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High-speed cold centrifugation of milk modifies the microbiota, the ripening process and the sensory characteristics of raw-milk hard cheeses

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ABSTRACT

The microbial population of raw milk plays a crucial role in the development of distinctive traits of raw-milk cheeses particularly appreciated by consumers. It was previously demonstrated that the microbial population of raw milk is modified by a high-speed centrifugation (also called bactofugation) conducted at 39 °C. The aim of the present study was to evaluate the effects of this process, performed once or twice, on the microbial, compositional, biochemical, and sensory characteristics of the derived hard cheeses. Experimental and control cheesemaking were conducted in parallel at a cheese factory during a 13-month period. Cheeses were analysed after 9, 15 and 20 months of ripening for microbial count, composition, proteolysis extent, volatile compounds, and sensory profile. Results evidenced that experimental cheeses were characterized by lower numbers of viable lactobacilli respect to control. Experimental cheeses also showed differences in the progress of primary and secondary proteolysis which, in turn, caused different patterns of free amino acids at all ripening times. Experimental cheeses had significantly lower content of esters and were differentiated from control for some traits by assessors. In conclusion, use of high-speed centrifugation of milk shall be discouraged if characteristic traits of raw-milk cheeses, particularly PDO cheeses, want to be retained.

1. Introduction

Grana Padano (GP) is a Protected Designation of Origin (PDO) hard cheese manufactured from partially skimmed raw milk added with a natural whey starter. Consequently, in the complex microbial community of this cheese, an equilibrium occurs between lactic acid bacteria (LAB) coming from two different sources: the autochthonous species present in raw milk, representing the non-starter LAB (NSLAB), and those present in the natural whey starter (SLAB). Species so joined in the vat milk undergo a selection during the whole cheesemaking process and those that survive then can grow in the cheese during ripening (Gatti et al., 2014; Giraffa, 2021). Thus, although it is composed of many species, the LAB community in raw milk cheeses is characteristic of the cheese type and regulates the biochemical mechanisms responsible for the maturation (Coelho et al., 2022). As the ripening of GP cheese lasts over 9–12 months, the proteolytic events prevail and bring to a specific profile of the compounds deriving from casein breakdown (Masotti et al., 2013). The free amino acids (FAA), that account for around 20% of cheese protein, proved to have a characteristic pattern in GP at selected ages (Hogenboom et al., 2017). The FAA also represent precursors of flavour compounds distinctive of this cheese type (Lazzi et al., 2016). Unfortunately, hard cheeses may suffer from the late blowing defect caused by butyric acid fermentation driven from spore-former *Clostridium* species. This defect is characterized by formation of holes and cracks, accompanied by an undesirable flavour. Clostridia spores survive the cheese manufacturing, making this defect extremely difficult to eradicate (D'Incecco et al., 2018). Centrifugation of milk at high centrifugal force (8,000–10,000 *g*) is widely used in the dairy sector, especially to produce non-PDO cheeses, since it allows an effective removal of bacterial spores (Guinee and O'Callaghan, 2010), and

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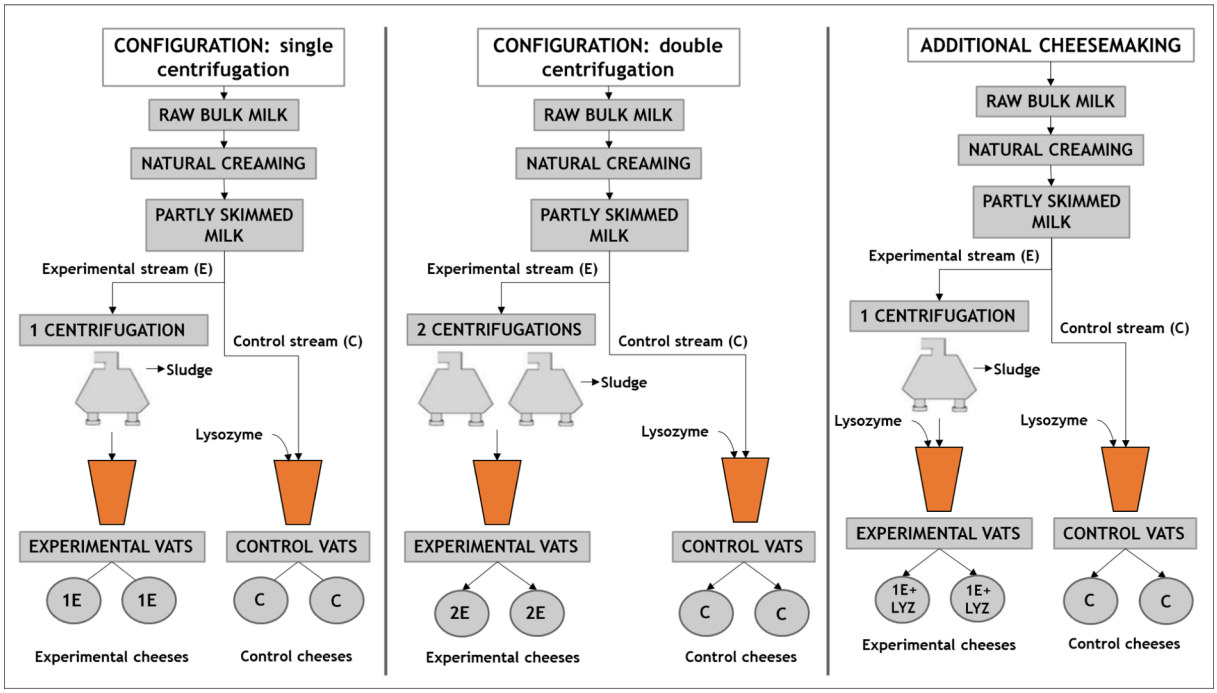

Fig. 1. Experimental design showing the production of experimental (E) and control (C) cheeses during the single (1E) and double (2E) configuration as well as in the additional cheesemaking. Appropriate controls were produced within each configuration.

decreases both bacterial and somatic cell counts (Sant'Ana, 2014). Modern high-speed centrifuges, also named Bactofuge (TetraPak, Sweden) or bacteria-removing centrifuge (Westfalia-GEA, Germany), operate at 50–60 °C to guarantee high efficiency in spore removal, although the characteristics of cells and spores, primarily density but also size and shape, also play a role in determining the final efficiency of the process (Te Giffel and Van Der Horst, 2004). Due to the operation temperature, such centrifugation process shall not be adopted in the manufacture of raw milk cheeses, because raw milk shall not be heated to more than 40 °C by definition (Regulation (EC) No 853/2004). The feasibility of using a low-temperature (39 °C) centrifugation for milk intended to produce raw milk hard cheeses has been recently investigated (D'Incecco et al., 2020a). The study demonstrated that milk centrifugation at 39 °C allowed a spore removal efficiency of 95.6% with an increase up to 98.2% with a double centrifugation. However, this process caused a decrease in cheese yield in the range 2.5–3.5%, depending on single or double centrifugation, but more remarkably, the centrifugation process was able to modify the typical LAB community. In particular, the following differences were observed in milk that underwent centrifugation compared with the non-centrifuged milk: (i) a lower concentration of LAB; (ii) a lower presence of rod-shaped bacteria; (iii) a faster acidification rate. Overall, these differences showed that centrifugation of milk caused the preferential removal of rod-shaped mesophilic bacteria, typically representing NSLAB and thus most important for the ripening process of raw milk cheeses. In fact, the typical NSLAB of GP cheese were identified in the mesophilic species belonging to genus *Lacticaseibacillus* (previously *Lactobacillus*) *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* that can catabolize peptide and FAA through different degradation pathways (Gobbetti et al., 2015).

