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Research paper

Enhancing "Ewiss" cheese production using autochthonous lactic acid bacteria and *Propioniciclava flava* as an alternative to commercial propionic acid bacteria

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ABSTRACT

This study explored enhancing Ewiss cheese's regional identity by using native starter cultures to develop a "regional cultured Ewiss cheese" (RCEC). Lactic acid bacteria (LAB) were previously isolated from local cheeses and selected from the University of Palermo culture collection (*Streptococcus thermophilus* strains RC-UNIPASAAFM00189, RC-UNIPASAAFM00233, RC-UNIPASAAFM00239, and RC-UNIPASAAFM00249), while *Propioniciclava flava* (RC-UNIPASAAFM01343) was isolated during this study. LAB were added as defined whey starters post-pasteurization; *Pc. flava* was introduced as an adjunct culture at pH 6.4. Microbial dynamics were monitored throughout cheese ripening. Thermophilic LAB dominated early stages; propionic acid bacteria increased after 9 months. Metataxonomics analysis revealed *Streptococcus* and *Lactobacillus* as dominant. Thirty-one volatile organic compounds were identified, with propionic acid most abundant. A control cheese using commercial cultures was produced for sensory comparison. Sensory analysis revealed that RCEC made with autochthonous cultures scored higher than the control in terms of overall acceptability. These results support Sicilian dairy diversification and valorisation.

1. Introduction

Sheep's milk cheese is rich in essential nutrients and valued for its distinctive sensory characteristics, often perceived as more natural and healthful than cow's milk products (Caprioli et al., 2020; Tribst et al., 2020). In Mediterranean regions, dairy sheep farming plays a vital socio-economic role, particularly in marginal rural areas (Koluman & Paksoy, 2024; Tumino et al., 2022). The majority of ewe's milk produced in Italy is traditionally used for the production of raw milk Pecorino cheeses (Centorotola et al., 2021), which are obtained through traditional artisanal cheese making methods that, while contributing to the development of typical sensory characteristics, may represent a source of microbiological risk (Todaro et al., 2011). The use of selected starter cultures can mitigate these risks (Holzapfel, 2002). In response to evolving consumer preferences for safe, sustainable, and geographically distinctive dairy products (Scano & Caboni, 2021) recent efforts in Sicily have focused on diversifying sheep cheese production. This has led to the development of new cheeses such as Quadrello di Ovino, Ovino Belmontese, Gran Ovino, and Ewiss (Gaglio et al., 2019, 2024; Garofalo et al., 2021, 2024). Among these, Ewiss is a medium-aged slicing cheese inspired by Swiss-type technology, incorporating *Streptococcus thermophilus* and *Propionibacterium freudenreichii* to drive lactic and propionic fermentation, respectively (Fröhlich-Wyder & Bachmann, 2007; Garofalo et al., 2024).

However, the use of commercial starter cultures, often selected from a limited strain pool, can lead to homogenized flavor profiles and weaken the link between cheese and its territory of origin (Montel et al., 2014). To reinforce this connection, the present study aimed to design a defined whey starter culture (DWSC) for Ewiss cheese using autochthonous lactic acid bacteria (LAB) strains and to explore the potential of

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Propioniciclava flava, a novel member of the Propionibacteriaceae family recently isolated from raw sheep's milk, as an alternative to *Pb. freudenreichii*. While *Pc. flava* has been detected in dairy environments (Wenning et al., 2020), its role in cheese fermentation remains uncharacterized.

This study represents the first attempt to apply *Pc. flava* in Swiss-type cheese production at industrial scale, evaluating its compatibility with LAB cultures and its impact on the microbiological, physicochemical, and sensory properties of a newly formulated regional cultured Ewiss cheese (RCEC). By doing so, it addresses the scientific gap concerning the dairy potential of *Pc. flava* and contributes to the development of territorially distinctive sheep cheeses.

2. Materials and methods

2.1. Isolation of propionic acid bacteria (PAB) from milk samples

Raw sheep's milk samples were collected from three dairies producing traditional Sicilian cheeses, located in the provinces of Agrigento and Palermo (Sicily, Italy). Following collection, three bulk milk samples from each dairy were transported under refrigerated conditions to the Agricultural Microbiology of Laboratories at the University of Palermo (Italy) for two consecutive weeks. The milk samples (1 mL) were serially diluted in 9 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy), with further dilutions performed at a 1:10 ratio.

Cell suspensions were plated on PAL PROPIOBAC PLUS (Laboratories Standa, Caen, France) agar medium, added with 7 mL of selective supplement, and incubated at 30 °C for 6d under anaerobic conditions using the ATCO® Biocult anaerobic generator placed in the ATCO® Biocult P incubation bags (Laboratories Standa). Presumptive PAB colonies from the highest dilutions of milk samples were isolated. Beige to dark orange-red colonies, surrounded by a yellow halo (Turgay et al., 2020), were picked up from PAL PROBIOBAC PLUS and cultivated in sodium lactate broth (SLB), prepared as described by Østlie et al. (1995). After purification, presumptive PAB were preliminarily tested for their general characteristics: Gram type was determined using a 3 % (w/v) KOH solution (Gregersen, 1978), and the catalase test was carried out as described by Chester (1979). Gram-positive and catalase-positive isolates were examined by microscopy to identify their morphology. Furthermore, the isolates were analysed for growth at pH 7.5 and in the presence of 1 % NaCl (w/v) as reported Wenning et al. (2020).

2.2. Genotypic differentiation and identification of propionic acid bacteria

A representative group of isolates underwent genetic characterization. Presumptive PAB colonies were cultured overnight in SLB at 30 °C, and genomic DNA was extracted from pelleted cells using the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Strain differentiation was achieved through random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis, as described by Gaglio et al. (2017). PCR products were separated by electrophoresis on 2 % (w/v) agarose gels (Gibco BRL, Cergy Pontoise, France) and visualised by UV trans-illumination. PAB with different RAPD-PCR profiles were further sequenced by 16S rRNA, following the approach of Weisburg et al. (1991). After confirming the molecular size of the amplicons (approximately 1600 bp) on agarose gels, PCR products were purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy) and sequenced with the same primers used for PCR amplification at AGRIVET (University of Palermo, Italy). The resulting sequences were uploaded to BLAST for searching the GenBank/EMBL/DDBJ database.

2.3. Defined whey starter culture and propionic acid bacterial inoculum preparation

Defined whey starter culture (DWSC) was produced using starter LAB (SLAB) cultures consisting of four strains of *Streptococcus thermophilus* (RC-UNIPASAAFM00189, RC-UNIPASAAFM00233, RC-UNIPASAAFM00239, and RC-UNIPASAAFM00249). These strains, originally isolated from PDO Sicilian cheeses (Gaglio et al., 2014), were selected for their desirable dairy-related characteristics, including acidification capacity, antibacterial activity, autolytic behaviour, and

