A gas chromatography-mass spectrometry untargeted metabolomics approach to discriminate Fiore Sardo cheese produced from raw or thermized ovine milk

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ABSTRACT

Thermization is a sub-pasteurization heat treatment of cheese milk (at 57–68°C for 15–30 s) aimed to reduce the number of undesirable microbial contaminants with reduced heat damage to the indigenous milk enzymes. In this work, the effects of milk thermization on the compositional parameters, proteolysis indices, free fatty acid levels, and low molecular weight metabolite profiles of ovine cheese were studied. Cheese samples at different ripening stages and produced in 2 different periods of the year were analyzed. While the effects of milk thermization on cheese macro-compositional parameters and free fatty acid levels were not evident due to the predominant effects of milk seasonality and cheese ripening stage, the gas chromatography-mass spectrometry based metabolomics approach of ovine cheese produced from raw and thermized milk highlighted strong differences at the metabolite level. Discriminant analysis applied to gas chromatography-mass spectrometry data provided an excellent classification model where cheese samples were correctly classified as produced from raw or thermized milk. The metabolites that mostly changed due to the thermization process belonged to the classes of free amino acids and saccharides. Gas chromatography-mass spectrometry-based metabolomics has proven to be a valid tool to study the effect of mild heat treatments on the polar metabolite profile in ovine cheese.

Key words: Fiore Sardo, heat treatment, ewe milk, partial least squares-discriminant analysis

INTRODUCTION

Sardinia (Italy) plays a relevant role in European Union sheep milk production of which 70% is transformed into 3 Protected Designation of Origin (**PDO**) cheeses: Pecorino Romano, Pecorino Sardo, and Fiore Sardo. According to the PDO specification, beyond intrinsic technological aspects, these cheeses differ in the milk heat treatments. In particular, Fiore Sardo cheese must be produced with raw ovine milk, whereas for Pecorino Romano and Pecorino Sardo cheese, heat treatment of milk is allowed. In European Union ovine cheese production, raw milk is used for several PDO cheeses such as Fiore Sardo and Pecorino di Filiano (Italy), Ossau-Iraty and Roquefort (France), and Roncal and Idiazabal (Spain).

In the dairy industry, milk is generally thermally treated to reduce the levels of viruses, bacteria, molds, yeasts, and protozoa. Common heat treatments are in order of increasing severity: thermization, pasteurization, extended shelf life treatment, UHT treatment, and in container sterilization. (Hilton and Lewis, 2017). These treatments have been shown to modify milk characteristics such as microbiota, carbohydrates, proteins, and lipids but also the enzymatic activity, renneting attitude, and the nutritional and sensory properties of dairy products (Vélez et al., 2010; Piga et al., 2013; Gathercole et al., 2017).

In the cheese industry, the thermal treatment of milk is a common practice. Generally, thermization and pasteurization are used to reduce undesirable microflora and pathogens and to lessen cheese technological defects. Usually, for semi-hard and hard ovine cheese production milk thermization is used. Few studies are available on the influence of thermization on ovine cheese characteristics. Though, milk thermization treat-

ments may have negative effects on desirable lactic acid bacteria (**LAB**) and on the biodiversity of the natural microflora of milk, and by inactivating thermolabile enzymes, it can also introduce numerous changes to milk components (Gaya et al., 2005; Raynal-Ljutovac et al., 2007). For example, in Greek hard cheese, the thermization process inactivated potentially protective LAB strains and enhanced the capability of potentially pathogenic enterococci to grow in the fresh cheese curds (Gaya et al., 2005). Microstructural differences were observed when comparing Pecorino cheese produced from raw or thermized milk (Mulas et al., 2013). Similarly, in PDO Fiore Sardo cheese, Pirisi et al. (1999) reported a greater calcium content and a more elastic cohesive texture in cheese produced from raw milk when compared with thermized milk. Currently, European Council regulations [EC Regulation 853/2004 (EC, 2004); EEC Directive 92/46 (EEC, 1992)] indicate the alkaline phosphatase test as an official method to detect milk heat treatments. The usefulness and reliability of this enzymatic test is a matter of debate (Scintu et al., 2000; Rankin et al., 2010) because of the false positives due to a possible reactivation of the enzyme in periods following the thermal treatment (Lyster and Aschaffenburg, 1962; Murthy et al., 1976; Farkye and Imafidon, 1995).

To the best of our knowledge, no official analytical methods or biomarkers able to discriminate between cheese obtained from raw or thermized milk are available at the moment. This is particularly important for PDO ovine cheeses as Fiore Sardo where the use of raw milk is mandatory and when fraudulent milk thermization procedures could be implemented.

Metabolomics is one of the -omic sciences for studying the pool of metabolites (metabolite profile) in a set of biological samples using high-throughput analytical platforms together with multivariate statistical tools that can be easily applied to dairy products (Afshari et al., 2018). Food metabolites include AA, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, inorganic species, and other components with nutritional properties. Applied to the characterization of milk and dairy products, GC-MS-based metabolomics has proven to be a robust approach thanks to its high sensitivity and ability to snap-shot a wide range of metabolites within a single analysis (Gowda and Djukovic, 2014). Our research group has conducted several studies on milk and dairy products with this approach and has found associations between metabolite profile and macroscopic data (Caboni et al., 2016; Pisano et al., 2016; Scano et al., 2014).

The aim of this work was to investigate, using a metabolomics approach, the modifications of the metabolite profile in ovine cheese caused by a milk ther-

mization process and to assess whether the metabolite profile can be used as a biomarker for milk thermization in ovine cheese. To this goal, the hydrophilic low-molecular weight metabolites, detected by GC-MS, were studied in ovine cheese samples obtained from either raw or thermized milk. Milk collection and cheese manufacturing were performed in different periods of the year to reduce seasonal variability.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were of analytical grade. Methanol, chloroform, hexane, trichloroacetic acid, pyridine, O-methylhydroxylamine hydrochloride, N-methyl-N-(trimethylsilyl)trifluoroacetamide, potassium chloride, phosphotungstic acid hydrate, and 2,2,3,3-D_4-succinic acid were purchased from Sigma-Aldrich (Milano, Italy). Bi-distilled water was obtained with a MilliQ purification system (Millipore, Milan, Italy).

Cheese Production and Sampling

Bulk milk samples from mature Sarda ewes were collected in December 2016, and February, April, and May 2017, leading to 8 cheesemaking trials (I, II, III, and IV, for raw and heated milk, respectively) from the experimental farm of Agris Sardegna and processed into Fiore Sardo cheese type following the process reported in Figure 1. Each time, 120 kg of sheep raw bulk milk was split in 2 aliquots: 60 kg of raw milk (**RM**) and 60 kg of thermized milk (**HM**). Thermal treatment occurred in a batch-wise process by using an infrared tubular exchanger (model Stoutz-Actinator, ACTINI Group, Evian-les-Bains, France), in which milk was heated at 68°C for 30 s and quickly cooled down to 38°C. The process was repeated under the same conditions to obtain 4 replicates for each milk treatment in the pilot plant of Agris Sardegna. To eliminate the effects of vat and cheesemaker, 2 vats of cheese, 1 with RM and 1 with HM alternately, were simultaneously used and the dairyman was the same for all cheesemaking. Six cheeses samples of approximately 1.5 to 1.7 kg were produced in each process, for a total of 24 raw milk cheeses (**RC**) and 24 heated milk cheeses (**HC**). For each production, 3 cheese loaves were sampled at 105 and 180 d of ripening, respectively.

