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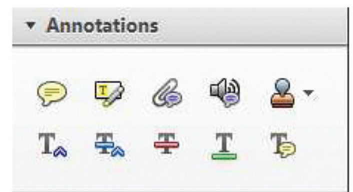

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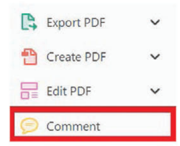

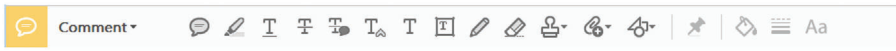

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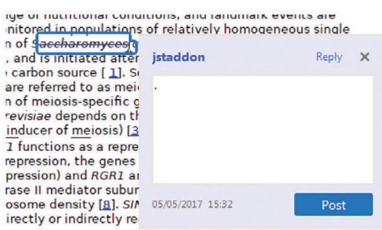

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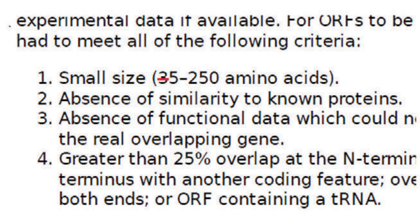

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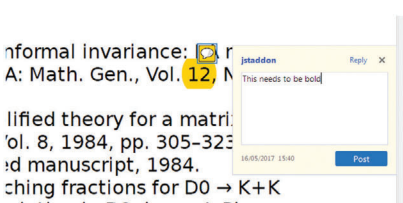

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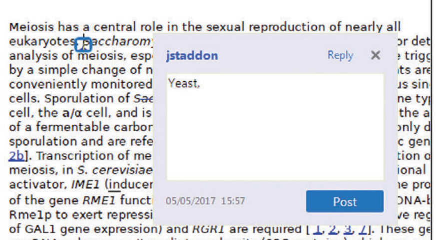

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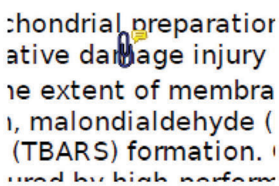

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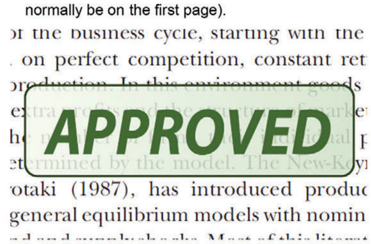

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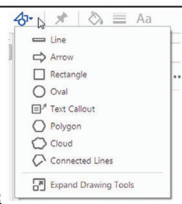

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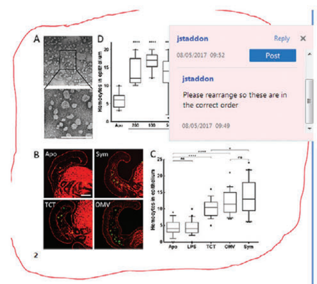

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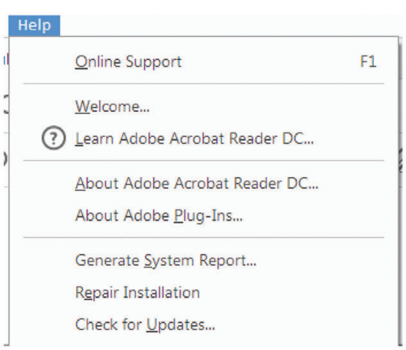

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Effects of adding solid and molten chocolate on the physicochemical, antioxidant, microbiological, and sensory properties of ewe's milk cheese

Mansour Rabie Ashkezary, Adriana Bonanno, Massimo Todaro, Luca Settanni, Raimondo Gaglio, Aldo Todaro, Marco Alabiso, Giuseppe Maniaci, Francesca Mazza, and Antonino Di Grigoli

Abstract: A novel dairy product, namely, "chocolate cheese," was produced with two typical Sicilian food products: Pecorino cheese, processed from ewe's milk, and Modica chocolate. The cheese, manufactured with 0%, 5%, 10%, and 15% (w/w) solid or molten chocolate, was evaluated after 0, 2, 4, and 6 weeks of vacuum storage for its nutritional and health properties. The addition of chocolate reduced the pH, protein, fat, and ash; the addition of 5% or 10% molten chocolate reduced hardness (N/mm^2). The addition of either solid or molten chocolate resulted in a slight increase ($P < 0.1038$) in the total polyphenol content, a higher oleic acid content, and less oxidative stability. The microbiological profile showed that the total mesophilic count and the number of mesophilic coccus lactic acid bacteria (LAB) were approximately equal (about 10^8 CFU/g) in all cheese. The survival of the microorganisms was affected by both the chocolate added and the storage time. Chocolate cheese stored for 6 weeks had less Enterobacteriaceae than control cheese, whereas yeasts were detected at higher cell densities in the former cheese. Filamentous fungi were undetectable in some cheese. Differences were also observed in the number of mesophilic rod LAB, which increased progressively over time in all cheese, and in Enterobacteriaceae, yeasts, and filamentous fungi, which decreased during storage. Descriptive and hedonic sensory tests and principal component analysis showed that fresh cheese and cheese stored for 2 weeks, including 5% molten chocolate, were the most preferred by evaluators. Based on these results, chocolate cheese has the potential to be appreciated in the market for its nutritional, health, and sensory properties.

Keywords: cheese storage, fatty acids, oxidation, PGI Modica chocolate, polyphenols

Practical Application: Chocolate cheese, made by combining two typical Sicilian foods, Pecorino cheese and Modica chocolate, is proposed as a novel dairy product. The highest sensory acceptance was obtained with the addition of 5% molten chocolate and storage for 2 weeks. Given its improved antioxidant properties, healthier fat, and sensory properties, chocolate cheese has the potential to be appreciated in the market, especially by young consumers.

1. INTRODUCTION

Modifying food products in response to consumer demand is crucial to valorizing local raw materials and typical and niche products, such as ewe's milk cheese. New packaging strategies, food formulations, and product shapes or sizes, as well as the addition of ingredients to fortify products, are perceived as innovations to traditional foods (Gellynck & Kühne, 2008).

Pecorino Siciliano is a pressed hard cheese produced in Sicily from raw ewe's milk transformed by traditional wooden equipment without the addition of a starter culture. It is consumed fresh, within a few days of production or after a certain ripening time; when aged for almost four months, it might receive a Protected Designation of Origin (PDO) status if the producing factory is included in the consortium for the protection of this traditional cheese.

Cheese is a rich source of protein, fat-soluble vitamins, and minerals such as calcium, magnesium, and phosphorus. It also contains functional ingredients such as bioactive peptides and rumenic acid (C18:2 c9 t11), the main isomer of conjugated linoleic acids (CLA) that has anti-obesity, antidiabetes, and anticancer potential (Parodi, 2009). The microbial activity that takes place during cheese making plays an important role in the development of the organoleptic properties and nutritive value of cheese. In particular, lactic acid bacteria (LAB) naturally present in milk or added as a starter culture generate volatile organic compounds during ripening, thus contributing to the aromatic profile of the cheese (Guarrasi et al., 2017).

Recently, to meet consumers' demand for healthier and more functional food, researchers in the field of nutrition have investigated bioactive dietary compounds. Among these compounds, plant polyphenols are being carefully investigated for their valuable contributions to human health, mainly due to their antioxidant, antiproliferative, antitumoral, and anti-inflammatory properties (Cutrim & Cortez, 2018). In cheese, phenolic compounds are of limited importance because they occur in small amounts (O'Connell & Fox, 2001) and thus have little antioxidant activity (Han et al., 2011). Accordingly, several studies have focused on fortifying cheese with phenolic compounds to enhance its antioxidant and sensory properties (Cutrim & Cortez, 2018; Han et al., 2011). Cocoa is a known source of polyphenols; it is particularly rich in catechins, which belong to the chemical group of

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flavonoids and have antioxidant effects (Erdem et al., 2014). Thus, chocolate represents a natural source of bioactive compounds with health-promoting properties (Alañón, Castle, Siswanto, Cifuentes-Gómez, & Spencer, 2016), such as a reduced risk for cardiovascular disease and cancer, as well as considerable prebiotic activity (Hu et al., 2016).

Cutrim and Cortez (2018) reported that various macro-biomolecules (carbohydrates, proteins, and lipids) have protective effects on polyphenols during gastrointestinal transit, favoring the bioavailability and bioaccessibility of polyphenols as well as their bioactivity. Lamothe, Langlois, Bazinet, Couillard, and Britten (2016) studied the *in vitro* digestion kinetic of polyphenols from a green tea extract incorporated into dairy matrices, observing improved stability and antioxidant activity due to the formation of a polyphenol–protein complex. As chocolate is a natural source of polyphenols, and cheese consists mostly of proteins, its inclusion in ewe's milk cheese could enhance the absorption of polyphenols from cheese.

In this research, Pecorino cheese was produced with the addition of solid or molten chocolate obtained from bars of Modica chocolate, one of the most appreciated products of Sicilian pastry (Lanza, Mazzaglia, & Pagliarini, 2011), recently recognized as a Protected Geographical Indication (PGI) product. Samples of the resulting chocolate cheese were evaluated for their physical, chemical, oxidative, microbiological, and sensory properties.

