CHEMICAL-NUTRITIONAL COMPOSITION, MICROBIOLOGICAL ANALYSIS AND VOLATILE COMPOUND CONTENT OF FOSSA CHEESE RIPENED IN DIFFERENT PITS

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ABSTRACT

Fossa cheese samples were ripened for 90 days in two different pits and analysed to evaluate the influence of the environment on the chemical and nutritional characteristics.
The significant changes were recorded only for certain parameters, particularly the contents of fatty acids and volatile molecules. In the fatty acid profiles, the sum of monounsaturated fatty acids showed a significant decrease in the mature cheeses due to a strong decrease in oleic acid. Even the sum of polyunsaturated fatty acids and the ratio between the sums of saturated fatty acids and polyunsaturated fatty acids decreased after ripening in both pits.
HP-SPME-GC/MS analysis allowed the identification of 77 volatile compounds that increased in the cheese samples during ripening.
The results of this study indicated that there are substantial differences between the chemical and chemical/physical parameters, and certain fatty acids of the just curdled cheese samples and the cheese ripened in the two pits showed different geological-geochemical parameters.

Keywords: Fossa cheese, microclimate conditions, cheese composition, microbiological analysis, volatile compounds

1. INTRODUCTION

Fossa cheese, literally pit cheese, is a typical Italian product of the Montefeltro area, specifically from Talamello, Sogliano al Rubicone and other towns located in a small geographical area (the Emilia Romagna and Marche regions of central Italy) (GOBBETTI *et al*., 1999; BARBIER *et al*. 2012).
The cheese is variously produced from sheep, bovine or a mix of sheep and bovine milk; it is a hard cheese, produced in limited quantities (approximately 200 tons/year), and it has great economic importance in its market niche. The first phase of maturation takes place at dairy farms for a period of approximately 60 days at 6-14°C and a relative humidity of 75-92 %, after which it is aged for a period of 90-100 days in pits dug in tuffaceous rock sanitized by fire and smoke. Wooden boards are laid on the bottom of the pit forming a floor, and the pit walls are lined with a 15-20-cm-thick layer of straw before the cheeses are placed inside. The pits are then filled with the cheeses and hermetically sealed from August to November (AVELLINI *et al*., 1999). The denomination of protected origin "Formaggio di Fossa di Sogliano al Rubicone and Talamello" is reserved for cheese that meets the requirements of this specification.
When put on the market, the "Fossa cheese from Sogliano al Rubicone and Talamello" DOP has the following characteristics: the colour of the outer part of the finished product varies from ivory white to amber yellow. At the end of ripening, the cheese exhibits irregular forms, with typical bumps and depressions. The cheese surface is primarily wet and greasy and, in some cases, may be covered by butterfat and mould that is easily scraped off. The presence of small cracks and possible yellow ochre stains, more or less intense, on the surface, fits all the characteristics of the product. A skin is absent or barely visible.
The internal consistency is easily friable, with a white or slightly yellow amber colour. The smell is typical and lingering, sometimes intense, with a rich aroma reminiscent of woodland undergrowth with hints of mould and truffles. The aging process gives the product its unique, highly appreciated flavour, which is different from that of cheeses not aged in pits.
The geological characteristics of the pits play a key role in the process of cheese ripening and affect the quality.
The present study aimed to test the influence of the geological-geochemical nature of two different pits on the chemical, microbiological, nutritional and olfactory properties of a product in the Italian culinary tradition, very peculiar in its organoleptic characteristics, which are intimately related to the process of ripening (GOBBETTI *et al*., 1999).
The two geographical areas in which cheese ripening took place were those of Talamello (Province of Rimini, Emilia-Romagna Region) and Cartoceto (Pesaro-Urbino Marche Region).
The first area is characterized by soils composed of bipolar cross-laminated sandstone-type medium- to coarse-grained "fishbone" material, belonging to the formation of "Arenarie di Monte Perticara" (Pliocene).
The second area is characterized by soils composed of very thick arenaceous layers intercalated with thin pelitic layers belonging to "Formazione a Colombacci" (Miocene superiore).

2. MATERIAL AND METHODS

2.1. Cheese samples

The Fossa cheese was produced in a single cheesemaking process (Valmetauro Fattorie Marchigiane - Amandola, FM, Italy) at the same dairy farm and ripened for 60 days. After this period, two Italian companies located in Emilia Romagna (Talamello) and Marche (Cartoceto) provided the cheese ripening environment as imposed by the Consortium of Fossa cheese regulations (GOBBETTI *et al*., 1999); the samples previously ripened at the dairy farm were equally distributed in the companies' respective pits for 90 days. The analyses were performed in triplicate on each different batch consisting of three distinct samples. In this paper, the following samples were analysed: sheep milk, cheese after curdling, cheese ripened at the dairy farm for sixty days and after ripening in two different pits for ninety days.
All samples were homogenized with a laboratory mixer (MSM87160 MaxoMixx, Bosch GmbH, Germany) before being subjected to the chemical analyses. All analyses were performed in triplicate.

2.2. Microclimate measurements

Microclimate measurements were carried out using relative humidity and temperature sensors (WM33 and 52, Michell Instruments) located at the surface and the bottom of each pit. A digital signal converter and a PC transformed the sensor signal to be compatible with a specific software program developed to acquire and store data. To analyse the microclimate dynamics inside the pits regularly, the downloaded data were controlled weekly using a UMTS/GPRS modem and the remote control software "TeamViewer".

2.3. Chemical-physical analysis

2.3.1 Dry matter method

The free water content in the samples was determined by drying an aliquot (5 g) of the sample to a constant weight in an oven at 105°C. The weight loss corresponded to the loss of moisture. The result was expressed as a percentage (AOAC, 1990).

2.3.2 Ash methods

The sample (approximately 2 g) was dried in an oven at 100°C and thereafter calcinated in a muffle at 525°C; the weight obtained after calcination was the ash content (AOAC, 1990).

2.3.3 pH determination

The pH determination was performed by potentiometric analysis. The pH of the milk was measured without dilution; regarding the cheese samples, 100 mL of distilled water previously brought to a boil was added to 10 g of cheese, and the mixture was vortexed on a magnetic plate for 15 min.
The mixture was centrifuged for 5 min and left to decant to separate the supernatant. Finally, the pH of the supernatant was measured (AOAC, 1990).

