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Characterisation of Conciato Romano: one of the oldest Italian cheeses

Giuseppe Blaiotta [a], Raffaele Marrone [b], Maria Aponte [a, *], Maria Francesca Peruzy [b], Giorgio Smaldone [a], Lucia Vollano [b], Nicoletta Murru [b, c]

[a] Department of Agricultural Sciences, University of Naples "Federico II", Via Università 100, 80055, Portici (Na), Italy
[b] Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Via Delpino 1, 80137, Napoli, Italy
[c] Task Force on Microbiome Studies, University of Naples "Federico II", Naples, Italy

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ABSTRACT

Conciato Romano is believed to be the oldest Italian ewe cheese; it is manufactured according to an unusual technology, ending with a long ripening in earthenware jars. Cheese was monitored during production and ripening in two different seasons: winter and spring. According to the data obtained, the ripening in jars prevents lipid oxidation, and ensures the safety of the product. Coagulase-positive staphylococci, *Salmonella* and *Listeria* spp. were never retrieved, while *Enterobacteriaceae* and *Escherichia coli* disappeared during the ripening, likely due to the antimicrobial activity of the tanning of the cheeses. Mesophilic lactic acid bacteria dominated the entire production process, with *Lactiplantibacillus plantarum* subsp. *plantarum* and enterococci accounting for about 26% and 30%, respectively, of the total cultures isolated (203). Total monounsaturated fatty acids and ω-3 and ω-6 polyunsaturated fatty acids were higher in cheeses produced during spring, while the atherogenic index was significantly lower. Outcomes of texture analysis confirmed seasonal differences, stressing the role of the animals' feeding regimen.

1. Introduction

Conciato Romano is believed to be the oldest Italian cheese and, in spite of the name, it dates back to the Samnite civilisation. In fact, the ripening and curing techniques suggest ancient practices, at the dawn of agro-pastoral civilisation (Caporaso, Armento, & Sacchi, 2015).

Conciato Romano cheese is manufactured by a short supply chain system, in tiny but precisely demarcated area of the Caserta province in the South of Italy. It is produced by coagulating sheep milk with rennet paste. The unusual aspect of the production technology is the management of the cheeses after a first ripening in the so-called 'fuscella' moulds. In fact, cheeses are washed with the cooking water of 'pettole', a traditional homemade pasta. Once dried, cheeses are tanned with a blend of oil, white wine, chili pepper, and 'piperna', a type of wild thymus (*Thymus serpyllum* L.), hence the name Conciato (concia is the Italian for tanning). Cheeses are then left to ripen in terracotta jars and stored in the dark. The vessels are moved periodically (every 10 days) to ensure uniformity of ripening, for an aging period of almost 6 months and up to 2 years. Cheese ripening is carried on by the metabolic activity of natural occurring microflora, since no starter culture is added. The production process is summarised in Fig. 1. As matter of fact, cheese organoleptic properties proved to be affected by herbs recurring where animals graze and by the typical tanning treatment of the cheese (Caporaso et al., 2015; Mormile et al., 2013).

Small ruminant food products are gaining major interest for their nutritional properties. Sheep milk cheeses provide an interesting nutritional regime for both children and adults and also supply essential nutrients, such as minerals and vitamins (Albenzio et al., 2016). Moreover, consumers exhibited growing interest in additional milk and cheese quality parameters, mainly related to environmental and animal welfare aspects (Canfora, 2016).

Conciato Romano was the first product of the Caserta province (Campania Region) to become a Slow Food Presidium back in 2000. In spite of its long history, the cheese is now produced by very few farms, thus becoming a further local production sentenced to extinction. Conciato Romano is undeniably a high added-value niche cheeses and, in spite of the originality of the manufacturing process and despite its popularity, has never been characterised, except from one study focused on its volatile profile (Caporaso et al., 2015).

In general, the study of the microbiological characteristics of cheeses is fundamental to improve their quality in accordance with

* Corresponding author. Tel.: +44 0812539398.
E-mail address: aponte@unina.it (M. Aponte).

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current legislation and to preserve, at the same time, the microbial biodiversity and their characteristic nature. For this reason, the aim of the present survey was to evaluate Conciato Romano cheese during its production and ripening under a microbiological, physico-chemical and rheological point of view. Moreover, to point out the effect of the animal feeding on the cheese characteristics, manufacturing was monitored in two different seasonal conditions: winter and spring.

2. Material and methods

2.1. Cheese manufacturing and sampling

Two Conciato Romano cheese manufactures, carried out according to the traditional technology detailed in Fig. 1, were monitored in winter (Trial I) and spring (Trial II) season in a dairy plant located in Caserta (Southern Italy). According with the strong traditional character of the cheese and with the usual dairy farm size, 10 L of raw milk were processed for each production. A total of seven samples were collected for each production: milk (M), curd (C), whey (W), "primosale" (i.e., fresh, lightly salted cheese) 24 h after production (Cf_0), cheese after 25 days of drying (Cf_{25}) and cheese after 60 (Cf_{60}), 80 (Cf_{80}) and 120 (Cf_{120}) days of ripening in jar. Samples were transported at 4 °C to the laboratory and analysed within few hours.

2.2. Physicochemical analysis

Moisture, N total and NaCl content were determined according to the guidelines of the Association of Official Analytical Chemists (AOAC, 2000a,b,c). pH was measured in a slurry prepared by mixing 10 g of chopped cheese in 10 mL of deionised water. Water activity (a_w) was determined by AQUALAB dew point water activity meter 4 TE (Pullman, WA, USA). Cheese fat content was measured using the Gerber method (AOAC, 2012).

The extent of lipolysis in cheese during maturation was evaluated as free fatty acid (FFA) liberation by titration with 0.05 N ethanolic KOH according to Nunez, Garcia-Aser, Rodriguez-Martin, Medina, and Gaya (1986). Lipid oxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) according to Rosmini et al. (1995); TBARS were recorded at 538 nm using a Jasco V-530 spectrophotometer (Jasco, Tokyo, Japan). Results were expressed as mg malon dialdehyde kg^{-1} sample (ppm).

Fatty acid profiles were determined by gas chromatography according to the method proposed by Bligh and Dyer (1959), modified according to Marrone et al. (2014). A Dani GC 1000 DPC gas chromatograph (DANI Instruments SpA) with capillary column SP-2380 fused silica, 100 m × 0.25 mm ID, 0.25 μm film thickness and flame ionisation detection was used. Fatty acid methyl esters were identified by comparison of the retention times of the peaks in the sample with previously run pure standard compounds (Sigma, St Louis, MO, USA). Fatty acids were expressed as percentage of the sum of total FA in the analysed sample. The FA contents were expressed as weight percentages, % w/w (g FA 100 g^{-1} of fat). From the fatty acid profile, atherogenic (AI) and thrombogenic index (TI) were calculated (Ulbricht & Southgate, 1991).

