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Chemical, microbiological, textural, and sensory characteristics of pilot-scale Caciofiore cheese curdled with commercial *Cynara cardunculus* rennet and crude extracts from spontaneous and cultivated *Onopordum tauricum*

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ABSTRACT

The aim of this study was the chemical, microbiological, textural, and sensory characterization of pilot-scale prototypes of an Italian ewe's raw milk cheese (Caciofiore) curdled with commercial *Cynara cardunculus* rennet, used as a control, and crude extracts obtained from flowers of either spontaneous or cultivated *Onopordum tauricum*. Hence, the control and experimental cheese prototypes produced in two rounds of cheesemaking trials were assayed, at the end of their 60-day maturation, for the following features: pH, titratable acidity, dry matter, fat, total and soluble nitrogen (TN and SN, respectively), ash, salt, protein, lactose, viable plate counts and composition of the bacterial and fungal populations, color, texture, volatile organic compounds (VOCs), and olfactory attributes by sensory analysis (the latter for the sole prototypes curdled with the commercial rennet and the extract obtained from cultivated *O. tauricum*). The data overall collected showed a very low impact of the type of thistle rennet on the analyzed cheese traits, with significant differences being exclusively found for SN/TN%, titratable acidity, color, and adhesiveness. By contrast, a higher impact of the cheesemaking round was seen, with significant differences being observed for salt content, load of presumptive lactobacilli, thermophilic cocci, and *Escherichia coli*, and levels of the following VOCs: 2,3-butanedione, 2-pentanone, 1-butanol, 2-heptanone, 3-methyl-1-butanol, 2-heptanol, 2-nonanone, dimethyl trisulfide, 2-methyl propanoic acid, butanoic acid, and 3-methyl butanoic acid. Sensory analysis revealed a strong ewe's cheese odor, accompanied by other olfactory notes, such as pungent, sour curd, sweet, and Parmesan cheese-like notes, in all the analysed cheese prototypes. Moreover, key odor active compounds, including butanoic acid, ethyl butanoate, 2,3-butanedione, 1-octen-3-one, and dimethyl trisulfide, were identified by GC-olfactometry analysis. Regarding the odor attributes as determined by sensory analysis, again the type of rennet had an almost negligible impact, with significant differences being only perceived for 1 or 2 out of 20 odor attributes, depending on the analytical conditions applied. Although some aspects deserve further investigation, the results herein collected confirm that *O. tauricum* can be regarded as an alternative source of thistle rennet for the manufacture of Caciofiore cheese, and more in general, Mediterranean ewe's milk cheeses.

1. Introduction

The use of vegetable extracts for milk coagulation is known since Roman times, and after two millennia, it still represents a valuable solution for the manufacturing of cheeses with unique and distinctive sensory attributes as well as of vegetarian-friendly alternatives to

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cheeses made with animal rennet (Silva & Malcata, 2005). Extracts obtained through maceration of thistle stamens in water are those most exploited, especially in the Iberian Peninsula, for the manufacture of traditional ewe's milk cheeses (Cardinali et al., 2021, 2022a; Fernández-Salguero, Tejada, & Gómez, 2002; Rampanti et al., 2023a), many of which are registered as Protected Designation of Origin (PDO) cheeses. Among thistle crude extracts, those obtained from *Cynara* spp. have been comprehensively investigated, given their suitability for cheesemaking, especially when using ewe's milk (Roseiro et al., 2003).

Within this genus, *Cynara cardunculus* L. is regarded as a model for the in-depth research to date conducted on the extraction, isolation, and characterization of proteases responsible for milk coagulation; these latter correspond to two aspartic proteinases, namely cardosin A and cardosin B, the first with a cleavage specificity like chymosin (Phe105-Met106 of κ-casein) and the latter acting like pepsin (Alavi & Momen, 2020).

Among thistles, *Onopordum* is a further genus within the Asteraceae family that grows spontaneously in typical habitats of the Mediterranean area constituted by ruderal, uninhabited, and decaying areas, as well as margins of paths. In 2020, for the very first time *Onopordum tauricum* Willd. has been investigated for its potential as a new source of vegetable rennet (Mozzon et al., 2020). One year later, the results of cultivation trials aimed at identifying the effect of two different planting densities on the flower differentiation, rennet production, and weed control of *O. tauricum* suggested the potentiality of this thistle as a new herbaceous crop in low-input cultivation systems (Zenobi et al., 2021). In a further research study (Foligni et al., 2022), the higher clotting performance of crude extracts obtained from flowers of cultivated than spontaneous *O. tauricum* was demonstrated. The latter study also reported the purification of tauricosin, a heterodimeric protein consisting of two subunits with molecular weights of 32 and 9.6 kDa, respectively, which hydrolyzes κ-casein similarly to chymosin and has a more intense proteolytic activity towards β- and α-casein (Foligni et al., 2022). Finally, the impact of crude extracts prepared from flowers of either spontaneous or cultivated *O. tauricum* on the microbial dynamics of pilot-scale raw ewe's milk cheese manufactures was investigated by Rampanti et al., 2023b. In this latter study, Caciofiore was chosen as a model cheese, given its traditional manufacture with crude extracts from spontaneous local thistles (Cardinali et al., 2017).

Caciofiore cheese owes its name to the union of two terms: "cacio", from latin *caseus*, meaning cheese, and "fiore", from latin *flos,* meaning flower. This cheese has deep historical roots, being known since Roman times. Indeed, in 50 A.D., Lucio Giunio Moderato Columella mentions the use of vegetable coagulants obtained from flowers of wild thistles and other essences in his treatise *De re rustica*.

Different regional versions of Caciofiore cheese are currently manufactured in Central Italy using ewe's milk curdled with thistle rennet, including "Caciofiore di Columella" and "Caciofiore Aquilano", manufactured in the Lazio and Abruzzo Regions, respectively. Both these cheeses are included in the national list of "traditional agri-food products" (PAT), whose processing, preservation, and ripening methods are practiced in a given territory, according to traditional rules and consolidated over time for a period of no less than twenty-five years (Renna et al., 2018). In the Marche Region, a further version of Caciofiore is manufactured in the Sibillini Mountains using thistle rennet (Cardinali et al., 2016, 2017). The manufacture of this specialty cheese, known as "Caciofiore della Sibilla", is limited to late summer when flowers of spontaneous local thistles are manually harvested and the stamens macerated in water prior to filtration with a cotton cloth and infusion into the milk (Cardinali et al., 2016, 2017).

The aim of the present study was the chemical, microbiological, textural, and sensory characterization of pilot-scale prototypes of Caciofiore cheese curdled with commercial *C. cardunculus* rennet, used as a control, and crude extracts obtained from flowers of either spontaneous or cultivated *O. tauricum*. Hence, the control and experimental cheese prototypes produced in two rounds of cheesemaking trials were assayed, at the end of their 60-day of maturation, for the following features: pH, titratable acidity, dry matter, fat, total and soluble nitrogen (TN and SN, respectively), ash, salt, protein, lactose, viable plate counts, composition of the bacterial and fungal populations, color, texture, volatile organic compounds (VOCs) by Gas Chromatography-Mass Spectrometry (GC–MS) (for all the prototypes) and GC-olfactometry (GC-O) (for the sole prototypes curdled with the commercial rennet and the extract obtained from flowers of spontaneous *O. tauricum*), and olfactory notes (for the sole prototypes curdled with the commercial rennet and the extract obtained from flowers of cultivated *O. tauricum*). The results overall collected were statistically evaluated to assess the effect of the type of rennet and the cheesemaking round on the analyzed cheese features.

2. Materials and methods

2.1. Cheesemaking and sampling

Experimental and control Caciofiore (CF) cheese prototypes were produced at a family-run dairy farm located in Pieve Torina (MC, Italy) according to a traditional method without any addition of starter cultures. Briefly, after filtration, ewe's raw milk from Sopravissana breed was heated to 37 °C prior to rennet addition; after coagulation (ca. 40 min.), the curd was broken with a wooden whisk until rice-size grains were obtained. Hence, the curd was placed in round perforated plastic molds (diameter 8 cm) and manually pressed to drain the whey. After dry salting, cheese wheels were ripened for 60 days at 12 °C and 70% of relative humidity (RH). Two rounds (R) of cheese-making trials were carried out in July (R1) and September (R2) 2022, respectively. In both rounds, control cheese prototypes (C) were manufactured with commercial *C. cardunculus* rennet (Dairen, Dairy and Food, Castel San Pietro Terme, BO, Italy), whereas in R1 and R2, experimental cheese prototypes were produced with a reconstituted extract prepared from flowers of spontaneous (OtS) and cultivated (OtC) *O. tauricum* thistles, respectively, as previously described by Mozzon et al. (2020).

Flower heads of spontaneous *O. tauricum* thistles were manually harvested from the Sibillini mountains area in July-August 2019, whereas those from cultivated *O. tauricum* thistles were collected in July-August 2020 from the crop produced at the "Pasquale Rosati" experimental farm of the Polytechnic University of Marche in Agugliano (AN), Italy (43°32′ N, 13°22′ E, at an altitude of 100 m above sea level and a slope gradient of 10%) (Zenobi et al., 2021).

For each round, cheesemaking trials were repeated twice using two batches (B) of raw ewe's milk. At the end of their 60-day maturation, the cheese prototypes were transported to the laboratory under refrigeration (+4 °C) and immediately subjected to the analyses described in the following paragraphs.

2.2. Physico-chemical analyses

The pH of each cheese wheel was measured using a pH-meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy). The titratable acidity was determined in 10 g of cheese paste homogenized with 90 mL of deionized water and expressed as % of lactic acid equivalents, as described by Rampanti et al., 2023b. Water activity (a_w) was measured with an AwTherm apparatus (Rotronic, Bassersdorf, Switzerland) on ~ 5 g of grated cheese paste placed in the sample holder supplied with the instrument. Dry matter, fat, total and soluble nitrogen (TN and SN, respectively), and ash content (%) were determined as previously described by Tejada, Abellán, Cayuela, Martínez-Cacha, & Fernández-Salguero, 2008a; Tejada, Abellán, Prados, & Cayuela, 2008b; protein content was estimated by multiplying TN by a factor of 6.38. NaCl concentration (%) was assessed using a salinity meter (LAQUAtwin salt-22, HORIBA, Ltd., Kyoto, Japan). Lactose content was measured using a commercial kit (K-LOLAC, Megazyme, Bray, Ireland) following the manufacturer's instructions. For each of the parameters listed above,

three independent measurements were performed, and the results were reported as mean ± standard deviation.

2.3. Viable plate counting

Ten g of cheese paste was homogenized with 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 (Osimani et al., 2009). Ten-fold dilutions were prepared with the same diluent. Aliquots of the homogenates and ten-fold dilutions were inoculated in duplicate on the opportune growth media for the enumeration by viable plate counting of the following microbial groups: (i) total mesophilic aerobes on Plate Count Agar (PCA) (VWR International, Milan, Italy) incubated at 30 °C for 48 h; (ii) presumptive lactobacilli on de Man, Rogosa, and Sharpe (MRS) agar (VWR) incubated at 30 °C for 48 h; (iii) presumptive mesophilic and thermophilic cocci on M17 agar (Liofilchem, Roseto degli Abruzzi, Italy) incubated at 22 and 45 °C, respectively, for 48 h; (iv) Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBGA) (VWR) incubated at 37 °C for 24 h; (v) coliforms and *Escherichia coli* on Chromogenic Coliform Agar (CCA) (VWR) incubated at 37 °C for 24 h; (vi) yeasts and molds on Rose Bengal (RB) agar (VWR) incubated at 25 °C for 72 h. The results were expressed as the Log of Colony-Forming Units (CFU) per gram of each sample and reported as mean ± standard deviation. Cheese samples were also tested for *Listeria monocytogenes* (AFNOR BIO 12/11–03/04), *Salmonella* spp. (AFNOR BIO 12/16–09/05) and staphylococcal enterotoxins (A, B, C, D, E) (UNI, EN, ISO, 2017).

2.4. Metataxonomic analysis

Cell pellets obtained by centrifugation of 1.5 mL of first decimal dilutions (10^{-1}) were processed using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) for the extraction of the total microbial DNA. The resulting extracts were checked for DNA quantity and purity by Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA), and the DNA was quantified and standardized by using the Qubit dsDNAkits (Thermo Fisher, Monza, Italy).

An amplicon-based sequencing approach was used to analyze the V3-V4 region of the 16S rRNA gene using primers and procedures previously described by Klindworth et al. (2013). The D1-D2 domain of the 26S rRNA gene was amplified according to Mota-Gutierrez et al. (2019). The Illumina metagenomic pipeline was adopted for amplicons purification, indexing, and pooling.

Illumina MiSeq platform with V2 chemistry was used to generate 250-bp paired-end reads, and the raw *fastq* files were analyzed by QIIME2 software (Bolyen et al., 2019). Briefly, primers were removed by Cutapter whereas the DADA2 algorithm was used to denoise the reads using a 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 ten read counts in at least two samples were excluded to increase the confidence of sequence reads. BLASTn tool was used to confirm the taxonomic assignment.

The raw read data were deposited in the Sequence Read Archive of NCBI under the bioproject accession number PRJNA822519.

2.5. Texture

Texture profile analysis (TPA) of cheeses was performed according to Joshi et al. (2004) using a texture analyzer (TA-XT2, Stable Micro Systems, Godalming, Surrey, UK) with a 25 kg force load cell. A double bite compression cycle was carried out with a rest of 5 s between bites on cylindrical specimens (2 cm diameter and 2 cm height) obtained from the cheese samples left at room temperature for 15 min after being removed from the refrigerator. Test conditions were as follows: P/36 probe, 36 mm diameter cylinder; crosshead speed 1.0 mm s^{-1}; and compression ratio of 70% from the specimen original height. For each cheese sample 10 measurements were carried out and the results were reported as mean ± standard deviation.

