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Metagenomic, microbiological, chemical and sensory profiling of Caciocavallo Podolico Lucano cheese

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ABSTRACT

In this study, Caciocavallo Podolico Lucano (CPL) cheese was deeply characterized for its bacterial community, chemical composition and sensory aspects. The entire cheese making process (from milk collection to ripened cheese) was performed by strictly applying the traditional protocol for CPL production in four dairy factories (A–D) representative of the production area. The vat made of wood represents the main transformation tool for CPL cheese production and the biofilms hosted onto the internal surfaces of all vats analyzed in this study were dominated by lactic acid bacteria. Total mesophilic microorganisms present in bulk milk (4.7–5.0 log CFU/ml) increased consistently after contact with the wooden vat surfaces (5.4–6.4 log CFU/ml). The application of Illumina sequencing technology identified barely 18 taxonomic groups among processed samples; streptococci and lactobacilli constituted the major groups of the wooden vat biofilms [94.74–99.70 % of relative abundance (RA)], while lactobacilli dominated almost entirely (94.19–100 % of total RA) the bacterial community of ripened cheeses. Except coagulase positive staphylococci, undesirable bacteria were undetectable. Among chemical parameters, significant variations were registered for unsaturated, monounsaturated, polyunsaturated fatty acids and antioxidant properties (significantly lower for CPL cheeses produced in factory B). The cheeses from factories A, C and D were characterized by a higher lactic acid and persistence smell attributes than factory B. This work indicated that the strict application of CPL cheese making protocol harmonized the main microbiological, physicochemical and sensory parameters of the final cheeses produced in the four factories investigated.

1. Introduction

Caciocavallo Podolico cheese is made from raw cows' milk in four regions (Apulia, Basilicata, Calabria and Campania) of southern Italy. This cheese is produced applying the characteristic stretching technology including the acidification of the curd and the subsequent scalding and molding to the final pear shape (Licitra & Carpino, 2014). Alike other traditional southern Italian stretched cheeses, such as PDO Ragusano and Caciocavallo Palermitano (Licitra et al., 2007; Settanni et al., 2012), Caciocavallo Podolico is a semihard cheese produced with wooden tools without the inoculation of starter cultures. The cheeses under Caciocavallo Podolico (CP) denomination are all produced from the milk of Podolica breed cows, but are further differentiated based on their production area: "CP Dauno" is produced in Apulia region; "CP Lucano" (CPL) in Basilicata region; "CP of Calabria" in Calabria region; and "CP of Campania" in Campania region. Depending on inoculants, rennet, curd acidification, stretching conditions, salting, ripening conditions and duration CP cheeses processed in distant areas are particularly different (Uzun et al., 2020). In addition, the transformation traditions of a given production territory provide uniqueness to the final products. CPL cheese is included in the list of Traditional Agri-Food Products (TAP) by the Italian Ministry of Agriculture, Food Sovereignty and Forestry (G.U.R.I., 2021). This stretched cheese is obtained from the transformation of the raw milk of autochthonous Podolica breed cows reared at pasture year-round without any supplementation and it is round shaped with a soft, creamy white consistency, sweet taste

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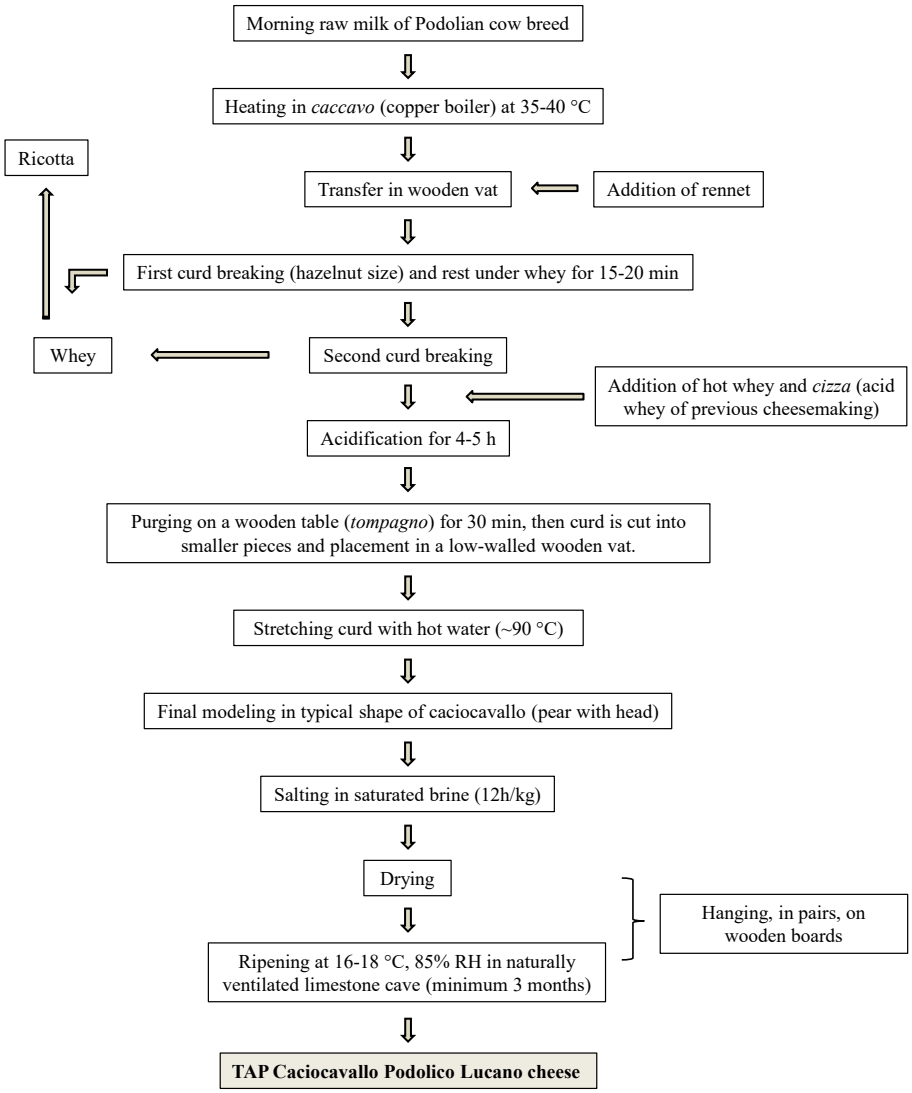

Fig. 1. Flowsheet of Traditional Agri-Food Products (TAP) Caciocavallo Podolico Lucano cheese production.

and delicate flavour. The minimum ripening time is three months and assumes a firmer structure and a darker yellow color with time; aging can be prolonged until three years (Claps, 2001).

The production of raw milk cheeses processed with wooden tools without starter addition in Sicily region relies on the indigenous 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) as well as on the environmental contaminants of the dairy factory (Cruciata, Gaglio, Todaro, & Settanni, 2019), and a similar trend is expected also for CPL cheese. Among the microorganisms responsible for the biochemical events occurring during cheese making and ripening, lactic acid bacteria (LAB) are of paramount importance. Specifically, dairy LAB necessary to produce a given ripened cheese belong to two groups: starter LAB defining for curd acidification; and, non-starter LAB that drive the ripening process (Barbaccia et al., 2020) and determine the typical sensory traits of the final cheese (Guarrasi et al., 2017).

Several works evidenced the key role played by bacterial biofilms associated to the wooden equipment during cheese production. In particular, the wooden vats used for milk coagulation have been thoroughly characterized and several works revealed the presence of dairy LAB (Licitra et al., 2007; Lortal et al., 2009; Scatassa et al., 2015; Sun & D'Amico, 2021). The species identified are both starter and non-starter LAB (Carpino et al., 2017; Di Grigoli et al., 2015). These bacteria are adsorbed and trapped onto the surface of the wooden vats because wood is a porous structure (Cruciata et al., 2019) and can easily form the typical aggregates of biofilms thanks to their extracellular polysaccharides (EPS) (Vert et al., 2012), but so far, no information is available on the bacterial biofilms of the wooden vats used to process CPL cheese.

In order to provide in-depth insights on the microbial populations characterizing CPL cheese production, in this study the wooden vats used for cheese making and the final cheeses, produced in four dairy factories applying the traditional cheese making protocol, were investigated for total microbial diversity, chemical composition, and sensory traits.

2. Materials and methods

2.1. Cheese production and sample collection

Four dairy factories (A–D) producing CPL cheese, located in the cities of Castelgrande and Muro Lucano (Potenza province, southern Italy), were monitored during the entire transformation process from milking until 4-month ripening. Cheese making was performed following the

traditional production protocol established with TAP disciplinary for protection of CPL cheese using raw cows' milk processed in wooden vats without the addition of starter cultures and curdled with animal rennet paste (B.U.R., 1999). The flowsheet of CPL cheese production is reported in Fig. 1. The wooden vats (WV.CPL-A – WV.CPL-D) were all made of ash-leaved maple (*Acer negundo* L.) wood and used for one year in factories A – C and four years in factory D.

