.0001	0.0001	<0.0001	0.0001	<0.0001
SC-A	7.3 ± 0.1 b	<2	<1 c	<1 b	<2 b	<2	<2	<2	<2	8.5 ± 0.2 a	7.6 ± 0.4 b	5.9 ± 0.1 b	4.4 ± 0.5	3.6 ± 0.2 c	3.3 ± 0.2 c	<2b
SC-B	8.2 ± 0.2 a	<2	<1 c	<1 b	<2 b	<2	<2	<2	<2	7.8 ± 0.2 b	8.4 ± 0.3 a	4.5 ± 0.3 c	4.9 ± 0.3	5.2 ± 0.1 ab	5.0 ± 0.1 a	2.8 ± 0.2a
SC-C	7.5 ± 0.2 b	<2	2.7 ± 0.0 b	2.8 ± 0.2 a	2.2 ± 0.1 a	<2	<2	<2	<2	8.5 ± 0.1 a	8.2 ± 0.1 ab	3.8 ± 0.2 d	4.3 ± 0.5	5.0 ± 0.1 b	3.8 ± 0.1 b	<2b
SC-D	8.4 ± 0.1 a	<2	3.7 ± 0.2 a	2.8 ± 0.2 a	2.7 ± 0.2 a	<2	<2	<2	<2	7.6 ± 0.0 b	8.1 ± 0.0 ab	7.3 ± 0.2 a	5.1 ± 0.1	5.4 ± 0.0 a	3.3 ± 0.1 c	<2b
SEM	0.14	–	0.49	0.23	0.62	–	–	–	–	0.13	0.11	0.41	0.14	0.22	0.21	0.37
p-Value	<0.0001	–	<0.0001	0.001	<0.0001	–	–	–	–	0.0001	0.026	<0.0001	0.096	<0.0001	<0.0001	<0.0001
RC-A	8.0 ± 0.4 a	<2	<1	<1	<2	<2	<2	<2	<2	7.2 ± 0.1 ab	8.5 ± 0.1 a	7.3 ± 0.2 b	8.1 ± 0.4 a	4.3 ± 0.2 b	<2	<2
RC-B	8.1 ± 0.2 a	<2	<1	<1	<2	<2	<2	<2	<2	7.6 ± 0.7 ab	8.0 ± 0.0 ab	7.2 ± 0.3 b	7.8 ± 0.2 a	6.2 ± 0.4 a	<2	<2
RC-C	6.9 ± 0.3 b	<2	<1	<1	<2	<2	<2	<2	<2	6.8 ± 0.5 b	7.7 ± 0.5 b	6.4 ± 0.0 c	7.1 ± 0.1 b	5.8 ± 0.2 a	<2	<2
RC-D	7.9 ± 0.1 a	<2	<1	<1	<2	<2	<2	<2	<2	8.1 ± 0.0 a	8.2 ± 0.1 ab	8.2 ± 0.2 a	7.8 ± 0.1 a	4.4 ± 0.3 b	<2	<2
SEM	0.16	–	–	–	–	–	–	–	–	0.18	0.11	0.20	0.12	0.26	–	–
p-Value	0.002	–	–	–	–	–	–	–	–	0.031	0.030	<0.0001	0.005	<0.0001	–	–

Results indicate mean values ± S.D. of four plate counts (carried out in duplicates for two independent productions). Units are log CFU/cm^2 for vat surfaces; log CFU/mL for milk samples; log CFU/g for curd, acidified curd, stretched curd and ripened cheeses. Data within a column followed by different letters are significantly different according to Tukey's test ($p < 0.05$).
Abbreviations: PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for total mesophilic microorganisms; PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for total psychrotrophic microorganisms; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; VRBA, violet red bile agar for coliforms; HEA, hektoen enteric agar for *E. coli* (red colonies) and *Salmonella* spp. (black colonies); BP, baird-parker agar for CPS, coagulase-positive staphylococci; LSAB, *Listeria* selective agar base for L. *monocytogenes*; PAB, *Pseudomonas* agar base for pseudomonads; M17 44 °C, medium 17 agar incubated at 44 °C for thermophilic coccus LAB; M17 30 °C, medium 17 agar incubated at 30 °C for mesophilic coccus LAB; WBAM, whey-based agar medium for thermophilic rod LAB; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; KAA, kanamycin aesculin azide agar for enterococci; YPD, yeast peptone dextrose agar for yeasts; PDA, potato dextrose agar for molds. WV, wooden vat; BM, bulk milk; MAC, milk after contact; C, curd; AC, acidified curd; SC, stretched curd; RP, ripened cheese; A–D, dairy factory A–dairy factory D; SEM, standard error of mean.