Milk and Cheese Composition, Nitrogen Fractions, and Free Fatty Acid Levels

Samples of raw and thermized milk were analyzed for fat, proteins, casein, lactose content, and SCC using a

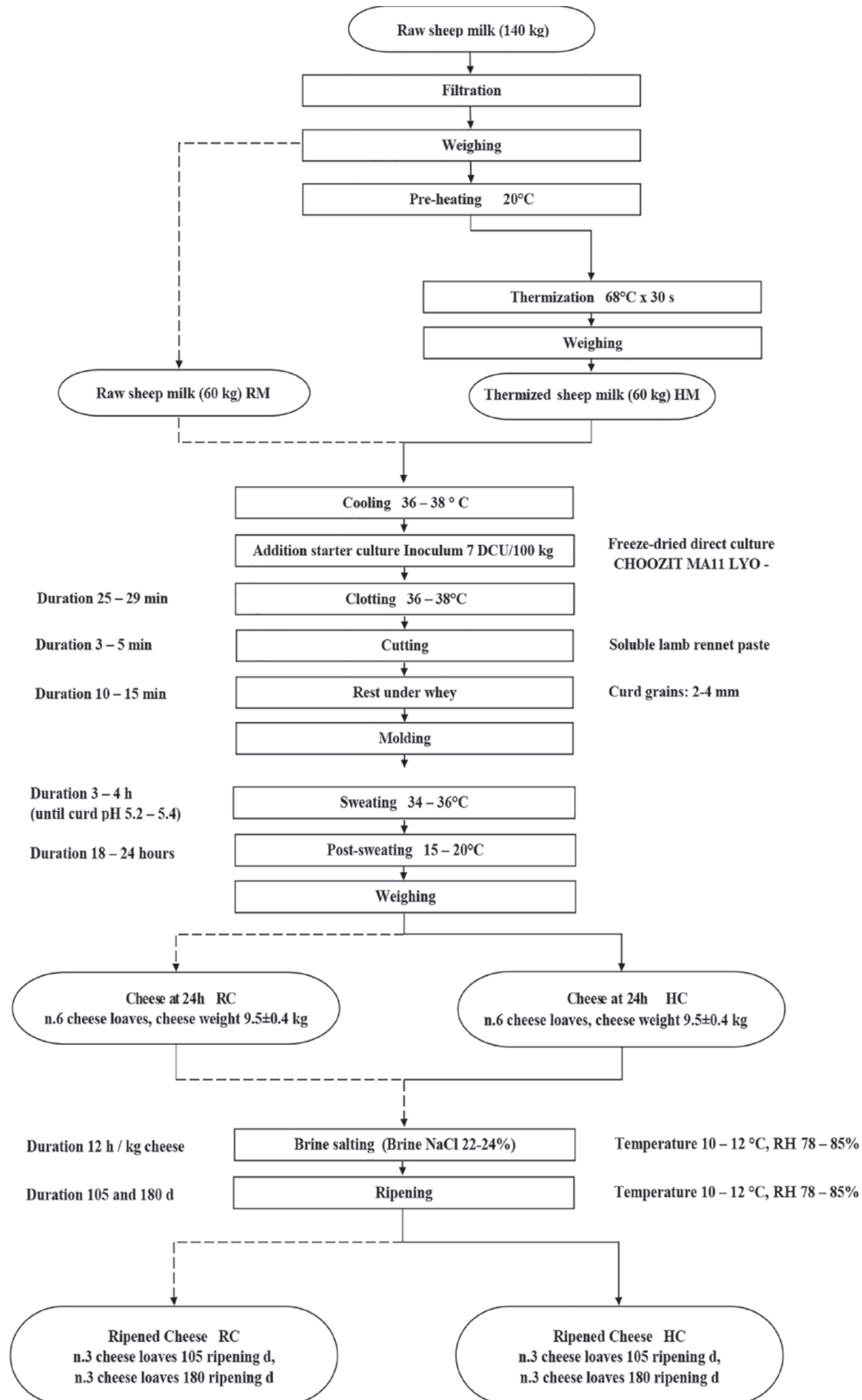CHEESE METABOLITE CHANGES AFTER MILK THERMIZATION

Figure 1. Fiore Sardo cheese production flowchart. RC = cheese from raw milk; HC = cheese from thermized milk; HM = thermized milk; RM = raw milk; RH = relative humidity; DCU = direct culture unit. Choozit MA11 LYO 50 DCU manufactured by Danisco (Copenhagen, Denmark).

Combifoss FT+ (Foss, Hillerød, Denmark), pH using a Crison Basic 20+ pH meter (Crison Instruments S.A., Alella, Spain), and TS (ISO 6731; ISO, 2010). Cheese samples at 105 and 180 d of ripening were analyzed for pH, DM (ISO 5534; ISO, 2004), fat (Soxhlet, 1879), total nitrogen (**TN**; ISO 8968–1; ISO, 2014), soluble nitrogen at pH 4.6, soluble nitrogen in 12% trichloroacetic acid (**SN-TCA**), and soluble nitrogen in 10% phosphotungstic acid (**SN-PTA**; Gripon et al., 1975). Sodium chloride was determined by potentiometric titration with $AgNO_3$ (ISO 5943; ISO, 2006; automatic titrator Mettler-Toledo DL55, Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Protein content in cheese was calculated as follows: protein = [(TN) × 6.38].

Cheese free fatty acid (**FFA**) levels were determined as previously described by Addis et al. (2005).

Sample Preparation for GC-MS

Milk and shredded cheese samples were stored into sterile plastic Falcon tubes at −20°C before analysis. Milk and cheese samples were thawed on ice. Milk samples were sonicated for 15 min and an aliquot of 100 µL was poured in a 1.5-mL Eppendorf tube. Finely shredded cheese samples were weighed (50 mg) and transferred into sterile tubes. To each milk sample, 250 µL of methanol and 125 µL of chloroform were added (Caboni et al., 2016). Samples were sonicated for 15 min, and 380 µL of chloroform and 90 µL of aqueous KCl 0.2 M were added and finally vortexed for 10 s. Samples were then centrifuged at 15,294 × g for 10 min at 4°C (Eppendorf 5810R, Milan, Italy). A volume of 200 µL of both aqueous and organic layer was extracted from each sample and moved into different 1.5 sterile glass vials and dried under a gentle nitrogen stream. The aqueous layer was derivatized first using 50 µL of methoxyamine hydrochloride dissolved in pyridine at 10 mg/mL, homogenized for 20 s, and kept at room temperature for 17 h. Then, 100 µL of N-methyl-N-(trimethylsilyl)trifluoroacetamide was added and samples were vortexed. After 1 h, 600 µL of hexane containing 5 mg/L of 2,2,3,3-D$_4$-succinic acid was added as the internal standard and homogenized again before GC-MS analysis. Cheese samples were treated with 200 µL of methanol, 250 µL of chloroform, and 10 µL of 1% aqueous trichloroacetic acid. Samples were vortexed 4 times for 15 s every 15 min. Then, 760 µL of chloroform and 90 µL of aqueous KCl 0.2 M were added and vortexed for 10 s. Samples were then centrifuged at 15,294 × g for 10 min at 4°C. A volume of 100 µL of the aqueous phase was evaporated under a nitrogen stream and derivatized as previously described (Caboni et al., 2016).

GC-MS Analysis

The derivatized samples were analyzed with a Hewlett Packard 6850 Gas Chromatograph, 5973 mass selective detector, and 7683B series injector (Agilent Technologies, Palo Alto, CA), using helium as the carrier gas at 1.0 mL·min^{-1} flow. One microliter of sample was injected in the split-less mode and resolved on a 30 m × 0.25 mm × 0.25 µm DB-5MS column (Agilent Technologies). Inlet, interface, and ion source temperatures were 250, 250, and 230°C, respectively. Oven starting temperature was set to 50°C, final temperature to 230°C with a heating rate of 5°C min^{-1} for 36 min and then for 2 min at constant temperature. Electron impact mass spectra were recorded from m/z 50 to 550 at 70 eV. The resulting data were analyzed using MSD ChemStation software (Agilent Technologies). The identification of metabolites was performed by comparison with instrumental analytical standards injected database and by metabolite mass spectra comparison with the NIST14 library of the National Institute of Standards and Technology (Gaithersburg, MD). The GC-MS spectra deconvolution was performed through NIST14 library's AMDIS tool. For each metabolite, the GC-MS peak area related to the most abundant and representative mass fragment was calculated using MSD ChemStation software.