2.4. Determination of the total protein content

The total nitrogen content of the samples was determined with the Kjeldahl method **(**AOAC, 1990).
One gram of homogenized sample was digested inside an appropriate reaction tube with 10 mL of sulfuric acid (96 %), 5 mL of hydrogen peroxide (30 %) and a catalyst based on copper sulphate pentahydrate and heated at a high temperature (250°C) to destroy all the organic material. Afterwards, 50 mL of distilled water and 50 mL of sodium hydroxide (30 %) were added. Adding an excess of sodium hydroxide solution, the ammonium ions were released in the form of ammonia, distilled and added to a boric acid solution. The ammonia content was determined with a volumetric acid solution or by back titration with a sodium hydroxide solution of a known concentration. The percentage of total protein was calculated using a conversion factor of 6.38.

2.5. Determination of the sodium chloride content (the Mohr method)

Approximately 2 g of the dried (in an oven at 105°C until constant weight) sample (cheese and milk) was added to 40 mL of bi-distilled water for 2 h under stirring at room temperature, followed by centrifugation for 10 min at 2683 *g*, and finally filtered.
The pH of the solution was adjusted with 0.1 N sodium hydroxide up to a value of 8.0. Twenty millilitres of distilled water containing 5 drops of a 5 % K_2CrO_4 indicator was added to the mixture, which was then titrated with 0.1 N silver nitrate until the colour changed (from white to brick red).
Twenty millilitres of sample with a few drops of indicator was titrated with silver nitrate until the colour changed from yellow to red brick (Johnson and Olson, 1985). When the silver chloride was completely precipitated, the excess of titrant formed a silver chromate precipitate, which indicated the end point.

2.6. Extraction of the total lipids from milk

After vortexing for 90 seconds, 300 mL of a solution composed of a dichloromethane:ethanol mix in a 2:1 ratio (v/v) was added to 30 g of sample, and the mixture was centrifuged for 10 min at 2683 *g*. The supernatant was removed, and the extraction was repeated twice (STEFANOV *et al*., 2010).
The lower organic phase was recovered and filtered into a round bottom flask, and the dichloromethane was removed using a rotary evaporator at 35°C (model Hei-VAP Value; Heidolph, Schwabach, Germany). The residue was placed in a drier and regularly weighed until a constant value was reached.

2.7. Extraction of the total lipids from cheese

Forty millilitres of hydrochloric acid (25 %) and 40 mL of ethanol (95 %) were added at approximately 12 g of sample. The mixture was stirred for 30' in a water bath at 50°C. After cooling, 100 mL of a solution of n-heptane:diethyl ether (1:2, v/v) was added, and the mixture was maintained under stirring for 15 min at room temperature.
Then, the mixture was allowed to decant, and the supernatant (organic phase) was recovered. The extraction procedure was carried out three times, and the supernatant was gathered. The solvent was removed by a rotary evaporator (model Hei-VAP Value;

Heidolph, Schwabach, Germany) (ROMANO *et al*., 2011). The residue was placed in a drier and regularly weighed until a constant value was reached.

2.8. Fatty acid methyl ester analysis

Fatty acid methyl esters (FAMEs) were prepared according to AOAC 996.06 (2011). Briefly, to 200 mg of a lipid extract, 2 mL of a 1.25 M HCl/CH_3OH solution was added and the mixture was heated for 60 min at 90°C. Then, the methyl esters were extracted with 1 mL of *n*-hexane and 1 μL of the methyl esters was injected in a TRACE GC Ultra gas chromatograph (Thermo Scientific, Waltham, MA, USA) equipped with a flame ionization detector (FID) and SP-2560 capillary column (100 m × 0.25 mm × 0.20 μm, Supelco, Bellefonte, PA, USA).
Helium was used as the carrier gas with a constant flow rate of 1.5 mL min^{-1}. The samples were introduced with a split-splitless injection system in split mode (ratio 1:100) using an AS 3000 autosampler (Thermo Scientific, Waltham, MA, USA). The operating conditions that were followed corresponded to those observed by SIANO *et al*. (2016).
The ramp started at a temperature of 140°C, which was stabilized for 5 min; the temperature was increased at a rate of 4°C per minute up to a temperature of 240°C for 15 min. The run lasted 45 min. The temperature of the injector and detector was 260°C.
To perform the qualitative and quantitative analysis, the retention times of the fatty acids detected in the cheese samples were compared with those of a mixture of fatty acid methyl esters (FAME Mix-37, Supelco, Bellefonte, PA, USA).

2.9. Microbiological analysis

A microbial analysis of the milk used in the Fossa cheesemaking process was not performed because, according to the production regulations, the milk had been pasteurized at 72°C for 15 min, cooled, inoculated with the selected starter and added to the rennet calf powder.
Under sterile conditions, 10 g of each cheese sample was placed in sterile stomacher bags, 90 mL of a sterile peptone-saline solution (bacteriological peptone 0.1 %; NaCl 0.85 %) was added, and the mixture was homogenized in a Stomacher apparatus (Lab-Blender 400, PBI International, Italy). The homogenates were serially diluted 10-fold. To count the bacterial population, the following media, and temperature and time conditions of incubation were used.
One millilitre of each dilution was inoculated on MRS agar and M17 agar plates (Oxoid, Thermo Fisher, Italy), incubated under anaerobic conditions (Anaerogen, Oxoid, Thermo Fisher, Italy) at 28°C for 72 h *Lactobacillus* and *Lactococcus* spp. in Plate Count Agar (PCA) (Oxoid, Thermo Fisher, Italy) and incubated at 28°C for 72 h, to enumerate the total microbial mesophilic bacteria (TMC). The sulphite-reducing clostridia (SRCs) on SPS agar plates incubated under anaerobic conditions at 28°C for 5 days were counted; the total and faecal coliforms were evaluated on Violet Red Bile Glucose agar and Violet Red Bile Lactose Agar (Oxoid, Thermo Fisher, Italy) after incubation at 36 and 44°C, respectively, for 48 h. In addition, 100 μL of the diluted solution was streaked onto mannitol salt agar plates (Oxoid, Thermo Fisher, Italy) and incubated at 28°C for 3-5 days to count *Micrococcaceae* and on YPD plates (yeast extract 1 %; bacteriological peptone 2 %; dextrose 2 %; and agar 2 %), incubated at 28°C for 3-5 days, for the detection of yeasts and moulds.
To evaluate the bacterial load of the Fossa cheese, the plates that contained between 15 and

300 colonies were counted. The values obtained were expressed as the colony forming units of a gram of sample (CFU/g). The microbial counts were carried out in triplicate.