In each factory, cheese production was followed twice at 15-d interval, both during May 2021. The wooden vat biofilms were sampled before milk contact applying the brushing recovery method described by Didienne et al. (2012) using 100 cm^2 sterile plastic squares (Biogenetics s.r.L., Padua, Italy). Bulk milk (500 L) was sampled before and after filling in the wooden vat. During the contact with the vat surfaces, milk was kept for 5 min under gentle manual agitation performed with the typical wooden stick used for curd breaking. Milk coagulation occurred with 30 g of lamb rennet paste (Camoscio® CSO 95/75, DSM Food Specialties, Segrate, Italy) per 100 L of milk and the resulting milk coagulum was disrupted manually. The resulting curd was then left to acidify until a pH (5.2–5.4) necessary for the stretching phase that lasted about 10–15 min. The acidified curd was finally molded. Salting was performed just after shaping in saturated brine. Ripening occurred for four months at 16–18 °C and 85 % relative humidity in naturally ventilated limestone caves.

Samples of wooden vat biofilms, bulk milk before and after wood contact, curd, acidified curd, stretched curd and 4-month ripened cheeses were transported to the laboratories of Agricultural Microbiology of University of Palermo under refrigeration using insulated boxes containing reusable ice packs.

2.2. Microbiological analyses

All samples collected during CPL cheese production, including vat biofilms taken before milk transfer, as well as 4-month ripened cheeses were subjected to the decimal serial dilution. Wooden vat biofilms (cells released from toothbrush and gauze) and milk samples were directly diluted in Ringer's solution, while curd and cheese samples (15 g) were first homogenized in 2 % (*w/v*) sodium citrate solution (135 ml) by means of 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) spread on plate count agar (PCA) supplemented with 1 g/L skimmed milk and incubated for 72 h at 30 °C; total psychrotrophic microorganisms (TPM) spread on PCA plus skimmed milk, incubated for 7 d at 7 °C; members of the Enterobacteriaceae family poured in violet red bile glucose agar (VRGBA), incubated for 24 h at 37 °C; coliforms poured in violet red bile agar (VRBA), incubated for 24 h at 37 °C; *Escherichia coli* and *Salmonella* spp. spread on Hektoen enteric agar (HEA), incubated for 24 h at 37 °C; coagulase-positive staphylococci (CPS) spread on Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen (RPF), incubated for 48 h at 37 °C; *Listeria monocytogenes* spread on *Listeria* selective agar base (LSAB) added with SR0140E supplement, incubated for 48 h at 37 °C; pseudomonads spread on *Pseudomonas* agar base (PAB) supplemented with cephaloridine sodium fusidate cetrimide (CFC), incubated for 48 h at 25 °C; thermophilic and mesophilic coccus LAB poured in M17 agar, 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; enterococci spread on kanamycin esculin azide (KAA) agar, incubated for 24 h at 37 °C; yeasts spread on yeast peptone dextrose (YPD), incubated for 48 h at 28 °C; molds spread on potato dextrose agar (PDA), incubated for 7 d at 25 °C. Growth of fungi on M17, WBAM and MRS was prevented by cycloheximide (10 mg/ml) addition, while YPD and PDA were supplemented with chloramphenicol (0.1 mg/ml) to inhibit the growth of bacteria. 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 (Milan, Italy). All microbiological counts were carried out in duplicates for all samples at each collection time.

2.3. Culture-independent analysis of total bacterial community

2.3.1. MiSeq library preparation and Illumina sequencing

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing by Illumina MiSeq system (Illumina, USA) were performed at the Sequencing Platform of Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Briefly, 464-nucleotide sequences from bacterial 16S rRNA gene V3-V4 region (Baker, Smith, & Cowan, 2003; Claesson et al., 2010), corresponding to *Escherichia coli* positions 341 to 805, were amplified from each sample. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

2.3.2. Illumina data analysis and sequences identification by QIIME2

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 & Standley, 2013; Price, Dehal, & Arkin, 2009). Bacterial 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 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 PRJNA924124.

2.4. Chemical analyses of cheeses

Proximate composition of cheeses (moisture, protein, fat, lactose, ash and NaCl) and fatty acid profile as Saturated Fatty Acids, (SFA), Unsaturated Fatty Acids (UFA), Monounsaturated Fatty Acids (MUFA) and Polyunsaturated Fatty Acids (PUFA) were analyzed in duplicate with the FoodScan-TM2 using mid-infrared spectroscopy (MIRS) prediction models developed and commercialized by FOSS (FOSS Analytical, Italy).

2.5. Cheese antioxidant properties

Samples of milk before and after wood contact, curd and cheeses for antioxidant property determinations were stored at −80 °C until analyses. Extracts of milk, curd and cheeses were performed according to the methods of Rashidinejad, Birch, Sun-Waterhouse, and Everett (2013) with some modifications. Briefly, 8.5 ml of milk and 0.5 g of curd

Table 1
Microbial loads[a] of samples collected through Caciocavallo Podolico Lucano cheese productions.

