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Effect of Ripening Time and Seasonal Changes on Microbial and Physicochemical Properties of Inland Pecorino Abruzzese Cheese

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HIGHLIGHTS

- Milking season greatly affect the characteristics of the Pecorino Abruzzese cheese.
- Cheese produced in springtime, called Marzolino, has a better quality profile than that produced in autumn.
- Ripening time has a positive effect on physicochemical parameters of the Pecorino Abruzzese cheese.

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Acronyms and abbreviations
CFU=Colony Forming Unit
DM=Dry Matter
PCA=Principal Component Analysis
TN=Total Nitrogen
Urea-PAGE=Urea-Polyacrylamide Gel Electrophoresis
WSN=Water-Soluble Nitrogen

ABSTRACT

Background: Hand-made cheeses are usually prepared following dissimilar procedures which influence the quality and the organoleptic properties of the products. Objective of the present study was to evaluate how manufacturing season and ripening time affect hand-made Pecorino Abruzzese cheese.

Methods: Microbiological and physicochemical characteristics were investigated on raw milk cheeses produced in spring and autumn sampled at different ripening times (20, 60, 120, 210, and 300 days). Statistical analysis was done using SPSS software version 21.

Results: Spring Marzolino cheeses showed better quality than those produced in autumn, with higher contents of protein, moisture, and Water-Soluble Nitrogen/Total Nitrogen (WSN/TN); and lower content of fat and salt. Besides, Marzolino samples exhibited an extensive α_{S1}-casein proteolysis, slight hydrolysis of β-casein, low levels of γ-casein, and the occurrence of heterogeneous mixtures of proteolytic products as well as more complex microbial populations. At 20 days of ripening, all spring-cheese microbial groups presented in a remarkably high number than that presented in autumn, whereas enterococci populations were significantly higher in autumn cheeses than in spring ones (7 and 6 log Colony Forming Unit/g for autumn and spring, respectively). Ripening demonstrated a positive effect, in both productions, by increasing the concentration of the physicochemical parameters and a decrease of microbial populations of 1-3 log units.

Conclusion: Marzolino cheeses, manufactured in springtime, had better quality profile than those manufactured in autumn which this finding could be utilized to set up marketing strategies.

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Introduction

Pecorino Abruzzese, a traditional ewes' milk cheese product of Abruzzo (Central Italy), is usually prepared following different procedures which may be dissimilar according to manufacturing tradition and production

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scale. Pecorino Abruzzese is semi-hard or hard ewes' milk cheese, usually made from raw/heated milk which is inoculated with natural cultures, "scotta fermentato" or commercial starters (Schirone et al., 2012).

The presence of typical and different microbial population is characteristic of artisanal cheeses, due to geographic area of origin and raw material (Centi et al., 2017; Poznanski et al., 2004; Schirone et al., 2012). Raw milk microorganisms play a fundamental role in the development of the organoleptic characteristics of Pecorino cheese during ripening (Castro et al., 2016).

Marzolino cheese, the nickname utilized to indicate the Pecorino cheese produced in L'Aquila province in springtime, is made with whole milk and is produced soon after Easter (between March and April) without the addition of starter cultures when ewes feed on freshly grown grasses. This artisanal cheese, produced only in small artisanal dairy units, is very popular in the highlands of the Abruzzo region. It is eaten mostly after a 120-day of ripening, but is often eaten fresh, not aged at all, or even after a seasoning of up to 300 days.

It is well known the influence of season on the volatile fraction and sensory characteristics of cheeses, especially of those made from raw milk without the addition of starter cultures. Besides, cheese ripening is a process which involves a series of microbiological, biochemical, and chemical reactions strongly influenced by the manufacturing process like composition, moisture, pH, salt, and eventually microbiota, starter, and lastly nonstarter microorganisms. Cheese ripening involves a complex series of biochemical events, including glycolysis, lipolysis, and proteolysis that leads to the characteristic taste, aroma, and texture of each cheese variety. Lipolysis is the process responsible for the flavor development during ripening and depends mostly on the activity of microbial lipases (Fernández-Garcia et al., 2004; Khattab et al., 2019; McSweeney, 2004).

The characterization of Pecorino Abruzzese produced in L'Aquila province is important to establish the peculiar traits of this cheese, and to differentiate it from similar products. A previous study showed microbiological, compositional, biochemical, and sensorial characteristics of Pecorino Abruzzese cheeses to describe the status of Protected Designation of Origin (PDO; Centi et al., 2017). The purpose of the present investigation was to study the effect of seasoning on the characteristics of Pecorino Abruzzese cheese and, in particular, the one produced in the spring season called Marzolino, which is locally much appreciated for its peculiar characteristics, and little studied so far. In this research, we analyzed the biochemical and microbiological characteristics of this particular cheese in comparison with the same product manufactured in autumn and we compared two cheeses during the ripening period ranging from 20 to 300 days.

Materials and methods

Samples

Analyses were conducted on raw milk and cheese manufactured between March-April (spring) and in late October (autumn). Milk and cheese samples were supplied by a dairy factory located in the L'Aquila province in the highland Abruzzo region, Italy. A total amount of 10 batches of Pecorino Abruzzese manufactured in 2014, 5 in spring (Marzolino) as well as 5 in autumn, were analyzed.

Cheese was manufactured by raw milk heating at 36-38 °C. Natural whey culture "scotta fermentato" was added to the milk and after 30 min, milk was coagulated at 35 °C by adding paste lamb rennet. The mixture was kept 20-30 min and placed in wicker molds for 12 h for whey removal. The cheeses were then coated with coarse salt and left to ripe at 10-15 °C and 85% relative humidity in an industrial chamber (Centi et al., 2017). The cheeses ripening time ranged from 20 to 300 days. At the end of the maturation, cheeses were round in shape, 15 cm high and 20 cm in diameter, and weighed about 2 kg. Figure 1 shows the flow diagram of the cheese manufacturing procedure.

Cheeses were sampled *in situ* (i.e. in cheese factory) at 20, 60, 120, 210, and 300 days of ripening. For each sample, three replications were collected. Each replication was divided in two parts including one for the microbiological analyses as well as one for the physicochemical analyses. Samples were carried to the laboratory in sterile and refrigerated containers (4 °C), and analyzed.

Microbiological analysis

Cheeses and milk microbiological analyses were performed within 24 h from sampling. After removing the rind, 25 g fractions from each sample were obtained by cutting slices, each containing equal amount of the innermost, intermediate, and outermost parts. The sub-samples (25 g) were diluted in 225 ml of sodium citrate solution (2% w/v) and homogenized with Stomacher Lab-Blender 400 (PBI International Milan, Italy) in sterile conditions.