2.6. Color

Color measurement was performed onto three 1 cm thick slices at 10 superficial points per slice with the reference illuminant D65 (standard daylight) and observer angle of 10°. Color parameters in the CIELab color space, L* (lightness, from 0% to 100%, dark to light), a* (from − 60 to 60, green to red), and b* (from − 60 to 60, blue to yellow) were measured by using a Konica Minolta CM-400 spectrophotometer equipped with SpectraMagic NX VA.9 software (Konica Minolta, Osaka, Japan). The color parameters C* (Chroma) and h° (Hue angle) were calculated as follows: $C^* = (a^*2 + b^*2)^{1/2}$, $h^\circ = \arctan(b^* / a^*)$.

2.7. VOCs quantitative determination

Cheese aliquots (60 g) were collected from each cheese wheel after the cheese rind was discarded by removing a 1 cm slice from the surface. Cheese samples were frozen with liquid nitrogen, quickly grounded into a fine powder using a batch mill (IKA A11, IKA-Werke GmbH & Co. KG, Darmstadt, Germany), and stored at −80 °C until analysis.

Isolation of VOCs was performed by headspace solid-phase microextraction (HS-SPME). In detail, 1.5 g of cheese powder was transferred in a 15 mL vial capped with a PTFE/silicone septum (Supelco, Sigma-Aldrich Italy) for HS-SPME. The extraction was carried out by exposing a 85 μm CAR/PDMS fiber (Supelco, Sigma-Aldrich, Italy) to the headspace of the cheese powder for 60 min, while keeping the extraction temperature at 30 °C with a water bath. Prior to extraction, each fiber was preconditioned for 10 min in the injector port of the gas-chromatograph at 240 °C. At the end of the extraction, the fiber was immediately inserted into the GC split-splitless injection port, for the desorption step, and the GC run was started. The same fiber was used for all the analyses. A triplicate extraction of each cheese sample was carried out.

GC–MS analyses were performed with an Agilent 6890 GC 5973 N MS system equipped with a quadrupole mass filter for mass spectrometric detection (Agilent Technologies, Santa Clara, CA). Extracted volatiles were desorbed from the fiber for 5 min within the GC injector provided with a 0.75 mm glass liner suitable for SPME and operating at 240 °C in the splitless mode. Then, the fiber was kept within the injector for 7 additional min, in purge mode (75 mL min^{-1} purge flow), to remove residual substances adsorbed on the fiber, so to minimize carryover effects. Carryover and peaks arising from the SPME fiber were checked by frequently running blank samples.

A DB-Wax column (0.25 mm i.d. × 60 m, 0.5 μm film thickness) (J &W Agilent Technologies - Santa Clara, CA) was used for VOCs separation. The following operating conditions were applied: injector temperature of 240 °C and oven temperature from 40 °C (2 min) to 240 °C (10 min) at 6 °C min^{-1} (total run time of 45 min). Helium was used as carrier gas at a constant flow of 1.5 mL min^{-1} corresponding to a linear velocity of 31 cm s^{-1}. The MS detector operated in the electron ionization mode at 70 eV; transfer line, source, and quadrupole temperatures were set at 250, 230, and 150 °C, respectively. Detection was performed in the full scan mode, over the mass range 33–350 amu, for identification purposes.

Identification of compounds was carried out by comparing mass spectra and linear retention indices (LRI) of cheese VOCs with spectra and retention indices of authentic standards (Merck Life Science S.r.l., Milan, Italy). The list of compounds for which an authentic standard was used for identification is given in Table S1 (Supplementary material). When authentic standards were not available, tentative identification was accomplished by comparison with information reported in the NIST/EPA/NIH EI Mass Spectral Library (Version 2.4, 2020) or in the

literature (Table S1). A standard solution of linear alkanes (C7–C30) was run under the same chromatographic conditions as the samples to determine the LRI of the detected compounds.

Concentration levels of cheese VOCs were expressed as the equivalent concentration of a suitable standard. In detail, for the semi-quantitative determination of each compound, the ratio between the peak area of chromatographic signals (signal of the Total Ion Current or of an appropriate extracted ion) of the target analyte and the peak area of the most appropriate external standard was calculated, and the concentration of the targeted analyte (mg kg^{-1}) was calculated according to the following equation:

$[C_n] = \frac{Area_n}{Area_S} \times [S]$.

where $[C_n]$ is the concentration (mg kg^{-1}) of the targeted compound, [S] is the concentration (mg kg^{-1}) of the external standard in a solution of pure compounds in deodorized olive oil, *n* is the target compound, and S is the external standard. For each target compound, the most appropriate external standard was that one which gave rise to the most repeatable ratio of peak areas (Table S1).

For GC-O analysis, isolation of VOCs from cheese samples was carried out by the same HS-SPME conditions, except for exposure time that was set at 16 h (overnight), at ambient temperature (~22 °C) according to the method proposed by Frank et al. (2004). GC-MS-O analyses were performed on an Agilent 6890 GC 5973 N MS system (Agilent Technologies, Palo Alto, CA) and an Olfactory Detector Port ODP 3 (Gerstel, Mülheim an der Ruhr, Germany). GC separation was performed by a DB-Wax column, under the same conditions as above, and also by a DB1-MS column (0.25 mm i.d. × 60 m, 0.5 μm film thickness) (J &W Agilent Technologies – Santa Clara, CA), under the following conditions: injector temperature of 240 °C; oven temperature from 40 °C (2 min) to 170 °C at 4 °C min^{-1}, then to 280 °C (6 min) at 20 °C min^{-1} (total run time of 46 min). Helium was used as carrier gas at a constant flow of 1.5 mL min^{-1} corresponding to a linear velocity of 31 cm s^{-1}. The effluent carrier gas was split 1:1 into the MS detector and the ODP 3 port using a column flow splitter . The MS detector was set as described above. The ODP 3 settings were: transfer line and mixing chamber at 250 °C and 160 °C, respectively, with N_2 as makeup gas. The olfactory analyses were carried out by two experienced sniffers (Raffo et al., 2018, and 2020), who had been trained by analysis of standard pure compounds (purchased from Merck Life Science S.r.l., Milan, Italy) and samples collected from different cheese types. One sample of Caciofiore cheese curdled with the experimental rennet (OtS) and one sample manufactured with the commercial vegetable coagulant (C1), both produced at the first round of cheesemaking, were analyzed in triplicate by each sniffer. Identification of odorants was carried out by comparing mass spectra, linear retention indices (LRI), and odor quality at the sniffing port as determined on cheese samples, with spectra, retention indices, and odor quality obtained from standard pure compounds when available.

2.8. Olfactory attributes

Cheese odor was analyzed following the general guidance described in the International Organization for Standardization (ISO) standards for sensory profiling and detection and recognition of food odors (International Organization for Standardization, 2016 and 2018) on the sole prototypes curdled with the commercial rennet and the extract obtained from cultivated *O. tauricum*. The evaluation panel was formed by six expert assessors with more than 20 years of experience in sensory testing on several food products, including cheese and, especially, ewe's milk cheese (Mughetti et al., 2012). Trained assessors referred to a list of twenty olfactory descriptors that they selected and defined during two preliminary analysis sessions. The intensity of descriptors was measured on an unstructured linear scale (150 mm, anchors described) with values between 0 and 9 corresponding to "absent" and "intense", respectively. Data were collected by an automatic system for the acquisition of the assessors' scores (FIZZ, Biosystems, France). Cheeses identified with a three-digit code were evaluated in individual sensory booths under white light. For the evaluation, each assessor received cheese cubs of 1 cm side, obtained from 1 cm thick slices. Cheese samples were analyzed under two different test conditions: (i) maintaining of the cheese samples at room temperature (20 ± 2 °C); (ii) warming of the cheese samples to 36 ± 2 °C and crumbling with a fork to promote the release of volatile compounds. While the first mode of sample presentation corresponds to that commonly adopted, the second mode was selected to evaluate cheese odor under conditions comparable to those occurring in the mouth during chewing to promote the release of aroma compounds. Cheese samples were analyzed in monadic order balanced across the assessors to avoid carryover effects. For each cheese, two analytical replicates obtained from the same cheese wheel were analyzed by the panel.

2.9. Statistical analysis

The Tukey-Kramer's Honest Significant Difference (HSD) test ($\alpha = 0.05$) was carried out to evaluate differences within cheese samples by one-way ANOVA using the software JMP® Version 11.0.0 (SAS Institute Inc., Cary, NC). A Principal Component Analysis (PCA) was performed on VOCs profile data: the dataset used for PCA was obtained by first averaging replicate determinations and then applying auto-scaling (standard deviation, n-1), as pre-treatment. PCA was performed by the XL Stat (ver. 2020.1.1; Addinsoft, New York, NY) software package.

Alpha and beta diversity indices of bacterial and fungal populations were calculated through the diversity script of QIIME2. Differences between alpha and beta diversity parameters and ASVs frequency were analyzed by non-parametric Kruskall-Wallis test in R environment.

Pairwise Spearman's non-parametric correlations performed by the psyc package of R were used to study the relationships between the microbiota (bacteria and fungi) and VOCs.

3. Results

3.1. Physico-chemical features

The results of the physico-chemical analyses are reported in Table 1. Briefly, dry matter (DM) ranged between 67.99 ± 1.58 (C2.2) and 71.82 ± 0.33 (C1.2) g 100 g^{-1} of cheese, with overall mean values attesting at 70.56 ± 1.40 (C1) *vs* 70.38 ± 0.58 (OtS) g 100 g^{-1} of cheese and 69.36 ± 1.86 (C2) *vs* 71.02 ± 0.55 (OtC) g 100 g^{-1} of cheese, in R1 and R2 cheeses, respectively.

Fat content was in the range 37.20 ± 4.29 (C2.2) and 51.67 ± 1.13 (OtS2), with overall mean values attesting at 45.10 ± 3.82 (C1) *vs* 48.43 ± 3.69 (OtS) and at 41.86 ± 6.01 (C2) *vs* 47.22 ± 3.32 (OtC) g 100 g^{-1} of DM in R1 and R2 cheeses, respectively.

Ash content was comprised between 7.29 ± 0.11 (C1.1) and 8.48 ± 0.02 (OtC1) g 100 g^{-1} of DM, with overall mean values attesting at 7.44 ± 0.21 (C1) *vs* 7.68 ± 0.49 (OtS) and at 7.67 ± 0.30 (C2) *vs* 8.15 ± 0.37 (OtC) g 100 g^{-1} of DM in R1 and R2 cheeses, respectively.

As for protein content (g 100 g^{-1} DM), mean values attesting at 43.40 ± 3.95 (C1) *vs* 42.52 ± 1.44 (OtS) and at 44.75 ± 1.02 (C2) *vs* 43.24 ± 1.07 (OtC) g 100 g^{-1} of DM were found in R1 and R2 cheeses, respectively.

As revealed by one-way ANOVA, no significant differences ($P < 0.05$) were seen in DM, fat, ash, and protein content of the cheese prototypes, depending on either the type of thistle rennet used or the round of cheesemaking trials.

By contrast, SN/TN% was significantly lower (about 5%) in the experimental than the control cheese prototypes irrespective of the cheesemaking round, with mean values attesting at 20.44 ± 1.28 (C1), 15.45 ± 2.20 (OtS), 18.28 ± 1.22 (C2), and 13.39 ± 1.83 (OtC) , respectively, whereas for salt content, the cheesemaking round affected the differences found among the cheese prototypes ($P < 0.05$), with

Table 1
Physico-chemical parameters of Caciofiore cheese prototypes.