Sample	Growth media															
	PCA-SkM 30 °C	PCA-SkM 7 °C	VRBGA	VRBA	HEA-E	HEA-S	BP	LSAB	PAB	M17 30 °C	M17 44 °C	MRS	WBAM	KAA	YPD	PDA
WV. CPL-A	4.3 ± 0.2 C	<1 B	<1	<1	<1	<1	<1	<1	<1	1.9 ± 0.0 C	3.6 ± 0.4	2.7 ± 0.1 B	3.1 ± 0.1 B	<1	<1 C	<1 B
WV. CPL-B	4.2 ± 0.1 C	<1 B	<1	<1	<1	<1	<1	<1	<1	3.4 ± 0.1 B	3.5 ± 0.1	2.8 ± 0.0 B	2.9 ± 0.1 B	<1	1.7 ± 0.0 B	1.8 ± 0.2 A
WV. CPL-C	5.3 ± 0.2 A	5.4 ± 0.1 A	<1	<1	<1	<1	<1	<1	<1	4.4 ± 0.1 A	3.8 ± 0.4	3.3 ± 0.1 A	3.5 ± 0.2 A	<1	2.2 ± 0.1 A	<1 B
WV. CPL-D	4.8 ± 0.1 B	<1 B	<1	<1	<1	<1	<1	<1	<1	3.4 ± 0.3 B	3.4 ± 0.2	3.4 ± 0.0 A	3.7 ± 0.1 A	<1	1.9 ± 0.3 AB	1.9 ± 0.3 A
P value	0.001	0.001	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	0.001	0.454	0.001	0.001	n.e.	0.001	0.001
BM. CPL-A	4.9 ± 0.0	2.0 ± 0.1 B	<1 B	2.3 ± 0.1 A	<1	<1	2.0 ± 0.0 C	<1	<1	5.0 ± 0.0 B	4.9 ± 0.0 A	3.0 ± 0.1 B	<1	3.0 ± 0.0 C	2.6 ± 0.0 B	<1 B
BM. CPL-B	4.7 ± 0.0	<1 C	<1 B	<1 D	<1	<1	2.8 ± 0.0 A	<1	<1	4.1 ± 0.0 C	3.7 ± 0.0 B	<1 C	<1	3.2 ± 0.1 B	1.3 ± 0.1 D	<1 B
BM. CPL-C	5.0 ± 0.8	3.9 ± 0.1 A	1.4 ± 0.1 A	1.9 ± 0.1 B	<1	<1	2.7 ± 0.0 A	<1	<1	5.4 ± 0.1 A	4.9 ± 0.0 A	<1 C	<1	<1 D	3.0 ± 0.0 A	0.8 ± 0.2 A
BM. CPL-D	4.7 ± 0.1	<1 C	1.3 ± 0.2 A	1.1 ± 0.1 C	<1	<1	2.2 ± 0.1 B	<1	<1	3.9 ± 0.1 D	3.8 ± 0.1 B	3.8 ± 0.1 A	<1	4.1 ± 0.0 A	2.0 ± 0.0 C	<1 B
P value	0.707	0.001	0.001	0.001	n.e.	n.e.	0.001	n.e.	n.e.	0.001	0.001	0.001	n.e.	0.001	0.001	0.001
MAC. CPL-A	5.6 ± 0.2 ab	3.8 ± 0.2 a	2.3 ± 0.0 A	2.3 ± 0.2 A	<1	<1	2.0 ± 0.1 b	<1	<2 B	6.2 ± 0.0 A	6.4 ± 0.0 A	4.2 ± 0.0 C	5.3 ± 0.0 A	3.1 ± 0.1 A	3.3 ± 0.2 A	2.6 ± 0.3 a
MAC. CPL-B	5.4 ± 0.1 bc	3.7 ± 0.1 ab	<1C	<1C	<1	<1	2.3 ± 0.3 ab	<1	<2 B	5.3 ± 0.0 C	5.9 ± 0.1 B	4.5 ± 0.1 B	5.1 ± 0.1 B	1.7 ± 0.1 B	2.7 ± 0.0 B	2.8 ± 0.1 a
MAC. CPL-C	6.4 ± 0.7 a	3.7 ± 0.1 ab	2.2 ± 0.2 A	2.5 ± 0.0 A	<1	<1	2.5 ± 0.0 a	<1	<2 B	6.1 ± 0.0 B	5.7 ± 0.2 B	5.4 ± 0.0 A	4.9 ± 0.0 C	1.7 ± 0.0 B	3.3 ± 0.2 A	2.3 ± 0.2 ab
MAC. CPL-D	5.9 ± 0.0 ab	3.4 ± 0.1 b	1.1 ± 0.1 B	1.9 ± 0.1 B	<1	<1	2.1 ± 0.0 ab	<1	1.9 ± 0.1 A	5.4 ± 0.1 C	5.7 ± 0.1 B	4.5 ± 0.0 B	4.8 ± 0.0 C	1.8 ± 0.1 B	2.2 ± 0.1 C	2.1 ± 0.1 b
P value	0.03	0.05	0.001	0.001	n.e.	n.e.	0.019	n.e.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.01
C.CPL-A	6.1 ± 0.0 C	3.1 ± 0.2 B	4.1 ± 0.1 A	3.7 ± 0.1 A	<2B	<2	5.0 ± 0.0 D	<2	<2 B	7.3 ± 0.3 A	7.3 ± 0.1 A	5.4 ± 0.1 C	5.8 ± 0.1 B	4.3 ± 0.2 B	3.7 ± 0.1 C	3.2 ± 0.0 B
C.CPL-B	6.6 ± 0.2 B	2.1 ± 0.1 C	<1 D	<1 D	<2B	<2	5.7 ± 0.0B	<2	<2 B	7.1 ± 0.1 A	7.1 ± 0.2 A	5.7 ± 0.0 B	6.2 ± 0.0 A	3.9 ± 0.1 C	4.2 ± 0.1 B	3.7 ± 0.2 A
C.CPL-C	6.8 ± 0.1 AB	4.7 ± 0.6 A	3.2 ± 0.1 B	3.1 ± 0.0 B	<2B	<2	5.2 ± 0.1 C	<2	<2 B	6.5 ± 0.1 B	6.6 ± 0.1 B	5.5 ± 0.0 BC	5.7 ± 0.2 B	<2 D	4.6 ± 0.1 A	3.3 ± 0.2 B
C.CPL-D	7.1 ± 0.0 A	5.1 ± 0.1 A	2.0 ± 0.0 C	2.7 ± 0.1 C	2.1 ± 0.1 A	<2	6.3 ± 0.0 A	<2	3.1 ± 0.1 A	7.3 ± 0.2 A	7.1 ± 0.2 A	6.7 ± 0.1 A	6.5 ± 0.2 A	5.1 ± 0.0 A	4.1 ± 0.1 B	3.1 ± 0.1 B
P value	0.001	0.001	0.001	0.001	0.001	n.e.	0.001	n.e.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
AC. CPL-A	5.7 ± 0.1 C	<2 B	<1C	<1C	<2B	<2	4.5 ± 0.1 B	<2	<2 B	8.4 ± 0.0 B	8.3 ± 0.1 B	7.0 ± 0.0 A	6.9 ± 0.0 A	4.1 ± 0.0 B	3.2 ± 0.2 C	<2 C
AC. CPL-B	7.1 ± 0.0 A	<2 B	<1C	<1C	<2B	<2	5.6 ± 0.2 A	<2	<2 B	8.4 ± 0.0 B	8.5 ± 0.0 B	7.1 ± 0.2 A	6.7 ± 0.0 B	4.0 ± 0.2 B	3.6 ± 0.1 B	3.0 ± 0.1 A
AC. CPL-C	7.1 ± 0.0 A	3.1 ± 0.1 A	2.7 ± 0.1 B	3.2 ± 0.1 A	<2B	<2	5.5 ± 0.3 A	<2	<2 B	8.9 ± 0.1 A	8.9 ± 0.1 A	6.0 ± 0.1 C	5.9 ± 0.1 C	3.2 ± 0.2 C	4.1 ± 0.0 A	<2 C
AC. CPL-D	6.8 ± 0.2 B	<2 B	4.7 ± 0.2 A	2.5 ± 0.1 b	2.2 ± 0.1 A	<2	6.0 ± 0.1 A	<2	2.7 ± 0.3 A	7.9 ± 0.0 C	7.6 ± 0.1 C	6.6 ± 0.2 B	6.5 ± 0.1 B	4.7 ± 0.1 A	4.1 ± 0.0 A	2.7 ± 0.1 B
P value	0.001	0.001	0.001	0.001	0.001	n.e.	0.001	n.e.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SC.CPL-A	5.5 ± 0.1 C	<2 B	<1	<1	<2	<2	3.3 ± 0.2 B	<2	<2	7.3 ± 0.2 C	7.8 ± 0.1 B	6.1 ± 0.1 C	6.5 ± 0.2 B	3.8 ± 0.1 B	<2	<2
SC.CPL-B	6.3 ± 0.2 B	<2 B	<1	<1	<2	<2	4.1 ± 0.0 A	<2	<2	7.5 ± 0.0 BC	7.8 ± 0.1 B	7.3 ± 0.2 A	7.2 ± 0.0 A	3.9 ± 0.0 B	<2	<2
SC.CPL-C	6.5 ± 0.1 B	<2 B	<1	<1	<2	<2	3.5 ± 0.2 B	<2	<2	7.8 ± 0.0B	7.9 ± 0.1 B	6.1 ± 0.1 C	6.4 ± 0.1 B	3.2 ± 0.2 C	<2	<2
SC.CPL-D	8.6 ± 0.1 A	2.7 ± 0.1 A	<1	<1	<2	<2	4.1 ± 0.0 A	<2	<2	8.5 ± 0.1 A	8.7 ± 0.1 A	6.8 ± 0.1 b	7.2 ± 0.0 A	5.1 ± 0.0 A	<2	<2
P value	0.001	0.001	n.e.	n.e.	n.e.	n.e.	0.001	n.e.	n.e.	0.001	0.001	0.001	0.001	0.001	n.e.	n.e.

(*continued on next page*)

Table 1 (*continued*)

Sample	Growth media															
	PCA-SkM 30 °C	PCA-SkM 7 °C	VRBGA	VRBA	HEA-E	HEA-S	BP	LSAB	PAB	M17 30 °C	M17 44 °C	MRS	WBAM	KAA	YPD	PDA
RC. CPL-A	7.4 ± 0.2 BC	<2	<1	<1	<2	<2	<2	<2	<2	6.9 ± 0.0 B	7.1 ± 0.1 B	7.2 ± 0.1 B	6.9 ± 0.1B	3.5 ± 0.1C	<2	<2
RC. CPL-B	8.0 ± 0.1 A	<2	<1	<1	<2	<2	<2	<2	<2	7.2 ± 0.2 B	6.5 ± 0.1 C	7.7 ± 0.0 A	7.5 ± 0.1 A	2.9 ± 0.3 D	<2	<2
RC. CPL-C	7.2 ± 0.2 C	<2	<1	<1	<2	<2	<2	<2	<2	6.5 ± 0.2 C	6.5 ± 0.1 C	6.7 ± 0.0 C	6.2 ± 0.3 C	5.2 ± 0.1 B	<2	<2
RC. CPL-D	7.7 ± 0.1 AB	<2	<1	<1	<2	<2	<2	<2	<2	7.6 ± 0.1 A	7.4 ± 0.0 A	7.7 ± 0.0 A	7.3 ± 0.2 AB	5.8 ± 0.1 A	<2	<2
P value	0.001	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	0.001	0.001	0.001	0.001	0.001	n.e.	n.e.

Loads are reported as log CFU/cm^2 for vat surfaces, log CFU/ml for milk samples, and log CFU/g for curds and cheeses. Results indicate mean values ± S.D. of four plate counts (carried out in duplicates for two independent productions). A, B, C, D capital letters and a, b, small letters in column within the same sample indicate significant differences for $P < 0.001$, $0.01 < P < 0.05$, respectively.

Abbreviations: PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for detection of total mesophilic microorganisms; PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for detection of total psychrotrophic microorganisms; VRBGA, violet red bile glucose agar for detection of Enterobacteriaceae; VRBA, violet red bile agar for detection of total coliforms; HEA-E, hektoen enteric agar for detection of *E. coli* (red colonies); HEA-S, hektoen enteric agar for detection of *Salmonella* spp. (black colonies); BP, baird-parker agar for detection of coagulase-positive staphylococci; LSAB, *Listeria* selective agar base for detection of *L. monocytogenes*; PAB, *Pseudomonas* agar base for detection of pseudomonads; 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 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; KAA, kanamycin aesculinazide agar for detection of enterococci; YPD, yeast peptone dextrose agar for detection of yeasts; PDA, potato dextrose agar for detection of molds; WV, wooden vat; BM, bulk milk; MAC, milk after contact with wooden vat surface; C, curd; AC, acidified curd; SC, stretched curd; RP, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D; n.e., not evaluated.

or cheese were suspended in 25 ml of a 95 % methanol aqueous solution supplemented 1 % HCl and homogenized at 12,000 rpm using an Ultra-Turrax homogenizer (T 25 D, IKA WERKE, Staufen, Germany). The suspension from each sample was kept at 40 °C for 30 min in a water bath and vortexed for 30 s every 10 min. The mixture was cooled and filtered with cheese cloth and the residues were washed with 1 ml of the same solution. The filtrate was centrifuged at 7000*g* for 10 min at 4 °C, and the supernatant was kept at −80 °C until analyses. All samples were analyzed in duplicate for their antioxidant properties, measuring total phenolic content (TPC), ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) as described by Di Trana et al. (2022b).