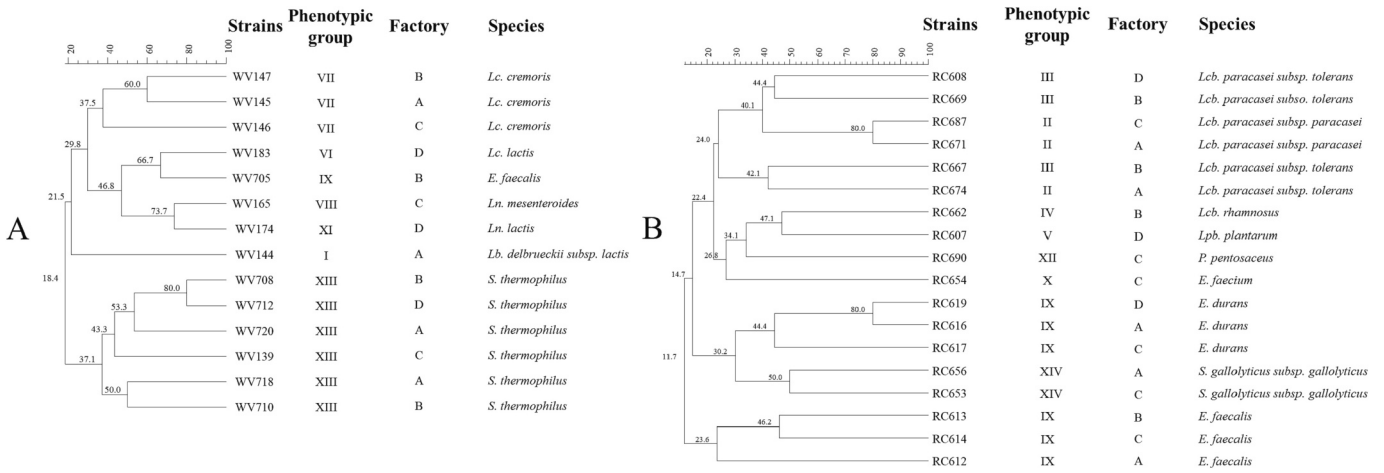

Fig. 2. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains from wooden vats (A) and 3-month ripened PDO Provola dei Nebrodi cheeses (B) generated with the primers M13, AB106 and AB111. Abbreviations: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Lcb.*, *Lacticaseibacillus*; *Lpb.*, *Lactiplantibacillus*; *Ln.*, *Leuconostoc*; *P.*, *Pediococcus*; *S.*, *Streptococcus*.

plantarum (group V), *Lacticaseibacillus rhamnosus* (group IV), *Pediococcus pentosaceus* (group XII) and *Streptococcus gallolyticus* subsp. *gallolyticus* (group XIV).

3.3. Characterization of cheese microbiota by Illumina analysis

The distribution of the relative abundances (%) of the bacterial OTUs identified by MiSeq Illumina within the wooden vat biofilms and 3-month ripened cheeses collected during PDO Provola dei Nebrodi cheese from four distinct factories are reported in Fig. 3. A total of 24 taxonomic groups, mainly identified at genus level, were detected. Although streptococci, lactococci, lactobacilli, leuconostocs, trichococci and other LAB constituted the majority of the total relative abundance % of the wooden vat (69.01–97.58 %) and cheese (81.57–99.87 %) bacterial communities, their proportions differed among cheese factories. In particular, the wooden vats of the factories A and C were dominated by