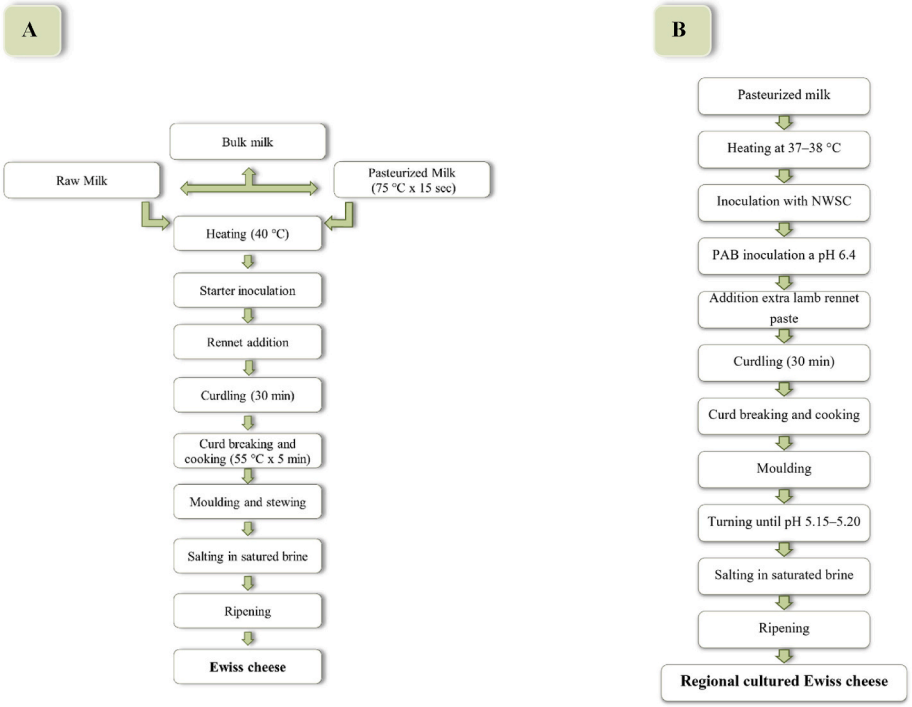

Fig. 1. Flowsheet Ewiss cheese production highlighting the sequential production steps and the key stages and operations: pasteurized milk collection, inoculation with defined whey starter culture, curd formation, and ripening. **A**, commercial Ewiss cheese (from Garofalo et al., 2024); **B**, regional cultured Ewiss cheese.

diacetyl production. They are currently maintained in the bacterial culture collection of the Department of Agricultural, Food and Forest Sciences at the University of Palermo, Italy. Each strain was individually grown in M17 broth (Oxoid, Basingstoke, UK) and incubated overnight at 44 °C. Cells were centrifuged at 5000 g × 5min, washed twice in Ringer's solution (Oxoid), and resuspended in the same solution to reach an optical density at 600 nm (OD600) of ca. 1.00, corresponding to a cell density of approximately 8 log CFU/mL, as determined spectrophotometrically (model 6400 spectrophotometer; Jenway Ltd., Dunmow, UK). Each strain was then inoculated to a final density of about 6 log CFU/mL in a whey-based medium (WBM), prepared as described by Settanni et al. (2012). Briefly, non-acidified cow's milk whey was sterilized (15min, 121 °C), clarified by centrifugation (10,000 g, 15min), re-sterilized, and mixed (10:3) with a sterile solution containing peptone (1 % w/v), yeast extract (0.4 % w/v), and agar (5 % w/v). The medium was adjusted to pH 5.4 with 5 mol/L lactic acid before autoclaving. After 24h of incubation at 44 °C, the DWSC containing the multi-strain culture was used for cheese making.

To promote propionic fermentation, the strain *Propioniciclava flava* RC-UNIPASAAFM01343 isolated in this study was used as adjunct culture. This strain was reactivated in SLB for 72h at 30 °C. The cells were centrifuged and washed as previously described. The strain was then inoculated to a final density of about 4 log CFU/mL.

2.4. Cheese production and sample collection

Cheese production was carried out in an industrial setting at the "Cooperativa Agricola Tumarrano" located in Cammarata (Agrigento, Italy). In detail, raw ewe's milk (1000L) from the Sicilian native "Valle del Belice" breed was collected from various dairy farms. The bulk milk was pasteurized at 72 °C for 15s in a P7550/2 pasteurizer (Technolat S. p.a.; Nocera Inferiore, Italy); once the temperature reached 37–38 °C, it was divided into two 500L aliquots. One 500L milk volume was transformed following the procedure described by Garofalo et al. (2024) to obtain the commercial Ewiss cheese (CEC) using defined strains of *S. thermophilus* (Lyobac-D GDA, ALCE International srl, Quistello, Italy) and *Propionibacterium freudenreichii* (Lyobac-D Propionibacterium, ALCE International srl), while the second 500L volume was inoculated with 8L of DWSC under continuous stirring and, when the pH reached 6.4, 50 mL of *Pc. flava* RC-UNIPASAAFM01343 (final cell density of 4 log CFU/mL) was added to promote propionic fermentation and obtain regional cultured Ewiss cheese (RCEC). Coagulation occurred within 30 min using 245g of extra lamb rennet paste (Caglificio Clerici, Cadorago, Italy; title 1:10,000). The curd was broken into rice-sized and gradually cooked, increasing the temperature by one degree per minute until reaching 45 °C. The curd was separated from the whey and left to drain on a cotton cloth for 15min. It was then transferred into a cylindrical plastic mould (Ø 30 cm × h 20 cm) pressed by hand and turned every 10min until the pH reached 5.15 to 5.20. The cheeses were salted in saturated brine at 8–12 °C for 7d. Following this, the cheese underwent a ripening process beginning with 14 days at 18–20 °C to facilitate acidification. This was followed by a 6-week incubation at approximately 20–22 °C to encourage the development of PAB, and finally a maturation phase at 10–12 °C until the completion of the 9-month ripening period. Finally, the cheeses were ripened for up to 9 months (Fig. 1). Cheese productions were carried out in three separate trials, each conducted in a consecutive month (from February to April), resulting in three experimental replicates. For each trial, nine cheeses were produced, representing nine technical replicates per production month. In total, 27 CEC and 27 RCEC were made. These cheeses were then assigned to different ripening durations: nine cheeses (three from each production month both for CEC and RCEC) were ripened for 3 months, other nine cheeses for 6 months, and the remaining nine cheeses for 9 months. The samples were collected at various stages: pasteurized milk (PM), inoculated milk with DWSC (IM), cooked curd (CC) and cheeses (CEC and RCEC) at 3, 6 and 9 months of ripening. These samples were transported in insulated plastic boxes with reusable ice packs to the Laboratories of Agricultural Microbiology at the University of Palermo for immediate analysis. Considering the previous data on CEC provided by Garofalo et al. (2024), in this work, CEC were only used to compare sensory attributes of 9-month ripened cheeses. Thus, all measurements refer to RCEC production.

The pH was monitored throughout the cheesemaking process (from milk to curd) using a portable HI 9025 pH meter (Hanna Instrument, Ann Arbor, MI, USA). Additionally, the cheese temperature during ripening (up to the 9-month) was monitored using Thermo Button 22 L 8 K data loggers (Plug & Track by Proges Plus, Willems, France) which were inserted into the core of the curds at molding and recorded hourly for the first day and then every 24 h.

2.5. Microbiological analysis by culture-dependent approach

One milliliter of liquid samples (PM, IM) was directly serially diluted in Ringer's solution (Sigma-Aldrich). Meanwhile, 25g of curd and cheese were aseptically transferred into a sterile bag (BagFilter P, Interscience, Saint Nom, France) and diluted with 225 mL of 2 % (w/v) Na-citrate solution (Sigma Aldrich). These samples were then homogenized in a stomacher BagMixer 400 (Interscience) at maximum speed for 2min. Further serial decimal dilutions were carried out in Ringer's solution.