2.6. Volatile organic compound emission

Headspace solid phase microextraction SPME (DVB/CAR/PDMS, 50 mm, Supelco) fiber was used to extract the volatile organic compounds (VOC) from cheeses. VOC profile was determined using a gas chromatograph (Agilent 6890) equipped with a mass spectrometry (MS) detector (Agilent 5975c) and a DB-624 capillary column (Agilent Technologies, 60 m, 0.25 mm, 1.40 μm). Cheese samples (5 g) were chopped, placed in a 25 ml glass vial and exposed to SPME-fiber under continuous stirring at 60 °C for 15 min. SPME fiber thermal desorption was performed through a splitless GC injector at 250 °C for 1 min. Chromatographic separation was achieved with helium carrier gas at 1 ml/min and an oven temperature program with a 5 min isotherm at 40 °C followed by a linear temperature increase of 5 °C min up to 200 °C, where it was held for 2 min. VOC analysis was performed in MS full scan mode applying interface temperature at 230 and acquisition mass range between 40 and 400 amu. Each VOC component was identified by MS comparison with spectral data (NIST library). The relative proportions of the identified components were shown as percentages obtained by normalizing the area of the GC–MS peaks with the total area of the significant peaks. Three replicates were performed for each sample.

2.7. Sensory analysis

Sensory analysis of the CPL cheeses was performed to grasp the differences among the cheeses produced in the four factories. In individual cubicles at the CREA-ZA, the labelled cheese samples (1 cm per side) were presented to nine judges, following the ISO 8589 (2007) indications, in a random order on a white paper plate. The judges evaluated appearance attributes on a whole slice of each cheese. All sensory attributes were evaluated on a graduated scale from 0 (extremely low) to 8 (extremely high).

2.8. Statistical analysis

Data of microbiological, chemical and volatile organic compounds were analysed using the statistical software package Systat 13 (Systat, 2009). All data were tested for the distribution of the variables with the Shapiro–Wilk test and analysed with ANOVA procedure. The model included the factory (4 levels = A, B, C and D) as fixed factor and means were compared by Tukey's test. For the sensory data analysis, smell, taste, structure and acceptability, the panellist effect was also introduced into the statistical model. Least square means were reported, and differences were considered significant at $P < 0.05$.

In addition, an explorative multivariate analysis was performed to investigate the relationship among cheeses. A hierarchical cluster analysis (HCA) (joining, tree clustering) was carried out for grouping the cheeses according to their dissimilarity, measured by Euclidean distances, whereas cluster aggregation was based on the Ward's method (Martorana et al., 2015). In particular, the relationships between sensory attributes of the cheeses produced in the four factories and microbiological-chemical-VOCs data were evaluated. Graphic construction was achieved by using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA).

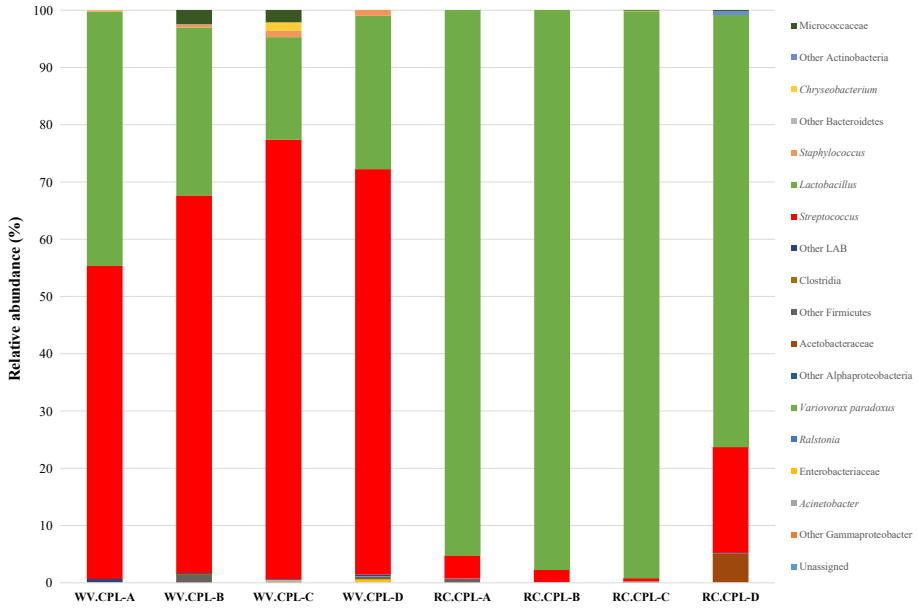

Fig. 2. Relative abundances (%) of the taxonomic groups identified by MiSeq Illumina in wooden vat biofilms and ripened Caciocavallo Podolico Lucano (CPL) cheeses. Abbreviations: WV, wooden vat; RC, ripened cheese; A–D, factories A–D.

3. Results and discussion

3.1. Viable levels of microorganisms

The levels of the viable microbial groups of wooden vat biofilms and all samples collected during cheese making, as well as, ripened CPL cheeses are reported in Table 1. Wooden vat biofilms are generally developed after the contact with cheese whey (Gaglio et al., 2016a; Sun & D'Amico, 2023). The vats used to process CPL cheese hosted levels of TMM in the range 4.2–5.3 log CFU/cm^2. Only sample WV.CPL-C showed detectable levels of TPM (5.4 log CFU/cm^2). Except mesophilic LAB cocci of WV.CPL-A biofilm (1.9 log CFU/cm^2), LAB onto the wooden vat surfaces were generally in the range 10^3–10^4 CFU/cm^2. The highest LAB levels were registered for mesophilic LAB cocci of WV.CPL-C biofilm counted at 4.4 log CFU/cm^2, explaining the high TPM levels associated to this wooden vat biofilm. High levels of TPM are often detected in wooden vat biofilms used to process PDO traditional Sicilian cheeses (Gaglio et al., 2016a; Settanni et al., 2013), and this finding is imputable to the psychrotrophic behaviour of LAB (Pothakos, Samapundo, & Devlieghere, 2012). Except WV.CPL-C, LAB counted at the highest levels in the other vats were generally thermophilic (cocci in WV.CPL-A and WV.CPL-B or rods in WV.CPL-D). The bacterial levels registered on the wooden vats used for CPL cheese production are similar to those generally reported for the wooden vats used for curdling raw cow's milk for the production of Caciocavallo Palermitano (Scatassa et al., 2015), PDO Ragusano (Licitra et al., 2007) and PDO Salers cheeses (Didienne et al., 2012).

All undesired groups represented by members of Enterobacteriaceae family, in particular, coliforms, and specifically *E. coli* and *Salmonella* spp., as well as CPS, *L. monocytogenes* and pseudomonads, generally associated with poor hygiene of dairy productions (Claeys et al., 2013), were below the detection limit in all wooden vat biofilms. These samples showed also undetectable levels of enterococci. Yeasts were uncountable in sample WV.CPL-A and counted at very low levels in the other samples, while molds were detected at low levels only in samples WV.CPL-B and WV.CPL-D (1.8 and 1.9 log CFU/cm^2, respectively). Except smear-ripened cheeses, yeasts and molds cause spoilage in other cheese typologies (Geronikou, Larsen, Lillevang, & Jespersen, 2022; Izzo, Mikušová, Lombardi, Sulyok, & Ritieni, 2022).

Bulk milk before transformation hosted almost 10^5 CFU/ml of TMM and increased until 5.4 – 6.4 log CFU/ml after contact with the wooden vat surfaces. The increase of TMM levels of milk is an expected phenomenon after rest in wooden vats (Didienne et al., 2012). Half of the factories (A and D) showed detectable levels of LAB rods in bulk milk, but after contact with the wooden surfaces this group increased until almost 5.0 log CFU/ml in all factories. No big differences were found for the levels of CPS, Enterobacteriaceae, yeasts and molds before and after wooden surface contact, while the average level of enterococci decreased. Regarding pseudomonads, these bacteria were not detected in wooden vat biofilms or in bulk milk, but the levels registered in milk after interaction with WV.CPL-D were 1.9 log CFU/ml. The contact with wood did not affect the detectability of *Salmonella* spp., *L. monocytogenes* and *E. coli*. Cruciata et al. (2018) demonstrated that the attachment of these human pathogens to the inner vat surfaces is hindered by the LAB biofilms.

A general increase of almost all microbial groups investigated was observed after curdling. On average, this increase was about 1 log cycle and, except *E. coli* for factory D (2.1 log CFU/g), the groups undetectable in milk were still below the detection limit in curds. The acidification of the curds, occurred during almost 4–5 h, determined the decrease of molds in all factories, and for factories A and C they decreased below the detection level. The acidification process did not kill members of Enterobacteriaceae family, coliforms and *E. coli* and well as pseudomonads, but inhibited their growth. All LAB groups (including enterococci) increased their levels, but those dominating the acidification process were mesophilic and thermophilic LAB cocci (on average, 8.4 and 8.3 log CFU/g, respectively). This behavior was expected considering the results registered during Caciocavallo Palermitano cheese production (Settanni et al., 2012).

The stretching operation, typical of Caciocavallo cheese making, exerted a definite sanitizing effect on the curds of all factories. Curd stretching is performed after acidification and consists on the scalding of the curd at approximately 85–95 °C to allow molding (Licitra et al., 2017). The thermal shock applied with stretching determined a further reduction (until below the detection levels) of almost all undesired microbial groups still present in curds after acidification. However, CPS, derived from bulk milk, were still detected in stretched curds and their levels were quite consistent (3.3–4.1 log CFU/g). These results are not surprising; De Andrade et al. (2022) reported that the treatment at 95 °C for a few minutes is not enough to inactivate completely CPS. However,

after 4-month ripening, also CPS levels decreased below the detection limit and these results comply with the Commission Regulation 2073/2005 on "microbiological criteria for foodstuff" (EC N° 2073/2005, 2005), highlighting the high hygienic standards of ripened CPL cheeses. Among the pro-technological groups, even though all LAB levels slightly decreased, once again both mesophilic and thermophilic LAB cocci groups dominated the microbial community. Only enterococci were not affected by the thermal treatment applied during stretching. The thermal resistance of enterococci at the temperatures applied during milk pasteurization is known (Barbaccia et al., 2022; García-González et al., 2022).