Based on previous evidence of the impact of single and double cold centrifugation on cheese milk properties (D'Incecco et al., 2020a), the aim of the present work was to integrate knowledge on the topic by evaluating the behaviour of ripening process of the derived cheeses. Both experimental (from centrifuged milk) and control cheeses were analysed for LAB count, gross composition, proteolysis extent, volatile compounds, and sensory properties throughout a 20-month ripening period.

2. Materials and methods

2.1. Experimental design, cheese manufacture and sampling

Experimental (E) and control (C) cheeses were produced at a factory of Grana Padano (GP) cheese using the raw milk received daily from three local farms. The graphic illustration of the experimental design used in this study is outlined in Fig. 1. After natural creaming at 8–12 °C for around 10 h, the skimmed milk was divided in two streams: one followed the traditional GP cheesemaking including the addition of lysozyme (25 g/vat) to produce control cheeses (C), while the second underwent to either one or two centrifugation steps and was destined to production of the experimental cheeses (no lysozyme added), named as 1E and 2E, respectively. The sludge discharged during the centrifugation steps was eliminated to accomplish with the manufacture of raw milk cheese. Two centrifuges (one-phase CSI-230–01-772, Westfalia-GEA, Germany) were set to operate, either individually or in series, at 39 °C and a flow rate of 21,000 L/h. After centrifugation, milk was held in a degassing tank at ~ 13 °C for 4 h and then conveyed to the vats. A total of 40,000 L milk was worked daily in 20 vats for the experimental stream and 20 vats for the control one. The cheesemaking conditions were those described by D'Incecco et al., (2020a). Five sessions of two weeks each were carried out using both 1E and 2E configurations. A total of 11,200 cheeses were produced (two cheeses per vat) and ripened in the same room for up to 20 months. Cheese sampling was carried out at 9-, 15- and 20-months ripening for a total of 320 cheeses.

Additional cheesemaking were carried out to assess the role of lysozyme. During manufacturing, experimental milk underwent to a single centrifugation and lysozyme (25 g/vat) was added to vat milk as for control. Cheeses underwent ripening up to 9 months.

All cheeses were preliminary inspected by X-ray imaging (TDI Packsys, IL, USA) and no defects such as holes or cracks due to butyric fermentation were detected. For cheese sampling, a representative wedge (around 500 g) was taken from the wheel, the rind (5-mm thick) was removed, and the cheese was finely grated using a domestic grinder.

The samples were kept frozen and destined to chemical analyses. A second wedge was vacuum packed and sent to the sensory laboratory. A third slice was immediately sampled for microbiological analyses.

2.2. Lactic acid bacteria in cheese

2.2.1. Cultivable bacteria count

Ten grams of grated cheese were suspended in 90 mL of sterile trisodium citrate solution (20 g/L; pH 7.5) (Sigma–Aldrich, St. Louis, USA) and homogenized at 230 rpm for 2 min by means of Stomacher® 400 Circulator (VWR International Srl, Milan, Italy). The following ten-fold serial dilutions were performed in quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom). Samples were plated on acidified MRS agar (pH 5.4) and incubated at 30 °C for 48 h in anaerobic conditions for the count of mesophilic lactobacilli. A total of 252 cheeses were analysed.

2.2.2. DNA extraction

Grated cheese (0.5 g) was suspended in 1 mL of trisodium citrate solution (20 g/L; pH 7.5) (Sigma–Aldrich, St. Louis, USA) and homogenized in a Mini-Beadbeater™ (BioSpec Products, Bertlesville, OK USA) at max speed for 1 min. Samples were centrifuged at 14,000 rpm for 5 min; 800 μL of supernatant were treated with Maxwell® RSC PureFood Pathogen Kit (Promega Corporation, Madison, WI, USA) for automated DNA purification following the manufacturer instruction. DNA quantification was done using Quantus™ Fluorometer with Quantifluor® ONE dsDNA System (Promega Corporation, Madison, WI, USA).

2.2.3. Real time quantitative PCR

Real-time quantitative polymerase chain reaction (RT qPCR) was carried out using a QuantStudio® 3 (Thermo Fisher Scientific, Milan, Italy) and the PowerUpSYBRGreen Master Mix (Applied Biosystems, Milan, Italy) using the specific primers for the target *spxB* gene, as previously described by Savo Sardaro et al. (2016), that allowed the specific quantification of LAB belonging to the *Lacticaseibacillus* species, namely *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamosus*. Furthermore, the *Limosilactobacillus fermentum* species was quantified by using the specific primers (Agrimonti et al., 2019). Samples were analysed in triplicate.

2.3. Cheese composition analysis

Moisture, protein, fat, and ash contents of cheese were determined by using the Standards ISO 5534:2004, ISO 27871:2011, ISO 1735:2004, and IDF 27:1964, respectively. The moisture in nonfat substance (MNFS) of cheese was calculated as 100 × moisture content/(100 − fat content). Analyses were carried out in duplicate.

2.4. Casein degradation by capillary zone electrophoresis

The progression of ripening was evaluated by capillary zone electrophoresis (CZE) adopting the conditions described by D'Incecco et al., (2020b). Grated cheese (1 g) was dispersed in 10 mL of sample buffer and kept at room temperature for 4 h. The sample buffer was prepared by dissolving 7.44 g of disodium ethylenediaminetetracetic acid dihydrate, 6.06 g of tris(hydroxymethyl)aminomethane, 2.64 g of 3-(N-morpholino)propane sulfonic acid and 0.77 g of DL-dithiothreitol in 200 mL of urea solution (60% w/v) in MilliQ water (Millipore, Milford, USA) and adding 300 mL of urea solution (60% w/v) containing 0.15% (w/v) of methylhydroxyethylcellulose (MHEC). The sample buffer and urea/MHEC solution were treated with mixed-bed ion-exchange resin (AG501-X8, Bio-Rad Laboratories, Segrate, Italy). The separation buffer (pH 3.0 ± 0.1) was prepared by dissolving 4.38 g of citric acid monohydrate and 0.59 g of trisodium citrate dihydrate in 40 mL of MilliQ water and adding 60 mL of urea/MECH solution. All buffers were filtered with 0.45 μm regenerated cellulose membrane filter (Agilent Technologies, Milan, Italy) prior to use. All chemicals were of analytical grade (Merck Life Science, Milan, Italy). Samples were further diluted (1:5) with the sample buffer and filtered with 0.22 μm polyvinylidene difluoride membrane filter (Merck Life Science) prior to injection. A P/ACE™ MDQplus capillary electrophoresis equipment (AB Sciex, Milan, Italy) was used, including a UV detector set at 214 nm and equipped with a hydrophilically coated capillary column (50 μm i.d., 0.05 μm coating, 500 mm effective length, 100 × 800 μm slit opening) (DB-WAX 126–7012, J&W Agilent Technologies). Separation was carried out at 45 °C using a linear gradient from 0 to 30 kV in 4 min, followed by constant voltage at 30 kV for 36 min. The peak area ratios between selected casein or peptide fractions were calculated, considering the normalized peak area (peak area counts/migration time). The following ratios between casein fractions were considered:

$$\alpha_{s1}\text{-I}/\alpha_{s1} = (\alpha_{s1}\text{-CN-I 8P})/(\alpha_{s1}\text{-CN 8P}) \quad (1)$$

$$\alpha_{s0}\text{-I}/\alpha_{s0} = (\alpha_{s0}\text{-CN-I 9P})/(\alpha_{s0}\text{-CN 9P}) \quad (2)$$

$$\alpha_s(f1\text{-}23)/\alpha_s(1+0) = (\alpha_{s1}\text{-CN}(f1\text{-}23)/(\alpha_{s1}\text{-CN 8P} + \alpha_{s0}\text{-CN 9P}) \quad (3)$$

$$\alpha_{s1}\text{-PL}/\alpha_s(1+0) = \alpha_{s1}\text{-CN}(f88\text{-}199)/(\alpha_{s1}\text{-CN 8P} + \alpha_{s0}\text{-CN 9P}) \quad (4)$$

$$\Sigma\gamma/\Sigma\beta = (\gamma1\text{-CN A1} + \gamma1\text{-CN A2} + \gamma2\text{-CN A} + \gamma3\text{-CN A} + p\gamma3\text{-CN A})/(\beta\text{-CN B} + \beta\text{-CN A1} + \beta\text{-CN A2}) \quad (5)$$

Analyses were carried out in duplicate.

2.5. Free amino acids evaluation

Accumulation of free amino acids during ripening was evaluated by ion-exchange chromatography using the method proposed by Hogenboom et al., (2017). Briefly, grated cheese (1.5 g) was dissolved in trisodium citrate buffer (0.2 N; pH 2.2), homogenized and then deproteinated with 5-sulfosalicylic acid (7.5% w/v). The obtained solution (10 mL) was added with 2 mL of a solution of Norleucine (600 mg/L) as an internal standard and diluted to 100 mL with trilithium citrate buffer (0.2 N; pH 2.2). The final solution was filtered on 0.2 μm cellulose acetate filter (Merck Life Science) prior to injection. The chromatographic conditions were those recommended by the manufacturer. A multipoint calibration was used for quantitation purposes. Analyses were carried out in duplicate.

2.6. Evaluation of volatile compounds SPME GC–MS

The volatile compounds were determined by a headspace solid phase micro extraction (SPME) technique followed by gas chromatography-mass spectrometry (GC–MS). A gas chromatograph Trace 1300 coupled with a TSQ 8000 Evo Mass Spectrometer (Thermo Scientific, Milan, Italy) and equipped with an automatic autosampler (Gerstel MPS) was used. Grated cheese (2 g) was weighted into 20 mL glass vial and added with 20 μL of 2-methyl pentanol (50 μg/mL; Sigma-Aldrich, Milan, Italy) as internal standard. Samples were equilibrated at 45 °C for 30 min and then exposed to the Carboxen-polydimethylsiloxane-divinylbenzene fibre (CAR/PDMS/DVB 50/30 μm × 1 cm, 23 Ga) (Supelco, Bellefonte, PA, USA) for 30 min. The injector was set at 250 °C and the injection mode was splitless for 0.75 min with a desorption time of 15 min. The gas-chromatographic separation was carried out with a Mega-WAX MS column (30 m × 0.25 mm × 0.25 μm) (Mega Srl, Milano, Italy) using helium as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially set at 40 °C and held for 5 min, then ramped at 5 °C/min up to 220 °C and held for 15 min. The temperatures of the transfer line and the source were 250 °C and 200 °C, respectively. The mass spectrometer operated in electron ionization mode at 70 eV using full scan mode, registering the m/z from 40 Da up to 250 Da. The ions used for identification were chosen according to the NIST MS Search 2.0 library and validated by external standard comparisons of ion fragmentation patterns. Relative abundance of each volatile compound was

Table 1
Gross composition (g/100 g) of experimental and control cheeses at different ripening times.

Configuration	Ripening (months)	Sample	Moisture	Protein	Fat	Ash	MNFS[1]
1E	9	C	33.53 ± 0.68^{a}	31.96 ± 0.55^{a}	28.80 ± 0.83^{a}	4.06 ± 0.15^{a}	47.10 ± 1.00
		E	33.46 ± 0.66^{A}	32.10 ± 0.41^{A}	28.23 ± 0.47**^{A}	4.03 ± 0.15^{A}	46.60 ± 0.80*
	15	C	32.09 ± 0.65^{b}	32.20 ± 0.78^{a}	29.22 ± 0.83^{ab}	4.23 ± 0.10^{b}	45.30 ± 0.70
		E	31.98 ± 0.72^{B}	32.49 ± 0.69^{A}	29.11 ± 0.63^{B}	4.21 ± 0.11^{B}	45.10 ± 1.00
	20	C	31.26 ± 0.81^{c}	33.10 ± 0.72^{b}	29.67 ± 0.47^{b}	4.37 ± 0.33^{b}	44.40 ± 1.00
		E	30.77 ± 0.44^{C}	33.69 ± 0.91^{b}	29.83 ± 0.67^{c}	4.20 ± 0.10^{B}	43.90 ± 0.70
2E	9	C	33.78 ± 0.84^{a}	31.87 ± 0.53^{a}	29.16 ± 0.91^{a}	4.11 ± 0.16^{a}	47.70 ± 0.90
		E	33.30 ± 0.81^{A}	32.19 ± 0.48*^{A}	28.70 ± 0.60*^{A}	4.13 ± 0.22^{A}	46.70 ± 1.00***
	15	C	32.27 ± 0.65^{b}	32.70 ± 0.82^{b}	28.81 ± 0.64^{a}	4.28 ± 0.16^{b}	45.30 ± 0.80
		E	32.28 ± 0.76^{B}	32.99 ± 0.60^{B}	28.69 ± 0.57^{A}	4.23 ± 0.14^{A}	45.30 ± 1.00
	20	C	31.46 ± 0.81^{c}	32.83 ± 0.29^{b}	29.36 ± 0.66^{a}	4.30 ± 0.10^{b}	44.50 ± 1.10
		E	31.34 ± 0.68^{C}	33.33 ± 0.46**^{B}	29.31 ± 0.94^{A}	4.24 ± 0.15^{A}	44.30 ± 1.10

Results of control (C) and experimental (E) cheeses are expressed as mean ± standard deviation. E cheeses were produced with single (1E) or double (2E) centrifugation of milk in parallel with the corresponding control cheeses. Difference between C and E cheeses within 1E or 2E configuration at each ripening time is indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; t-Test). Difference of each parameter during ripening for each type of cheese is indicated by different superscripts (ANOVA): small letters refer to C cheeses and capital letters refer to E cheeses. [1] MNFS = moisture in non-fat substance.

determined through the ratio of the target compound areas and the internal standard area (A/A_{is}). Analyses were carried out in duplicate on 20-months ripened 1E cheeses.

