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Onopordum platylepis (Murb.) Murb. as a novel source of thistle rennet: First application to the manufacture of traditional Italian raw ewe's milk cheese

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ABSTRACT

In this study, for the very first time, aqueous extracts obtained from flowers of spontaneously grown or cultivated *Onopordum platylepis* (Murb.) Murb. thistles were used as coagulating agents for the pilot-scale manufacture of *Caciofiore*, a traditional Italian raw ewe's milk cheese. Cheese prototypes were compared to control cheeses curdled with a commercial thistle rennet obtained from flowers of *Cynara cardunculus* L. After 45 days of ripening under controlled conditions, both the experimental and control cheese prototypes were analyzed for: cheese yield, physico-chemical (pH, titratable acidity, a_w, proximate composition), morpho-textural (color and texture), and microbiological parameters (viable cell counts and species composition assessed by Illumina sequencing), as well as volatile profile by SPME-GC–MS. Slight variations in titratable acidity, color, and texture were observed among samples. Based on the results overall collected, neither the yield nor the proximate composition was apparently affected by the type of thistle coagulant. However, the experimental cheese prototypes curdled with extracts from flowers of both spontaneous or cultivated thistles showed 10 % higher values of water-soluble nitrogen compared to the control prototypes. On the other hand, these latter showed slightly higher loads of presumptive lactococci, thermophilic cocci, coliforms, and eumycetes, but lower counts of *Escherichia coli*. No statistically significant differences were revealed by the metataxonomic analysis of the bacterial and fungal biota. Though most volatile organic compounds (VOCs) were consistent among the prototypes, significant variability was observed in the abundance of some key aroma compounds, such as butanoic, hexanoic, and octanoic acids, ethanol, propan-2-ol, isobutyl acetate, 2-methyl butanoic acid, and 3-methyl butanal. However, further investigations are required to attribute these differences to either the type of coagulant or the metabolic activity of the microorganisms occurring in the analyzed cheese samples. The results overall collected support the potential exploitation of *O. platylepis* as a novel source of thistle coagulant to produce ewe's milk cheeses.

1. Introduction

Cheese is part of the Mediterranean diet and cultural heritage; its consumption is appreciated worldwide, thus exhibiting significant economic potential for the dairy sector (Laranjo & Potes, 2022; Sgroi et al., 2022). Cheese varieties differ in the origin of milk, coagulation and rennet type, and microorganisms involved in fermentation and maturation (McSweeney et al., 2017). The production of cheese often relies on traditional raw materials and technological processes that are closely tied to the unique heritage of dairies situated in specific geographical regions. This general rule also applies to cheeses made with vegetable rennet. For example, cheeses coagulated with thistles are characteristic of specific regions in the Mediterranean basin, where various types of thistles, including well-known species like *Cynara cardunculus* L. and *Cynara humilis* L., grow naturally. These thistles have been utilized for centuries to prepare extracts used as clotting agents, typically for raw ewe's milk cheeses (Cesaro et al., 2023; Roseiro et al., 2003). The longstanding tradition associated with these cheeses is recognized and valorized through the protected designation of origin (PDO) granted by the European Union. Among the most renowned cheeses that obtained

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the PDO status, *Torta del Casar* in Spain, *Queso Serra da Estrela* in Portugal, and *Fiore Sardo* in Italy are included (eAmbrosia, 2022). Besides enhancing the unique sensory properties of these traditional products (Cardinali et al., 2017), thistle rennet also holds significant value as a vegetarian-friendly alternative to animal rennet, aligning cheese with vegetarian dietary choices (Alavi & Momen, 2020).

Thistles are flowering plants belonging to the Asteraceae family that can grow in marginal areas under challenging environmental conditions since they need minimal requirements in terms of agronomic inputs. For this reason, in recent years, the interest in the exploitation of thistles for their industrial applications has increased (Mandim et al., 2023; Zenobi et al., 2021). Traditionally, thistle-curdled cheeses are manufactured using aqueous extracts of flowers harvested from wild plants (Merchán et al., 2024; Rampanti, Raffo et al., 2023). Consequently, the cultivation of thistles for the production of vegetable rennet represents a promising sustainable solution for conserving biodiversity, by ensuring consistent supply of thistle flowers, and valorizing marginal areas.

The term "thistle" encompasses numerous species classified under the genera *Carduus*, *Carlina*, *Carthamus*, *Cirsium*, *Cynara*, *Echinops*, *Onopordum*, *Scolymus*, *Silybum*, and *Sonchus* (Aquilanti et al., 2011). Besides the widely recognized species *Cynara cardunculus* L., commonly known as wild cardoon, other thistles, including *Carlina acanthifolia* All., *Cynara humilis* L., *Cynara scolymus* L., *Cirsium vulgare*, *Onopordum acanthium* L., *Onopordum tauricum* Willd., *Onopordum turcicum*, and *Silybum marianum* L. proved to be sources of extracts containing aspartic proteases (APs) with milk clotting activity (MCA) (Ben Amira et al., 2017; Cardinali et al., 2016; Mozzon, Foligni, Mannozzi et al., 2020).

Onopordum platylepis (Murb.) Murb. (basionym *Onopordum nervosum* subsp. *platylepis* Murb.) is a thistle species endemic to Tunisia, where it grows wild in Northern and Central areas and more rarely in the South (Hachicha et al., 2007). It is characterized by an erect and spiny stem (50–150 cm) and flower heads blooming from May to July (Hachicha et al., 2007). The properties of the aqueous extracts obtained from *O. platylepis* flowers have recently been investigated by Bande-De León et al. (2023) and Essaidi et al. (2023). In more detail, the MCA of *O. platylepis* extracts was found to be enhanced by high temperature (70 °C) and $CaCl_2$ concentration (60 mM), and low pH (5.5) (Bande-De León et al., 2023). Furthermore, *O. platylepis* extracts also showed antioxidant activity, with no differences between cultivated and spontaneous thistles (Essaidi et al., 2023). Compared to the extracts of *C. cardunculus* and *C. humilis*, those obtained from *O. platylepis* showed a lower proteolytic activity (PA) and a lower MCA/PA ratio (Bande-De León et al., 2023). Although excessive proteolytic activity and low MCA/PA ratio represent the major drawbacks of vegetable rennet due to the development of bitter taste and cheese texture defects during storage or ripening (Ben Amira et al., 2017), several other factors, including the protein composition of milk (Wedholm et al., 2006) and breed (Martins et al., 2009), affect the clotting of milk. However, to date, no data are currently available in the scientific literature regarding cheese produced with *O. platylepis*.

Based on these premises, the objective of this study was to explore the potentiality of *O. platylepis* for the manufacturing of *Caciofiore*, a typical Italian cheese traditionally made with raw ewe's milk coagulated with thistle rennet. To assess the suitability of *O. platylepis* for cheese production and investigate the prospective of this species as a crop for vegetable rennet production, pilot-scale cheesemaking trials were conducted using either the extracts obtained from flowers of spontaneous or cultivated thistles, or a commercial *C. cardunculus* rennet as a control. After 45 days of maturation, ripened cheeses were analyzed for their physico-chemical and morpho-textural parameters, composition of the bacterial and fungal biota, and volatile organic compounds (VOCs). The results collected were comparatively evaluated to determine the impact of the new milk coagulants on the assayed cheese traits.

2. Materials and methods

2.1. Preparation of thistle-based coagulants, cheesemaking, and yield determination

Flowers from spontaneously grown or cultivated *O. platylepis* thistles were manually collected in spring 2021 from North Tunisia, as previously detailed by Essaidi et al. (2023). The purple tubular flowers were separated from the receptacles, air-dried, and shipped to Italy for preparation of thistle rennet, according to the procedure previously described by Rampanti, Belleggia et al. (2023). Briefly, the flowers were macerated in demineralized water (1:10 w v^{-1}) at 4 °C for 24 h; the resulting aqueous extract was filtered through a muslin cloth and centrifugated at 5,000 × g. Finally, the clear supernatant was collected and freeze-dried (VirTis Advantage benchtop freeze dryer, Steroglass S.r.L., Perugia, Italy) prior to its reconstitution.