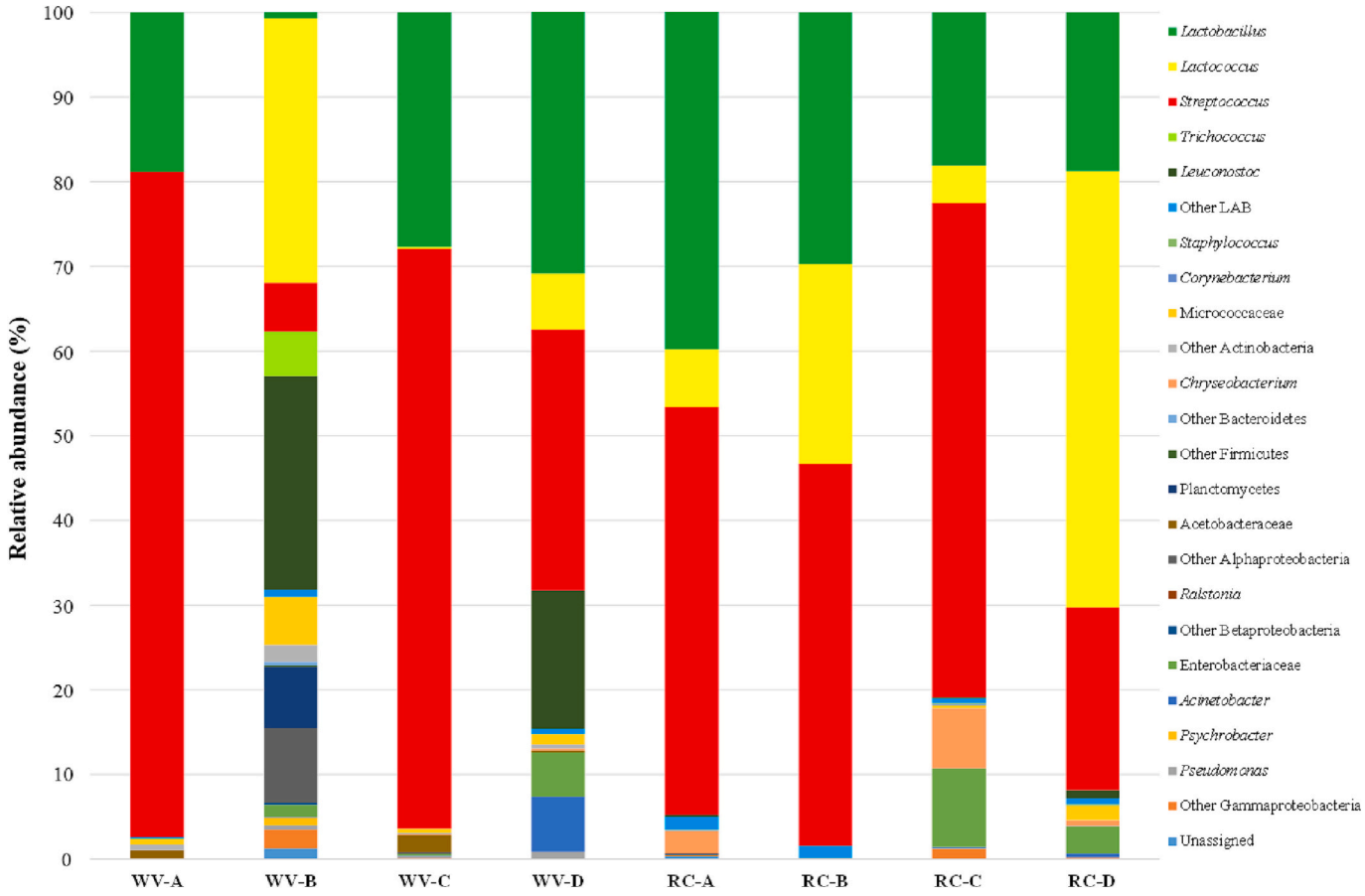

Fig. 3. Relative abundances (%) of bacterial taxa identified by MiSeq Illumina in wooden vat biofilms and ripened PDO Provola dei Nebrodi cheeses. Abbreviations: WV, wooden vat; RC, ripened cheese; A–D, factories A to D.

Streptococcus (78.57 and 68.53 %, respectively) and *Lactobacillus* (18.84 and 27.70 %, respectively), that of factory B by *Lactococcus* (31.26 %), *Leuconostoc* (25.24 %) and *Trichococcus* (5.26 %), while the vat biofilm of the factory D by *Lactobacillus* (31.10 %), *Streptococcus* (30.83 %), *Leuconostoc* (16.36 %) and *Lactococcus* (6.56 %). Wooden vat biofilms also hosted low levels of Micrococcaceae, Enterobacteriaceae and other OTUs allotted, basically, into α- and γ-proteobacteria. Unlike wooden vat composition, ripened cheeses showed a more uniform situation in terms of relative abundance (%) of LAB. In general, the relative abundance (%) of lactobacilli detected in cheeses from factories A and B was higher than that observed for the corresponding wooden vats. Regarding lactococci, their relative abundance (%) registered in cheeses from factories A, C and D, was much higher than that of the wooden vat biofilms. Streptococci relative abundance (%) of the cheeses from factory B was 9-fold higher than that displayed by the wooden vat used for cheese making. Enterobacteriaceae were found only in cheese samples from the factories C and D, while *Chryseobacterium* in those from factories A, C and, at very low level, from factory D.