2. MATERIALS AND METHODS

2.1 Cheese making and sampling

Cheese was produced from the raw bulk milk of Valle del Belice ewes in a dairy factory located in Santa Margherita di Belice (Province of Agrigento, Sicily). Three experimental cheese-making trials, representing three replicates, were performed over three consecutive weeks in March 2017. Cheese making followed the protocol for PDO Pecorino Siciliano cheese production. Briefly, raw ewe's milk (200 L) was heated to 35 to 37 °C, and then 50 g lamb rennet paste (titre 1:4,000) was added. Coagulation was complete within 45 to 60 min. The coagulum was cut into pea-size pieces and covered gradually with 10% (v/v) hot water (75 °C) to facilitate syneresis. Fourteen curd aliquots, 2 kg each, were hand pressed into cylindrical, perforated plastic molds to drain the residual whey. According to the experimental plan, PGI Modica chocolate, in solid or molten form, was incorporated into the curd to evaluate its effects during storage, especially in terms of sensory attributes and consumer preference. Solid or molten chocolate was added at 5%, 10%, and 15% (w/w), and control cheese was produced without the addition of chocolate. All cheese was left under hot whey (70 °C) for 20 min and then transferred onto a flat surface for draining.

Bars of PGI Modica chocolate were purchased from a retail market. Molten chocolate, prepared by melting the bars in a water bath (45 °C), was added to the curd with a pastry syringe, overlaying layers of cheese curd and thin layers of molten chocolate. Solid chocolate was in the form of 0.75 mL volume pearls obtained by melting the chocolate bars in water bath and then solidifying the chocolate in the mold at 4 °C; the solid chocolate was incorporated to the curd using the same procedure as described for the molten chocolate.

All trials were performed in duplicate, for a total of 14 cheeses produced each cheese-making day. Then 24 hr after manufacture, each cheese was cut in half, and the four halves (two halves for each of the two cheeses of a kind produced each day) were immediately sampled and then vacuum packaged and stored (in a cellar at 16 °C) for 2, 4, or 6 weeks.

Table 1–Chemical composition and coagulation traits of bulk milk used for cheese making.

	Unit	Means	SD	CV%
Lactose	%	4.81	0.095	1.96
Fat	%	6.34	0.312	4.93
Protein	%	5.94	0.180	3.03
Casein	%	4.61	0.137	2.96
Urea	mL/dL	30.8	4.58	14.8
Somatic cells count	1000 n/mL	885	157	17.7
pH		6.68	0.010	0.150
Titratable acidity	SH/50 mL	5.23	0.850	16.3
Coagulation time (r)	min	23.6	0.425	1.80
Curd firming time (k_{20})	min	2.19	0.035	1.60
Curd firmness (a_{30})	mm	41.0	2.94	7.17

Abbreviations: SD, standard deviation; CV = coefficient of variation.

2.2 Milk analyses

Bulk milk used for cheese making was sampled before the addition of rennet. Milk samples were analyzed for lactose, fat, protein, casein, and somatic cell count with the infrared method (Combifoss 6000; Foss Electric, Hillerød, Denmark). Urea content was analyzed with the enzymatic method with different pHs (CL-10 Plus; Eurochem, Rome, Italy). pH was measured with a HI 9025 pH meter (Hanna Instruments, Ann Arbor, MI, USA), and titratable acidity was determined according to the Soxhlet–Henkel method (°SH 50/mL; Table 1). Milk coagulation ability was evaluated after the addition of 0.2 mL aqueous rennet (1:15,000; Chr. Hansen, Parma, Italy) solution (0.8:100) to 10 mL milk at 35 °C by Formagraph (Foss Electric), measuring the coagulation time (r, min), curd firming time (k_{20}, min), and curd firmness after 30 min (a_{30}, mm).

2.3 Cheese analyses

2.3.1 Physical and chemical traits. pH in samples of cheese and chocolate (filtered solution derived from chocolate powder diluted in boiling water [1:9 w/v]) was measured directly with a HI 9025 pH meter equipped with a spear electrode FC 200 (Hanna Instruments). Cheese hardness was measured with the Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy) as the maximum resistance to compression (compressive stress [N/mm^2]) of samples (2 cm × 2 cm × 2 cm) kept at room temperature (about 22 °C). Cheese was analyzed for dry matter (DM), fat, protein (N × 6.38), ash, and NaCl content according to International Dairy Federation standards (IDF, 4A:1982, 5B:1986, 25:1964, 27:1964, 17A:1972) after freeze drying. PGI Modica chocolate was analyzed for DM, protein (N × 6.25), ether extract, reducing sugars, and ash (AOAC, 2000).

2.3.2 Cheese and chocolate fatty acid composition. Cheese fatty acid (FA) composition was determined on freeze-dried samples. Each sample (100 mg) was directly methylated in 1 mL hexane with 2 mL 0.5 M $NaOCH_3$ at 50 °C for 15 min, followed by 1 mL 5% HCl in methanol at 50 °C for 15 min, according to the bimethylation procedure described by Lee and Tweed (2008). FA methyl esters were recovered in hexane (1.5 mL). Then 1 μL each sample was injected by automatic sampler into an HP 6890 gas chromatography system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA). Separation of FA methyl esters from cheese samples was performed with a capillary column 100 m in length with an internal diameter of

Table 2–Chocolate composition (mean ± SD).

	g/100 g FA	g/kg chocolate
pH		5.24 ± 0.057
Dry matter		988 ± 6.43
Protein		85.0 ± 8.87
Fat		256 ± 5.59
Sugars		494 ± 16.3
Ash		22.6 ± 3.54
Polyphenols		17.3 ± 1.63
Fatty acids (FA):		
C12:0	0.084 ± 0.001	0.204 ± 0.005
C14:0	0.129 ± 0.010	0.314 ± 0.029
C16:0	27.0 ± 0.169	65.8 ± 1.60
C16:1	0.244 ± 0.003	0.595 ± 0.020
C17:0	0.236 ± 0.010	0.574 ± 0.012
C18:0	34.9 ± 0.097	85.1 ± 1.81
~~C18:1*c9*,~~ OA	32.4 ± 0.071	78.9 ± 1.69
~~C18:1*c11*~~	0.479 ± 0.006	1.17 ± 0.012
C18:2 *n*-6, LA	2.98 ± 0.006	7.25 ± 0.148
C20:0	1.10 ± 0.002	2.67 ± 0.057
C18:3 *n*-3, ALA	0.229 ± 0.005	0.557 ± 0.024
C22:0	0.186 ± 0.002	0.453 ± 0.013
Saturated FA	63.7 ± 0.075	155 ± 3.43
Unsaturated FA	36.3 ± 0.075	88.5 ± 1.89

Abbreviations: OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid.

0.25 mm and a film thickness of 0.25 µm (CP-Sil 88; Chrompack, Middelburg, the Netherlands). The conditions used for gas chromatography were previously described by Bonanno et al. (2013). Each FA was identified using a FA methyl ester hexane mix solution (Nu-Check-Prep, Elysian, MN, USA), individual standards of C15:0 *iso*, C15:0 *anteiso*, C17:0 *iso*, and C17:0 *anteiso* (Larodan Fine Chemicals AB, Malmö, Sweden) and, for CLA isomers, a standard mixture of methyl esters of C18:2 *c9t11* and C18:2 *c10t12* (Sigma–Aldrich, Milan, Italy). Results were expressed as g/100 g FA. The health profile of the cheese fat was evaluated using the health-promoting index reported by Chen et al. (2004) and the ratio of hypocholesterolemic FA to hypercholesterolemic FA proposed by Santos-Silva, Bessa, and Santos-Silva (2002).

The chocolate FA composition (Table 2) was determined in triplicate on freeze-dried samples (50 mg) using one-step extraction and transesterification (Sukhija & Palmquist, 1988), with C23:0 as the internal standard (Sigma–Aldrich), following the same procedure of FA identification described for cheese.

2.3.3 Oxidation products, polyphenol content, and antioxidant activity. The primary lipid oxidation of cheese fat was assessed on freeze-dried samples by determining the peroxide value (POV, meq O_2/kg fat) (IDF, 74A:1991). Moreover, thiobarbituric acid-reactive substances (TBARs), expressed as µg malonylaldehyde (MDA)/kg DM, representing the products of secondary lipid oxidation, were measured as reported by Tarladgis, Watts, Younathan, and Dugan (1960) and modified by Mele et al. (2011). The POV and TBARs were determined on three reads per sample.

Cheese and chocolate extracts were prepared, as described by Rashidinejad, Birch, Sun-Waterhouse, and Everett (2013) with slight modifications, to determine the total content of phenolic compounds and to measure cheese antioxidant activity by determining the trolox equivalent antioxidant capacity (TEAC). Both polyphenols and TEAC were identified on three replicates per sample. Briefly, freeze-dried and milled samples (0.5 g) were homogenized for 30 s and then extracted for 30 min with 25 mL methanol (95% v/v aqueous solution) containing 1% HCl at 50 °C on an orbital shaker at 200 rpm. The mixture was cooled and filtered through cheesecloth, and the residues were washed with 1 mL of the same solvent (95% methanol aqueous solution with 1% HCl) and finally centrifuged at 7,000 rpm for 10 min at 9 °C.

The total concentration of polyphenols in sample extracts was determined according to the Folin–Ciocalteau colorimetric method, as reported by López-Andrés et al. (2014). The results were expressed as ~~gram~~ gallic acid equivalent (GAE)/kg DM. The antioxidant activity of the cheese extracts was investigated by TEAC assay, as explained by Re et al. (1999), and expressed as mmol trolox equivalent/kg DM.