Cheesemaking trials for the manufacture of *Caciofiore* were conducted in September 2022 at a family-run dairy located in the Marche region (Central Italy) following a traditional process previously described by Rampanti et al. (2023). Sopravissana raw ewe's milk (9 L) was divided into three aliquots; two aliquots were coagulated with the extract obtained from flowers of spontaneous (OP_S) or cultivated (OP_C) thistles, respectively, whereas the third aliquot was coagulated with a commercial *C. cardunculus* rennet (CC), used as a control. For the experimental extracts, 8 g of freeze-dried extract were reconstituted in demineralized water (1:10 w v^{-1}) and added to the milk. Commercial vegetable rennet was used according to the manufacturer's instructions. Cheesemaking was repeated twice, using two different batches of milk (B1 and B2, respectively), as outlined in Fig. 1. After coagulation, the curd was cut into rice grain-sized pieces and transferred to perforated plastic molds (diameter: 6 cm; height: 5.5 cm). Cheese wheels were ripened under controlled conditions (12 °C and 70 % of relative humidity) for 45 days. At the end of ripening, mature cheeses were transported to the laboratory under refrigerated conditions (+4 °C) and subjected to analysis. Actual yield was calculated as the percentage ratio between the obtained 45 days-ripened cheese and the initial milk weight (kg 100 kg^{-1} milk) (Aldalur et al., 2021).

2.2. Physico-chemical analysis

The pH of each cheese sample was measured by a pH-meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy). Titratable acidity (TA) was determined in a solution obtained from 10 g of cheese homogenized with 90 mL of deionized water, subsequently titrated with NaOH 0.1 M to a pH of 8.3; the results were expressed as % of lactic acid equivalents, as detailed by Rampanti, Belleggia et al. (2023). Water activity (a_w) was measured on grated cheese with a HygroPalm23-AW equipped with an HC2-AW probe (Rotronic,

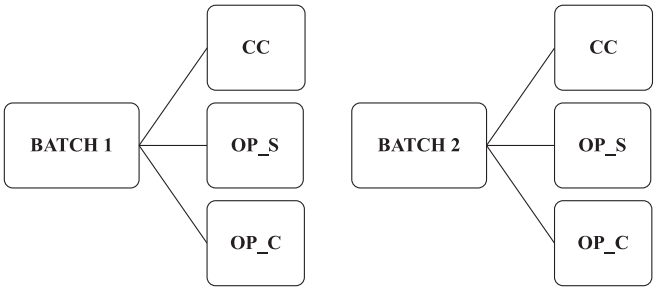

Fig. 1. Experimental plan of cheesemaking trials. 9 L of milk were divided into three aliquots respectively coagulated with a commercial vegetable rennet extracted from *C. cardunculus* (CC), and the extracts from flowers of spontaneous (OP_S) and cultivated *O. platylepis* (OP_C) thistles. Cheesemaking trials were repeated twice, with two different batches of milk (B1 and B2, respectively).

Bassersdorf, Switzerland). Proximate composition, including dry matter, fat, protein, and ash content (%), was determined as previously detailed by Tejada, Abellán, Prados, et al. (2008). Water-soluble and non-protein nitrogen fractions (WSN and NPN, respectively) were measured following the procedures described by Tejada, Abellán, Cayuela, et al. (2008). For the parameters listed above, the results were expressed as mean ± standard deviation of two biological (B1 and B2) and three technical replicates.

2.3. Morpho-textural analysis

Color measurements were performed using a Chroma Meter CR-200 (Minolta, Osaka, Japan) with a D65 illuminant. Color was determined on 1-cm thick slices according to the CIELab color space (L*, lightness; a*, redness/greenness; b*, blueness/yellowness).

Cheese texture was measured on cylindrical specimens (height: 20 mm, diameter: 20 mm) using a CT3-4500 texture analyzer (Brookfield Engineering Laboratories Inc., Middleboro MA, USA) with a 4500 g load cell. A 36-mm-diameter cylindrical probe (mod. TA-AACC36) was used in a double compression test (TPA) at 1 mm s^{-1} using a non-destructive deformation (2 mm) (Belleggia et al., 2024). Both color and texture measurements were conducted in triplicate for each biological replicate (B1 and B2), and the results were expressed as mean ± standard deviation.

2.4. Microbiological analysis

Ten grams of each cheese sample were homogenized in 90 mL of sterile peptone water (bacteriological peptone, 1 g L^{-1}, Oxoid, Basingstoke, UK) with a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm. The cheese homogenates were serially diluted in the same diluent; aliquots of each dilution were inoculated in duplicate on the opportune growth media for viable counting of the following microbial groups: total mesophilic aerobes on Plate Count Agar (PCA) (VWR International, Milan, Italy) incubated at 30 °C for 48 h; presumptive lactobacilli on de Man, Rogosa, Sharp (MRS) (VWR) agar incubated at 30 °C for 48 h; presumptive mesophilic and thermophilic cocci on M17 agar (Liofilchem, Roseto degli Abruzzi, Italy) incubated at 22 and 45 °C, respectively, for 48 h; Enterobacteriaceae in Violet Red Bile Glucose Agar (VRBGA) (VWR) incubated at 37 °C for 24 h; coliforms and *Escherichia coli* in Chromogenic Coliform Agar (CCA) (VWR) incubated at 37 °C for 24 h; yeasts and molds on Rose Bengal (RB) (VWR) agar incubated at 25 °C for 72 h. The results were expressed as the Log of colony-forming units (cfu) per gram of sample and reported as mean ± standard deviation of two biological replicates, each including two technical replicates.

2.5. DNA extraction, sequencing, and metataxonomic analysis

The E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) was used for the extraction of the total microbial DNA from the cell pellets obtained by centrifugation of 1.5 mL of the first decimal dilution (10^{-1}) of the cheese samples. DNA quantity and purity of the resulting extracts were checked by Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA); DNA extracts were quantified and standardized by using the Qubit dsDNA Assay kit (Thermo Fisher, Monza, Italy). An amplicon-based sequencing approach was used to analyze the V3-V4 regions of the 16S rRNA gene using primers and procedures previously described by Klindworth et al. (2013), whereas the D1-D2 domain of the 26S rRNA gene was amplified according to Mota-Gutierrez et al. (2019). The obtained PCR products were purified, tagged, and pooled following the Illumina metagenomic pipeline. The Illumina MiSeq platform (Illumina, San Diego, California, USA) with V2 chemistry generated raw fastq files composed by 250-bp paired-end reads. The data were elaborated by QIIME 2 software (Bolyen et al., 2019). Briefly, Cutapter and DADA2 algorithms were respectively used to remove primer sequences and to denoise to obtain reads using the q2-dada2 plugin in QIIME 2 (Callahan et al., 2016). Taxonomy classification was performed against the Greengenes (for bacteria) or SILVA database (for fungi) by means of the QIIME2 feature-classifier. Amplicon sequence variants (ASVs) with less than five read counts in at least two samples were excluded to increase the confidence of sequence reads. The raw reads data were deposited in the Sequence Read Archive of NCBI under the BioProject accession number PRJNA1054101.

2.6. Determination of volatile compounds by SPME GC–MS

Solid phase microextraction (SPME) was used to collect the volatile components of 45-day-ripened cheese prototypes; in detail, a 10 mL glass vial was filled with 0.5 g of ground cheese, and a DVB/PDMS 65 μm fibre (Supelco/Sigma-Aldrich, Milan, Italy) was exposed into the headspace for 45 min at 50 °C, as previously described by Belleggia et al. (2020).

For the determination of the volatile profile, a Trace 1300 gas chromatograph coupled with a ISQ 7000 single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used. The system was equipped with a Zebron ZB-5 ms capillary column 30 m × 0.25 mm i.d., 0.25 μm film thickness (Phenomenex, Torrance, CA, USA). The operative conditions previously reported by Foligni et al. (2022) were used. The identification of volatile compounds was performed by matching the mass spectral data with the NIST/EPA/NIH Mass Spectral Library 2020 and the chromatographic behaviour with published Kovats retention indices (RIs) according to Mozzon et al. (2020). An automated spreadsheet was used to simplify the calculation of RIs of the unknown components (Maoloni et al., 2021).

2.7. Statistical analysis

The differences among cheese samples were assessed by one-way ANOVA coupled with Tukey – Kramer's (HSD) Post Hoc test ($\alpha = 0.05$) using the software JMP® Version 11.0.0 (SAS Institute Inc., Cary, NC).