Ripened CPL cheese microbiological characteristics were highly similar among the four dairy factories. Basically, among the 16 microbial groups investigated by plate counts only TMM and all LAB groups, including enterococci, were detected. Regarding enterococci the cheeses produced in factories A and B (3.5 and 2.9 log CFU/g, respectively) showed levels lower than those produced in factories C and D (5.2 and 5.8 log CFU/g, respectively). These bacteria play several positive roles during cheese fermentation; in particular, they are involved in the development of the organoleptic characteristics and, generally, contribute consistently to the typicality of the ripened cheeses (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Concerning safety aspects, enterococci represent a risk for consumers when they show multidrug resistance and/or virulence traits (Gaglio et al., 2016b). However, the Commission Regulation 1441/2007 of 5 December 2007 does not set limits for their presence in cheeses (EC N° 1441/2007, 2007). No big differences were found between LAB cocci and LAB rods; in particular, rod groups increased with ripening following the common LAB evolution during cheese production (Samelis & Kakouri, 2022).

3.2. Analysis of microbiotas by Illumina analysis

Microbiotas associated with the wooden vats and ripened CPL cheeses were deeply studied by a next generation sequencing (NGS) approach that represents a routine investigation to provide a deep description of the microbial composition and evolution of complex environments (Jagadeesan et al., 2019). The relative abundances (%) of the bacterial OTUs resulting from MiSeq Illumina analysis of the biofilms associated with the wooden vats used to process CPL cheeses and the final 4-month ripened cheeses are distributed according to the bar plot of Fig. 2. The bacterial diversity displayed by both wooden biofilm and cheese biotas is quite limited; barely 18 taxonomic groups, mainly at family and genus levels, were identified. RA % of all samples analyzed was mostly represented by LAB: 94.74–99.70 % in wooden vat biofilms; 94.19–100 % in ripened cheeses. LAB proportions among the four dairy factories varied and these differences can be imputed to the environmental conditions, efficacy of brushing during cleaning and, especially, different bulk milks processed by each factory (Seale, Bremer, Flint, Brooks, & Palmer, 2015). The vast majority of LAB were classified as lactobacilli and streptococci; in particular, *Lactobacillus* species dominated ripened cheeses ranging from 75.56 % in RC.CPL-D and even up to 98.99 % in RC.CPL-C. However, following the reclassification of Zheng et al. (2020), the group of lactobacilli includes several genera in addition to *Lactobacillus*. Wooden vat biofilms showed a major presence of *Streptococcus* species, whose RA % ranged between 54.70 and 76.76 %. These results follow a general trend observed for the wooden vats used to process other stretched cheeses in South Italy (Lortal et al., 2009; Scatassa et al., 2015). The consistent presence of lactobacilli explains the high increase in numbers registered for this group in milk after contact with the wooden vat for all factories.

Ripened cheeses hosted negligible levels (<1 % of total bacterial diversity) of Micrococcaceae and other Actinobacteria, Firmicutes other than LAB, *Variovorax*, and Ralstonia. The presence of Acetobacteriaceae was only detected in RP.CPL-D and accounted for 5.06 %. All taxonomic groups detected in cheeses were also detected within wooden vat biofilms and, in addition, low percentages of *Chryseobacterium*, *Staphylococcus*, Enterobacteriaceae and *Acinetobacter* were also identified, especially from the wooden vat used in factory C. The presence of these bacteria is quite common on the surfaces of the wooden vats used to process milk into cheese (Sun & D'Amico, 2021).

Although culture-independent methods are being used as the sole tools to characterize complex matrix microbiota (Marino et al., 2019), these relatively new technologies are still evolving and not yet standardized (Zapka et al., 2017). Furthermore, culture-independent methods alone might not provide all information about microbiota behavior, like viability of dominant strains. For this reason, culture-based assessments are still particularly useful to study cheese microbiotas. Thus, in the present study, both culture-dependent and –independent approaches were combined and provided important information in terms of dominance of desired dairy LAB and absence of potentially harmful *E. coli* and CPS as well as pathogenic species, such as *L. monocytogenes* and *Salmonella* spp. both in wooden vats and ripened CPL cheeses. These results confirmed that wooden tools might definitely contribute to the safety of the final traditional cheeses made from raw milk (Cruciata et al., 2019).

Table 2
Gross chemical composition (g/100 g of cheese) and fatty acids profile (mg/100 g fat) of Caciocavallo Podolico Lucano cheeses.

Parameters	Samples				SEM	P value
	RC.CPL-A	RC.CPL-B	RC.CPL-C	RC.CPL-D		
Moisture	33.62 C	33.99 B	30.85 D	35.11 A	0.065	0.001
Protein	34.56 C	33.92 B	36.96 A	34.40 C	0.105	0.001
Fat	26.36 C	26.31 C	27.94 A	25.16 B	0.097	0.001
Lactose	0.69 A	1.30 A	0 B	0 B	0.167	0.001
Ash	4.77 B	4.47 B	5.81 A	5.87 A	0.076	0.001
NaCl	1.98 A	1.82 AC	1.53 BC	1.89 A	0.071	0.011
SFA	13.60	13.25	13.83	13.56	0.191	0.281
UFA	5.62 C	5.15 D	6.37 A	6.07 B	0.035	0.001
MUFA	5.08 B	4.85 C	5.44 A	5.43 A	0.040	0.001
PUFA	0.97 C	0.85 D	1.29 A	1.15 B	0.024	0.001

Results indicate the mean values of determinations carried out in duplicate for each of the two independent productions. A, B capital letters indicate significant differences for $P < 0.01$.
Abbreviations: RC, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D; SEM = standard error of mean; SFA = Saturated fatty acids; UFA = Unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

3.3. Chemical composition and fatty acid profile of cheese samples

Gross chemical composition and fatty acid profile of CPL cheese determined by FOSS are reported in Table 2. This methodology was utilized to predict dairy chemical composition and fatty acid content in several studies (Soyeurt, Dardenne, Dehareng, Bastin, & Gengler, 2008; De Marchi, Toffanin, Cassandro, & Penasa, 2014; Penasa, Tiezzi, Gottardo, Cassandro, & De Marchi, 2015; Gottardo et al., 2017). The factory affected all parameters of chemical composition ($P < 0.01$) and, except SFA, fatty acid profile ($P < 0.001$). The cheeses produced in factory C showed higher protein and fat contents than the rest of cheeses. These macromolecules are both considered indicators of nutrition quality and energy availability. Protein fraction, mainly casein, represents an excellent source of essential amino acids and bioactive peptides exerting beneficial effect on the human body (Zimecki & Kruzel, 2007). The absence of lactose in CPL cheeses of factory C provides these products with added value, since lactose in hard and long-term ripened cheeses is generally absent or present at very low concentrations and can be consumed by individuals suffering primary lactose intolerance (Silanikove, Leitner, Merin, & Prosser, 2010). Fat fraction of cheeses produced in factory C was characterized by the highest PUFA and MUFA contents that are considered of health value (Dewhurst, Shingfield, Lee, & Scollan, 2006); increasing the level of PUFA in the consumer diet is an

Table 3
Antioxidant capacity of samples collected through Caciocavallo Podolico Lucano cheese productions.

Parameters	Factories	Samples			
		BM. CPL	MAC. CPL	C.CPL	RC.CPL
TPC (g GAE/kg)	A	0.212 b	0.154 b	2.795	3.714 c
	B	0.236 a	0.203 a	2.791	4.043 ab
	C	0.235 a	0.149 b	2.946	4.219 a
	D	0.200 b	0.202 a	2.656	4.007 b
	SEM	0.005	0.013	0.051	0.049
	P Value	0.011	0.030	0.069	0.008
FRAP (mmol$FeSO_4$/kg)	A	0.588	0.565	1.692	3.354 a
	B	0.590	0.504	1.237	2.471 c
	C	0.688	0.577	1.618	2.918 b
	D	0.620	0.659	1.565	3.218 ab
	SEM	0.029	0.037	0.139	0.089
	P Value	0.194	0.063	0.244	0.008
TEAC (mmolTrolox/kg)	A	5.648 A	2.740 c	18.535	47.563 b
	B	3.007 B	3.572 a	18.760	39.701 c
	C	3.282 B	3.079 b	16.813	57.995 a
	D	2.902 B	3.402 a	23.401	51.964 ab
	SEM	0.155	0.102	2.154	1.910
	P Value	0.001	0.004	0.301	0.011

Results indicate the mean values of the determinations carried out in duplicate for each of the two independent productions. A, B capital letters and a, b, c small letters in column within the same sample indicate significant differences for $P < 0.001$, $0.01 < P < 0.05$, respectively.
Abbreviations: BM, bulk milk; MAC, milk after contact with wooden vat surface; C, curd; RP, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D; TPC = total phenolic content; GAE = gallic acid equivalent; FRAP = ferric reducing ability power; TEAC = Trolox equivalent antioxidant capacity; SEM = standard error of mean.

important nutritional recommendations are to (Department of Health, 1994). Chemical composition and fatty acid profile of CPL cheese highlighted the uniqueness of this traditional cheese. These results showed a great variability due to the numerous factors characterizing the dairy factories, such as quality and quantity of forage available for grazing, pasture biodiversity, soil type, geographical position and climate. Furthermore, the artisanal techniques applied during cheese making and ripening play a relevant role in definition of chemical and fatty acid profiles. The average values of protein, fat, lactose, ash and salt of the CPL cheeses, ripened for four months, produced in the four factories were close to those reported by Di Trana, Claps, Quaranta, Salvia, and Lovallo (2022a) for the CPL ripened 6 months and made at early spring. Regarding fatty acid profile, PUFA content of the cheeses manufactured in this study confirm that grazing is closely related to higher PUFA intake (Chilliard et al., 2007). The extensive rearing system of Podolica cattle is a peculiar characteristics of this traditional cheese production system; consequently the differences in PUFA among factories are related to the type of plants ingested and their development stage (Chilliard et al., 2007). In Alpine cheeses produced in summer and winter, Danieli et al. (2022) reported a factory effect in MUFA and PUFA contents.