2.3.4 Microbiological profile. Cheese was analyzed for the main microbial groups. Each sample (15 g) was homogenized in 135 mL sodium citrate solution (2% w/v) with a stomacher (BagMixer 400; Interscience, Saint Nom, France) for 2 min at the maximum speed and then diluted serially (1:10). The cell suspensions were plated and incubated as follows: total mesophilic microorganisms were spread-plated on plate count agar supplemented with 1 g/L skimmed milk and incubated aerobically at 30 °C for 72 hr; mesophilic coccus LAB were pour-plated on M17 agar and incubated anaerobically at 30 °C for 48 hr; mesophilic rod LAB were plated on de Man Rogosa Sharpe (MRS) agar acidified at pH 5.4 with lactic acid (5 mol/L) and incubated anaerobically for 48 hr at 30 °C; Enterobacteriaceae were pour-plated on double-layered violet red bile glucose agar (VRBGA) and incubated aerobically at 37 °C for 24 hr; total yeasts were spread-plated on yeast peptone dextrose agar (YPDA) and incubated aerobically at 30 °C for 48 hr; filamentous fungi were cultivated on potato dextrose agar (PDA) supplemented with chloramphenicol at 0.1% and incubated aerobically at 25 °C for 7 days. Microbiological counts were performed in duplicate. All media were purchased from Oxoid (Milan, Italy).

2.3.5 Sensory analyses. Sensory properties of the cheese were evaluated with descriptive sensory analyses (ISO, 2003) and hedonic tests. Descriptive sensory analyses involved seven untrained evaluators (three women and four men ages 30 to 50 years) selected among volunteers of the Department SAAF at the University of Palermo for their cheese consumption habits. For all panel sessions, samples of approximately 10 g each were placed in randomly coded disposable containers with a three-digit number. All samples were set out at room temperature (about 20 °C) for 30 min before tasting. The evaluators investigated 15 descriptors regarding aspect (color and structure uniformity), smell (strength of odor, odor of butter, odor of milk, and unpleasant odor), taste (salty, sweet, acid, bitter, and spicy), and consistency (chewiness, solubility, and grittiness following mastication); moreover, the evaluators were asked to score the distribution of the chocolate and their overall satisfaction. The evaluators drank water after tasting each cheese. All evaluators, after tasting, scored the quality descriptors using a 90-mm visual analogue scale anchored on the left by *dislike* (low quality) and on the right by *like* (high quality); the results are expressed as the distance (mm) of the marks from the left end of the line. Moreover, the evaluators rated their overall acceptance of the product. During the same sessions, tests were performed to evaluate overall appreciation of the cheese on a 9-point hedonic scale (9 = like extremely; 1 = dislike extremely). The evaluators evaluated five attributes: taste, texture, mouthfeel, overall appearance, and overall liking.

2.4 Statistical analyses

The generalized linear model procedure in SAS 9.2 (SAS Institute, 2010) was used to analyze the data, including the effects of cheese-making day (three levels), chocolate added (CHO; seven levels: control, 5% solid, 10% solid, 15% solid, 5% molten, 10%

molten, and 15% molten), and storage time (STT; four levels: fresh, 2 weeks, 4 weeks, and 6 weeks) and the interaction CHO × STT. When a statistically significant effect ($P \leq 0.05$) was detected, means were compared using P-values adjusted according to the Tukey–Kramer multiple comparisons test. Correlations between parameters were determined with Pearson correlation analyses. The principal component analysis (PCA) was performed with the PRINCOMP procedure in SAS 9.2 (SAS Institute, 2010) on the means of the 15 sensory descriptors together with chocolate distribution, overall satisfaction, and overall acceptance to assess their importance in explaining the sensory differences among the cheese due to the different amounts of chocolate added and storage times.

3. RESULTS AND DISCUSSION

3.1 Physical and chemical traits of milk, chocolate, and cheese

The chemical and coagulation parameters of the bulk milk used for cheese making (Table 1) were quite close to the mean values observed in milk produced in Sicily by Valle del Belice ewes during spring, when pasture feeding is widely available (Todaro, Bonanno, & Scatassa, 2014). Modica chocolate supplemented to curds (Table 2) showed a pH of 5.24, slightly lower than the value (5.92) reported by Lanza et al. (2011) for the same chocolate type. The chocolate in the present study had high fat and sugar, together accounting for 75% of the content, and a total polyphenol content of 17.3 g/kg. The chocolate FA profile was characterized by high saturated fatty acids (SFA, 64%) due to the contribution of stearic acid (C18:0) and palmitic acid (C16:0); most unsaturated fatty acids (UFA, 89%) were represented by oleic acid (C18:1), which helps reduce low-density lipoprotein cholesterol if present in high amounts in the diet (Molkentin, 2000).

As expected, almost all chemical parameters of the chocolate cheese differed from those of the control cheese, and the chemical profile of the cheese changed over time (Table 3).

The addition of 5% and 10% chocolate reduced DM independent of its physical state (solid or molten). An unexpected finding is that when chocolate was added at the highest percentage, the DM content was comparable to that of the control cheese. Adding the chocolate presumably reduced the capacity of the curd to release whey; however, this effect was negligible with the highest amount of chocolate, which, with 99% of DM, could have masked the lower whey loss. Ribeiro et al. (2016) also detected greater moisture in cheese produced with rosemary extract. Opposite trends were observed in other studies, in which the addition of phenolic extracts reduced the moisture content of cheese (Cutrim & Cortez, 2018). The slight but significant reduction in DM during storage might have been due to the fact that the vacuum packaging limited water evaporation, as observed by Duval et al. (2018). The protein, fat, and ash contents decreased significantly and regularly in direct relation to the percentage of chocolate, due to the lower amounts of these components in the PGI Modica chocolate than in the control cheese. A slight increasing trend was observed in these same components during storage; however, these changes were not statistically significant for protein, and in all samples a reduction in fat was observed at 6 weeks of storage, presumably linked to bacterial activity in degrading FA as energy source. Only the NaCl content was not significantly ($P > 0.05$) affected by the addition of chocolate, although a decreasing trend was observed for the chocolate cheese. Moreover, NaCl increased with storage time in most cheese, following the common trend observed during ripening (Todaro et al., 2017b).

Both the chocolate added and storage time significantly decreased the pH of the cheese. The effect of the chocolate was minimal, as its pH was 5.24. However, these results agree with those obtained in studies on the effects of catechins on the pH of cheese (Rashidinejad et al., 2013; Rashidinejad, Birch, & Everett, 2016). According to those studies, the decrease in pH depends on the reaction between sodium ions present in the cheese matrix and phenolic OH groups. Nevertheless, similar behavior of phenolic compounds in the chocolate has to be ruled out because of the decreasing NaCl content, and then the reduction in sodium ions. However, polyphenols in chocolate, such as those in tea (Najgebauer-Lejko, Sady, Grega, & Walczycka, 2011) and apple (Sun-Waterhouse, Zhou, & Wadhwa, 2012), support growth in LAB, as shown in Section 3.2 of this paper; thus, the relative lactic acid production should have contributed to decreasing the pH of the cheese. LAB activity might also explain the lower pH observed during storage (Rashidinejad et al., 2013).

In this study, cheese hardness (Table 3), measured as resistance to compression (N/mm^2), decreased with 5% and 10% molten chocolate compared to the control, especially after 4 and 6 weeks of storage. Chocolate is polymorphic and can crystallize in five or six different crystalline forms. Moreover, when reheating, stable crystals are converted into unstable ones. To make the molten chocolate, the solid chocolate was heated; this change in temperature could have transformed the chocolate crystals from a stable form, with a melting point at a temperature above 30 °C, to an unstable form, with a melting point at a lower temperature (Afoakwa, Paterson, & Fowler, 2007). Given this presumably lower melting point, the molten chocolate in the cheese could have been softer at the normal ambient temperature, leading to a decrease in the hardness of the cheese samples. However, an increase in hardness was recorded with 15% molten chocolate, probably because, at this percentage, the chocolate was distributed in thicker layers, which resulted in greater solidification at room temperature. It is interesting that there was a significant positive correlation between hardness and cheese DM ($r = 0.6674$, $P < 0.0001$), which confirms the results of previous studies (Ayad, Awad, Attar, De Jong, & El-Soda, 2004; Ren, Chen, Chen, Miao, & Liu, 2013). Storage time affected cheese hardness, which, although it showed an irregular trend, was lower at 6 weeks than in the fresh cheese. This result contradicts those showing greater hardness for 60-day ripened Pecorino cheese than fresh cheese (Santillo & Albenzio, 2008). Many factors can affect the hardness and stretch of cheese during storage; for example, increased proteolysis and moisture result in less hardness (Guinee et al., 2004; Lucey, Johnson, & Horne, 2003). Therefore, in this study, the decreasing DM observed during storage, favored by the packaging condition, could have contributed to reducing cheese hardness. However, the change in hardness over time was not comparable for all cheese; indeed, a decrease in hardness was evident in cheese with added molten chocolate, especially 5% and 10% molten chocolate, which was characterized by lower DM after 4 and 6 weeks of storage.

3.2 Oxidation products

Table 3 reports the effects of chocolate and storage time on the development of the POV and TBARs in cheese. The POV, an index of the initial stages of lipid oxidation, ranged from 0.64 to 2.69 meq O_2/kg fat, which is comparable to previous reports for cheese (Branciari et al., 2015; Kristensen, Hansen, Arndal, Trinderup, & Skibsted, 2001; Mele et al., 2011). The significant interaction between the studied factors ($P = 0.0191$) can be as-

Table 3–Effect of chocolate addition and storage time on chemical composition, pH, hardness, oxidation products, and antioxidant activity of cheese (n = 3).