Differences in alpha diversity indices of bacterial and fungal populations were analyzed by Bray Curtis comparison between the cheese samples. The alpha diversity parameters of bacterial and fungal populations were calculated through the diversity script of QIIME2.

A Principal Component Analysis (PCA) was carried out on VOCs data using the software JMP® Version 11.0.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Physico-chemical profile and yield

The results of proximate composition and physico-chemical analysis are reported in Table 1.

Concerning proximate composition, no statistically significant differences were found among the samples. In detail, the dry matter (DM) ranged between 68.69 ± 2.83 (CC) and 69.66 ± 2.23 g 100 g^{-1} of cheese (OP_C). A higher variability in fat content was observed, with this parameter ranging from 34.85 ± 5.58 (CC) and 40.75 ± 3.47 g 100 g^{-1} of DM (OP_S). Ash content values of approximately 8 g 100 g^{-1} DM were found in all the samples. The protein content ranged between 45.18 ± 2.22 (OP_S) and 48.60 ± 2.18 g 100 g^{-1} of DM (CC). Concerning nitrogen fractions, the cheese prototypes clotted with the two *O. platylepis* extracts (OP_S and OP_C) showed significantly higher WSN with respect to the control cheese (CC) prototypes clotted with the commercial thistle rennet. By contrast, no significant differences were found in NPN values (g 100 g^{-1} of total nitrogen). Comparable a_w and pH values of approximately 0.91 and 5.3, respectively, were found in both control and experimental cheese prototypes. Conversely, OP_C showed a significantly lower TA value (0.63 ± 0.05) compared to OP_S and CC (0.75 ± 0.08 and 0.77 ± 0.08 % lactic acid equivalents, respectively).

Table 1
Proximate composition and physico-chemical parameters of *Caciofiore* cheese samples.

	CC	OP_S	OP_C
Dry matter [1]	68.69 ± 2.82[a]	69.66 ± 2.23[a]	68.77 ± 1.64[a]
Fat [2]	34.85 ± 5.58[a]	40.75 ± 3.47[a]	38.69 ± 10.60[a]
Ash [2]	8.02 ± 1.13[a]	8.26 ± 0.35[a]	8.39 ± 0.51[a]
Protein [2]	48.60 ± 2.18[a]	45.18 ± 2.22[a]	46.75 ± 3.17[a]
WSN [3]	17.33 ± 0.74[b]	28.09 ± 3.12[a]	25.79 ± 4.31[a]
NPN [3]	16.17 ± 2.01[a]	13.33 ± 1.36[a]	13.02 ± 1.37[a]
a_w	0.906 ± 0.01[a]	0.912 ± 0.00[a]	0.910 ± 0.00[a]
pH	5.32 ± 0.07[a]	5.33 ± 0.06[a]	5.35 ± 0.05[a]
TA [4]	0.77 ± 0.08[a]	0.75 ± 0.08[a, b]	0.63 ± 0.05[b]

The results are expressed as mean ± standard deviation of two biological and three technical replicates. Within each row, values with different superscript letters are significantly different according to the Tukey – Kramer's (HSD) test ($p < 0.05$).
CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from flowers of cultivated *O. platylepis* thistles. WSN: water-soluble nitrogen; NPN: non-protein nitrogen; a_w: water activity; TA: titratable acidity.
[1] Expressed as g 100 g^{-1} of cheese.
[2] Expressed as g 100 g^{-1} of dry matter.
[3] Expressed as g 100 g^{-1} of total nitrogen.
[4] Expressed as % of lactic acid equivalents.

Concerning cheese yield, no statistically significant differences related to thistle rennet type were observed; values of 15.64 ± 0.17, 16.75 ± 1.02, and 16.68 % ± 0.05 (average of B1 and B2) were found for cheese manufactured with CC, OP_S, and OP_C.

3.2. Color and texture

The results of color and texture parameters are reported in Table 2.

As for color parameters, the lightness (L*) attested between 68.77 ± 1.85 (OP_C) and 70.58 ± 4.62 (CC), with no statistically significant differences between control and experimental cheese prototypes. CC showed a lower redness/greenness (a*) value (−1.84 ± 0.13) compared to both OP_S and OP_C (−0.62 ± 0.13 and −0.69 ± 0.32, respectively). Blueness/yellowness (b*) values of 16.62 ± 0.38 (CC), 15.54 ± 0.62 (OP_S), and 15.39 ± 0.42 (OP_C) also revealed statistically significant differences between control and experimental cheese samples.

Regarding texture, except for hardness, no statistically significant differences were observed among the cheese prototypes. Specifically, OP_C showed significantly lower hardness values (18.53 ± 4.58 N) compared to CC and OP_S (39.60 ± 6.91 and 41.37 ± 4.81 N, respectively). Both control and experimental cheese prototypes were characterized by adhesiveness values ranging from 0.11 ± 0.02 to 0.12 ± 0.03, springiness ranging from 1.78 ± 0.05 to 1.85 ± 0.10, and cohesiveness ranging from 0.81 ± 0.05 to 0.90 ± 0.03.

Table 2
Color and texture parameters of *Caciofiore* cheese samples.

	CC	OP_S	OP_C
Color parameters			
*L**	70.58 ± 4.62[a]	68.92 ± 1.60[a]	68.77 ± 1.85[a]
*a**	−1.84 ± 0.13[b]	−0.62 ± 0.13[a]	−0.69 ± 0.32[a]
*b**	16.62 ± 0.38[a]	15.54 ± 0.62[b]	15.39 ± 0.42[b]
Texture parameters			
Hardness (N)	39.60 ± 6.91[a]	41.37 ± 4.81[a]	18.53 ± 4.58[b]
Adhesiveness	0.11 ± 0.04[a]	0.12 ± 0.03[a]	0.11 ± 0.02[a]
Springiness	1.78 ± 0.05[a]	1.80 ± 0.08[a]	1.85 ± 0.10[a]
Cohesiveness	0.86 ± 0.07[a]	0.81 ± 0.05[a]	0.90 ± 0.03[a]

The results are expressed as mean ± standard deviation of two biological and three technical replicates. Within each row, values with different superscript letters are significantly different according to the Tukey – Kramer's (HSD) test ($p < 0.05$).
CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from flowers of cultivated *O. platylepis* thistles. L* value describes the lightness; a* value describes the redness/greenness; b* describes the blueness/yellowness.

3.3. Viable counting

The results of viable counting are reported in Table 3.

In more detail, all the cheese prototypes showed similar loads of total mesophilic aerobes, ranging between 8.72 ± 0.14 (OP_C) and 8.93 ± 0.19 Log cfu g^{-1} (CC). Viable counts in the same order of magnitude were found for presumptive lactobacilli in all the samples (average of 8.81 Log cfu g^{-1}). As for presumptive lactococci, CC showed a slightly higher load (8.85 ± 0.19 Log cfu g^{-1}) compared to OP_S and OP_C (8.64 ± 0.15 and 8.46 ± 0.06 Log cfu g^{-1}, respectively). Viable counts of presumptive thermophilic cocci were comprised between 6.09 ± 0.16 (OP_C) and 7.09 ± 0.17 Log cfu g^{-1} (CC), with statistically significant differences between control and experimental cheese prototypes. No statistically significant differences emerged in the loads of Enterobacteriaceae, attesting between 5.09 ± 1.17 (CC) and 5.65 ± 0.16 Log cfu g^{-1} (OP_C). Conversely, significant differences emerged in the counts of coliforms and *E. coli*; in detail, OP_S showed the lowest load of coliforms (4.97 ± 0.72 Log cfu g^{-1}), whereas significant lower values of *E. coli* were found in CC compared to the experimental cheese prototypes. As for eumycetes, significantly lower values of molds were observed in OP_C (1.29 ± 0.35 Log cfu g^{-1}) compared to OP_S and CC; CC showed a statistically significant higher load of yeasts (5.53 ± 0.05 Log cfu g^{-1}) compared to the experimental cheese prototypes.