R	B	Sample	Dry matter[1]	Fat[2]	Protein[2]	SN/TN%	Ash[2]	Salt[1]	a_w	pH	Titratable acidity [3]
1	1	C1.1	69.30 ± 0.18	42.72 ± 3.25	45.98 ± 4.35	19.67 ± 1.44	7.29 ± 0.11	1.93 ± 0.15	0.909 ± 0.02	5.11 ± 0.02	1.00 ± 0.04
	2	C1.2	71.82 ± 0.33	47.48 ± 2.97	40.82 ± 0.19	21.22 ± 0.47	7.59 ± 0.17	1.88 ± 0.12	0.899 ± 0.00	5.10 ± 0.03	1.06 ± 0.01
		mean C1	70.56 ± 1.40	45.10 ± 3.82	43.40 ± 3.95	20.44 ± 1.28[a]	7.44 ± 0.21	1.88 ± 0.13[b]	0.904 ± 0.01	5.11 ± 0.02	1.03 ± 0.05[a]
	1	OtS1	69.92 ± 0.43	45.19 ± 1.12	42.15 ± 0.96	13.61 ± 0.14	8.12 ± 0.10	2.10 ± 0.20	0.897 ± 0.00	5.06 ± 0.03	0.78 ± 0.03
	2	OtS2	70.84 ± 0.18	51.67 ± 1.13	42.88 ± 1.97	17.29 ± 1.38	7.23 ± 0.03	1.77 ± 0.06	0.905 ± 0.01	5.06 ± 0.09	0.82 ± 0.02
		mean OtS	70.38 ± 0.58	48.43 ± 3.69	42.52 ± 1.44	15.45 ± 2.20[b]	7.68 ± 0.49	1.93 ± 0.23[b]	0.901 ± 0.01	5.06 ± 0.06	0.80 ± 0.03[b]
2	3	C2.1	70.74 ± 0.68	46.52 ± 2.60	43.96 ± 0.38	17.23 ± 0.43	7.89 ± 0.18	2.27 ± 0.15	0.896 ± 0.00	4.99 ± 0.03	0.97 ± 0.02
	4	C2.2	67.99 ± 1.58	37.20 ± 4.29	45.55 ± 0.74	19.33 ± 0.51	7.45 ± 0.20	2.10 ± 0.26	0.915 ± 0.01	5.18 ± 0.03	0.98 ± 0.03
		mean C2	69.36 ± 1.86	41.86 ± 6.01	44.75 ± 1.02	18.28 ± 1.22[a]	7.67 ± 0.30	2.18 ± 0.21[a, b]	0.906 ± 0.01	5.08 ± 0.11	0.98 ± 0.03[a]
	3	OtC1	71.08 ± 0.44	48.94 ± 2.89	43.29 ± 0.82	11.72 ± 0.16	8.48 ± 0.02	2.57 ± 0.15	0.886 ± 0.00	5.09 ± 0.02	0.81 ± 0.03
	4	OtC2	70.95 ± 0.74	45.49 ± 3.21	43.19 ± 1.48	15.06 ± 0.22	7.83 ± 0.14	2.20 ± 0.17	0.909 ± 0.01	5.09 ± 0.02	0.82 ± 0.04
		mean OtC	71.02 ± 0.55	47.22 ± 3.32	43.24 ± 1.07	13.39 ± 1.83[b]	8.15 ± 0.37	2.38 ± 0.25[a]	0.898 ± 0.01	5.09 ± 0.01	0.81 ± 0.03[b]

The results are expressed as mean ± standard deviation of three independent measurements. Within each column, overall mean values with different superscript letters are significantly different ($P < 0.05$).
R Round of cheesemaking trials.
B Batch of ewe's raw milk.
SN /TN% soluble nitrogen/total nitrogen %
C1.1 Control cheese prototype manufactured in round 1 with milk batch 1 and commercial *C. cardunculus* rennet.
C1.2 Control cheese prototype manufactured in round 1 with milk batch 2 and commercial *C. cardunculus* rennet.
OtS1 Experimental cheese prototype manufactured in round 1 with milk batch 1 and the crude extract from flowers of spontaneous *O. tauricum*.
OtS2 Experimental cheese prototype manufactured in round 1 with milk batch 2 and the crude extract from flowers of spontaneous *O. tauricum*.
C2.1 Control cheese prototype manufactured in round 2 with milk batch 3 and commercial *C. cardunculus* rennet.
C2.2 Control cheese prototype manufactured in round 2 with milk batch 4 and commercial *C. cardunculus* rennet.
OtC1 Experimental cheese prototype manufactured in round 2 with milk batch 3 and the crude extract from flowers of cultivated *O. tauricum*.
OtC2 Experimental cheese prototype manufactured in round 2 with milk batch 4 and the extract from flowers of cultivated *O. tauricum*.
[1] expressed as g 100 g^{-1} of cheese.
[2] expressed as g 100 g^{-1} of dry matter.
[3] expressed as % of lactic acid equivalents.

overall mean values attesting at 1.88 ± 0.13 (C1), 1.93 ± 0.23 (OtS), 2.18 ± 0.21(C2), and 2.38 ± 0.25 (OtC) g 100 g^{-1} of cheese, respectively.

A_w ranged between 0.886 and 0.915, whereas mean pH values attesting at 5.11 ± 0.02 (C1) *vs* 5.06 ± 0.06 (OtS), and at 5.08 ± 0.11 (C2) *vs* 5.09 ± 0.01 (OtC), were found in R1 and R2 cheeses, respectively, with no statistical differences found among the cheese prototypes depending on either the type of rennet or the cheesemaking round. By contrast, analogously to SN/TN% , TTA of the control cheese prototypes curdled with commercial thistle rennet was higher than that of the cheese prototypes curdled with crude extracts from flowers of either spontaneous or cultivated *O. tauricum*, irrespective of the cheesemaking round.

Finally, all the cheese prototypes were characterized by a lactose content < 0.01 g 100 g^{-1} of cheese.

3.2. Viable plate counts

Table 2 shows the results of viable plate counting. Loads of total mesophilic aerobes ranged from 7.90 ± 0.04 (OtS1) to 8.68 ± 0.03 (OtS2) Log CFU g^{-1}, with overall mean values attesting at 8.33 ± 0.39 (C1) *vs* 8.28 ± 0.44 (OtS) and at 8.44 ± 0.16 (C2) *vs* 8.12 ± 0.12 (OtC) Log CFU g^{-1} in R1 and R2 cheeses, respectively.

For presumptive lactobacilli, viable counts were comprised between 7.72 ± 0.07 (OtS1) and 8.61 ± 0.13 (C2.2) Log CFU g^{-1}, with R2 cheeses showing significantly higher overall mean counts than R1 cheeses, irrespective of the type of thistle rennet used.

No statistically significant differences were seen in the load of presumptive lactococci among pilot cheeses, irrespective of the type of thistle rennet used and the cheesemaking round or batch of milk, with mean viable counts attesting at 8.02 ± 0.61 (C1), 8.03 ± 0.50 (OtS), 8.20 ± 0.15 (C2), and 7.86 ± 0.14 (OtC) Log CFU g^{-1}.

Regarding presumptive thermophilic cocci, R2 cheeses showed a significantly higher load of this microbial group than R1 cheeses, with these latter showing mean viable counts of 6.99 ± 0.18 (C1) and 7.07 ± 0.10 (OtS). A significant difference between C2 and OtC cheeses was also seen, with mean viable counts attesting at 8.16 ± 0.16 and 7.73 ± 0.12 Log CFU g^{-1}, respectively.

No statistically significant differences were seen among the cheese prototypes for both Enterobacteriaceae and coliforms, whereas a significantly higher load of *Escherichia coli* was seen in R2 than R1 cheeses, with no effect of the type of thistle rennet assayed on the level of these microorganisms. Similarly, no statistically significant differences were seen for eumycetes, with viable counts ranging from 2.43 ± 0.02

Table 2
Viable counts (expressed as Log cfu g^{-1} ± standard deviation of duplicate independent experiments) of Caciofiore cheese prototypes.

R	B	Sample	Total mesophilic aerobes	Presumptive lactobacilli	Presumptive lactococci	Presumptive thermophilic lactococci	Enterobacteriaceae	Coliforms	*Escherichia coli*	Molds	Yeasts
1	1	C1.1	7.99 ± 0.02	7.85 ± 0.07	7.49 ± 0.02	6.96 ± 0.30	5.00 ± 0.12	4.24 ± 0.09	5.05 ± 0.01	2.94 ± 0.01	4.32 ± 0.02
	2	C1.2	8.68 ± 0.03	7.96 ± 0.13	8.54 ± 0.02	7.02 ± 0.09	4.98 ± 0.09	< 1	4.88 ± 0.05	2.84 ± 0.02	3.86 ± 0.00
		mean C1	8.33 ± 0.39	7.91 ± 0.11[b]	8.02 ± 0.61	6.99 ± 0.18[c]	4.99 ± 0.09	2.12 ± 2.45	4.97 ± 0.11[b]	2.89 ± 0.06	4.09 ± 0.26
	1	OtS1	7.90 ± 0.04	7.72 ± 0.07	7.61 ± 0.05	7.05 ± 0.16	4.90 ± 0.01	4.44 ± 0.06	4.93 ± 0.07	3.26 ± 0.02	4.69 ± 0.03
	2	OtS2	8.66 ± 0.06	7.88 ± 0.04	8.46 ± 0.13	7.10 ± 0.04	5.22 ± 0.08	< 1	4.86 ± 0.06	2.49 ± 0.02	3.53 ± 0.08
		mean OtS	8.28 ± 0.44	7.80 ± 0.10[b]	8.03 ± 0.50	7.07 ± 0.10[c]	5.06 ± 0.19	2.22 ± 2.56	4.89 ± 0.07[b]	2.88 ± 0.45	4.11 ± 0.67
2	3	C2.1	8.55 ± 0.07	8.27 ± 0.04	8.09 ± 0.09	8.12 ± 0.15	6.08 ± 0.18	< 1	6.25 ± 0.08	2.45 ± 0.02	4.76 ± 0.07
	4	C2.2	8.33 ± 0.14	8.61 ± 0.13	8.31 ± 0.13	8.21 ± 0.23	4.95 ± 0.01	4.84 ± 0.00	5.28 ± 0.17	2.54 ± 0.05	4.18 ± 0.03
		mean C2	8.44 ± 0.16	8.44 ± 0.21[a]	8.20 ± 0.15	8.16 ± 0.16[a]	5.51 ± 0.66	2.42 ± 2.80	5.77 ± 0.77[a]	2.50 ± 0.06	4.47 ± 0.34
	3	OtC1	8.02 ± 0.04	8.26 ± 0.02	7.74 ± 0.03	7.65 ± 0.11	5.82 ± 0.19	< 1	5.93 ± 0.10	2.43 ± 0.02	4.87 ± 0.03
	4	OtC2	8.23 ± 0.02	8.27 ± 0.02	7.98 ± 0.05	7.82 ± 0.04	5.13 ± 0.09	4.50 ± 0.02	5.64 ± 0.01	2.55 ± 0.03	4.23 ± 0.02
		mean OtC	8.12 ± 0.12	8.27 ± 0.02[a]	7.86 ± 0.14	7.73 ± 0.12[b]	5.48 ± 0.42	2.25 ± 2.60	5.78 ± 0.18[a]	2.49 ± 0.07	4.55 ± 0.37

Within each column, overall mean values with different superscript letters are significantly different ($P < 0.05$).
R Round of cheesemaking trials.
B Batch of ewe's raw milk.
For legend of samples see Table 1 for reference.

(OtC1) to 2.94 ± 0.01 (C1.1) Log CFU g^{-1} for molds, and from 3.53 ± 0.08 to 4.87 ± 0.03 Log CFU g^{-1} for yeasts.

Finally, *L. monocytogenes*, *Salmonella* spp., and staphylococcal enterotoxins were never detected.

3.3. Metataxonomic analysis

A total of 125.440 denoised reads (15.960 reads on average per sample) for bacteria and 427.000 (53.375 reads on average per sample) for fungi were analyzed, with a coverage greater than 99%. Alpha and beta diversity did not show any significant difference among the cheese prototypes based on the type of thistle rennet. The results of the metataxonomic analysis of bacterial DNA are shown in Fig. 1. A neat predominance of *Lactococcus lactis* was seen in all the cheese prototypes, with relative frequencies ranging from 55.72 to 82.93 %, irrespective of the round of cheesemaking, the batch of raw milk, and the type of thistle rennet. This species was followed by *Lactiplantibacillus plantarum*, again detected in all the cheese prototypes at a relative frequency ranging from 11.37 to 30.78%. Within lactic acid bacteria, minority taxa were also found, including *Lacticaseibacillus zeae* (from 0.22 to 3.40 % of the relative frequency), *Levilactobacillus brevis* (from 0.33 to 1.95 % of the relative frequency), *Leuconostoc mesenteroides* (from 0.01 to 4.60 % of the relative frequency), *Latilactobacillus sakei* (from 0.07 to 0.61% of the relative frequency), *Enterococcus* spp. (from 0.54 to 5.98 % of the relative frequency), and *Weissella* spp. (from 0.22 to 1.81 % of the relative frequency). Potential pathogens as well as spoilage or adventitious microorganisms were also sporadically detected at very low relative frequencies, including *Aerococcus* spp., *Serratia* spp., *Staphylococcus sciuri*, *Staphylococcus aureus*, *Staphylococcus equorum*, and *Staphylococcus succinus*.

Fig. 2 shows the results of metataxonomic analysis of fungal DNA. As a rule, no significant differences were seen in the composition of the core mycobiota of the control and experimental cheeses, irrespective of the round of cheesemaking, the batch of milk, and the type of thistle rennet. In detail, a neat predominance of *Galactomyces* spp. (from 0.23 to 52.67 % of the relative frequency), *Debaryomyces hansenii* (from 0.04 to 44.21 % of the relative frequency) and *Kluyveromyces marxianus* (from 0.43 to 85.56 % of the relative frequency) was observed, followed by *Geotrichum* spp. (from 0.12 to 30.20 % of the relative frequency), *Saccharomyces cerevisiae* (from 0.02 to 11.54 % of the relative frequency), and *Kazachstania unispora* (from 0.02 to 8.88 % of the relative frequencies). A plethora of taxa including species ascribed to *Pichia* (*P. cactophila*, *P. ethanolica*, *P. fermentans*, and *P. kudriavzevii*), *Candida* (*C. parapsilosis* and *C. sake*), *Kurtzmaniella zeylanoides*, *Wickerhamiella pararugosa*, and *Penicillium* spp. were also detected at various relative frequencies in most cheeses. Beyond the microorganisms listed above, minority taxa were sporadically detected at very low relative frequencies, being *Yarrowia lipolytica*, *Rhodotorula mucilaginosa*, *Magnusiomyces capitatus*, *Filobasidium magnus*, *Diutina catenulata*, *Clavispora lusitaniae*, *Clavispora intermedia*, *Alternaria* spp., *Aureobasidium proteae*, *Cladosporium* spp., *Cutaneotrichosporon curvatus,* and *Torulaspora quercuum*.