	Storage time (STT)		Chocolate addition (CHO) Control	5% SC	10% SC	15% SC	5% MC	10% MC	15% MC	SEM	Significance ($P<$) CHO	STT	CHO × STT
Dry matter (DM), %	Fresh	57.1^{AB}	57.6	57.2	56.8	57.8	56.3	56.3	57.9	0.433	<0.0001	0.0005	0.9460
	2 weeks	57.4^{A}	58.5	56.5	57.6	58.2	56.6	57.0	57.6				
	4 weeks	56.7^{BC}	57.5	56.8	56.6	57.5	55.4	56.0	56.9				
	6 weeks	56.5^{C}	57.0	56.3	56.2	57.5	55.4	56.2	56.8				
	Total		57.6^{ab}	56.7^{cd}	56.8^{bcd}	57.8^{a}	55.9^{d}	56.4^{cd}	57.3^{abc}				
Protein, % DM	Fresh	43.7	47.9^{a}	45.3^{ab}	43.0^{bcd}	40.9^{cd}	45.4^{ab}	43.5^{bc}	39.5^{d}	0.693	<0.0001	0.2727	0.6196
	2 weeks	43.8	48.5^{a}	45.6^{ab}	42.7^{bc}	39.2^{c}	45.6^{ab}	43.0^{bc}	41.7^{c}				
	4 weeks	44.3	48.1^{a}	45.2^{ab}	43.9^{bc}	40.9^{c}	45.9^{ab}	43.7^{bc}	42.6^{bc}				
	6 weeks	44.1	48.2^{a}	45.5^{ab}	43.6^{bc}	41.1^{c}	46.1^{ab}	43.2^{bc}	40.7^{c}				
	Total		48.2^{a}	45.4^{b}	43.3^{c}	40.5^{d}	45.8^{b}	43.3^{c}	41.1^{d}				
Fat, % DM	Fresh	39.4^{B}	40.4	39.8	37.9	39.0	40.7	40.3	37.8	0.828	<0.0001	<0.0001	0.1773
	2 weeks	40.6^{AB}	42.5^{a}	42.2^{a}	41.8^{a}	39.4^{ab}	40.7^{ab}	40.1^{ab}	37.3^{b}				
	4 weeks	41.5^{A}	42.5	42.5	42.0	41.4	42.6	39.4	40.0				
	6 weeks	39.5^{B}	40.8	40.4	39.7	38.5	38.8	39.6	38.9				
	Total		41.6^{a}	41.2^{ab}	40.4^{ab}	39.6^{bc}	40.7^{ab}	39.8^{abc}	38.5^{c}				
Ash, % DM	Fresh	5.67^{B}	6.20^{a}	5.82^{ab}	5.75^{ab}	5.30^{b}	5.76^{ab}	5.67^{ab}	5.21^{b}	0.117	<0.0001	<0.0001	0.7874
	2 weeks	5.72^{B}	6.28^{a}	5.86^{abc}	5.46^{bc}	5.26^{c}	5.93^{ab}	5.66^{abc}	5.59^{bc}				
	4 weeks	6.02^{A}	6.48^{a}	6.11^{abc}	5.90^{abc}	5.60^{c}	6.29^{ab}	5.99^{abc}	5.77^{bc}				
	6 weeks	5.96^{A}	6.50^{a}	6.24^{ab}	5.75^{bc}	5.49^{c}	6.15^{ab}	5.92^{abc}	5.70^{bc}				
	Total		6.36^{a}	6.00^{b}	5.71^{c}	5.41^{d}	6.03^{b}	5.81^{bc}	5.56^{cd}				
NaCl, % DM	Fresh	0.462	0.467	0.543	0.607	0.249	0.510	0.470	0.390	0.132	0.1164	0.0932	0.8847
	2 weeks	0.613	0.845	0.585	0.440	0.580	0.700	0.555	0.585				
	4 weeks	0.605	0.757	0.620	0.507	0.543	0.623	0.603	0.583				
	6 weeks	0.626	0.817	0.720	0.350	0.527	0.623	0.800	0.543				
	Total		0.721	0.617	0.476	0.475	0.614	0.607	0.525				
pH	Fresh	6.21^{A}	6.40	6.20^{A}	6.09^{A}	6.16^{A}	6.14	6.18^{A}	6.27^{A}	0.075	<0.0001	<0.0001	0.8802
	2 weeks	6.13^{A}	6.26	6.07^{AB}	6.02^{AB}	6.16^{A}	6.18	6.09^{A}	6.17^{AB}				
	4 weeks	5.84^{B}	6.11	5.72^{B}	5.81^{AB}	5.80^{AB}	5.87	5.79^{AB}	5.78^{BC}				
	6 weeks	5.76^{B}	6.06^{a}	5.72^{Bab}	5.65^{Bb}	5.73^{Bab}	5.79^{ab}	5.62^{Bb}	5.76^{Cab}				
	Total		6.21^{a}	5.93^{b}	5.89^{b}	5.96^{b}	5.99^{b}	5.92^{b}	5.99^{b}				
Hardness, N/mm^2	Fresh	1.09^{A}	1.09	1.03	1.15	1.22	0.98	1.03	1.11	0.079	<0.0001	0.0336	0.4103
	2 weeks	1.00^{B}	1.14	0.96	0.95	1.16	0.85	0.89	1.06				
	4 weeks	1.09^{A}	1.25^{ab}	1.05^{ab}	1.09^{ab}	1.31^{a}	0.84^{b}	0.82^{b}	1.28^{a}				
	6 weeks	0.99^{B}	1.16^{ab}	1.02^{ab}	1.11^{ab}	1.22^{a}	0.76^{b}	0.76^{b}	0.93^{ab}				
	Total		1.16^{ab}	1.01^{bc}	1.07^{ab}	1.23^{a}	0.86^{c}	0.88^{c}	1.09^{ab}				
POV, meq O_2/kg fat	Fresh	1.17	0.82^{Bb}	0.96^{ab}	1.07^{ABab}	0.85^{ab}	0.69^{Bab}	2.11^{Aa}	1.70^{a}	0.465	0.9847	0.4017	0.0191
	2 weeks	1.57	2.54^{Aa}	2.20^{a}	1.75^{ABa}	1.85^{a}	1.43^{ABa}	0.54^{Ba}	0.66^{a}				
	4 weeks	1.51	1.50^{ABab}	1.38^{ab}	0.80^{Bb}	0.81^{b}	2.69^{Aa}	1.76^{ABab}	1.65^{ab}				
	6 weeks	1.51	0.64^{Bb}	1.71^{ab}	2.12^{Aa}	1.64^{ab}	1.26^{Bab}	1.59^{ABab}	1.57^{ab}				
	Total		1.38	1.56	1.44	1.29	1.52	1.50	1.40				
TBARs, µg MDA/kg DM	Fresh	32.7^{A}	20.1^{c}	29.2^{bc}	31.2^{ac}	35.6^{ac}	26.9^{bc}	39.5^{ab}	46.8^{a}	2.89	<0.0001	0.0225	0.1936
	2 weeks	30.3^{AB}	18.0^{b}	25.5^{ab}	33.4^{ab}	38.8^{a}	27.8^{ab}	32.0^{ab}	36.5^{a}				
	4 weeks	28.6^{B}	11.5^{c}	21.7^{bc}	31.1^{ab}	42.9^{a}	21.6^{bc}	34.3^{ab}	37.2^{ab}				
	6 weeks	28.3^{B}	15.7^{b}	27.3^{ab}	33.7^{a}	33.0^{a}	22.6^{ab}	31.2^{ab}	34.6^{a}				
	Total		16.3^{d}	25.9^{c}	32.3^{b}	37.6^{ab}	24.7^{c}	34.3^{ab}	38.8^{a}				
Total phenolic compounds, g GAE/kg DM	Fresh	4.10	3.40	5.70	4.54	4.46	1.99	2.59	6.05	2.18	0.1038	0.7420	0.5155
	2 weeks	4.17	3.31	1.68	3.23	8.68	2.65	6.79	2.86				
	4 weeks	4.06	1.95	2.97	3.18	3.38	3.55	7.78	5.64				
	6 weeks	5.17	1.06	5.56	3.42	3.34	4.59	7.75	10.48				
	Total		2.43	3.98	3.59	4.96	3.19	6.23	6.26				
TEAC, mmol trolox equivalent/kg DM	Fresh	20.2^{AB}	4.14^{b}	12.8^{ab}	16.6^{ab}	31.4^{ab}	11.6^{ab}	18.7^{ab}	46.3^{a}	7.07	<0.0001	0.0012	0.9625
	2 weeks	29.8^{A}	14.3	23.6	27.1	48.9	22.7	31.0	41.1				
	4 weeks	25.0^{A}	7.35	13.7	24.3	35.4	15.9	40.2	38.3				
	6 weeks	14.4^{B}	3.10	6.71	13.6	29.5	3.12	19.6	25.1				
	Total		7.23^{c}	14.2^{bc}	20.4^{bc}	36.3^{a}	13.3^{bc}	27.4^{ab}	37.7^{a}				

Abbreviations: SEM, standard error of mean; SC, solid chocolate; MC, molten chocolate; POV, peroxide value; TBARs, thiobarbituric acid-reactive substances, MDA, malonylaldehyde; GAE, gallic acid equivalent; TEAC, trolox equivalent antioxidant capacity.

^{A,B,C}Values in the same column with different superscripts differ significantly (P < 0.05).