Statistical Data Analysis

Physico-chemical and FFA data of cheeses were analyzed with the statistical package Minitab 16 (Minitab 16 Statistical Software, 2010, Minitab Inc., State College, PA). General linear model analysis was used to verify the effects of the thermization (F, 2 levels) and the effect of period (F, 4 levels). The comparison between means was performed using Tukey's significant difference test ($P < 0.05$). After GC-MS analysis of milk samples, a 8 × 71 data matrix was obtained. For each sample the total sum of metabolites area was calculated. Matrix was then normalized row wise by dividing each metabolite's area for the corresponding sum value. The same procedure was applied for the 48 × 64 cheese data matrix. Multivariate statistical analysis of GC-MS data was performed as implemented in SIMCA-P+ software (version 14.1, Umetrics, Umeå, Sweden). Prior to multivariate statistical analysis, variables (metabolites) were mean centered and scaled to unit variance column wise, and row-wise normalization adopted. Principal component analysis was performed to investigate sample distributions, deviating features and common trends. The partial least squares-discriminant analysis and its orthogonal variant (**OPLS-DA**) were performed for

classification of samples and identification of the most discriminant variables. Classificatory and predictive powers were verified based on the goodness of fit (R^2Y) and goodness of prediction, determined through cross validation (Q^2Y). Models were tested for overfitting by permutation tests (n = 400), and negative values of the obtained intercept ($Q^2_{intercept}$) indicate more robust models. The variable importance in projection (**VIP**) score summarizes the contribution of each variable to the model. The VIP scores in the predictive component were analyzed and only those metabolites having VIP values >1 were considered as discriminant between the classes (Eriksson et al., 2006).

RESULTS AND DISCUSSION

Milk Composition Analysis

Milk composition data of RM and HM are reported in Supplemental Table S1 (https://doi.org/10.3168/jds .2018-15885). Fat and protein levels follow the normal seasonal variation observed for Sardinian sheep milk (Carta et al., 1995). Fat content displayed a minimum on February and increased in the following months, in comparison the protein content showed a slower increase from December to May as expressed by the fat to protein ratio. The SCC were found to be higher in January and February. The thermization treatment did not affect the milk composition in any of the considered production periods.

Cheese Macro Composition Analysis and Proteolysis Indices

In Tables 1 and 2 we reported the physico-chemical composition and proteolysis indices (SN/TN, SN-TCA/TN, and SN-PTA/TN) for cheeses, at 105 and 180 d of ripening. In cheese at 105 d of ripening (Table 1), the milk thermization treatment did not affect the pH value, moisture, fat, and protein content in any considered production period. Regardless of the heat treatment of cheese milk, the production period affected the pH and the main macro-composition. Cheeses produced in February and May reached, after 105 d of ripening, a pH value significantly higher than those produced in December and April. The cheeses produced in February were characterized by a higher moisture content compared with those produced in April and May ($P <$ 0.01, Table 1). Moreover, the cheese fat content reflects the trend of fat and the fat/protein-ratio in the milk, decreasing significantly from December to February and increasing from February to May ($P <$ 0.001), and the protein content increasing significantly from

Table 1. Cheese physico-chemical parameters, macro-constituents, and proteolysis indices of cheese at 105 d of ripening (mean ± SD)[1]

Item	December RC	December HC	February RC	February HC	April RC	April HC	May RC	May HC	Sig. T	Sig. P	Sig. T × P
pH	4.95[c] ± 0.04	5.02[cd] ± 0.04	5.22[a] ± 0.03	5.16[ab] ± 0.03	4.97[d] ± 0.05	4.99[d] ± 0.04	5.21[a] ± 0.03	5.11[bc] ± 0.01	NS	**	***
Moisture (%)	32.5[ab] ± 1.2	31.9[ab] ± 0.4	32.7[a] ± 1.5	33.0[a] ± 1.0	30[bc] ± 1	31[bc] ± 3	29.2[c] ± 0.4	29.3[c] ± 0.6	NS	**	NS
Fat (%)	35.8[a] ± 0.7	36.0[a] ± 0.4	30.3[c] ± 0.2	30.5[c] ± 0.3	33[b] ± 1	32[bc] ± 1	34.5[a] ± 0.4	34.5[a] ± 0.3	NS	***	NS
Protein (%)	26.3[c] ± 0.2	26.3[c] ± 0.2	29.1[ab] ± 0.6	29.0[ab] ± 0.9	30.4[a] ± 0.2	30.2[ab] ± 0.4	29.3[ab] ± 0.2	28.8[b] ± 0.6	NS	***	NS
SN/TN	24.5[a] ± 0.3	23.1[ab] ± 0.6	24.8[a] ± 0.7	23.3[ab] ± 0.7	22.0[a] ± 0.4	21.8[b] ± 1.1	21.5[b] ± 0.8	19.0[c] ± 0.4	***	***	NS
SN-TCA/TN	18.7[bc] ± 1.1	18.7[bc] ± 0.6	20.9[a] ± 0.5	19.0[abc] ± 0.3	20[ab] ± 1	19[bc] ± 1	17.4[cd] ± 0.5	16.7[d] ± 0.1	**	***	NS
SN-PTA/TN	9.2[ab] ± 0.5	8.6[b] ± 0.6	9.5[ab] ± 0.1	9.0[b] ± 0.7	9.9[ab] ± 0.2	8.9[b] ± 1.0	10.7[a] ± 0.5	8.5[b] ± 0.5	***	NS	NS
NaCl (%)	1.66[b] ± 0.02	1.82[b] ± 0.09	1.9[b] ± 0.1	2.2[a] ± 0.1	1.9[b] ± 0.1	1.9[b] ± 0.1	1.70[b] ± 0.07	1.75[b] ± 0.02	**	***	*

a–dMeans in the same row with different superscript letters are statistically different.

[1]RC = cheese from raw milk; HC = cheese from thermized milk; T = thermal treatment; P = period of production; SN = soluble nitrogen; TN = total nitrogen; TCA = trichloroacetic acid; PTA = phosphotungstic acid.

NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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Table 2. Cheese physico-chemical parameters, macro-constituents, and proteolysis indices at 180 d of ripening (mean ± SD)[1]

Item	December RC	December HC	February RC	February HC	April RC	April HC	May RC	May HC	Significance T	Significance P	Significance T × P
pH	4.99^{cd} ± 0.06	5.08^{bc} ± 0.08	5.23^{ab} ± 0.03	5.13^{abc} ± 0.02	5.18^{ab} ± 0.08	5.25^{a} ± 0.03	4.88^{d} ± 0.05	4.92^{d} ± 0.07	NS	***	*
Moisture (%)	26.6^{ab} ± 0.2	26.4^{abc} ± 0.4	26.4^{abc} ± 0.7	27.7^{ab} ± 0.2	26.0^{bc} ± 1.4	28.3^{a} ± 0.5	24^{c} ± 1	26^{bc} ± 1	**	**	NS
Fat (%)	38.6^{a} ± 0.3	38.4^{a} ± 0.2	33.6^{c} ± 0.3	33.2^{c} ± 0.6	34.1^{c} ± 0.8	32.9^{c} ± 0.4	37.0^{b} ± 0.6	35.8^{b} ± 0.3	**	***	NS
Protein (%)	28.6^{d} ± 0.3	29.2^{d} ± 0.3	32.3^{a} ± 0.3	31.8^{abc} ± 0.1	32.2^{ab} ± 0.7	31.5^{abc} ± 0.3	30.8^{bc} ± 0.5	30.6^{c} ± 0.9	NS	***	NS
SN/TN	26.3^{a} ± 0.5	25.0^{ab} ± 1.3	26.0^{a} ± 1.4	24.9^{ab} ± 0.6	22^{b} ± 3	25^{ab} ± 1	22^{b} ± 1	22^{b} ± 1	NS	***	NS
SN-TCA/TN	20.4^{abc} ± 2.1	21.7^{ab} ± 0.7	23.0^{a} ± 1.3	21.2^{abc} ± 0.2	19.1^{c} ± 1.9	20.5^{abc} ± 0.6	18^{bc} ± 2	17^{c} ± 1	NS	***	NS
SN-PTA/TN	13.0^{a} ± 0.3	12.3^{ab} ± 0.7	12.1^{ab} ± 0.3	11.2^{bc} ± 0.4	10.8^{c} ± 0.6	10.7^{c} ± 0.2	9.9^{cd} ± 0.1	9.2^{d} ± 0.6	**	***	NS
NaCl (%)	1.92^{b} ± 0.04	1.94^{b} ± 0.01	2.01^{ab} ± 0.04	2.23^{a} ± 0.07	1.88^{b} ± 0.09	1.78^{b} ± 0.04	1.8^{b} ± 0.1	1.9^{b} ± 0.1	NS	***	*

a–d Means in the same row with different superscript letters are statistically different.

[1] RC = cheese from raw milk; HC = cheese from thermized milk; T = thermal treatment; P = period of production; SN = soluble nitrogen; TN = total nitrogen; TCA = trichloroacetic acid; PTA = phosphotungstic acid.

NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

December to February remaining almost unchanged in the following months.

The values shown in Table 1 reflect, in general, a weak proteolytic ripening, characteristic of this type of cheese and comparable to what was reported by Pirisi et al. (1999). Contrary to what was observed by these authors, who did not find significant differences in the proteolysis indices of Fiore Sardo produced from raw or thermized milk, we observed, in general, a more pronounced proteolysis in RC compared with HC cheese at 105 d of ripening. This trend was significant for the SN/TN (21.5 vs. 19.0; $P < 0.001$) and SN-PTA/TN (10.7 vs. 8.5; $P < 0.001$) ratios in cheese produced in May. The heat treatment on milk, besides reducing the bacterial load, may have induced a partial denaturation of the enzymes responsible for the proteolysis of cheese, in particular, bacterial peptidases, mainly produced by the microorganisms of the native milk microflora, which are responsible for secondary proteolysis and therefore for the development of the SN-PTA fraction. In cheese at 105 d of ripening, a positive correlation was found between cheese moisture, SN/TN ($R^2 = 0.76$; $P < 0.001$) and SN-TCA/TN ($R^2 = 0.60$; $P < 0.01$). Moisture, ensuring a greater water activity, was able to favor the enzymatic degradation of proteins. No correlation between proteolysis and NaCl content was found. The highest NaCl content was found for HC produced in February, the highest moisture content of cheese produced in February may have also favored a better dissolution and permeation of sodium chloride.

In 180-d ripened cheeses, excluding samples produced in December, HC showed a higher moisture content when compared with RC and this difference was statistically significant for the samples produced in April ($P < 0.01$, Table 2). A possible explanation is that the milk heat treatment promotes the interactions between caseins and the whey proteins. The latter has a marked ability to retain the water that is then incorporated into the protein matrix, thus conferring greater moisture to the cheese.

Due to natural water evaporation, samples at 180 d compared with those at 105 d showed a decrease in moisture content with subsequent higher levels of fat and proteins. In fact, for RC samples, from 105 to 180 d, the loss of moisture, as well as the concentration of fat and proteins, was significant for all the periods of production considered, whereas for HC samples it was significant only for the months of December and February.

In general, an increase of the proteolytic indices is observed during cheese ripening (Santiago-López et al. 2018). In RC samples, when 105-d samples are compared with 180 d, the increase of the SN-PTA/TN value was statistically significant ($P < 0.01$) in the cheese

produced in December and February. In HC samples, the increase of the proteolytic indices was always statistical significant, except for the SN/TN ratio in the cheese produced in December and February and for the SN-TCA/TN and SN-PTA/TN ratios for the cheese produced in May.

Cheese FFA Profiles

During cheese ripening, the progress of lipolysis and FFA catabolism are the main factors responsible for the flavor development (Collins et al., 2003). In Tables 3 and 4 we reported the levels of FFA determined in the RC and HC samples at 105 and 180 d of ripening, respectively. The FFA were grouped into 3 main groups based on the chain length of carbon atoms: short-chain (C4:0, C6:0, C8:0, and C10:0), medium-chain (C12:0, C14:0, and C16:0), and long-chain (C18:0, C18:1, C18:2, and C18:3) fatty acids. At 105 d, thermization had statistically significant effects only on short-chain FFA, whereas at 180 d all the FFA levels were affected by the heat treatment. When compared with the HC, the RC samples at 105 d of ripening showed an overall higher content of the short-chain FFA. Similar results were found by Pinna et al. (1999) on Fiore Sardo cheese samples obtained from raw and thermized at 65°C milk. This fact can be explained by a partial inactivation of the endogenous lipoprotein lipase (**LPL**) due to the thermization milk treatment (Driessen, 1989; Sousa and Malcata, 1997). As highlighted by Ha and Lindsay (1993), the native milk lipase is characterized by a certain selectivity for the sn-1 and sn-3 positions of the triglycerides where the short-chain fatty acids, predominantly C4:0 and C6:0, are esterified. At 105 d of ripening, the medium- and long-chain fatty acid contents were not significantly different between RC and HC, which could be explained by hypothesizing a selectivity of LPL to the sn-1 and sn-3 positions of triglycerides and to the fatty acid chain length. In fact, the latter fatty acid can exert a steric hindrance to the enzyme's activity on the triglyceride.

After 180 d of ripening (Table 4), RC cheeses are characterized by a significantly higher lipolysis when compared with HC cheeses and the differences were statistically significant for all FFA.

Except for samples produced in December, as ripening progresses, these differences in the FFA content between RC and HC are emphasized. In fact, in 180-d ripened cheeses not only short-chain fatty acids but also the medium- and long-chain fatty acids are higher in RC than in HC cheeses. Probably, after a first phase of the lipolysis process, in which the external and sterically less encumbered position of the triglyceride (the sn-3) was preferentially hydrolyzed, the LPL, with the

Table 3. Free fatty acid levels (mmol/kg of cheese) determined on raw milk cheese (RC) and heated milk cheese (HC) at 105 d of ripening (mean ± SD)

Free fatty acids	December		February		April		May		Significance[1]		
	RC	HC	RC	HC	RC	HC	RC	HC	T	P	T × P
C4:0	$3.7^a \pm 0.3$	$2.6^b \pm 0.3$	$1.3^{cde} \pm 0.1$	$1.05^{de} \pm 0.03$	$1.5^{cd} \pm 0.1$	$0.84^e \pm 0.04$	$1.75^c \pm 0.04$	$1.2^{de} \pm 0.1$	***	***	**
C6:0	$0.9^a \pm 0.2$	$0.6^b \pm 0.1$	$0.5^{bc} \pm 0.1$	$0.34^c \pm 0.01$	$0.51^{bc} \pm 0.01$	$0.29^c \pm 0.03$	$0.52^{bc} \pm 0.01$	$0.37^c \pm 0.02$	***	***	NS
C8:0	$0.4^a \pm 0.1$	$0.3^a \pm 0.1$	$0.28^{ab} \pm 0.04$	$0.20^b \pm 0.01$	$0.26^{ab} \pm 0.01$	$0.18^b \pm 0.01$	$0.265^{ab} \pm 0.004$	$0.21^b \pm 0.01$	**	***	NS
C10:0	$0.6^{ab} \pm 0.1$	$0.6^{ab} \pm 0.1$	$0.7^a \pm 0.1$	$0.47^c \pm 0.00$	$0.68^a \pm 0.02$	$0.43^c \pm 0.05$	$0.65^{ab} \pm 0.01$	$0.49^{bc} \pm 0.03$	***	NS	**
Short chain	$5.6^a \pm 0.7$	$4.2^b \pm 0.6$	$2.7^{cde} \pm 0.3$	$2.05^{de} \pm 0.05$	$2.7^{cd} \pm 0.1$	$1.7^e \pm 0.2$	$3.2^c \pm 0.1$	$2.3^{cde} \pm 0.1$	***	***	NS
C12:0	$0.36^{ab} \pm 0.04$	$0.4^a \pm 0.1$	$0.37^{ab} \pm 0.04$	$0.26^b \pm 0.01$	$0.40^{ab} \pm 0.02$	$0.26^b \pm 0.03$	$0.41^{ab} \pm 0.01$	$0.32^{ab} \pm 0.02$	*	NS	**
C14:0	$0.7^{abc} \pm 0.1$	$0.9^a \pm 0.2$	$0.6^{bcd} \pm 0.1$	$0.45^d \pm 0.02$	$0.7^{abc} \pm 0.1$	$0.5^{cd} \pm 0.1$	$0.81^{ab} \pm 0.01$	$0.7^{abc} \pm 0.1$	NS	***	**
C16:0	$1.6^{abc} \pm 0.1$	$1.9^a \pm 0.5$	$1.1^{bcd} \pm 0.1$	$0.9^d \pm 0.1$	$1.4^{abcd} \pm 0.2$	$1.1^{cd} \pm 0.1$	$1.9^a \pm 0.1$	$1.7^{ab} \pm 0.1$	NS	***	*
Medium chain	$2.6^{ab} \pm 0.3$	$3.3^a \pm 0.8$	$2.1^{bc} \pm 0.2$	$1.6^c \pm 0.1$	$2.5^{abc} \pm 0.3$	$1.9^{bc} \pm 0.2$	$3.09^a \pm 0.1$	$2.7^{ab} \pm 0.2$	NS	***	NS
C18:0	$0.5^{ab} \pm 0.1$	$0.7^a \pm 0.4$	$0.22^b \pm 0.05$	$0.2^b \pm 0.1$	$0.28^{ab} \pm 0.03$	$0.2^b \pm 0.1$	$0.30^{ab} \pm 0.04$	$0.3^{ab} \pm 0.1$	NS	**	NS
C18:1 cis-9	$0.9^{ab} \pm 0.1$	$1.1^a \pm 0.4$	$0.5^{bcd} \pm 0.1$	$0.36^d \pm 0.05$	$0.5^{bcd} \pm 0.1$	$0.42^{cd} \pm 0.03$	$0.74^{abcd} \pm 0.02$	$0.8^{abc} \pm 0.1$	NS	***	NS
C18:2 cis-9,cis-12	$0.04^{bc} \pm 0.02$	$0.02^{bc} \pm 0.00$	$0.01^c \pm 0.01$	$0.02^c \pm 0.02$	$0.03^c \pm 0.02$	$0.02^c \pm 0.01$	$0.07^{ab} \pm 0.01$	$0.09^a \pm 0.01$	NS	***	NS
C18:3 cis-9,cis-12, cis-15	$0.04^{ab} \pm 0.03$	$0.00^b \pm 0.00$	$0.01^b \pm 0.00$	$0.01^{ab} \pm 0.00$	$0.033^{ab} \pm 0.002$	$0.02^{ab} \pm 0.00$	$0.07^a \pm 0.01$	$0.06^a \pm 0.02$	NS	**	NS
Long chain	$1.4^{ab} \pm 0.1$	$1.8^a \pm 0.8$	$0.8^b \pm 0.1$	$0.6^b \pm 0.1$	$0.8^b \pm 0.1$	$0.68^b \pm 0.03$	$1.2^{ab} \pm 0.1$	$1.3^{ab} \pm 0.2$	NS	***	NS
Total	$9.7^a \pm 1.0$	$9.3^{ab} \pm 1.5$	$5.5^{cd} \pm 0.6$	$4.3^d \pm 0.3$	$6.2^{cd} \pm 0.5$	$4.3^d \pm 0.3$	$7.5^{bc} \pm 0.2$	$6.3^{cd} \pm 0.5$	**	***	NS