Serial dilutions in sterile quarter-strength Ringer's solution were plated on selective media to enumerate the following microorganisms: aerobic mesophilic bacteria on plate count agar at 30 °C for 24-48 h; lactococci and streptococci on M17 agar, respectively at 30 °C and 44 °C for 24-48 h; mesophilic and thermophilic lactobacilli on De Man, Rogosa and Sharpe (MRS) agar at 30 °C and 44 °C, respectively, for 48 h in anaerobic conditions (Schirone et al., 2011). Bacterial counts were determined as log Colony Forming Unit (CFU)/g.

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Citrate-fermenting bacteria were grown on MRS agar with the addition of 2% calcium citrate (Panreac, Barcelona, Spain) incubated at 30 ℃ for 48 h in aerobiosis. As the media utilized were not highly selective, plate counts were confirmed by microscopic observation. Enterococci were cultured on Slanetz-Bartley agar plates and incubated at 37 °C for 48 h. Yeasts were grown on Yeast Peptone Dextrose agar added with chloramphenicol (0.15g/L; Sigma-Aldrich, St. Louis, MO, USA) at 25 ℃ for 3-5 days (Schirone et al., 2011).

All media were purchased from Oxoid, Basingstoke, Hampshire, England. Viable microbial counts were performed in triplicate.

Compositional analysis

Fat, protein, and lactose were investigated in milk samples by infrared analysis (Milko Scan, model: Foss 4000, Foss Food Technology, Denmark) calibrated for sheep milk. Methods proposed by the Association of Official Analytical Chemists and to the International Dairy Federation Standard were employed to determine Dry Matter (DM), fat, and casein (AOAC, 1990, 1999; IDF, 1990). Milks' pH was determined by inserting directly pH probe in the sample. Cheese samples were analyzed for moisture, pH, and NaCl (IDF, 1988, 1989; ISO, 2004), total protein, and Water-Soluble Nitrogen (WSN; IDF, 1993), and fat content (AOAC, 1999). The analysis were performed in triplicate for each cheese sample (n=5).

Assessment of proteolysis

Both pH 4.6-soluble and -insoluble fractions of the cheeses were prepared according to Sousa and McSweeney (2001). The pH 4.6-insoluble fraction was utilized for electrophoretic analysis. Total Nitrogen (TN) of the soluble fraction (pH 4.6) was determined by the macro-Kjeldahl method (IDF, 1964) and proteins were calculated from the total N concentrations using the standard factor of 6.38. Ethanol (70%) was added to aliquots of the pH 4.6-soluble fraction which was in turn fractionated into ethanol-soluble and ethanol-insoluble fractions.

The fraction containing the ethanol-soluble peptides (ethanol-soluble sub-fraction) and that one containing the ethanol-insoluble peptides (ethanol-soluble sub-fraction) were obtained following the procedures suggested by Shakeel-Ur-Rehman et al. (1998). Both fractions of the cheeses (at 20, 60, 120, 210, and 300 days of ripening) were analyzed by Urea-Polyacrylamide Gel Electrophoresis (Urea-PAGE) according to the method of Andrews (1983).

The gels were stained according to Blakesley and Boezi (1977). The ethanol-insoluble and soluble fractions of the pH 4.6 soluble fraction of Pecorino Abruzzese cheese were also analyzed by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC, Varian Associates Inc. Walnut Creek CA, USA). Preparation and elution of the samples were carried out in acetonitrile gradient according to method of Sousa and McSweeney (2001). Monitoration of the eluates was performed spectrophotometrically at 214 nm. Total free amino acids were determined by the trinitrobenzenesulphonic acid method (Polychroniadau, 1988), while individual free amino acids were measured according to the method proposed by Fenelon et al. (2000).

Fatty acids

The extraction and determination of free fatty acids were performed according to the procedure described by De Jong and Badings (1990). This method, suitable to the analysis of the short-medium-long chain fatty acids, was utilized for cheese samples during ripening at different seasons.

The samples were analyzed by Gas-Chromatograph (Model Star 3400CX) equipped with a Flame Ionization Detector (FID) interfaced Star Chromatography Workstation 5.0 software for system control and data acquisition (Varian Analytical Instruments, Harbor City, California, USA). The free fatty acids were separated on a Wall-Coated Open Tubular (WCOT) fused-silica capillary column (Chrompack, FFAP-CB) for free fatty acid analysis (DF 0.3, Varian).

Statistical analysis

Means and Standard Deviations (SD) were calculated for each experimental parameter. For each experimental parameter, the results obtained from autumn and spring seasons of production were compared. The effects of the days of ripening on the results of microbiological profile, chemical composition, and total amount of free amino acids were also considered, comparing the results of 20 days of ripening with those obtained at 120 days (that matches with the common final ripening time for ewe's milk cheeses) and the latter with the results of 300 days of ripening (maximum seasoning times). Mean values and the standard deviations comparisons and *p* values calculations were carried out by unpaired t-tests, using GraphPad Prism 6 for Windows Version 6.1 (GraphPad Software, San Diego, California, USA). Data from the RP-HPLC chromatograms of pH 4.6 ethanol-soluble and insoluble fractions were analyzed using multivariate statistical techniques to analyze the patterns of proteolysis and the volatile profile in Abruzzese cheese during ripening. Data for multivariate statistical analysis of the chromatograms were obtained by visually recognizing similar peaks in the chromatograms and the peak heights as variables as described by Pripp et al. (1999).

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Principal Component Analysis (PCA) was performed by using a covariance matrix and carried out using SPSS software, version 21 for Windows 8 (SPSS Inc., Chicago, Illinois, USA).