Cell suspensions were plated on several media: Plate Count Agar (PCA) with 1 g/L skim milk for total mesophilic microorganisms (TMM), incubated for 72h at 30 °C, or for total psychrotrophic microorganisms (TPM), incubated for 7d at 7 °C; de Man-Rogosa-Sharpe (MRS) agar, incubated for 48h at 30 °C and 44 °C for mesophilic and thermophilic LAB rods; Medium 17 (M17) agar, incubated for 48h at 30 °C and 44 °C for mesophilic and thermophilic LAB cocci; kanamycin aesculin azide (KAA) agar, incubated for 48h at 37 °C for enterococci; sodium lactate agar (SLA) with 4 μg/mL of cloxacillin for PAB, incubated for 72h at 30 °C; *Pseudomonas* agar base (PsAB) supplemented with cephaloridine sodium fusidate cetrimide (CFC), incubated for 48h at 25 C for *Pseudomonas* spp.; violet red bile glucose agar (VRBGA), incubated for 24h at 37 °C to detect total coliforms; Baird Parker (BP) agar with rabbit plasma fibrinogen (RPF) supplement, incubated for 48h at 37 °C to identify coagulase-positive staphylococci (CPS); dicloran rose bengal chloramphenicol (DRBC) agar, incubated for 5d at 25 °C for yeasts; potato dextrose agar (PDA), incubated for 7d at 25 °C for molds; hektoen enteric agar (HEA), incubated for 24h at 37 °C for *Escherichia coli* and *Salmonella* spp; and *Listeria* selective agar base (LSAB) with SR0140E supplement, incubated for 24h at 37 °C for *Listeria monocytogenes*. These analyses were performed in duplicate using media purchased from Oxoid.

2.6. Culture-independent analysis of total bacterial community

Bacterial diversity was assessed using the 16S rRNA gene. Primers targeting the variable V3-V4 region of the 16S rRNA gene (*Escherichia coli* position 341–805) were used: forward primer 341F (CCTACGGGNGGCWGCAG) and reverse primer 806R (GACTACNVGGGTWTCTAATCC) (Galli et al., 2023). Barcodes were added to the forward primer to differentiate samples. To avoid preferential sequencing of smaller amplicons, the amplicons were purified using the Agencourt AMPure kit (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions. DNA quantification was performed using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Milano, Italy). At each sampling point (3, 6, and 9 months of ripening), DNA was extracted from three cheeses, each representing a different production month (from February to April). The DNA from these three cheeses was then pooled together, resulting in a single composite DNA sample per ripening stage. This pooled sample was treated as representative of an individual cheese at that specific stage of ripening. DNA quality and purity of the library were evaluated with the High Sensitivity DNA Kit (Agilent, Palo Alto, CA, USA) using the Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA).

Library preparation and pair-end sequencing were carried out at the Genomic Platform – Fondazione Edmund Mach (San Michele a/Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, San Diego, USA) according to standard laboratory protocols.

2.7. Illumina data analysis and sequences identification by QIIME

Raw paired-end FASTQ files were demultiplexed using idemp (available at https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). The sequences underwent quality filtering, trimming, de-noising, and merging using DADA2 (Galli et al., 2023). Chimeric sequences were identified and removed using DADA2's consensus method. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree, utilizing the alignment and phylogeny plugins (Galli et al., 2023).

Taxonomic and compositional analyses for bacteria were conducted using the feature-classifier plugins (available at 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 trimmed to the V4 region of 16S rDNA bound by the 341F/805 R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by MiSeq Illumina sequencing has been deposited in the NCBI Sequence Read Archive (SRA) and is available under Ac. No. PRJNA1264968.

2.8. Physicochemical composition and texture analysis of cheese

Cheese samples, collected at 3, 6 and 9 months of ripening, were analysed for dry matter (DM), protein, fat, ash content and total acidity following AOAC methods (2012a, 2012b, 2012c, 2012d). For each ripening period, the homogenized cheese samples were assessed for pH using a portable Hanna HI98161 pH meter (Hanna Instruments, Woonsocket, RI). The color of the cheeses was evaluated using a CR-400 tristimulus chromometer (Konica Minolta, Osaka, Japan). Color values, L^* (lightness), a^* (redness/greenness), and b^* (yellowness/blueness) for the cheese samples were measured from the inner surfaces of the cheese, and chroma values were calculated as: $Chroma = \sqrt{a^2 + b^2}$ according to the standard Commission Internationale de l'Eclairage (CIE, 1986). The salt content of the cheese samples was determined using the Mohr method according to Hooi et al. (2004). Briefly, chloride ions were titrated with 0.1 N $AgNO_3$ in the presence of potassium chromate (K_2CrO_4) as an indicator until the appearance of a reddish-brown end point indicating the formation of Ag_2CrO_4, while nitrogen fractions were determined following the IDF standard (IDF, 1993).

Textural parameters of cheese samples, cut into cubes of 3 cm per side, including hardness, adhesiveness, chewiness, and cohesiveness, were measured using a texture analyzer (TA-XTplusC; Stable Micro Systems, Godalming, UK) by applying two compression cycles at a constant crosshead speed of 2 mm/s. Textural data were recorded as force (in newtons), and the parameters were obtained from TPA curves using Exponent software (version 6.0.6.0, Stable Micro Systems).

All analyses were performed in duplicate for three independent samples of each batch of cheese, with a total of four readings taken for each treatment.

2.9. Free fatty acid, organic acid and volatile composition of cheese

The composition of free fatty acid (FFA), including both short- and long-chain FFA, was analysed in cheese at 9 months of ripening by the capillary gas chromatographic method. Ten grams of grated samples were treated as described by De Jong and Badings (1990) with some modification. One microliter of the sample was injected with a split ratio of 1:40 through a GC-MS/MS (Agilent, 7890B GC -7010B MS) with a flame ionization detector with an autosampler (Gerstel, Germany). The separation of the fatty acids was achieved in a capillary Agilent J&W DB-WAX column (60m × 0.25 μm × 0.25 μm) using helium as the carrier gas (1 mL/min). The oven temperature was held at 50 °C for 1 min, raised to 200 °C at a rate of 25 °C/min held for 10 min and then to 230 °C at a rate of 3 °C/min held this temperature for 26 min. The inlet temperature and detector were set to 250 °C and 300 °C, respectively. Fatty acid identification was verified by comparing the sample peak retention times with the reference standards (Supelco 37 Component FAME Mix, Sigma-Aldrich). The results were expressed as the relative percentage of total identified fatty acids.

Volatile organic compounds in cheese samples were determined using headspace solid-phase microextraction (HS-SPME) and analysed by gas chromatography (Agilent 7890B GC) coupled with mass spectrometry (7010B MS, Agilent Technologies). Samples were pre-incubated at 30 °C for 15min to facilitate volatilization, after which a Carboxen™/PDMS StableFlex™ fiber was exposed to the headspace for 30min to adsorb volatile compounds. Desorption was carried out for 5min using a splitless injector, with subsequent injection into a capillary column (60 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA). The oven temperature was programmed as follows: initial ramp from 40 °C to 90 °C at 3 °C/min, followed by an isothermal hold at 130 °C for 4min with a ramp of 4 °C/min, and a final increase to 240 °C at 5 °C/min, held for 8min. Helium was used as the carrier gas at a constant flow of 1 mL/min. Mass spectrometric data were acquired in scan mode within a range of 30–600 m/z, with a split ratio of 1:10. Compound identification was performed using the NIST mass spectral library, and results were expressed as percentages of the total peak area of relevant signals (Gioacchini et al., 2010).