[a-e]Means in the same row with different superscript letters are statistically different.

[1]T = thermal treatment; P = period of production.

NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4. Free fatty acid levels (mmol/kg of cheese) determined on raw milk cheese (RC) and heated milk cheese (HC) at 180 d of ripening (mean ± SD)

Free fatty acids	December RC	December HC	February RC	February HC	April HC	April RC	May HC	May RC	Significance[1] T	P	T × P
C4:0	5.3^a ± 0.4	4.8^a ± 0.6	3.3^b ± 0.3	2.1^c ± 0.1	3.1^b ± 0.2	1.6^c ± 0.1	3.3^b ± 0.2	2.1^c ± 0.2	***	***	NS
C6:0	1.5^{ab} ± 0.1	1.3^{ab} ± 0.2	1.3^{ab} ± 0.1	0.67^c ± 0.04	1.2^{ab} ± 0.2	0.51^c ± 0.03	1.1^b ± 0.1	0.7^c ± 0.1	***	***	*
C8:0	0.6^{ab} ± 0.1	0.59^{ab} ± 0.02	0.7^a ± 0.1	0.34^c ± 0.02	0.6^{ab} ± 0.1	0.28^c ± 0.02	0.5^b ± 0.1	0.36^c ± 0.04	***	***	***
C10:0	1.0^{bcd} ± 0.2	1.04^{bc} ± 0.04	1.7^a ± 0.1	0.8^{cd} ± 0.04	1.7^a ± 0.3	0.65^d ± 0.04	1.3^b ± 0.1	0.8^{cd} ± 0.1	***	*	***
Short chain	8.5^a ± 0.8	7.8^{ab} ± 0.8	7.0^{abc} ± 0.6	3.6^d ± 0.1	6.6^{bc} ± 0.8	3.1^d ± 0.1	6.1^c ± 0.5	3.9^d ± 0.3	***	***	**
C12:0	0.6^{bc} ± 0.1	0.59^{bc} ± 0.02	1.0^a ± 0.1	0.46^c ± 0.03	1.0^a ± 0.2	0.39^c ± 0.03	0.8^{ab} ± 0.1	0.5^c ± 0.1	***	NS	***
C14:0	1.2^{bc} ± 0.2	1.3^{bc} ± 0.1	1.6^{ab} ± 0.2	0.8^c ± 0.1	2.1^a ± 0.4	0.8^c ± 0.1	1.7^{ab} ± 0.2	1.1^{bc} ± 0.1	***	NS	***
C16:0	2.5^{bc} ± 0.4	2.6^{bc} ± 0.2	3.0^{ab} ± 0.4	1.6^c ± 0.1	3.8^a ± 0.7	1.5^c ± 0.1	3.4^{ab} ± 0.3	2.4^{bc} ± 0.4	***	NS	***
Medium chain	4.4^{bc} ± 0.7	4.4^{bc} ± 0.3	5.6^{ab} ± 0.7	2.9^c ± 0.2	6.9^a ± 1.3	2.7^c ± 0.2	5.8^{ab} ± 0.5	4.0^{bc} ± 0.5	***	NS	***
C18:0	0.8^a ± 0.1	0.7^{ab} ± 0.1	0.6^b ± 0.1	0.31^{cd} ± 0.02	0.6^{ab} ± 0.1	0.3^d ± 0.1	0.5^{bc} ± 0.1	0.6^{bc} ± 0.1	***	***	*
C18:1 cis-9	2.1^a ± 0.3	2.1^a ± 0.6	1.8^{ab} ± 0.1	0.9^c ± 0.1	1.8^{ab} ± 0.4	0.6^c ± 0.1	1.3^{abc} ± 0.2	1.2^{bc} ± 0.3	***	**	*
C18:2 cis-9,cis-12	0.16^{ab} ± 0.03	0.17^{ab} ± 0.04	0.13^{ab} ± 0.02	0.07^b ± 0.02	0.2^a ± 0.1	0.07^b ± 0.02	0.12^{ab} ± 0.04	0.08^{ab} ± 0.05	**	*	NS
C18:3 cis-9,cis-12, cis-15	0.16^a ± 0.02	0.17^a ± 0.04	0.12^{abc} ± 0.03	0.05^c ± 0.01	0.15^{ab} ± 0.04	0.06^{bc} ± 0.05	0.09^{abc} ± 0.02	0.04^c ± 0.04	**	***	NS
Long chain	3.3^a ± 0.4	3.2^a ± 0.8	2.6^{ab} ± 0.2	1.3^c ± 0.1	2.7^{ab} ± 0.6	1.1^c ± 0.2	2.1^{abc} ± 0.2	1.9^{bc} ± 0.4	***	***	*
Total	16^a ± 2	15.4^a ± 0.5	15.3^{ab} ± 1.5	8.1^b ± 0.3	16^a ± 3	6.8^b ± 0.6	14^a ± 1	10^b ± 1	***	***	***

a–d Means in the same row with different superscript letters are statistically different.

[1] T = thermal treatment; P = period of production.

NS, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.

ripening progress, was able to hydrolyze also the less accessible sites (sn-2 and sn-1) where the medium- and long-chain fatty acids are preferentially esterified.

At 105 d of ripening, the month of production had a significant effect on the FFA content on both RC and HC samples. A greater lipolysis was observed in the cheese produced in the month of December, followed by those produced in May, April, and February, and following the same trend as the fat content. This observation is supported by the strong correlation with these parameters ($R^2 = 0.85$, $P < 0.001$).

At 180 d compared with 105 d, we observed a generally and statistically significant increase of FFA ($P < 0.001$) for both RC and HC samples, except for the C18:0 levels in the HC samples. This fact is probably due to the cheese-making technology, which involves the use of lamb paste rennet, which contains lipolytic enzymes of both pregastric and gastric origin that catalyzed the hydrolysis of triglycerides. Overall, differences in the FFA profile between the HC and RC samples, especially in the short-chain fatty acids, can lead to different flavor development.