3.4. Antioxidant properties of cheeses

Results concerning the effect of dairy factory on cheese antioxidant properties are reported in Table 3. In bulk milk before and after wood contact, TPC and TEAC were affected by factory factors ($P < 0.05$ and $P < 0.001$, respectively), while no significant differences were recorded for FRAP assay. Concerning curd, no effect of factory was observed on any quantified parameters. On the contrary, production factory affected TPC, FRAP and TEAC values of CPL cheeses ripened for four months ($P < 0.008$ and $P < 0.01$, respectively).

Phenolic compounds are substances produced by several plants that are beneficial to the human health (Di Lorenzo, Colombo, Biella, Stockley, & Restani, 2021). The presence of these compounds originating from forage species (De Feo et al., 2006; Di Trana et al., 2015), natural pastures (Chavez-Servin et al., 2018; Hilario, Puga, Ocana, & Romo, 2010) and aromatic plants (Branciari et al., 2015; Garcia, Rovira, Boutoial, & Lopez, 2014) has been observed in milk and dairy products. Therefore, a strong relationship between a given breeding environment (plant biodiversity and plant availability) and the dairy products processed from milk of cows reared in that area is established. Furthermore, the bioavailability of phenolic compounds is connected to the physiology of ruminant digestion and to the degree of polymerization of polyphenols (Bravo, 1998), which act on the degradation and absorption of these compounds and/or their metabolites (Di Lorenzo et al., 2021; Tufarelli, Casalino, D'Alessandro, & Laudadio, 2017). This complex mechanism underlies the degree of transfer of these bioactive molecules from the animal diet to milk and, finally, cheese. Thus, each factory characterized by its own pasture determines the uniqueness of the final cheeses.

In this study, the factory as a whole affected TPC of milk before and after contact with the wooden vat and CPL cheese ripened for four months. The highest TPCs were observed in factories B and C both for bulk milk and for CPL cheeses, but not for bulk milk after contact with the wooden vat, because the samples from factory C showed the lowest TPC value (Table 3). The effect of the feeding system, implemented in various factories, was highlighted on the TPC content in the milk of cows reared on intensive rotational grazing, semi-intensive conventional grazing and conventional grazing (Kuhnen et al., 2014). TPC values registered in this study are not always comparable with those from cheeses obtained with the same production technique and ripening. Even though TPC values measured for CPL cheeses were in the same range of those recorded for Caciocavallo Palermitano cheese (3.52–4.65 g GAE/kg) (Di Trana et al., 2022b), they were higher than those reported for stretched cheese Casizolu del Montiferru (2.98–3.65 g GAE/kg).

The antioxidant capacity of dairy products is mainly related to caseins, β-carotene, uric acid, vitamin E, phenols, whey protein and microbiological profile (Fardet & Rock, 2018; Khan, Bule, Rahman Ullah, Asif, & Niaz, 2019), and it can hinge on cheese making procedure (Lucas et al., 2006), type of coagulant (Pattorn & Hongsprabhas, 2013), conditions and duration of ripening (Gupta, Mann, Kumar, & Sangwan, 2009). In the present study, FRAP assay and TEAC (ABTS assay) were used to determine the antioxidant capacity of CPL cheese. FRAP assay did not indicate great specificity for antioxidant compound content in milk and curd of different factories (Table 3); no difference was found among factories for this parameter. In CPL cheeses 4-month ripened, the antioxidant capacity evaluated by FRAP test showed the highest values in cheeses from factories A and D, and this result is in agreement with what was observed by Danieli et al. (2022) who highlighted the factory effect on Alpine cheeses produced in winter. Comparing CPL cheeses to other traditional southern Italian cheeses, FRAP values were higher than those of 6-month ripened Casizolu del Montiferru cheese (1.69–2.08 mmol $FeSO_4$/kg) and 2-month ripened Caciocavallo Palermitano cheese (1.84–2.00 mmol $FeSO_4$/kg) (Di Trana et al., 2022b).

The bulk milk before wood contact of factory A showed the highest TEAC, while among bulk milks after wood contact, the highest TEAC values were detected in factories B and D. A similar trend was registered for TPC values. Regarding curd samples, no significance was observed for the factory factor. In addition, differences were found for TEAC when comparing the factories; CPL cheeses produced in factory C showed TEAC values 32 %, 18 % and 10 % higher than those registered for the cheeses produced in factories B, A and D, respectively (Table 3). The factory effect on the TEAC seems reasonably explained by the cows' feeding regimes adopted in spring-summer and by plant biodiversity and plant availability in the different area of grazing. The highest values of

Table 4
Volatile organic compounds emitted from Caciocavallo Podolico Lucano cheeses.

Chemical compounds	Samples				P value
	RC.CPL-A	RC.CPL-B	RC.CPL-C	RC.CPL-D	
Acids					
Acetic acid	13.78 ± 2.62	14.51 ± 3.01	15.31 ± 2.30	12.25 ± 1.96	0.523
Butanoic acid	32.15 ± 4.82	35.11 ± 5.69	26.87 ± 4.03	30.63 ± 5.21	0.305
Pentanoic acid	0.34 ± 0.07 A	n.d. B	0.28 ± 0.05 A	0.29 ± 0.06 A	0.001
Exanoinc acid	42.06 ± 6.73	30.77 ± 5.91	40.26 ± 6.04	37.77 ± 6.42	0.214
Octanoic Acid	5.82 ± 0.81 a	3.00 ± 0.57 b	5.70 ± 1.09 a	5.48 ± 1.05 a	0.015
Nonanoic Acid	0.64 ± 0.13 B	2.11 ± 0.4 A	0.25 ± 0.05 B	0.25 ± 0.04 B	0.001
Esters					
Butanoic acid ethyl ester	0.27 ± 0.06 B	1.67 ± 0.31 A	0.75 ± 0.11 B	2.01 ± 0.44 A	0.001
Hexanoic acid ethyl ester	2.37 ± 0.44 C	6.54 ± 1.16 AB	5.17 ± 0.98 B	8.80 ± 1.38 A	0.001
Heptanoic acid, 2 methyl-2-butyl ester	n.d. B	0.53 ± 0.08 A	n.d. B	n.d. B	0.001
octanoic acid ethyl ester	0.24 ± 0.05 C	0.50 ± 0.09 BC	0.60 ± 0.12 AB	0.86 ± 0.16 A	0.001
Alcohol					
1 butanol 3 methyl (isoamyl alcohol)	1.08 ± 0.21 a	1.34 ± 0.2 a	1.08 ± 0.17 a	0.62 ± 0.1 b	0.007
1 hexanol/ 1 pentanol 4 methyl	0.53 ± 0.1 B	0.54 ± 0.09 B	2.22 ± 0.37 A	0.40 ± 0.07 B	0.001
2 heptanol	0.21 ± 0.03 B	1.20 ± 0.24 A	0.42 ± 0.07 B	0.33 ± 0.06 B	0.001
1 octanol	n.d. B	n.d. B	0.32 ± 0.05 A	n.d. B	0.001
Ketons					
2 Pentanone	0.22 ± 0.05 A	0.10 ± 0.02 B	0.07 ± 0.01 B	0.03 ± 0.01 B	0.001
2 Heptanone	0.17 ± 0.09 B	0.50 ± 0.02 A	0.13 ± 0.02 B	0.07 ± 0.02 B	0.001
2 Nonanone	n.d. C	1.25 ± 0.17 A	0.44 ± 0.07B	n.d. C	0.001
Aldeydes					
2-Octenal	0.06 ± 0.01 C	0.21 ± 0.04 A	0.08 ± 0.01 BC	0.14 ± 0.02 B	0.001
Nonanal	0.06 ± 0.01 B	0.12 ± 0.02 A	0.04 ± 0.01B	0.06 ± 0.01 B	0.001

Results indicate the mean percentage values of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant peaks in all samples) × *l*00. A, B capital letters and a, b small letters on the row indicate significant differences for $P < 0.001$, $0.01 < P < 0.05$, respectively.
Abbreviations: RC, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D; n.d., not detectable.