^{a,b,c,d}Values in the same row with different superscripts differ significantly (P < 0.05).

cribed to the different trends recorded in the cheese during storage. Indeed, among the fresh cheese, the POV was higher in cheese made with 10% and 15% molten chocolate; after 2 weeks, these values decreased because of the known instability of the peroxides, then increased again. However, at 6 weeks, the lowest POV was observed in the control cheese, although this value differed significantly only from the cheese with 10% solid chocolate. TBARs, which are products of secondary lipid oxidation, varied greatly

Table 4–Effect of chocolate addition and storage time on fatty acid profile (g/100 g FA) and health indexes of cheese fat (n = 3).

	Storage time (STT)		Chocolate addition (CHO) Control	5% SC	10% SC	15% SC	5% MC	10% MC	15% MC	SEM	Significance (P<) CHO	STT	CHO × STT
Saturated FA, SFA	Fresh	70.0^{A}	70.1	70.5	70.0	69.8	70.1	69.9	69.3	0.268	0.0001	0.0033	0.7738
	2 weeks	69.5^{B}	69.8	70.1	69.6	68.9	69.5	69.4	69.1				
	4 weeks	69.7^{AB}	70.2	70.0	69.7	69.6	69.6	69.5	69.3				
	6 weeks	69.5^{B}	69.9	69.8	69.2	68.7	69.6	69.3	69.7				
	Total		70.0^{a}	70.1^{a}	69.6^{ab}	69.2^{b}	69.7^{ab}	69.6^{ab}	69.3^{b}				
Monounsaturated FA	Fresh	22.8^{B}	22.2^{b}	22.2^{b}	22.8^{ab}	23.3^{ab}	22.5^{b}	22.9^{ab}	23.9^{a}	0.210	<0.0001	0.0319	0.4998
	2 weeks	22.9^{AB}	22.1^{c}	22.3^{bc}	23.0^{abc}	23.9^{a}	22.7^{bc}	23.1^{abc}	23.3^{ab}				
	4 weeks	23.0^{AB}	22.1^{b}	22.6^{ab}	23.0^{ab}	23.4^{a}	22.9^{ab}	23.2^{ab}	23.6^{a}				
	6 weeks	23.2^{A}	22.4^{b}	22.6^{b}	23.3^{ab}	24.2^{a}	22.9^{b}	23.3^{ab}	23.4^{ab}				
	Total		22.2^{e}	22.5^{e}	23.0^{c}	23.7^{a}	22.8^{cd}	23.1^{bc}	23.5^{ab}				
Polyunsaturated FA, PUFA	Fresh	7.21^{B}	7.70	7.33	7.16	6.96	7.32	7.20	6.82	0.188	<0.0001	0.0033	0.9834
	2 weeks	7.60^{A}	8.12	7.55	7.45	7.22	7.83	7.45	7.56				
	4 weeks	7.33^{B}	7.66	7.33	7.35	6.99	7.54	7.23	7.17				
	6 weeks	7.39^{AB}	7.69	7.54	7.52	7.12	7.53	7.35	6.94				
	Total		7.80^{a}	7.44^{abc}	7.37^{bc}	7.07^{c}	7.56^{ab}	7.31^{bc}	7.12^{c}				
Unsaturated FA, UFA	Fresh	30.0^{B}	29.9	29.5	30.0	30.2	29.8	30.1	30.7	0.268	0.0001	0.0033	0.7738
	2 weeks	30.5^{A}	30.2	29.9	30.4	31.1	30.5	30.5	30.9				
	4 weeks	30.3^{AB}	29.8	30.0	30.3	30.4	30.4	30.4	30.7				
	6 weeks	30.5^{A}	30.1	30.2	30.8	31.3	30.4	30.7	30.3				
	Total		29.1^{b}	29.9^{b}	30.4^{ab}	30.8^{a}	30.3^{ab}	30.4^{ab}	30.7^{a}				
UFA/SFA	Fresh	0.429^{B}	0.426	0.419	0.429	0.433	0.426	0.430	0.443	0.005	0.0001	0.0034	0.7583
	2 weeks	0.439^{A}	0.432	0.426	0.437	0.451	0.439	0.440	0.447				
	4 weeks	0.435^{AB}	0.424	0.428	0.435	0.437	0.438	0.438	0.443				
	6 weeks	0.440^{A}	0.431	0.432	0.445	0.455	0.437	0.442	0.435				
	Total		0.429^{b}	0.426^{b}	0.437^{ab}	0.444^{a}	0.435^{ab}	0.438^{ab}	0.442^{a}				
n-6/n-3	Fresh	3.72	3.76	3.66	3.71	3.82	3.61	3.75	3.76	0.083	0.0178	0.0571	0.6704
	2 weeks	3.64	3.60	3.47	3.53	3.76	3.66	3.64	3.81				
	4 weeks	3.70	3.65	3.55	3.75	3.72	3.72	3.68	3.79				
	6 weeks	3.76	3.56	3.37	3.83	3.84	3.71	3.86	3.79				
	Total		3.64^{ab}	3.60^{b}	3.71^{ab}	3.79^{a}	3.68^{ab}	3.73^{ab}	3.79^{a}				
Health-promoting index	Fresh	0.402^{B}	0.382^{c}	0.384^{bc}	0.403^{abc}	0.417^{ab}	0.392^{bc}	0.404^{abc}	0.427^{a}	0.006	<0.0001	0.0404	0.6019
	2 weeks	0.409^{AB}	0.382^{b}	0.390^{b}	0.410^{ab}	0.439^{a}	0.340^{b}	0.416^{ab}	0.433^{a}				
	4 weeks	0.406^{AB}	0.380^{b}	0.392^{ab}	0.405^{ab}	0.422^{a}	0.402^{ab}	0.414^{ab}	0.426^{a}				
	6 weeks	0.411^{A}	0.385^{c}	0.397^{bc}	0.415^{abc}	0.443^{a}	0.401^{bc}	0.419^{abc}	0.420^{ab}				
	Total		0.382^{f}	0.391^{ef}	0.409^{cd}	0.430^{a}	0.399^{de}	0.413^{bc}	0.426^{ab}				
HH	Fresh	0.543	0.498	0.516	0.548	0.570	0.527	0.545	0.596	0.029	0.0046	0.8525	0.3729
	2 weeks	0.540	0.489	0.513	0.548	0.593	0.520	0.554	0.565				
	4 weeks	0.546	0.501	0.527	0.543	0.580	0.532	0.561	0.580				
	6 weeks	0.533	0.506	0.530	0.559	0.603	0.535	0.564	0.434				
	Total		0.499^{b}	0.521^{b}	0.550^{ab}	0.586^{a}	0.529^{ab}	0.556^{ab}	0.544^{ab}				

Abbreviations: SEM, standard error of mean; SC, solid chocolate; MC, molten chocolate.
Health-promoting index = (n-3 PUFA + n-6 PUFA + MUFA)/(C12:0 + 4 × C14:0 + C16:0) (Chen et ~~al.,~~ 2004). ~~HH = hypocholesterolemic FA/Hypercholesterolemic FA ratios =~~ (C18:1 c9 + C18:2 n-6 + C20:4 n-6 + C18:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3)/(C14:0 + 16:0) (Santos-Silva et al., 2002).
[A,B]Values in the same column with different superscripts differ significantly (P < 0.05).
[a,b,c,d,e,f]Values in the same row with different superscripts differ significantly (P < 0.05).

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among the cheese types during storage, from 11.5 to 46.8 μg MDA/kg DM. The level of TBARs increased with the addition of chocolate and decreased over time spent in storage so that, during storage, the lowest value was observed in the control cheese. The level of TBARs is an important index of quality, as products resulting from lipid oxidation may lead to food safety concerns, such as a loss of nutrients or flavor defects (Fox, Guinee, Cogan, & McSweeney, 2000). The increase in UFA, especially oleic acid, due to the addition of chocolate was likely responsible for the increase in TBARs. The decrease in oxidation products over time suggests a certain stability of the cheese during storage independent of the presence of chocolate. The lack of contribution of ripening to cheese oxidation has already been recognized, especially when cheese is stored at 20 °C or lower (Kristensen et al., 2001), as in this study. The level of TBARs in this investigation is comparable to or lower than that observed for other Italian cheese (Branciari et al., 2015; Mele et al., 2011).

3.3 Polyphenol content and antioxidant activity

The total phenolic compounds (TPC) and TEAC of the cheese are also shown in Table 3. As expected, increasing the chocolate resulted in a significant increase in TEAC regardless of the physical state of the chocolate. In contrast, the differences in TPC among samples were not significant (P = 0.1038), although increasing the chocolate resulted in an increase in TPC, with values quite close to those predicted (3.97, 5.49, and 6.94 g GAE/kg DM with 5%, 10%, and 15% ~~g~~ both solid and molten chocolate, respectively) on the basis of the polyphenol content of the chocolate (17.3 g/kg). Results on the enrichment of cheese with TPC from chocolate are consistent with results on the addition of polyphenol-rich dietary sources, such as cocoa, tea, wine, soy products, and fruits ~~to cheese~~ (Cutrim & Cortez, 2018; Rashidinejad et al., 2016). However, the dissimilar trends in TPC and TEAC observed in this study were confirmed by the weak but significant correlation between these two parameters (r = 0.2463, P = 0.0239), which suggests that

Table 5–Effect of chocolate addition and storage time on microbiological profile (log CFU/g) of cheese (*n* = 3).