3.4. Physicochemical parameters of cheese samples

The physicochemical composition of PDO Provola dei Nebrodi cheese is reported in Table 2. Considering gross composition, dry matter (DM), fat and proteins were in the range 59.88–61.16 %, 34.84–39.94 and 45.83–50.46 g/100 g DM, respectively. The effect of dairy factories was significant only for protein content. The differences for fat content were not significant probably due to the high variability, as evidenced by the high SEM value. N soluble/N total ratio ranged between 10 and 14 %. Salt percentage was non influenced by dairies and ranged between 4.49 and 4.85 on DM, that correspond to 2.6–2.8 % on fresh cheese. Cheese hardness was measured for minimum of 8.30 to a maximum of 10.47 kg/cm^2.

Table 2
Physicochemical analysis of PDO Provola dei Nebrodi cheeses.

Parameters	Samples				SEM	*p*-Value
	RC-A	RC-B	RC-C	RC-D		
Dry matter (DM) (%)	60.63	59.88	61.08	61.16	0.745	0.639
Fat (g/100 g DM)	39.94	34.84	37.00	36.65	1.430	0.233
Protein (g/100 g DM)	45.83 D	50.46 A	49.04 B	47.92C	0.551	0.017
Soluble N/total N (%)	10.59	14.06	13.95	13.10	1.390	0.377
Ash (g/100 g DM)	7.55	8.47	8.10	8.51	0.496	0.558
NaCl (g/100 g DM)	4.32	4.39	4.69	4.59	0.244	0.698
a_w	0.970	0.960	0.968	0.957	0.006	0.449
Hardness (kg/cm^2)	8.30	10.47	8.60	10.30	2.039	0.822
TEAC (mmol Trolox/kg DM)	12.29	18.40	17.93	18.06	3.205	0.541
TBARS, mg MDA/kg ss	0.44	0.87	0.69	0.38	0.141	0.186
Outside color						
L*, lightness	59.55	59.24	59.15	62.67	1.427	0.365
a*, redness	−3.61	−4.48	−3.51	−4.20	−0.429	0.423
b*, yellowness	13.01	18.25	8.68	16.52	3.409	0.335
Chroma	13.54	18.80	9.38	17.04	3.370	0.338
Hue angle	−0.302	−0.255	−0.417	−0.255	0.060	0.318
Inside color						
L*, lightness	82.87	78.56	74.30	79.43	2.892	0.346
a*, redness	−3.51	−4.25	−3.76	−4.06	0.400	0.617
b*, yellowness	18.18	23.62	13.70	23.68	3.645	0.296
Chroma	18.52	24.01	14.21	24.03	3.64	0.303
Hue angle	−0.196	−0.186	−0.278	−0.172	0.057	0.110

Abbreviations: RP, ripened cheese; A–D, dairy factory A–dairy factory D; SEM, standard error of mean. On the row, values with different letters are significant A, B, C: $p \leq 0.01$.

Color parameters were determined outside and inside PDO Provola dei Nebrodi cheese samples. Although dairy factories did not affect the color of the cheeses, evident differences were found between the readings inside and onto the surface of Provola cheese. Inside Provola cheese color was lighter and yellower than surface, while redness index did not change, consequently chroma detected at the inside of cheese was higher than cheese surface.

3.5. Antioxidant properties and fatty acid profile of cheeses

Cheese antioxidant properties evaluated on PDO Provola dei Nebrodi cheeses (Table 2) did not show differences among dairy farms. Similarly, also TBARS determination results were not statistically different among the four dairy factories investigated. Regarding cheese FA composition (Table 3), the effect of dairy factories resulted statistically significant only for a few FA detected, in particular, Myristic acid (C14) and the total amount of Monounsaturated FA (MUFA). Cheeses made in dairy factory C showed higher values of Myristic FA ($p < 0.05$) and lower MUFA than cheeses processed in other dairy factories ($p < 0.05$).