2.10. Determination of the profile of volatile molecules

The extraction of volatile compounds was carried out using the headspace solid-phase microextraction technique (HS-SPME) combined with gas chromatography paired with mass spectrometry (HS-SPME-GC/MS). Five grams of sample (pre-equilibrated to 45°C for 10 min) was weighed in vials of 20 mL containing 5 μL of 3-octanol (internal standard, 100 mg/L standard solution) and the volatile compounds (VOCs) were extracted from the samples by a fibre DVB/CAR/PDMS; the compounds were held for 45 min and block heated to 45°C in the headspace of the sample. The analysis of the volatile compounds was performed using an Agilent 7890A/5975C GC/MS with a Gerstel MPS2 autosampler, using an INNOWax capillary column (30 m × 0.25 mm × 0.50 μm) and the following temperature programme: 40°C for 2 min, 5°C/min to 230°C for 10 min. The injector, quadrupole, source and transfer line temperatures were 240, 150, 230 and 200°C, respectively. The electron ionization mass spectra in full-scan mode were recorded at an electron energy level of 70 eV in the range of 20-400 amu (2 s/scan). The volatile molecules were identified by comparing the recorded values to the mass spectra present in the Wiley 07/NIST 98 libraries and the retention index present in the database or in the literature. Afterwards, to calculate the RI value of the compounds, the *n*-alkanes (C5-C25) were also analysed under the same conditions using GC-MS (VAN DEN DOOL and KRATZ, 1963). The results were expressed as the relative peak area (RAP) with respect to the internal standard.

2.11. Statistical analysis

The experimental data (the moisture content, ash, pH, lipid content and sodium chloride concentration) were statistically analysed using Statistica software version 10 (Statsoft, Tulsa, USA). One-way repeated measures analysis of variance (RM ANOVA) was used to estimate the significant differences during the manufacture and ripening of the cheeses. To isolate the group or groups that differed from the others, multiple comparisons versus control group (the Holm-Sidak method) were used. ANOVA followed by Kruskal-Wallis one-way analysis of variance on ranks was used to estimate the differences in the fatty acid content ($P < 0.05$). The averages and the standard deviations were calculated with Microsoft Office Excel 2016.

3. RESULTS AND DISCUSSION

3.1. Microclimate measurements

Microclimate measurements were performed during the cheese ripening in both pits (78 days, in the *Talamello* pit and in *Cartoceto* pit). The results are shown in Figs. 1 and 2. Different microclimate characteristics were found between the pits. In *Talamello,* the pit temperature remained constant over time at both depths (18°C at the surface and 20-21°C at the bottom), while in the *Cartoceto* pit, the temperature increased over time (increasing from 12°C to 23°C at the surface and from 17°C to 27°C at the bottom). On the other hand, the relative humidity was variable at all depths and in both pits. In the *Talamello* pit, the

surface values were in the range of 88 to 99 % and the bottom values varied between 85 and 90 %, whereas in the *Cartoceto* pit, the relative humidity ranged between 90 and 99 % at the surface and between 80 and 88 % at the bottom.

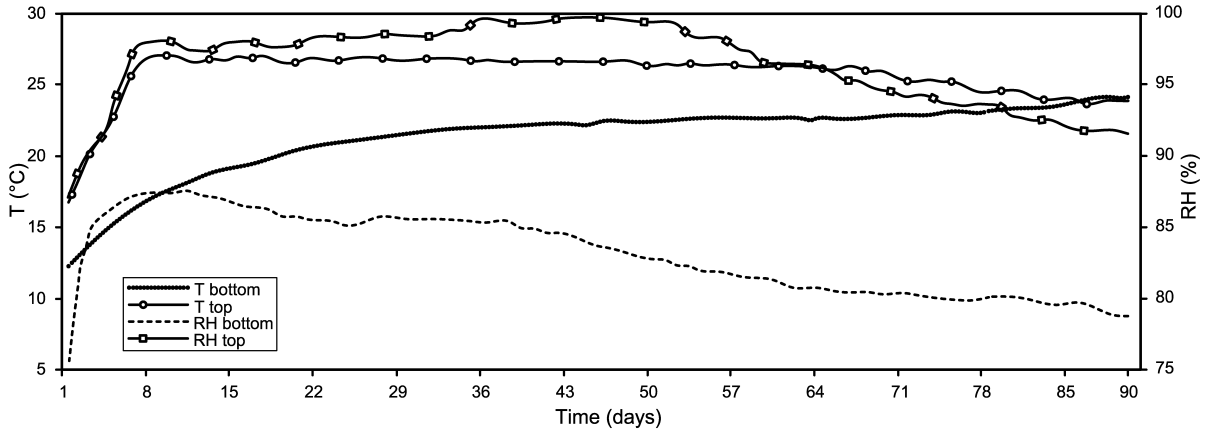

Figure 1. Trend of the temperature and humidity inside the Talamello pit.

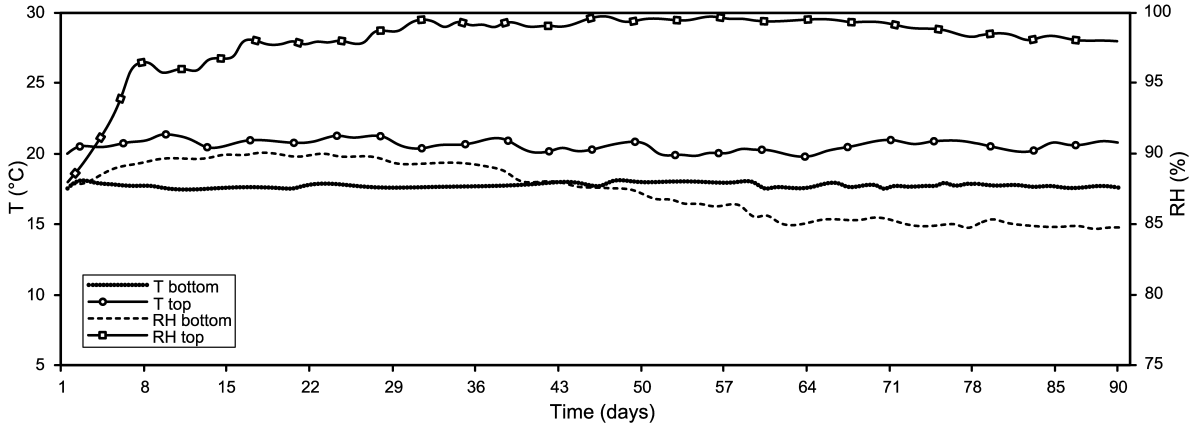

Figure 2. Trend of the temperature and humidity inside the Cartoceto pit.