TPC and TEAC observed in CPL cheeses produced in factory C reflect the positive correlation reported by Přikryl et al. (2018) between TPC and antioxidant capacity of cheese. TEAC values in CPL cheeses (Table 3) are close to those reported for Caciocavallo Palermitano cheese (46.8–52.4 mmol/kg) but much higher than those characterizing Casizolu del Montiferru cheese (10.3–18.9 mmol/kg) (Di Trana et al., 2022b).

3.5. Cheese VOC profiles

VOC profiles generated from cheese samples were identified by SPME-GC–MS; results are reported in Table 4. VOC belonging to fatty acids, alcohols, esters, aldehydes and ketones were detected in all cheese samples. For all factories, the main VOC class found in cheese samples was free fatty acids (FFA), specifically hexanoic and butanoic acids showed the highest values in all cheeses. This finding is not surprising; indeed, hexanoic and butanoic acids are generally recognized in cow's milk cheeses (Wolf, Perotti, & Zalazar, 2011; Sulejmani & Hayaloglu, 2020). Short-chain FFA may generally result from lipolysis of milk fats due the action of the lamb rennet used for curdling and, partly, also to the activity of raw milk lipoprotein lipase (McSweeney & Sousa, 2000). Even acetic acid was found in comparable proportions among the cheeses from the four dairy factories; this compound can be originated by the carbohydrate (primarily lactose) catabolism of LAB under anaerobic conditions (Rehman et al., 2000).

After FFA, esters represented the second major class of compounds found in the volatile fraction of cheese samples. Within this class, ethyl hexanoate showed the highest area value, followed by ethyl butanoate, ethyl octanoate and ethyl decanoate. These compounds have floral and fruity notes and may contribute to cheese aroma by minimizing the sharpness of fatty acids and the bitterness of amines (Pinho, Pérès, & Ferreira, 2003). Similar ester profiles were also observed in other cheeses such as "Provola dei Nebrodi" (Ziino, Condurso, Romeo, Giuffrida, & Verzera, 2005). Aldehydes, alcohols and ketones are the most poorly represented classes in all cheeses. Among the four cheese productions, statistical analysis showed slight variations. In particular, pentanoic acid was not detected in cheeses from factory B, while nonanoic acid concentration was higher than that of the other cheeses. Among esters, hexanoic acid ethyl ester in cheese from factory A was detected at low concentrations, while the highest were registered for production D. Heptanoic acid, 2 methyl-2-butyl ester was identified only for the cheeses processed in factory B. Regarding alcohols, isoamyl alcohol was less abundant in production D, but comparable values were displayed by the other cheeses. Similarly, 1-hexanol was most abundant in cheese from factory C while comparable levels were registered in the other cheeses. Among ketones, production A differed for the highest content of 2-pentanone, while production B showed the highest 2-heptanone content. Finally, 2-nonanone was detected exclusively in cheeses from factories B and C. Generally, the aromatic profiles of the cheeses did not greatly differ among the four productions, especially with regard to the volatile compounds present at low levels. The production process may affect the content of esters that are mainly produced by enzymatic or chemical reaction of fatty acids with primary alcohols during ripening (Engels, Dekker, De Jong, Neeter, & Visser, 1997), whereas the presence of aldehydes, alcohols and ketones is directly influenced by the enzymatic activities of microorganisms (Muñoz, Ortigosa, Torre, & Izco, 2003).

3.6. Sensory analysis

CPL is a high quality cheese with an intense and complex flavour. Fig. 3 reports the Kiviat graphic representation of the sensory characteristics evaluated on the CPL cheeses produced in four factories. Farm management, animal diet (Carpino et al., 2004), cheesemaking technology, chemical and microbiological characteristics of raw milk (Martin, Verdier-Metz, Buchin, Hurtaud, & Coulon, 2005) influence cheese sensory properties. As the factories were located in the same territory but on different altitude, the sensory attributes could present a variability linked to the different floristic composition of pasture. Except for persistence, all smell sensory descriptors of the cheeses were affected by factory. Acid smell attribute, typical of lactic and citric acids, was evident only in cheeses produced in factory D. The pungent aroma is a sharp smelling or irritating with a physically penetrating sensation in the nasal cavity (Murray & Delahunty, 2000). Curioni and Bosset (2002) reported that the odour pungent descriptor in cheeses could be linked to the following volatile compounds: propanoic acid, ethanoic acid when pungent is vinegar-like, 3-methylbutanal and sulfur compounds

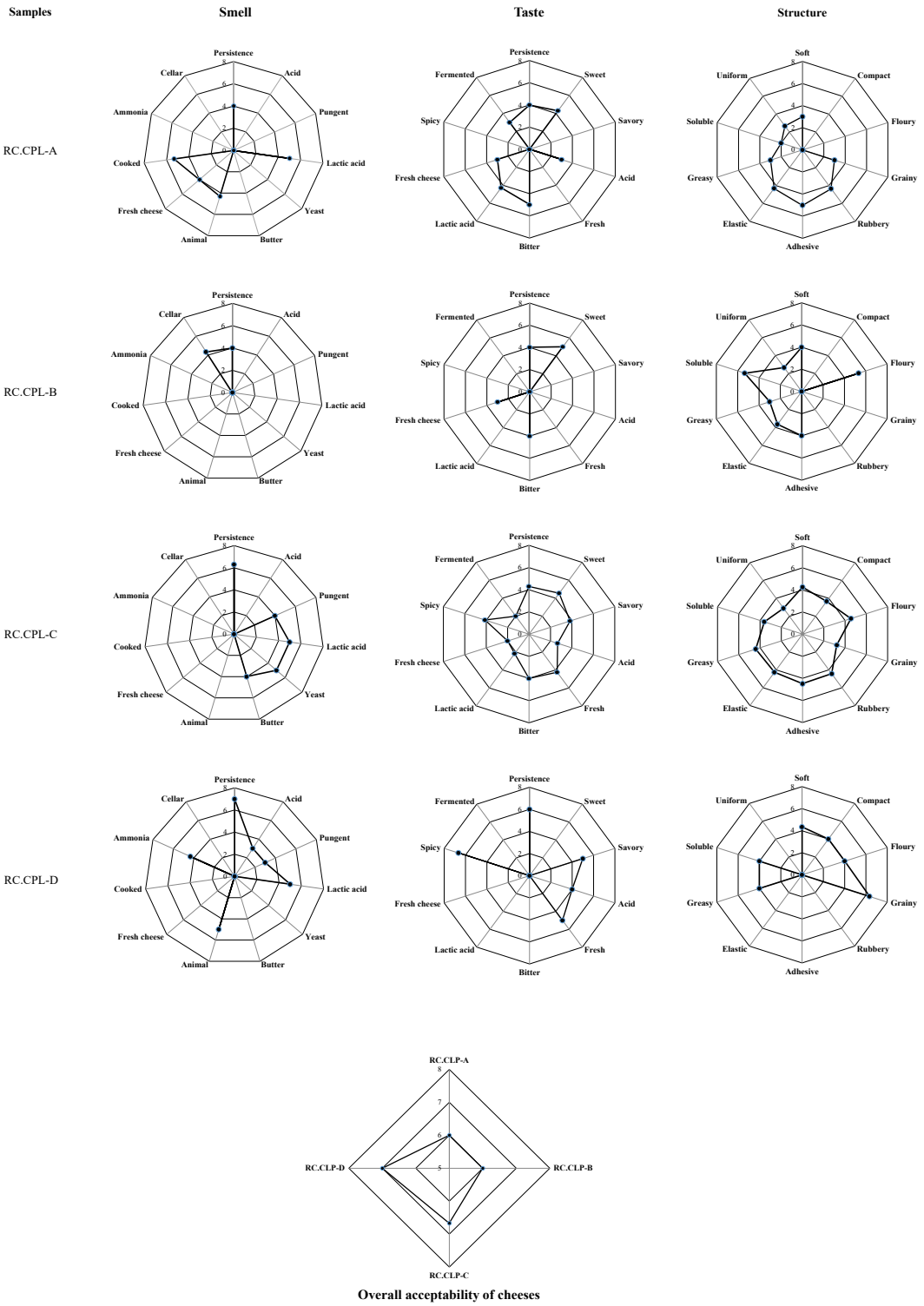

Fig. 3. Kiviat diagrams corresponding to the descriptive sensory analysis of Caciocavallo Podolico Lucano (CPL) cheeses. Abbreviations: RC, ripened cheese; A–D, factories A–D.

products from Strecker degradation, in particular 3-methylthiopropanal which imparts a pungent acrid odour. In our work, VOCs responsible for pungent aroma were not detected, but this attribute characterized the cheeses from factories C and D. Fermented aroma could be associated with a long-ripened cheese (Santillo et al., 2012) and except for the cheeses from factory B, was revealed in all cheeses. Butter odour has been associated with δ-dodecalactone and decanoic acid in Grana Padano, with 2-methylpropanoic acid in Gruyère cheese and with 2,3-butanedione (diacetyl) in Camembert, Cheddar and Emmental (Curioni & Bosset, 2002). Furthermore, butter smell linked to 3-hydroxy-2-butanone was detected in Ragusano cheese obtained from cows fed on pasture, and not from cows that received a total mixed ration (Carpino et al., 2004). In our study, none of these VOCs was detected and all cows were fed on pasture, but the cheese produced in factory C were scored positive for butter smell. Animal odour was reported in bovine and water buffalo Mozzarella due to the presence of aldehydes nonanal, in

Roncal cheese connected to tetradecanoic acid and in water buffalo Mozzarella linked to ketons 2-heptanone (Curioni & Bosset, 2002). In our study, animal smell was perceived in cheeses from factories A and D but does not seem to be related to nonanal and 2-heptanone emitted at the highest levels from the cheeses produced in factory B which resulted negative for this attribute. Fresh cheese smell attribute was linked to the presence of ethyl-3-methylbutanoate, and identified in Mozzarella bovine (Curioni & Bosset, 2002). Cooked smell, related with S-methional compounds was found in Grana Padano and Cheddar (Curioni & Bosset, 2002). In our study however, this was only evident in cheese produced in factory A and was not correlated with the sulphur compounds.