3.6. Sensory analysis

Results concerning the sensory analysis of the PDO Provola dei Nebrodi cheese are reported in Fig. 4. Among appearance attributes only color was affected by dairies effect ($p < 0.05$) and cheese produced by the dairy factory B showed the highest score. Oiliness and homogeneity attributes were similar in all cheeses. Holes attributes ranged between 1.49 and 1.61. No significant differences were found in aroma attributes between the dairy factories, with pasture attribute ranging from 4.49 to 4.82. Most of the taste attributes were significantly different among the dairies. In particular B and D cheeses showed higher overall intensity, salty and spicy attributes compared with the others ($p < 0.0001$, $p < 0.05$ and $p < 0.01$ respectively). Regarding bitterness, no significant difference was found, and C cheese showed the highest score. As to texture, cheeses produced by A and C dairies tended to be less hard than B and D cheeses, with a significant difference ($p < 0.01$). Chewiness and adhesiveness did not show significant difference between the dairies, ranging from 10.52 to 11.15 and from 4.6 to 4.84 respectively.

4. Discussion

The majority of traditional and the totality of PDO Sicilian cheeses are produced from raw milk kept into a wooden vat without the addition of starter cultures (Gaglio et al., 2021c). However, curd acidification and the development of the final cheese characteristics are ensured by the LAB biofilms present onto the wooden vat surface (Di Grigoli et al., 2015; Licitra et al., 2007; Scatassa et al., 2015; Settanni et al., 2012), but so far, these biofilms have not been characterized yet for the wooden vats used to process PDO Provola dei Nebrodi cheese. Since 2020, this cheese achieved the European quality status of "protected denomination of origin" (PDO) and the cheese making protocols have been standardized among dairies. Thus, previous data available on this cheese were influenced by different protocols and data need to be revised. The four factories chosen for the present study were all characterized by the use of douglas fir wooden vats, activated contemporarily three years before, to exclude variability of data based on the different vat age.

The viable counts estimated on wooden vat biofilms differed among the four dairy factories for almost all microbial groups investigated. These differences are imputable to several factors, such as environmental conditions, efficacy of brushing during cleaning and, especially, different bulk milks processed by each factory. *E. coli*, *Salmonella* spp., *Listeria monocytogenes* and CPS, generally associated with poor hygiene

Table 3
Fatty acid composition (g/100 g FA) of PDO Provola dei Nebrodi cheeses.

Fatty acids	Samples				SEM	*p*-Value
	RC-A	RC-B	RC-C	RC-D		
C4	2.70	2.77	2.74	2.91	0.261	0.95
C6	2.27	2.36	2.42	2.30	0.134	0.86
C8	1.45	1.58	1.56	1.43	0.065	0.36
C10	3.15	3.62	3.53	3.01	0.230	0.31
C11	0.34	0.45	0.38	0.35	0.053	0.52
C12	3.54	4.13	3.89	3.29	0.355	0.44
C13	0.18	0.29	0.20	0.18	0.048	0.55
C14	11.59 b	12.10 ab	12.73 a	11.11 b	0.282	0.05
C15:iso	0.27	0.29	0.33	0.38	0.294	0.16
C14:1 t	0.55 b	0.66 ab	0.63 ab	0.75 a	0.033	0.05
C14:1 c	0.76	1.05	0.94	0.85	0.161	0.66
C15	1.06	1.33	1.15	1.47	0.145	0.29
C15:1 t	0.37	0.31	0.36	0.31	0.038	0.30
C16	30.16	26.88	31.97	28.89	1.487	0.24
C16:1	0.38	0.51	0.36	0.52	0.050	0.16
C17 anteiso	0.17	0.20	0.16	0.23	0.030	0.39
C16:1 t	1.70	1.85	1.92	1.71	0.187	0.80
C17	0.69	0.69	0.68	0.84	0.041	0.12
C17:1	0.23	0.22	0.21	0.25	0.026	0.75
C18	10.27	8.00	8.78	9.92	1.330	0.64
C18:1 t6	0.44 ABb	0.59 Aa	0.40 ABb	0.38 Bb	0.32	0.03
C18:1 t11 TVA	1.16	2.12	1.23	2.73	0.73	0.26
C18:1 t9	0.24	0.44	0.19	0.25	0.069	0.20
C18:1 c6 n12	0.45	0.99	0.45	0.63	0.169	0.21
C18:1 c9 n9	20.61	17.51	18.23	18.17	1.03	0.30
C18:1 c11	0.68 a	0.63 ac	0.53 bc	0.57 b	0.023	0.03
C18:2 t	0.12	0.44	0.10	0.19	0.112	0.26
C18:2 n6 LA	2.05	1.96	1.62	1.47	0.177	0.21
C20	0.18	0.13	0.17	0.20	0.026	0.27
C18:3 n6 GLA	0.14	0.12	0.12	0.18	0.013	0.08
C20:1 n11	0.33	0.72	0.25	0.99	0.246	0.26
C18:3 n3 ALA	0.42	1.30	0.46	1.03	0.314	0.27
C22	0.07	0.10	0.07	0.12	0.019	0.35
C20:3 n6 DGLA	0.09	0.09	0.08	0.07	0.013	0.64
C20:4 n6	0.13	0.12	0.14	0.10	0.007	0.16
Altri	1.06	2.45	1.00	0.07	0.469	0.21
SFA	68.10	64.93	70.76	66.68	1.448	0.16
MUFA	27.91 a	28.59 a	25.70 b	28.22 a	0.528	0.05
PUFA	2.94	4.03	2.53	3.05	0.551	0.37