2.7. Cheese sensory analysis

The descriptive sensory profile was evaluated for both E and C cheeses at 9, 15 and 20-mo ripening to assess whether they could be discriminated based on the intensity of selected attributes. A panel of 10 trained experts randomly selected among 35 (22 women and 13 men), aged between 26 and 59 years, was encouraged to develop a common vocabulary for the description of smell, taste and texture of cheeses by the consensus method (ISO Standard 11035, 1994). The following descriptors were considered: colour, sapidity, bitterness, texture, sour taste, saltiness, astringency, spiciness, solubility, friability, hardness, smell intensity, granular structure, and texture. During the formal

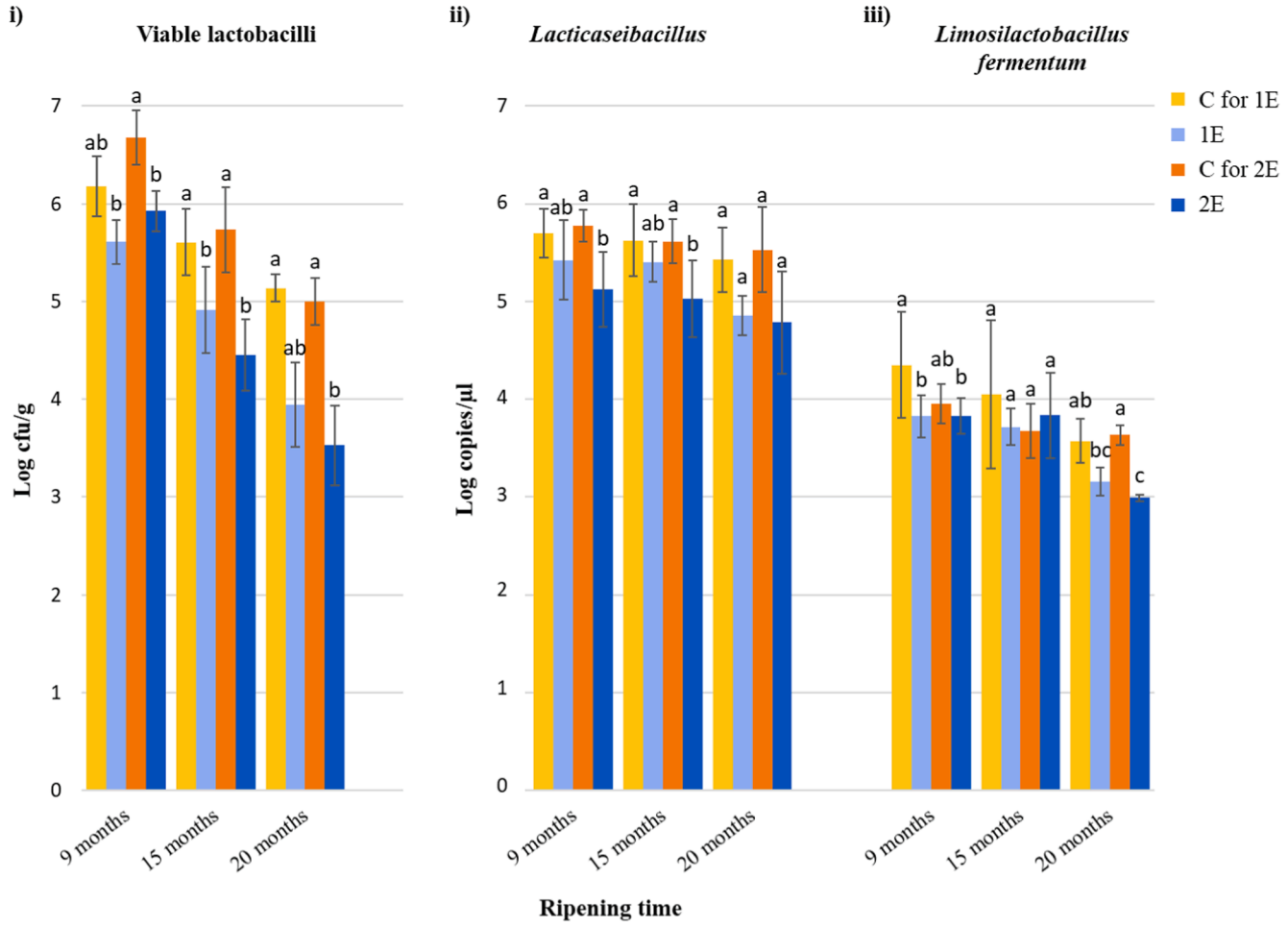

Fig. 2. Plate count of viable lactobacilli in MRS agar in panel i) and *Lacticaseibacillus* and *Limosilactobacillus fermentum* quantification in panels ii) and iii), in cheeses at different ripening times. Statistical differences between control (C) and experimental (E) cheeses among both configurations (1E and 2E) at each ripening time are indicated by different letters (ANOVA; $p < 0.05$).

Table 2
Casein fraction ratios and total content of free amino acids (FAA) in experimental and control cheeses at different ripening times.

Configuration	Ripening (months)	Sample	α_{s1}-I/α_{s1} (1)	α_{s0}-I/α_{s0} (2)	α_s (f1-23)/$\alpha_{s(1+0)}$ (3)	α_{s1}-PL/$\alpha_{s(1+0)}$ (4)	Σγ/Σβ (5)	FAA (g/100 g protein)
1E	9	C	0.58 ± 0.10^{a}	0.48 ± 0.13^{a}	0.21 ± 0.05^{a}	0.29 ± 0.08^{a}	1.63 ± 0.32^{a}	18.67 ± 1.21^{a}
		E	0.58 ± 0.12^{A}	0.50 ± 0.12^{A}	$0.15 \pm 0.04^{***A}$	0.30 ± 0.07^{A}	1.65 ± 0.41^{A}	19.13 ± 0.65^{A}
	15	C	0.48 ± 0.10^{b}	0.37 ± 0.09^{b}	0.23 ± 0.05^{a}	0.39 ± 0.06^{b}	2.36 ± 0.43^{b}	21.32 ± 1.64^{b}
		E	0.47 ± 0.06^{B}	0.36 ± 0.08^{B}	$0.18 \pm 0.04^{***B}$	$0.44 \pm 0.07^{*B}$	2.47 ± 0.44^{B}	20.98 ± 1.29^{B}
	20	C	0.37 ± 0.06^{c}	0.29 ± 0.08^{b}	0.22 ± 0.04^{a}	0.44 ± 0.08^{b}	2.71 ± 0.40^{c}	21.44 ± 2.19^{b}
		E	0.35 ± 0.09^{C}	0.30 ± 0.12^{B}	$0.18 \pm 0.04^{*AB}$	$0.51 \pm 0.07^{*C}$	2.91 ± 0.50^{C}	21.69 ± 1.81^{B}
2E	9	C	0.65 ± 0.17^{a}	0.59 ± 0.15^{a}	0.23 ± 0.06^{a}	0.29 ± 0.05^{a}	1.50 ± 0.26^{a}	20.05 ± 0.94^{ab}
		E	0.60 ± 0.14^{A}	0.54 ± 0.12^{A}	$0.16 \pm 0.03^{***A}$	0.30 ± 0.06^{A}	1.56 ± 0.27^{A}	19.94 ± 0.90^{A}
	15	C	0.49 ± 0.08^{b}	0.32 ± 0.08^{b}	0.23 ± 0.04^{a}	0.41 ± 0.07^{b}	2.43 ± 0.49^{b}	19.37 ± 1.34^{a}
		E	0.46 ± 0.07^{B}	0.33 ± 0.08^{B}	$0.18 \pm 0.04^{***A}$	0.44 ± 0.08^{B}	2.36 ± 0.33^{B}	$20.29 \pm 0.98^{*AB}$
	20	C	0.39 ± 0.05^{b}	0.28 ± 0.05^{b}	0.22 ± 0.04^{a}	0.43 ± 0.06^{b}	2.76 ± 0.50^{b}	21.15 ± 1.82^{b}
		E	0.38 ± 0.07^{B}	0.29 ± 0.05^{B}	$0.18 \pm 0.03^{*A}$	0.48 ± 0.11^{B}	2.78 ± 0.67^{C}	20.95 ± 1.48^{B}