2.3. Instrumental texture and colour determinations

The texture parameters were determined using the S texturometer EZ-Test Shimadzu (Shimadzu Corporation, Tokyo, Japan), by carrying out the texture profile analysis (TPA) described by Bourne (2002). Two cheese wheels were collected under sterile conditions and processed after removing a 0.5 cm layer from the surface. Five (one on the middle and four on different parts of the cheese surface) replicas per sample were carried out. Penetration tests were performed at room temperature (20 ± 2 °C) using a stainless cylindrical probe of 25 mm (relative size of the plate to the sample 5:1) at a constant rate of 50 mm s^{-1} of crosshead speed and compressing samples to 80% using two compression cycles. From the force versus time texturograms, eight parameters were obtained: hardness, fracturability, adhesiveness, springiness, cohesiveness, gumminess, chewiness and resilience. The interpretation of these texture parameters was made according to Armero and Collar (1997) and Bara-Herczegh, Horvath-Almassy, Csanadi, and Orsi (2002).

Colour analyses were performed using a colorimeter CR 300 (Minolta, Osaka, Japan). The L*, a*, and b* colour measurements were determined according to the CIELAB colour space, were L* a* b*correspond to light/dark (0% dark to 100% light), green/red (−60% green to 60% red), and blue/yellow (−60% blue to 60% yellow) chromaticity, respectively. Longitudinal cheese samples, measuring 1 cm thick, were taken by duplicate, exposing the internal surface of the cheese, and five measurements were carried out at different points of the exposed surface.

2.4. Microbiological analyses

Samples were homogenised in sterile quarter strength Ringer's solution (Oxoid, Basingstoke, UK) with a StomacherLab-Blender400 (Seward Medical, London, UK) for 2 min, serially diluted and spread-plated in duplicate for both microbial enumeration and isolation. The following media and incubation conditions were used: total viable count (TVC) at 30 °C and at 4 °C (ISO, 2003); total coliforms (ISO, 2006), *Escherichia coli* (ISO, 2001a), *Enterobacteriaceae* (ISO, 2004a), coagulase-positive staphylococci (ISO, 2004b), yeasts on Rose-Bengal chloramphenicol agar containing chloramphenicol selective supplement (Oxoid) at 22 °C for 5 days, sulphite-reducing clostridia on SPS agar (Oxoid) at 43 °C for 24 h, enterococci on kanamycin aesculin azide agar base (Oxoid) with kanamycin selective supplement (KAA, Oxoid) at 37 °C for 24–48 h, lactococci on M17 Agar (Oxoid) at 30 °C for 72 h under anaerobic conditions, and mesophilic and thermophilic lactobacilli on MRS agar (Oxoid) at 30 °C for 5 days and on Rogosa agar (Oxoid) at 42 °C for 72 h, respectively, in both cases under anaerobic conditions (Anaerogen kit, Oxoid). Finally, each sample was screened for the presence of *Salmonella* spp. (ISO, 2001b) and *Listeria monocytogenes* (ISO, 1996).

2.5. Lactic acid bacteria isolation and identification

A total of 142 colonies were randomly picked from MRS, M17, KAA, and Rogosa agar plates, seeded with the highest sample dilutions, to analyse dominant LAB species. Each colony was purified by repetitive streaking on the same media, and incubating at 30 or 37 °C for 48 h. All isolates were characterised by Gram staining, catalase activity, and spore formation. All isolates were stored at −25 °C in MRS broth added of sterile glycerol (20%).

Cultures were identified by means of molecular techniques. DNA from cocci-shaped LAB was obtained by InstaGeneTM Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's recommendations. The protocol described by Aponte et al. (2012) was adopted for DNA extraction by presumptive lactobacilli. All reagents were provided by Merck (Milan, Italy).

Cocci-shaped LAB were identified by 16S-23Sr DNA spacer region PCR amplification using primers described by Jensen and Straus (1993). Cocci characterised by a spacer of 360 bp were reported as belonging to the genus *Streptococcus* spp. and identified at species level by carrying out species-specific PCR for *St. thermophilus* (Lick, Keller, Bockelmann, & Heller, 1996). Rod-shaped LAB were reported to the genus formerly known as *Lactobacillus* spp., by means of 16S-23S rDNA spacer analysis. Prior to identification at species level, lactobacilli strains were

analysed by RAPD-PCR (Random Amplified Polymorphic DNA) with M13-R2 (5'GGAAACAGCTATGACCATGA3') primer according to the protocol described by Rossetti and Giraffa (2005). Gels were analysed using the Bionumerics software, version 5.1 (Applied Maths, Kortrijk, Belgium). RAPD-PCR patterns were grouped by means of cluster analysis with the Pearson's product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA). The profiles generated by both primers were concatenated by means of Bionumerics software to obtain a single dendrogram. Strains representative of the obtained patterns' groups were identified by sequencing the 5′ end of the 16S rDNA. PCR amplification of the16S rDNA gene was performed according to Weisburg, Barns, Pelletier, and Lane (1991). The 16S rDNA PCR fragments were purified from agarose gel 1.5% (w/v) by Qiaquick Gel Extraction Kit (Qiagen, Milan, Italy) according to the supplier's instructions. The DNA sequences were determined by the dideoxy chain termination method (Sanger, Nicklen, & Coulson, 1977) using the forward primers (fD1) described by Weisburg et al. (1991). Research for DNA similarity was performed with the National Centre of Biotechnology Information GenBank.

2.6. Statistical analysis

Analyses, when not otherwise specified, were performed in triplicate. Analysis of variance was made on data collected for each stage of ripening using the General Linear Model procedure of NCSS (East Kaysville, Utah, USA). Turkey test was used for comparison of data. Level of significance was set for $p < 0.01$.

3. Results and discussion

3.1. Physicochemical analyses

According to pH data, the acidification rate varied according to the season. In Trail I, carried out during winter, the pH started