Abbreviations: RP, ripened cheese; A–D, dairy factory A–dairy factory D; SEM, standard error of mean; TVA, trans vaccenic acid; LA, linoleic acid; GLA, gamma linoleic acid; ALA, alfa linolenic acid; DGLA, dihomo-γ-linolenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. On the row, values with different letters are significant a, b, c: $p \leq 0.05$.

of dairy productions (Claeys et al., 2013), were never detected. The attachment of these human pathogenic dairy bacteria has been proven to be hindered by the LAB biofilms colonizing the inner vat surfaces (Cruciata et al., 2018). Microbes from cheese whey are able to form stable biofilms on new wooden vats within a few days from the first contact (Gaglio et al., 2016; Sun and D'Amico, 2022).

In view of the Commission Regulation 853 (2004) establishing that the maximum level of total bacteria at 30 °C is 100.000 per ml for raw cow's milk, the levels of TMM in the raw milks processed in this study have to be considered high and only milk from factory D complied fully with the Eurpoean regulation. However, this regulation refers to the raw cow's milk intended for the manufacture of products by a process that does not involve any heat treatment. In case of PDO Provola dei Nebrodi cheese, the production process includes a heat treatment step because stretching is carried out at 80 °C. Furthermore, in our study, the dominant groups of the microbial community of the raw milk from all factories were LAB. As matter of fact, after 3-month ripening, all potentially harmful microorganisms were below the detection limit and these results complies with the Commission Regulation 2073 (2005) on "microbiological criteria for foodstuff".

The isolates collected after plate counts were identified in order to identify the viable populations dominating the wooden vat biofilms and the final ripened cheeses. The species found (*Lactococcus lactis*, *Streptococcus thermophilus*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, *Enterococcus faecalis* and leuconostocs) were already detected in Provola dei Nebrodi cheeses not processed according to PDO protocol (Cronin et al., 2007; Randazzo et al., 2021) and other raw cow's milk stretched cheeses produced in Sicily (Licitra et al., 2007; Scatassa et al., 2015). As matter of fact, vat biofilms mainly hosted *S. thermophilus* and *Lc. lactis*, the most common starter LAB for cheese processing, while cheese isolated mainly belonged to the nonstarter LAB species *Lcb. pacaracasei* and enterococci (Settanni and Moschetti, 2010). These data showed a lack of correspondence between dominant cheese and vat LAB; the only species found at dominant level in both groups was *E. faecalis*, but the RAPD profile of the only wooden vat isolate WV705, associated to factory B, was not superimposable to that of the strain RC613 identified from ripened cheeses produced in the same factory. Indeed, the microbiological characterization of the open-topped table (mastredda) used to acidify Provola dei Nebrodi curd evidenced the presence of several dominating LAB in viable form (Busetta et al., 2021), indicating that, for this cheese, not only the wooden vat but also mastredda biofilms provide LAB for cheese fermentation. When wooden surfaces represent the major source of LAB for the spontaneous fermentation no additional cultures are required (Sun and D'Amico, 2021).

The microbial community of the four wooden vat biofilms and the final cheeses was studied through a polyphasic phenotypic/genotypic culture-dependent approach combined with a next generation sequencing (NGS) technique. The characterization of isolates based on phenotypic tests followed by strain typing and 16S rRNA gene sequencing is a routine methodology applied to identify the dominant strains in dairy products (Franciosi et al., 2015; Rossetti et al., 2008), but also to evaluate their physiological and ecological functions (Rosselló-Mora and Amann, 2001). In particular, strain typing by RAPD-PCR analysis is a common technique to discriminate among LAB isolated from food matrices (Fusco et al., 2019). Regarding NGS approach, the study of total DNA from complex matrices became a routine activity to deeply describe microbial composition and evolution (Ercolini, 2013) and also to track the source of microbial communities in cheese facilities (Sun and D'Amico, 2021). In our study, MiSeq Illumina technology confirmed the dominance of streptococci, lactococci, lactobacilli and leuconostocs in the inner wooden vat surfaces, but also evidenced differences among cheese microbiota of the four dairy factories.