	Storage time (STT)		Chocolate addition (CHO)								Significance (*P*<)		
			Control	5% SC	10% SC	15% SC	5% MC	10% MC	15% MC	SEM	CHO	STT	CHO × STT
Total mesophilic count	Fresh	8.65	8.45	8.64	8.86	8.32	8.83	8.62	8.79	0.2030	0.3853	0.6814	0.9224
	2 weeks	8.64	8.58	8.85	8.62	8.65	8.85	8.52	8.40				
	4 weeks	8.60	8.30	8.63	8.95	8.56	8.56	8.66	8.56				
	6 weeks	8.73	8.76	8.83	8.77	8.72	8.76	8.63	8.65				
	Total		8.52	8.74	8.80	8.56	8.75	8.61	8.60				
Mesophilic coccus LAB	Fresh	8.60	8.49	8.58	8.82	8.57	8.63	8.58	8.52	0.1861	0.6740	0.8242	0.9992
	2 weeks	8.51	8.49	8.59	8.51	8.48	8.65	8.48	8.40				
	4 weeks	8.58	8.29	8.69	8.67	8.48	8.66	8.73	8.53				
	6 weeks	8.54	8.52	8.66	8.53	8.53	8.51	8.54	8.46				
	Total		8.45	8.63	8.63	8.51	8.61	8.58	8.48				
Mesophilic rod LAB	Fresh	6.02^{C}	6.76^{Ba}	6.63^{Ba}	6.95^{Ba}	5.48^{Bb}	5.00^{Cb}	5.85^{Cab}	5.48^{Bb}	0.1908	0.0003	<0.0001	0.0002
	2 weeks	7.61^{B}	7.30^{A}	7.86^{A}	7.87^{AB}	7.66^{A}	7.48^{BC}	7.30^{B}	7.83^{A}				
	4 weeks	8.34^{A}	7.96^{A}	8.33^{A}	8.64^{A}	8.25^{A}	8.60^{A}	8.48^{A}	8.13^{A}				
	6 weeks	8.41^{A}	8.30^{A}	8.51^{A}	8.56^{A}	8.52^{A}	8.34^{AB}	8.43^{A}	8.22^{A}				
	Total		7.58^{abc}	7.83^{ab}	8.01^{a}	7.48^{bc}	7.36^{c}	7.51^{bc}	7.41^{bc}				
Enterobacteriaceae	Fresh	4.28^{A}	4.04	4.95^{A}	4.59^{A}	4.58^{A}	3.85	4.10	3.86^{A}	0.2087	<0.0001	<0.0001	<0.0001
	2 weeks	3.96^{B}	4.46	3.62^{B}	3.80^{AB}	3.76^{A}	4.22	3.86	4.01^{A}				
	4 weeks	3.04^{C}	4.58^{a}	2.86^{BCb}	2.32^{Cb}	2.48^{Bb}	3.40^{ab}	3.02^{b}	2.60^{Bb}				
	6 weeks	3.09^{C}	3.83^{a}	2.00^{Cc}	2.60^{BCbc}	2.48^{Bbc}	3.51^{ab}	3.60^{ab}	3.58^{ABab}				
	Total		4.23^{a}	3.36^{b}	3.33^{b}	3.32^{b}	3.74^{b}	3.64^{b}	3.51^{b}				
Yeasts	Fresh	3.84^{A}	3.15^{c}	3.36^{c}	3.52^{c}	3.70^{Abc}	3.61^{Abc}	4.83^{Aa}	4.70^{Aab}	0.1943	<0.0001	<0.0001	0.0004
	2 weeks	3.37^{B}	2.78	3.40	3.58	3.76^{A}	3.04^{AB}	3.40^{B}	3.63^{AB}				
	4 weeks	2.51^{D}	2.48	3.00	2.90	2.00^{B}	2.00^{B}	2.70^{B}	2.48^{C}				
	6 weeks	2.97^{C}	2.48	2.60	2.95	3.32^{A}	3.04^{AB}	3.15^{B}	3.26^{BC}				
	Total		2.72^{c}	3.09^{abc}	3.24^{ab}	3.20^{ab}	2.92^{bc}	3.52^{a}	3.52^{a}				
Filamentous fungi	Fresh	3.69^{A}	3.46^{Ab}	2.60^{Ac}	2.00^{Ac}	3.43^{b}	4.81^{Aa}	4.77^{Aa}	4.78^{Aa}	0.1186	<0.0001	<0.0001	<0.0001
	2 weeks	2.38^{B}	2.48^{Bb}	2.00^{Ab}	2.30^{Ab}	3.28^{Aa}	2.30^{Cb}	2.30^{Bb}	2.00^{Cb}				
	4 weeks	1.96^{C}	2.00^{Bc}	$<2^{Bd}$	$<2B^{d}$	$<2^{Cd}$	3.69^{Ba}	2.30^{Bbc}	2.70^{Bb}				
	6 weeks	1.47^{D}	2.00^{Ba}	$<2^{Bb}$	$<2B^{b}$	2.00^{Ba}	2.30^{Ca}	$<2^{Cb}$	$<2^{Db}$				
	Total		2.48^{b}	1.65^{d}	1.58^{d}	2.43^{c}	3.28^{a}	2.59^{bc}	2.62^{bc}				

Abbreviations: SEM, standard error of mean; SC, solid chocolate; MC, molten chocolate; LAB, lactic acid bacteria.
^{A,B,C}Values in the same column with different superscripts differ significantly ($P < 0.05$).
^{a,b,c,d}Values in the same row with different superscripts differ significantly ($P < 0.05$).

polyphenols are not the sole contributors to antioxidant activity in the cheese. This antioxidant activity could be partly attributed to peptides, which exhibit antioxidant activity and derive from microbial proteolysis (Gupta, Mann, Kumar, & Sangwan, 2009), which in turn could have been favored by the presence of polyphenols in the chocolate. In fact, although several microorganisms are inhibited by polyphenols, some LAB are resistant and grow in their presence (Tabasco et al., 2011). Also, storage time affected TEAC but not TPC. In the first 4 weeks, however, TEAC increased, but later it decreased strongly independent of cheese type. The loss of antioxidant activity was probably due to further proteolysis involving peptides during cheese storage, in accordance with the findings of Gupta et al. (2009).

3.4 Cheese ~~FAs~~

Table 4 and Tables S1, S2, and S3 report the FA profile of the cheese samples. On the whole, the highest amount of FA was SFA (70%), followed by monounsaturated fatty acid (MUFA, 23%) and polyunsaturated fatty acids (PUFA, 7%) (Table 4). C16:0 (palmitic acid) was the most abundant molecule at 23%, followed by C18:1 (oleic acid), C14:0 (myristic acid), and C18:0 (stearic acid) at approximately 15%, 11%, and 9%, respectively (Tables S1 and S3). Analogous FA profiles have been observed in other studies (Bonanno et al., 2016; Todaro et al., 2017a).

As expected, increasing the amount of chocolate in the cheese, regardless of its form, reduced most FA, with the exception of those present in high concentrations in chocolate, such as C16:0, C18:0, C20:0, C18:1, and C18:2 *n*-6 (linoleic acid; Table 2), which increased significantly in the chocolate cheese (Tables S1 and S2). However, especially due to the increase in oleic acid (Table S2), the chocolate cheese showed an increase in UFA, which reduced the amount of SFA (Table 4) despite the high content of palmitic acid. Thus, the addition of chocolate contributed to improving the calculated health indices (Table 4). In contrast, because of the transfer of linoleic acid, the *n*-6/*n*-3 ratio increased (Table 4), although it was constantly below the threshold (≤5) recommended by FAO/WHO (1994) in the human diet for the prevention and treatment of chronic disease.

Dietary fat is essential for optimal performance and the desired balance of fats in the cells. However, SFA, which are mainly found in animal originated food, are harmful to humans because of their association with increased triglycerides in the blood and their relation to hypertension. In contrast, UFA are beneficial for the prevention of cardiovascular disease (Simpoulos, 1999). Thus, adding chocolate conferred more beneficial properties on the cheese fat.

Storage time did not have notable effects on the cheese FA profile, as found in previous studies (Bonanno et al., 2013). Indeed, although the effect of storage was significant for most FA, the change observed over time was limited and was quite similar among the different cheese types. However, most of the chocolate cheese aged 6 weeks had slightly increased UFA, which significantly improved the UFA/SFA ratio and health-promoting indices (Table 4).

Table 6–Effect of chocolate addition and storage time on scores of sensory traits of cheese evaluated by descriptive analysis.[1].