3.4. Color and texture

The results of instrumental measurement of color and texture are shown in Table 3. Regarding the first parameter, the cheese prototypes curdled with crude extracts from either spontaneous or cultivated *O. tauricum* differed significantly from those curdled with commercial thistle rennet. More specifically, the experimental cheese prototypes (OtS1, OtS2, OtC1, and OtC2) were consistently darker than the control ones (C1.1, C1.2, C2.1, and C2.2), as suggested by lower L^* mean values. The experimental cheese prototypes were also less green and more yellow than the control prototypes, as suggested by less negative a^* and higher b^* mean values, respectively. If the cheese prototypes curdled

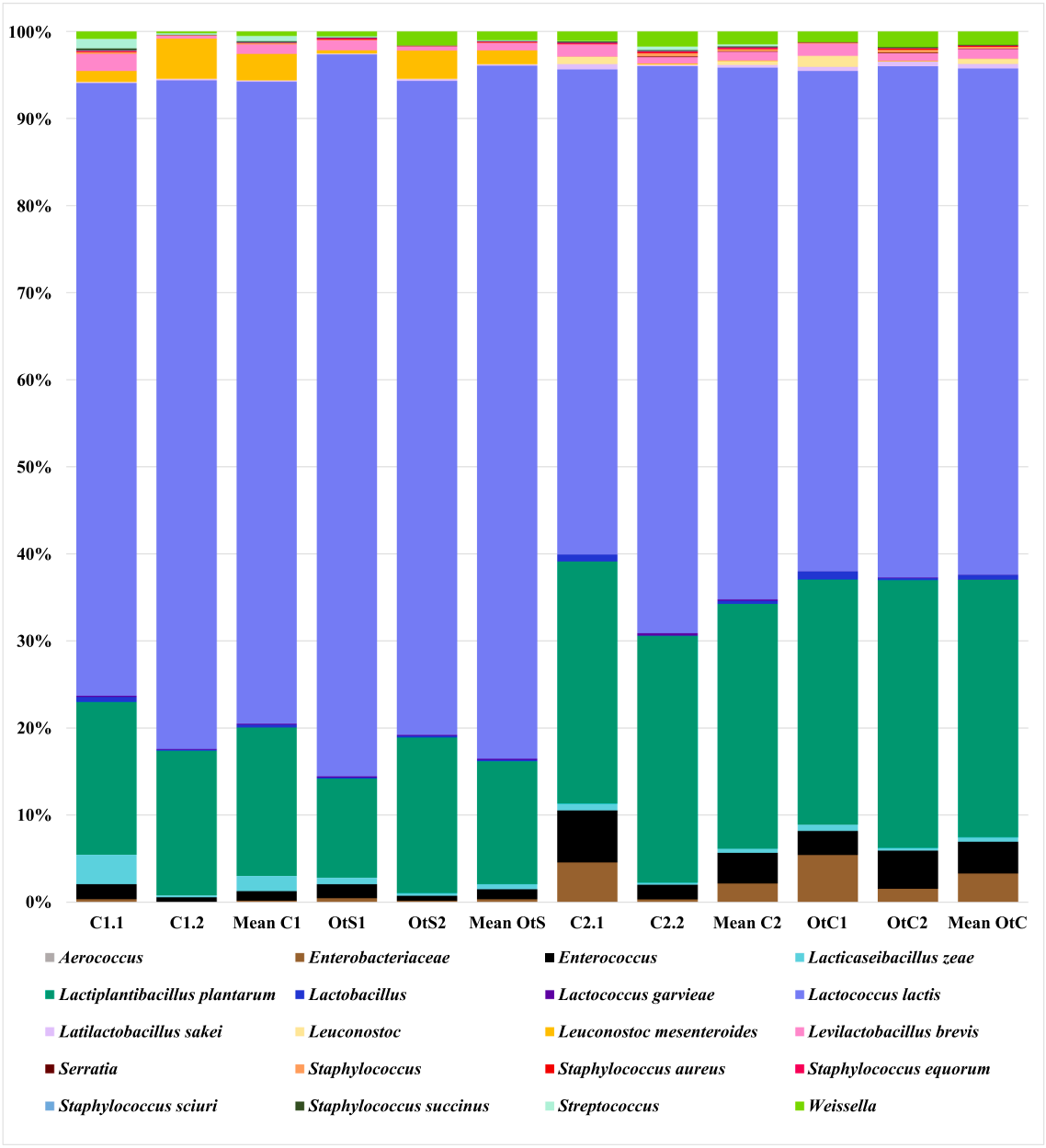

Fig. 1. Relative frequency of the bacterial ASVs detected by Illumina sequencing in Caciofiore cheese prototypes. Only ASVs with an incidence above 1% in at least two samples are shown. For legend of samples see Table 1 for reference.

with spontaneous and cultivated *O. tauricum* were comparatively evaluated, the first group was characterized by less negative *a** , more positive b* , and more positive C* mean values than the second group.

Regarding texture, a significant rennet-related effect was neatly seen for adhesiveness, with the control cheese prototypes showing a higher intensity (indicated by more negative values) than the prototypes curdled with *O. tauricum* (either spontaneous or cultivated). For some other textural parameters, such as hardness, fracturability, springiness, cohesiveness, gumminess, and chewiness, the differences found could not be directly related to either the type of thistle rennet used or the cheesemaking round. Finally, for cohesiveness and chewiness, no significant differences emerged among the cheese prototypes. If the sole prototypes made in the second round of cheesemaking trials are concerned, the control prototypes showed lower values for hardness, fracturability, springiness, and gumminess than the prototypes curdled with *O. tauricum*.

3.5. VOCs

Thirty-one volatile compounds were identified in the control and experimental cheeses; they belonged to the chemical classes of alcohols (9 compounds), carboxylic acids (7), esters (7), ketones (5), sulfur compounds (2), and aldehydes (1) (Table S1).

The one-way ANOVA of the VOCs dataset highlighted the occurrence of some significant differences between the cheese prototypes made in the two cheesemaking rounds (Table 4), with levels of 2,3-butanedione (diacetyl), 2-heptanone, 2-heptanol, 2-nonanone, and butanoic acid being significantly higher in R1 than R2 cheeses and, conversely, with levels of 2-pentanone, 1-butanol, 3-methyl-1-butanol, dimethyl trisulfide, and branched-chain fatty acids (2-methyl propanoic and 3-methyl butanoic acid) being significantly lower in R1 than R2 cheeses. By contrast, no differences were seen among the cheese prototypes, depending on the type of thistle rennet used.

Fig. 3 shows the biplot merging the score plot and loadings plot of the

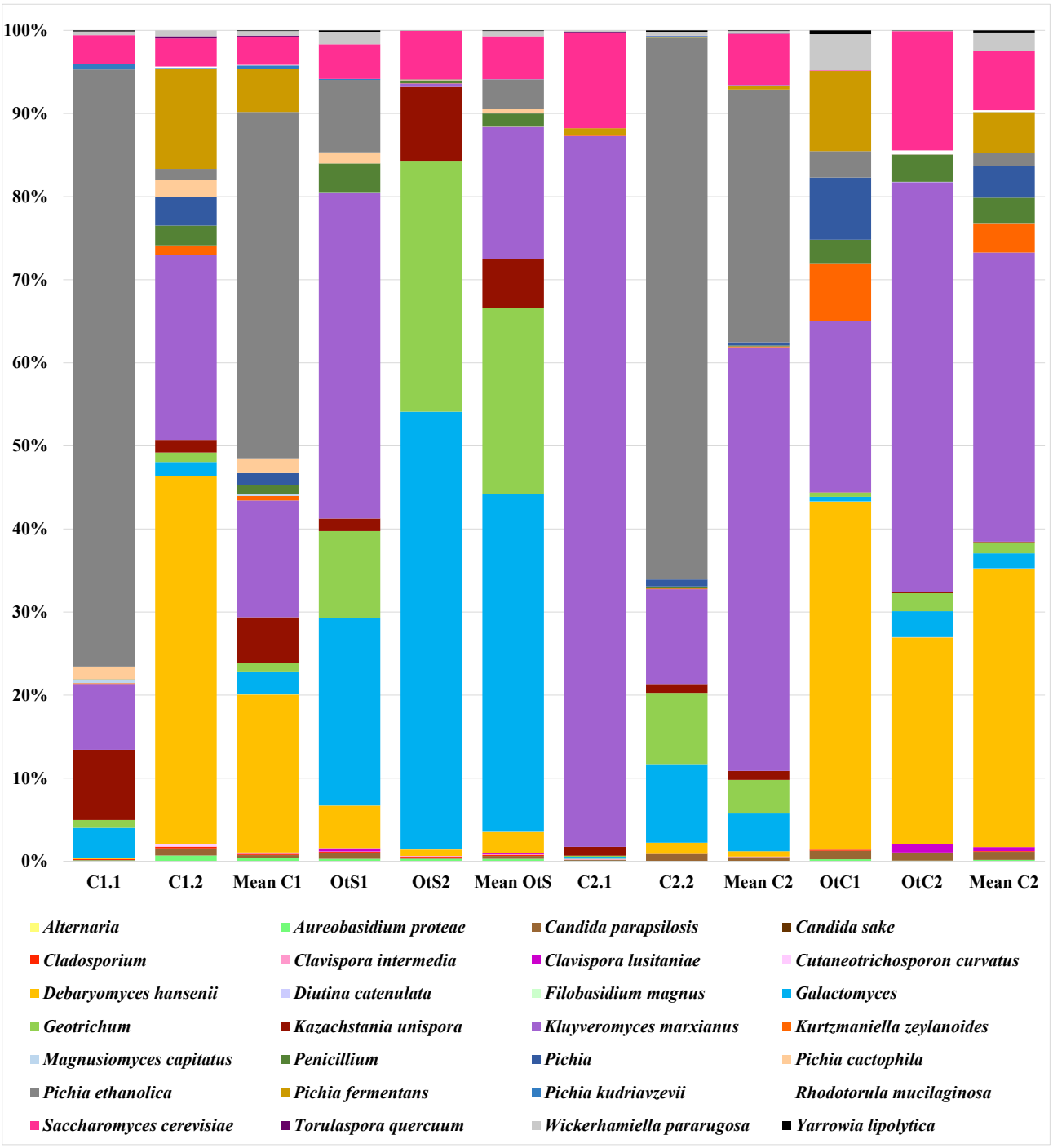

Fig. 2. Relative frequency of the fungal ASVs detected by Illumina sequencing in Caciofiore cheese prototypes. Only ASVs with an incidence above 1% in at least two samples are shown. For legend of samples see Table 1 for reference.

PCA performed onto VOCs quantitative data. PC1 and PC2 explained 42 and 28% of the total variance, respectively, with PC1 neatly separating the cheese prototypes produced in the first cheesemaking round (C1.1, C1.2, OtS1, and OtS2, all characterized by negative scores) from those manufactured in the second cheesemaking round (C2.1, C2.2, OtC1, and OtC2, all characterized by positive scores). Among R2 cheese prototypes, PC2 separated those curdled with the commercial rennet (C2.2, C2.1, both characterized by higher positive scores) from those curdled with the crude extract obtained from cultivated *O. tauricum* (OtC1 and OtC2, both characterized by lower positive scores). By contrast, for R1 cheese prototypes, a higher effect of the milk batch rather than the type of rennet was observed, based on PC2 scores.

Moreover, based on the PCA biplot graph, the formation of ethyl esters and acetic esters was apparently associated, similarly to the formation of branched chain fatty acids and the corresponding alcohols and esters, whereas the formation of linear ketones (2-pentanone, 2-heptanone and 2-nonanone) was apparently associated to that of butanoic acid.

Figure S1 (Supplementary material) shows the results of correlation analysis between the ASVs and VOCs. A direct association between *Lc. lactis* and 2-nonanone and butanoic acid was observed; *L. plantarum* was positively associated with 1-butanol, 2 methyl propanoic acid, and

Table 3
Color and texture parameters of Caciofiore cheese prototypes.

