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Application of whole genome sequenced selected *Pediococcus acidilactici* to tailor the making of the spreadable fresh ewe's milk "Quadrello di Ovino" cheese to the production area

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ABSTRACT

The aim of this study was to utilize *Pediococcus acidilactici* strains as starter microorganisms in producing the fresh ewe's cheese "Quadrello di Ovino". Whole-genome analysis confirmed the absence of virulence factors (such as hemolysins) and genes conferring resistance to commonly used antibiotics indicated by the European Food Safety Authority. A control trial was conducted with commercial freeze-dried starters (CP), against the novel experimental cheese (EXP) inoculated with pediococci. Cheeses from both the control and experimental production showed high levels of lactic acid bacteria (LAB). Mesophilic LAB were present at 7.09 and 8.50 log CFU/g, respectively, while thermophilic LAB were found at 6.95 and 8.46 log CFU/g, respectively. Both cheeses showed no presence of spoilage or pathogenic microorganisms according to plate counts. While there were no significant differences in fat and protein content between them, the EXP cheese had a slightly higher protein content (16.85 %). Additionally, both cheeses had a complex profile of volatile organic compounds, with higher monounsaturated fatty acids (oleic acid) and polyunsaturated fatty acids (linoleic and linolenic acids) content in the EXP cheese at 26.80 %, 2.61 %, and 0.70 %, respectively. From a sensory perspective, the EXP cheese showed a diminished persistence of the taste typical of ewe's milk and a reduction in the unpleasant animal odour commonly found in cheeses made from small ruminants, while enhancing paste homogeneity and odor intensity. These observations indicate the product's promising potential, considering the increasing demand in local and foreign markets for spreadable cheeses with creamy consistence.

1. Introduction

Most cheeses produced today worldwide rely on the inclusion of specific starter microorganisms. These "starters" initiate cheese fermentation by consuming lactose and converting it into lactic acid (Herreros, Fresno, Prieto, & Tornadijo, 2003). Commonly referred to as "primary starters", these microorganisms typically belong to the lactic acid bacteria (LAB) genera, including *Streptococcus*, *Lactococcus*, *Lactobacillus* and related genera, as well as *Leuconostoc* (Parente & Cogan, 2004). However, in cheesemaking, alongside starter cultures, non-starter cultures play a significant role. These non-starters contribute to the development of flavourful organoleptic and sensory characteristics (Gaglio et al. 2020).

When incorporating new LAB as a starter, prioritizing food safety is crucial. Although the use of starter cultures has a long history, recent research indicates that microorganisms within these cultures may acquire antibiotic resistance genes. This development raises concerns about the potential antibiotic resistance of bacteria associated with LAB (Federici et al., 2014; Kastner et al., 2006). It is worth noting that the antibiotic resistance observed in starter culture bacteria does not directly endanger consumers, as these bacteria are not pathogenic. However, they can serve as environmental reservoirs for antibiotic

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resistance determinants. Consequently, caution should be exercised when using bacteria with antibiotic resistance genes in food production. Furthermore, stress factors during food production and storage may influence the antibiotic resistance profile of these microorganisms (Papadimitriou et al. 2016). While the development of antibiotic resistance in starter cultures is not a critical concern, it remains an important consideration that cannot be overlooked (Zarzecka, Zadernowska, & Chajęcka-Wierzchowska, 2020). All microorganisms used in food production must be classified as GRAS (Generally Recognised as Safe). Specifically, they should lack pathogenicity factors, antibiotic resistance, or virulence genes. Simultaneously, they must contribute to technological aspects without posing any risk to consumer health. This concept aligns with the European Food Safety Authority (EFSA) 2020 guidelines, which mandate comprehensive genome investigations of microorganisms to mitigate potential risks to consumers. Biological sciences play a crucial role in food safety (Capozzi, Fragasso, & Russo, 2020). The EFSA introduced the Qualified Presumption of Safety (QPS) to assess the risk of microorganisms used in products, based on taxonomic identification, knowledge analysis, safety concerns evaluation, and intended use. Microbial strains meeting these criteria and aligning with a QPS cluster are exempt from further safety evaluations, while others undergo full safety assessment (Koutsoumanis et al., 2023).

In an effort to innovate the ewe's milk product sector, Quadrello di Ovino cheese is a newly created spreadable fresh sheep milk cheese. It is made from pasteurized milk inoculated with *Streptococcus thermophilus* starter cultures (Garofalo et al., 2021). Briefly, the cheese-making process involves heating the milk to 38 °C and adding the starter culture approximately 40 min before the addition of liquid rennet. After coagulation, the curd is cut into approximately 3 cm cubes, placed in cuboid molds, and steamed. During steaming, the cheeses are turned upside down and left to acidify until reaching a pH of 5.4. The cheeses are then refrigerated for around 24 h, immersed in saturated brine for a few minutes, and stored under refrigeration for 4 d before being packed in plastic boxes sealed with transparent film. The decision to use *S. thermophilus* as the starter culture stems from the adaptation of the production process of cow's milk "Crescenza" cheese to create Quadrello di Ovino cheese. For Crescenza cheese, a commercial thermophilic culture preparation containing *S. thermophilus* is recommended (Tidona et al., 2020).

This research aimed to utilize specific strains of *Pediococcus acidilactici* as starter culture in Quadrello di Ovino cheese production. The strains used in this study were isolated from the Sicilian dairy environment and had demonstrated intriguing acidifying capabilities. Interestingly, *P. acidilactici* are not commonly employed as primary starters in cheesemaking, making them an excellent subject for exploration. Indeed, as noted in previous studies (Pavlatou et al., 2023; Wang et al., 2023), the inclusion of *P. acidilactici* strains can affect the physicochemical characteristics of cheese, such as pH, acidity, and moisture content, which are crucial for determining cheese quality. This addition not only enhances the overall quality, taste, and texture but also results in a higher moisture content, bringing the cheese closer to its bovine counterpart in texture. The primary goal was to investigate whether these strains could impart unique sensory properties to the final cheese, thereby establishing a connection between the product and its geographical origin. Before their use in cheesemaking, the *P. acidilactici* strains underwent whole-genome sequencing (WGS) analysis to assess their safety. The resulting cheese underwent thorough assessment, covering microbiological, physicochemical characteristics, composition, and sensory aspects.

2. Materials and methods

2.1. Whole-genome sequencing analysis of Pediococcus acidilactici strains

The total genomic DNA was extracted from *P. acidilactici* RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035 from 1.8 mL of broth culture using the Wizard Genomic DNA Purification System kit (Promega Italia, Milan, Italy), following the manufacturer's instructions. The DNA quality was estimated by Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and NanoDrop One/OneC Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Library preparation and sequencing were performed by Centro Piattaforme Tecnologiche (CPT University of Verona, Italy). In detail, libraries were obtained using KAPA PCR-Free Kit (Roche Diagnostics SpA, Monza, Italy) according to the manufacturer's instructions; Illumina MiSeqDX in paired-end 150 mode was employed as platform sequencing. The raw reads were demultiplexed and adapters were masked by CPT tools. The *de novo* assembly was performed with SPAdes using default options [version 3.13.0; (Bankevich et al., 2012)]. The quality of the genome assembly was assessed with the software QUAST (Quality Assessment Tool for Genome Assemblies) [version 5.0.2; (Gurevich, Saveliev, Vyahhi, & Tesler, 2013)] and BUSCO (Benchmarking Universal Single-Copy Orthologs) [version 4.1.4; (Manni, Berkeley, Seppey, & Zdobnov, 2021)] using 'lactobacillales_odb10′ as lineage dataset. Genome annotation was performed with RAST (Rapid Annotation using Subsystem Technology) (https://rast.nmpdr.org/rast.cgi) which was used to obtain the subsystem category distribution (Overbeek et al., 2014).