2.10. Sensory analysis

After 9 months of ripening, the sensory characteristics of RCEC were assessed in accordance with ISO 8589:2007 sensory evaluation guidelines. These cheeses were evaluated alongside the CEC. Additionally, commercial Swiss cheese (CSC) (Emmentaler, Switzerland), purchased from a supermarket (Conad Società Cooperativa, Palermo) was included in the sensory analysis as a reference model to provide a broader context for comparison. Cheese cubes, each measuring 1 cm per side, were placed in 250 mL plastic cups and presented to a panel of 20 judges (13 women and 7 men, age ranging 24–63 years), recruited from students and staff at the Department of Agricultural, Food and Forest Science at the University of Palermo. All panellists were specifically trained for cheese attribute evaluation following the ISO 8589:2007 indications. Panellists evaluated 16 sensory descriptors including rind color, size of eyes, eyes distribution (appearance), elasticity, firmness (texture), odour intensity, butter odour, milk odour, unpleasant odour (odour), saltiness, sweetness, bitterness, typical Swiss cheese flavour, persistence of taste, unpleasant flavours (taste), and overall acceptability (Garofalo et al., 2024). Each attribute was scored on a 9-point linear scale, with 1 representing low perception and 9 representing high perception. Judging was carried out in individual rooms using an iPad connected to the Smart Sensory Box software (Smart Sensory Solutions S. r. l., Sassari, Italy).

2.11. Statistical analysis

All microbiological, physicochemical, and sensory data were analysed using one-way analysis of variance (ANOVA) with pairwise comparisons performed using Tukey's test at a significance level of $p \leq 0.05$. Cluster analysis was used to identify the distribution of VOCs emitted by the RCEC produced with autochthonous LAB and PAB strains. The analysis was conducted using XLSTAT software version 2020.3.1 (Addinsoft, New York, NY, USA).

Table 1
Microbial evolution during regional cultured Ewiss cheese production.[a].

Growth media	Samples						Statistical significance[b]
	PM	IM	CC	RCEC 3M	RCEC 6M	RCEC 9M	
PCA-SkM 30 °C	2.9 ± 0.2 d	7.0 ± 0.1 c	7.3 ± 0.1 bc	7.7 ± 0.1 ab	8.1 ± 0.1 a	8.0 ± 0.2 a	***
PCA-SkM 7 °C	2.1 ± 0.1 e	2.6 ± 0.2 d	4.7 ± 0.2 a	3.6 ± 0.2 b	3.4 ± 0.1 bc	3.0 ± 0.2 cd	***
M17 30 °C	2.8 ± 0.1 e	6.3 ± 0.2 d	7.0 ± 0.2 bc	7.6 ± 0.3 ab	7.7 ± 0.2 a	6.7 ± 0.4 cd	***
M17 44 °C	1.9 ± 0.1 c	7.1 ± 0.3 ab	7.2 ± 0.3 ab	7.4 ± 0.2 ab	7.7 ± 0.2 a	6.8 ± 0.2 b	***
MRS 30 °C	2.8 ± 0.1 e	5.2 ± 0.2 d	5.8 ± 0.2 c	6.9 ± 0.2 b	7.9 ± 0.3 a	7.5 ± 0.1 a	***
MRS 44 °C	1.8 ± 0.2 e	5.1 ± 0.2 d	5.9 ± 0.2 c	6.7 ± 0.1 b	7.8 ± 0.1 a	6.9 ± 0.2 b	***
SLA	<2 e	4.2 ± 0.2 d	5.7 ± 0.3 c	8.7 ± 0.3 a	8.4 ± 0.1 a	7.8 ± 0.2 b	***
KAA	<1 c	3.7 ± 0.2 ab	4.0 ± 0.3 a	3.5 ± 0.2 b	3.9 ± 0.1 ab	3.7 ± 0.2 ab	***
VRBGA	<1	<1	<1	<1	<1	<1	n.s.
(*E. coli*)	<1	<1	<2	<2	<2	<2	n.s.
Salmonella spp	<1	<1	<2	<2	<2	<2	n.s.
BP	<1	<1	<2	<2	<2	<2	n.s.
LSAB	<1	<1	<2	<2	<2	<2	n.s.
PsAB	<1	<1	<2	<2	<2	<2	n.s.
DRBC	<1	<1	<2	<2	<2	<2	n.s.
PDA	<1	<1	<2	<2	<2	<2	n.s.

Abbreviations: PM, pasteurized milk; IM, inoculated milk after addition of DWSC; CC, cooked curd; RCEC 3M, Regional cultured Ewiss cheese ripened 3 months; RCEC 6M, RCEC ripened 6 months; RCEC 9M, RCEC ripened 9 months; PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for total mesophilic microorganisms; PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for total psychrotrophic microorganisms; M17 30 °C, medium 17 agar incubated at 30 °C for mesophilic coccus LAB; M17 44 °C, medium 17 agar incubated at 44 °C for thermophilic coccus LAB; MRS 30 °C, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; MRS 44 °C, de Man-Rogosa-Sharpe agar medium for thermophilic rod LAB; SLA, sodium lactate agar medium for propionic acid bacteria; KAA, kanamycin aesculin azide agar for enterococci; VRBGA, violet red bile glucose agar for Enterobacteriaceae; HEA, hektoen enteric agar for *E. coli* (red colonies) and *Salmonella* spp (black colonies); BP, baird-parker agar for CPS, coagulase-positive staphylococci; LSAB, *Listeria* selective agar base for *L. monocytogenes*; PsAB, *Pseudomonas* agar base for pseudomonads; DRBC, dicloran rose bengal chloramphenicol agar for yeast; PDA, potato dextrose agar for molds; n.s., not significant.
Units are log CFU/mL for liquid samples and log CFU/g for solid samples. Results indicate mean values ± S.D. of six plate counts (carried out in duplicate for three independent productions).
Data within a row followed by same letter are not significantly different according to Tukey's test ($p \leq 0.05$). *P*-value: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

3. Results and discussion

3.1. Microbiological counts, differentiation and identification of PAB

Microbiological analyses were performed to detect the presence of PAB and estimate their levels in raw sheep's milk samples collected from different Sicilian farms. The goal was to add autochthonous PAB to the LAB already present in the Culture Collection of the SAAF Department, originating from dairy products of the Sicily region. The presumptive PAB, enumerated on PAL PROBIOBAC PLUS agar medium, ranged between 3.8 and 4.1 log CFU/mL in the analysed milk samples. This indicates a consistent presence of PAB in sheep's milk and confirms the findings of Bücher et al. (2024), who analysed PAB in various raw milk samples, including conventional milk, hay milk, organic milk, and organic hay milk.

Based on color and shape, at least five colonies from each milk sample were randomly selected from selective culture media. A total of 210 colonies of presumptive PAB were isolated. Of these, only 65 isolates were considered putative PAB, being Gram-positive and catalase positive. After microscopic inspection, 25 isolates were found to be non-motile and showed the typical short rod shape of PAB. All these strains were tested for growth at pH 7.5 and in the presence of 1 % NaCl, but only 12 exhibited these characteristics. These 12 isolates were subjected to RAPD pattern analysis for PAB typing. This approach identified four distinct RAPD profiles (Fig. S1), and these strains were then processed for species identification through 16S rRNA gene sequencing. The sequencing results identified only one strain as belonging to the Propionibacteriaceae family, specifically *Propioniciclava* flava (Acc. No. PV636770). This species is commonly found in raw milk and dairy environments, as noted by Wenning et al. (2020); however, it has not yet been evaluated as a selected culture for dairy applications, and its specific role in Swiss-type cheese production remains unestablished.