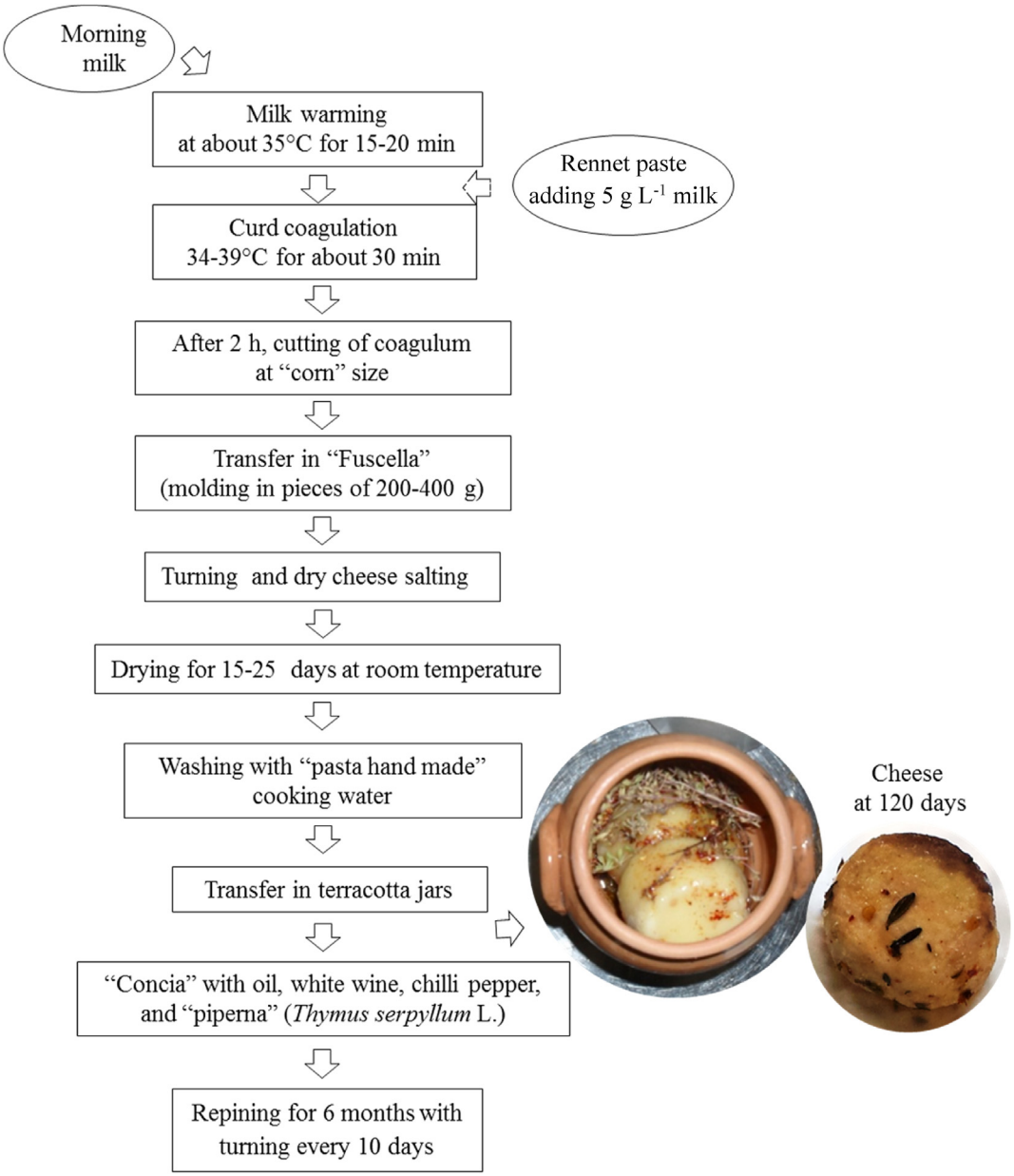

Fig. 1. Flow chart of the Conciato Romano cheese-making process.

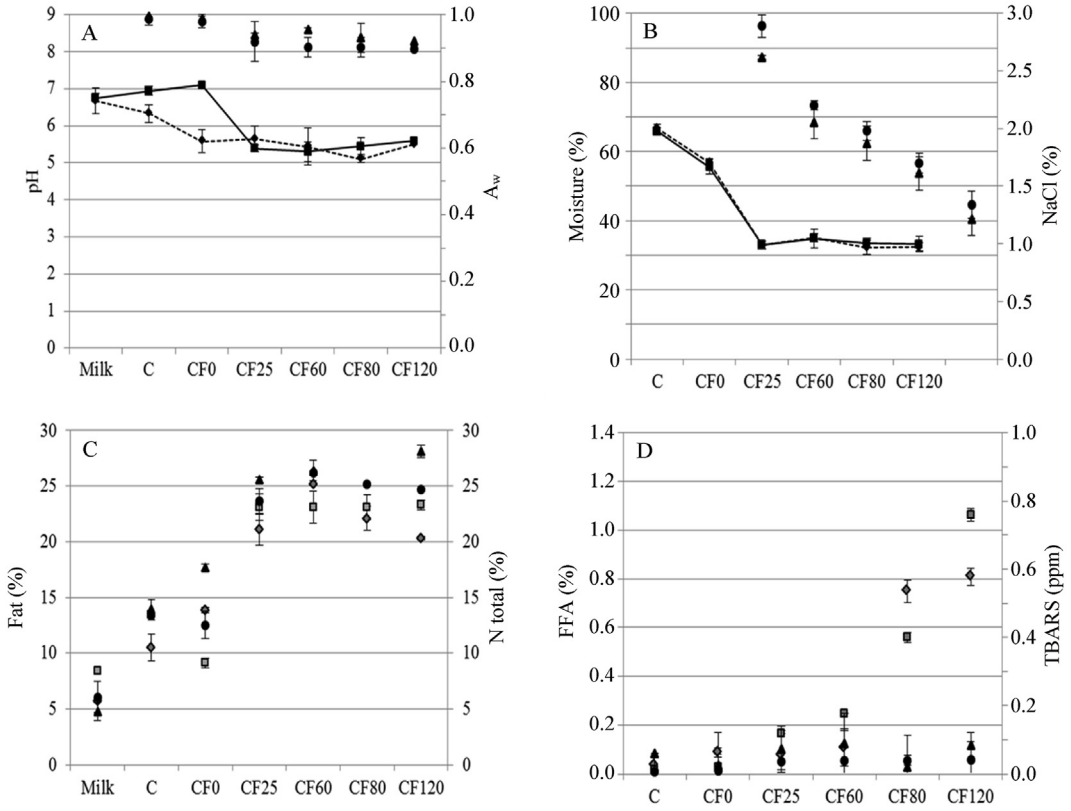

Fig. 2. Evolution during the manufacturing and ripening of Conciato Romano cheese produced in winter (Trial I) and spring (Trial II) of: A, pH (I, ◆; II, ■) and a_w (I, ▲; II, ●); B, moisture (I, ◆; II, ■) and NaCl (I, ▲; II, ●); C, N total (I, ▲; II, ●) and fat content (I, ◆; II, ■); D, FFA (I, ◆; II, ■) and TBARS (I, ▲; II, ●). C, curd; Cf_0, "primosale"; Cf_{25}, cheese after 25 days of drying; Cf_{60}, Cf_{80}, and Cf_{120}, cheese after 60, 80 and 120 days, respectively, of ripening in jar.

to drop only during the first 25 days of drying (Fig. 2). The a_w decreased during the drying and then stayed almost constant at about 0.91 up to the end of monitoring (Fig. 2). Moisture decrease perfectly reflected the dynamics of the cheese manufacture, with values remaining constant during the storage in jars (Fig. 2). Moreover, no apparent differences could be noticed between the two productions. NaCl content drastically diminished from 2.7% in the "primosale" (25th day) to around 1% in cheese at the end of ripening. Such a drop is likely due to the cheese washing with the cooking water of hand-made pasta, and to the slow salt release in the tanning during the ripening in jars (Fig. 1). Fat percentage was around 20% at the end of drying (25th day), while total N constantly increased up to 25–30% at the end of sampling. As expected, fat and total N did not change during the ripening in jar. Fat hydrolysis continued up to the end of ripening but the rate of lipid oxidation did not increase, as revealed by the TBARS values (Fig. 2). The protection from light and oxygen provided by the jar is likely to prevent lipid rancidity. TBARS values of the spring production (Trial II) were constantly higher than those recorded for the winter cheese. The effect of feeding based on fresh forages affected the fatty acid composition of sheep milk by increasing the content of polyunsaturated fat (Addis et al., 2005), which are known to be more susceptible to oxidation.