R	B	Sample	Color parameters					Texture parameters						
			*L**	*a**	*b**	*C**	*h*°	*Hardness (N)*	*Fracturability (N)*	*Adhesiveness*	*Springiness*	*Cohesiveness*	*Gumminess (N)*	*Chewiness (N)*
1	1	C1.1	73.1 ± 2.6	−2.5 ± 0.1	18.0 ± 1.1	18.2 ± 1.1	97.9 ± 0.6	85.7 ± 12.9	40.9 ± 7.5	−1.1 ± 0.7	0.3 ± 0.1	0.1 ± 0.0	10.7 ± 1.7	3.5 ± 1.4
	2	C1.2	70.3 ± 3.8	−2.1 ± 0.3	16.9 ± 1.1	17.0 ± 1.1	97.3 ± 1.1	74.2 ± 9.9	39.2 ± 3.5	−1.3 ± 0.8	0.2 ± 0.0	0.1 ± 0.0	8.6 ± 1.8	2.1 ± 0.7
		mean C1	71.7 ± 3.5^{a,b}	−2.3 ± 0.3^{c}	17.4 ± 1.2^{c}	17.6 ± 1.2^{c}	97.5 ± 0.9^{a}	80.0 ± 12.7^{a,b}	40.0 ± 5.8^{a}	−1.2 ± 0.7^{b}	0.3 ± 0.1^{b}	0.1 ± 0.0	9.6 ± 2.0^{b}	2.8 ± 1.3^{b}
	1	OtS1	68.7 ± 2.9	−0.8 ± 0.2	19.9 ± 0.9	19.9 ± 0.9	92.3 ± 0.7	79.9 ± 8.2	40.1 ± 3.8	−0.4 ± 0.5	0.3 ± 0.1	0.1 ± 0.0	10.2 ± 1.8	3.6 ± 1.0
	2	OtS2	68.4 ± 3.3	−0.2 ± 0.2	22.3 ± 0.9	22.3 ± 0.9	90.6 ± 0.5	70.8 ± 12.4	40.2 ± 8.0	−0.5 ± 0.6	0.3 ± 0.0	0.1 ± 0.0	8.5 ± 1.8	2.4 ± 0.6
		mean OtS	68.6 ± 3.0^{b}	−0.5 ± 0.3^{a}	21.1 ± 1.5^{a}	21.2 ± 1.5^{a}	91.4 ± 1.0^{c}	75.3 ± 11.2^{b}	40.2 ± 6.1^{a}	−0.4 ± 0.6^{a}	0.3 ± 0.1^{b}	0.1 ± 0.0	9.4 ± 1.9^{b}	3.0 ± 1.0^{b}
2	1	C2.1	76.2 ± 3.4	−2.0 ± 0.2	18.1 ± 1.5	18.2 ± 1.5	96.4 ± 0.5	68.5 ± 8.3	33.6 ± 3.8	−1.5 ± 1.0	0.3 ± 0.2	0.1 ± 0.0	8.3 ± 1.3	2.7 ± 1.4
	2	C2.2	73.2 ± 5.3	−2.8 ± 0.3	18.2 ± 1.6	18.4 ± 1.6	98.7 ± 1.4	62.8 ± 11.3	29.8 ± 5.5	−0.9 ± 0.9	0.2 ± 0.0	0.1 ± 0.0	6.6 ± 1.6	1.6 ± 0.5
		mean C2	74.7 ± 4.7^{a}	−2.4 ± 0.4^{c}	18.1 ± 1.6^{c}	18.3 ± 1.5^{c}	97.6 ± 1.5^{a}	65.5 ± 10.2^{c}	31.6 ± 5.0^{b}	−1.2 ± 1.0^{b}	0.3 ± 0.1^{b}	0.1 ± 0.0	7.4 ± 1.7^{c}	2.1 ± 1.1^{b}
	1	OtC1	72.1 ± 3.4	−1.6 ± 0.2	18.6 ± 1.4	18.6 ± 1.4	95.1 ± 0.6	87.1 ± 4.6	46.5 ± 3.5	−0.9 ± 0.6	0.4 ± 0.1	0.1 ± 0.0	11.7 ± 1.1	5.0 ± 1.3
	2	OtC2	68.7 ± 4.2	−1.3 ± 0.2	20.0 ± 1.0	20.0 ± 1.0	93.9 ± 0.6	85.8 ± 15.7	41.2 ± 6.4	−0.2 ± 0.4	0.4 ± 0.1	0.1 ± 0.0	12.0 ± 3.6	4.8 ± 2.1
		mean OtC	70.4 ± 4.2^{b}	−1.5 ± 0.3^{b}	19.3 ± 1.4^{b}	19.3 ± 1.4^{b}	94.5 ± 0.9^{b}	86.4 ± 11.6^{a}	43.7 ± 5.8^{a}	−0.5 ± 0.6^{a}	0.4 ± 0.1^{a}	0.1 ± 0.0	11.8 ± 2.7^{a}	4.9 ± 1.8^{a}

Values of color and texture parameters are expressed as mean ± standard deviation. Within each column, overall mean values with different superscript letters are significantly different ($P < 0.05$).
R Round of cheesemaking trials.
B Batch of ewe's raw milk.
For legend of samples see Table 1 for reference.

dimethyl trisulfide; *L. brevis* with hexanal; *Enterococcus* spp. with 2-methyl propanoic acid, 3-methyl butanoic acid, dimethyl trisulfide, and ethyl-3-methyl butanoate; and *Leuc. mesenteroides* with 2-nonanone, 2-pentanone, and butanoic acid. Regarding fungi, a positive correlation between *Penicillium* spp. and hexanoic acid and octanoic acid was found, as well as *K. unispora* with 2-propanol, *Geotricum* spp. and *Galactomyces* spp. with 2-propen-1-ol, ethanol, ethyl acetate, and ethyl butanoate.

By GC-O qualitative determinations it was also possible to identify some of the key odorants of the Caciofiore cheese manufactures produced at the first round of cheesemaking using the commercial rennet and the crude extract obtained from flowers of spontaneous *O. tauricum*.

Twenty odorant areas were detected and for twelve of them, it was possible to associate the corresponding aroma compound (Table 5). Common cheese odorants were identified: ethyl butanoate, pentanoate and hexanoate, among the fruity ethyl esters, butanoic acid among carboxylic acids, the buttery 2,3-butanedione, along with hexanal (green odor note), 2-heptanone (blue cheese note), 3-methyl-1-butanol (solvent, glue odor), 1-octen-3-one (mushroom odor note), dimethyl trisulfide (garlic odor), acetic acid (vinegar odor), and 2-nonenal (solvent, acrid odor). Some of these compounds, such as ethyl pentanoate, 1-octen-3-one, and 2-nonenal, were not determined by the semi-quantitative method used for the analysis of VOCs (Table 4), because they were not detected in the cheese prototypes by the MS detector. This was consistent with their presence at a relatively low level. However, these compounds are characterized by a very low odor threshold, thus it was possible to detect their presence by GC-O analysis, and this result suggested their significant contribution to the global cheese odor. It was not possible to identify the odorant compound for eight odorant areas that were preliminarily characterized by odor note and retention index. Most of the odorant areas were detected in both the experimental and control cheese prototypes herein assayed, with differences observed only for two unknown odorants (no. 2 and 12).

3.6. Odor attributes

A list of twenty odor sensory attributes was developed to accurately describe the odor sensory profile of Caciofiore cheese during preliminary analyses by the sensory panel, and these attributes were measured on the control and experimental cheese prototypes produced in the second round of cheesemaking trials (Table 6). These attributes were measured by the panelists under two different conditions for the maintenance of the cheese samples: at ambient temperature and at 36 °C after crumbling the cheese specimen with a fork. Being not possible to evaluate odor attributes during chewing, and hence their contribution to cheese flavor, due to a high load of Enterobacteriaceae, the second mode of cheese odor measurement was developed to provide information on cheese odor as it develops in the mouth during chewing of cheese. The odor of the assayed cheeses was dominated by an ewe's cheese note, followed by pungent, sour curd, sweet, raw milk, cheese rind, and Parmesan cheese-like notes. The remaining odor attributes were perceived at a relatively low intensity level. Some odor notes, such as fatty, sweet, and cheese rind, tended to be perceived at a higher intensity when the cheese was evaluated at 36 °C rather than room temperature, whereas the opposite was observed for the raw milk note. The ANOVA

Table 4
Volatile organic compounds (VOCs) level (expressed as equivalent concentration mg kg^{-1} of a suitable standard) of Caciofiore cheese prototypes.

N°	Compound	C1.1	C1.2	**mean C1**	OtS1	OtS2	**mean OtS**	C2.1	C2.2	**mean C2**	OtC1	OtC2	**mean OtC**
1	Ethyl acetate	15.3 ± 1.3	49.3 ± 5.5	32.3 ± 19.0	21.1 ± 1.3	64.4 ± 5.6	42.8 ± 24.0	31.9 ± 2.7	14.7 ± 0.9	23.3 ± 9.6	36.8 ± 1.6	46.2 ± 2.4	41.5 ± 5.4
2	2-Butanone	41.6 ± 1.9	26.8 ± 3.5	34.2 ± $8.5^{a,b}$	35.8 ± 3.2	55.9 ± 3.0	45.9 ± 11.3^{a}	38.3 ± 1.7	31.0 ± 1.0	34.6 ± $4.2^{a,b}$	31.1 ± 0.0	13.8 ± 0.3	22.4 ± 9.5^{b}
3	2-Propanol	21.3 ± 3.6	6.8 ± 1.7	14.1 ± 8.3^{a}	4.6 ± 1.4	7.8 ± 1.2	6.2 ± 2.1^{b}	6.5 ± 0.8	6.5 ± 0.3	6.5 ± 0.6^{b}	4.9 ± 0.8	5.0 ± 0.2	4.9 ± 0.5^{b}
4	Ethanol	90.2 ± 5.2	106.1 ± 10.3	98.1 ± $11.4^{a,b}$	89.0 ± 6.2	133.7 ± 6.8	111.4 ± 25.2^{a}	97.6 ± 2.8	66.2 ± 1.2	81.9 ± 17.3^{b}	109.6 ± 2.2	110.0 ± 2.0	109.8 ± 1.9^{a}
5	n-Propyl acetate	0.9 ± 0.1	1.6 ± 0.1	1.3 ± 0.4^{c}	1.3 ± 0.1	2.5 ± 0.1	1.9 ± $0.7^{b,c}$	3.7 ± 0.2	2.2 ± 0.2	3.0 ± $0.8^{a,b}$	5.7 ± 0.1	2.9 ± 0.2	4.3 ± 1.6^{a}
6	2,3-Butanedione	29.7 ± 2.9	10.8 ± 1.7	20.3 ± 10.5^{a}	27.1 ± 3.7	5.6 ± 0.9	16.3 ± $12^{a,b}$	7.7 ± 1.3	5.4 ± 0.4	6.6 ± 1.5^{b}	3.9 ± 0.3	3.9 ± 0.4	3.9 ± 0.3^{b}
7	2-Pentanone	3.7 ± 0.1	3.4 ± 0.2	3.6 ± 0.2^{b}	4.3 ± 0.1	4.2 ± 0.1	4.3 ± 0.1^{a}	1.4 ± 0.1	1.1 ± 0.0	1.2 ± 0.2^{c}	0.9 ± 0.0	1.6 ± 0.1	1.3 ± 0.4^{c}
8	*sec*-Butylacetate	2.5 ± 0.1	7.2 ± 0.6	4.9 ± 2.6	3.1 ± 0.3	9.6 ± 0.5	6.4 ± 3.6	4.6 ± 0.1	2.2 ± 0.1	3.4 ± 1.3	4.3 ± 0.3	4.5 ± 0.3	4.4 ± 0.3
9	2-Butanol	566 ± 26.6	637.3 ± 44.1	601.6 ± 50.9	535.1 ± 26.4	795.8 ± 29.9	665.4 ± 145	659.9 ± 2.8	653.3 ± 8.2	656.6 ± 6.6	667.3 ± 7.4	517.7 ± 2.2	592.5 ± 82.1
10	Ethyl butanoate	5.2 ± 0.2	8.9 ± 0.4	7.1 ± $2.1^{a,b}$	5.7 ± 0.2	13.7 ± 0.5	9.7 ± 4.4^{a}	4.2 ± 0.0	2.9 ± 0.2	3.5 ± 0.7^{b}	5.3 ± 0.1	6.6 ± 0.4	6.0 ± $0.8^{a,b}$
11	Ethyl-3-methyl butanoate	1.2 ± 0.1	1.1 ± 0.1	1.1 ± $0.1^{b,c}$	0.8 ± 0.1	0.6 ± 0.0	0.7 ± 0.1^{c}	2.6 ± 0.1	1.7 ± 0.2	2.1 ± 0.5^{a}	1.3 ± 0.0	1.5 ± 0.1	1.4 ± 0.1^{b}
12	Dimethyl disulfide	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{b}	0.0 ± 0.0	0.6 ± 0.1	0.3 ± 0.4^{a}	0.2 ± 0.0	0.3 ± 0.1	0.3 ± $0.1^{a,b}$	0.2 ± 0.1	0.2 ± 0.0	0.2 ± $0.1^{a,b}$
13	Hexanal	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.5 ± 0.3	0.7 ± 0.4	0.6 ± 0.3	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
14	2-Methyl-1-propanol	4.9 ± 0.4	0.3 ± 0.0	2.6 ± 2.6^{b}	5.0 ± 0.3	0.2 ± 0.0	2.6 ± 2.6^{b}	5.4 ± 0.1	10.8 ± 0.5	8.1 ± 3.0^{a}	2.6 ± 0.1	8.1 ± 0.2	5.4 ± $3.0^{a,b}$
15	2-Propen-1-ol	0.9 ± 0.1	0.8 ± 0.1	0.9 ± $0.1^{a,b}$	0.8 ± 0.1	2.0 ± 0.1	1.4 ± 0.7^{a}	0.5 ± 0.1	0.7 ± 0.0	0.6 ± 0.1^{b}	0.9 ± 0.1	0.8 ± 0.0	0.9 ± $0.1^{a,b}$
16	2-Pentanol	40.1 ± 5.2	4.0 ± 0.1	22.1 ± 20.1	44.3 ± 2.2	3.6 ± 0.3	23.9 ± 22.3	25.5 ± 2.7	37.5 ± 0.7	31.5 ± 6.8	23.6 ± 2.2	29.8 ± 1.1	26.7 ± 3.7
17	Isoamyl acetate	10.2 ± 0.8	13.3 ± 1.9	11.7 ± 2.1	10.0 ± 0.8	18.5 ± 1.6	14.2 ± 4.8	15.0 ± 2.1	23.2 ± 3.0	19.1 ± 5.1	9.1 ± 1.3	24.6 ± 3.1	16.9 ± 8.7
18	1-Butanol	1.8 ± 0.1	0.3 ± 0.0	1.1 ± 0.8^{b}	1.8 ± 0.1	0.4 ± 0.0	1.1 ± 0.8^{b}	4.3 ± 0.2	3.7 ± 0.1	4.0 ± 0.4^{a}	4.8 ± 0.2	2.8 ± 0.1	3.8 ± 1.1^{a}
19	2-Heptanone	2.0 ± 0.1	3.5 ± 0.3	2.7 ± 0.8^{a}	2.6 ± 0.1	2.4 ± 0.4	2.5 ± 0.2^{a}	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1^{b}	0.6 ± 0.1	1.1 ± 0.1	0.8 ± 0.3^{b}
20	3-Methyl-1-butanol	95.6 ± 5.8	6.3 ± 0.2	51.0 ± 49.0^{b}	99.9 ± 5.0	5.3 ± 0.1	52.6 ± 51.9^{b}	93.5 ± 1.9	157.3 ± 2.5	125.4 ± 35^{a}	54.2 ± 0.9	128.0 ± 3.2	91.1 ± $40.5^{a,b}$
21	Ethyl hexanoate	0.7 ± 0.0	1.2 ± 0.0	0.9 ± 0.2	0.7 ± 0.0	2.6 ± 0.6	1.7 ± 1.1	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	2.3 ± 0.2	1.7 ± 0.6
22	2-Heptanol	3.0 ± 0.1	4.2 ± 0.1	3.6 ± 0.7^{a}	3.1 ± 0.2	3.1 ± 0.4	3.1 ± 0.3^{a}	1.5 ± 0.3	2.8 ± 0.2	2.1 ± 0.7^{b}	1.5 ± 0.2	1.6 ± 0.2	1.6 ± 0.2^{b}
23	2-Nonanone	2.9 ± 0.4	4.1 ± 1.0	3.5 ± 1.0^{a}	4.4 ± 0.7	3.0 ± 0.4	3.7 ± 0.9^{a}	1.1 ± 0.3	1.1 ± 0.1	1.1 ± 0.2^{b}	2.0 ± 0.2	1.7 ± 0.3	1.8 ± 0.3^{b}
24	Dimethyl trisulfide	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{b}	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0^{a}
25	Acetic acid	374.2 ± 21.0	360.6 ± 8.6	367.4 ± 16.2^{b}	411.1 ± 69.6	445.7 ± 26.7	428.4 ± 50.8^{b}	380.3 ± 34.0	403.5 ± 14.9	391.9 ± 26.7^{b}	463.2 ± 43.5	585.9 ± 38.1	524.5 ± 76.5^{a}
26	Propanoic acid	1.8 ± 0.2	0.8 ± 0.2	1.3 ± 0.5^{b}	1.4 ± 0.2	1.8 ± 0.3	1.6 ± 0.3^{b}	3.1 ± 0.2	3.6 ± 0.3	3.3 ± 0.3^{a}	1.5 ± 0.3	2.2 ± 0.2	1.8 ± 0.4^{b}
27	2-Methyl propanoic acid	2.3 ± 0.1	2.0 ± 0.2	2.2 ± 0.2^{c}	1.9 ± 0.2	1.1 ± 0.1	1.5 ± 0.4^{c}	5.2 ± 0.2	6.8 ± 0.7	6.0 ± 1.0^{a}	3.7 ± 0.5	5.4 ± 0.6	4.5 ± 1.0^{b}
28	Butanoic acid	78.0 ± 5.2	78.3 ± 3.8	78.2 ± 4.1^{a}	83.5 ± 13.0	74.1 ± 2.9	78.8 ± 9.8^{a}	58.0 ± 1.7	64.1 ± 4.7	61 ± 4.6^{b}	63.4 ± 8.4	69.3 ± 5.4	66.4 ± 7.1^{b}
29	3-Methyl butanoic acid	7.5 ± 0.7	4.5 ± 0.5	6.0 ± $1.7^{b,c}$	6.0 ± 1.0	1.9 ± 0.1	3.9 ± 2.3^{c}	14.6 ± 0.4	15.3 ± 1.6	14.9 ± 1.1^{a}	6.8 ± 1.3	8.6 ± 0.9	7.7 ± 1.4^{b}
30	Hexanoic acid	15.9 ± 1.5	21.9 ± 3.1	18.9 ± 3.9	18.1 ± 2.6	20.8 ± 1.5	19.4 ± 2.4	17.7 ± 1.4	22.2 ± 2.0	20.0 ± 2.9	19.2 ± 2.1	25.3 ± 1.9	22.2 ± 3.8
31	Octanoic acid	1.7 ± 0.2	2.7 ± 0.4	2.2 ± 0.6^{b}	2.1 ± 0.2	2.7 ± 0.4	2.4 ± $0.4^{a,b}$	1.8 ± 0.1	3.1 ± 0.2	2.5 ± $0.7^{a,b}$	2.6 ± 0.1	4.5 ± 0.4	3.6 ± 1.1^{a}