3.4. Composition of bacterial and fungal populations

Concerning the composition of bacterial and fungal populations, no significant differences emerged in the alpha diversity indices resulting from the metataxonomic analysis (data not shown). A core of microbial species was stably detected in all the cheese prototypes. Furthermore, both the bacterial (Fig. 2) and fungal (Fig. 3) populations dominating in the cheese prototypes clotted with the *O. platylepis* extracts (OP_S and OP_C) exhibited a high similarity in the distribution of ASVs compared to the control (CC). By considering the composition of the bacterial biota, *Lactococcus lactis* represented the dominant species in both the experimental and control cheese prototypes, with relative frequencies exceeding 50 % of the bacterial ASVs. *Serratia* spp. was the second most abundant taxon in OP_S and OP_C, with relative frequencies of 29.41 and 34.97 %, respectively. In CC, *Serratia* spp. represented 9.41 % of the

Table 3
Viable counts of *Caciofiore* cheese samples.

	CC	OP_S	OP_C
Total mesophilic aerobes	8.93 ± 0.19[a]	8.75 ± 0.19[a]	8.72 ± 0.14[a]
Presumptive lactobacilli	8.93 ± 0.16[a]	8.64 ± 0.26[a]	8.87 ± 0.16[a]
Presumptive lactococci	8.85 ± 0.19[a]	8.64 ± 0.15[a,b]	8.46 ± 0.06[b]
Presumptive termophilic cocci	7.09 ± 0.17[a]	6.34 ± 0.31[b]	6.09 ± 0.16[b]
Enterobacteriaceae	5.09 ± 1.17[a]	5.63 ± 0.16[a]	5.65 ± 0.16[a]
Coliforms	6.10 ± 0.27[a]	4.97 ± 0.72[b]	5.22 ± 0.30[a,b]
Escherichia coli	3.60 ± 0.66[b]	5.85 ± 0.08[a]	5.87 ± 0.18[a]
Molds	2.66 ± 0.04[a]	2.12 ± 0.54[a]	1.29 ± 0.35[b]
Yeasts	5.53 ± 0.05[a]	1.38 ± 1.59[b]	2.59 ± 0.26[b]

Values of viable counts are expressed as Log cfu g^{-1} ± standard deviation of two biological and two technical replicates. Within each row, values with different superscript letters are significantly different according to the Tukey – Kramer's (HSD) test ($p < 0.05$).
CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from flowers of cultivated *O. platylepis* thistles.

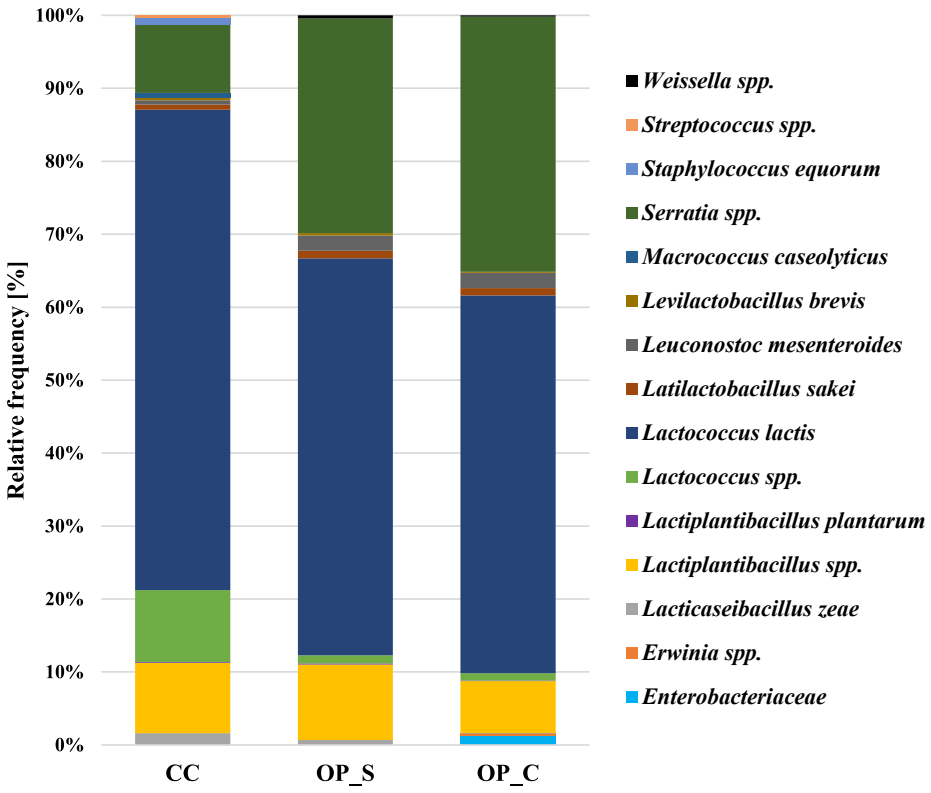

Fig. 2. Bacterial amplicon sequence variants (ASVs) of *Caciofiore* cheese samples. CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from flowers of cultivated *O. platylepis* thistles.

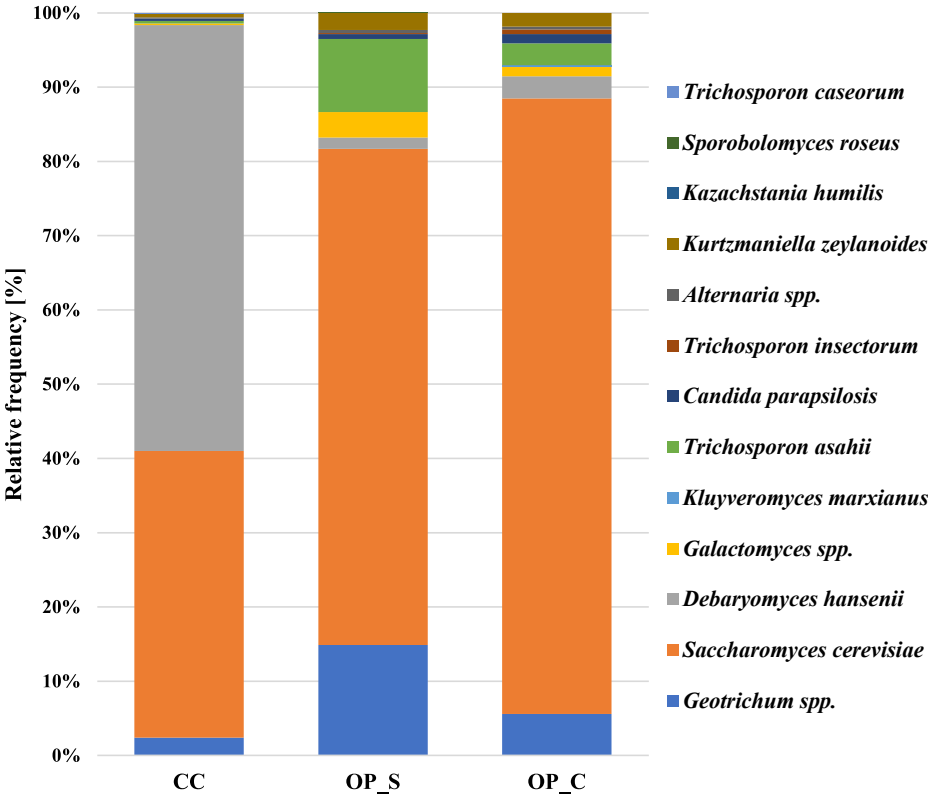

Fig. 3. Fungal amplicon sequence variants (ASVs) of *Caciofiore* cheese samples. CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from flowers of cultivated *O. platylepis* thistles.

relative frequency. *Lactiplantibacillus* spp. was detected in all the cheese prototypes at a relative frequency ranging from 7.08 to 10.34 %. Within lactic acid bacteria, minority taxa were also found in all the samples, including *Lacticaseibacillus zeae* (ranging from 0.13 and 1.59 % of the relative frequency), *Lactiplantibacillus plantarum* (0.09 to 0.14 of the relative frequency), *Latilactobacillus sakei* (0.72 to 1.04 % of the relative frequency), *Leuconostoc mesenteroides* (0.58 to 2.10 % of the relative frequency), and *Levilactobacillus brevis* (0.15 to 0.36 % of the relative frequency). Experimental cheese prototypes also showed the occurrence of *Weissella* spp., with relative frequencies of 0.43 and 0.19 % in OP_S and OP_C, respectively. In CC, three taxa not detected in the cheese prototypes clotted with *O. tauricum* were found, including *Streptococcus* spp. (0.38 % of the relative frequency), *Macrococcus caseolyticus* (0.68 % of the relative frequency), and *Staphylococcus equorum* (0.83 % of the relative frequency). Members of the family Enterobacteriaceae, including the genus *Erwinia*, were found exclusively in OP_C, with relative frequencies of 1.25 and 0.28 %, respectively.