Results for fatty acids (FAs), which is the sum of saturated, monounsaturated and polyunsaturated FAs (∑SFAs, ∑MUFAs and ∑PUFAs, respectively) as well as other fat indices in milk and cheese along ripening are reported in Table 1. ∑SFAs did not change by passing from milk to cheese. Within ∑MUFAs, palmitoleic acid (C 16:1 n-7), proved to be the most abundant, accounting for 1.7% of FAs (data not shown). Moreover, the tanning, which contains olive oil, did not modify the FAs profile. When compared with the number of variations associated to ripening, manufacturing exerts a poor relevance on the cheese fat composition, although noticeable changes can occur in the conjugated linoleic acid (CLA) positional and geometrical isomers during this phase (Marrone et al., 2014). Rumenic acid (cis-9, trans-11–18:2) content enhanced by about 0.2% with ripening in both productions (data not shown).

At the end of the monitoring, ∑MUFAs, ω-3 and ω-6 were statistically different ($p < 0.05$) in the productions carried out in the two seasons with higher values for cheeses produced during spring (Trial II). Such differences are to be put in relation with animals' feeding that in Campania is generally semi-extensive, with pasture playing a crucial role during the warm season. A large body of literature reports a "season" effect on the FA composition of the milk fat, with a higher PUFA and CLA concentration in spring, due to the corresponding PUFA increment in the fresh forage (Serrapica et al., 2020). On the other end, CLAs, even if more abundant in spring cheeses, did not experience significant differences (Table 1). Such results perfectly match those reported by Tsiplakou, Kominakis, and Zervas (2008) for ewe milk collected during winter months from sheep kept indoors and fed with alfalfa hay plus concentrates or from sheep grazing native pastures from April onwards. According to authors, the diet had a significant effect on FA profile, with grazing causing higher proportions of unsaturated FAs.

The AI characterises the atherogenicity of dietary fat: higher AI values are assumed to be more detrimental to human health. In the human diet, lipids (particularly saturated FAs) are known to contribute to coronary diseases. On the contrary, some unsaturated

Table 1
Sum of fatty acids and the atherogenic and thrombogenic indices of samples collected during Conciato Romano cheese manufacturing.[a]

Parameter	Trial	Sample						
		M	C	Cf_0	Cf_{25}	Cf_{60}	Cf_{80}	Cf_{120}
∑SFAs	I	66.19 ± 1.02	64.98 ± 0.89	65.82 ± 0.35	65.39 ± 2.71	66.62 ± 3.02	66.66 ± 2.90	67.30 ± 3.01
	II	67.13 ± 1.67	65.52 ± 1.91	65.95 ± 2.03	65.54 ± 2.56	66.07 ± 0.87	66.25 ± 1.14	67.15 ± 0.99
∑MUFAs	I	28.01 ± 0.32	29.06 ± 1.15	27.44 ± 2.02	28.55 ± 0.28	27.98 ± 0.15	25.90 ± 0.56	26.63 ± 2.17^{A}
	II	28.24 ± 0.99	30.10 ± 0.14	28.32 ± 1.34	28.98 ± 1.56	28.56 ± 1.35	26.95 ± 1.49	28.70 ± 2.14^{B}
∑PUFAs	I	3.33 ± 0.03	3.35 ± 0.00	3.92 ± 0.56	3.45 ± 0.02^{A}	3.72 ± 0.23	4.13 ± 0.12	3.68 ± 0.01
	II	3.51 ± 0.90	3.38 ± 0,12	3.93 ± 0.01	3.76 ± 0.01^{B}	3.93 ± 0.00	4.09 ± 0.23	3.79 ± 0.34
ω-3	I	1.47 ± 0.00^{a}	1.46 ± 0.01	1.57 ± 0.03^{a}	1.45 ± 0.00	1.63 ± 0.02^{a}	1.69 ± 0.00	1.54 ± 0.01^{a}
	II	1.51 ± 0.01^{b}	1.46 ± 0.00	1.65 ± 0.01^{b}	1.47 ± 0.04	1.57 ± 0,00^{b}	1.66 ± 0.00	1.61 ± 0.03^{b}
ω-6	I	1.86 ± 0.04^{A}	1.89 ± 0.01^{a}	2.35 ± 0.02^{A}	2.01 ± 0.03^{A}	2.09 ± 0.04^{A}	2.45 ± 0.00	2.13 ± 0.02^{a}
	II	2.00±0–01^{B}	1.92 ± 0.00^{b}	2.28 ± 0.01^{B}	2.30 ± 0.00^{B}	2.36 ± 0.03^{B}	2.44 ± 0.01	2.17 ± 0.03^{b}
CLAs	I	1.49 ± 0.00^{A}	1.45 ± 0.01	1.91 ± 0.05	1.60 ± 0.03^{A}	1.62 ± 0.05^{A}	1.87 ± 0.03	1.64 ± 0.04
	II	1.57 ± 0.01^{B}	1.44 ± 0.04	1.89 ± 0.06	1.87 ± 0.00^{B}	1.94 ± 0.03^{B}	1.85 ± 0.01	1.69 ± 0.00
AI	I	2.69 ± 0.05^{A}	2.48 ± 0.00	2.61 ± 0.03	2.44 ± 0.05	2.60 ± 0.01^{A}	2.77 ± 0.04	2.95 ± 0.01^{A}
	II	2.62 ± 0.06^{B}	2.34 ± 0.07	2.57 ± 0.03	2.45 ± 0.00	2.52 ± 0.02^{B}	2.67 ± 0.04	2.68 ± 0.02^{B}
TI	I	1.62 ± 0.00	1.54 ± 0.01^{a}	1.62 ± 0.00^{A}	1.57 ± 0.04	1.61 ± 0.02^{A}	1.69 ± 0.04	1.72 ± 0.00^{a}
	II	1.60 ± 0.02	1.49 ± 0.00^{b}	1.55 ± 0.01^{B}	1.52 ± 0.03	1.54 ± 0.03^{B}	1.60 ± 0.00	1.59 ± 0.03^{b}

[a] Abbreviations are: ∑SFA, sum of saturated fatty acids; ∑MUFA, sum of medium chain unsaturated fatty acids; ∑PUFA, sum of polyunsaturated fatty acids; ω-3 and ω-6, ω-3and ω-6 polyunsaturated fatty acids, respectively; CLAs, conjugated linoleic acids; AI, atherogenic index; TI, thrombogenic index; M, milk; C, curd; Cf_0, "primosale"; Cf_{25}, cheese after 25 days of drying; Cf_{60}, Cf_{80}, and Cf_{120}, cheese after 60, 80, and 120 days of ripening, respectively, in jar. Data (mean ± sd) are reported as percentage of total FAs; lowercase and uppercase superscript letters indicate statistical differences at $p < 0.01$ and $p < 0.05$, respectively, based on Turkey test for trial variables.