Values are expressed as mean ± standard deviation. Within each row, overall mean values with different superscript letters are significantly different ($P < 0.05$). For legend of samples see Table 1 for reference.

analysis highlighted that the type of rennet had an almost negligible influence on the intensity of the perceived odor attributes, with 1 (cheese rind) or 2 (fruity and Parmesan cheese) out of twenty odor attributes significantly differing, depending on the conditions applied prior to the analysis.

4. Discussion

In the Mediterranean area, since ancient civilizations, flowers from spontaneous thistles are macerated in water for the preparation of extracts to be exploited in cheesemaking. However, in recent years, the need to respond to a growing consumer demand for thistle curdled cheeses and, hence, to standardize the cheesemaking process with thistle

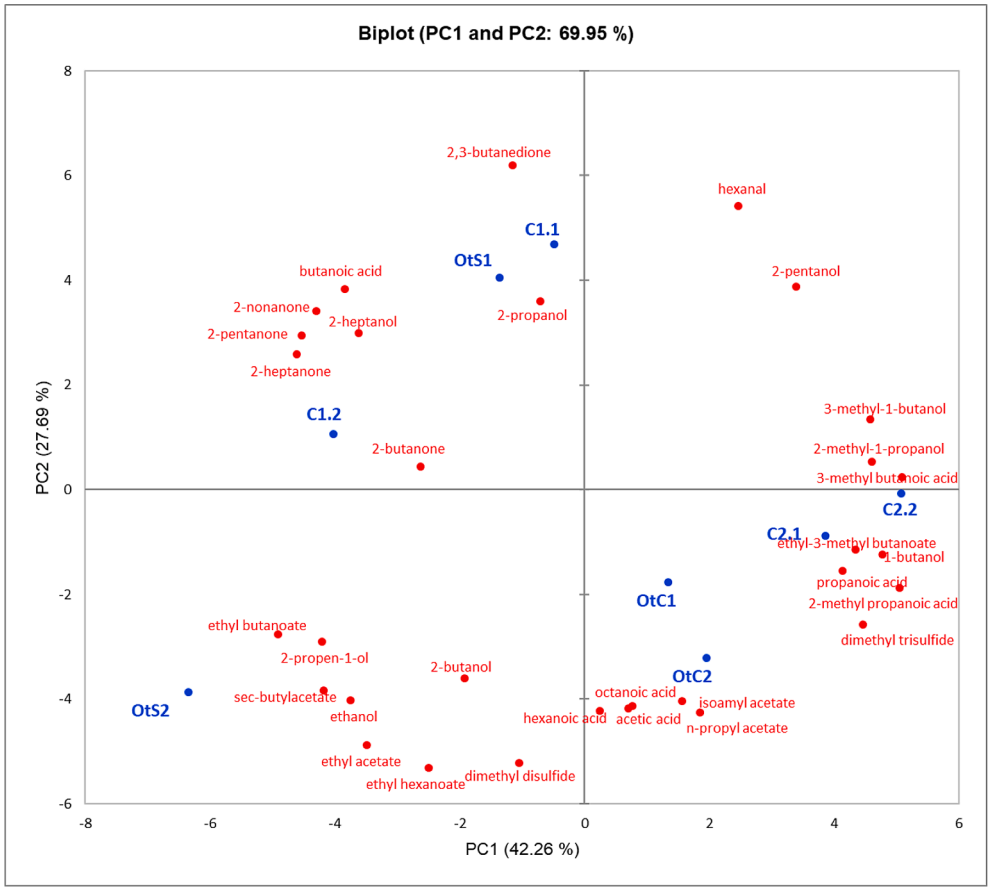

Fig. 3. Principal Component (PC) analysis of volatile organic compounds (VOCs) levels in Caciofiore cheeses curdled with thistle rennet obtained from flowers of spontaneous and cultivated *Onopordum tauricum* and a commercial coagulant extracted from flowers of *Cynara cardunculus*. Biplot of the first two PCs. Red labels represent the volatile compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Key aroma compounds identified by GC-olfactometry in Caciofiore cheese prototypes manufactured in the first round of cheesemaking trials.

N°	Retention index		Compound	Odor impression	Identification method	Chese prototype	
	DB-Wax	DB-1 MS				C1	OtS
1	983	507	2,3-butanedione	buttery	RI, MS, odor	×	×
2	1018	–	unknown	vegetable, harsh	–	×	
3	1032	–	unknown	sweaty, harsh	–	×	×
4	1047	770	ethyl butanoate	fruity, apple	RI, MS, odor	×	×
5	1076	–	unknown	sweet	–	×	×
6	1098	772	hexanal	green	RI, MS, odor	×	×
7	1112	–	unknown	cardboard, fatty	–	×	×
8	1142	–	ethyl pentanoate	fruity, sweet, melon	RI, odor	×	×
9	1201	800	2-heptanone	blue cheese	RI, MS, odor	×	×
10	1220	720	3-methyl-1-butanol	glue, solvent	RI, MS, odor	×	×
11	1248	967	ethyl hexanoate	fruity, pineapple	RI, MS, odor	×	×
12	1255	–	unknown	sweaty, fatty	–		×
13	1318	943	1-octen-3-one	mushroom	RI, odor	×	×
14	1374	–	unknown	fatty, French fries	–	×	×
15	1390	–	unknown	cucumber, fatty	–	×	×
16	1427	934	dimethyl trisulfide	garlic	RI, MS, odor	×	×
17	1461	640	acetic acid	vinegar	RI, MS, odor	×	×
18	1459	–	unknown	sour curd	–	×	×
19	1572	1127	2-nonenal	solvent, acrid	RI, odor	×	×
20	1646	787	butanoic acid	ewe's cheese, sweaty	RI, MS, odor	×	×

Compound identification based on retention index (RI), mass spectrum (MS) or odor evaluation (odor) at the GC-O port.
C1 Control cheese prototypes manufactured in round 1 with commercial *C. cardunculus* rennet.
OtS Experimental cheese prototypes manufactured in round 1 with the extract from flowers of spontaneous *O. tauricum*.

rennet, especially by modern, large-scale cheese processors (Ordiales et al., 2013), has stimulated the research efforts towards the cultivation of *C. cardunculus* for biomass production (Gominho et al., 2011).

Given this premise, the present study summarizes some outputs of the recently concluded EU funded project VEGGIE-MED-CHEESES (https://veggiemedcheeses.com/), which was aimed at demonstrating

Table 6
Olfactory attributes of Caciofiore cheese prototypes, as measured by sensory analysis.