Propionibacterium freudenreichii undoubtedly remains the reference species for Swiss-type cheese production due to its well-characterized metabolic activity, particularly its role in propionate and CO_2 production, which contribute to eye formation and flavour development. In contrast, *Pc. flava* is a recently identified member of the Propionibacteriaceae family with no prior application in cheesemaking and limited metabolic characterization. In this study, we explored its industrial-scale use not following traditional metabolic screening, but in response to local cheesemakers' interest in developing a geographically distinctive ewe's milk cheese. This reverse approach aimed to assess the technological feasibility and potential synergy of *Pc. flava* with lactic acid bacteria (LAB) cultures under real production conditions.

3.2. Evolution of microbial populations during production, acidification and ripening of regional cultured Ewiss cheese

Table 1 reports the levels of various microbial groups investigated in this study, from pasteurized milk to cheeses after 9 months of RCEC ripening. Notably, CPS, *L. monocytogenes* and *Salmonella* spp., which are crucial for monitoring food hygiene and safety standards (EFSA, 2005), were not detected in any of the analysed samples. In pasteurized ewe's milk, the levels of total mesophilic microorganism (TMM) were similar to those of mesophilic coccus and rod LAB, at 2.9 log CFU/mL. These findings are consistent with those reported by Garofalo et al. (2024) during the production of Ewiss cheese with pasteurized milk and commercial LAB and PAB. Following the addition of DWSC, thermophilic coccus LAB increased to 7.1 log CFU/mL, aligning with previous observations by Gaglio et al. (2019) in "Grana Ovino" cheese production. In addition, the levels of PAB rose to 4.2 log CFU/mL. After curdling, a general increase in most of the microbial groups analysed was observed, with the curd showing high levels of TMM (7.3 log CFU/g). This increase is generally expected after whey draining (Todaro et al., 2024). As ripening progressed, PAB reached their highest levels in cheeses at 3 months of ripening but remained high (7.8 log CFU/g) even in the 9-month samples, consistent with trends observed in Swiss cheeses (Fröhlich-Wyder & Bachmann, 2004). The addition of DWSC did not negatively affect the growth and survival of *Propioniciclava flava* used as a adjunct culture. After 6 months of ripening, cheeses exhibited high levels of TMM, thermophilic and mesophilic coccus LAB, approximately 8.0 log CFU/g. These results align with those previously reported by

Table 2
Alpha diversity indices.

Parameters	Samples		
	RCEC 3M	RCEC 6M	RCEC 9M
Obs OTUs	104	189	181
Shannon index	0.499	0.626	0.632
Evenness	7.452	8.277	8.243

Abbreviations: RCEC 3M, regional cultured Ewiss cheese ripened 3 months; RCEC 6M, RCEC ripened 6 months; RCEC 9M, RCEC ripened 9 months; Obs OTUs, observed operational taxonomic units.

various authors for the production of several hard cheeses (De Pasquale et al., 2016).

Regarding the acidification process, the pasteurized milk had an initial pH of 6.70, while DWSC reached a pH of 4.13 due to the multi-strain DWSC. After inoculation, PM achieved a pH of 6.40 and underwent rapid acidification; CC reached a pH of 6.15 at molding. Cheeses, soon after molding, reached a temperature of 45.5 °C. After 24 h from production, the core temperature of the cheese dropped to 15.5 °C. The cheeses were then stored in a ripening chamber under controlled conditions, with temperatures maintained between 13 and 15 °C and relative humidity kept between 85 and 90 %. Throughout the 9-month ripening period, the internal temperature remained almost stable, ranging from 13.7 to 14.1 °C.

3.3. Characterization of cheese microbiota during ripening

This study undertook a comprehensive analysis of the bacterial community of RCEC at different ripening stages using a next generation sequencing (NGS) approach, which is a widely adopted routine method for in-depth profiling of food microbial ecosystems (Jagadeesan et al., 2019). NGS approach was combined with culture-based microbiological counts. In our analysis OTUs with a relative abundance (RA) > 0.1 % were considered abundant bacterial communities (Logares et al., 2014). Across the three samples, a total of 720,440 raw sequences were generated. Clustering at 97 % similarity enabled the calculation of alpha diversity indices, including observed OTUs, Shannon diversity, and Simpson's evenness (Table 2). The analysis showed a gradual increase in microbial richness and diversity from RCEC 3M to RCEC 9M, evidenced by the rise in observed OTUs (104–181) and Shannon index values (0.499–0.632), indicating progressive microbial maturation. Simpson's evenness also showed a slight increase, suggesting a more balanced taxonomic distribution in later samples. The elevated diversity observed at RCEC 6M and RCEC 9M may point to the development of a more complex and functionally stable microbiota compared to earlier stages. Nonetheless, due to the limited sample size and absence of biological replicates, these results should be interpreted with caution.

The classification results are presented in a bidimensional bar graph (Fig. 2). Eight taxonomic groups, primarily at the genus level were identified.

The NGS analysis revealed that the bacterial community in RCEC was dominated by LAB, particularly *Streptococcus* and *Lactobacillus*, across all ripening stages. The high RA% of *Streptococcus*, ranging from 26.79 % at 3 months to 49.86 % at 9 months, corresponds well with the use of *S. thermophilus* as a starter culture (Settanni & Moschetti, 2014). The persistence of this genus at later stages, despite the expected decline in viable cells, is likely due to the detection of residual DNA from dead or non-culturable cells, a known limitation of DNA-based methods, already registered in Sicilian cheeses (Todaro et al., 2024).

In parallel, microbiological counts confirmed the presence of high levels of thermophilic and mesophilic LAB cocci, approximately 8.0 log CFU/g after 6 months of ripening. This aligns with the NGS data, supporting the dominance of LAB throughout maturation. The presence of *Lactobacillus* (43.44 % in RCEC 3M to 37.58 % in RCEC 9M) in the NGS profile, despite not being part of the starter culture, is consistent with the survival and growth of thermoduric strains naturally present in pasteurized milk, which survived heat treatment and proliferate during ripening. These strains are known to withstand heat treatment and proliferate under the selective conditions of cheese ripening such as low pH and high salt concentrations (Perin et al., 2017). Thus, the DWSC should not be considered a source of lactobacilli, as it was prepared after sterilization of whey prior to *S. thermophilus* inoculation.