The assembly datasets obtained for *P. acidilactici* RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035 have been deposited at DDBJ/EMBL-ENA/GenBank under the accession number JBINAC000000000 and JBIUGK000000000, respectively.

2.2. Taxonomic identification and genome-based safety assessment

The Average Nucleotide Identity (ANI; (Goris et al., 2007)), was calculated using the ANI Calculator in EzBiocloud (https://www.ezbiocloud.net/tools/ani) while digital DNA-DNA hybridization (dDDH; (Auch, von Jan, Klenk, & Goker, 2010)) was determined using GGDC (Genome to Genome Distance Calculator 3.0; (Meier-Kolthoff, Carbasse, Peinado-Olarte, & Goker, 2022)).

The annotated sequences of both strains were employed to query the Comprehensive Antibiotic Resistance Database (CARD, version 3.2.7; https://card.mcmaster.ca/) through the Resistance Gene Identifier tool (RGI, version 6.0.2; (Alcock et al., 2023)) selecting only "Perfect" and "Strict" hits and to query ResFinder 4.1 database (https://cge.food.dtu.dk/services/ResFinder/; (Florensa, Kaas, Clausen, Aytan-Aktug, & Aarestrup, 2022)) with 90 % as percentage of identity and 60 % as query coverage for acquired antimicrobial resistance genes.

The presence of the genes encoding for histidine and tyrosine decarboxylases was investigated in the two *Pediococcus* genomes. BLASTn was performed using the following sequences (GenBank ID) as queries: AF446085.5 (*Levilactobacillus brevis* IOEB 9809); AF354231.1 (*Enterococcus faecalis* JH2-2); AB125629.1 (*Tetragenococcus muriaticus*); U58865.1 (*Oenococcus oeni* 9204); J02613.1 (*Ligilactobacillus saerimneri* 30A); AJ749838.1 (*Lentilactobacillus buchneri* B301); AY651779.1 (*Lentilactobacillus hilgardii* IOEB 0006).

VFDB [Virulence Factor DataBase; (Liu, Zheng, Zhou, Chen, & Yang, 2022)] was employed for virulence factor analysis, focusing the search on the enzymes and toxins of *Enterococcus* (the phylogenetically closest LAB) reported in VFDB.

2.3. Experimental plan and starter preparation

Drawing inspiration from the research conducted by Garofalo et al. (2021), this study devised two distinct cheese production approaches. The control production (CP) involved incorporating freeze-dried commercial starter formulations CRBS7, composed of one defined strains of *Streptococcus salivarius* subsp. *thermophilus*, purchased from Calza Clemente (Acquanegra Cremonese, Italy). The lyophilised starter was reactivated following the manufacturer's instructions. Specifically, 5

units were reactivated in 2 L of pasteurized ewe's milk through manual agitation for 10 min, after which they were directly added to the pasteurized ewe's milk. The experimental production (EXP) was carried out by inoculating the two selected strains of *P. acidilactici* (RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035), as previously described. These distinct approaches aimed to explore the impact on cheese quality and characteristics and the experimental plan is described in Fig. 1.

The two bacterial strains were revitalized in a suitable culture broth (M17) medium (Oxoid, Milan, Italy) and incubated at 37 °C for 24 h. Subsequent refreshments were performed to generate an adequate pellet volume for inoculation. Following this, two consecutive washes with Ringer's solution (0.9 % v/v) were carried out using a Neya 16R centrifuge (Securlab SRL, Rome, Italy) at 7000 rpm for 2 min to remove any remnants of the broth medium. The milk starter culture (MSC) was then prepared by inoculating approximately 10^6 colony-forming units (CFU)/mL of the washed cells from both strains into whole UHT ewe's milk (Leeb Vital, Wartberg an der Krems, Austria) and then incubated at 37 °C for 24 h.

2.4. Cheesemaking and sample collection

Cheese production followed the methodology outlined by Garofalo et al. (2021) using ewe's milk sourced from various dairy farms. This milk is daily transported to the "Melia S.r.l." factory (Sicily, Italy) via tanker trucks maintained at controlled temperatures. Subsequently, the milk underwent pasteurization in a PASTMATIK pasteurizer (Magnabosco S.r.l., Zugliano, Italy) for 15 s at 71 °C. After heat treatment, the milk was cooled to 38 °C and inoculated with the starter strains. The same protocol used to make "Quadrello di Ovino" cheese (Garofalo et al., 2021) was then repeated. The cheesemaking trials, CP and EXP, were conducted on the same day using the same bulk milk to accurately assess the impact of the different starter cultures. The productions were then repeated one month later to serve as experimental replicates. Batch samples of raw sheep's milk (RM), pasteurized milk (PM), inoculated milk (IM), whey (W) and curd (C) were sampled under aseptic conditions during each day of cheesemaking in both trials. All samples were transported under cooled conditions to the Agricultural Microbiology Laboratory at the University of Palermo. Finally, the cheeses (Ch) were sampled after 4 d of refrigerated storage.

2.5. Microbiological analyses

One mL of liquid samples (RM, PM, IM, W) was aliquoted and subjected to ten-fold dilutions in Ringer's solution prepared at 0.9 % (v/v) salt. Additionally, 15 g of solid samples (C and Ch) were first initially homogenized with 135 mL of an isotonic sodium citrate solution prepared at 0.2 % (v/v) using a Bag-Mixer 400 paddle homogenizer (Interscience, Saint Nom, France). The homogenizer operated at maximum speed for 2 min. Subsequently, the solid samples underwent the dilution procedure in Ringer's solution, effectively reducing the initial density by an order of magnitude. Cell suspensions from RM and PM were subjected to plate counts to enumerate several microbial groups: Total Mesophilic Microorganisms (TMM) on Plate Cout Agar (PCA), incubated at 30 °C for 72 h; pseudomonads on *Pseudomonas* Agar Base (PAB) supplemented with Cephaloridine–Fucidin–Cetrimide (CFC), incubated at 25 °C for 48 h; enterococci on Kanamycin Aesculin Azide (KAA) agar, incubated at 37 °C for 24 h; coagulase-positive staphylococci (CPS) on Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen, incubated at 37 °C for 24 h. *Listeria monocytogenes* was cultured on *Listeria* Selective Agar Base (LSAB) supplemented with SR0140E, incubated at 37 °C for 24 h; *Escherichia coli* and *Salmonella* spp. were grown on Hektoen Enteric Agar (HEA) at 37 °C for 24 h. Unicellular fungi were cultivated on Yeast Peptone Dextrose (YPD) supplemented with chloramphenicol (0.1 mg/mL) to inhibit bacterial growth. These cultures were incubated at 30 °C for 48 h. Potato Dextrose Agar (PDA) was used for filamentous fungal growth and incubated at 30 °C for 7 d. Additionally, lactic acid bacteria (LAB), total coliforms, and members of the Enterobacteriaceae family were included. Mesophilic LAB rods were cultured on de Man-Rogosa-Sharpe (MRS) agar acidified with 5 M lactic acid to pH 5.4, and incubated at 30 °C for 48 h. Thermophilic LAB rods were grown on Whey-Based Agar Medium (WBAM), following the preparation described by Settanni et al. (2012), and incubated at 44 °C for 48 h. Mesophilic and thermophilic LAB cocci were cultured on M17 agar medium, with mesophilic LAB incubated at 30 °C for 48 h and thermophilic LAB at 44 °C for 48 h. Enumeration of members of the Enterobacteriaceae family and coliforms was performed on Violet Red Bile Glucose Agar (VRBGA) and Violet Red Bile Agar (VRBA), respectively, both incubated at 37 °C for 24 h.

The group of LAB was incubated under anaerobic conditions using anaerobic jars (AnaeroGen, Thermo Fisher Scientific, Waltham, MA, USA). The growth medium for LAB was supplemented with cycloheximide (10 mg/mL) to prevent fungal growth. Samples from IM, W, C and Ch were specifically analysed for TMM and mesophilic and thermophilic cocci LAB as described above. All growth media and supplements were sourced from Oxoid (Milan, Italy). Microbiological counts were carried out in duplicate for all samples collected at any time.