FAs in milk have protective effects against the risk of cardiovascular diseases, including CLA, MUFAs (in particular oleic acid) and PUFAs (Tsiplakou et al., 2008). AI was significantly lower ($p < 0.05$) in cheese produced in spring (II) by sheep pasture-fed compared with the respective produced during winter. The TI was lower in Conciato of Trial II as well ($p < 0.01$). Both indices were favourable from a health point of view. As reported by other authors (Caporaso et al., 2015; Marrone et al., 2014; Mormile et al., 2013), pasture quality, traditional cheese-making and ripening may ensure a high nutritional value to ewes' milk cheeses.

3.2. Microbiological analyses

The main microbial groups were monitored throughout the manufacturing and ripening of the two cheeses batches, namely those produced in winter (Trial I) and spring (Trial II) (Figs. 3 and 4). Coagulase-positive staphylococci and sulphite-reducing clostridia were always beyond the level of detection, while *Salmonella* and *Listeria* spp. were never retrieved (Data not showed). The absence of *Staphylococcus aureus* and *Listeria* spp. is in accordance with Aktypis et al. (2018), who studied an ovine cheese supplemented

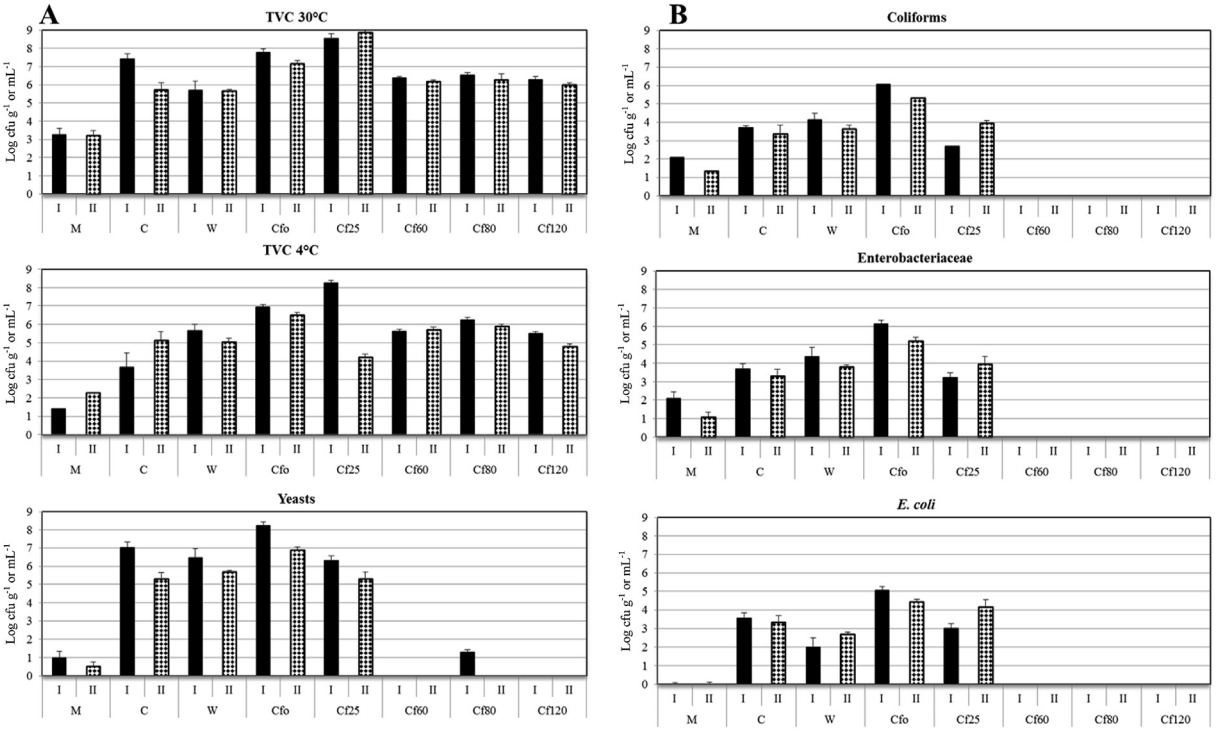

Fig. 3. Evolution of (A) TVCs at 30 °C and 4 °C and yeasts and (B) coliforms, *Enterobacteriaceae* and *E. coli* during the manufacturing and ripening of Conciato Romano cheese in produced in winter (Trial I; black bars) and spring (Trial II; diamond bars). Results are reported as log cfu mL or g^{-1}. M, milk; C, curd; W, whey; Cf_0, "primosale"; Cf_{25}, cheese after 25 days of drying; Cf_{60}, Cf_{80}, and Cf_{120}, cheese after 60, 80 and 120 days, respectively, of ripening in jar.

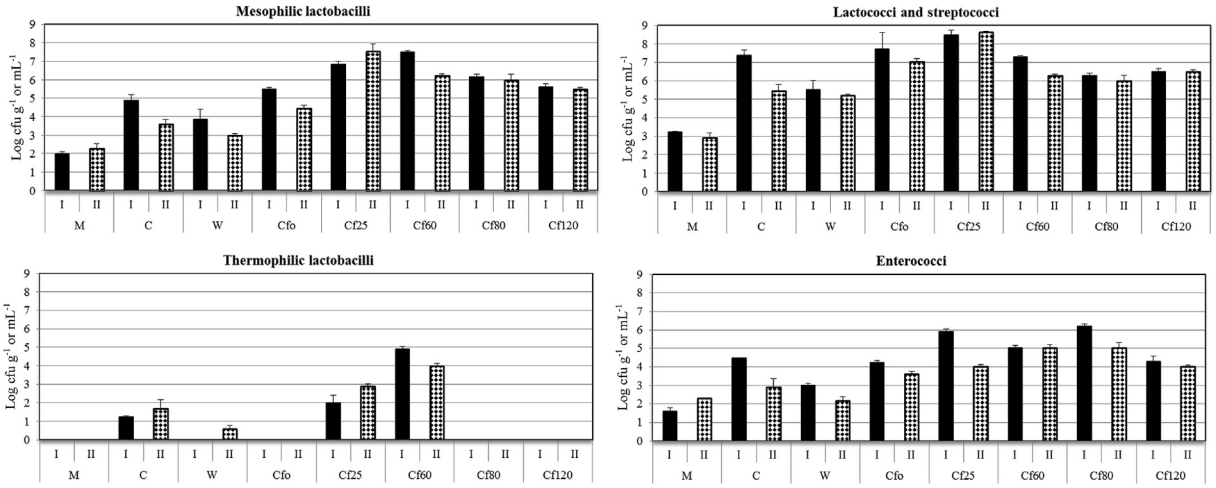

Fig. 4. Evolution of lactococci, streptococci, enterococci, mesophilic and thermophilic lactobacilli during the manufacturing and ripening of Conciato Romano cheese in produced in winter (Trial I; black bars) and spring (Trial II; diamond bars). Results are reported as log cfu mL or g^{-1}. M, milk; C, curd; W, whey; Cf_0, "primosale"; Cf_{25}, cheese after 25 days of drying; Cf_{60}, Cf_{80}, and Cf_{120}, cheese after 60, 80 and 120 days, respectively, of ripening in jar.

with saffron, as well as with Tarakci, Durmaz, Sagun, and Sancak (2005), who studied a traditional Turkish herby cheese.