The detection of *Leuconostoc mesenteroides* (8.18 % in RCEC 9M) further supports the dynamic nature of the ripening microbiota. This species contributes to flavour development through the metabolism of

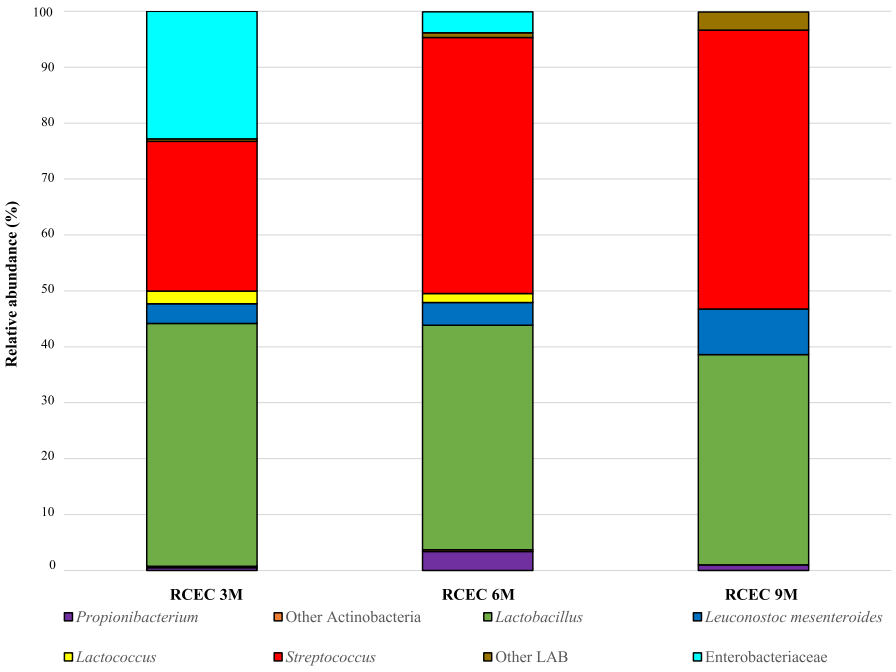

Fig. 2. Relative abundances (%) of bacteria identified by MiSeq Illumina in regional cultured Ewiss cheeses after 3, 6 and 9 months of ripening. Abbreviations: RCEC 3M, regional cultured Ewiss cheese ripened 3 months; RCEC 6M, RCEC ripened 6 months; RCEC 9M, RCEC ripened 9 months.

Table 3
Physicochemical parameters of cheeses.

Parameters	Samples			p value
	RCEC 3M	RCEC 6M	RCEC 9M	
Color				
Lightness L*	81.40 ± 0.76 a	79.17 ± 0.51 a	79.40 ± 1.43 a	n.s
Redness a*	−4.95 ± 0.22 a	−4.29 ± 0.45 ab	−3.48 ± 0.15 b	*
Yellowness b*	15.22 ± 0.65 a	16.00 ± 0.69 a	17.06 ± 054 a	n.s
pH	4.49 ± 0.09 a	5.18 ± 0.14 b	5.73 ± 0.02 c	***
Acidity	1.63 ± 0.04 a	2.14 ± 0.16 a	2.41 ± 0.49 a	n.s
Dry matter (%)	64.82 ± 0.28 c	70.61 ± 0.24 b	73.88 ± 0.60 a	***
Fat in DM (%)	55.15 ± 0.30 b	58.07 ± 0.19 a	53.47 ± 0.44 c	***
Protein in DM (%)	28.59 ± 1.04 a	28.44 ± 1.80 a	28.19 ± 0.37 a	n.s
Salt in DM (%)	3.53 ± 0.06 a	3.23 ± 0.03 b	2.92 ± 0.02 c	***
Ash in DM (%)	6.35 ± 0.05 a	6.16 ± 0.06 b	6.42 ± 0.01 a	**

Results indicate mean values ± S.D. of six determinations (carried out in duplicate for three independent productions). Abbreviations: RCEC 3M, regional cultured Ewiss cheese ripened 3 months; RCEC 6M, RCEC ripened 6 months; RCEC 9M, RCEC ripened 9 months; DM, dry matter; n.s., not significant. Data within a row followed by same letter are not significantly different according to Tukey's test ($p \leq 0.05$). *P*-value: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

residual carbohydrates and amino acids, producing key volatile compounds (Marino et al., 2019). Although not quantified in culture-based assays, its presence in the NGS data suggests a functional role in the later stages of ripening (Alegría et al., 2013).

Importantly, Enterobacteriaceae were only detected at 3 months and were absent by 9 months, indicating a decline likely driven by environmental stressors (low pH, high salt, reduced water activity) (Dahl et al., 2000; Maifreni et al., 2013), and the potential antimicrobial activity of LAB. This observation is supported by both NGS and the known inhibitory effects of LAB metabolites such as organic acids, hydrogen peroxide, and bacteriocins (Aljewicz & Cichosz, 2017; de Souza de Azevedo et al., 2020).

Finally, while we acknowledge that direct molecular evidence would provide more definitive proof of *Pc. flava* survival and activity, our current findings offer indirect support. The stable LAB counts and the absence of spoilage-associated taxa in the NGS data suggest a favorable microbial balance consistent with the expected contribution of the adjunct culture. Although *Propioniciclava* was not explicitly identified among the dominant OTUs, its metabolic contribution may be masked by lower abundance or limitations in taxonomic resolution. Nonetheless, the overall microbial stability and high LAB viability suggest a favorable environment for its persistence and activity, as generally reported in hard cheeses (De Pasquale et al., 2016).

3.4. Physicochemical composition of cheese

The physicochemical quality of RCEC produced with autochthonous bacteria is summarized in Table 3. Color parameters remained mostly stable during ripening, except for a significant decrease in a*, indicating reduced redness. This trend, consistent with Todaro et al. (2017), may be linked to moisture loss and pigment degradation rather than carotenoid concentration alone (Fox et al., 2017). Core compositional parameters (fat, protein, salt, ash) showed minimal variation, while pH and acidity increased progressively, as also observed in Apuseni cheese (Mureşan et al., 2021). Texture analysis (Table 4) revealed notable increases in hardness, chewiness, and cohesiveness over time, aligning with findings in other hard sheep cheeses (Renes et al., 2021).

3.5. Free fatty acid and volatile organic profile of cheese

Ovine dairy products are indeed recognized for their high nutritional value, including bioactive compounds, among which some FAs like conjugated linoleic acid (CLA) and omega-3s have been studied for health benefits (e.g., anti-inflammatory and anti-carcinogenic properties) (Moatsou & Sakkas, 2019). However, the complexity of cheese depends on several factors, including the microbiological and chemical composition of the milk, cheesemaking technology, ripening time, and dairy conditions (Manuelian et al., 2017). Table 5 presents the FFA composition of ripened cheese. SFAs predominated in the cheese samples, with long-chain FAs, especially palmitic acid (30.56 %), being the most abundant at the end of ripening. Monounsaturated fatty acids (MUFA) constituted about 25 % of the total FAs, primarily due to the concentration of oleic acid (19.17 %), which is known for its anticancer, anti-inflammatory, and antiatherogenic properties (Machado et al., 2023). In contrast, polyunsaturated fatty acids (PUFA) made up the

Table 5
Free fatty acid profile of ripened cheese.

Free fatty acid (%)	RCEC 9M
Short- and medium-chain SFA	
Caproic acid (C6)	2.54 ± 0.53
Caprylic acid (C8)	2.34 ± 0.41
Capric acid (C10:0)	7.10 ± 0.96
Lauric acid (C12:0)	4.14 ± 0.41
Myristic acid (C14:0)	13.12 ± 0.70
Long-chain SFA	
Pentadecanoic acid (C15:0)	1.58 ± 0.03
Palmitic acid (C16:0)	30.56 ± 0.44
Stearic acid (C18:0)	10.91 ± 0.20
MUFA	
Palmitoleic acid (C16:1)	1.22 ± 0.02
Oleic acid (C18:1 cis)	19.17 ± 0.22
Oleic acid (C18:1 trans)	2.21 ± 0.04
PUFA	
Linoleic acid (C18:2)	2.95 ± 0.02
Linolenic acid (C18:3 n3)	1.07 ± 0.02

Results indicate mean values ± S.D. of six determinations (carried out in duplicate for three independent productions). Individual free fatty acid levels were reported as percentages of the total free fatty acids. Abbreviations: RCEC 9M, regional cultured Ewiss cheese ripened 9 months; SFA, saturated fatty acids; FA, fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 4
Textural properties of cheeses.