2.6. Physical analysis

The interior surface of both control and experimental cheese productions were used to assess color parameters according to the Commission Internationale de l'Èclairage standard (CIE, 1986), L* a* b* system. These parameters include: lightness (L*) which ranges from 0 (black) to 100 (white); redness (a*), varying from red (+a) to green (−a); and yellowness (b*), spanning from yellow (+b) to blue (−b). To determine the pH value, a portable Hanna HI98161 pH meter (Hanna Instruments, Woonsocket, RI) was immersed in a homogenized cheese sample. Measurements for both pH and color were taken from three distinct areas, and the results were averaged. The hardness of cheeses resulting from both CP and EXP productions was evaluated using an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy). The maximum compressive strength (N/mm^2) was measured for samples previously cut into dimensions of 3 cm × 3 cm × 3 cm and kept at room temperature (22 °C).

2.7. Chemical analysis

The total dry matter and ash contents of the cheeses were determined using the gravimetric method (AOAC 2012d, 2012b). The fat content was assessed through the Gerber method (AOAC 2012c). The acidity levels were measured using the titrimetric method and expressed as a percentage of lactic acid (AOAC 2012a). The salt content was determined using the Mohr titration method (Kelrich, 1990). Protein content was established according to IDF standards (2008). The nitrogen content of the samples was determined using the Kjeldahl method, and the total protein ratio was calculated by multiplying the nitrogen value by a factor of 6.38.

Total free fatty acids were quantified by de-emulsifying the samples and separating the free fat with diethyl ether. After sufficient treatment with the solvent, the liquid was passed through Whatman No. 113 filter paper and collected in a flask. Diethyl ether was then removed from the mixture using a rotary evaporator under vacuum, and total free fatty acids were determined by titration with KOH prepared in 0.1 N ethyl alcohol using a 1 % phenolphthalein indicator. Results are expressed as grams of oleic acid per 100 g of cheese fat (De Jong & Badings, 1990).

The fatty acid composition of the cheeses was analyzed using Gas Chromatography–Mass Spectrometry (7890B GC-7010B MS, Agilent Technologies Inc., Santa Clara, CA). Grated cheese samples weighing 10 g underwent fatty acid esterification following the method outlined by De Jong and Badings (1990) with modifications. Specifically, a 1 μL aliquot of the sample, with a split ratio of 1:40, was injected into a GC–MS/MS system equipped with a flame ionization detector. The fatty

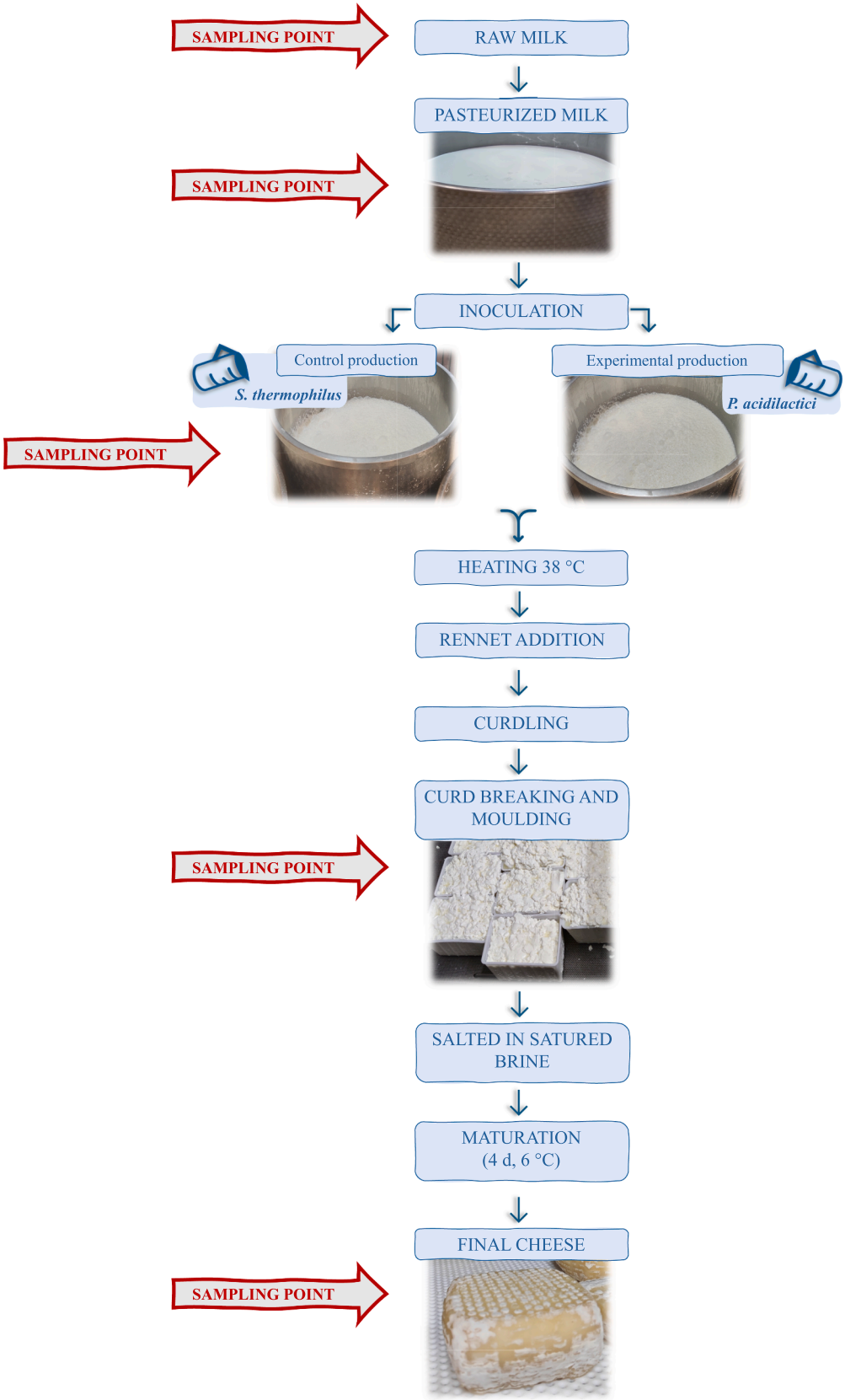

Fig. 1. Graphical representation of cheese production and sampling plan.

acids were separated using a capillary Agilent J&W DB-WAX column (60 m × 0.25 μm × 0.25 μm) with helium as the carrier gas flowing at a rate of 1 mL/min. The oven temperature program started at 50 °C for 1 min, increased to 200 °C at a rate of 25 °C/min, held for 10 min, then increased to 230 °C at a rate of 3 °C/min, and maintained at this temperature for 26 min. The injector and detector temperatures were set to 250 °C and 300 °C, respectively. Fatty acids were identified by comparing the retention times of sample peaks with those of reference standards (Supelco 37 Component FAME Mix, Sigma-Aldrich).

2.8. Cheese proteolysis

Total nitrogen (TN), water-soluble nitrogen (WSN), trichloroacetic acid soluble nitrogen (TCASN) and phosphotungstic acid soluble nitrogen (PTASN) contents of samples were calculated by the methods described in Bütikofer, Rüegg, and Ardö (1993). Proteolytic maturation parameters were calculated from TN, WSN, TCASN, and PTASN values using the following equations:

$$\textit{Ripening extension index } (REI) = WSN/TN \times 100;$$

$$\textit{Ripening depth index } (RDI) = TCASN/TN \times 100;$$

$$\textit{Free amino acid index } (FAAI) = PTASN/TN \times 100$$

In addition to PTASN values, the total free amino acid (FAA) content of cheese samples was quantified using the 2,4,6-trinitrobenzenesulphonic acid (TNBS) method. TNBS reacts with primary amines, producing a yellow color, and the absorbance of this color was measured at 420 nm (Salum, Govce, Kendirci, Bas, & Erbay, 2018). Through this method, the total amino acid content in cheeses can be better quantified because there are only free amino acid groups (Sousa, Ardö, & McSweeneyet, 2001).