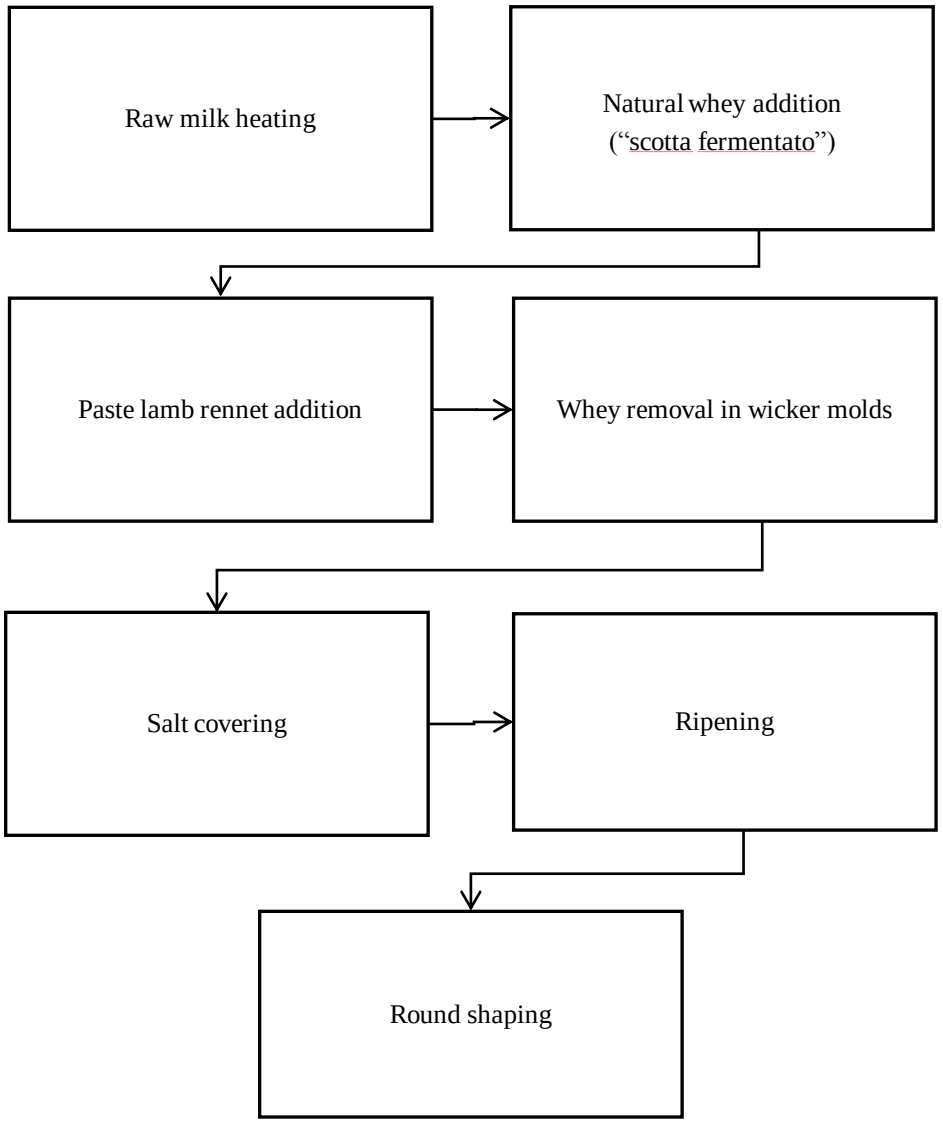


Figure 1: Flow diagram of the process used to produce Pecorino Abruzzese cheese

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Results

The microbial counts concerning both raw milk and Pecorino Abruzzese cheese are shown in Table 1 and Table 2, respectively. Opposite to enterococci, both raw milk and cheese samples produced in spring exhibit a remarkably higher number (p<0.05) of microbial groups than that produced in autumn. At 20 days ripening time of the samples, about 7 log CFU/g enterococci were found in cheese manufactured during the autumn compared to those, approximately 6 log CFU/g, recorded

in spring season. Moreover, for both the investigated seasons, it should be noted that with increasing ripening time, microbial counts decreased by 1-3 log units departing from the early stage until middle stage (20 *vs.* 120) and late stage of maturation of cheese (20 *vs.* 300). Notably, the microbial group of lactobacilli and lactococci corresponding to 300 days values differentiated between 0.49 log CFU/g for mesophilic lactobacilli, 0.06 log CFU/g for thermophilic lactobacilli and 0.52 log CFU/g for lactococci (p<0.001), whereas mesophilic lactobacilli and lactococci populations, dominate during more than half the ripening period (Table 2).

Table 1: Cell numbers (mean±SD $\log_{10}$ CFU/ml) of the principal microbial groups found in raw milk

Microorganism	Spring	Autumn	*P* value
Aerobic mesophilic bacteria	5.67±0.02	4.47±0.03	<0.0001
Thermophilic lactobacilli	4.85±0.03	3.93±0.6	0.0568
Mesophilic lactobacilli	5.24±0.04	4.24±0.3	0.0046
Thermophilic cocci	5.36±0.03	4.57±0.02	<0.0001
Mesophilic cocci	5.51±0.01	5.23±0.02	<0.0001
Yeasts	4.83±0.01	4.39±0.01	<0.0001
Enterococci	4.20±0.03	5.41±0.02	<0.0001

Table 2: Effects of production season on the microbiological profile (mean±SD log CFU/g) of Pecorino Abruzzese cheese

	Days of ripening					*P* value	*P* value
	20	60	120	210	300	20 *vs.*120	20 *vs.* 300
Aerobic mesophilic bacteria							
Spring	8.78±0.01	8.7±0.01	8.6±0.02	8.27±0.01	7.28±0.07	0.0002	<0.0001
Autumn	8.38±0.03	8.35±0.03	8.32±0.1	8.05±0.02	7.38±0.01	0.3759	<0.0001
P value	<0.0001	<0.0001	0.0089	<0.0001	0.0705		
Total coliform							
Spring	6.29±0.12	2.49±0.13	ND*	ND	ND	-	-
Autumn	3.82±0.03	2.24±0.13	ND	ND	ND	-	-
P value	<0.0001	0.0781	-	-	-		
Staphylococci							
Spring	6.85±0.16	5.08±0.06	4.82±0.2	4.16±0.18	3.32±0.03	0.0002	<0.0001
Autumn	5.53±0.19	5.35±0.05	5.72±0.02	4.87±0.03	4.21±0.03	0.1601	0.0003
P value	0.0007	0.0039	0.0015	0.0026	<0.0001		
Enterococci							
Spring	6.47±0.09	4.2±0.05	6.94±0.02	6.01±0.02	5.54±0.19	0.0009	0.0016
Autumn	7.01±0.05	6.95±0.02	6.02 ±0.07	5.47±0.11	5.25±0.03	<0.0001	<0.0001
P value	0.0008	<0.0001	<0.0001	0.0011	0.0593		
Mesophilic lactobacilli							
Spring	8.53±0.05	8.12±0.07	7.98±0.14	7.81±0.05	7.21±0.01	0.0031	<0.0001
Autumn	8.33±0.13	8.00±0.01	7.46±0.09	7.21±0.02	6.72±0.02	0.0007	<0.0001
P value	0.0677	0.0424	0.0057	<0.0001	<0.0001		
Thermophilic lactobacilli							
Spring	8.09 ±0.03	8.05±0.06	7.79±0.04	7.72±0.08	6.6±0.03	0.0005	<0.0001
Autumn	8.01±0.03	7.76±0.04	7.65±0.02	7.09±0.02	6.54±0.03	<0.0001	<0.0001
P value	0.0309	0.0022	0.0056	0.0002	0.0705		
Thermophilic streptococci							
Spring	8.30±0.07	8.25±0.18	7.86±0.04	7.22±0.1	6.53±0.02	0.0007	<0.0001
Autumn	8.17±0.07	8.10±0.04	7.56±0.01	6.99±0.06	6.16±0.05	0.0002	<0.0001
P value	0.0853	0.2316	0.0002	0.0268	0.0003		
Lactococci							
Spring	8.60±0.08	8.23±0.05	8.42±0.04	7.92±0.07	7.27±0.03	0.0252	<0.0001
Autumn	8.17±0.15	7.83±0.06	7.51±0.04	6.39±0.05	6.75±0.12	0.0018	0.0002
P value	0.0119	0.0009	<0.0001	<0.0001	0.0019		