Casein fraction ratios are calculated on corrected peak area. Results of control (C) and experimental (E) cheeses are expressed as mean ± standard deviation. E cheeses were produced with single (1E) or double (2E) centrifugation of milk in parallel with the corresponding control cheeses. Difference between C and E cheeses within each configuration at each ripening time is indicated by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001; t-Test). Difference of each parameter during ripening for each type of cheese is indicated by different superscripts (ANOVA): small letters refer to C cheeses and capital letters refer to E cheeses. (1)(2)(3)(4)(5) Equations presented in section 2.4.

evaluation, the perceived intensity of each descriptor was evaluated by using a seven-point scale ranging from 0 (not perceived) to 7 (highest intensity) following the procedure of the ISO Standard 13299 (2016). Samples were identified using random three-digit numbers and served individually in lidded cups to minimize any sensorial changes during evaluation. Cheese portions (2x2x8 cm) were given to assessors after conditioning at room temperature (23 ± 2 °C). Crackers and plane water were provided to cleanse mouth after each sample assessment. Four cheeses were assessed in each session. Scores were analysed using Tukey's HSD by comparing E and C cheeses at each ripening time.

2.8. Statistical treatment of data

Both statistical difference between E and C cheeses at each ripening time (*t*-Test; two-tailed distribution) and statistical difference among ripening times within each cheese group (one-way ANOVA, Tukey's test) were evaluated using the SPSS Win 12.0 software Version 27 (SPSS, IBM Corp., Chicago, IL). Principal component analysis (PCA) was conducted after the Bartlett's test of sphericity was verified ($p < 0.05$) and using only components with eigenvalue > 1. Only variables with loading > 0.7 were selected: Ser, Gln, Cit, Orn, Arg and viable lactobacilli count. The SPSS Win 12.0 software Version 27 was used. Before PCA, data were normalized using the standardize function. Differences at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant.

3. Results and discussion

According to a recent study, the major impact of cold centrifugation on milk debacterization was remarkably related to the preferential removal of rod-shaped bacteria with a consequent imbalance in the microbial population of milk (D'Incecco et al., 2020a). Starting from these observations, the present work investigated the effects of this altered microbial population of milk on the ripening of the derived cheeses, with a particular focus on the proteolytic pathways, and thus on their final chemical and sensory features. In fact, the selective removal of *Lacticaseibacillus* species from milk might alter both primary and secondary proteolysis in cheeses, possibly affecting the availability of flavour precursors (Ardö, 2021).

The gross composition of 1E, 2E and C cheeses was preliminary determined at different ripening times (Table 1). As expected, moisture significantly decreased during ripening, similarly in E and C cheeses. At 9 months of ripening, fat content was significantly ($p < 0.05$) lower in E cheeses and, therefore, moisture in non-fat substance (MNFS) was significantly lower as well. Non-systematic differences were observed for the protein content and none for the ash content.

3.1. Viable LAB in cheese

Viable lactobacilli were quantified by means of plate count in elective agar medium (Reuter, 1985). The results showed a decrease in the total concentration of lactobacilli during ripening of C cheeses (Fig. 2-i), confirming the trend already observed in GP throughout a 15-month ripening period (Santarelli et al., 2013; Pogačić et al., 2013). As expected, viable cell counts in the C cheeses (green bars) decreased during the ripening time but, interestingly, viable LAB were still present in a concentration of about 5 log cfu/g after 20 months. The same trend was observed for the E cheeses where, however, the concentration of viable LAB cells was significantly lower throughout the entire ripening period, regardless whether obtained from milk submitted to one (1E) or two (2E) centrifugation steps. In fact, after 20-month ripening, live cells were 6.4% in 1E cheeses and 3.4% in 2E cheeses compared to the respective C cheeses (Fig. 2-i). Thus milk centrifugation implies that fewer cells are able to solubilize the cheese matrix, metabolize soluble molecules and produce aroma compounds (Lazzi et al., 2016; Gobbetti et al., 2015).

Considering that *Lacticaseibacillus* is known to be the genus more involved in aroma formation, its presence in cheeses was quantified by RT qPCR (Sardaro et al., 2016). The results are shown Fig. 2-ii as log DNA copies/μL. Values were in line with those obtained by plate counts, confirming a significantly lower concentration of the genus *Lacticaseibacillus* in the E cheeses, especially 2E, as compared to the C cheeses of the same age. After 20 months of ripening, this decrease reached 73% for 1E and 54% 2E. It is known that centrifugal separation efficiency also depends on characteristics of microbial cells such as density, size, and shape (Gésan-Guiziou, 2010). In general, rod-shaped bacteria are preferentially removed compared to cocci bacteria. Faccia et al., (2013) observed values of "microbial growth decrease" index of 33% for rod-shaped LAB and of 26% for coccus-shaped ones in milk bactofuged at 55 °C. The reduction of LAB produced by milk centrifugation impacted on two different aspects at the same time in the ripened cheeses: one was negative, as it implied the selective removal of *Lacticaseibacillus* species, the other one was positive, as *L. fermentum* was removed as well. Remarkably, the reduction of the *Lacticaseibacillus* genus was up to 73% in E cheeses, compared to C cheeses, after 20 months of ripening. The reduced concentration of this genus in the ripened cheese may impair the formation of the complex flavour which is typical of raw-milk cheese varieties. *L. rhamnosus, L. casei* and *L. paracasei* are in fact NSLAB species well known for their capability to better adapt and grow during even prolonged ripening periods (Bottari et al., 2018; Lazzi et al., 2016).