3.2. Chemical-nutritional composition

The average values (± SD) of the pH, the moisture, lipid, and protein content, and the ash and salt concentration of the Fossa cheese samples are shown in Table 1. RM ANOVA showed significant differences between treatments ($F = 18.642$ with 3 degrees of freedom, $P = 0.004$). Multiple comparisons versus control group (the Holm-Sidak method) showed significant differences in the comparison of "cheese ripened at the dairy farm for sixty days" versus "cheese from the *Talamello* site" ($P = 0.004$) and "cheese ripened at the dairy farm for sixty days" versus "cheese from the *Cartoceto* site" ($P = 0.027$); in contrast, there was no statistically significant difference in the comparison between "cheese from the *Talamello* site" versus "cheese from the *Cartoceto* site" ($P > 0.05$).

The pH of the milk was 6.51, while the pH of the cheese curds decreased to 5.43, a value similar to that of the Talamello-ripened cheese and slightly different from that of the Cartoceto-ripened cheese (5.19).
The protein, lipid, ash and NaCl content of the cheese after ripening in the two pits increased significantly with respect to the cheese curd values, but no significant differences were recorded between the ripened cheese in the two pits and the cheese ripened at the dairy farm for sixty days.

Table 1. The gross composition of the Fossa cheese during production and ripening.

	Milk	Cheese after curdling	Cheese ripened at the dairy farm for sixty days	Cheese from the *Talamello* site	Cheese from the *Cartoceto* site	Statistical significance
pH	6.51±0.02	5.43±0.04	5.42±0.01	5.42±0.27	5.19±0.15	ns
Moisture (%)	83.02±4.98	42.33±1.52 ^{a}	35.46±1.28 ^{b}	34.33±6.71 ^{b}	32.59±3.81 ^{c}	**
Lipid[1] (%)	33.92±5.71	49.26±2.75 ^{a}	52.13±4.19 ^{b}	51.25±2.40 ^{b}	51.10±2.20 ^{b}	*
Protein[1] (%)	32.27±0.59	32.35±5.77 ^{a}	42.10±3.95 ^{b}	40.61±6.58 ^{c}	40.32±3.50 ^{c}	*
Ash[1] (%)	2.35±1.88	5.10±0.78 ^{a}	5.77±1.08 ^{b}	8.14±0.61 ^{c}	8.58±0.67 ^{c}	*
NaCl[1] (%)	0.00±0.00	2.25±0.00 ^{a}	4.91±0.01 ^{b}	6.85±0.56 ^{c}	6.67±0.50 ^{c}	*

a, b, c, d: The different letters in the same row indicate the statistically significant differences ($P<0.05$).
ns = not significant, *$P<0.05$; **$P<0.01$. [1]The concentrations are expressed in terms of the dry matter.

The fatty acid contents in the cheese samples during the production and ripening, together with the results of the variance analysis, are shown in Table 2. Significant differences between the free fatty acid contents of the Fossa cheese samples during the production time and the ripening phase ($F = 81.093$ with 20 degrees of freedom, $P \leq 0.001$) were observed.
For all types of cheese, the major saturated fatty acids (SFA) were myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acid; all the cheese showed highly significant different values ($P < 0.001$) during the production time. Moreover, cheese ripening in the *Talamello* pit resulted in high values of SFA compared to cheese from the *Cartoceto* pit.
Three monounsaturated fatty acids (MUFA) were identified: myristoleic (C14:1), palmitoleic (C16:1), and oleic (C18:1 ω-9,*cis*) acid. The most important differences observed in the content of MUFA were the strong decreases during ripening, up to values of approximately 15 %.
Among the polyunsaturated fatty acids (PUFA), only linoleic (C18:2 ω-6,*cis*) and linolenic (C18:3 ω-3) acid were identified, but only the C18:2 ω-6,*cis* content was significantly different during ripening ($P < 0.001$).
In particular, the fatty acid profile of the cheese after ninety days of ripening in the pits showed a percentage increase of the butyric, caproic, caprylic and capric acid content compared to the cheese after sixty days of ripening at the dairy farm, with respect to just the cheese curds, confirming the fact that the short-chain fatty acids are produced by lipolysis phenomena; in parallel, a decrease in the percentage of long chain fatty acids (oleic, linoleic and linolenic acids) in the cheese after sixty days at the dairy farm was observed compared to the cheese after ninety days of ripening in the pits, with respect to

just the cheese curds. The values of the long chain fatty acid content were almost constant except for oleic acid and palmitic acid, as suggested by various authors (OLMEDO and COLL-HELLIN, 1976; NAJERA *et al*., 1993), highlighting the higher values in linoleic and linolenic acids.
The sum of saturated fatty acids showed no substantial differences between the milk and mature cheese, even if there was a decrement measured in the cheese curds.
Instead, the sum of MUFA showed a significant decrease from 21.39 % in the milk to approximately 16.70 % in the ripened cheeses. According to ALEWIJN *et al*. (2005), this decrease is due to the conversion of fatty acids into aldehydes, ketones, and lactones as a result of β-oxidation followed by decarboxylation. The most significant decrease was observed for the oleic and stearic acids, the main components of the total fatty acids in the analysed samples.
Even the sum of PUFA and the ratio between the sums of PUFA and SFA decreased after ripening in the pits at both sites.

Table 2. Concentration of fatty acids in the milk and Fossa cheese expressed in %.