2.9. Volatile organic compounds analysis

The volatile organic compounds in the cheeses were determined using the headspace solid-phase microextraction method (HS-SPME) and analyzed via Gas Chromatography (7890B GC, Agilent) coupled with mass spectrometry (7010B MS, Agilent). Initially, the samples were heated to 30 °C for 15 min, allowing the volatile compounds to be adsorbed onto a coated fiber (Carboxen TM/PDMS StableFlexTM) for 30 min. Subsequently, the samples were desorbed for 5 min through a splitless GC injector and injected into a capillary column (60 m × 0.25 mm × 0.25 μm, J&W Scientific-Folsom, USA). The column temperature was programmed to increase gradually from 40 °C to 90 °C at a rate of 3 °C per min, followed by an isothermal hold at 130 °C for 4 min with a ramp of 4 °C per min. Afterwards, the temperature was further raised to 240 °C at a rate of 5 °C per min and maintained for 8 min. Helium served as the carrier gas at a flow rate of 1 mL/min. The acquisition was conducted under scanning conditions within a mass range from 30 to 600 *m/z*. The partition ratio was 1:10. Identification of volatile compounds was accomplished using the NIST library, and the results were expressed as percentages of the peak area relative to the total area of significant peaks (Gioacchini, De Santi, Guescini, Brandi, & Stocchi, 2010).

2.10. Sensory analysis

A total of 12 assessors (comprising five men and seven women, aged between 26 and 63 years) evaluated CP (control production) and EXP (experimental production) cheese samples for their sensory attributes. These assessors received specific training at the Department of Agricultural, Food, and Forestry Sciences of the University of Palermo, following the guidelines outlined in ISO 8589-2007. Cheese samples were cut into 3 cm × 3 cm × 3 cm pieces 24 h before sensory evaluation, transferred to sterile plastic cups (Anicrin, Scorzè, Italy), and refrigerated. Approximately one hour before evaluation, the cheese cubes were allowed to equilibrate to room temperature and were presented to the judges in plastic dishes labeled with three random digits. Panelists cleansed their palates between samples with unsalted crackers and water and were unaware of the identity of the tested samples. Using tablets, the panelists assessed 16 descriptive attributes on a growing intensity score ranging from 1 to 9. These attributes covered various aspects, including appearance (color and uniformity of the paste), odor (intensity of odor, butter odor, milk odor, and unpleasant odor), taste (salty, sweet, sour, bitter, spicy, taste persistence and unpleasant aromas), and mouthfeel (chewability, solubility, and grittiness after chewing) characteristics. The sensory evaluation of the cheeses was compared to that of commercial cow's Crescenza (C–C) purchased in a retail market. Panelists rated all attributes using a 9-cm line scale that ranged from 'low' at 0 to 'high' at 9, moving from left to right. The ratings provided by the panelists were then averaged.

2.11. Statistical analysis

Microbiological, physical and chemical data as well as VOCs were examined by one-way analysis of variance (ANOVA) with the software XLStat version 7.5.2 for Excel (Addinsoft, New York, NY, USA). Differences in means were determined by Tukey's test at $p < 0.05$. To analyse the sensory data the panellist effect was included in the statistical model. Least squares means were reported, and all differences were considered significant at $p < 0.05$.

3. Results and discussions

3.1. General features of Pediococcus acidilactici RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035 genomes and genome-based safety assessment

The genome sequence of *P. acidilactici* RC-UNIPASAAFM00032 consisted of 28 contigs (24 contigs ≥ 1000 bp) and a size of 1.98 Mb, its GC content was 41.9 %, and the N50 value was 162631 bp, whereas the genome of *P. acidilactici* RC-UNIPASAAFM00035 consisted of 63 contigs (44 contigs ≥ 1000 bp) with a size of 2.06 Mb, a GC content of 42.1 %, and an N50 value of 122047 bp. Based on BUSCO, the genomes resulted complete and in single copy at 99.5–99.8 %, respectively, (Tables S1 and S2) confirming that the assemblies obtained are of high-quality. A total of 1955 and 2150 genes were predicted for RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035, respectively; most of the genes were related to Carbohydrates (306 and 281), followed by Protein metabolism (199 and 210), DNA metabolism (107 and 121) and Cell Wall and Capsule (107 and 127, respectively). Digital DNA-DNA hybridization (dDDH) and the Average Nucleotide Identity (ANI) were calculated to precisely determine the species designation and both RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035 showed the highest dDDH (90,50 % and 74 %, respectively) and ANI values (98.86 % and 97.10 % respectively) with the genome of *P. acidilactici* DSM 20284^T (acc. Number: AEEG00000000.1), thus confirming species identification.

As for safety assessment, the *in silico* search of antibiotic resistance genes was performed using the databases CARD (Alcock et al., 2023) and ResFinder 4.1 (Florensa et al., 2022), as indicated in the EFSA guidance (2024). ResFinder returned no hits, while CARD's RGI algorithm found three putative antibiotic-resistance genes (cut-off: STRICT) both in RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035 genomes: *qac*G – ARO:3007015 (48,11 % similarity in both the genomes) and *sdr*M – ARO3007013, (34,73–34,81 % similarity, respectively), coding two efflux pumps involved in sanitizing agent resistance; and *van*T – ARO:3002972 (31,42–31,52 % identity, respectively), involved in vancomycin resistance by altering the target of the antibiotic. These three traits do not pose a risk related to the safety of the two strains: the gene *van*T is at the basis of the intrinsic resistance towards vancomycin, a feature already well explored for most lactobacilli and pediococci

(Campedelli et al., 2019) and for which EFSA does not require a further investigation at phenotypic level (Rychen et al., 2018); while the other two genes code for efflux pumps that are not specific for any antibiotics.

As for biogenic amines, none of the genes responsible for tyrosine and histidine decarboxylases (related to tyramine and histamine production, respectively) were detected. Finally, the VFDB search did not retrieve any genes that could be associated to virulence factors. The targeted analysis of genes associated with hemolysis revealed the presence of a gene in both genomes annotated as "predicted membrane protein hemolysin III homolog." This gene is present in several *P. acidilactici* genomes and is widely distributed within the Lactobacillaceae family (data not shown), indicating that it is associated with a membrane protein involved in intracellular trafficking, secretion, and vesicular transport rather than being a safety-related trait. Furthermore, the assessment of plasmids and other mobile genetic elements was conducted to predict the overall stability of the genomes. Neither the genome of strain RC_UNIPASAAFM00032 nor the genome of strain RC_UNIPASAAFM00035 displayed the presence of plasmids. However, both genomes were found to harbor genes associated with prophages. Transposable elements were not identified in either genome.

Overall, the genomes obtained provided valuable insights into the biology, metabolism, and potential applications of *P. acidilactici* RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035, in particular, the *in silico* safety assessment showed that these two strains can be considered safe for their use as starter or adjunct cultures in food fermentations. WGS analysis of *P. acidiliactici* species often reveals significant genetic diversity, mainly due to variable genomes, mobile genetic elements, and hypothetical genes acquired through horizontal gene transfer. Li et al. (2021) demonstrated, through comparative genomics, how *P. acidilactici* adapts to the host environment. Furthermore, different *P. acidilactici* strains can metabolize diverse carbon sources, enhancing the adaptability of this species and survival across different environments.