As far as fungi are concerned, a core mycobiota composed of *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, and *Geotrichum* spp., and representing more than 80 % of the relative abundance in all the cheese prototypes, was observed. Of note, *D. hansenii* was particularly abundant in CC (57.36 % of the relative frequency) compared to OP_S and OP_C (1.55 and 2.96 % of the relative frequency, respectively). In the experimental cheese prototypes, a neat predominance of *S. cerevisiae* was revealed. *Galactomyces* spp., *Trichosporon asahii*, *Candida parapsilosis*, *Alternaria* spp., and *Kurtzmaniella zeylanoides* were also detected at various relative frequencies (ranging from 0.17 to 9.86 %) in all the cheese prototypes. Minority taxa were sporadically detected at very low relative frequencies (<0.5 %); these included *Kazachstania humilis* and *Trichosporon caseorum* in CC, *Sporobolomyces roseus* in OP_S, and *Kluyveromyces marxianus* in OP_C.

3.5. Volatile profile

The volatile profile analysis allowed 29 compounds to be identified (Table 4): 10 acids (propanoic acid, 2-methyl; butanoic acid; butanoic acid, 3-methyl; butanoic acid, 2-methyl; hexanoic acid; heptanoic acid; octanoic acid; nonanoic acid; decanoic acid; dodecanoic acid); 6 esters (isobutyl acetate; butanoic acid, 1-methylpropyl ester; ethyl hexanoate; octanoic acid ethyl ester; decanoic acid ethyl ester; dodecanoic acid ethyl ester); 3 alcohols (ethanol; propan-2-ol; 1-butanol, 3-methyl); 4 ketones (2-butanone; 3-hydroxybutan-2-one; 2-heptanone; 2-nonanone); 1 aromatic hydrocarbon (1,3-di-*tert*-butylbenzene); 4 aldehydes (butanal, 3-methyl; benzaldehyde; phenylacetaldehyde; dodecanal); 1 alkane (dodecane). Almost 70 % of the identified VOCs were observed in both control and experimental samples. As depicted in Fig. 4, the ketone group was the most abundant in all the cheese prototypes (approx. 50 % of the relative abundance). In CC, ketones were followed by acids (approx. 24 %), esters (approx. 20 %), and alcohols (approx. 10 %); both aldehydes and alkanes reached values < 1 %. In OP_S, acids (approx. 25 %) were followed by aldehydes (approx. 11 %). Both ester and alcohol groups represented approximately 9 % of the total areas. In OP_C, alcohols showed higher values compared to the other samples (approx. 18 % of the total areas), followed by acids (approx. 15 %), and esters (approx. 7 %). Aldehydes and alkanes represented approximately 3 % and 0.5 % of the total areas, respectively. In more detail, the most abundant VOCs were 2-butanone, ethanol, butanoic acid, ethyl hexanoate, and butanoic acid, 3-methyl. Significantly higher levels of butanal, 3-methyl, isobutyl acetate, butanoic acid, 2-methyl, hexanoic acid, and octanoic acid ethyl ester were observed in OP_S. Instead, OP_C showed a higher level of octanoic acid compared to CC and OP_S, while CC showed a higher level of 3-hydroxybutan-2-one compared to OP_S and OP_C.

Fig. 5 shows the biplot (score plot and loadings plot) of the PCA conducted on VOCs data. Specifically, PC1 and PC2 accounted for 64.5 % and 35.5 % of the total variability, respectively. PC1 neatly separated

Table 4
Volatile compounds detected in *Caciofiore* cheese samples.

Name	CAS-NUMBER	Flavor note	CC	OP_S	OP_C
Acid					
butanoic acid	107–92-6	Rancid, cheesy, putrid, sweaty	12864 ± 919^{a}	7901 ± 1326^{b}	12998 ± 1286^{a}
butanoic acid, 2-methyl	116–53-0	Fruity, sour, sweaty	245 ± 203^{b}	7721 ± 1026^{a}	405 ± 114^{b}
butanoic acid, 3-methyl	503–74-2	Swiss, cheese, waxy, sweaty	2451 ± 1079^{a}	9993 ± 8631^{a}	2877 ± 690^{a}
heptanoic acid	111–14-8	Rancid, sour, fatty odor	190 ± 190^{a}	14 ± 15^{a}	71 ± 16^{a}
decanoic acid	334–48-5	Rancid	0 ± 0^{a}	1 ± 1^{a}	0 ± 0^{a}
dodecanoic acid	143–07-7	Like oil of bay	1 ± 1^{a}	0 ± 1^{a}	1 ± 1^{a}
hexanoic acid	142–62-1	Pungent, blue cheese, goat-like	2244 ± 295^{b}	11420 ± 1148^{a}	41 ± 32^{c}
nonanoic acid	112–05-0	Coconut, fatty odor	1 ± 1^{a}	0 ± 0^{a}	12 ± 14^{a}
octanoic acid	124–07-2	Goaty, waxy, soapy, rancid	216 ± 248^{b}	430 ± 59^{b}	1067 ± 278^{a}
propanoic acid, 2-methyl	79–31-2	Rancid butter	97 ± 12^{a}	49 ± 14^{b}	0 ± 0^{c}
Alcohol					
1-butanol, 3-methyl	123–51-3	Fruity, alcohol	54 ± 25^{b}	1 ± 1^{b}	174 ± 92^{a}
ethanol	64–17-5	Alcohol, mild	4413 ± 753^{b}	103701 ± 5201^{a,b}	20094 ± 8407^{a}
propan-2-ol	67–63-0	Rubbing alcohol	3140 ± 989^{a}	3063 ± 1131^{a}	772 ± 109^{b}
Aldehyde					
benzaldehyde	100–52-7	Sweet, strong almond odor	20 ± 23^{b}	361 ± 83^{a}	48 ± 5^{b}
butanal, 3-methyl	590–86-3	Dark chocolate, malt, green	363 ± 104^{c}	16361 ± 733^{a}	3005 ± 695^{b}
dodecanal	112–54-9	Fatty odor	0 ± 0^{a}	13 ± 14^{a}	1 ± 0^{a}
phenylacetaldehyde	122–78-1	Pungent green floral, hyacinth-like	1 ± 1^{a}	60 ± 65^{a}	31 ± 36^{a}
Alkane					
dodecane	112–40-3		461 ± 418^{a,b}	1 ± 1^{b}	637 ± 92^{a}
Aromatic hydrocarbon					
1,3-di-*tert*-butylbenzene	1014–60-4		1 ± 1^{a}	133 ± 152^{a}	1 ± 1^{a}
Ester					
butanoic acid, 1-methylpropyl ester	819–97-6		6425 ± 7293^{a}	2734 ± 551^{a}	143 ± 42^{a}
decanoic acid ethyl ester	110–38-3		15 ± 17^{b}	9 ± 11^{b}	41 ± 2^{a}
dodecanoic acid ethyl ester	106–33-2	Fruity, floral	5 ± 6^{a}	0 ± 0^{a}	3 ± 3^{a}
ethyl hexanoate	123–66-0	Pineapple, apple powerful	6904 ± 1015^{a}	3711 ± 403^{a}	6722 ± 5129^{a}
isobutyl acetate	110–19-0	Fruity, floral	1668 ± 274^{b}	5436 ± 1390^{a}	643 ± 68^{b}

(*continued on next page*)

Table 4 (*continued*)

Name	CAS-NUMBER	Flavor note	CC	OP_S	OP_C
		banana-like			
octanoic acid ethyl ester	106–32-1		241 ± 280[b]	1396 ± 296[a]	1 ± 1[b]
Ketone					
2-butanone	78–93-3	Acetone, etheric	29710 ± 26484[a]	65966 ± 42424[a]	63094 ± 26266[a]
2-heptanone	110–43-0	Floral, fruity	933 ± 826[a]	627 ± 71[a]	1129 ± 619[a]
2-nonanone	821–55-6	Musty, fruity, floral	903 ± 1043[a]	437 ± 90[a]	10 ± 12[a]
3-hydroxybutan-2-one	5077–67-8	Buttery	3873 ± 1187[a]	532 ± 203[b]	1 ± 1[b]

Values are expressed as mean ± standard deviation of chromatographic peak areas (arbitrary units × 10^4); n = 4. Means with different superscript letters in the same row are statistically different according to the Tukey – Kramer's (HSD) test ($p < 0.05$). CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from cultivated *O. platylepis* thistles.