* ND: not detected; *P* values within rows refers to statistical difference between the ewe's milk cheeses produced in different seasons (spring *vs*.autumn); *P* values in right columns refers to statistical differences between the ewe's milk cheeses at different stages (20 *vs*.120 days and 20 *vs*.300 days).

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The physicochemical characteristics of ewe's milk are shown in Table 3. The milk produced in spring was lower in lactose (p<0.001) as well as DM (p<0.001), and higher in protein (p<0.05), and pH (p<0.01) than that produced in autumn. No statistical significant differences were found among seasons for fat and casein (p>0.05). The physicochemical characteristics of Pecorino Abruzzese cheeses manufactured at different seasons are summarized in Table 4. The comparison between the data collected in spring with those collected in autumn, at 20 days ripening time, demonstrated an higher content of proteins (43.0% *vs.* 26.4%; p<0.001), fat (61.4% *vs.* 45.6%; p<0.001), moisture (47.1% *vs.* 34.4%; p<0.001), and WSN/TN (40.6% *vs.* 24.6; p<0.001).

Table 3: Physicochemical characteristics of ewe's milk in different seasons

	Spring (mean±SD)	**Autumn (mean±SD)**	***P* value**
pH	6.73±0.07	6.44±0.08	0.0091
Dry matter (%)	19.75±0.09	20.81±0.10	0.0001
Fat (%)	7.00±0.06	6.92±0.06	0.1778
Lactose (%)	4.01±0.04	4.61±0.09	<0.0001
Protein (%)	6.04±0.06	5.79±0.08	0.0123
Casein (%)	4.72±0.11	4.52±0.08	0.0635

Table 4: Effects of production season on the chemical composition of the Pecorino Abruzzese cheeses during ripening

	Days of ripening					***P* value**	***P* value**
	20	60	120	210	300	**20 *vs.* 120**	**20 *vs.* 300**
pH							
Spring	5.5±0.2	5.2±0.1	5.4±0.3	5.3±0.1	5.4±0.1	0.6560	0.4818
Autumn	5.4±0.1	5.6±0.2	5.7±0.1	5.4±0.1	5.3±0.3	0.0213	0.6130
P value	0.4818	0.0363	0.1757	0.2879	0.6130		
Moisture (%)							
Spring	47.1±0.2	31.0±1.7	23.6±1.9	25.7±3.2	18.8±3.3	<0.0001	0.0001
Autumn	34.0±0.8	28.1±0.6	20.5±0.4	19.7±0.9	18.0±1.2	<0.0001	<0.0001
P value	<0.0001	0.0495	0.0506	0.0353	0.7133		
Protein on DM (%)							
Spring	43.0±0.3	38.7±0.6	37.8±0.4	40.2±0.2	41.4±0.6	<0.0001	0.0145
Autumn	26.4±0.4	27.4±0.3	26.3±0.4	34.7±0.2	36.0±0.6	0.7747	<0.0001
P value	<0.0001	<0.0001	<0.0001	<0.0001	0.0004		
Fat on DM (%)							
Spring	61.4±0.8	49.6±0.1	47.8±0.1	49.1±0.1	46.7±0.3	<0.0001	<0.0001
Autumn	45.6±0.6	50.9±0.1	48.1±0.2	48.0±0.2	49.2±0.9	0.0024	0.0045
P value	<0.0001	<0.0001	0.0808	0.0010	0.0103		
WSN/TN on DM (%)							
Spring	40.6±1.4	33.2±0.9	35.0±1.3	42.0±0.7	46.5±1.1	0.0071	0.0046
Autumn	24.6±1.1	28.2±1.6	29.8±0.8	33.3±1.4	38.1±1.8	0.0027	0.0004
P value	<0.0001	0.0092	0.0041	0.0007	0.0023		
Salt on DM (%)							
Spring	5.7±0.2	4.7±0.3	6.6±0.1	7.0±0.3	7.0±0.1	0.0022	0.0005
Autumn	6.0±0.8	6.4±0.6	7.1±0.4	6.9±0.1	7.6±0.1	0.1002	0.0264
P value	0.5628	0.0118	0.1036	0.6130	0.0018		

P values within rows refers to statistical difference between the ewe's milk cheeses produced in different seasons (spring *vs.* autumn). *P* values in right columns refers to statistical differences between the ewe's milk cheeses at different stages of ripening (20 *vs.* 120 days and 20 *vs.* 300 days).
WSN/TN=Water-Soluble Nitrogen/Total Nitrogen
DM=Dry Matter.

In spring, from 20 to 120 days, salt content increased (p<0.01) while for moisture, protein, fat, and WSN/TN a significantly decrease was detected (p<0.001). In spring, from 20 to 300 days, all the physicochemical characteristics showed a decrease in values as ripening time increase (p<0.001), while WSN/TN and salt (p<0.001) increased. In autumn, moisture decreased during ripening (from 20 to 300 days), while proteins, fat, and WSN/TN increased (p<0.001). The salt contents of the cheese during ripening were not significant (p>0.05). Comparisons of the

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salt content of autumn and spring cheeses were in general not significant corresponding to the same ripening time. For all sampling periods, cheeses manufactured in spring had the highest content of WSN/TN (p<0.001 for 20 *vs.* 120 days; p<0.01 for 20 *vs.* 300 days). Moreover, at the end of the maturation period (300 days), fats and proteins decreased in cheeses produced in the spring season compared with those produced during the autumn season.