3.2. Monitoring of microbiological levels in Quadrello di Ovino cheese

Fig. 2 reports the results of ewes' milk plate count level before and after pasteurisation. Initially, raw ewe's milk exhibited a TMM load of 5.86 log CFU/mL, a value consistent with findings from Pisano, Fadda, Deplano, Corda, and Cosentino (2006) in their study on Fiore Sardo cheese, an ewe's milk cheese produced in Sardinia. Following the sanitization treatment, the TMM load decreased by approximately 2 log cycles. Among spoilage microorganisms, pseudomonads were found in higher cell densities in milk compared to total coliforms and Enterobacteriaceae members (5.67, 4.80 and 4.42 log CFU/mL respectively). The thermal sanitation significantly impacted the initial level of spoilage microorganisms. Specifically, pseudomonads decreased by approximately 3 log cycles, while total coliforms and members of the Enterobacteriaceae family decreased by approximately 4 log cycles. A similar trend was observed for pathogenic microorganisms (such as CPS, *E. coli*, and *Salmonella* spp.) and unicellular and filamentous fungi. Interestingly, *L. monocytogenes* was never found even in raw milk samples.

The results of the plate counts carried out throughout cheese production from inoculated milk to final cheeses are reported in Table 1. Within LAB, mesophilic cocci were the most highly counted after sanitisation (3.09 log CFU/mL), consistent with the findings in the work of Garofalo et al. (2023). During fermentation of both productions, mesophilic coccus LAB remained dominant, with counts of 7.65 and 7.86 log CFU/g in the C CP and C EXP samples, respectively. Enumeration of inoculated milk samples showed no significant differences in the detected LAB levels between CP and EXP. However, a slightly higher count was observed in the control production.

After refrigerated storage for 4 days, the Quadrello di Ovino cheese was analyzed. The plate count results revealed statistically significant differences between the two productions. Specifically, the EXP production exhibited levels of approximately 10^8 CFU/g for mesophilic and thermophilic coccus LAB. This suggests that pediococci may play an interesting role as starters for fresh ovine cheeses. In contrast, the results

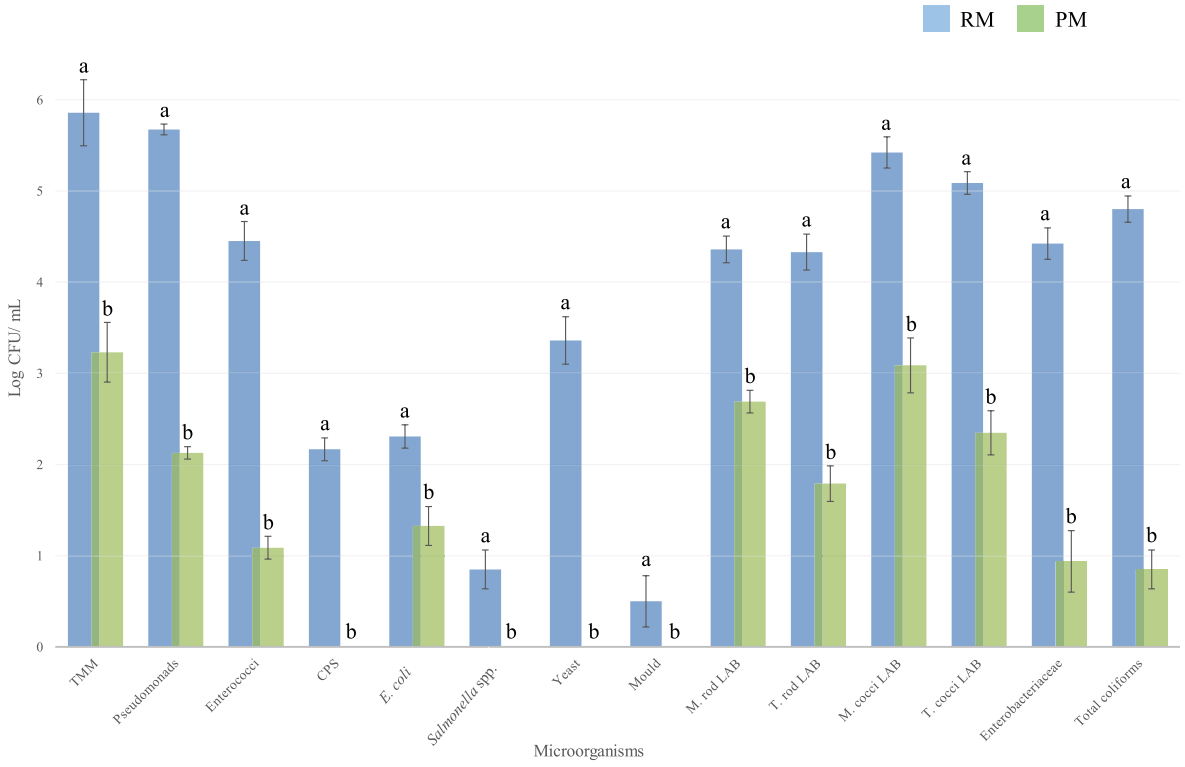

Fig. 2. Microbial level of milk samples. Results indicate mean values ± S.D. of four plate counts (carried out in duplicate for two independent productions). Abbreviations: RM, raw milk; PM, pasteurized milk; TMM, total mesophilic microorganisms; CPS, coagulase-positive staphylococci; *E.*, *Escherichia*; M., mesophilic; T., thermophilic; LAB, lactic acid bacteria. a, b = $p < 0.05$.

Table 1
Microbial loads of samples.

Samples	Microorganisms		
	Total mesophilic microorganisms	Mesophilic cocci LAB	Termophilic cocci LAB
Inoculated milk			
Control production	7.32	7.36	6.24
Experimental production	7.42	6.95	5.85
SEM	0.04	0.07	0.08
p value	0.556	0.058	0.166
Curd			
Control production	7.88[b]	7.65	6.45
Experimental production	8.51[a]	7.86	6.71
SEM	0.09	0.04	0.05
p value	0.008	0.106	0.125
Whey			
Control production	5.55[b]	4.41	4.39
Experimental production	6.64[a]	4.57	4.47
SEM	0.16	0.06	0.03
p value	0.004	0.485	0.425
Cheese			
Control production	7.16[b]	7.09[b]	6.95[b]
Experimental production	8.74[a]	8.50[a]	8.46[a]
SEM	0.23	0.20	0.23
p value	0.003	0.001	0.017

Results indicate mean values of four plate counts (carried out in duplicates for two independent productions) and are expressed as log CFU/mL for inoculated milk and whey samples and as log CFU/g for curd and cheese samples.
Abbreviations: LAB; lactic acid bacteria; SEM, standard error of the mean.
On the column: a, b = $p < 0.05$.

of LAB in the CP production align with those reported in the work of Natrella, Gambacorta, Squeo, and Faccia (2023) for Canestrato Pugliese cheese.

3.3. Physical properties of Quadrello di Ovino cheese

The physical characteristics of the cheeses are summarized in Table 2. The values of pH differed between CP and EXP cheeses. Notably, the EXP cheese exhibited higher acid values than the CP cheese. Interestingly, there was no significant diversity between the cheese made with selected pediococci and the commercial Crescenza purchased from a local supermarket. The three-color parameters lightness (L*), redness (a*), and yellowness (b*) were not affected by the two starter cultures. Furthermore, both CP and EXP cheeses showed no differences compared to the commercial Crescenza in terms of color. Color plays a significant role in consumer acceptance, especially for fresh cheeses like Crescenza and Stracchino. For instance, an intensification of yellow coloration can be negatively perceived by consumers (Marcuzzo, Peressini, & Sensidoni, 2013).