N°	Olfactory attribute	Room temperature						36 °C					
		C2.1	C2.2	Mean C2	OtC1	OtC2	Mean OtC	C2.1	C2.2	Mean C2	OtC1	OtC2	Mean OtC
1	Global odour	7.2 ± 0.7	6.8 ± 0.6	7.0 ± 0.7	7.5 ± 0.5	6.6 ± 0.5	7.0 ± 0.7	7.1 ± 0.5	6.8 ± 0.6	6.9 ± 0.6	7.1 ± 0.4	7.0 ± 0.4	7.0 ± 0.4
2	Ewe's cheese	6.7 ± 0.7	5.5 ± 0.5	6.0 ± 0.9	6.7 ± 0.5	5.4 ± 0.4	6.0 ± 0.8	6.5 ± 0.8	5.6 ± 0.5	6.0 ± 0.8	6.4 ± 0.5	5.9 ± 0.4	6.1 ± 0.5
3	Vegetable	0.9 ± 0.7	1.2 ± 0.5	1.0 ± 0.6	0.8 ± 0.5	0.8 ± 0.6	0.8 ± 0.5	0.8 ± 0.7	0.8 ± 1.1	0.8 ± 0.9	0.4 ± 0.7	0.6 ± 0.6	0.5 ± 0.6
4	Raw milk	1.1 ± 0.5	1.6 ± 0.6	1.4 ± 0.6	0.5 ± 0.7	1.2 ± 0.7	0.9 ± 0.7	0.3 ± 0.5	0.9 ± 0.8	0.6 ± 0.7	0.4 ± 0.5	0.8 ± 0.7	0.6 ± 0.6
5	Fatty	0.9 ± 0.8	1.0 ± 0.5	0.9 ± 0.6	1.0 ± 0.5	0.9 ± 0.6	1.0 ± 0.5	2.0 ± 0.8	2.0 ± 0.3	2.0 ± 0.6	2.3 ± 0.6	1.8 ± 0.7	2.0 ± 0.7
6	Sour curd	3.4 ± 0.5	2.7 ± 0.6	3.0 ± 0.7	4.1 ± 0.5	2.6 ± 0.5	3.2 ± 0.9	3.1 ± 0.6	3.1 ± 0.6	3.1 ± 0.6	3.2 ± 0.3	2.7 ± 0.4	2.9 ± 0.5
7	Pungent	4.9 ± 0.7	3.4 ± 0.6	4.1 ± 1.0	5.5 ± 0.9	2.8 ± 0.6	4.0 ± 1.5	4.1 ± 0.5	3.3 ± 0.5	3.7 ± 0.6	4.7 ± 0.5	3.0 ± 0.3	3.8 ± 0.9
8	Sweet	2.1 ± 0.5	2.3 ± 0.4	2.2 ± 0.5	1.7 ± 0.5	2.0 ± 0.4	1.9 ± 0.5	2.4 ± 0.6	2.6 ± 0.5	2.5 ± 0.5	2.2 ± 0.6	2.0 ± 0.4	2.1 ± 0.5
9	Fruity	0.8 ± 0.7	1.0 ± 0.8	0.9 ± 0.7	0.6 ± 1.3	0.9 ± 0.5	0.8 ± 0.9	0.6 ± 0.7	0.9 ± 1.1	0.8 ± 0.9^{A}	0.4 ± 0.8	0.3 ± 0.6	0.4 ± 0.7^{B}
10	Citrus fruit	0.0 ± 0.0	0.5 ± 0.9	0.3 ± 0.7	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0	0.5 ± 1.1	0.3 ± 0.8	0.0 ± 0.0	0.2 ± 0.4	0.1 ± 0.3
11	Cut grass	0.3 ± 0.7	0.4 ± 0.7	0.3 ± 0.6	0.0 ± 0.1	0.3 ± 0.4	0.2 ± 0.3	0.1 ± 0.3	0.2 ± 0.6	0.1 ± 0.5	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.1
12	Mushroom	0.9 ± 0.9	0.5 ± 0.7	0.7 ± 0.8	1.1 ± 0.5	0.9 ± 0.9	1.0 ± 0.8	0.6 ± 0.6	0.7 ± 0.9	0.6 ± 0.8	0.6 ± 0.6	0.5 ± 0.8	0.6 ± 0.7
13	Hazelnut	1.0 ± 0.6	0.9 ± 0.5	1.0 ± 0.6	0.3 ± 0.4	1.2 ± 0.4	0.8 ± 0.6	1.4 ± 0.5	0.9 ± 0.7	1.1 ± 0.7	0.9 ± 0.8	1.3 ± 0.5	1.1 ± 0.7
14	Cocoa	0.4 ± 0.5	0.1 ± 0.3	0.2 ± 0.4	0.1 ± 0.1	0.2 ± 0.5	0.2 ± 0.4	0.4 ± 0.5	0.1 ± 0.3	0.2 ± 0.4	0.2 ± 0.6	0.5 ± 0.8	0.4 ± 0.7
15	Cookie	0.0 ± 0.0	0.2 ± 0.3	0.1 ± 0.2	0.1 ± 0.3	0.3 ± 0.5	0.2 ± 0.4	0.4 ± 1.0	0.3 ± 0.6	0.3 ± 0.8	0.0 ± 0.0	0.4 ± 0.5	0.2 ± 0.4
16	Bread crust	0.4 ± 0.5	0.2 ± 0.4	0.3 ± 0.4	0.3 ± 0.4	0.5 ± 1.1	0.4 ± 0.8	0.7 ± 0.8	0.8 ± 0.7	0.7 ± 0.7	0.7 ± 0.7	1.0 ± 1.0	0.9 ± 0.8
17	Parmesan cheese	2.1 ± 0.4	1.8 ± 0.5	2.0 ± 0.5	2.3 ± 0.7	1.7 ± 0.5	2.0 ± 0.7	2.6 ± 0.5	2.3 ± 0.4	2.4 ± 0.4^{A}	2.1 ± 0.3	1.9 ± 0.4	2.0 ± 0.3^{B}
18	Smoked	0.3 ± 1.0	0.3 ± 0.7	0.3 ± 0.8	0.6 ± 0.7	0.8 ± 1.3	0.7 ± 1.0	0.5 ± 1.3	0.5 ± 0.8	0.5 ± 1.0	0.4 ± 0.9	0.7 ± 1.3	0.6 ± 1.1
19	Mold	0.5 ± 0.5	0.2 ± 0.4	0.3 ± 0.5	0.2 ± 0.6	0.3 ± 0.6	0.2 ± 0.6	0.3 ± 0.5	0.1 ± 0.4	0.2 ± 0.4	0.2 ± 0.5	0.1 ± 0.2	0.1 ± 0.4
20	Cheese rind	0.3 ± 0.6	0.2 ± 0.3	0.3 ± 0.4^{b}	0.8 ± 0.4	0.9 ± 0.8	0.8 ± 0.6^{a}	1.2 ± 1.0	0.8 ± 0.7	1.0 ± 0.9	2.1 ± 0.7	1.2 ± 0.4	1.6 ± 0.7

Values are expressed as overall mean ± standard deviation. Within each row and temperature condition, overall mean values with different superscript letters (in lower and upper case for room temperature and 36 °C, respectively) are significantly different ($P < 0.05$).
For legend of samples see Table 1 for reference.

the exploitability of underused thistle species, including *O. tauricum*, for the manufacturing of Mediterranean ovine cheeses. In such a project, spontaneous thistle populations growing in marginal Mediterranean areas were sampled at different growth stages (flowering and seed production). In summer 2019, stamens from flowers harvested from the spontaneous thistle populations were macerated in water according to a traditional procedure; hence, the resulting crude extracts (including OtS) were thoroughly analyzed for the assessment of their milk clotting activity and chemical properties (Mozzon et al., 2020). In summer 2021, these crude extracts were exploited in a first round of pilot-scale cheesemaking trials aimed at manufacturing Mediterranean ewe's milk cheeses, including Caciofiore. In the meanwhile, seeds collected from the spontaneous thistle populations under study underwent germinations tests, followed by transplantation of seedlings with 3–4 true leaves into demo fields in Italy and Tunisia for production of thistle crops (Zenobi et al., 2021). In summer 2022, the crude extracts (including OtC) obtained from flowers of these new crops were assayed in a second round of cheesemaking trials aimed at manufacturing the same Mediterranean cheeses previously produced with extracts from flowers of spontaneous thistles. The experimental design described above was basically based on: (i) the progressive availability of thistle flowers from spontaneous and, later on, cultivated thistles, spanning from summer 2019 to summer 2022; (ii) the low number of available capitula from both the spontaneous and cultivated thistle populations, due to on the one hand the need to protect the Mediterranean floral species under study and, on the one other hand, to the low number of available plants at the demo field, respectively.

The results herein presented refer to the characterization of the thistle curdled Caciofiore cheese prototypes manufactured from summer 2021 to summer 2022 in two rounds of cheesemaking trials, aimed at: for round 1, preliminary demonstrating the exploitability of spontaneous *O. tauricum* thistles as a source of thistle rennet for the manufacturing of Caciofiore cheese, compared to *C. cardunculus* rennet; and, for round 2, demonstrating the exploitability of cultivated *O. tauricum* thistles as a source of thistle rennet for the production of Caciofiore cheese, again compared to *C. cardunculus* rennet.

To this end, an interdisciplinary approach was adopted for the chemical, microbiological, textural, and sensory characterization of the control and experimental Caciofiore cheese prototypes.

Regarding water content (100 - DM%), at the end of their 60 day-maturation, all the cheese prototypes herein analyzed could be classified as hard cheeses.

Mean values of pH and titratable acidity of the cheese prototypes were comparable to those previously found by Rampanti et al., 2023b in other thistle curdled Caciofiore cheese manufactures. For pH, no significant differences were found among the cheese prototypes, whereas

for titratable acidity, significantly higher mean values were seen in the control cheese prototypes than those curdled with the two *O. tauricum* crude extracts. Titratable acidity and pH are both measures of acidity, with pH reflecting the concentration of hydrogen ions that are free in solution and not associated with salts or proteins and titratable acidity measuring free hydrogen ions plus hydrogen ions associated with organic acids and proteins.

Hence, titratable acidity can be intended as a measurement of milk or whey buffer capacity as well as of acid development due to the activity of lactic acid bacteria.

The discrepancies herein observed between titratable acidity and pH might likely be ascribed to differences in the acid dissociation constant (pKa) and thus in the buffering capacities of the acids produced by the different strains dominating in the control rather than the experimental cheese prototypes.

For pH values, those recorded in the cheese prototypes herein analyzed fell in the range of 5.0 – 5.3, which is recognized as the optimum for most hard cheeses (Fox et al., 2017a). A proper acid development is fundamental for the driving of the cheesemaking process, since it affects multiple reactions, including: the solubilization of colloidal calcium phosphate, which in turn has an impact on the level of calcium in the cheese curd and hence the ratio of soluble to colloidal calcium (these latter influencing texture and functionality of cheese); the activity of the coagulant during cheese manufacture and ripening; the syneresis and, therefore, the moisture of cheese; the activity of several enzymes, which in tun affecs cheese flavor and quality; the growth of spoilage microorganisms and pathogens.

For dry matter and fat content, the data herein collected were comparable to those found by Centi et al. (2017) in 60-day ripened Pecorino Abruzzese cheese. Even protein content was equivalent to that measured by Manca et al. (2023) in Fiore Sardo PDO cheese (an ewe's milk cheese curdled with animal rennet) and by Tofalo et al. (2015) in Pecorino di Farindola cheese (a further traditional Italian ewe's milk cheese curdled with animal rennet), respectively. However, slightly higher values than those reported for other Italian ewe's milk cheeses (Aquilanti et al., 2013; Di Cagno et al., 2007) were observed, likely due to the high protein content of ewe's raw milk from Sopravissana breed, attesting at ~ 6 % (Boselli et al., 2014). Though fat and protein are recognized as the most variable compounds in milk, from both a qualitative and quantitative point of view, as a consequence of season, feeding, genotype, breed, and lactation stage (Nayik et al., 2022), in the present study no significant differences were seen in the cheese prototypes made with different batches of Sopravissana ewe's raw milk.

For SN/TN%, in both rounds of cheesemaking trials herein carried out, the prototypes curdled with crude extracts of *O. tauricum* (either spontaneous or cultivated) showed a lower ratio of soluble nitrogen (SN) to total nitrogen (TN) than the prototypes curdled with the commercial *C. cardunculus* rennet. The SN/TN% ratio is traditionally considered a "ripening index" for measuring the proteolysis extent (Galán et al., 2012); it is particularly useful for comparing cheeses curdled with animal and vegetable rennet. As a previously reported trend, milk coagulation with crude extracts from *C. cardunculus* flowers leads to a higher cheese SN content compared to animal rennet (Fernández-Salguero & Sanjuán, 1999; Galán et al., 2008, 2012; Tejada et al., 2008b). Given this premise, the data herein collected seem to suggest a greater proteolytic activity and a broader specificity of *C. cardunculus* proteases on caseins compared with *O. tauricum* proteases. To date, aspartic proteases with milk clotting activity from either *C. cardunculus* (cardosin A, cardosin B, cyprosin 1, 2 and 3) (Folgado & Abranches, 2020; Shah et al., 2014) or *O. tauricum* (tauricosin) (Foligni et al., 2022; Mozzon et al., 2020) have been widely investigated; however, no data are currently available on a direct comparison between caseinolytic proteases from these two thistle species.

Regarding salt content, mean values of the cheese prototypes manufactured in R2 were slightly higher than those manufactured in R1. As recently suggested by Santapaola et al. (2021), the manual procedure for the rubbing of dry salt over the whole cheese surface, herein applied in both the pilot scale rounds of cheesemaking, can lead to the occurrence of slight differences among cheese wheels. In addition to this, further factors are known to affect the salt content of cheese, including curd moisture, pH, and fat content (Giroux et al., 2022).

Though salt is the main component of ash, no significant differences were seen between R2 and R1 cheese prototypes for the latter parameter. Moreover, in agreement with what suggested by Giroux et al. (2022) about the linear correlation between cheese moisture and salt content, no significant differences were seen in mean a_w values recorded in R1 and R2 cheeses.

Lactose is the fermentation substrate of lactic acid bacteria, which metabolize this carbon source into lactic acid and additional metabolites, depending on their homo- or heterofermentative pathway (McSweeney, 2004). As expected, a very low lactose content (<0.01 g 100 g^{-1}) was found in all the 60-day ripened cheese manufactures herein analyzed, thus allowing the indication "lactose free" to be adopted for all the cheese prototypes, based on the thresholds defined by the European Food Safety Authority (EFSA) (EFSA, 2010).

As far as the instrumental measurement of color attributes is concerned, for all the colorimetric coordinates, the values herein recorded were like those observed in other semi-hard or hard raw ewe's milk cheeses with a similar ripening time (Ávila et al., 2017; Cardinali et al., 2022b). As a general trend, the color of cheese paste, corresponding to the inner portion of the cheese wheels, was darker in the experimental than the control cheese prototypes. This finding might be tentatively attributed to the darker color of the two *O. tauricum* crude extracts in respect with the commercial rennet, as distinctly observed during cheesemaking (data not available). A higher yellowness and a lower greenness were also observed in the experimental than the control cheese prototypes, again as a secondary effect of the occurence of dark brown constituents in the two *O. tauricum* extracts. As a rule, cheese yellowness is mainly associated to carotenoids, fat-soluble yellow pigments, whose level in the milk mainly depends on their level in forages (Nozière et al., 2006). Of note, no significant differences in color parameters of Serra de Estrela PDO cheese manufactures curdled with thistle rennet from two flower ecotypes had previously been found by Correia et al. (2014). By comparing the sole cheese prototypes curdled with rennet from spontaneous and cultivated *O. tauricum*, significant differences could also be appreciated in the values of CIELab coordinates; this latter finding might be likely ascribed to slight variations in the composition of the two experimental crude extracts, in turn attributable to the manual procedure used for the preparation of these extracts.

As far as the data from instrumental measurement of texture are concerned, a low impact of the type of thistle rennet emerged, with the sole adhesiveness being significantly higher in the control than the experimental cheese prototypes curdled with crude extracts from *O. tauricum* (either spontaneous or cultivated). At this regard, a high uniformity in the textural properties of Serra da Estrela cheese manufactures curdled with thistle rennet from two flower ecotypes has previously been reported by Correia et al. (2014).