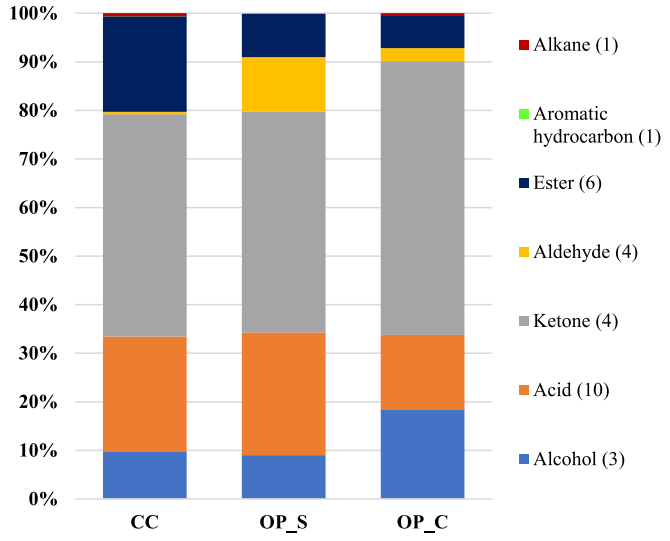

Fig. 4. Relative abundances of VOCs classes found by GC–MS in *Caciofiore* cheese samples. CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from flowers of cultivated *O. platylepis* thistles.

experimental cheese produced using the extract from flowers of spontaneous *O. platylepis* thistles (OP_S) from both control (CC) and experimental cheese produced using the extract from flowers of cultivated *O. platylepis* thistles (OP_C). Meanwhile, CC and OP_C were neatly separated by PC2. The level of butanoic acid, higher in CC and OP_C cheeses, resulted negatively correlated with butanoic acid, 2-methyl, butanoic acid, 3-methyl, and butanal, 3-methyl. Moreover, hexanoic acid, mostly associated with OP_S, showed a high negative correlation with 2-heptanone, ethyl hexanoate, dodecane, and dodecanoic acid.

4. Discussion

This work presents the very first application of aqueous extracts derived from *O. platylepis* to the small-scale manufacture of ewe's milk cheese, and the characterization of the resulting cheese prototypes. Cheese yield is a pivotal indicator of production efficiency, subject to variation based on several factors related to the origin of milk and technological parameters (Fox et al., 2017). Ewe's milk typically contains higher total solids than cow's and goat's milk, thus leading to higher cheese yields (Silanikove et al., 2016). Nevertheless, the influence of other factors, including breed, feeding practices, lactation period, technological parameters and even type of milk coagulant, may also affect cheese yield. As far as rennet is concerned, a high degree of proteolysis results in the formation of low molecular weight hydrophobic peptides, which in turn are associated with a diminished yield (Jacob et al., 2010). Remarkably, the experimental coagulants obtained from *O. platylepis* herein assayed exhibited cheese yields comparable to that achieved with the commercial coagulant derived from *C. cardunculus* (~16 %). Furthermore, similar values of dry matter, fat, ash, and protein content were observed in both the control (CC) and experimental cheese prototypes (OP_S and OP_C), suggesting that the proximate composition was unaffected by the type of coagulant, as previously reported in the literature (Rampanti, Raffo et al., 2023; Soodam et al., 2015; Vioque et al., 2000). On the contrary, a notable variability was seen in nitrogen fractions when comparing cheeses manufactured with different coagulants (Fernández-Salguero & Sanjuán, 1999; Galán et al., 2008, 2012; Tofalo et al., 2015). Water-soluble nitrogen (WSN) and non-protein nitrogen (NPN) content are considered as indices of ripening and ripening depth respectively, the former indicating the extent of proteolysis related to casein hydrolysis and the latter referring to the concentration of small peptides (2–20 residues) and free amino acids resulting from the metabolic activity of microorganisms (Tejada, Abellán, Cayuela et al., 2008). The results collected in the present study show that both the experimental cheese prototypes manufactured with flowers of spontaneous (OP_S) and cultivated (OP_C) *O. platylepis* thistles were characterized by a higher WSN content (approximately + 10 g 100 g^{-1} of total nitrogen), and thus, a higher degree of proteolysis, than control cheese prototypes (CC). According to the available literature comparing nitrogen fractions of cheese manufactured with animal rennet and thistle coagulants (especially derived from *C. cardunculus*), as a general trend, the latter exhibits higher proteolysis (Galán et al., 2008, 2012; Zikiou & Zidoune, 2019). By contrast, scarce information is available about cheese manufactured with other thistle coagulants.

Ewe's milk cheeses manufactured with *C. humilis* and *O. tauricum* showed a lower WSN/TN ratio compared to cheese obtained with *C. cardunculus* (Rampanti, Raffo et al., 2023; Vioque et al., 2000).

Although no significant differences were seen in the pH of cheese prototypes herein assayed, OP_C showed a significantly lower TA with respect to CC and OP_S. Similar evidence emerged in a previous study, where ewe's milk cheese prototypes clotted with extracts from spontaneous or cultivated *O. tauricum* showed lower TA values than control cheese prototypes clotted with the same commercial *C. cardunculus* rennet herein assayed (Rampanti, Raffo, et al., 2023).

In milk and dairy products, TA measures undissociated acidic molecules, including citrates, phosphates, and proteins (Calamari et al., 2016) and organic acids produced by lactic acid bacteria. Thus, variation in TA can result from differences in the levels of organic acids produced by microorganisms during fermentation and ripening, as well as from differences in the concentration of nitrogen fractions, in turn, determined by the type of coagulant, among possible factors.

Concerning color assessment, the type of coagulant did not affect L^* (lightness); for this color attribute, the values herein recorded were in accordance with those reported for other ewe's milk cheeses (Bennato et al., 2023; Todaro et al., 2024).

Conversely, significant differences were seen between the experimental and control cheese prototypes for other color parameters. Similarly to what was reported by Rampanti, Raffo et al. (2023), the cheese prototypes clotted with *O. platylepis* showed lower a^* values and, hence, higher greenness compared to the control cheeses. OP-curdled cheeses