The hardness parameters of Crescenza-type cheeses made using the two different starters did not exhibit significant differences, and they were also comparable to the commercial counterpart. This finding is encouraging for promoting the use of indigenous starters. Notably, the low hardness values, averaging 0.05 N/mm^2, can be attributed to several factors. Cheese texture is primarily influenced by moisture, fat content, and the integrity of the protein matrix (Alinovi et al., 2018). High moisture levels, combined with elevated fat content and reduced proteolytic activity, contribute to a softer cheese texture (Giha, Ordoñez, & Villamil, 2021).

Table 2
Physicochemical composition of Quadrello di Ovino cheeses.

Parameters	Samples			SEM	p value
	Commercial Crescenza	Control production	Experimental production		
Color					
L*	92.19	88.79	92.28	0.73	0.280
a*	2.38	2.94	2.60	0.08	0.086
b*	13.43	14.92	12.49	0.58	0.490
pH	5.22[a]	4.88[b]	5.25[a]	0.04	<0.0001
Hardness (N/mm^2)	0.035	0.043	0.067	0.00	0.078
Dry matter (%)	43.16[c]	45.49[b]	49.07[a]	0.66	<0.0001
Fat on dry matter (%)	60.24	62.65	62.16	0.68	0.587
Ash on dry matter (%)	2.07[b]	2.11[b]	3.34[a]	0.16	<0.0001
Titrable acidity (%)	1.02[c]	1.59[a]	1.20[b]	0.06	<0.0001
Salt (%)	1.46[b]	1.47[b]	2.39[a]	0.12	<0.0001
Protein (%)	15.39	14.81	16.85	0.32	0.111

Results indicate mean values of four determinations (carried out in duplicate for each of the two independent cheese-making).
Abbreviations: SEM, standard error of the mean.
On the row: a, b, c = $p < 0.05$.

3.4. Cheese chemical content and free fatty acids composition

The chemical characteristics of the cheeses are summarized in Table 2. Notably, the fat and protein content remained unaffected by the type of starter culture used. However, significant differences were observed in terms of total dry matter and titratable acidity across all cheeses. Additionally, the EXP cheese exhibited higher levels of ash and salt content. It is worth noting that these values fall within the typical range encountered in other fresh ewe's cheeses (Kaminarides, Moschopoulou, & Karali, 2019; Salum et al., 2018).

The total fatty acid amounts, along with the fatty acid profile, are detailed in Table 3. While no statistically significant differences were observed in the overall fatty acid content among the cheeses, variations were evident in their specific fatty acid profiles. Saturated fatty acids (SFAs), such as palmitic acid (C16:0) and myristic acid (C14:0), are naturally abundant in ewe's milk (De La Fuente et al. 2009). The nutritional value of fat in ewe's milk is highly regarded due to the potential health benefits associated with fatty acids like conjugated linoleic acid (Govari, Iliadis, Papageorgiou, & Fletouris, 2020). Our study aligns with this, revealing palmitic and myristic acid levels of 26.31 and 10.37, respectively, in the CP cheese, and 25.99 and 9.42 in the EXP cheese. Additionally, CLA was present at similar levels in both CP and EXP productions (2.50 and 2.61, respectively). Short-chain fatty acids (SCFAs; C4:0–C10:0) significantly contribute to the final taste of cheese (Voblikova, Permyakov, Rostova, Masyutina, & Eliseeva, 2020). In our investigation, caproic, caprylic, and capric acid exhibited significantly higher concentrations in the CP cheese compared to the EXP cheese. A similar trend was observed for medium-chain fatty acids (lauric, myristic, and pentadecanoic acid), which were more abundant in the CP cheese. On the other hand, the EXP cheese displayed higher concentrations of stearic acid (14.35), linolenic acid (0.70), and oleic acid (26.80). Notably, oleic acid belongs to the group of medium-chain fatty acids (MUFAs). These MUFAs play a crucial role; they are directly absorbed and transported to the liver, where rapid metabolism enhances the thermogenic effect induced by the diet (Aoyama, Nosaka, & Kasai, 2007). Interestingly, the fatty acid profile of the EXP cheese sample closely resembles that of other fresh cheeses (Felicio et al., 2016).

In cheese production and ripening, three primary biochemical events

Table 3
Total free fatty acid amount (%) and fatty acid profile (%) of Quadrello di Ovino cheeses.

Parameters	Samples			SEM	p value
	Commercial Crescenza	Control production	Experimental production		
Free fatty acid (% in dry matter)	1.49	2.68	1.83	0.19	0.123
Caproic acid (C6)	2.23^{a}	2.30^{a}	1.84^{b}	0.06	0.009
Caprylic acid (C8)	1.49^{b}	1.96^{a}	1.58^{b}	0.06	0.005
Capric acid (C10:0)	3.67^{c}	5.40^{a}	4.44^{b}	0.20	0.001
Lauric acid (C12:0)	4.39^{a}	3.46^{b}	2.94^{c}	0.16	<0.0001
Myristic acid (C14:0)	13.20^{a}	10.37^{b}	9.42^{c}	0.43	<0.0001
Pentadecanoic acid (C15:0)	1.43^{a}	1.24^{b}	1.18^{c}	0.03	<0.0001
Palmitic acid (C16:0)	35.68^{a}	26.31^{b}	25.98^{c}	1.19	<0.0001
Palmitoleic acid (C16:1)	1.79^{a}	1.49^{b}	1.48^{b}	0.04	<0.0001
Stearic acid (C18:0)	10.18^{c}	13.10^{b}	14.35^{a}	0.47	<0.0001
Oleic acid (C18:1 Δ^{c9})	21.36^{c}	25.07^{b}	26.80^{a}	0.61	<0.0001
Oleic acid (C18:1 Δ^{t9})	1.68^{c}	6.16^{b}	6.67^{a}	0.60	<0.0001
Linoleic acid (C18:2)	2.90^{a}	2.50^{b}	2.61^{b}	0.05	<0.0001
Linolenic acid (C18:3)	0.48^{c}	0.66^{b}	0.70^{a}	0.03	<0.0001

Results indicate mean values of four determinations (carried out in duplicate for each of the two independent cheese-making).
Abbreviations: SEM, standard error of the mean.
On the row: a, b, c = $p < 0.05$.

play crucial roles: glycolysis, proteolysis, and lipolysis. These events directly or indirectly influence the chemical composition, sensory characteristics, and overall quality, including flavour and texture, of dairy products (Zeppa, Conterno, & Gerbi, 2001). During production, starter bacteria convert lactose into lactic acid (Izco, Tormo, & Jiménez-Flores, 2002). For the fresh cheeses manufactured in our study, which have a limited maturation period of just a few days, lactic acid was the sole organic acid detected by HPLC. Its concentration was measured at 3147.92 and 2630.10 ppm in CP and EXP samples, respectively.

The concentrations of glucose, a hydrolysis product of lactose, were higher in the EXP samples (720.966 ppm) compared to the CP samples (243.880 ppm).

3.5. Cheese proteolysis

Proteolysis, a crucial biochemical process, significantly impacts the organoleptic qualities of cheese. It is influenced by residual enzymes from curd, milk proteinases, and proteolytic enzymes from both starter and non-starter bacteria (Dimitrova, Mondeshka, Hristov, Stoycheva, & Markov, 2023). The results of the proteolytic parameters of Quadrello di Ovino cheese samples are presented in Table 4. To specify the degree of proteolysis, one straightforward approach is to examine the fractions of soluble nitrogen. Additionally, water-soluble nitrogen (WSN) provides insights into proteolysis. WSN quantification involves assessing coagulant and plasmin activity, as well as measuring small peptides and amino acids within WSN (Sousa et al., 2001). WSN, TCASN, and PTASN values were in the ranges 0.18–0.45 %, 0.05–0.21 % and 0.04–0.13, respectively. Based on these data, three indices were calculated: REI, ranged from 7.56 to 16.92 %; RDI, ranged from 2.10 to 9.24 %, and FAAI, ranged from 0.78 to 5.62 %. Total free amino acid (FAA) content in the cheese samples ranged from 0.39 to 1.50 mg Leu/g cheese. These ranges, however, appear lower than those reported in the current literature. This discrepancy can be partly attributed to the nature of Crescenza, which is a fresh cheese with limited proteolytic activity. Its positively perceived lactic and slightly acidic taste may be linked to the survival of a high density of starter cells within the cheese matrix (Juan, Ferragut, Buffa, Guamis, & Trujillo, 2007).