GC-MS Metabolomics

Representative chromatograms of RC and HM samples at 105 and 180 d of ripening are shown in Figure 2. Table 5 shows the metabolites identified in cheese samples (i.e., AA, hydroxylated short-chain fatty acids, polyols, and mono- and disaccharides). With respect to the original milk, cheese samples showed a different profile of AA, hydroxylated short-chain fatty acids, and sugars. From the mass spectra analysis of each chromatographic peak we were able to annotate 82 metabolites, whereas 30 were considered unknowns.

Principal component analysis was first carried out on GC-MS cheese data. Two strong outliers (samples HC11180 and RC33180) were found and therefore excluded from the statistical analysis. Therefore, a pairwise OPLS-DA "RC vs. HC" was carried out on 46 samples, and results indicated a good fitting and an excellent classification power ($R^2Y = 0.98$, $Q^2Y = 0.90$, classification 100%). The score plot is shown in Figure 3 and the most discriminant metabolites obtained from the analysis of the VIP values are reported in Table 6. Significant changes in cheese carbohydrates were observed with galactose as the most upregulated metabolite when HC samples were compared with RC samples. This observation agrees with previous studies by Olano and Calvo (1989) and Troyano et al. (1996) that reported the formation kinetics of galactose and tagatose during the heating of milk at various temperatures. Another upregulated annotated metabolite was gluconic acid, a weak organic acid found in dairy products po-
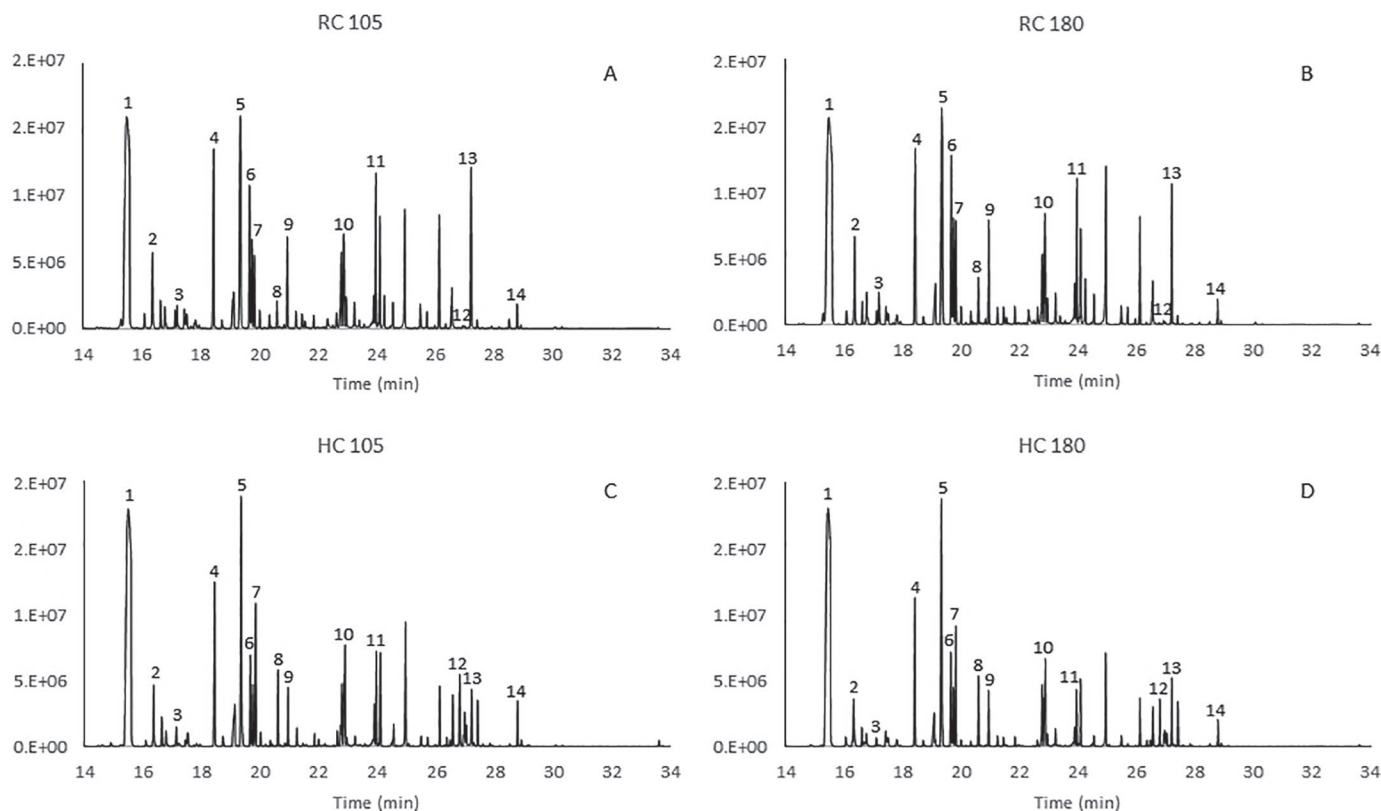
Figure 2. Gas chromatography-mass spectrometry representative chromatograms of cheese samples with significant peak identification: (A) raw milk cheese (RC) at 105 d, (B) RC at 180 d, (C) heated milk cheese (HC) at 105 d, and (D) HC at 180 d of ripening. 1. Lactic acid, 2. alanine, 3. β-lactic acid, 4. phosphate, 5. leucine, 6. isoleucine, 7. proline, 8. serine, 9. threonine, 10. pyroglutamic acid, 11. glutamic acid, 12. lysine, 13. galactose, 14. *myo*-inositol.

tentially formed from the oxidation of glucose. We also found that the serine was a metabolite upregulated in HC samples. It is well known that milk heat treatments cause the denaturation of whey proteins determining an increase of serine, serine phosphate, glycosylated serine, and cysteine residues (Durazzo, 2017). Interestingly, level of fucose, a deoxyhexose metabolite, was found to be higher in cheeses obtained from thermized milk than those from raw milk. Moreover, we found higher levels of *myo*-inositol, a sugar-like carbohydrate, in cheeses obtained from thermized milk. Other upregulated discriminants, not detected in milk, were not annotated metabolites (unknowns) that shared a common fragmentation pattern ascribable to mono- or disaccharides (Table 5). The presence of different saccharides among the upregulated metabolites in HC samples leads us to hypothesize a lower consumption of these molecular components by microorganisms, the presence of which was depleted in the original milk by the heat treatment. The most downregulated metabolite in cheese obtained from thermized milk was α-hydroxybutyric acid. In semi-hard-type and hard-type cheeses, hydroxy acids are considered as neither flavor compounds nor flavor

precursor compounds and originate from the conversion of caseins to small peptides and AA (Smit et al., 2000). The latter are primarily needed by LAB for protein synthesis and are enzymatically transformed intracellularly by aminotransferases to α-keto acids and then to hydroxy acids, CoA-esters, and aldehydes by dehydrogenase and decarboxylase enzymes. The increased AA conversions and the production of α-hydroxy acids, α-keto acids, and carboxylic acids production was confirmed for different cheeses by Yvon et al. (1998) and Banks et al. (2001) after adding α-ketoglutarate to the curd. The pathways that lead to the formation of hydroxyl acids from the corresponding α-ketoacids reduce the levels of α-ketoacids to produce volatile cheese flavors.