Considering the physicochemical composition of the cheeses gross parameters are in accordance with previous studies performed on Provola dei Nebrodi cheeses at 90 days of ripening (Condurso et al., 2006; Cronin et al., 2007). The differences encountered among the different productions depend on the high variability of dairy transformations. Indeed, even though PDO Provola dei Nebrodi cheese production process is applied uniformly among the various dairy factories that adhere to PDO production regulations, cheese differences depend primarily on differences in bulk milk composition, but also on the craftsmanship of this cheese production, performed manually by the cheese makers. N soluble/N total ratio and salt percentage of PDO Provola dei Nebrodi cheese were similar to those registered for other stretched cheeses (Bonanno et al., 2013; Di Trana et al., 2022; Licitra et al., 2000), while hardness is higher than that displayed by other stretched cheeses at the same ripening period (Bonanno et al., 2013). Although color parameters at the rind of PDO Provola dei Nebrodi cheese is similar to those of other

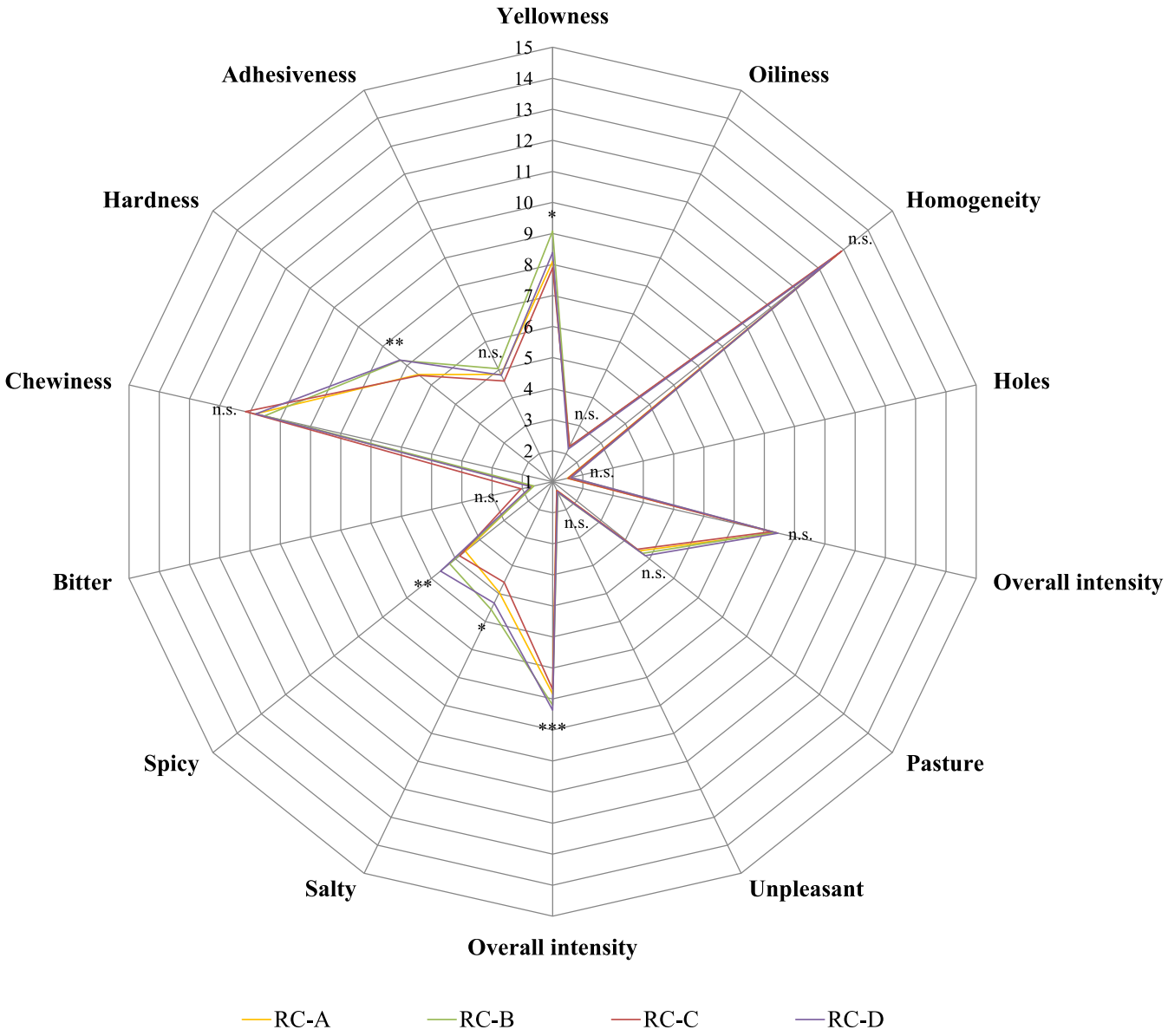

Fig. 4. Spider diagrams of descriptive sensory analysis of PDO Provola dei Nebrodi cheese. Abbreviations: RC, ripened cheese; A–D, factories A to D. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant.

Sicilian stretched cheeses, the inside was yellower (Bonanno et al., 2013).

Several factors affect cheese antioxidant properties, mainly caseins, β-carotene, uric acid, vitamin E, phenols, whey protein, and folate (microbial influence) (Fardet and Rock, 2018). Moreover, TEAC value is influenced by the season of production, with higher values registered for summer cheeses (Revilla et al., 2016), and ripening time, since ripened cheeses show higher antioxidant activity than fresh cheeses (Gupta et al., 2009). The absence of statistical differences between PDO Provola dei Nebrodi cheeses produced in the four dairy factories followed in this study is not only due to the application of the same production protocol, but probably also to the very similar techniques of livestock applied. A comparison of data among various studies is difficult, due to the different methods used for cheese antioxidant assays (Di Trana et al., 2022). The level of TBARs recorded in this study is quite comparable or slightly lower than that observed for other Italian cheeses (Garofalo et al., 2021; Ponte et al., 2022). So far, PDO Provola dei Nebrodi cheese FA profile was only determined by Condurso et al. (2006) who analyzed cheeses produced before the adoption of PDO production protocol. FA profile showed as saturated FA resulted between 65 and 70 % of the total FA detected, confirming data displayed by other raw cow's milk stretched cheeses (Esposito et al., 2014; Maniaci et al., 2021; Pistoia et al., 2015). Our cheeses were produced during February and March when pasture availability is limited in Nebrodi area and the feeding of lactating cows includes hay and concentrate. Cows' diet might explain the high percentages of saturated FA in PDO Provola dei Nebrodi cheeses analyzed and also the higher percentage of myristic FA and the lower percentage of MUFA of the cheeses produced in dairy C due to the feeding of the lactating cows mainly with hay and concentrates (Chion et al., 2010; Coppa et al., 2011; Maniaci et al., 2021).