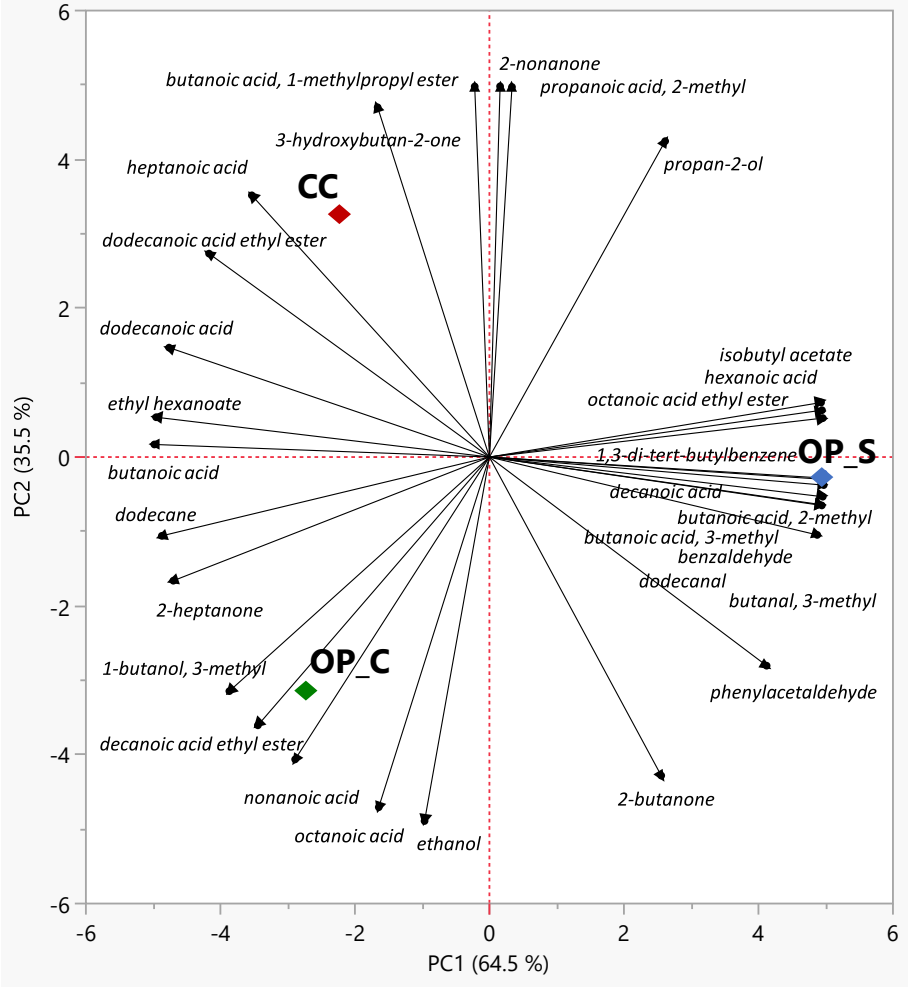

Fig. 5. Principal Component Analysis (PCA) of volatile organic compounds (VOCs) in *Caciofiore* cheese prototypes. CC: control cheese manufactured with the commercial *C. cardunculus* extract; OP_S: experimental cheese manufactured with the extract from flowers of spontaneous *O. platylepis* thistles; OP_C: experimental cheese manufactured with the extract from flowers of cultivated *O. platylepis* thistles.

were also less yellow, as indicated by lower b^* values compared to the control. As previously suggested by Rampanti, Raffo et al. (2023), the color variation between control and experimental cheeses is likely due to the different color of the flower extracts, being darker in the two *O. platylepis* extracts in respect with the commercial *C. cardunculus* rennet. Changes in color attributes may be perceived as a defect by consumers (Wang et al., 2023). Nevertheless, this issue can be more pronounced in cheeses characterized by a white-paste color, typically found in fresh or brined cheese, whereas it can be less noticeable in ripened cheeses that undergo spontaneous color modifications due to chemical changes occurring during maturation (Sardiñas-Valdés et al., 2021).

Concerning texture, the use of the experimental extract from flowers of cultivated *O. platylepis* thistles resulted in statistically lower hardness values in OP_C compared to CC and OP_S cheese samples. Hardness values are known to correlate closely with moisture content (Abarquero et al., 2024). However, no differences in the moisture content emerged in the cheese prototypes analyzed in this study. Changes in cheese hardness are also associated with the proteolytic activity of milk coagulants (Mohsin et al., 2024). Despite the water-soluble nitrogen values indicating a higher degree of proteolysis in the experimental cheeses compared to the control, the disparity in hardness values between OP_S and OP_C suggests that this factor may not fully explain the observed differences. Further investigation into proteolysis and sensory analysis will be necessary in the future to comprehensively understand the factors contributing to the texture variations observed in the cheese prototypes herein studied.

As far as microbial viable counts are concerned, loads of total mesophilic aerobes in both control and experimental cheese prototypes were similar to those reported for other raw ewe's milk thistle-curdled cheeses (Cardinali et al., 2021; Rampanti, Raffo et al., 2023). For presumptive lactobacilli, counts of the same order of magnitude were found in the assayed cheese prototypes, irrespective of the type of coagulant. In more detail, the loads of this microbial group were comparable to those found in Portuguese thistle-curdled cheeses (Cardinali, Foligni, Ferrocino, Harasym, Orkusz, Franciosa, et al., 2022; Cardinali, Foligni, Ferrocino, Harasym, Orkusz, Milanović, et al., 2022). Differences likely related to the type of coagulant also emerged for other microbial groups, including presumptive lactococci, presumptive thermophilic cocci, coliforms, *E. coli*, and eumycetes. As a general trend, the control cheeses (CC) showed slightly higher loads of lactic acid bacteria compared to both OP_S and OP_C. Variations in the load of lactic acid bacteria in cheeses manufactured with different milk coagulants have previously been reported (Mohsin et al., 2023; Rampanti, Raffo et al., 2023). However, in the case of raw milk cheeses, particularly those without the addition of any starter cultures, these differences are more likely ascribable to the microbiological quality of the milk and to the process parameters rather than the type of coagulant (Rampanti, Belleggia et al., 2023).

Concerning molds, viable counts were below 3 Log cfu g^{-1} in all the

prototypes, in accordance with the results reported by Torkar & Vengušt (2008). Both the experimental cheese prototypes clotted with the extracts from spontaneous and cultivated *O. platylepis* showed lower loads of yeasts compared to the controls and other ewe's milk cheeses (Gardini et al., 2006; Rampanti, Belleggia et al., 2023). No differences emerged in the counts of Enterobacteriaceae, attesting at approximately 5 Log cfu g^{-1} in both control and experimental cheese prototypes. The occurrence of Enterobacteriaceae in raw milk cheeses is highly documented and is a subject of controversy (Metz et al., 2020; Montel et al., 2014). In fact, although the activity of these microorganisms has been associated with increased proteolysis, lipolysis, and development of volatile aroma compounds (Irlinger et al., 2012), certain species within this bacterial family include potential pathogens, raising concerns for public health (Bovo Campagnollo & Sant'Ana, 2022). Among Enterobacteriaceae, coliforms and *E. coli* are regarded as indicators of a lack of hygiene and fecal contamination of milk and dairy products. Milk usually contains < 2 Log cfu g^{-1} of both coliforms and *E. coli*; in the early stages of ripening, the loads of these indicator bacteria increase, usually followed by a decline through maturation (Metz et al., 2020). In the present study, excessive levels of these bacteria were found at the end of ripening in both the control and experimental cheese prototypes, thus indicating a potential exposure of milk or cheese to unhygienic conditions (International Life Sciences Institute, 2011). However, even based on what was found in previous similar investigations (Rampanti, Belleggia et al., 2023), a coagulant-related effect on the high loads of Enterobacteriaceae at the end of ripening can be excluded.

In recent years, the use of high throughput sequencing technologies and metataxonomic analysis has significantly increased to validate the results derived from conventional culture-dependent techniques (Quigley et al., 2013). Moreover, the application of these novel techniques allowed the comprehension of the role of the microbiota, as well as the interactions among species within the cheese matrix, to be achieved (Ferrocino et al., 2022).

In the present study, metataxonomic analysis did not allow *E. coli* to be detected in any of the assayed cheese prototypes, in apparent contrast with the results of viable counting. However, this evidence is in accordance with the results previously reported by Rampanti, Belleggia et al. (2023) and Rampanti, Raffo et al. (2023). *Serratia* spp. emerged as a predominant genus among the assayed cheese samples. *Serratia* is a genus recently reclassified in the family Yersiniaceae within the order *Enterobacterales* (Adeolu et al., 2016). Some species within this genus, notably *Serratia marcescens* and *Serratia liquefaciens*, have previously been associated with mastitis in dairy cows (Friman et al., 2019) and, more rarely, in ewes (Tzora & Fthenakis, 1998). The occurrence of *Serratia* spp. in raw milk (Ercolini et al., 2009; Rampanti, Belleggia et al., 2023) and cheeses (Biolcati et al., 2022; Ordiales et al., 2013) has been widely documented. Some species within this genus exhibit high metabolic activities, such as proteolytic and lipolytic activities (Baglinière et al., 2017; Mladenović et al., 2018). However, they may also be associated with undesirable cheese defects, such as pink discoloration (Martelli et al., 2020).