3.6. VOC profile of cheeses

The analysis of volatile organic compounds (VOCs) in cheeses was conducted using SPME-GC/MS, and the results are presented in Fig. 3. A total of 53 volatile compounds were identified, categorized into nine phytochemical clusters: acids, alkanes, alcohols, aldehydes, amines, ketones, esters, lactones, and terpenes. The overall content of VOCs varied significantly among the cheeses. Interestingly, five compounds were shared by all three cheeses; one acid (isovaleric acid), three alcohols (phenylethyl alcohol, [S,S]-2,3-butanediol and [R,S]-2,3-butanediol) and one ester (ethyl caproate). Phenylethyl alcohol, in particular, plays a key role in shaping the aromatic profile of ewe's milk cheeses. Its delicate rose-like scent adds to the sensory experience of these cheeses (Kırmacı, Hayaloğlu, Özer, Atasoy, & Levent, 2015).

In both commercial Crescenza cheese and EXP Quadrello ovino, alcohols emerged as the most abundant class of compounds. Numerous studies have highlighted that alcohols play a pivotal role in shaping the chemical landscape of certain ewe's milk cheeses, potentially influencing their flavor profiles (Bintsis & Robinson, 2004; Sulejmani, Hayaloglu, & Rafajlovska, 2014). However, in CP Quadrello di Ovino

Table 4
Proteolytic parameters of Quadrello di Ovino cheeses.

Parameters	Samples			SEM	p value
	Commercial Crescenza	Control production	Experimental production		
Total nitrogen	2.41	2.32	2.64	0.05	0.107
Water-soluble nitrogen (%)	0.18^{c}	0.21^{b}	0.45^{a}	0.03	<0.0001
Trichloroacetic acid-soluble nitrogen (%)	0.05^{c}	0.21^{a}	0.15^{b}	0.02	<0.0001
Phosphotungstic acid-soluble nitrogen (%)	0.04^{c}	0.13^{a}	0.10^{b}	0.01	<0.0001
Total free amino acid (mg leucine g^{-1} cheese)	0.39^{c}	1.47^{a}	0.90^{b}	0.12	<0.0001
Ripening extension index (%)	7.56^{c}	9.04^{b}	16.92^{a}	1.10	<0.0001
Ripening depth index (%)	2.10^{c}	9.24^{a}	5.57^{b}	0.78	<0.0001
Free amino acid index (%)	0.78^{c}	5.62^{a}	3.64^{b}	0.53	<0.0001

Results indicate mean values of four determinations (carried out in duplicate for each of the two independent cheese-making).
Abbreviations: SEM, standard error of the mean.
On the row: a, b, c = $p < 0.05$.

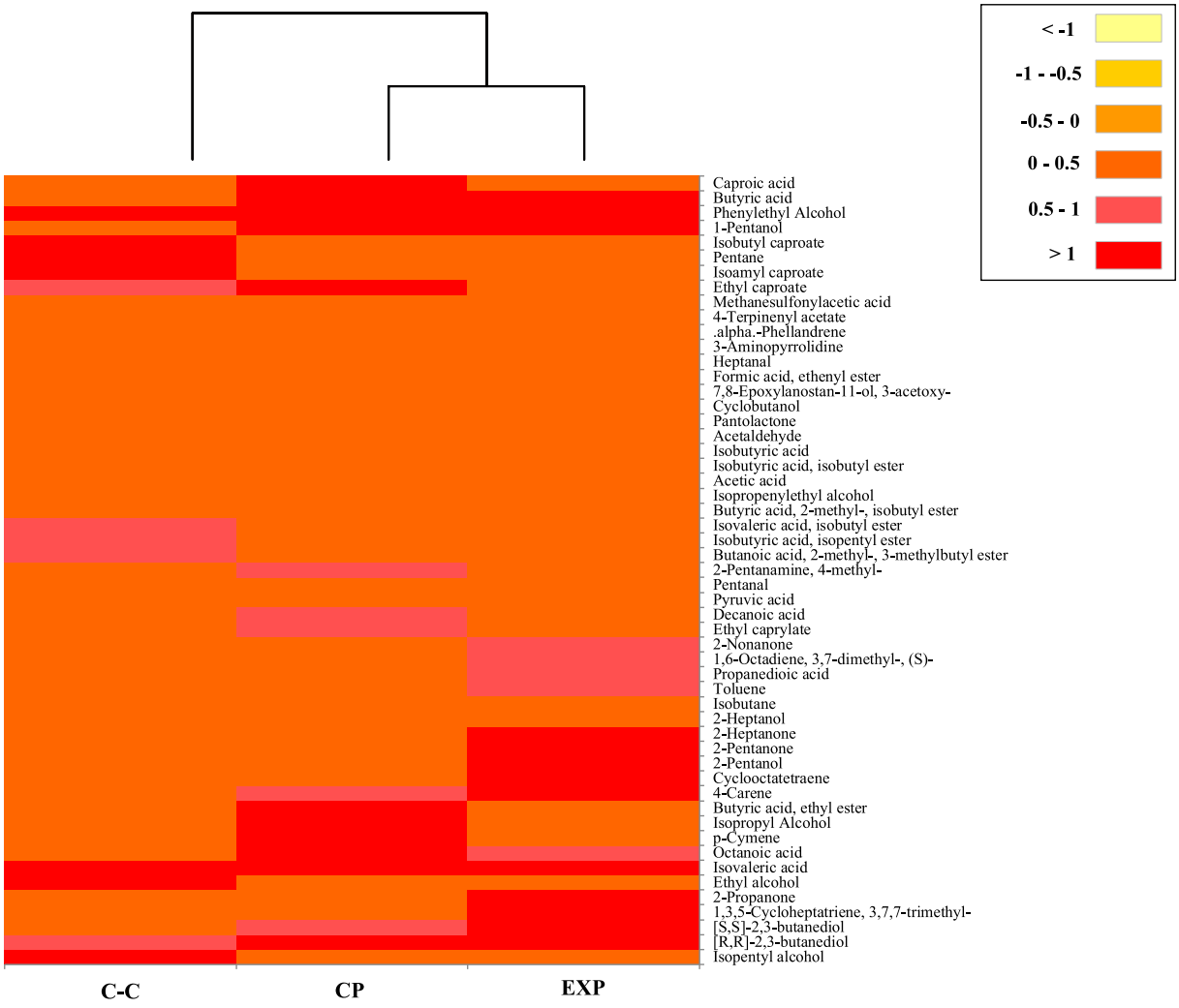

Fig. 3. Heat map of VOCs emitted from Quadrello di Ovino cheeses. Abbreviations: C–C, commercial Crescenza; CP, control production made with freeze-dried starter culture (CRBS7); EXP, experimental production made with selected strains (RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035).

cheese, a different trend emerged. Acids dominated the volatile organic compounds, with butyric acid and caproic acid occurring in higher proportions (36.57 % and 19.21 %, respectively). Butyric acid, notorious for its rancid cheese-like odor, significantly contributes to the flavor of cheeses such as Camembert, Cheddar, Grana Padano, Gruyère, Pecorino, Ragusano, and Roncal (Curioni & Bosset, 2002). Interestingly, amines, aldehydes, and lactones were negligible in all three cheeses examined. On the other hand, alkanes and ketones were significantly present in the EXP cheese (10.22 % and 13.26 %, respectively), directly contributing to its flavor profile (Tekin & Güler, 2019). Esters, however, took center stage in the aromatic ensemble. They were predominantly detected in both commercial Crescenza and CP ovine cheese. Among these esters, ethyl caproate and isoamyl caproate stood out with the highest concentrations. Notably, ethyl caproate also holds the title of the most abundant ester in Pecorino Romano (Di Cagno et al., 2003) and Canestrato Pugliese cheeses (Piombino, Pessina, Genovese, Lisanti, & Moio, 2008). Esters, being a common volatile fraction in cheese, significantly contribute to the overall aroma. Their high volatility at ambient temperatures and low perception threshold make them essential players in the sensory experience (Garde, Carbonell, Fernández-García, Medina, & Nuñez, 2002).