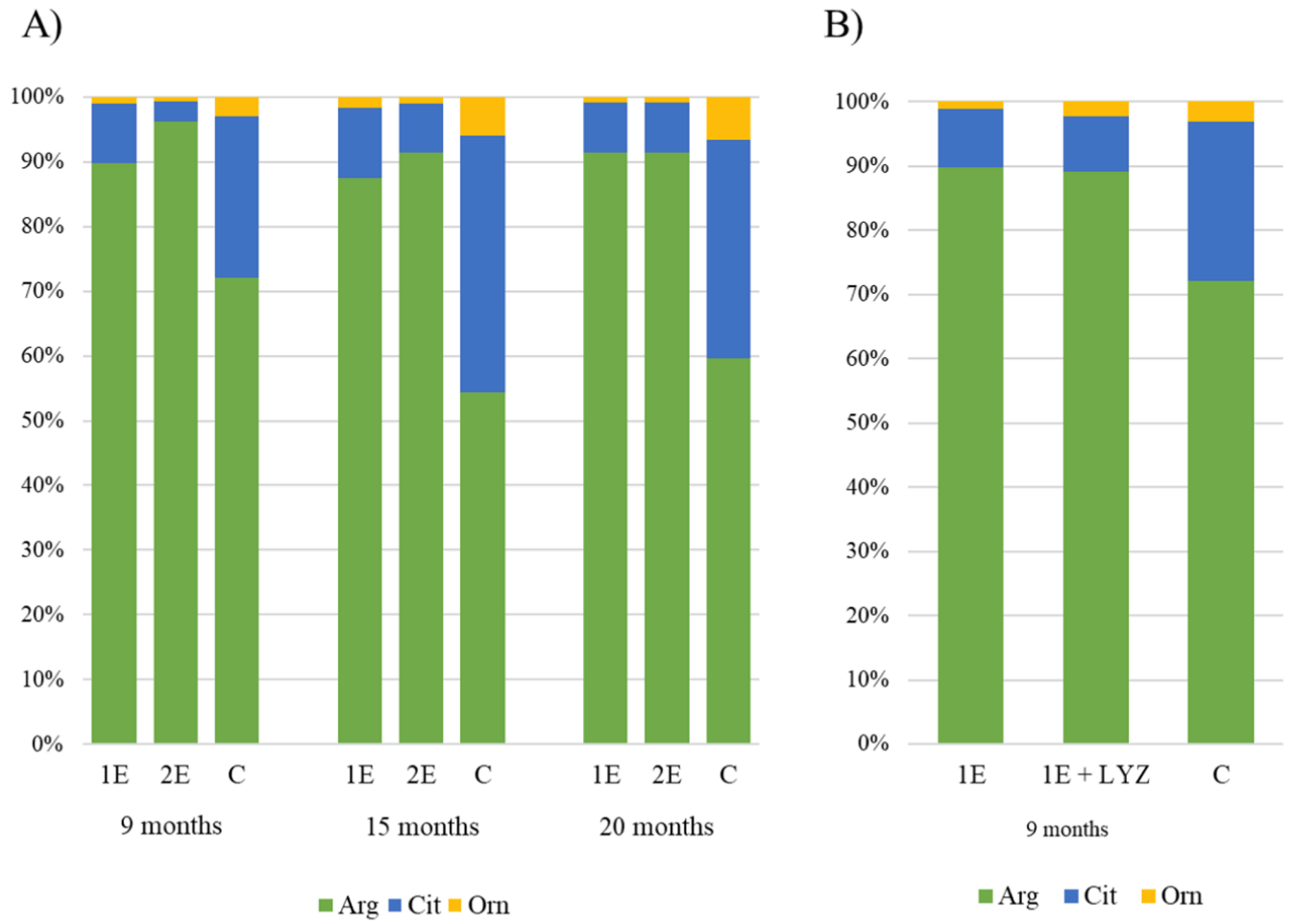

Fig. 3. A) Relative content of arginine (Arg), citrulline (Cit) and ornithine (Orn) in experimental cheeses produced with either single (1E) or double (2E) centrifugation of milk in parallel with the corresponding control (C) cheeses at different ripening times. **B)** Relative content of Arg, Cit and Orn in C and 1E cheeses produced with (1E + LYZ) and without (1E) the addition of lysozyme and in parallel with a control cheese.

The heterofermentative *L. fermentum,* recently reclassified as *Limosilactobacillus fermentum,* is known for its functional properties (Ale et al 2020). However, its excessive growth during the aging of cheese leads to the production of gas and thus the formation of micro-holes which are undesired in cheeses like GP (Xue et al 2021). Interestingly, *L. fermentum* was always present in concentrations more than one log unit lower than *Lacticaseibacillus* in both C and E cheeses (Fig. 2-iii). However, compared to what observed for *Lacticaseibacillus*, the trend of *L. fermentum* in E cheeses (especially for 2E cheeses) was less regular.

3.2. Casein proteolysis and release of amino acids

The breakdown of casein in cheeses was monitored by capillary zone electrophoresis (CZE) (Table 2). This technique is particularly appropriate for monitoring primary proteolysis because it allows to separate the residual intact caseins as well as the main breakdown products (Recio et al., 2001; Børsting et al., 2012). Chymosin and plasmin are responsible for primary proteolysis in cheese through the preferential breakdown of α_{s1}- and β-CN, respectively. Beside the specific cleavage of κ-CN, chymosin splits the α_{s1}-CN into α_{s1}-CN (f1-23) and α_{s1}-I-CN (f24-199), both undergoing further hydrolysis during cheese ripening (Singh et al., 1997). Accordingly, the α_{s1}-I/α_{s}1 peak area ratio progressively decreased until 20 months of ripening likewise for C and E cheeses. A comparable behaviour was noted for the α_{s0}-I/α_{s0} ratio, with the α_{s0}-CN fraction differing from α_{s1}-CN for an additional phosphate group. Fragment (f1-23), which is common to α_{s1}-CN and α_{s0}-CN, is rapidly hydrolysed by starter proteinases, likely because of its small size (McSweeney, 2004) and this may explain why the α_s-CN (f1-23)/$\alpha_{s(1+0)}$ ratio remained almost stable during ripening. This was evidenced in both E and C cheeses, albeit the ratio values were lower ($p < 0.05$) in the former, possibly due to the hydrolytic activity of different microbial proteinases between the two cheese groups. The activity of plasmin is particularly relevant in cooked cheeses where it brings to accumulation of selected peptides that may represent characterizing indices (Upadhyay et al., 2006; Masotti et al., 2010; D'Incecco et al., 2020b). In the present study, plasmin activity was monitored on both α_{s1}-CN and β-CN through the values of α_{s1}-PL/$\alpha_{s\ (1+0)}$ and ɣ-CN/β-CN ratios, respectively (Table 2). Both ratios increased during ripening, regardless of whether E or C cheeses, with minor and not systematic differences. These trends suggested the ongoing plasmin activity without interfering effects of milk centrifugation. Further removal of somatic cells, with plasminogen-activator activity, by centrifugation is mitigated by the preliminary creaming step. Indeed, natural creaming of fat causes around 90% removal of somatic cells (Caplan et al., 2013). Accumulation of ɣ-CNs in cheese has been reported by other authors (Børsting et al., 2012; D'Incecco et al., 2020b; Upadhyay et al., 2006).

The progress of secondary proteolysis was studied by evaluating the content of FAA. In accordance with previous studies (Masotti et al., 2010), the total amount of FAA was already high (18–20% of cheese protein) in 9-month-old cheeses (Table 2). In the subsequent ripening period (9–20 months), the total FAA contents increased slightly, for both C and E cheeses, since FAA breakdown occurs together with the release.

The typical population of NSLAB of GP cheese, with many species belonging to genus *Lacticaseibacillus,* contributes to the metabolic

Table 3
Volatile compounds identified in experimental and control cheeses at 20-month ripening.

Chemical class	Volatile compound	1E	C	SL
Acids	acetic acid	2.05 ± 0.08	2.28 ± 0.16	n.s.
	butanoic acid	5.85 ± 0.24	5.91 ± 0.40	n.s.
	hexanoic acid	4.53 ± 0.22	4.61 ± 0.51	n.s.
	octanoic acid	0.11 ± 0.01	0.15 ± 0.02	*
Ketons	2-pentanone	0.82 ± 0.20	0.94 ± 0.13	n.s.
	2-heptanone	0.50 ± 0.03	0.57 ± 0.07	n.s.
	2-nonanone	0.26 ± 0.10	0.34 ± 0.10	n.s.
	2-undecanone	0.02 ± 0.01	0.03 ± 0.01	n.s.
Esters	ethyl butanoate	0.33 ± 0.01	0.70 ± 0.06	***
	ethyl hexanoate	0.13 ± 0.02	0.33 ± 0.03	***
	ethyl-S-lactate	0.01 ± 0.00	0.03 ± 0.00	***
	ethyl octanoate	0.02 ± 0.00	0.04 ± 0.01	***
Alcohols	ethanol	0.86 ± 0.06	0.99 ± 0.01	*
	2-pentanol	0.12 ± 0.01	0.30 ± 0.12	n.s.
	1-butanol	0.01 ± 0.00	0.02 ± 0.01	*
	2-heptanol	0.03 ± 0.00	0.06 ± 0.02	n.s.
	1-hexanol	0.01 ± 0.00	0.02 ± 0.01	n.s.