In our experimental conditions, the milk thermization process determined a lower level of α-hydroxybutyric acid and this fact is probably related to lower dehydrogenase enzyme activity of LAB when compared with raw milk. Interestingly, α-ketoglutarate can be also produced by a glutamate dehydrogenase rather than transamination, thus lower levels of glutamic acid found in thermized milk and cheese obtained from

Table 5. GC-MS metabolite characteristics

Metabolite	rt[1]	m/z	%	m/z	%	m/z	%	Dairy[2]
Lactic acid (2TMS)[3]	15.57	117	80	147	100	191	30	M, C
Acetic acid (2TMS)	15.85	147	100	117	20	205	10	C
Valine (1TMS)	16.14	72	100	55	20	75	25	C
Alanine (2TMS)	16.37	116	100	147	25	190	10	C
α-Hydroxybutyric acid (2TMS)	16.84	131	70	147	100	205	10	C
Glycine (2TMS)	16.93	102	100	147	70	204	20	M, C
Unknown #1	17.13	147	100	73	10	58	10	C
β-Lactic acid (2TMS)	17.22	177	20	147	100	219	25	C
Oxalic acid (2TMS)	17.27	133	40	147	100	220	25	M
Leucine (1TMS)	17.49	86	100	146	5	188	5	C
β-Hydroxybutyric acid (2TMS)	17.62	117	30	147	100	191	15	M
Isoleucine (1TMS)	17.82	86	100	75	30	146	10	C
Valine (2TMS)	18.54	144	100	73	50	218	25	M, C
α-Hydroxyisocaproic acid (2TMS)	18.72	159	100	103	70	147	90	C
α-Hydroxy-β-methylvaleric acid (2TMS)	18.76	159	100	103	20	147	70	C
γ-Hydroxybutyric acid (2TMS)	18.92	117	30	147	100	233	15	M
Urea 1 (2TMS)	19.20	189	60	73	25	147	100	M
Urea 2 (2TMS)	19.30	189	60	73	25	147	100	M
Leucine (2TMS)	19.32	158	100	147	30	102	10	C
Ethanolamine (3TMS)	19.36	174	100	100	15	147	25	M
Glycerol (3TMS)	19.36	205	60	147	100	117	30	C
Phosphate (3TMS)	19.48	299	100	211	10	314	15	M, C
Threonine (2TMS)	19.71	117	100	130	60	219	50	C
Acetoacetic acid (2TMS)	19.74	231	100	147	25	157	5	M
Isoleucine (2TMS)	19.78	158	100	147	30	218	15	M, C
Proline (2TMS)	19.87	142	100	73	60	216	10	M, C
Glycine (3TMS)	19.96	174	100	147	30	248	25	M
Unknown #2	19.99	256	100	73	85	107	65	M
Succinic acid (2TMS)	20.13	147	100	73	50	247	15	M, C
Glyceric acid (3TMS)	20.29	292	60	147	100	189	60	M, C
Uracil (2TMS)	20.48	241	100	147	50	255	60	M, C
Itaconic acid (2TMS)	20.53	147	100	73	70	259	15	M, C
Citraconic acid (2TMS)	20.63	259	15	73	60	147	100	M
Fumaric acid (2TMS)	20.65	245	100	73	60	147	60	M
Serine (3TMS)	20.73	204	100	147	30	218	65	M, C
Nonanoic acid (1TMS)	20.82	215	100	117	70	129	35	M, C
Threonine (3TMS)	21.08	218	100	117	65	291	35	M, C
Methionine (1TMS)	21.46	104	100	56	90	61	90	C
Aspartic acid (2TMS)	21.56	160	100	130	50	117	30	C
Cystathionine (2TMS)	22.14	75	100	147	95	56	70	C
Capric acid (1TMS)	22.16	229	50	73	100	117	60	M
Unknown #3	22.18	147	100	262	25	365	10	M
Aminomalonic acid (3TMS)	22.25	218	75	147	100	320	35	M
Malic acid (3TMS)	22.47	233	35	147	100	245	25	M
Unknown #4	22.63	116	28	243	100	128	45	C
Erythritol (4TMS)	22.66	217	60	147	100	205	40	M, C
Aspartic acid (3TMS)	22.74	232	100	147	30	100	30	C
Adipic acid (2TMS)	22.76	111	50	147	100	172	20	M
Methionine (2TMS)	22.77	128	50	147	20	176	100	C
γ-Aminobutyric acid (3TMS)	22.89	304	30	174	100	147	60	C
Hydroxyproline (3TMS)	22.96	230	100	75	50	140	20	M
Glycil-glutamic acid (2TMS)	22.96	174	90	158	30	84	100	C
Pyroglutamic acid (2TMS)	22.99	156	100	147	30	258	10	M, C
N-Acetyl-glutamic acid (2TMS)	23.13	174	85	75	85	84	100	M
Creatinine (3TMS)	23.35	115	90	73	100	329	25	M
α-Hydroxyglutaric acid (3TMS)	23.57	129	100	147	65	247	65	M
β-Phenyl-lactic acid (2TMS)	23.57	193	70	147	100	220	15	C
α-Ketoglutaric acid (2TMS)	23.62	198	60	147	100	288	20	M
Asparagine (2TMS)	23.79	159	100	147	40	116	50	C
Glutamic acid (3TMS)	24.12	246	100	128	25	147	30	M, C
Phenylalanine (2TMS)	24.25	192	60	91	20	218	100	M
Ribose (4TMS)	24.35	217	70	103	100	307	50	M, C
Unknown #5 (pentose, 4TMS, MEOX)[3]	24.51	103	100	217	60	307	40	C
Asparagine (3TMS)	24.55	231	70	188	40	116	100	C
Fucose 1 (4TMS, MEOX)	25.06	117	100	147	25	160	15	C
Arabitol (5TMS)	25.17	217	100	103	60	307	30	M
Fucose 2 (4TMS, MEOX)	25.22	117	100	147	30	160	15	M, C

Continued

Table 5 (Continued). GC-MS metabolite characteristics

Metabolite	rt[1]	m/z	%	m/z	%	m/z	%	Dairy[2]
Orotic acid (3TMS)	25.57	254	100	147	20	357	50	M
cis-Aconitic acid (3TMS)	25.61	229	40	147	100	375	30	M
Glycero-phosphate (2TMS)	25.70	357	100	299	90	445	20	M
Azelaic acid (2TMS)	25.98	317	100	201	70	129	75	C
Citric acid (4TMS)	26.33	273	100	363	30	465	20	M
Unknown #6 (hexose, 5TMS)	26.48	204	100	191	40	217	30	C
Unknown #7	26.60	217	100	191	20	204	60	M
N-Acetyl-lysine (3TMS)	26.59	174	100	156	15	147	20	C
Unknown #8	26.60	217	100	191	20	204	60	M
Galactose (5TMS, MEOX)	26.80	319	100	205	75	103	20	M, C
Unknown #9 (hexose, 5TMS, MEOX)	26.83	103	100	217	70	307	50	M
Unknown #10 (hexose, 5TMS, MEOX)	26.93	103	100	217	75	307	50	M
Tyrosine (2TMS)	26.98	179	100	208	15	310	10	C
Unknown #11 (hexose, 5TMS, MEOX)	26.99	319	100	205	90	160	40	M
Unknown #12 (hexose, 5TMS, MEOX)	27.10	319	100	205	75	160	40	M
Lysine (4TMS)	27.21	174	100	230	25	317	75	M, C
Unknown #13 (hexose, 5TMS, MEOX)	27.28	319	100	205	90	160	33	M
Dulcitol (6TMS)	27.29	217	100	205	60	319	56	C
Unknown #14 (hexose, 5TMS)	27.59	217	100	147	30	129	15	C
Tyrosine (3TMS)	27.66	218	100	179	16	280	16	M
Unknown #15	27.83	147	100	217	50	319	40	M
Gluconic acid (6TMS)	28.15	333	100	292	90	205	80	M, C
Pantothenic acid (3TMS)	27.92	291	100	420	75	247	50	C
Scyllo-inositol (6TMS)	28.44	318	100	217	90	191	50	M, C
Unknown #16 (hexose, 5TMS, MEOX)	28.91	319	100	205	70	147	60	C
N-Acetyl-glucosamine (4TMS, MEOX)	28.97	202	80	205	100	319	80	M
Myo-inositol (6TMS)	29.05	305	100	318	60	217	90	M, C
Unknown #17 (hexose, 6TMS)	29.13	319	100	205	70	147	80	C
Unknown #18 (hexose, 6TMS)	29.18	319	100	205	40	147	55	C
Unknown #19 (hexose, 6TMS)	29.42	319	100	205	60	147	60	M
Unknown #20 (hexose, 6TMS)	29.47	319	100	205	60	147	60	M
Tryptophan (2TMS)	30.05	202	100	160	5	73	90	C
Dulcitol (6TMS)	31.65	217	100	205	75	319	60	M, C
Mannitol (6TMS)	31.73	217	100	205	70	319	40	M
Unknown #21 (disaccharide, 8TMS)	33.24	204	100	217	30	191	25	C
Unknown #22 (disaccharide, 8TMS, MEOX)	33.61	204	100	217	70	361	75	C
Unknown #23 (disaccharide, 8TMS)	33.69	204	100	191	30	361	10	M
Unknown #24 (disaccharide, 8TMS, MEOX)	33.74	204	100	217	60	361	60	C
Unknown #25 (disaccharide, 8TMS, MEOX)	34.07	361	100	204	90	319	40	M
Unknown #26 (disaccharide, 8TMS)	34.14	191	50	204	100	217	40	C
Unknown #27 (disaccharide, 8TMS, MEOX)	34.26	361	100	204	90	319	40	M
Unknown #28 (disaccharide, 8TMS, MEOX)	34.32	361	75	204	100	319	30	M
Unknown #29 (disaccharide, 8TMS, MEOX)	34.37	361	75	204	100	319	30	M
Unknown #30 (disaccharide, 8TMS)	34.69	204	100	361	30	191	65	M
Isomaltulose (8TMS, MEOX)	34.93	204	100	361	60	217	75	M

[1]rt = retention time (min).