The different levels of pasture in the diet might also explain the different color perceived by the panelists who were asked to judge the sensory characteristics of PDO Provola dei Nebrodi cheeses of the four factories. Indeed, Carpino et al. (2004) demonstrated that yellowness was more pronounced when PDO Ragusano cheese was transformed from milk of cows consuming fresh native pasture. However, the results from sensory evaluation were in contrast with those measured with colorimeter that found no significant differences among factories

probably because of adoption of a 15-points scale for the sensory evaluation and because this test was performed by trained personnel. The scores for holes are similar to those registered for other stretched cheeses reported in literature (Bonanno et al., 2013). This attribute is correlated with the microbiological quality of milk (Fox et al., 2004) and the low presence of holes is in accordance with the production regulation of PDO Provola dei Nebrodi cheese that tolerate only a few holes with small dimensions. Pasture attribute of the final cheeses was scored at high levels and this is a trait of volatile compounds usually correlated with fresh pasture plants (Carpino et al., 2004). Bitterness was scored almost at the same level for the four dairy factories. Fallico et al. (2005) reported that this attribute might depend on a difference in cheese making technology or salting brine which consequently influence the microbial growth during aging. Sensory evaluation found some of the cheeses to be quite hard and, in general, the judges confirmed the hardness registered instrumentally. In general, sensory analysis showed a good sensory profile of PDO Provola dei Nebrodi cheeses analyzed in this study, although it confirmed a high variability among farms characterized by traditional herd management and cheese making system.

In conclusion, the main microbiological parameters were almost comparable among the cheeses produced in different factories and also the physicochemical profiles and sensory attributes of the final cheeses were quite stable among dairy factories. This study has proven that the application of the same cheese making protocol among dairies producing PDO Provola dei Nebrodi cheese harmonized the microbial evolution and the final characteristics at ripening.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110188.

Declaration of competing interest

The authors declare that there is no conflict of interest for this research.

Data availability

Data will be made available on request.

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