	Storage time (STT)		Chocolate addition (CHO) Control	5% SC	10% SC	15% SC	5% MC	10% MC	15% MC	SEM	Significance ($P<$) CHO	STT	CHO × STT
Color	Fresh	28.4	6.40^{b}	25.5^{ab}	26.3^{ab}	37.8^{a}	32.3^{ab}	32.9^{ab}	38.1^{a}	4.982	<0.0001	0.0744	0.9511
	2 weeks	33.0	16.3	32.2	32.3	41.8	34.2	37.6	36.8				
	4 weeks	35.4	23.9	37.2	38.3	37.7	35.2	36.3	39.1				
	6 weeks	33.3	23.1	32.0	36.4	37.5	29.1	38.6	36.5				
	Total		17.4^{a}	31.7^{b}	33.3^{b}	38.7^{b}	32.7^{b}	36.3^{b}	37.6^{b}				
Structure uniformity	Fresh	42.8^{A}	74.7^{a}	42.6^{b}	42.8^{b}	31.8^{b}	46.3^{b}	31.9^{b}	29.6^{b}	4.698	<0.0001	0.0004	0.4591
	2 weeks	33.1^{B}	66.3^{a}	41.5^{b}	23.5^{b}	21.5^{b}	30.7^{b}	29.1^{b}	19.2^{b}				
	4 weeks	41.6^{A}	62.3^{a}	46.0^{ab}	33.2^{b}	33.5^{b}	44.0^{ab}	39.6^{ab}	32.9^{b}				
	6 weeks	37.2^{AB}	54.6^{a}	41.5^{ab}	33.9^{ab}	31.1^{b}	39.2^{ab}	31.6^{ab}	28.3^{b}				
	Total		64.5^{a}	42.9^{b}	33.3^{bcd}	29.5^{d}	40.1^{bc}	33.1^{cd}	27.5^{d}				
Unpleasant odor	Fresh	5.12^{AB}	3.21	3.00	7.00	6.00	4.64	5.87	6.16	2.445	0.0267	0.0218	0.7776
	2 weeks	4.75^{B}	3.75	2.85	3.99	6.42	2.85	3.79	9.62				
	4 weeks	6.58^{AB}	6.02	5.50	8.81	8.08	5.73	5.42	6.54				
	6 weeks	8.17^{A}	3.65	7.80	10.62	6.10	4.04	11.28	13.69				
	Total		4.16^{b}	4.79^{ab}	7.60^{ab}	6.65^{ab}	4.32^{ab}	6.59^{ab}	9.00^{a}				
Sweet	Fresh	20.8^{A}	6.78^{b}	15.7^{ab}	28.3^{a}	29.8^{a}	16.3^{ab}	21.9^{ab}	26.7^{a}	3.711	<0.0001	0.0007	0.8368
	2 weeks	15.6^{B}	14.1	12.4	15.1	20.8	14.3	13.4	19.3				
	4 weeks	14.6^{B}	9.53	11.6	16.7	21.6	11.0	12.0	19.6				
	6 weeks	13.4^{B}	9.05	11.3	14.9	18.6	11.7	12.6	15.4				
	Total		9.86^{d}	12.7^{c}	18.8^{abc}	22.7^{a}	13.3^{bcd}	15.0^{bcd}	20.2^{ab}				
Acid	Fresh	4.84^{B}	4.65	4.73	3.65	3.58	5.94	6.40	4.92	2.101	0.1097	<0.0001	0.5358
	2 weeks	5.11^{B}	4.42	4.82	4.95	8.18	3.78	3.78	5.82				
	4 weeks	6.99^{B}	3.89	11.0	8.30	5.92	4.84	8.87	6.18				
	6 weeks	10.4^{A}	4.65	12.7	12.7	11.3	8.06	11.3	12.2				
	Total		4.40	8.31	7.40	7.25	5.65	7.60	7.27				
Bitter	Fresh	6.56	4.47	5.07	7.40	8.40	6.54	8.20	5.80	2.310	0.0203	0.7486	0.9871
	2 weeks	5.89	2.54	6.41	4.07	7.91	7.07	8.31	4.94				
	4 weeks	5.36	1.50	4.31	4.62	8.41	5.91	5.17	7.63				
	6 weeks	6.42	1.17	7.33	6.98	6.78	5.96	7.32	9.40				
	Total		2.42^{b}	5.78^{ab}	5.77^{ab}	7.88^{a}	6.37^{ab}	7.25^{a}	6.94^{ab}				
Overall satisfaction	Fresh	51.0^{A}	63.3	48.4	50.9	49.4	53.3	43.1	48.5	4.653	<0.0001	<0.0001	0.5071
	2 weeks	51.0^{A}	63.8^{a}	52.3^{a}	49.1^{a}	50.3^{a}	48.9^{a}	54.6^{a}	38.5^{b}				
	4 weeks	43.1^{B}	63.4^{a}	38.1^{b}	42.0^{ab}	32.8^{b}	42.8^{ab}	44.3^{ab}	38.5^{b}				
	6 weeks	35.6^{C}	58.6^{a}	31.9^{b}	32.3^{b}	31.0^{b}	39.4^{ab}	29.6^{b}	26.1^{b}				
	Total		62.3^{a}	42.7^{b}	43.6^{b}	40.9^{b}	46.1^{b}	42.9^{b}	37.9^{b}				
Overall acceptance, %	Fresh	93.6^{A}	100	93.3	93.3	93.3	100	93.3	81.1	10.148	<0.0001	<0.0001	0.3995
	2 weeks	85.0^{AB}	99.3	86.0	79.3	79.3	92.7	79.3	79.3				
	4 weeks	76.1^{B}	99.4	78.0	78.0	49.4	79.3	78.0	70.8				
	6 weeks	51.1^{C}	93.6	46.6	34.8	34.8	72.2	46.6	28.9				
	Total		98.2^{a}	76.0^{bc}	71.4^{bc}	64.2^{c}	86.2^{ab}	74.3^{bc}	65.1^{c}				

Abbreviations: SEM, standard error of mean; SC, solid chocolate; MC, molten chocolate. [1] Scale 0 to 90 (0 = low quality; 90 = high quality).
[A,B,C]Values in the same column with different superscripts differ significantly ($P < 0.05$).
[a,b,c,d]Values in the same row with different superscripts differ significantly ($P < 0.05$).

3.5 Microbiological profile

The microbial populations in the cheese are reported in Table 5. Total mesophilic count (TMC) was measured at levels higher than 8 log CFU/g in all samples, and no significant differences were found between the control and chocolate cheese. Mesophilic coccus LAB was the dominant microbial group in all cheese, with levels of about 10^8 CFU/g being almost similar to those of TMC as generally observed for raw ewe's milk cheese produced in Sicily (Gaglio et al., 2019; Guarcello et al., 2016; Pino et al., 2017). Levels of coccus LAB were comparable among the cheese types and did not change during storage.

Counts of mesophilic rod LAB differed slightly among the chocolate cheese samples but increased until 2 log cycles over time in all samples. Levels of rod LAB were moderately lower (by 1 log unit) than those of coccus LAB, as observed in other similar Italian cheese (Caridi, Micari, Caparra, Cufari, & Sarullo, 2003; Guarcello et al., 2016), and levels of both coccus and rod LAB were on the same order of magnitude as those generally reported for cheese (Pisano, Fadda, Deplano, Corda, & Cosentino, 2006; Ricciardi, Blaiotta, Di Cerbo, Succi, & Aponte, 2014). However, in the present investigation, the larger difference between coccus and rod LAB in fresh samples, almost 2 log units, decreased after 6 weeks, when the rod LAB reached a concentration of about 10^8 CFU/g, similar to that of coccus LAB. In line with these results, in previous work on the microbial ecology of PDO Pecorino Siciliano cheese produced in different seasons (Vernile et al., 2006) and subjected to different salting technologies (Todaro et al., 2011), mesophilic coccus and rod LAB were found in approximately the same amounts. Because of their ability to ferment citrate, mesophilic rod LAB play an essential role in the maturation of cheese and are also involved in several enzymatic processes that occur during cheese ripening, including proteolysis (Crow, Curry, & Hayes, 2001).

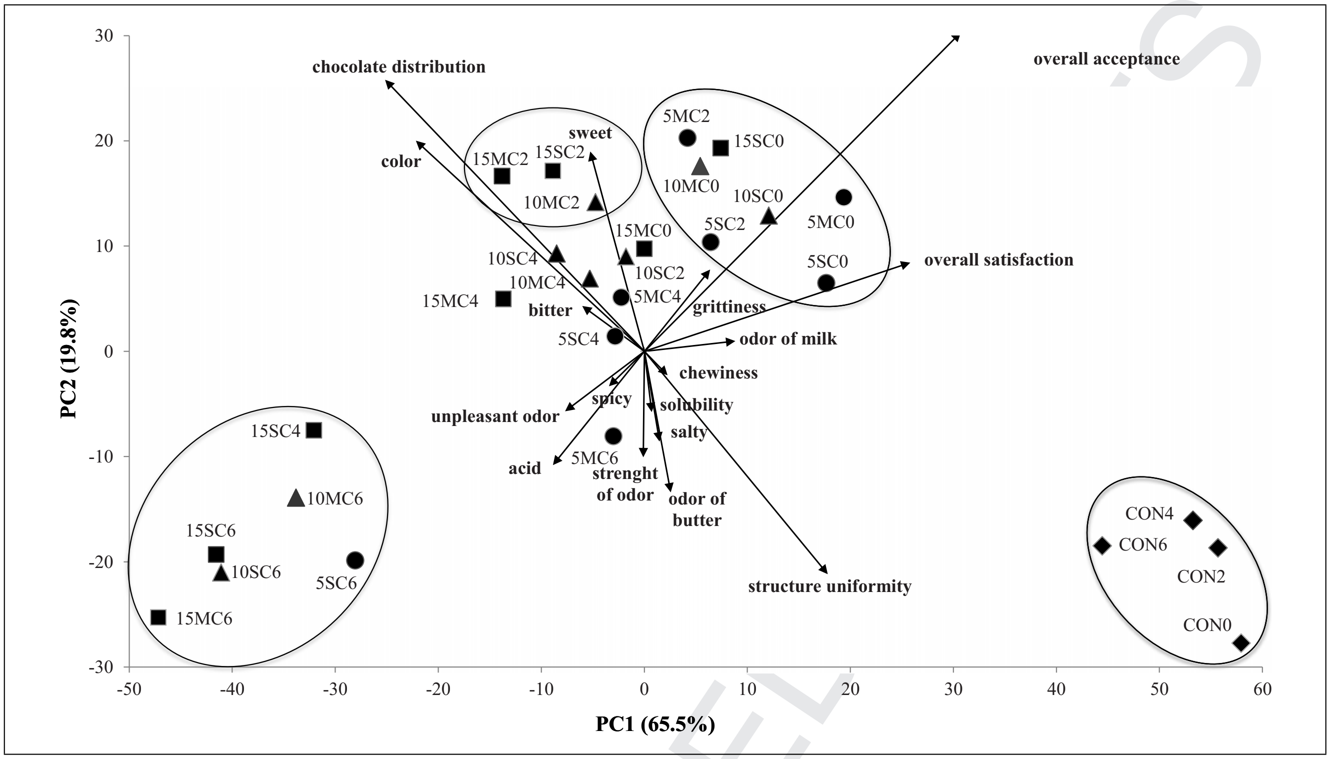

Figure 1–Score plot and loading plot from principal component analysis (PCA) of cheeses based on their sensory attributes
Note. ♦ control cheese; ● cheese with 5% chocolate; ▲ cheese with 10% chocolate; ■ cheese with 15% chocolate.
Abbreviations: CON, control without chocolate; SC, solid chocolate; MC, molten chocolate; 0, fresh cheese; 2, cheese stored for 2 weeks; 4, cheese stored for 4 weeks; 6, cheese stored for 6 weeks.