Results of control (C) and experimental (E) cheeses are expressed as peak area counts corrected on peak area of the internal standard and shown as mean ± standard deviation. E cheeses were produced with single (1E) centrifugation of milk in parallel with the corresponding control cheeses. SL, significance level for the differences between the two groups of samples according to the t-Test: n.s., not significant ($p > 0.05$); *, significant ($p < 0.05$); ***, highly significant ($p < 0.001$).

degradation of FAA in the late ripening, i.e. when intracellular aminopeptidases are released (Gobbetti et al., 2015). Therefore, the relative patterns of FAA were expected to be more informative in this study. In fact, the relative levels of some individual FAA were significantly ($p < 0.05$) different between E and C cheeses, regardless the ripening time (Table S1). In detail, Ser content was lower in all of the E cheeses, compared to the corresponding C cheeses, while the content of Ala had the opposite trend. Interestingly, Ala can be used by SLAB but can be also produced by homofermentative lactobacilli through Ser catabolism (Liu, 2003).

Furthermore, we observed a reduced conversion of Arg into Cit and Orn in E cheeses, compared to the respective C cheeses (Fig. 3A). Arg is a common source of energy for some NSLAB via the arginine deiminase (ADI) pathway. It generates one mole of ATP by deaminating one mole of Arg into Cit that can be further converted into Orn (Laht et al., 2002). The concurrent production of NH_3 is a route for pH control in acidic environment (Zúñiga et al., 1998). This finding may suggest that NSLAB removed by milk centrifugation included species able to adopt the ADI pathway. However, the absence of lysozyme in E cheeses could have reduced the microbial degradation of Arg into Cit (D'Incecco et al., 2016). Lysozyme is normally used in GP cheesemaking to prevent the cheese late blowing defect (D'Incecco et al., 2018). Thus, also the lack of this enzyme in E cheeses could be responsible for their higher content of Arg. To clarify this point, additional experimental cheeses were manufactured from centrifuged milk with and without the addition of lysozyme. The relative contents of Arg, Cit and Orn in the 9-month ripened cheeses evidenced that the ADI pathway was equally adopted in the E cheeses, regardless the presence lysozyme, and less than in C cheeses (Fig. 3B). This result indicated that the centrifugation of milk was clearly the factor responsible for the different FAA pattern. Clearly identifying the microbial metabolisms of FAA taking place during cheese ripening is difficult, also because pathways are interconnected with metabolism of C5 and C6 compounds, and of citric acid (Gobbetti et al., 2015).

3.3. Volatile compounds in 20-month ripened cheeses

Although some FAA resulting from casein breakdown may directly impact cheese flavour, they mostly represent precursors of other flavour compounds (McSweeney and Sousa, 2000). Formation of volatile compounds (VOCs) is often associated with the presence of NSLAB species which can have a great potential in producing flavour compounds (Gobbetti et al., 2015; Pellegrino et al., 2021). Thus, the VOCs profile of both E and C cheeses at 20 months of ripening was determined by SPME-GC (Table 3). Ethyl esters, in particular ethyl butanoate and ethyl hexanoate, were identified as the most important compounds in GP cheese flavour, due to their contribution to the characteristic fruity and cheesy notes of this cheese (Qian & Reineccius, 2003; Lazzi et al., 2016). As evidenced in Table 3, the content of ethyl esters was significantly lower in E samples than in C samples; the same trend was observed for compounds belonging to the other chemical classes in the table, although the differences were not always significant. These results indicated a depletion of VOCs in E cheeses, likely related to the lower content of the viable cells, and confirmed the important contribution the microbial species removed by milk centrifugation may give to cheese aroma development.

3.4. Descriptive sensory profile

The average profiles for 13 sensory traits of both E and C cheeses at the three ages (i.e. 9, 15 and 20 months) were obtained from the scores given by the assessors (Fig. 4). Three traits linked to mechanical response, i.e. texture, granular structure, and friability, showed lower average scores in E cheeses, compared to the respective C cheeses, indicating a partial loss of the characteristic structural features of hard

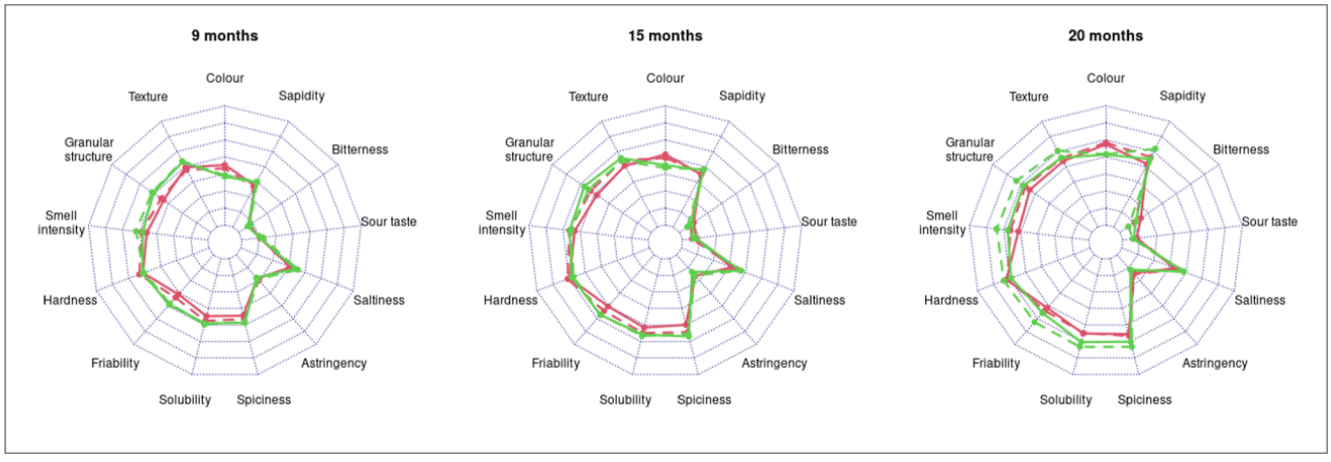

Fig. 4. Sensory profiles defined by a panel test for experimental (red) and control (green) cheeses at different ripening times. Experimental cheeses were produced with either single (solid line) or double (dashed line) centrifugation of milk in parallel with corresponding control cheeses.

Table 4
Significance levels of sensory profiles defined by the panel test for experimental cheeses compared with the respective control cheeses.

Trait	1E			2E		
	9 months	15 months	20 months	9 months	15 months	20 months
Colour	***	***	**	**	***	*
Sapidity	*	n.s.	n.s.	*	n.s.	*
Bitterness	n.s.	n.s.	n.s.	n.s.	*	*
Sour taste	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Saltiness	*	***	***	***	n.s.	*
Astringency	ns	*	n.s.	n.s.	n.s.	n.s.
Spiciness	***	**	*	n.s.	n.s.	**
Solubility	**	*	*	n.s.	n.s.	*
Friability	***	***	*	n.s.	n.s.	**
Hardness	n.s.	n.s.	n.s.	n.s.	*	n.s.
Smell intensity	n.s.	n.s.	*	n.s.	n.s.	**
Granular structure	***	***	n.s.	***	n.s.	n.s.
Texture	*	*	n.s.	***	n.s.	n.s.