The protein hydrolytic patterns were investigated by Urea-PAGE as function of ripening time and season. Urea-PAGE of pH 4.6-insoluble and ethanol-soluble N fraction are shown in Figure 2. The pH 4.6-insoluble N fraction (Figure 2A), exhibits some differences between the cheeses produced in spring and autumn. Proceeding from 60 days to the end of the ripening time (300 days), cheeses produced during spring showed a more pronounced hydrolysis of α_{S1}-casein and β-casein than that from autumn. Furthermore, α_{S1}-casein was degraded more extensively than β-casein. The pH 4.6 ethanol-soluble N fraction of the spring cheese shown in Figure 2B highlighted a more enhanced proteolytic process during the early stage of ripening than that of cheeses manufacture in autumn. The greater proteolytic activity of the spring cheeses is in line with a higher (p<0.01) content of the nitrogen fractions in the WSN/TN measurements compared to cheeses made in autumn (Table 4).

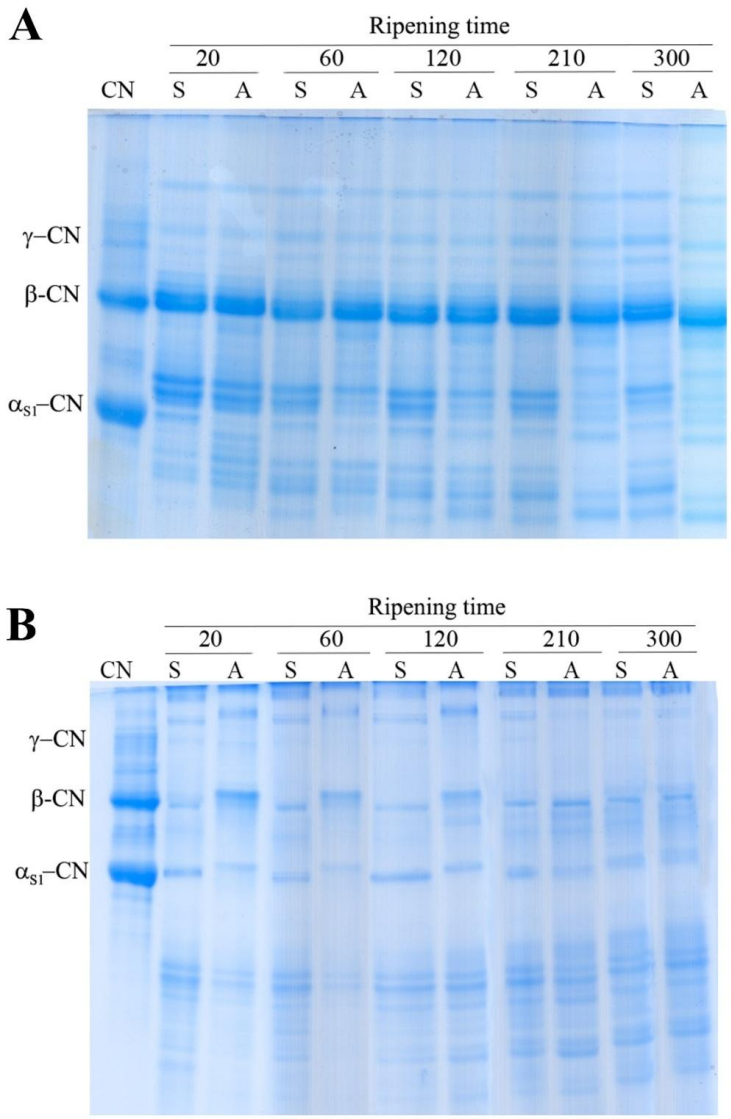


Figure 2: Urea- PAGE of pH 4.6- insoluble (A) and ethanol-soluble (B) fractions of Pecorino Abruzzese cheeses
Lanes: (CN) ovine Na-caseinate (standard); (S) spring production at 20, 60, 120, 240 and 300 days of ripening; (A) autumn production at 20, 60, 120, 240 and 300 days of ripening

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RP-HPLC peptide profiles of the insoluble/soluble ethanol fractions of the pH-4.6 soluble fractions of Pecorino Abruzzese cheese after 20, 60, 120, 210, 300 days of ripening time are shown in Figure 3A and 3B, respectively. Chromatograms of the ethanol-insoluble fractions of the cheeses manufactured in spring (Figure 3A left) and autumn (Figure 3A right) showed a highly complex peptide profile. For both seasons, many peptides were eluted just before 10 min and 70 min. Chromatogram of spring cheeses showed a large number of peaks. These peptides are referred as the ones eluted with low retention time (from 20 to 40 min-hydrophilic), late retention time (from 50 to 70 min-hydrophobic), and small hydrophilic peptides eluting from 2 to 10 min.