Other significant differences among the cheese types were detected for Enterobacteriaceae, yeasts, and filamentous fungi. In cheese stored for 6 weeks, chocolate favored the reduction of members of Enterobacteriaceae family, especially when it was used in solid form. Moreover, a positive increasing trend was noticed for yeasts (in YPDA), whose levels increased in line with the amount of chocolate in the cheese, probably linked to its sugar content. On the whole, filamentous fungi increased more in cheese with 5% molten chocolate than in cheese with 5% and 10% solid chocolate; however, after 6 weeks of storage, they disappeared from the majority of chocolate cheese.

With regard to the effect of storage time, significant reductions were detected by about 1 log cycle for Enterobacteriaceae (on VRBGA) and yeasts and by 2 log cycles for fungi. On the whole, these unwanted microbial populations were detected at relatively high levels, with the exception of yeasts, whose presence was in the same range as those reported in similar aged products (Pisano et al., 2006) and lower than those found in 3-month ripened Pecorino Siciliano cheese (Vernile et al., 2006). Indeed, the levels of Enterobacteriaceae in all samples, which ranged from 10^3 to 10^4 CFU/g, were higher than those reported in raw ewe's milk cheese (Pisano et al., 2006; Serio et al., 2005) but lower than those in cheese ripened for longer periods (Macedo, Tavares, & Malcata, 2004; Todaro et al., 2011), which ranged from 10^5 to 10^6 CFU/g.

In general, as cheese ripens, many microorganisms, such as Enterobacteriaceae, yeasts, and filamentous fungi, undergo a reduction in cell density. In particular, Enterobacteriaceae are not usually detected in the final product because they are gradually inhibited by the cheese stressing conditions (Dahl, Tavaria, & Malcata, 2000), such as the drop in pH, increase in NaCl concentration, decrease in water activity, and presence of bacteriocin and organic acids. Nevertheless, high numbers of Enterobacteriaceae have been reported in different Mediterranean cheese made from raw ewe's and goat's milk after 30 days of ripening (Macedo et al., 2004; Psoni, Tzanetakis, & Litopoulou-Tzanetaki, 2003), and their levels have been correlated with certain properties of artisanal cheese, such as aroma, taste, and texture (Dahl et al., 2000; Morales, Feliu, Fernandez-Garcia, & Núñez, 2004), contributing to sensory characterization of the products (Chaves-López et al., 2006).

However, in the present study, the decrease in Enterobacteriaceae and filamentous fungi and the increase in yeasts from fresh samples to cheese stored for 6 weeks differed among cheese types, which indicates that adding chocolate affected the evolution of these unwanted microorganisms during storage.

3.6 Sensory evaluation

Mean scores for the main cheese descriptors are reported in Table 6, and comprehensive descriptive analyses appear in Table S4. The chocolate added and storage time affected several sensory characteristics of the cheese; because the interaction between these factors was never significant, storage influenced the different cheese types similarly.

Adding chocolate resulted in a darker color and a less uniform structure (Table 6), but it did not affect the butter or milk odor (Table S4); moreover, an increase in unpleasant odor, sweetness, and bitterness resulted when the amount of chocolate increased (Table 6). The perceived sweetness could be attributed to the sugar in the PGI Modica chocolate, whereas the bitterness could be due to the phenolic compounds that occur naturally in cocoa beans, such as catechins, but also to alkaloids, such as theobromine and caffeine (Afoakwa, 2010). Contrary to expectations, the additive effect of sugar and fat from the chocolate was not able to mask or decrease the perception of bitterness, as has been observed for other dairy products, such as yogurt (Tuorila, Sommardahl, Hyvönen, Leporanta, & Merimaa, 1995).

During storage, the unpleasant odor, acidity, spiciness, and solubility increased, as well as the distribution of chocolate improved, but the uniformity of structure, milk odor, sweetness, and grittiness decreased (Tables 6 and S4). These results are consistent with the trend observed for pH. Indeed, decreasing the pH of the cheese during ripening increased perceptions of acidity, in line with Pettersen, Eie, and Nilsson (2005), who observed an increase in acidulous flavor in cream cheese during 6 months of storage.

The results of the descriptive analyses in terms of overall satisfaction and acceptance (Table 6) indicated that the evaluators appreciated the control cheese more than the chocolate cheese, as well as fresh cheese and cheese stored for 2 weeks. Among the chocolate cheese, there was greater acceptance of samples with 5% and 10% molten or solid chocolate; however, these values decreased greatly during storage, especially after 4 weeks, whereas acceptance of the control cheese remained almost unchanged over time.

The results of the hedonic tests (Table S4) confirmed those of the descriptive analyses. The control cheese and the chocolate cheese stored up to 2 weeks obtained higher hedonic evaluations by evaluators with regard to taste, texture, mouthfeel, overall appearance, and overall liking, whereas the cheese containing 15% solid or molten chocolate was less appreciated.

On the whole, among the chocolate cheese, cheese with 5% and 10% solid or molten chocolate and cheese stored up to 2 weeks received better evaluations for all attributes in both descriptive and hedonic tests. However, the evaluators expressed the greatest acceptance of cheese with 5% molten chocolate, either fresh or stored for 2 weeks. These results are in line with a recent review by Cutrim and Cortez (2018) showing that enriching dairy products with high levels of phenols generates unpleasant taste and, consequently, less consumer liking.

The plot generated by PCA, performed to evaluate the ability of sensory attributes to discriminate among cheese produced with different percentages of chocolate and storage times, is depicted in Figure 1. The first two principal components explained 85.3% of the variance. The first component (*x*-axis), accounting for 65.5% of the total variance, broadly separated the control cheese from the other cheese on the basis of the positive contribution of overall acceptance, overall satisfaction, structure uniformity, and, to a lesser extent, the odor of butter and milk. Among the chocolate cheese, unpleasant odor and acidity differentiated a group consisting mainly of cheese with 10% to 15% chocolate stored for 6 weeks. Chocolate distribution, color, and sweetness differentiated cheese with 10% to 15% chocolate stored for 2 weeks. Finally, sweetness, and especially overall acceptance and satisfaction, characterized almost all fresh cheese and cheese with 5% solid or molten chocolate stored for 2 weeks, which shared the same space, in accordance with the preferences of the evaluators that emerged in the descriptive and hedonic sensory tests.

4. CONCLUSIONS

In this study, chocolate cheese is proposed as an innovative dairy product obtained by combining two traditional foods, each characterized by complementary nutrition, health, and sensory effects. Adding chocolate resulted in cheese with improved antioxidant properties and healthier fat. Accordingly, chocolate cheese might have the potential to target consumers interested in preventing disease through a healthy diet. Among the chocolate cheese, higher sensory acceptance was found for cheese with 5% molten chocolate stored up to 2 weeks. On the whole, the results suggest avoiding high amounts of chocolate (>10%) and long vacuum packaging (>4 weeks) to preserve the consistency, oxidative stability, sensory attributes, as well as antioxidant activity of the product. Further studies are required to optimize the manufacturing and storage conditions of chocolate cheese from ewe's milk and PGI Modica chocolate, taking into account this first production attempt.

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AUTHOR CONTRIBUTIONS

MRA designed the experiment, performed the laboratory analyses and statistical analyses, interpreted the data, and drafted the work. AB designed the experiment, performed the statistical analyses, interpreted the data, drafted the work, and revised the paper. MT was associated with cheese-making trials and revising the paper. LS interpreted the microbiological data and revised the paper. RG performed the microbiological analyses, interpreted the microbiological data, and drafted the microbiological work. AT designed the experiment and performed the sensory analyses. MA was associated with interpretation of data, cheese-making trials, and revising the paper. GM was associated with cheese-making trials and laboratory analyses. FM performed the laboratory analyses. ADG was associated with the design of the experiment, cheese-making trials, and revising the paper.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Effect of chocolate addition and storage time on saturated fatty acids ~~(g (100 g)$^{-1}$ FA)~~ of cheese fat (n=3).

Table S2. Effect of chocolate addition and storage time on unsaturated fatty acids ~~(g (100 g)$^{-1}$ FA)~~ of cheese fat (n=3).

Table S3. Effect of chocolate addition and storage time on fatty acid profile ~~(g (100 g)$^{-1}$ FA)~~ and health indexes of cheese fat (n=3).

Table S4. Effect of chocolate addition and storage time on scores of sensory traits of cheese by descriptive analysis and hedonic test.

Integrated Food Science Q7