	Milk	Cheese after curdling	Cheese ripened at the dairy farm for sixty days	*Talamello* Cheese	*Cartoceto* Cheese	Statistical significance
Butyric C4:0	1.80±0.12	0.68±0.08	1.33±0.10	2.10±0.14	1.76±0.10	**
Caproic C6:0	1.92±0.15	1.04±0.12	1.61±0.09	2.17±0.11	1.77±0.12	**
Caprylic C8:0	1.96±0.09	1.42±0.10	1.92±0.12	2.51±0.13	2.10±0.10	**
Capric C10:0	6.06±0.25	5.13±0.26	6.24±0.26	8.10±0.22	6.86±0.24	**
Lauric C12:0	3.49±0.16	3.13±0.12	3.56±0.15	4.39±0.20	3.89±0.18	**
Myristic C14:0	10.89±0.24	10.19±0.28	10.96±0.28	11.28±0.22	11.33±0.24	**
Myristoleic C14:1	0.16±0.06	0.14±0.02	0.55±0.05	0.20±0.08	0.22±0.09	*
Pentadecanoic C15:0	1.25±0.18	1.28±0.14	1.22±0.10	1.23±0.11	1.38±0.14	**
Palmitic C16:0	26.38±0.16	25.74±0.08	26.42±0.36	25.49±0.32	25.10±0.48	**
Palmitoleic C16:1	1.06±0.10	1.39±0.05	0.99±0.08	0.76±0.10	0.79±0.12	*
Heptadecanoic C17:0	0.78±0.08	0.87±0.04	0.83±0.08	0.73±0.04	0.83±0.10	*
Stearic C18:0	11.83±0.66	11.56±0.78	11.56±0.65	9.98±0.88	10.29±0.87	**
Oleic C18:1 ω-9,*cis*	20.17±0.95	21.89±0.86	21.03±0.88	15.43±0.98	15.92±0.77	**
Linoleic C18:2 ω-6,*cis*	2.24±0.22	2.44±0.42	2.37±0.36	1.89±0.42	1.80±0.37	**
Arachidic C20:0	0.34±0.04	0.40±0.08	0.39±0.09	0.28±0.02	0.33±0.07	*
Linolenic C18:3 ω-3	1.47±0.21	1.54±0.23	1.44±0.26	0.89±0.23	1.15±0.18	ns
Σ-SFA	66.70±1.14	61.44±1.37	66.04±1.28	68.26±1.77	65.63±1.98	
Σ-MUFA	21.39±1.51	23.42±1.22	22.57±1.29	16.39±1.61	16.93±1.01	
Σ-PUFA	3.71±0.15	3.98±0.38	3.81±0.32	2.77±0.41	2.95±0.28	
Σ-PUFA/Σ -SFA	0.06	0.06	0.06	0.04	0.04	
Oleic C18:1 ω-9,*cis* Linoleic C18:2 ω-6,*cis*	9.00	8.97	8.87	8.18	8.83	

Note: *, ** indicate the significant differences during ripening, with P<0.05 and P<0.001; ns = not significant.

3.4. Microbiological analysis

The data on the microbial population of the different Fossa cheese samples are shown in Fig. 3.

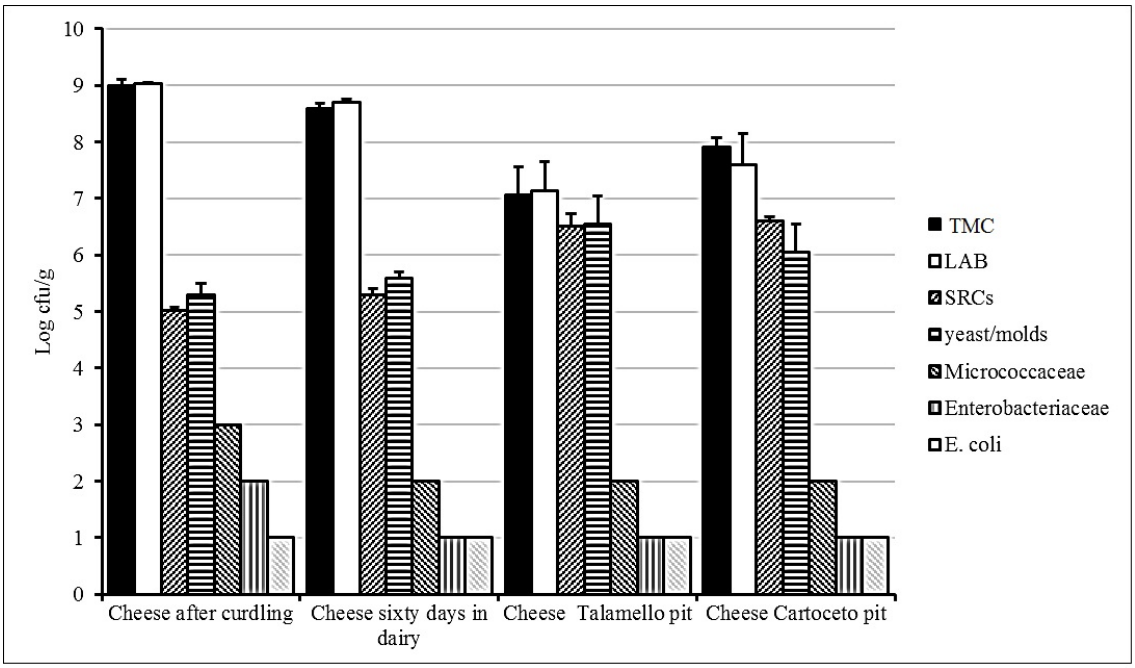

Figure 3. Bacterial dynamics of the principal microbial groups in the Fossa cheese during the manufacturing and ripening process in the pits located in different geographical areas. Data are the means±SD of the three cheese samples.

In summary, in the cheese ripened for 24 h and sixty days, the total microbial count (TMC) was between 10^9 and 10^8 CFU/g. However, the mesophilic lactic microflora count was approximately 10^9 and 10^7 CFU/g; in this case, the higher load of lactic acid bacteria (LAB) and, consequently, the higher total bacterial count were related to the addition of the starter during cheesemaking, and the content of sulphite-reducing clostridia (SRCs) was approximately 10^5 CFU/g. Instead, the contents of the yeast and moulds grown during ripening were approximately (on the order of) 10^4 and 10^5 CFU/g, respectively. A very low load of *Micrococcaceae* was detected in the Fossa cheese, as well as coliforms and *Escherichia coli.* Moreover, in the cheeses that were ripened for 3 months in underground pits (fossa) located at two different sites (*Talamello* and *Cartoceto*), the microbial population showed certain changes in the order of magnitude relative to the total mesophilic microbial count, which dropped from 10^9 to 10^7 CFU/g in the cheese of the Talamello pit, while the count decreased from 10^9 to 10^8 CFU/g in the Cartoceto cheese; the same trend was recorded for the lactic microflora (lactobacilli and lactococci).