Total mesophilic counts on PCA increased during cheese manufacturing and ripening. After the transferring in jars, counts stayed almost stable up to 4 months ($p < 0.05$). The same trend could be noticed for psychrophilic bacteria counted on the same medium after incubation at 4 °C, except for loads at the end of ripening, which were more variable, and generally almost one log lower if compared with the mesophilic bacteria. Counts on batches produced during winter (Trial I) were, in the case of psychrophilic bacteria, constantly higher than those recorded for samples collected along the spring production. This can be linked to the lower temperatures which might likely favour the psychrophilic microflora. Such differences appear to be reduced after the cheeses' transferring into the jars, as a result of the attainment of a constant temperature (Fig. 3A). Yeast counts reached a peak in the Primosale (Cf_0) and then decreased during the ripening up to disappear in cheeses in the jars (Fig. 3A). This behaviour could be put in relation to the general antimicrobial activity of the concia coupled to the anaerobic conditions in the jar. In addition to wine, several concia components, such as chilli, pepper and even thyme, are well known to possess antimicrobial features (Sherman & Billing, 1999). Likely due to the ability to grow at lower temperatures (Ferreira & Viljoen, 2003), yeast counts during winter (Trial I) resulted constantly higher than those recorded in the spring (Trial II) production, matching results reported by Salmeron, deVega, Perez-Elortondo, Albisu, and Barron (2002) for ewes' milk and by Caridi, Micari, Foti, Ramondino, and Sarullo (2003) for the artisanal Caprino d'Aspromonte cheese.

Table 2
Occurrence of different LAB species during cheesemaking and ripening of Conciato Romano cheese; samples collected during winter (I) and spring (II).[a]

Strain	M		C		Cf_0		Cf_{25}		Cf_{60}		Cf_{80}		Cf_{120}		Medium
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	
Enterococcus. faecalis					1	2			2	2					M17, 30 °C
Enterococcus faecium							3		2	1					
Enterococcus pseudoavium								1							
Enterococcus durans									1						
Lactococcus lactis	1	3	6		4		5	5		1	3		1	4	
Pediococcus. pentosaceus											4	2			
Streptococcus thermophilus									3	3	5				
Enterococcus durans								1							KAA, 37 °C
Enterococcus faecalis			8		1	2	5	3			5	1	4	2	
Enterococcus faecium						3	2	1		4			1	3	
Lacticaseibacillus rhamnosus										1					
Lacticaseibacillus casei								1							
Lactiplantibacillus plantarum									1						
Aerococcus viridans					1										
Ligilactobacillus acidipiscis											2				MRS, 30 °C
Levilactobacillus brevis									1						
Lacticaseibacillus casei									1	7		8			
Limosilactobacillus fermentum										5					
Lactiplantibacillus plantarum	1		4	3	1	4	2	8	8	1	4	1	4	3	
Levilactobacillus brevis								4		2					Rogosa, 42 °C
Limosilactobacillus fermentum										5					
Lactiplantibacillus plantarum			1	4				2	1	1					

[a] Abbreviations are: M, milk; C, curd; Cf_0, "primosale"; Cf_{25}, cheese after 25 days of drying; Cf_{60}, Cf_{80}, and Cf_{120}, cheese after 60, 80, and 120 days of ripening, respectively, in jar. Values are numbers of isolates of each sample.

Coliforms and *Enterobacteriaceae* counts indicated a low contamination of the milk, but during the cheese production both populations' levels increased (Fig. 3B). The same trend was described by Tabla, Gómez, Simancas, Rebollo, and Roa (2016) during manufacturing and ripening of semi–hard ewes' milk cheese. However, in their study, *Enterobacteriaceae* were still around 2 log cfu g^{-1} at the end of monitoring after 60 days, whilst in Conciato Romano, both coliforms and *Enterobacteriaceae* were no longer detectable during the ripening in jar (Fig. 3B). High *Enterobacteriaceae* counts after 60 days (>10^5 cfu g^{-1}) have been previously reported by several authors in ewe cheeses manufactured with raw milk (Ordiales et al., 2013; Rodríguez-Pinilla, Márquez, Tabla, Ramírez, & Delgado, 2015; Sousa & Malcata, 1996; Vioque et al., 2000). Various factors could justify the decline of both groups during ripening in jars; specifically, the antimicrobial effect of thyme against *Enterobacteriaceae* has been widely reported, even recently, when chitosan enriched with this spice was used to treat fermented sausages (Demirok Soncua, Özdemirab, Arslan, Küçükkaya, & Soyera, 2020). Given that the cheese physicochemical parameters like pH, moisture and salt were not different in Trials I and II during ripening, the antimicrobial nature of the concia is supposed to be the factor liable for such microbial group's reduction.

With reference to LAB, mesophilic rods and cocci counted on MRS and M17, respectively, dominated the entire production process, with counts around 6 log cfu g^{-1} up to the end of ripening (Fig. 4). Thermophilic lactobacilli on Rogosa agar were undetectable throughout the manufacturing and reached a peak only at the beginning of the ripening in jar. Enterococci loads recorded in samples of cheeses produced in winter, except in samples after 60 days of ripening in jar, were always significantly higher ($p < 0.05$) than those collected during spring (Fig. 4). Enterococci are recognised as well able to survive during milk refrigeration due to their psychrotrophic nature (Hanchi, Mottawea, Sebei, & Hammami, 2018). Enterococci constitute part of the typical microflora of ewe milk and play an important role in the late ripening of traditional cheeses from Mediterranean countries (Centi, Matteucci, Lepidi, Del Gallo, & Ercole, 2017; Mormile et al., 2016). Nevertheless, their role in cheese ripening remains controversial for the high rate of multi-resistant strains and the rapid acquisition of antimicrobials resistance that characterise this genus (Russo et al., 2018).