Lactic acid bacteria, primarily represented by *L. lactis*, accounted for more than half of the total ASVs in both the control and experimental cheese prototypes. *L. lactis* is commonly used as a starter culture to produce cheese and dairy products, thanks to its acidification activity and its acknowledged contribution to milk proteins breakdown (Cavanagh et al., 2015). This species is also frequently detected among the predominant bacterial species in artisanal cheeses produced without any starter culture (Pino et al., 2018). Bacterial taxa belonging to the Lactobacillaceae family, including *Lactiplantibacillus* spp., *L. plantarum*, *L. zeae*, *L. sakei*, *L. brevis*, and *L. mesenteroides* were equally distributed in all the cheese prototypes herein assayed. Their occurrence in thistle-curdled raw ewe's milk cheeses, as well as their potential contribution to cheese characteristics, are well documented (Cardinali et al., 2021; Cardinali, Foligni, Ferrocino, Harasym, Orkusz, Franciosa, et al., 2022; Cardinali, Foligni, Ferrocino, Harasym, Orkusz, Milanović, et al., 2022; Rampanti, Ferrocino et al., 2023). In Portuguese cheeses, *L. plantarum* and *L. brevis* were found to be involved in the production of free amino acids and carboxylic acid (Pereira et al., 2010). *L. zeae* is characterized by high proteolytic activity (Terzić-Vidojević et al., 2020), whereas some *L. sakei* strains can exhibit antimicrobial effects against pathogens (Martinez et al., 2015). *L. mesenteroides* can produce dextran from sucrose and buttery and yoghurt aromas (Nieto-Arribas et al., 2010). Of note, *L. plantarum* and *L. mesenteroides* are used as secondary/adjunct cultures in dairy fermentation for flavor development (Mayo et al., 2021). Other minority taxa, including *Weissella* spp., *Streptococcus* spp., *Erwinia* spp., *M. caseolyticus*, and *S. equorum* were sporadically detected in the cheese prototypes herein assayed. *Weissella* spp. and *Streptococcus* spp. are common taxa found in dairy products (Mayo et al., 2021). Adventitious bacteria, including *M. caseolyticus*, *Erwinia* spp., and *Staphylococcus* spp. have previously been detected in raw ewe's milk and cheeses by Rampanti, Belleggia, et al. (2023).

Most of the species revealed through metataxonomic analysis of the fungal ASVs were identified as yeasts. Yeasts are associated with the secondary microbiota involved during ripening, mainly in artisanal cheeses (Bintsis, 2021). Yeasts significantly contribute to the development of cheese aroma thanks to their deacidifying, proteolytic, and lipolytic activities (Fröhlich-Wyder et al., 2019). The predominant taxa found in the cheese prototypes analyzed in this study, including *S. cerevisiae*, *D. hansenii*, and *Geotrichum* spp., are commonly found in hard cheeses (Bintsis, 2021). *Galactomyces* spp., *Trichosporon asahii*, *C. parapsilosis*, and *K. zeylanoides* (formerly known as *Candida zeylanoides*) were also identified as minority taxa in all the cheese prototypes. *Galactomyces* is the teleomorphic genus of *Geotrichum*. While the presence and role of *Geotrichum* spp., such as *G. candidum*, in cheese, are widely acknowledged, scarce information is available regarding *Galactomyces* spp. (Perkins et al., 2020). Martin et al. (2023) showed that *T. asahii* was particularly abundant in *Canastra* cheese during the wet season. Both *C. parapsilosis* and *K. zeylanoides* were found as part of the raw ewe's milk and cheese mycobiota by Rampanti, Belleggia et al. (2023). *Candida* spp. can be associated with mycotic mastitis in small ruminants (Mousa et al., 2021), thus indicating the necessity for monitoring the health conditions of the animals. Additionally, a plethora of taxa was sporadically identified, each with ASVs < 1 %, including *K. marxianus*, *T. insectorum*, *T. caseorum*, *K. humilis*, *S. roseus*, and *Alternaria* spp. Among them, *K. marxianus* and *S. roseus* have been frequently isolated from cheese (Macedo et al., 1995; Tofalo et al., 2014).

Cheese aroma depends on complex biochemical pathways occurring during ripening, mainly lactate and citrate catabolism, lipolysis, and proteolysis. Apart from rennet pastes containing pre-gastric esterase, clotting extracts are not known to exhibit lipase activity (Mcsweeney & Sousa, 2000); consequently, the catabolism of triglycerides is mainly attributed to the lipase/esterase present in milk and in metabolic systems of the microbiota. The most abundant carboxylic acids revealed in this work, being butanoic, hexanoic, and octanoic acids, have previously been detected among the predominant VOCs in ewe's milk cheese (Randazzo et al., 2010; Santamarina-García et al., 2023). Besides directly impacting the aroma of cheese, free fatty acids can undergo other metabolic reactions that lead to the formation of other flavored compounds, such as methyl ketones through β-oxidation, and esters, resulting from reactions with alcohols (Mcsweeney & Sousa, 2000). 2-butanone, 2-heptanone, and 2-nonanone are frequently identified as dominant methyl ketones in ripened ovine milk cheeses (Cardinali et al., 2021; Gaglio et al., 2019; Santamarina-García et al., 2023). Ethyl esters, such as ethyl hexanoate, are recognized as key aroma compounds, imparting fruity and floral olfactory notes (Rampanti, Raffo et al., 2023; Santamarina-García et al., 2023). Ethanol, the most highly detected primary alcohol in cheese, is directly correlated with the formation of ethyl esters (Richoux et al., 2008). In accordance with Rampanti, Raffo, et al. (2023), higher levels of ethanol were found in the experimental cheese prototypes compared to the controls manufactured with the

extract from *C. cardunculus*. Nonetheless, ethanol in cheese is mainly generated through the fermentation of lactose by yeasts and leuconostocs (Ortigosa et al., 2001). The experimental cheeses also showed higher levels of the branched-chain aldehyde 3-methyl butanal likely resulting from leucine catabolism by lactic acid bacteria (Afzal et al., 2017). In semihard and hard cheeses, this compound is commonly associated with a malt/chocolate flavor (Afzal et al., 2017).

5. Conclusions

The results overall collected in the present investigation support the potential application of *O. platylepis* for the sustainable production of novel thistle-based milk coagulants.

A slight variability in TA, color, and texture parameters was observed between the control and experimental cheese prototypes manufactured with the aqueous extracts prepared by macerating flowers of spontaneous or cultivated *O. platylepis* thistles. However, the main difference was found in the value of water-soluble nitrogen, being 10 % higher in the experimental cheeses than in control ones. Although some differences emerged in the load of some microbial groups, such as presumptive thermophilic cocci, coliforms, *E. coli*, and eumycetes, no statistically significant differences in the bacterial and fungal biota were revealed through the metataxonomic analysis. Lactic acid bacteria, primarily represented by *L. lactis*, and yeasts such as *S. cerevisiae*, *Geotrichum* spp., and *D. hansenii*, dominated the bacterial and fungal biota, respectively. Most of the identified VOCs were shared among control and experimental cheese prototypes; nonetheless, a wide variability was observed in the abundance of some key aroma compounds, such as butanoic, hexanoic, and octanoic acids, ethanol, propan-2-ol, isobutyl acetate, 2-methyl butanoic acid, and 3-methyl butanal. However, further investigations are required to understand whether those differences are attributable to the type of coagulant and/or the metabolic activities of the bacterial and fungal biota. Moreover, these investigations could be further complemented by applying sensory analysis to the cheese prototypes, which was not conducted in the samples herein studied due to the high levels of Enterobacteriaceae. This approach will also allow to assess whether the detected variations in the volatile profile could also be perceived through tasting, and if the higher proteolysis found in the experimental cheeses results in sensory defects, such as bitterness.

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CRediT authorship contribution statement

Giorgia Rampanti: Writing – original draft, Formal analysis, Data curation. **Federica Cardinali:** Investigation, Formal analysis. **Cindy María Bande De León:** Writing – review & editing, Formal analysis. **Ilario Ferrocino:** Writing – review & editing, Formal analysis. **Irene Franciosa:** Writing – original draft, Formal analysis. **Vesna Milanović:** Investigation, Formal analysis. **Roberta Foligni:** Investigation, Formal analysis. **Luis Tejada Portero:** Writing – review & editing, Visualization, Resources, Methodology. **Cristiana Garofalo:** Writing – review & editing, Visualization, Resources. **Andrea Osimani:** Writing – review & editing, Visualization, Resources, Methodology. **Lucia Aquilanti:** Writing – review & editing, Visualization, Resources, Project administration, Methodology.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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