Adhesiveness is defined as "*the stickiness of a sample in the mouth throughout mastication*" (Civille and Szczesniak, 1973). In previous studies, cheese adhesiveness correlated positively with milk fat content (Nateghi et al., 2012; Zheng et al., 2016) and negatively with protein content (Zheng et al., 2016). Regarding this latter trait, an intense proteolysis is acknowledged to increase cheese adhesiveness (McSweeney, 2007). However, other variables, including temperature, level of potassium salts (El-Bakry et al., 2011), acidification rate, and even the type and amount of rennet used for cheesemaking (Pirisi et al. 2007) were suggested to have an impact on adhesiveness.

As a thistle curdled cheese, Caciofiore is expected to have high intensity of adhesiveness. However, excessively high adhesiveness might be also regarded as a moderate defect in hard and semi-hard ewe's milk cheeses (Zabaleta et al., 2016).

Of note, if the sole second round of cheesemaking trials is considered, texture measurements partly corresponded to the observed differences in SN/TN%; in fact, the control prototypes showed a softer texture than the experimental prototypes, in agreement with a greater extent of proteolysis in the former. The link between rennet proteolytic activity and cheese creaminess has previously been discussed in several studies highlighting that thistle curdled cheeses have a softer texture than those made with animal rennet (Alavi & Momen, 2020). Thus, the differences in the proteolytic activity of *C. cardunculus* and *O. tauricum* proteases could have led to some significant differences in the texture of the cheese prototypes herein analyzed. Certainly, other factors not examined in the present study might have contributed to the increased hardness of the experimental cheese prototypes in respect with the control prototypes, such as the level of solubilization of colloidal calcium phosphate.

Microbial viable counts recorded in the cheese prototypes herein analyzed were consistent with those previously reported by Rampanti et al., 2023b for further Caciofiore cheese manufactures curdled with *O. tauricum* crude extract. Counts of total mesophilic aerobes were also comparable to those previously reported in other ewe's milk cheese (Centi et al., 2017; Ordiales et al., 2013). High counts of presumptive mesophilic and thermophilic cocci, as well as of lactobacilli, were detected in all the cheese prototypes; this finding agrees well with viable counts of these microbial groups found in other raw ewe's milk cheeses manufactured without the addition of any starter cultures (Cardinali et al., 2021, 2022a, 2022b). Being part of the microbiota occurring on the external udder surface, on grassland, and in the faeces of homeothermic vertebrates, Enterobacteriaceae are commonly found in raw milk and raw milk cheeses (Montel et al., 2014; Tabla et al., 2016). Although some species in this family (e.g., *Psychrobacter celer* and *Hafnia alvei*) are known to contribute to cheese aroma by the enhanced production of volatile compounds (Irlinger et al., 2012), other species include pathogenic bacteria that may cause raw milk cheese-related illness (Yoon et al., 2016). Among Enterobacteriaceae, coliforms and *E. coli* are considered hygiene indicators; low loads of these bacteria are usually found in raw milk, with viable counts increasing and then declining in the early and late stage of cheese ripening, respectively (Metz et al., 2020). As for eumycetes, the results herein collected are in accordance with those previously reported by Cardinali et al. (2016) and Rampanti et al., 2023b for other manufactures of thistle curdled raw ewe's milk cheeses.

Over the past decades, DNA-based methodologies, such as Illumina sequencing, enabled a sensitive and in-depth analysis of the microbiota, thus allowing the characterization of complex food matrices, such as cheese (Biolcati et al., 2020). The metataxonomic analysis of bacterial DNA herein carried out clearly showed the dominance of *Lc. lactis* in all the cheese prototypes, irrespective of the round of cheesemaking trials, the batch of raw milk, and the type of coagulant. Within this species, *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* are commonly involved in dairy fermentations and used as starters because of their technological properties, mainly lactate production, casein breakdown, and production of flavor compounds from amino acids (Kazou, 2021). However, the occurrence of *Lc. lactis* is also widely documented in cheeses produced without the addition of any starter cultures to milk (Cardinali et al., 2017, 2021; 2022a; 2022b; Rampanti et al., 2023a, 2023b). *L. plantarum* was the second species in terms of relative frequency in all the cheese prototypes herein analyzed. This finding was not unexpected, since these facultatively heterofermentative lactobacilli have previously been isolated from several fermented foods, including cheese (Zago et al., 2011; Zheng et al., 2020). Selected strains of this species are also exploited as starters or probiotics by the food industry thanks to their multiple properties, including the antioxidant and antimicrobial activity, the improvement of nutritional quality and flavor of foods, as well as the beneficial effects exerted on human health (Echegaray et al., 2023). Other bacterial taxa were found by sequencing in all the cheese prototypes herein analyzed, including *L. zeae, L. brevis*, *Leuc. mesenteroides*, and *Enterococcus* spp.; again, these taxa are frequently detected in raw ewe's milk cheese (Cardinali et al., 2021; 2022a, 2022b; Rampanti et al., 2023a). Concerning the metataxonomic analysis of the fungal biota, a core of yeasts was detected in all the cheese prototypes, including *D. hansenii*, *K. marxianus, S. cerevisiae, Pichia* spp., *Geotrichum* spp., and *Galactomyces* spp.; these microorganisms, which constitute the secondary microbiota of several cheeses, play a key role during cheese ripening due to their multiple activities, including deacidification, release of compounds by autolysis, and development of flavor compounds (Fasoli et al., 2015; Gardini et al., 2006; Merchán et al., 2022).

Of note, higher relative frequencies of *Penicillium* spp. were seen in the cheese prototypes curdled with the two *O. tauricum* crude extracts compared to control cheese manufactures curdled with the commercial rennet; this finding undoubtedly deserves further research efforts, as many *Penicillium* species are known to produce mycotoxins (Ramos-Pereira et al., 2019).

Concerning aroma, previous investigations revealed that cheeses curdled with thistle rennet obtained from *C. cardunculus* were characterized by a more intense odor and a higher content of volatile fatty acids compared to cheeses curdled with animal rennet (Rincón et al., 2017; Tavaria et al., 2006; Tejada et al., 2006, and 2007). However, to the best of the authors' knowledge, no comparative data on odor of cheeses curdled with different types of thistle rennet are currently available. In the present study, no significant differences were found between the cheese prototypes curdled with commercial *C. cardunculus* rennet and *O. tauricum* crude extracts (either spontaneous or cultivated) based on VOCs profile and quantification, whereas a few differences emerged by comparing the two rounds of cheesemaking trials, likely because of the differences in the composition of the cheese microbiota.

High levels of ethanol, which reacts with fatty acids in the esterification reaction to produce ethyl esters (Fox et al., 2017a), were found, with a slight (but not statistically significant) higher content in the cheese prototypes curdled with O. *tauricum* extracts than the control ones. In cheese, ethanol can be produced by yeasts through alcoholic fermentation (Ortigosa et al., 2001) and, at a lesser extent, by facultative and obligate heterofermentative lactic acid bacteria, including leuconostocs (Vedamuthu, 1994) and lactobacilli such as *L. brevis* and *L. casei* (Belarbi et al., 2022); in addition, ethanol can also result from amino acids catabolism (McSweeney, 2004). Interestingly, the cheese prototypes manufactured with *O. tauricum* showed higher relative frequencies of *D. catenulate*, *Geotricum* spp., and *Galactomyces* spp. in respect with the control prototypes, with all these microorganisms being positively associated by correlation analysis with ethanol, ethyl butanoate, and ethyl hexanoate.

Branched-chain fatty acids (2-methyl propanoic and 3-methyl butanoic acid) are products of the metabolism of amino acids, and their accumulation is associated to an extensive breakdown of proteins (Mcsweeney & Sousa, 2000). In the present study, a significantly higher level of these compounds were observed in R2 than R1 cheeses. Of note, both 2-methyl propanoic and 3-methyl butanoic acids showed a positive correlation with Enterobacteriaceae, *Enterococcus* spp. and *L. plantarum*, all these microorganisms being known for affecting proteolysis (Morales et al., 2003; Terzić-Vidojević et al., 2021). Intriguingly, a significantly higher load of presumptive lactobacilli was seen in the R2 than R1 cheese prototypes.

The higher level of 3-methyl butanoic acid in R2 cheeses was also associated to the increased level of the derived ester ethyl-3-methyl butanoate. In addition, higher values of 2,3-butanedione were found in R1 cheeses; the occurrence of this ketone is usually associated with the metabolism of citrate by lactic acid bacteria. However, it has been observed that its concentration is higher during the early stage of ripening and decreases with time due to enzymatic activities that reduce it to acetoin, 2,3-butandiol, 2-butanone, and 2-butanol (Bontinis et al., 2012; Santamarina-García et al., 2023; Sözeri Atik et al., 2021).

Most of these volatiles, for which significant differences were observed between the R1 and R2 cheese prototypes, are generally reported as key odorants in several cheese types (Curioni & Bosset, 2002;

Kilcawley & O'Sullivan, 2017). However, all the differences herein observed did not produce marked changes in the odor attributes as perceived by sensory analysis. It is plausible that the extent of variation in the levels of aroma compounds of control *vs* experimental or R1 *vs* R2 cheese prototypes was not sufficient to produce a perceivable change in intensity of many of the olfactory attributes herein evaluated. Hence, the results of sensory analyses suggest that both the rennet and the conditions applied for cheese odor evaluation had an almost negligible impact on the sensory profile of the analyzed cheeses. According to the results of GC-O analyses and to what is generally known about cheese odorants, butanoic acid, which was perceived at a very high intensity at the GC-O olfactory port, was the compound that more strongly contributed to the ewe's cheese odor, but its content was not affected by the rennet type.

Ethyl esters are generally described as having a fruity odor; however apparently no correlations were seen between the comparable levels of these compounds in all the cheese prototypes and the higher intensity of the fruity odor perceived by the sensory panel in the control than experimental cheeses manufactured in the second round of cheesemaking. While 1-octen-3-one can be directly associated to a mushroom note, the contribution of other identified key odorants could be mixed with that of other compounds and not clearly related to any single odor attribute, as it frequently occurs in food flavor. Interestingly, some odor attributes could be associated to other still unidentified odorants, such as the unknown odorant no. 18, described as having a sour curd odor, which might have contributed to the sour curd note perceived and quantified by the panel test.

5. Conclusions

The results overall collected in this study provided a comprehensive characterization of raw ewe's milk cheeses manufactured in two separate rounds of cheesemaking trials, using different batches of Sopravissana ewe's raw milk curdled with *C. cardunculus* commercial thistle rennet and crude extracts obtained from either spontaneous or cultivated *O. tauricum*.

A high uniformity was seen among the cheese prototypes, with the major differences being apparently associated to the round of cheesemaking trials, rather than the type of thistle rennet used for milk coagulation.

This finding agrees well with a previous study, carried out onto pilot-scale manufactures of Serra de Estrela PDO cheese produced using thistle rennet from two flower ecotypes, where a very low effect of the type of rennet on the cheese chemical, microbiological, textural, and sensory traits were seen. The data herein collected are also in agreement with those obtained in a previous study aimed at evaluating the microbial dynamics of Caciofiore cheese manufactures curdled with extracts of flowers of spontaneous and cultivated *O. tauricum*, where the batch of milk had an apparently higher impact than the type of thistle rennet used.

Just a few significant rennet-dependent differences emerged by comparing the control and experimental cheese prototypes herein analyzed, with those curdled with either spontaneous or cultivated *O. tauricum* being characterized by a lower SN/TN% ratio (suggesting a lower proteolysis), a lower titratable acidity, and a darker color, this latter indicated by lower values for L* (lightness), less negative values for a* (green), more positive values for b* (yellow), and more positive values for C* (Chroma, relative saturation). Regarding texture, VOCs, and odor notes evaluated by a trained sensory panel, again no marked differences emerged between the control and experimental cheese prototypes, though those curdled with *O. tauricum* (either spontaneous or cultivated) showed a less intense adhesiveness and different levels of few cheese-associated VOCs.

Further investigations are undoubtedly needed for an exhaustive identification of the unknown odorants found in the present study. The sensory evaluation of taste and texture attributes is another issue to be complemented, which had been prevented in this work by the high loads of Enterobacteriaceae found in all the cheese prototypes.

To conclude, the data overall collected suggest that *O. tauricum* represents a valuable alternative to *C. cardunculus* for the manufacture of Caciofiore cheese and, potentially, other Mediterranean ewe's milk cheeses. Moreover, the comparable features of cheeses curdled with *C. cardunculus* rennet or crude extracts from both spontaneous and cultivated *O. tauricum* also demonstrate the potential of this still unexploited crop for the supply of sustainable and cruelty-free milk coagulants.

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CRediT authorship contribution statement

Giorgia Rampanti: Data curation, Formal analysis, Writing – original draft. **Antonio Raffo:** Data curation, Formal analysis, Writing – original draft. **Valentina Melini:** Formal analysis, Investigation. **Elisabetta Moneta:** Formal analysis, Investigation. **Nicoletta Nardo:** Formal analysis, Investigation. **Eleonora Saggia Civitelli:** Formal analysis, Investigation. **Cindy Bande-De Leon:** Formal analysis, Writing – review & editing. **Luis Tejada Portero:** Formal analysis, Writing – review & editing. **Ilario Ferrocino:** Formal analysis, Writing – review & editing. **Irene Franciosa:** Formal analysis, Writing – review & editing. **Federica Cardinali:** Formal analysis, Investigation. **Andrea Osimani:** Resources, Visualization, Methodology, Writing – review & editing. **Lucia Aquilanti:** Resources, Visualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113459.

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