The high relative humidity, fairly high temperature and size of the pit environment may influence the oxygen pressure during ripening and may affect the metabolic pathways of cheese microflora that characterize the finished product. Through enzymatic processes, the

elimination of fat and residual moisture occurs, and at the same time the drying of the product is limited (POZZETTO, 2000).
Conversely, the content of sulphite-reducing clostridia increased up to one order of magnitude, and the same trend was shown by yeast and mould contents, probably because of the cheese ripening process and the pit habitat benefitting their growth compared to the other microbial components. Finally, Enterobacteriaceae and *Escherichia coli* were not detected in the 10 g cheese samples. These results suggest that the content of Fossa cheese microflora during ripening decreased because the chemical-physical parameters changed. In fact, during maturation, the cheeses undergo a considerable decrease in weight (approximately 20 %) and take on irregular shapes. The surface of the shapes is wet and greasy, and in some cases, the surface is covered by mould; a skin is absent (GOBBETTI *et al*., 1999). The microflora involved in the Fossa cheese-making process were composed of starter cultures and native microflora that played important roles during the manufacturing of the cheese at the dairy farm and during its ripening in the pit. In particular, the starter microbiota carried out a rapid acidification by the production of lactic acid but also produced enzymes that are important for flavour development during ripening (LEROY and DE VUYST, 2004). Furthermore, non-starter lactic acid bacteria (NSLABs), which are complex mixtures of bacteria, yeasts and moulds, play an important role, together with environmental factors, in achieving the specific characteristics of cheese varieties (FOX and WALLACE, 1997; BERESFORD *et al*., 2001). Additionally, filamentous fungi may reach the cheeses in the environment of the natural caves during ripening (LOPEZ-DIAZ *et al*., 1996; BUDAK *et al*., 2016). The content of non-starter lactic acid bacteria usually increases from a low number in fresh curds to eventually dominate the microflora in the mature cheese because the bacteria tolerate the hostile environment during the cheese ripening well. Furthermore, the heterogeneity of the NSLAB strains together with a pool of enzymatic activities, such as proteolytic and lipolytic activities, may determine a higher complexity in cheese flavour (FOX *et al*., 1998; MCSWEENEY and SOUSA, 2000; DE ANGELIS *et al*., 2001).

3.5. Volatile compounds

The HP-SPME-GC/MS analysis of the cheese samples allowed the identification of 77 compounds belonging to seven groups of volatile compounds. In this work, we quantified 5 aldehydes, 16 ketones, 15 esters, 16 alcohols, 12 acids, 7 terpenes, 3 lactones and 3 S-compounds. The relative amounts of the individual compounds were expressed in terms of their relative peak area (RAP) (Table 3). The total quantities of the volatile compounds typically increase in almost all cheeses during ripening while the profile changes (MASSOURAS *et al*., 2006). Additionally, the addition of spice plants during cheese manufacturing enhances the volatile compounds both qualitatively and quantitatively (CAKIR *et al*., 2016). Aliphatic aldehydes (hexanal, heptanal and nonanal) are transitory compounds and do not accumulate in cheese because they are rapidly transformed to alcohols or to the corresponding acids (HAYALOGLU *et al*., 2007). Branched chain aldehydes are normally found in cheese, 3-methylbutanal is formed by the Strecker degradation of Leu amino acid (URBACH, 1995) and has been found to be a potent odour compound in different cheese varieties (CURIONI and BOSSET, 2002; HAYALOGLU *et al*., 2007). Benzaldehyde, mainly derived from the metabolism of phenylalanine in cheese, which has the aromatic note of bitter almond, is commonly found in cheese and is formed by the oxidative reactions of cinnamic acid or phenylacetaldehyde (MOLIMARD and SPINNLER, 1996).

Table 3. Relative peak area (area of the compound/IS area)×100±the standard deviation for the volatile compounds of the Fossa cheese (n = 3).

		Cheese after curdling	Cheese ripened at the dairy farm for sixty days	Cheese from the *Talamello* site	Cheese from the *Cartoceto* site	Odour description*
RI	*Aldehydes*					
1165	hexanal	13.7±0.5^{b}	38.7±0.2^{a}	nd	nd	Herbaceous
1264	heptanal	20.3±0.9^{b}	73.8±3.8^{a}	nd	nd	Sour milk
1466	nonanal	26.0±0.03^{a}	10.5±0.07^{b}	nd	nd	Floral, citrus
1009	butanal, 3-methyl	nd	nd	2.7±0.2^{b}	4.2±0.1^{a}	Mild, oil
1589	benzaldehyde	nd	nd	44.9±4.9^{a}	7.9±0.5^{b}	Sweet
	Ketones					
872	2-propanone	208.3±6.7^{b}	240.9±2.3^{a}	101.7±7.2^{d}	189.0±5.2^{c}	Apple, pear
993	2-butanone	20.8±0.1^{a}	44.5±0.5^{c}	33.4±0.5^{b}	41.3±2.0^{c}	Chemical, fruity
1066	2-pentanone	39.3±0.9^{d}	965.5±31.3^{a}	263.3±4.6^{c}	501.7±35.2^{b}	Sweet, floral
1069	2,3-butanedione	100.1±7.2^{a}	82.2±1.5^{b}	nd	nd	Fruity, buttery
1165	2-hexanone	nd	nd	12.2±0.4^{a}	7.6±0.2^{b}	Fruity, fungal
1261	2-heptanone	68.5±0.7^{d}	645.8±27.1^{c}	1254.7±19.1^{a}	1034.9±29.5^{b}	Blue cheese
1277	2-heptanone, 3-methyl	nd	nd	2.0±0.2^{a}	2.3±0.2^{a}	-
1303	5-hepten-2-one	nd	nd	13.1±0.4^{a}	2.9±0.1^{b}	-
1359	2-octanone	nd	nd	22.3±1.7^{b}	28.1±0.9^{a}	Dairy, waxy
1360	acetoin	342.8±18.6^{a}	159.1±7.1^{b}	4.7±0.2^{c}	7.3±0.03^{d}	Cream, dairy
1410	6-methyl-5-hepten-2-one	nd	2.7±0.04^{a}	1.1±0.1^{b}	2.5±0.1^{a}	Citrus
1461	2-nonanone	31.3±1.6^{d}	438.4±16.6^{c}	1139.6±81.4^{a}	1294.4±80.4^{a}	Fruity, floral
1497	5-nonen-2-one	nd	nd	2.1±0.2^{b}	3.8±0.2^{a}	Fruity
1512	8-nonen-2-one	nd	nd	67.6±3.6^{a}	94.9±2.6^{b}	Fruity, baked
1556	2-decanone	nd	nd	7.2±0.2^{a}	7.3±0.2^{a}	Orange, fatty
1663	2-undecanone	nd	17.9±0.1^{c}	56.8±1.1^{a}	43.5±0.8^{b}	Waxy, fruity
	Esters					
975	ethyl acetate	63.5±3.5^{a}	45.3±0.2^{b}	nd	3.2±0.2^{c}	Fruity
1047	propanoic acid, ethyl ester	13.7±0.2^{a}	7.3±0.08^{b}	nd	nd	Fruity
1137	butanoic acid, 2-methyl, methyl ester	2.5±0.1^{a}	3.7±0.1^{b}	nd	nd	Fruity
1124	butanoic acid, ethyl ester	19.3±0.4^{d}	23.7±0.8^{c}	63.7±0.8^{a}	45.1±0.6^{b}	Fruity, cheesy
1135	butanoic acid, 2-methyl, ethyl ester	6.7±0.5^{a}	6.5±0.4^{a}	nd	nd	-
1156	acetic acid, butyl ester	12.7±0.6^{a}	12.3±0.3^{a}	nd	nd	Fruity
1253	butanoic acid, 2-propenyl ester	nd	nd	0.9±0.1^{a}	1.1±0.03^{a}	Fruity, green
1286	butanoic acid, 1-methyl, butyl ester	nd	nd	1.0±0.1^{a}	0.8±0.01^{a}	Fruity
1310	hexanoic acid, ethyl ester	8.2±0.03^{d}	20.7±0.1^{c}	28.0±1.8^{a}	25.1±0.6^{b}	Sweet, pineapple
1390	butanoic acid, 3-methyl, butyl ester	2.8±0.02^{b}	3.4±0.1^{a}	nd	nd	Apple, fruity
1505	octanoic acid, ethyl ester	nd	7.2±0.06^{c}	24.1±0.8^{a}	9.7±0.4^{b}	Sweet, fruity
1701	decanoic acid, ethyl ester	nd	nd	33.8±1.8^{a}	13.6±0.3^{b}	Waxy, fruity
1423	formic acid, hexyl ester	4.0±0.3^{a}	4.1±0.1^{a}	nd	nd	Ethereal, sweet
1348	acetic acid, hexyl ester	11.6±0.5^{a}	16.3±0.1^{b}	nd	nd	Fruity, green
1942	butanoic acid, propyl ester	nd	nd	10.2±0.5^{b}	19.5±0.9^{a}	Sweet, fruity