Except for persistence, all taste sensory descriptors of cheeses were affected by factory. Sweet taste, typical of sucrose, characterized all cheeses except that produced in factory D. Furthermore, all cheeses except cheese D were characterized by bitter taste, typically associated to caffeine and quinine (Murray & Delahunty, 2000); this may related to the different cheesemaking technologies or brine (Fallico et al., 2005). The spicy or piquant note, irritating and aggressive taste perceived in the mouth or throat (Esposito et al., 2014), was evident in cheeses from factories C and D. Furthermore, this sensation is also a consequence of an intense lipolysis generating higher FFA (Santillo et al., 2012).

Regarding CPL cheese structure, the products from factories A and C showed the greatest number of attributes (soft, grainy, rubbery, adhesive, elastic and greasy). Rubbery, adhesive and elastic were significantly affected by the factory. From a structural point of view, the mechanical behavior of cheese is the result of complex interactions established between various components such as the resistance to deformation of the casein network (Lawrence, Gilles, & Creamer, 1983), humidity, minerals and fat (Masi & Addeo, 1986). The elasticity of some stretched curd cheeses is positively related to fat and moisture content and negatively to mineral content (Masi & Addeo, 1986). In fact, CPL cheeses produced in factory C with higher rubbery, adhesive and elastic attributes were characterized by higher protein, fat and lower salt and moisture contents, while the cheeses produced in factory A showed a lower protein and fat but a higher salt and humidity content and exhibited slightly lower scores for rubbery and elastic attributes. Elasticity was related to ripening and cheesemaking technology factors and, for Caciocavallo Palermitano it was correlated to the compressive stress (Bonanno et al., 2013). A relationship between rubbery attribute and the percentage of salt in brine has been detected in Caciocavallo dei Monti Dauni produced in different factories (Santillo et al., 2012). Buffa, Trujillo, Pavia, and Guamis (2001) and (Bonanno et al., 2013) proposed that cheese microbiota modifies the structure of cheese. The soft attribute has been related to cheese FA profile (Martin et al., 2005; Esposito et al., 2014) which, in turn, is closely connected to the nature and amount of fresh forage intake (Chilliard, Ferlay, & Doreau, 2001). The cheeses produced in factories C and D, a higher UFA content associated with a slightly higher softness score was observed. In all factories, Podolica breed cows used pasture characterized by plant biodiversity and availability with an impact on cheese UFA content.

In general, a good sensory profile was found in TAP CPL cheeses analyzed in this study. However the variability among factories, considered as a peculiarity of all TAP cheeses, was confirmed. However, although sensory evaluation indicated some differences among cheeses, sensory evaluation highlighted that the overall acceptability did not differ significantly ($p > 0.05$) among factories. Similar results were observed by Claps (2001), who evaluated the sensory profile and acceptability of CPL cheeses produced on mountain and hilly pastures.

3.7. Data correlation

With the aim of investigating the relationships between cheese sensory attributes and data from microbiological, chemical and VOC analyses, a HCA was performed. This analysis allows to differentiate the samples examined (cheeses) in accordance with their mutual

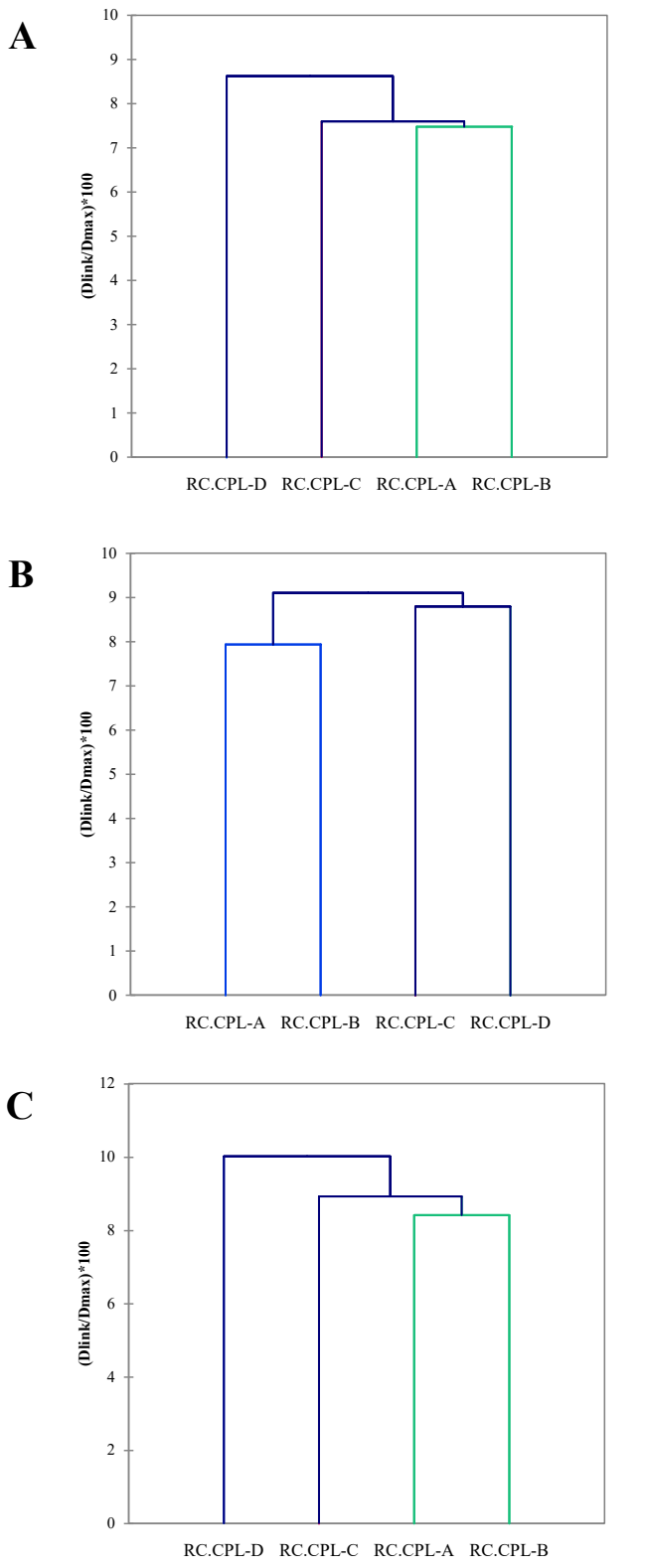

Fig. 4. Dendrograms resulting from HCA based on values of: A, sensory attributes and microbiological data; B, sensory attributes and chemical data; C, sensory attributes and VOCs data. The dissimilarity among cheeses was measured by Euclidean distance, while cluster aggregation was achieved by single linkage. Abbreviations: RC, ripened cheese; CPL, Caciocavallo Podolico Lucano; A–D, factories A–D.

dissimilarity and relationship and join together in a dendrogram from the closest one, i.e., the most similar, to the furthest apart, which is the most different (Guarcello et al., 2016). The three resulting clusters are reported in Fig. 4. This figure clearly shows levels of dissimilarity at around 8 % among the cheeses for all relationships evaluated. In detail, regarding the relationships among sensory attributes and microbiological-VOCs (Fig. 4 A and C) data, the cheeses from the factories A and B formed a single cluster and they were clearly separated from those produced in factory C and factory D. The HCA performed with sensory attributes and chemical data formed two main clusters (Fig. 4 B) one for the cheeses from the factories A and B and one for those produced in factory C and factory D.

4. Conclusions

CPL cheese is produced from raw milk without the addition of starter cultures. The use of the traditional wooden tools provide typicality to the final products. This work demonstrated that the transformation conditions determined the development and dominance of dairy LAB over undesired raw milk microbiota, even though the role of enterococci needs further investigation. The differences in terms of microbial levels and species composition revealed among the dairy factories object of the present study highlighted the strong link of the final cheeses with the production environment. Basically, the main microbiological parameters were highly comparable among the cheeses produced in different factories and also the physicochemical profiles, antioxidant properties and sensory attributes of CPL cheeses were quite stable among dairy factories. This study demonstrated that the slavish application of the traditional CPL cheese making protocol among dairies is the preferred strategy to harmonize the microbial evolution and to maintain almost constant the final characteristics of CPL cheese.

CRediT authorship contribution statement

Gabriele Busetta: Formal analysis, Investigation, Methodology. **Giuliana Garofalo:** Formal analysis, Investigation, Methodology. **Marcella Barbera:** Formal analysis, Investigation, Writing – original draft. **Adriana Di Trana:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Salvatore Claps:** Conceptualization, Data curation, Writing – original draft. **Carmela Lovallo:** Formal analysis, Investigation. **Elena Franciosi:** Formal analysis, Methodology, Software. **Raimondo Gaglio:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Luca Settanni:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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