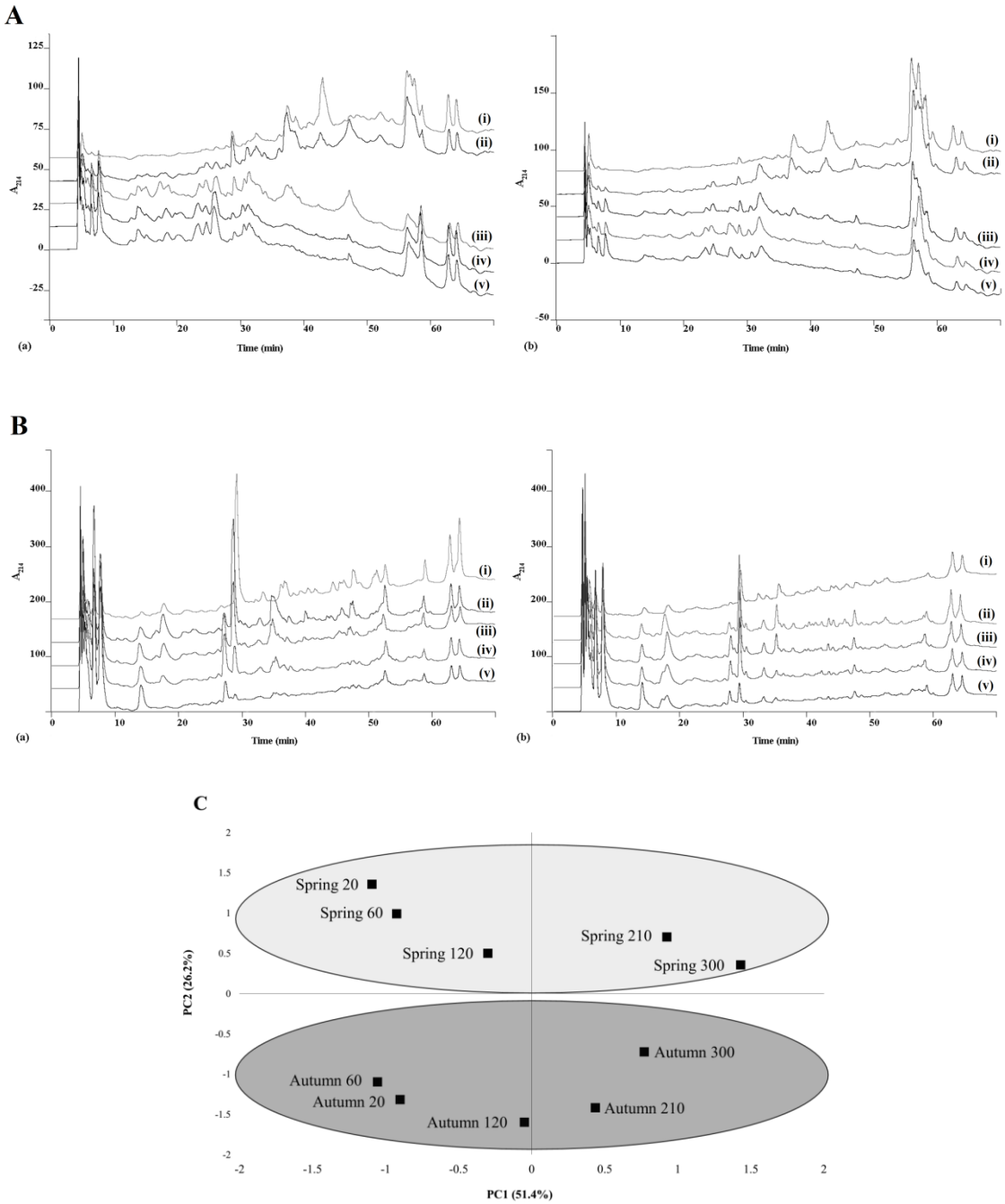


Figure 3: Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) chromatograms of the ethanol -insoluble (A), and -soluble fractions (B), from Pecorino Abruzzese cheeses manufactured in spring (left) and autumn (right) seasons at 20 days (i), 60 days (ii), 120 days (iii), 240 days (iv), and 300 days (v) of ripening. Score plot obtained by Principal Component Analysis of Reversed Phase-HPLC data of ethanol insoluble- and soluble-fractions from spring and autumn production of cheeses at 20, 60, 120, 240, and 300 days of ripening (C).

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Peptide chromatograms of the ethanol-soluble fractions shown in Figure 3B exhibited some differences between spring (Figure 3B left) and autumn (Figure 3B right). Although, differing in the peak areas, samples showed similar and rather complex peptide profiles that comprised peptides throughout the acetonitrile gradient. Furthermore, during ripening time (from 20 to 300 days) and for both seasons, a decrease was observed in proteolysis (Figures 3A and 3B).

Figure 3C shows the PCA of the RP-HPLC the insoluble/soluble ethanol fractions of spring and autumn production of cheeses at 20, 60, 120, 210, and 300 days ripening. The first two principal components represented the 77.6% of the total variance. The first Principal Component (PC1) accounted for 51.4% can be considered as the behavior of the chromatographic areas of most of the peptides and concentration of the free amino acids, which can be related to the ripening time. Second Principal Component (PC2) explained 26.2% of the total variance and differentiated the season of production.

The PCA plot shown in Figure 3C distinguishes clearly between cheeses produced in autumn (i.e. negative value of PC2) from those produced in spring (i.e. positive value of PC2) for all stages of ripening time. The ripening times also differ; the first 120 days are in the quadrants on the left, both for spring and autumn cheeses, while the subsequent times of seasoning are both located in the right quadrants.

The differentiation between cheeses produced in autumn and spring was confirmed in light of the higher spring-cheese concentration of free amino acids compared with the autumn one (Figure 4A). Figure 4B shows the composition of individual free amino acids corresponding to 120 days ripening. Cheeses produced in the spring season associated higher levels of some amino acids such as alanine, methionine, histidine, serine (p<0.05), valine (p<0.01), arginine, glutamic acid, leucine, phenylalanine, proline, threonine, and tyrosine (p<0.001); and as compared with those produced in autumn.