	Alcohols					
1023	ethanol	440.9±13.3^{a}	343.8±1.7^{b}	169.8±17.0^{c}	204.9±13.0^{d}	Alcoholic
1226	1-butanol	8.7±0.1^{a}	7.5±0.1^{b}	1.3±0.1^{d}	3.5±0.2^{c}	Banana, fusel
1230	isobutanol	nd	nd	3.4±0.3^{b}	9.4±0.1^{a}	Fusel
1208	2-pentanol	nd	nd	44.2±2.7^{b}	88.1±4.0^{a}	Mild, green
1284	3-methyl-1-butanol	12.0±0.1^{b}	42.3±0.2^{a}	2.07±0.1^{c}	1.9±0.1^{d}	Fusel, fermented
1297	1-hexanol	nd	nd	1.5±0.1^{a}	1.6±0.1^{a}	Green, fruit
1324	1-pentanol	25.6±0.9^{a}	21.3±0.5^{b}	2.6±0.2^{c}	0.9±0.02^{d}	Fusel, fermented
1391	2-heptanol	nd	nd	71.1±3.4^{a}	58.7±2.6^{b}	Fresh, lemon
1392	3-methyl-2-buten-1-ol	15.9±1.3^{a}	9.4±0.1^{b}	nd	nd	-
1518	1-octen-3-ol	7.7±0.03^{b}	11.2±0.1^{a}	nd	nd	Mushroom, earthy
1556	2-ethyl-hexanol	6.8±0.2^{a}	5.1±0.01^{b}	nd	nd	Sweet, fatty
1638	2,3-butandiol	18.4±0.9^{b}	68.5±0.4^{a}	nd	nd	Fruity, creamy
1640	2-octanol	nd	nd	1.5±0.1^{b}	5.6±0.3^{a}	Fresh, woody
1647	2-nonanol	nd	nd	51.4±3.9^{a}	41.9±1.2^{b}	Waxy, green
1721	2-furanmethanol	nd	nd	1.6±0.01^{b}	4.1±0.2^{a}	Burnt, sweet
1016	2-propanol	nd	nd	7.3±0.8^{b}	18.2±0.4^{a}	Must, woody
	Acids					
1524	acetic acid	141.4±4.9^{c}	249.3±16.5^{a}	95.9±3.2^{d}	154.0±5.8^{b}	Pungent, sour
1610	propanoic acid	nd	2.7±0.1^{c}	4.8±0.3^{b}	16.1±0.3^{a}	Acidic, dairy
1634	propanoic acid, 2-methyl	nd	nd	nd	5.5±0.2^{a}	Sour, cheese
1695	butanoic acid	132.2±8.9^{d}	1022.9±58.5^{c}	1386.9±78.5^{b}	1902.1±69.6^{a}	Sharp, cheese
1735	butanoic acid, 3-methyl	nd	nd	7.8±0.3^{a}	6.3±0.2^{b}	Cheesy, dairy
1736	pentanoic acid	nd	11.6±0.3^{c}	20.4±0.6^{a}	19.6±0.3^{b}	Sweet, rancid
1909	hexanoic acid	120.8±3.8^{d}	584.3±35.3^{c}	1337.4±72.9^{b}	2028.3±100.1^{a}	Sickening, sour
2012	heptanoic acid	5.9±0.03^{c}	6.3±0.05^{c}	12.3±0.6^{b}	15.1±0.4^{a}	Rancid, cheese
2117	octanoic acid	24.3±0.6^{d}	44.3±0.6^{c}	346.1±8.5^{b}	444.3±14.9^{a}	Fatty, waxy
2220	nonanoic acid	nd	nd	6.1±0.2^{a}	5.7±0.3^{a}	Waxy, dirty
2326	decanoic acid	nd	7.5±0.06^{c}	115.7±6.8^{a}	74.6±1.5^{b}	Fatty
2384	9-decenoic acid	nd	nd	3.1±0.03^{a}	2.7±0.03^{a}	Waxy, green
	Terpenes					
1119	dihydromyrcene	22.7±1.5^{a}	21.4±0.1^{a}	2.1±0.1^{c}	2.4±0.1^{b}	Citronellol, herbal
1178	p-menth-4(8)-ene	43.8±1.4^{a}	45.4±0.7^{a}	nd	2.7±0.2^{b}	-
1238	α-phellandrene	11.4±0.3^{a}	6.4±0.1^{b}	1.5±0.1^{c}	1.4±0.03^{c}	Citrus, lime
1272	limonene	152.6±12.7^{b}	360.6±7.6^{a}	6.3±0.2^{c}	2.3±0.1^{d}	Pine, peppery
1107	α -pinene	20.4±0.1^{a}	20.5±0.1^{a}	1.2±0.1^{c}	1.9±0.1^{b}	Woody, pine
1319	γ -terpinene	3.7±0.02^{a}	4.7±0.1^{b}	nd	nd	Citrus, lime
1195	sabinene	2.2±0.1^{b}	3.4±0.1^{a}	nd	3.7±0.04^{c}	Woody, citrus
	Lactones					
1766	γ-caprolactone	nd	5.4±0.5^{c}	7.0±0.3^{b}	13.0±0.5^{a}	Herbal, coconut
2246	δ-decalactone	nd	nd	2.6±0.3^{a}	4.2±0.2^{a}	Coconut, sweet
1974	γ-octalactone	nd	nd	nd	2.4±0.1^{a}	Sweet, coconut
	S-compounds					
761	dimethyl sulphide	35.9±0.1^{b}	44.1±0.2^{a}	nd	nd	Vegetable, dairy
1308	methyl propyl disulphide	7.4±0.03^{b}	12.8±0.5^{a}	nd	nd	Onion, radish
1958	dimethyl sulfone	10.3±0.3^{a}	6.6±0.1^{b}	0.03±0.00^{c}	2.2±0.1^{c}	Sulphurous, burnt