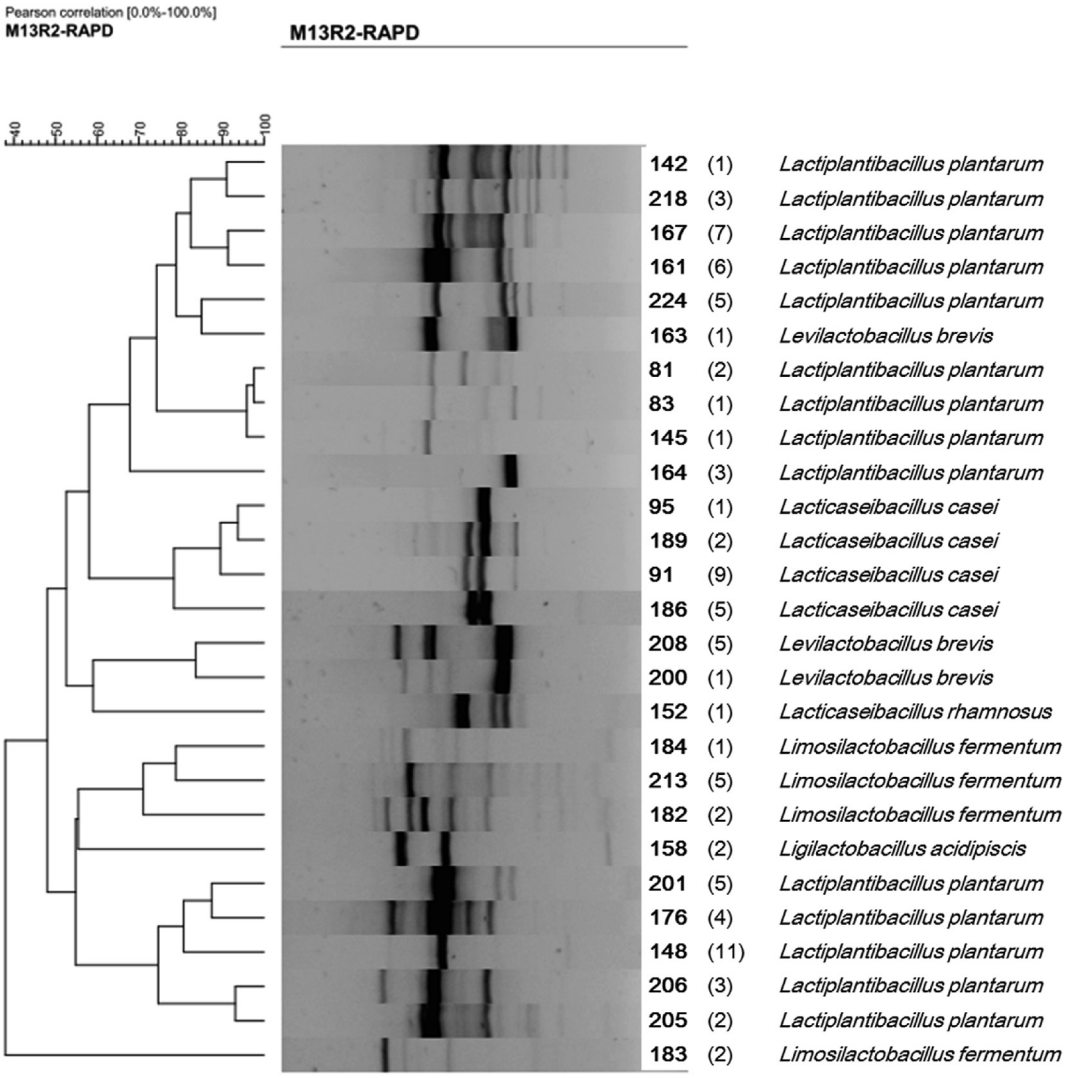

Fig. 5. Dendrogram obtained from M13-R2 RAPD-PCR fingerprints of 91 *Lactobacillus* spp. strains isolated during the production of Conciato Romano cheese. Patterns were grouped with the unweighted pair group algorithm with arithmetic averages (UPGM). The number of strains is given in parentheses.

3.3. LAB identification

Isolates from plates containing between 30 and 300 colonies were randomly picked from MRS, M17, KAA, and Rogosa agar plates. A total of 203 isolates were attributed to the LAB group since Gram positive, catalase negative and not spore-forming. By means of PCR spacer analysis, LAB were identified at species and subspecies level in the following cases: *Enterococcus faecalis* (two bands located at about 300 and 400 bp), *Enterococcus faecium* (two bands located at about 410 and 510 bp), *Enterococcus durans* (two bands at about 370 and 430 bp), and *Lactococcus lactis* subsp. *lactis* (one band located at about 380 bp). LAB characterised by spacer regions of 360 bp or 320 and 520 bp were assigned to the genus *Streptococcus* or ex-*Lactobacillus* spp., respectively (Aponte, Fusco, Andolfi, & Coppola, 2008). Sample sources, media and incubation conditions are reported in Table 2.

All 91 cultures exhibiting the typical spacer of lactobacilli were analysed by M13-R2-RAPD. Genotyping highlighted a low polymorphism, and 27 major profiles were recognised (Fig. 5). Strains representative of each pattern were submitted to 16S rDNA sequencing (Supplementary material Table S1), which was used even for 7 cultures whose spacer could not be led back to none amongst the known ones. According to outcomes such cultures were assigned to the species *Pediococcus* (*Pd.*) *pentosaceous* and *Aerococcus viridans* (Table 2).

With reference to lactobacilli, 53 out of 91 cultures could be reported to the species *Lactiplantibacillus plantarum* subsp. *plantarum*, formerly known as *Lactobacillus plantarum.* The genus *Lacticaseibacillus* spp. accounted for 8.87% of the total isolates with the species *Lacticaseibacillus casei* largely dominant: only one isolate out of 18 could be referred to the species *Lacticaseibacillus rhamnosus.*

This preliminary examination of the principal microbial species colonising *Conciato Romano* cheese revealed that LAB microflora is mainly composed by mesophilic lactobacilli, above all *Lb. plantarum* subsp. *plantarum*, which accounts for about 26% of total isolates, and enterococci, in the complex, 30% of the total cultures.

These results allow to further emphasise the microbiological quality of *Conciato Romano*, if compared with other Italian "Pecorinos", such as "Piacentinu Ennese" (Pino et al., 2019), "Pecorino Abruzzese" (Centi et al., 2017), "Pecorino Umbro" (Gobbetti, Corsetti, Smacchi, De Angelis & Rossi, 1997) and "Pecorino Sardo" (Mannu et al., 1999), whose microflora has been proved to be largely dominated by enterococci. The species *Lc. lactis* and *St. thermophilus* accounted for 16.26 and 5.42%, respectively.

3.4. Cheese texture and colour

Results about the texture evolution are reported in Table 3. The appearance of the inner part resulted modified: at the beginning, the cheese was characterised by a large number of medium size eyes but, as time passes, holes became smaller (data not shown).

Hardness, fracturability, chewiness, springiness, and gumminess constantly increased during the manufacturing in both batches. In the two manufacturing, the adhesiveness, after an initial drop, started to increase during the maturation in the jars, as even confirmed by resilience data. The cohesiveness, which account for the strength of the inner bonds, showed a variable trend, likely due to the unusual manufacturing process that includes the drying of the cheeses followed by a ripening in jars. In samples produced during winter, a decrease in gumminess, chewiness and springiness could be noted during the last forty days of storage in the jar.