[2]Class of samples (M = milk, C = cheese) where metabolites were found.

[3]TMS = trimethylsilyl derivate; MEOX = methyloxime derivate.

thermized milk may be also tightly correlated with a dramatically reduced enzymatic activity. On the other hand, pyroglutamic acid can be easily produced by thermophilic LAB as demonstrated by Mucchetti et al. (2002) and its levels should increase with the hard-type cheese ripening process. Considering that aspartic acid can be produced or consumed by LAB aminotransferases (Dudley and Steele, 2001) whereas asparagine is produced from aspartic acid through asparagine synthetase (Weimer, 2007), low levels of these 2 AA in cheese are a signature of reduced AA catabolism and consequently of a reduction in aroma compounds. Furthermore, we noted that 2 metabolites (i.e., α-ketoglutaric acid and α-hydroxyglutaric acid) were found only in milk samples. Same conclusions can be drawn for proline and lysine that are biosynthetically produced by arginine and glutamate metabolism and are catabolized in cheese to fatty acids (Dudley and Steele, 2001). Lyases are responsible for the conversion of AA. In cheese obtained from thermized milk we found lower levels of threonine, another flavor compound, and this fact can be related to the activity of threonine aldolase responsible to produce acetaldehyde and eventually ethanol (not detectable with our ana-

Table 6. Orthogonal least squares-discriminant analysis variable importance in projection (VIP) values of metabolites discriminant between heated milk cheese (HC) and raw milk cheese (RC) samples (model 1 in Table S2; https://doi.org/10.3168/jds.2018-15885)

Upregulated in HC	VIP	SE in cv[1]	Downregulated in HC	VIP	SE in cv
Galactose	1.67	0.21	α-Hydroxybutyric acid	1.55	0.38
Gluconic acid	1.56	0.53	Lysine	1.52	0.40
Fucose	1.49	0.47	Uracil	1.51	0.55
Serine	1.44	0.70	Isoleucine	1.24	0.60
Unknown 11	1.38	0.48	β-Lactic acid	1.23	0.50
Unknown 8	1.36	0.83	Glutamic acid	1.18	0.25
Unknown 12	1.34	0.57	Pyroglutamic acid	1.16	0.36
Unknown 10	1.23	0.65	Threonine	1.11	0.66
Myo-inositol	1.22	0.21	Aspartic acid	1.09	0.26
Lactic acid	1.07	0.58	Proline	1.01	0.64
Ribose	1.01	0.67	Asparagine	1.00	0.37
Unknown 9	1.01	0.55			

[1]Standard errors in cross validation.

lytical platform; Marshall and Cole, 1983). Uracil can be formed as a consequence of the enzymatic hydrolysis of uridine by LAB and nonstarter LAB microorganisms and lower levels of this metabolite found in thermized milk and cheese can be attributed to their lower enzymatic activity. Moreover, the strong correlations among AA and their concomitant downregulation in HC lead us to hypothesize an overall weaker proteolytic activity in HC when compared with RC, as found analyzing the proteolytic indices reported in Tables 1 and 2.

The effects of thermization on the ripening periods (i.e., 105 and 180 d) were also tested by OPLS-DA (i.e., models 2 and 3 reported in Supplemental Table S2; https://doi.org/10.3168/jds.2018-15885). The good performance of the statistical analysis indicates that HC samples were distinguishable from RC samples at both 105 and 180 d of ripening. The effects of ripening on the metabolite profile were also tested, comparing samples collected at 105 d of ripening with those at 180 d. The OPLS-DA results showed a good classification power (see model 4 in Supplemental Table S2; https://doi.org/10.3168/jds.2018-15885). Cheese samples at 180 d with respect to samples at 105 d, in accordance with a major proteolysis extent during ripening, showed
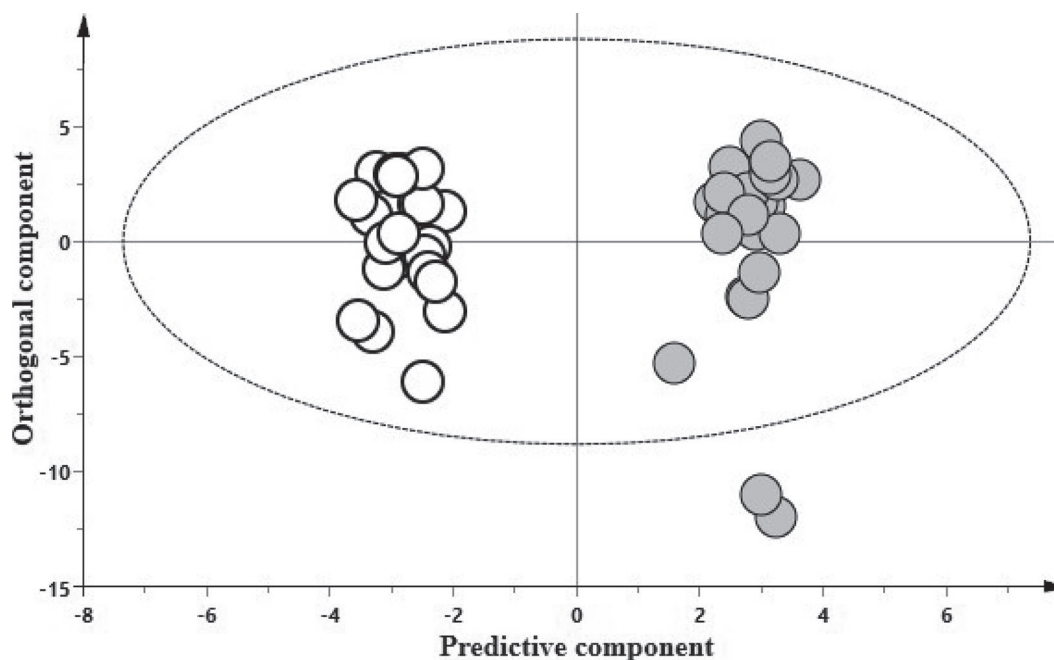

Figure 3. Orthogonal least squares-discriminant analysis score plot of GC-MS data of raw milk cheese (RC) versus heated milk cheese (HC) samples (model 1 in Table S2; https://doi.org/10.3168/jds.2018-15885). Empty circles represent RC samples, and filled circles represent HC samples.

higher levels of methionine, isoleucine, valine, and other free AA (Supplemental Table S3; https://doi.org/10.3168/jds.2018-15885). Moreover, in 180-d cheese, we found lower levels of inorganic phosphate, which was negatively correlated (r = −0.89) with protein levels known to increase during cheese ripening.

However, comparing results of Supplemental Table S2 (https://doi.org/10.3168/jds.2018-15885) and the quality of OPLS-DA models, we can state that thermization had a stronger effect on the polar fraction metabolite profile than the ripening stage.

CONCLUSIONS

In summary, for the samples herein studied, the effects of milk thermization on cheese macro-compositional parameters, proteolysis indices, and FFA levels were minimal compared with the predominant effects of milk seasonality and cheese ripening stage. On the contrary, the GC-MS polar metabolite profile analyzed by a multivariate statistical approach, considering the correlations among variables, highlighted all the concomitant changes of the metabolites due to milk thermization, thus giving an ampler picture of biomarker candidates and a more robust classification. The application of the proposed GC-MS multivariate analytical protocol may have potential application to protect PDO high-quality ovine cheeses, such as Fiore Sardo, Roquefort, Idiazabal, and Roncal from the fraudulent use of thermized milk.

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