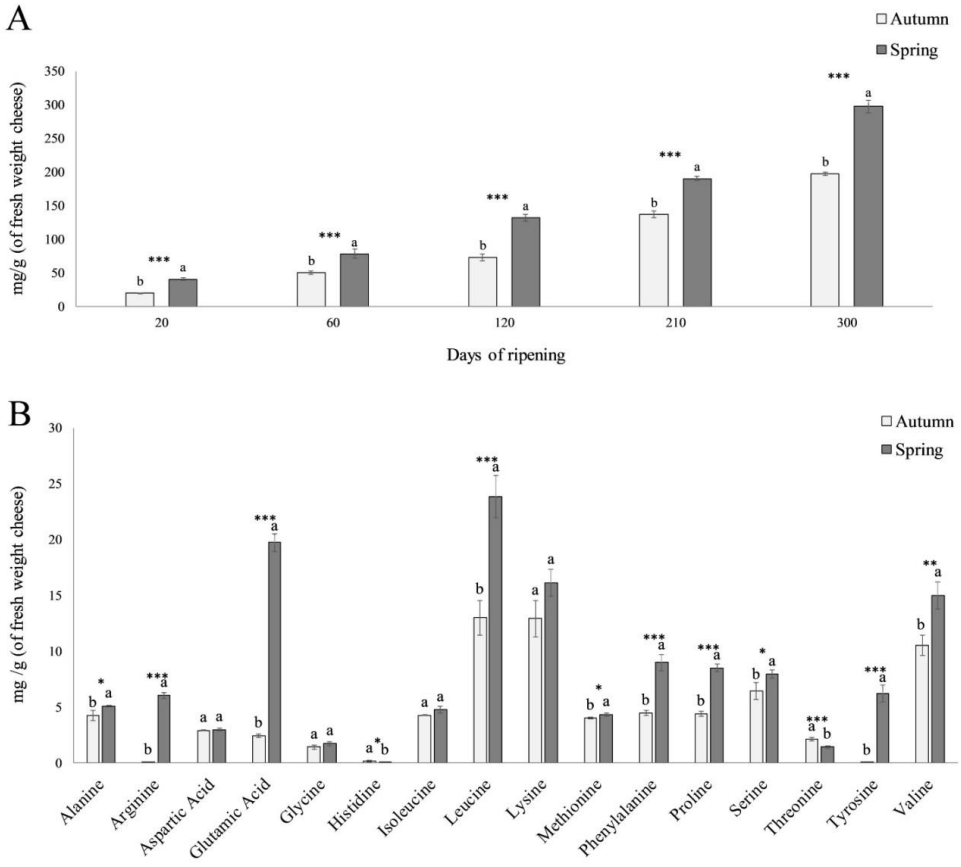


Figure 4: Effects of production season on amino acids of the Pecorino Abruzzese cheeses during ripening. A: total amount of free amino acids of the Pecorino Abruzzese cheeses during ripening (total free amino acids are expressed as mg leucine/g cheese). B: individual free amino acids (mg/g of fresh weight cheese±standard deviation) of cheese at 120 days ripening. Analyses were performed in triplicate for each batch of cheeses (n=5). Means followed by different letters are significantly different according to unpaired t-Tests comparison: *p>0.05; **p>0.01; ***p>0.001.

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The concentrations of total free fatty acids were also analyzed in spring and autumn Pecorino Abruzzese cheese at 120 days (Figure 5). The amounts of saturated short- and medium-chain fatty acids (C2:0-C14:0) were higher in cheese produced in early spring compared to that produced in autumn (p<0.001). By contrast, long fatty acids (C16:0, C18:1, C18:2, C18:3) showed a higher concentration in autumn (p<0.001).

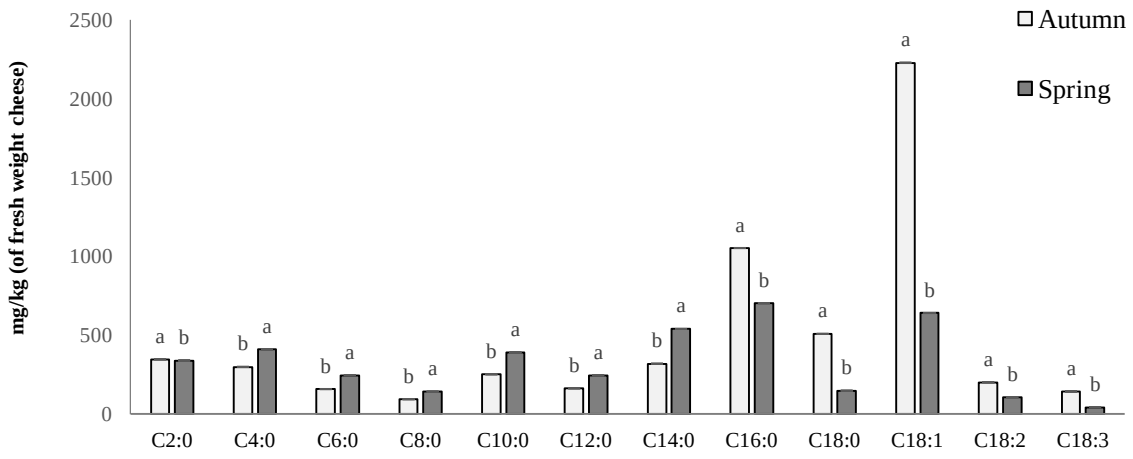


Figure 5: Effects of production season on carboxylic acids concentrations (mg/Kg of fresh weight cheese±standard deviations) of Pecorino Abruzzese cheese at 120 days of ripening. Analyses were performed in triplicate for each batch of cheeses (n=5). Means followed by different letters are significantly different according to unpaired t-Tests comparison (p<0.001).

Discussion

In this study, the characterization of the microbial groups present in autumn and spring (Marzolino) cheeses showed how the season influenced the composition and richness of the microbial groups of both milk and cheese. A great variation in the number and distribution of different microbial groups were observed in cheeses produced in spring compared to those produced in autumn.

We found a considerable difference for enterococci count in the samples. This group, as typical of ewe's milk microflora (Aquilanti et al., 2006; Quigley et al., 2011), constitute a considerable part of the cheese microbiota of Pecorino Abruzzese (Ogier and Serror, 2008; Schirone et al., 2012). During the ripening period, the microbial counts of pecorino Abruzzese decreased for all the examined groups. Also, for enterococci, a gradual decrease during cheese ripening was observed. Once again, enterococci play an important role in this process, contributing especially in the late ripening of several cheeses (Aquilanti et al., 2006). Two other microbial groups that perform an important function in the ripening are lactobacilli and lactococci. Indeed, these microorganisms can affect taste and flavor of cheese products (Hynes et al., 2003). Randazzo et al. (2010) recorded microbial communities' biodiversity changes during ripening of Pecorino Crotonese cheese.

An influence of manufacturing season on microbial quality of ewe's milk and cheese has been also observed by Barron et al. (2001). In their study, seasonal changes were reported in the microbiological composition of raw ewe's milk used for Idiazabal, a cheese made with ewe's milk in Spain. Moreover, artisanal cheeses' microbial populations are influenced not only by milk source, but also by hygienic conditions and manufacturing process (Martín-Platero et al., 2008). In the present research, spring and autumn ewe's milk were also different in terms of physicochemical characteristics. The differences described can be explained by seasonality and lactation according to previous researches. For instance, Sevi et al. (2004) reported that the physicochemical characteristics of ewe's milk are influenced by stage of lactation; in

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particular, the early lactation differs significantly from late lactation in terms of fat, protein, casein lactose, calcium, and phosphorous contents. Same research group also demonstrated that in some cases, solar radiation exposure and feeding time (morning *vs*. afternoon) influence milk composition of ewe Comisana breed. In particular, ewes exposed to morning solar radiation recorded a decrease of casein and fat yields, principally due to the lower yield of milk (-20%), which, in turn, negatively affected clot firmness (Sevi et al., 2001). According to Todaro et al. (2014), Sardinian sheep's milk produced in autumn had lower fat, protein, and casein contents, higher pH, lactose, and urea contents, and presented a reduction of clotting time with respect to summer milk.