3.7. Sensorial assessment of Quadrello di Ovino cheeses

The introduction of a new product on the market inevitably requires consumer approval. Consequently, a sensory analysis conducted by a panel of judges can yield valuable insights into the perception and acceptance of the product. In this study, sensory features of rated cheeses were visualized using a spider plot (Fig. 4). Except for the attributes intensity of odor, unpleasant odor, texture homogeneity, saltiness, and sweetness that were significantly different between CP and EXP cheeses, all other attributes did not show statistically significant differences ($p > 0.05$) according to Tukey's test. In particular, EXP cheese was characterized by lower unpleasant odour and sweet sensation and higher texture homogeneity and saltiness values than CP cheese. Sensory attributes of cheeses are generally influenced by the microbiological quality of the milk, the animals' diet, and dairy management practices (Todaro, Bonanno, & Scatassa, 2014).

The higher texture homogeneity of EXP cheese confirmed what reported previously for Quadrello di Ovino cheeses (Garofalo et al., 2021). The milder unpleasant odor detected in the EXP cheese is a positive aspect considering the strong flavours characterizing sheep milk cheeses. For these reasons, the majority of consumers prefer neutral flavors associated with cow's milk cheeses.

4. Conclusions

Compliance with food regulations necessitates a thorough examination of the entire genome of new strains to ensure the absence of virulence and antibiotic resistance genes. For this reason, *P. acidilactici*

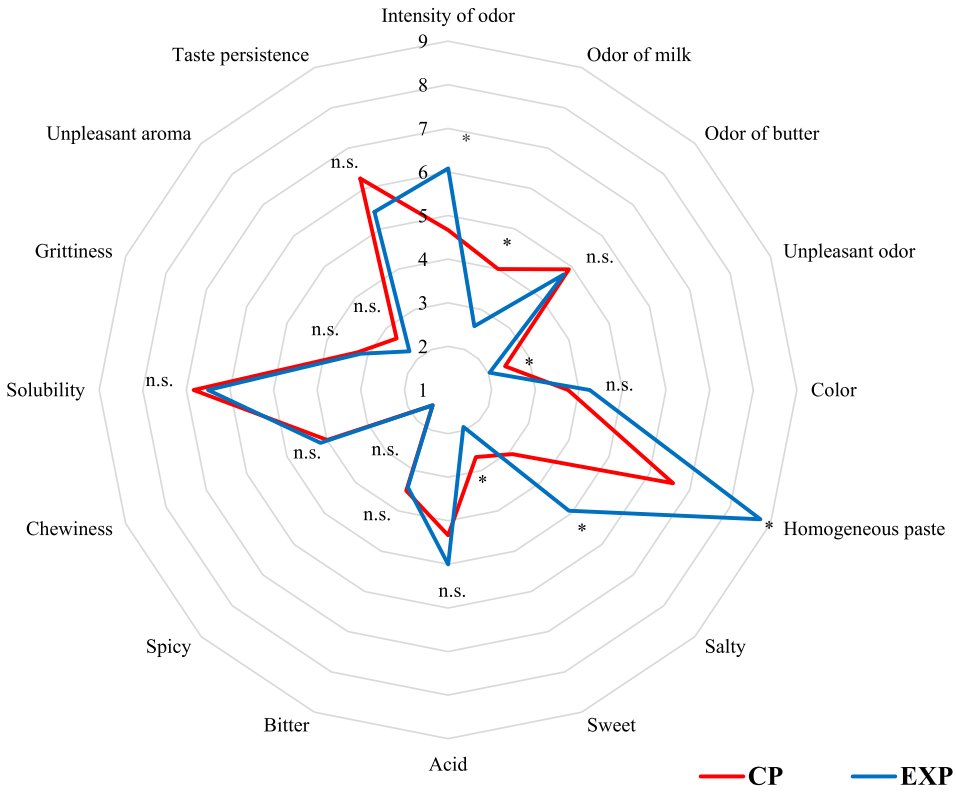

Fig. 4. Spider plot of sensory analysis of Quadrello di Ovino cheeses. Abbreviations: CP, control production made with freeze-dried starter culture (CRBS7); EXP, experimental production made with selected strains (RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035); n.s., not significant.

strains RC-UNIPASAAFM00032 and RC-UNIPASAAFM00035 were investigated by WGS. This analysis indicated that these two genomes do not harbor safety-related traits. The search within the CARD database identified only genes not specifically linked to antibiotic resistance (e.g., efflux pumps), and the analysis against the VFDB did not find any hits related to virulence factors (which was further confirmed by the targeted analysis of hemolysins). Additionally, no genes with decarboxylase activity for biogenic amine formation were detected. These findings confirmed the genomic safety of the *P. acidilactici* strains used, making them suitable candidates as starters in cheesemaking. Their industrial application resulted in the production of Quadrello di Ovino cheeses that were microbiologically safe and highly appreciated in sensory terms by judges. Furthermore, in this study, one of the most relevant aspects of using *P. acidilactici* is its nutritional impact, particularly the increase in stearic acid, oleic acid, and linoleic acid content, which are essential fatty acids for maintaining human health. Additionally, *P. acidilactici* influenced the aroma of the final cheeses, resulting in a wide variety of volatile compounds mainly derived from the microbial metabolism of residual lactose, the formation of free fatty acids, and the degradation of casein into various peptides and free amino acids. Judges noted a reduction in unpleasant odors and the persistence of animal taste in the mouth, usually associated with butyric acid and caproic acid, found in low percentages in the experimental cheese. Instead, higher levels of 1-pentanol, 2-pentanol, 2,3-butanediol, and 2-propanone, which impart sweet, fruity, and floral notes, were observed. Aware that the current production situation in the small ruminant sector has considerable room for improvement, with a significant increase in milk production (from 30 to 50 %) expected by 2030, and given that Italy is one of the four most specialized countries in this industry, innovation is crucial to remain competitive. Therefore, strengthening the connection with the local area through the use of autochthonous breeds and starters isolated within this context could be a highly appealing strategy for today's consumers. Considering this, the significance of this work lies in expanding the variety of fresh ewe's cheese through the use of typical non-starter microorganisms, which connected the product to its production area, basically within Palermo and Agrigento provinces. This provides a competitive advantage for local and international business opportunities for the island. Additionally, future research on Quadrello di Ovino cheese should focus on monitoring shelf life parameters, profiling amino acids, and applying custom packaging to maintain its sensory qualities.

CRediT authorship contribution statement

Giuliana Garofalo: Writing – original draft, Formal analysis, Data curation. **Tansu Taspinar:** Formal analysis, Data curation. **Ilaria Larini:** Formal analysis, Data curation. **Giovanna E. Felis:** Writing – review & editing, Conceptualization. **Gabriele Busetta:** Formal analysis. **Luca Settanni:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Maria T. Sardina:** Resources, Project administration, Funding acquisition. **Huseyin Erten:** Writing – review & editing, Methodology, Funding acquisition. **Giancarlo Moschetti:** Visualization, Methodology. **Elisa Salvetti:** Writing – review & editing, Validation, Conceptualization. **Raimondo Gaglio:** Writing – review & editing, Validation, Supervision, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.115696.

Data availability

Data will be made available on request.

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