Parameters	Samples			p value
	RCEC 3M	RCEC 6M	RCEC 9M	
Hardness (N)	119.32 ± 8.65 b	134.84 ± 24.45 b	313.89 ± 62.96 a	**
Adhesiveness (N.s)	−32.36 ± 31.46 a	−99.37 ± 69.00 a	−53.77 ± 55.91 a	n.s
Chewiness	52.31 ± 2.41 b	55.22 ± 10.76 b	146.96 ± 48.63 a	*
Cohesiveness	0.61 ± 0.05 a	0.51 ± 0.03 a	0.58 ± 0.09 a	n.s
Deformation (%)[a]	40.03 ± 0.03	40.03 ± 0.01	40.04 ± 0.01	

Results indicate mean values ± S.D. of six determinations (carried out in duplicate for three independent productions). Abbreviations: RCEC 3M, regional cultured Ewiss cheese ripened 3 months; RCEC 6M, RCEC ripened 6 months; RCEC 9M, RCEC ripened 9 months; n.s., not significant. Data within a row followed by same letter are not significantly different according to Tukey's test ($p \leq 0.05$). *P*-value: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

[a] % Deformation = (2 × deformation time [sec])/deformation height [mm].

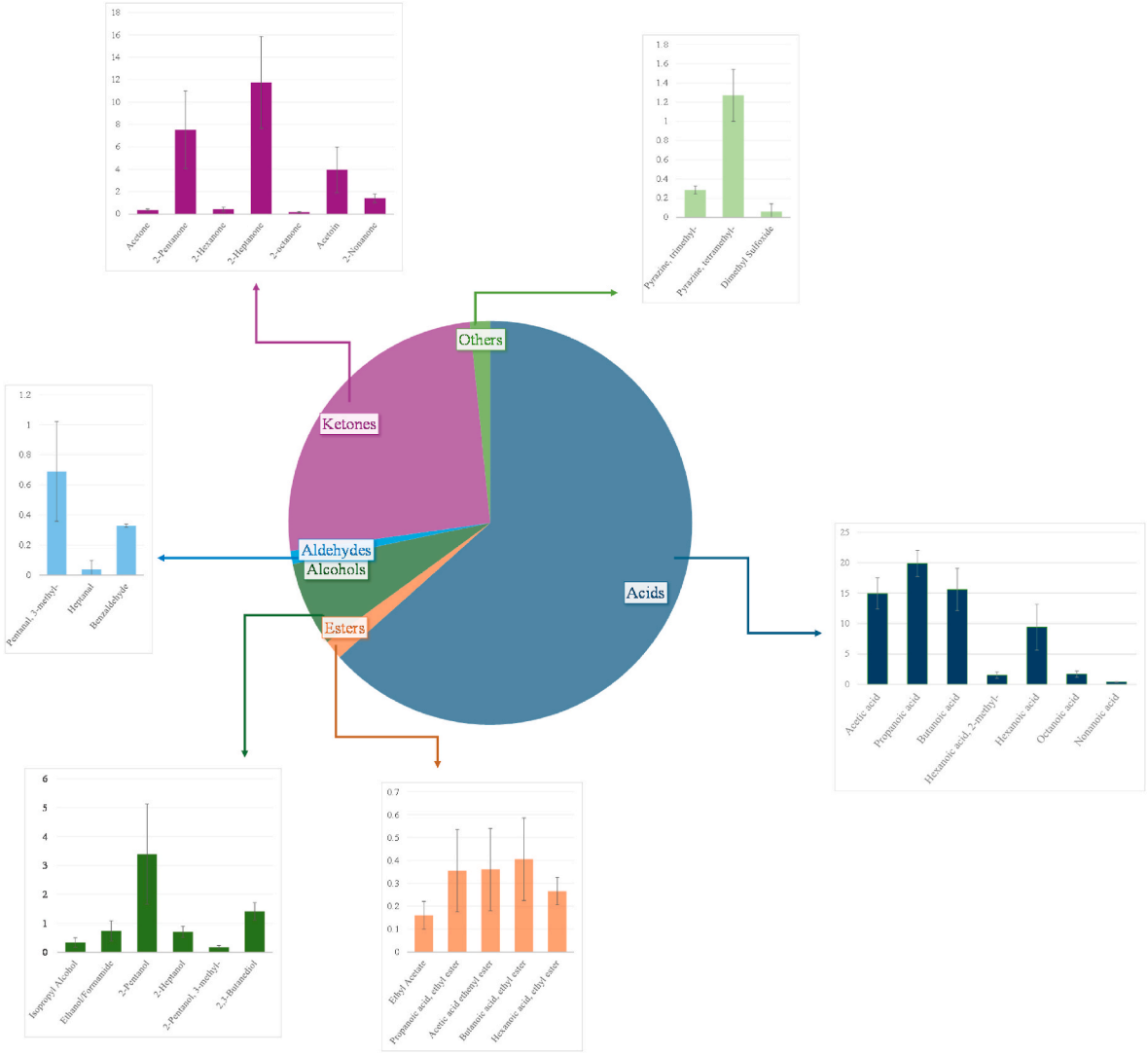

Fig. 3. Volatile organic compounds emitted from Regional cultured Ewiss cheese ripened 9 months. Results are the relative concentrations and indicate mean values ± S.D. of six determinations (carried out in duplicate for three independent productions).

smallest portion of the total FA pool. These findings are consistent with those reported for other sheep cheeses in the literature (Szterk et al., 2022; Tzamaloukas et al., 2021). When comparing this FFA profile with that of commercial Swiss cheese, a similar composition was observed (Białek et al., 2020).

VOCs identified in this study are reported in Fig. 3. Thirty-one compounds were detected in RCEC samples. The main classes of volatile components were acids (63.41 %) and ketones (25.61 %). Esters (1.55 %), alcohols (6.76 %) and aldehydes (1.06 %) accounted for a small percentage of the total area of detected compounds; however, they can contribute synergistically to the overall aroma of the cheese due to their low perception thresholds. Propionic acid was the most abundant compound, arising from propionic fermentation and responsible for the hazelnut aroma characteristic of Swiss-type cheeses (Fröhlich-Wyder & Bachmann, 2004). Compounds such as 2-pentanone, 2-heptanone, and 2-nonanone are linked to the lipolytic activity of microflora in cheese and are commonly reported as important volatile components in ewe's milk cheeses (Borowik et al., 2025). Additionally, the compounds detected in RCEC can be compared with the volatile profile of cow's milk Swiss cheeses (Xia et al., 2023).

The identified VOCs play key roles on the sensory characteristics of the resulting cheese. Among these, ketones, formed through β-oxidation of FFA and amino acid catabolism, are linked to blue cheese-like, fruity, or buttery aromas (Spinnler, 2025). Specifically, 2-heptanone and 2-nonanone are associated with nutty and sweet notes in Swiss-type cheeses (Curioni & Bosset, 2002). Esters, produced via esterification of alcohols and acids during ripening, contribute fruity, sweet, and pleasant aromas, with medium-chain ethyl esters particularly enhancing fruity and nutty notes. Alcohols, generated from amino acid metabolism and carbohydrate fermentation, impart alcoholic, solvent-like, or mildly fruity aromas. For instance, 2-heptanol may introduce vegetative or green notes. Aldehydes, arising from lipid oxidation and amino acid degradation, are often linked to green, grassy, almond, or nutty aromas, with benzaldehyde being a key contributor to almond-like notes. Acids, resulting from lipolysis and fermentation, can produce sharp, pungent, rancid, or sweaty aromas depending on their concentration. Notably, butanoic and hexanoic acids are strongly associated with the intense, pungent aroma characteristic of aged cheeses (Castada et al., 2019; Gasser et al., 2023; Tintrop et al., 2025). Lastly, pyrazines, typically formed through Maillard reactions during cheese aging, add roasted, nutty, or earthy nuances (Gao et al., 2024).