In the current study, the differences among spring and autumn milks resulted in different physicochemical characteristics of cheeses. All chemical parameters (i.e. DM, moisture, proteins, fat, and WSN/TN) were influenced by manufacturing period, as well as by ripening. Instead, pH variations were not dependent on ripening time and season; while, the fairly irregular salt contents were probably due to the salting technique utilized.

The proteolysis investigation through several indicator techniques allowed underlining that this process is influenced by cheese manufacturing period and ripening. WSN/TN, Urea-PAGE, and RP-HPLC results are good indexes of proteolysis. As revealed by Souza and McSweeney (2001), the comparison of proteolysis results obtained through WSN/TN, Urea-PAGE, and RP-HPLC allowed classifying several varieties of cheeses, both qualitatively and quantitatively.

The higher WSN/TN contents recorded in spring samples (with respect to the autumn ones) and its increase during ripening are indicative of a greater proteolytic activity. These findings are in accordance with literature data. A recent report about Serra da Estrela, a typical Portuguese cheese obtained from ewe's milk, reported an increase of WSN/TN index along ripening, starting from 9.5-11% up to 23.33-59.17% after a ripening time of 35-180 days (Inácio et al., 2020). Both proteolytic activity and WSN/TN could be linked to higher microbial loads in spring season and, in particular, to the higher levels of lactic acid bacteria. The proteolytic activity of lactic acid bacteria has been widely reported (Farahani et al., 2017) and is exploited in cheese industry. Using proteinases and peptidases, lactic acid bacteria release free amino acids from casein peptides. These amino acids are the substrates for secondary catabolic reactions of lactic acid bacteria that produce bioactive compounds which are important for human health (Renes et al., 2019).

The connection between microbial proteolytic activity and WSN/TN contents was also confirmed by the protein hydrolytic patterns by Urea-PAGE. Our hydrolytic pattern findings are in agreement with literature data. For example, Gobbetti et al. (2002) found that during ripening time of Caciocavallo Pugliese, α_{S1}-casein had a higher hydrolysis than β_{S1}-casein both in spring and autumn samples. Also, more consistent αs1-casein hydrolysis than β-casein was obtained during ripening PDO Fiore Sardo cheese (Di Cagno et al., 2003). The decreasing rate of proteolysis is common in ripening of cheese and is usually reported after 30 days. Aminifar et al. (2014) reported a marked proteolysis occurred only during the first month of ripening of Iranian Lighvan cheese, followed by a lower proteolytic activity until 90 days. More recently, Espinosa-Pesqueira et al. (2018) showed that in Spanish artisanal cheeses produced with ewe's milk, the proteolysis was intense during the first month of ripening, followed by a considerable decrease until 60 days.

The high levels of soluble nitrogen fractions found in spring cheeses are also in line with findings underlined by RP-HPLC peptide profiles. In fact, the chromatograms of spring cheeses indicated a heterogeneous mixture of proteolytic products and peptides. Thus, in light of the previously discussed WSN and Urea-PAGE, cheeses produced in spring are associated to a higher proteolytic activity than those manufactured in autumn. RP-HPLC findings also demonstrated a decrease of proteolysis rate during ripening. The differences among the samples on the basis of manufacturing period were well underlined by PCA of RP-HPLC data, which clearly divided spring samples from the autumn ones. This statistical analysis also allowed pointing out a remarkable contribution of ripening on peptide profiles. The different proteolytic behavior among spring and autumn samples was underlined also by total free and individual amino acids.

Recently, differences in the concentrations of free amino acids in sheep milk cheeses with different initial microflora have been reported by Renes et al. (2019). They underlined that concentration and type of free amino acids in cheese are strongly dependent on the enzymes specific substrates present in the proteolytic system of lactic acid bacteria. The most abundant free amino acids in all their cheeses batches were leucine, glutamic acid, phenylalanine, proline, alanine, as well as valine; broadly similar to our findings. Moreover, similar with the results of this study, they also reported that during ripening, the concentration of most of the free amino acids increased significantly. Usually, the proteolysis decrease phenomenon can be ascribed to the salt increase that plays an inhibitory effect on proteolytic enzymes (Gaiaschi et al. 2001) and influences negatively proteolytic bacteria (Kafili et al., 2009).

Fat's component of our cheese samples was influenced by the manufacturing period which is in agreement with Addis et al. (2015) who showed a relationship between fatty acid's chain-length and animal's diet regime in Pecorino Romano cheese. These researchers stated that

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the percentage of saturated short- and medium-chain fatty acid (C4:0 - C14:0) was higher in cheese produced in late winter and in early spring compared to that produced in summer. In our study, the period of cheese manufacture seems to affect the composition of free fatty acids in cheeses. Some factors such as season, feeding system, and microbial composition of the cheeses could explain the differences observed. Moreover, in line with what reported by Chilliard et al. (2003), the decline of the pasture quality could also explain both the decrease of fatty acids with chain length less than 16C atoms, and the increase of long-chain fatty acids (C18:0, C18:1). Despite the importance of lipolysis, its mechanisms have not been completely clarified and more studies are needed to characterize the enzymes involved and the main factors influencing their activity.

As described by Gaglio et al. (2019), the biochemical events occurring in the production of cheeses volatile compounds are really complicated. Lipolysis, proteolysis, and residual lactose, lactate, and citrate metabolism produce cheeses volatile compounds. Especially, the breakdown of triacylglycerols in free fatty acids by lipolysis is the key element in the production of cheese flavor (Collins et al., 2003). Lipolysis is mainly ascribed to microbial lipases activity. However, other enzymes take part to this process, including microbial and indigenous milk, added rennet pastes, starter strains, and other ripening microorganism's enzymes.

Conclusion

Inland Pecorino Abruzzese cheeses produced in central Italy were analyzed to assess the biochemical and microbiological characteristics as function of the manufacturing period and ripening time. Our data demonstrated that Marzolino cheeses, manufactured in springtime, had better quality profile than those manufactured in autumn. Ripening time increased WSN/TN, indicating a greater proteolytic activity and a decrease of the microbial population. The characterization of microbial communities in milks from different seasons and in cheeses during ripening allowed better understanding the production processes of these important Apennine cheeses. These processes are at the basis of the production of local food products and the development of high mountain sheep-farming.

Author contributions

C.E. conceptualized the study; V.C. conducted the experimental work; M.P. analyzed and interpreted the data; C.E., M.P., and F.M. wrote the manuscript; M.D.G. wrote, reviewed and edited the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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