1E = one centrifugation step; 2E = two centrifugation steps. p-values coming from Tukey's HSD test at each ripening time. n.s., not significant ($p > 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$).

cheeses, although hardness itself was not distinctive. The colour trait received higher scores (i.e. more intense yellowness) in E samples than in C samples. Typical flavours (smell intensity, sapidity, spiciness) were less perceived in E cheeses, in accordance with outcomes of VOCs analyses, and the differences with the C cheeses increased with the aging progress. Traits linked to flavour defects (bitterness, sour taste, astringency) were not influenced by milk centrifugation and supported the absence of defects in both cheese groups. The overall shape of the plots at the three stages of ripening was the same, although the differences on the most charactering traits for hard cheeses progressively increased.

The Tukey's HSD test on the scores assigned for the 13 sensory traits gave the p-values reported in Table 4. In one case (2E, 15 months) three traits resulted significantly different, but in all the other cases samples differed for 5–8 traits. Colour was a major driving trait, as it resulted significantly different in all six combinations of treatment and aging whereas no significant differences were perceived for sour taste. All in all, the analysis of sensory profiles indicated that the studied treatment introduced non-negligible changes in the final characteristics of the product.

3.5. Principal component analysis

Considering that a large multivariate dataset was monitored in this study, we evaluated which variables could better differentiate between E and C cheeses through the principal component analysis (PCA) (Fig. 5). Loadings were > 0.7 for six variables, i.e. Arg, Cit, Orn, Gln, Ser and viable lactobacilli counts, that brought to the cheese positioning in the vector space shown in Fig. 5A. Globally, the selected variables explained 81.8% of the total variability of the dataset. The E cheeses (empty circles), mostly grouped in the negative part of the component 1, while C cheeses (solid circles) distributed along its positive segment. Differently, cheeses progressively distributed along the component 2 depending on the ripening time, regardless the cheese type. The loading plot (Fig. 5B) revealed that Cit, Orn and Arg accounted for separation between E and C cheeses along component 1, in agreement with the differences observed for the ADI pathway utilization. Instead, Ser and Gln, identified as descriptors of proteolysis progression in raw-milk hard cheeses (Masotti et al., 2010; Hogenboom et al., 2017), and viable lactobacilli counts accounted for the spreading of cheeses along the component 2.

4. Conclusions

Although conducted at an unusual temperature (39 °C), the high-speed centrifugation of milk tested in this study caused a selection of microbiota of milk that undoubtedly reflected on that of cheese. The concentration of viable lactobacilli was lower in E cheeses throughout the entire ripening period but also species having less propensity to adopt the ADI pathway or metabolise FAA into VOCs were selectively retained. More in general, we have evidenced that removal of autochthonous NSLAB species could be potentially negative for any type of raw-milk cheese, because it influences microbial pathways operating in

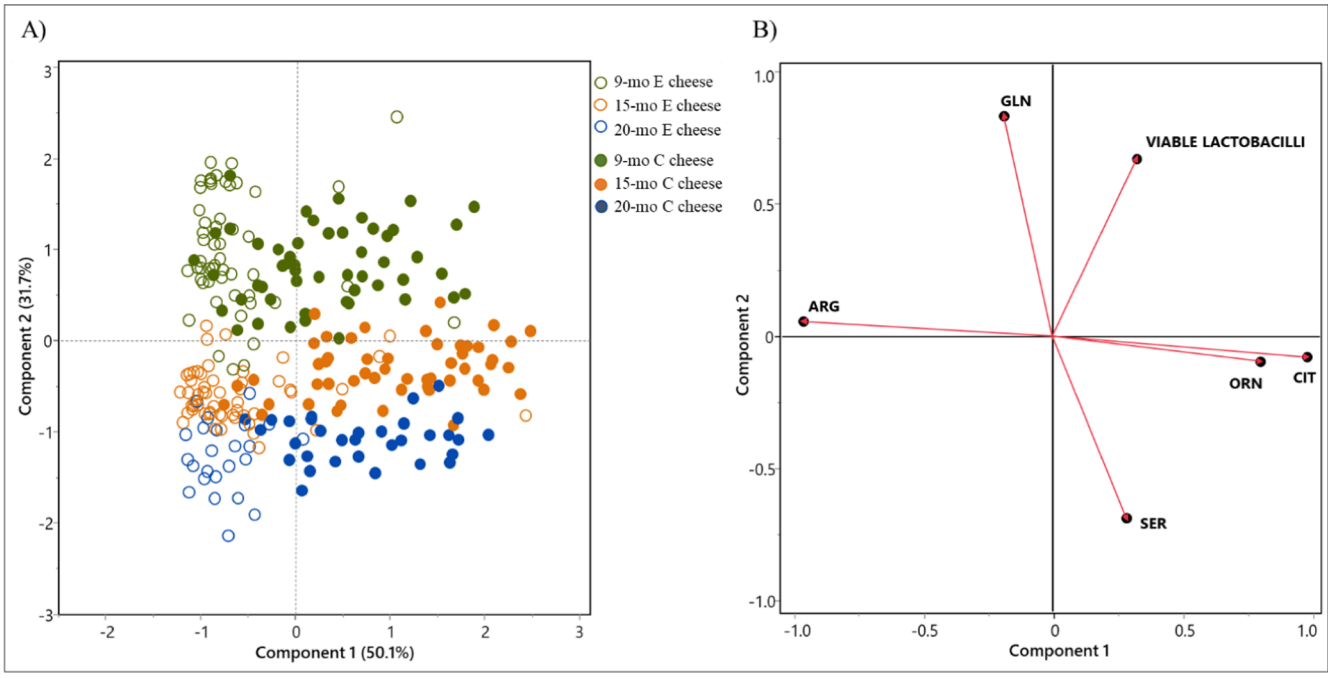

Fig. 5. Principal component analysis of experimental (empty circles) and control (solid circles) cheeses ripened for 9, 15 and 20 months. Panel A refers to score plot and panel B refers to loadings of single variables: Arg = arginine, Gln = glutamine, Cit = citrulline, Orn = ornithine, Ser = serine and viable lactobacilli.

cheese ripening. In this context, restrictions provided by specifications of raw-milk PDO cheeses aim to preserve the unique characteristics of those cheeses. Pre-treatments of raw milk that even partially modify its native microbiota may affect the subsequent ripening process of the cheese in various ways. We have demonstrated that high-speed centrifugation of milk, altered the typical FAA pattern as well as various sensory attributes of Grana Padano cheese thus impairing the unique characteristics deeply appreciated by consumers worldwide.

CRediT authorship contribution statement

Paolo D'Incecco: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Luca Bettera:** Formal analysis, Data curation, Writing – original draft. **Elena Bancalari:** Data curation, Writing – original draft. **Veronica Rosi:** Formal analysis. **Marta Sindaco:** Formal analysis, Data curation. **Serena Gobbi:** Formal analysis. **Paolo Candotti:** Formal analysis. **Nelson Nazzicari:** Data curation. **Sara Limbo:** Writing – review & editing. **Monica Gatti:** Conceptualization, Writing – review & editing. **Luisa Pellegrino:** Conceptualization, 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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113102.

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