In terms of VOC profiles, clear distinctions emerged between RCEC and CEC. Specifically, RCEC exhibited significantly higher levels of acetic acid, 2-pentanone, 2-heptanone, acetoin, 2-heptanol, and 2,3-butanediol, compounds typically associated with enhanced aromatic complexity and desirable flavour notes. In contrast, CEC showed greater

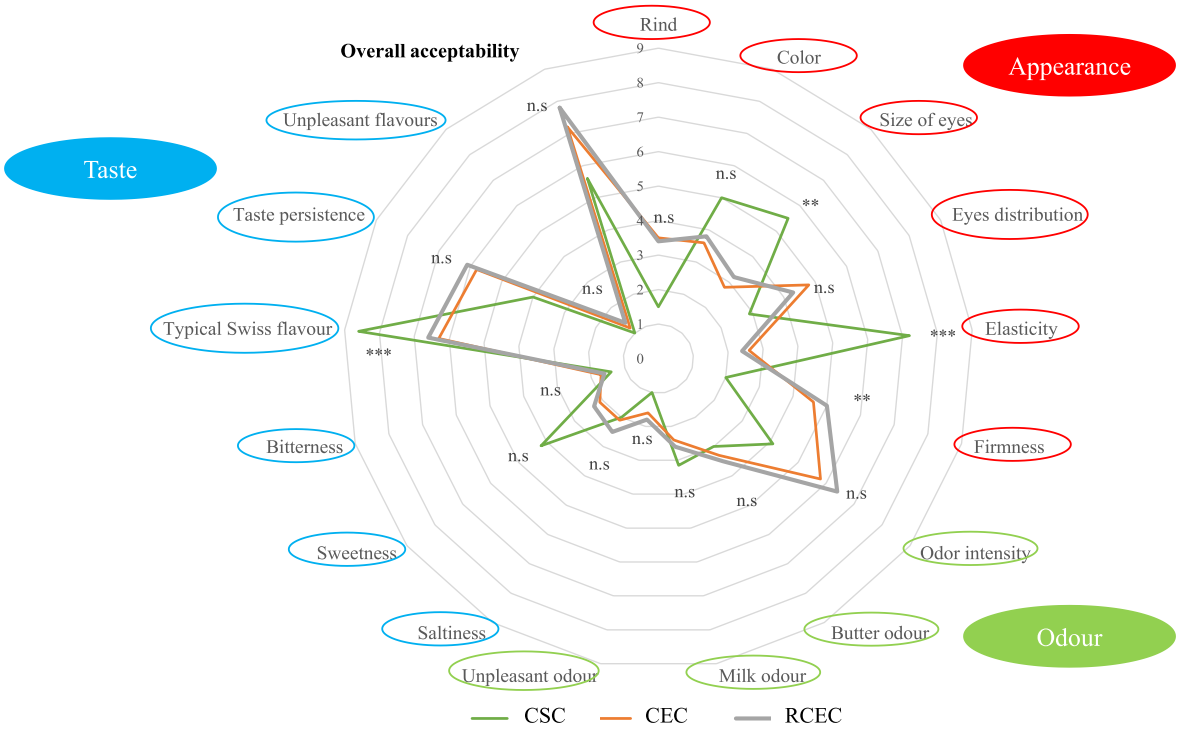

Fig. 4. Spider plot of sensory evaluation of cheese after 9 months of ripening. Abbreviations: CSC, commercial Swiss cheese; CEC, commercial Ewiss cheese made with commercial strains; RCEC, regional cultured Ewiss cheese made with autochthonous strains; **, $p \leq 0.01$; ***, $p \leq 0.001$; n.s., not significant. Results indicate mean values ± S.D. of six determinations (carried out in duplicate for three independent productions).

concentrations of butyric acid, octanoic acid, and, notably, propionic acid. These differences in VOC composition clearly demonstrate that RCEC developed a distinct volatilome, highlighting the unique metabolic contributions of the autochthonous microbial consortium compared to the commercial starter cultures.

3.6. Sensory analysis

The sensory profiles of the cheeses are illustrated in the spider plot (Fig. 4). Sensory assessments were conducted at the end of the ripening period. Comparative analysis revealed no statistically significant differences ($p > 0.05$) for several attributes between ewe's milk cheeses (RCEC and CEC) and the commercial Swiss reference (CSC). All cheese samples were free from off-flavours, and sensory variations were generally minor relative to the control cheeses. Notably, RCEC samples exhibited enhanced odour intensity, saltiness, firmness, and taste persistence compared to both CEC and CSC. In contrast, sweetness, milk odour, and the characteristic Swiss aroma were more pronounced in CSC, while CEC displayed a more uniform distribution of eyes. Statistically significant differences ($p < 0.01$) were observed in eye size, firmness, elasticity, and the intensity of the typical Swiss cheese flavour. Although not all differences reached statistical significance ($p > 0.05$), cheeses made from autochthonous strains received the highest overall acceptability rate from the panel.

4. Conclusions

This study conducted an extensive analysis of the microbiological analysis by dependent and independent approach, physicochemical, VOC profile, and sensory characteristics of RCEC. Microbiological analyses confirmed the hygienic and sanitary quality of RCEC, revealing high levels of LAB and the absence of spoilage and pathogenic microorganisms. For the first time, the bacterial community of Ewiss cheese typology has been thoroughly investigated by Illumina technology, that confirmed LAB dominance. From a physicochemical perspective, RCEC exhibited a lower salt content and a higher concentration of MUFA, particularly oleic acid, compared to the commercial Ewiss cheese. Although sensory evaluation did not detect statistically significant differences between the two cheeses, the VOC profile of RCEC was notably richer, likely reflecting the metabolic activity of the DWSC microbiota. Collectively, these findings support the feasibility of producing a 100 % Sicilian cheese applying Swiss cheese technology, thereby enhancing the agri-food heritage of the region and contributing to the economic and social vitality of the local dairy sector. Furthermore, while preliminary, our findings suggest that *Pc. flava* may offer novel opportunities for innovation in dairy fermentation, warranting further investigation into its safety, functionality, and sensory impact.

CRediT authorship contribution statement

Gabriele Busetta: Data curation, Formal analysis, Writing – original draft. **Giuliana Garofalo:** Data curation, Formal analysis, Writing – original draft. **Tansu Taspinar:** Data curation, Formal analysis. **Rosa Guarcello:** Data curation, Formal analysis. **Elena Franciosi:** Data curation, Formal analysis. **Maria Teresa Sardina:** Funding acquisition, Project administration, Resources. **Giancarlo Moschetti:** Supervision. **Luca Settanni:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Huseyin Erten:** Funding acquisition, Methodology, Writing – review & editing. **Raimondo Gaglio:** Conceptualization, Supervision, Validation, Writing – review & editing.

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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.idairyj.2025.106473.

Data availability

Data will be made available on request.

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