Mean data for the three batches of Fossa cheese, cheeses analysed in triplicate.
a, b, c, d: The different letters in the same row indicate the statistically significant differences ($P< 0.05$).
nd = not detected; RI= retention index; * www.thegoodscentscompany.com/

Ketones are formed by the enzymatic oxidation of fatty acids to keto-acids and their consequent decarboxylation to methyl ketones (MCSWEENEY and SOUSA, 2000). Moreover, the content of longer chain methyl ketones increases during cheese ripening. The ketones have distinctive odours and low perception thresholds. According to

GIOCCHINI *et al*. (2010), in Fossa cheese, the major ketones are 2-heptanone and 2-nonanone, which contribute to the aroma with blue cheese notes.
Different esters were detected in the volatile fraction of the Fossa cheese, namely, 7-ethyl ester, 1-propyl ester, 3-butyl ester, 2-hexyl ester, 1-propenyl ester and 1-methyl ester. Esterification reactions may occur between the short- to medium-chain fatty acids and the alcohols. Nevertheless, the esters might also be synthesized directly from triglycerides and alcohols via an alcoholysis reaction. In particular, the proliferation of esters after ripening is due to the presence of ethanol and the abundance of short-chain FFA. These compounds are probably the result of microbial metabolism of the fatty acids. They play an important role in the formation of the fruity feature and characterize the flavour of certain Italian cheeses (PANSERI *et al*., 2008). They also contribute to the balance of the flavour by minimizing the sharpness imparted by the free fatty acids. We observed a variability in the level of esters during ripening. The more representative esters in the Fossa cheese samples that increased were butanoic acid ethyl ester, hexanoic acid ethyl ester, octanoic acid ethyl ester, decanoic acid ethyl ester and butanoic acid propyl ester. Ethyl esters, due to their high content, probably contributed to the overall flavour of Fossa cheese because they have low detection thresholds (DELGADO *et al*., 2010).
The alcohol class showed higher variability during ripening, and the different patterns could be associated with the different metabolic pathways involved in the formation of alcohols in cheese, namely, lactose metabolism, methyl ketone reduction, amino acid metabolism and degradation of linoleic and linolenic acids (DELGADO *et al*., 2010).
Twelve acids were identified in the samples, and they had a positive contribution to the typical flavour in a majority of the cheeses (PANSERI*ET al*., 2008). During the ripening of cheese, carboxylic acids could originate from three main biochemical pathways: (i) lipolysis (hydrolysis of the triglycerides into free fatty acids), (ii) proteolysis (cracking of the caseins into peptides and amino acids) and (iii) lactose fermentation (CURIONI and BOSSET, 2002). Based on this information, we have found acids with a microbial origin (acetic acid and propanoic acid), acids with an origin of lipolysis (butanoic, pentanoic, hexanoic, heptanoic, octanoic, nonanoic and decanoic acid), and acids with an origin in amino acids (propanoic acid, 2-methyl- and 3-methylbutanoic acid).
Finally, the trend of the acids increased during the ripening process of the Fossa cheese. Hexanoic and butanoic acids were the most abundant acids identified. Due to their low aroma thresholds, they are considered important contributors to the flavour profile in a wide variety of cheeses (MOIO and ADDEO, 1998; DELGADO *et al*., 2010; DELGADO *et al*., 2011). The branched-chain fatty acids (propanoic acid, 2-methyl- and 3-methylbutanoic acid) are characteristic odour-active compounds that have an impact on goat and sheep milk cheeses.
Terpenes are important volatile compounds with origins in plants that constitute the forage mixture of pastures (DELGADO *et al*., 2011).
Three lactones, namely, γ-caprolactone, δ-decalactone, and γ-octalactone, were detected in the cheese ripened for 90 days, but γ-octalactone was only detected in the cheese from the Cartoceto pit. The lactones have fruity sweet, creamy, fermented notes, and they could contribute pleasant odour notes to the aroma of Fossa cheese (DELGADO *et al*., 2010). Moreover, three S-compounds were identified (dimethyl sulphide, methyl propyl disulphide and dimethyl sulfone), and these compounds decreased during ripening.
Finally, the cheese from the Cartoceto pit contained more volatile compounds than the cheese from the Talamello pit, in particular for the alcohol, acid and lactone classes.
The results of this study showed that there were substantial differences between the chemical and chemical/physical parameters, and many fatty acids of the just curdled

cheese samples and the cheese ripened for 90 days in the two pits were characterized by different geological-geochemical parameters.
Slight differences in the nutritional parameters between the cheeses ripened in the different pits could be identified in certain components of the fatty acids and in the content of certain groups of volatile molecules.
In particular, the cheese ripened in the Talamello pit showed high values of SFA compared to the cheese from the Cartoceto pit, while the sum of PUFA in the Cartoceto cheese was higher. The cheese from the Cartoceto pit had more volatile compounds than the cheese from the Talamello pit, in particular for the alcohol, acid and lactone class.
In conclusion, although the cheeses ripened in the two different pits had been prepared with the same milk, the pedo-climatic environment in the pits significantly influenced only certain nutritional parameters.

ACKNOWLEDGEMENTS

The authors thank Prof. Francesca Rosati (Sworn Translator) for her support in checking the manuscript for English form.

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Paper Received December 18, 2018 Accepted March 14, 2019