Table 3
Texture parameters of samples collected during Conciato Romano cheese manufacturing.[a]

Texture	Trial	Sample				
		Cf_0	Cf_{25}	Cf_{60}	Cf_{80}	Cf_{120}
Adhesiveness	I	-13.09^{ab}	-15.12^{ab}	-18.80^{a}	-19.40^{ab}	-1.52^{ab}
	II	-0.87^{b}	-7.26^{b}	-9.87^{b}	-2.72^{b}	-2.66^{b}
Hardness	I	6.69^{A}	8.71^{A}	16.26^{CD}	19.71^{BD}	22.76^{CD}
	II	1.77^{A}	8.13^{AC}	9.40^{AC}	11.76^{AC}	11.67^{ACD}
Fracturability	I	1.22	1.38	4.76	7.52	11.59
	II	0.00	2.08	3.40	5.40	6.75
Springiness	I	0.82^{B}	0.97^{B}	1.06^{A}	1.29^{B}	1.26^{B}
	II	0.91^{B}	0.83^{B}	0.88^{B}	0.88^{B}	1.03^{B}
Cohesiveness	I	0.41	0.44	0.24	0.34	0.26
	II	0.50	0.29	0.30	0.40	0.29
Gumminess	I	2.60	4.60	6.38	7.81	7.05
	II	0.88	2.37	2.85	4.72	5.74
Chewiness	I	2.10^{B}	4.43^{B}	8.92^{A}	9.47^{B}	7.90^{B}
	II	0.79^{B}	2.01^{B}	2.54^{B}	4.34^{B}	5.46^{B}
Resilience	I	0.02^{B}	0.04^{B}	0.64^{A}	0.65^{B}	0.62^{B}
	II	0.11^{B}	0.02^{B}	0.02^{B}	0.18^{B}	0.59^{B}

[a] Abbreviations are: M, milk; C, curd; Cf_0, "primosale"; Cf_{25}, cheese after 25 days of drying; Cf_{60}, Cf_{80}, and Cf_{120}, cheese after 60, 80, and 120 days of ripening, respectively, in jar. Lowercase and uppercase superscript letters indicate statistical differences at $p < 0.01$ and $p < 0.05$, respectively, based on Turkey test for trial variables.

Generally, hardness, chewiness, fracturability, springiness, gumminess and resilience values in spring samples were significantly lower than those recorded for samples produced during winter, while an opposite trend was exhibited by the hardness parameter (Table 3). Differences in the samples' texture parameters can be related to the effect of the different environmental conditions typical of the two seasons. As reported by Serrapica et al. (2020) for Pecorino Bagnolese, the higher temperatures of the spring may induce a proteolysis increasing, which elicit a lower hardness of the cheeses. Moreover, the warmer spring temperatures lower the cheeses water content, thus causing a lower adhesiveness. Biochemical changes occurring during cheese ripening, such as proteolysis and changes in the water availability for the chemical bindings are known to affect the cheese consistency, even if the mechanism still need to be fully understood (Lucey, Johnson, & Horne, 2003). In detail, the creation of ionic groups generated by peptide bonds breaking that can compete with proteins for the water, may justify the hardness increase and the resistance to deformation recorded during the cheeses ripening (Lawrence, Creamer, & Gilles, 1986). In addition, Combs Rankin, Stevenson, and Greenberg (2007) stated that Cheddar cheese made from milk of cows graze on pasture was consistently softer than that from milk of cows undergoing to typical winter feeding.

The colour coordinates (L*, a*, b*) are indices of colour dimensions, green to red (a*), blue to yellow (b*), and lightness (L*), which constitutes the balance of green, red, and blue. In both batches, the external appearance changed from a bright white colour in the fresh lightly salted cheese, to a pale yellow after 25 days of drying, likely due to the washing with pasta cooking water. During ripening in jar, for effect of the concia, colour turned step by step to a brownish tint. The dyeing effect of the wine-containing dressing is accounted by the slowly decrease of the L* parameter (Table 4). The effect of the wine is highlighted by the b* and a* values evolution as well: after raising both values dropped starting by the 60th day in jar. The colour parameters in the inner part exhibited a similar behaviour, except for the b* values of winter manufacturing: during the ripening in the jars, b* values remained almost constant (Table 4).

Table 4
Colour parameters of samples collected during Conciato Romano cheese manufacturing.[a]

Colour	Trial	Sample				
		Cf_0	Cf_{25}	Cf_{60}	Cf_{80}	Cf_{120}
Ext L*	I	87.14^{A}	63.41^{C}	79.42^{AC}	72.24^{ABC}	60.41^{BC}
	II	89.12^{A}	75.79^{ABC}	77.13^{ABC}	67.75^{B}	64.68^{B}
Ext a*	I	-0.05^{B}	0.35^{B}	3.46^{BC}	12.27^{B}	7.13^{C}
	II	-0.95^{B}	1.21^{B}	11.04^{A}	9.29^{A}	7.36^{C}
Ext b*	I	13.56^{D}	19.40^{BD}	24.49^{ABC}	33.87^{A}	25.96^{ABC}
	II	12.71^{D}	21.35^{BD}	28.72^{ABC}	29.06^{ABC}	27.39^{ABC}
Int L*	I	89.45^{ab}	75.60^{ab}	82.33^{ab}	89.30^{a}	74.68^{b}
	II	89.66^{a}	79.59^{ab}	86.09^{ab}	81.81^{ab}	78.81^{ab}
Int a*	I	-0.74^{BC}	-1.03^{B}	0.40^{BC}	-0.80^{BD}	0.84^{C}
	II	-0.56^{BC}	-0.96^{B}	0.57^{B}	2.57^{A}	3.03^{A}
Int b*	I	11.71^{B}	15.74^{B}	18.24^{A}	20.23^{A}	21.20^{A}
	II	11.24^{B}	17.99^{A}	22.37^{A}	22.44^{A}	21.94^{A}

[a] Abbreviations are: M, milk; C, curd; Cf_0, "primosale"; Cf_{25}, cheese after 25 days of drying; Cf_{60}, Cf_{80}, and Cf_{120}, cheese after 60, 80, and 120 days of ripening, respectively, in jar; Ext, external; Int, internal. Lowercase and uppercase superscript letters indicate statistical differences at $p < 0.01$ and $p < 0.05$, respectively, based on Turkey test for trial variables.

4. Conclusions

Local cheese production is an important economic activity, especially in Mediterranean countries (Caridi et al., 2003). The unusual cheese-making method of Conciato Romano cheese, more than other ewe cheeses, crafts a tight link with territory and its traditions. According to outcomes from the cheese characterisation, such production technology is able to preserve the nutritional value of the product and to ensure its safety. Ripening is believed to be a major factor in determining the quality of small ruminant dairies (Zabaleta et al., 2016) and, in such light, the approach of the ripening in jars might be profitably extended to other ewe cheeses. In addition, even if seasonality proved to be an important source of variation, the average hygienic and nutritional quality proved to be satisfactory in winter productions as well.

This work represents the first study focused on Conciato Romano cheese and may contribute to deepen the knowledge of its microbiological, biochemical and rheological features. The data collected can lay the foundations for the selection of LAB strains to be used in the management of the manufacturing process and for a preliminary characterisation to achieve a PDO status. The work was focused on the dominant lactic microflora, and a further effort, including omics techniques and integrated system biology, is undoubtedly required for the characterisation of non-starter lactic acid bacteria, whose role for the overall quality of cheese production has been recently pointed out by Gobbetti et al. (2018).

Author contributions

Maria Aponte: Investigation, review & editing. Giuseppe Blaiotta: Methodology & conceptualization. Raffaele Marrone: Methodology and analysis. Maria Francesca Peruzy: Data curation. Giorgio Smaldone: Experiments and analysis. Lucia Vollano: Experiments and analysis. Nicoletta Murru: Funding & supervision.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2021.105077.

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