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# Effect of adding solid and molten chocolate on the polyphenol content, chemical, textural, microbiological and sensory aspects of Pecorino Siciliano cheese during ripening

IL DOTTORE
**Mansour Rabie Ashkezary**

IL COORDINATORE
**Prof. Vincenzo Bagarello**

IL TUTOR
**Dott. Aldo Todaro**

CO TUTOR
**Prof. Adriana Bonanno**
**Prof. Baldassare Portolano**

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# 1. GENERAL INTRODUCTION

## 1.1. Cheese

Today, Italy has a greater range of cheese varieties than any other country, with the exception of France, and the same Italian cheeses, Gorgonzola and Grana (Parmigiano Reggiano and Grana Padano), rank among the famous international cheese varieties. While Roquefort and Stilton may challenge Gorgonzola as the prime blue-mould cheese, Grana cheeses are at the forefront of grating cheeses. Italy is also the principal producer of that rather unique family of cheeses, the Pasta filata or stretched curd cheese of which Provolone, Caciocavallo and Mozzarella are the best-known members. Italy is probably unique in using milk commercially from four species (cow, sheep, goat and buffalo); goat cheeses ('caprini' or 'di capra'), sheep cheeses ('pecorini' or 'di pecora'), buffalo cheeses ('bufalini' or 'di bufala') and cheeses from mixtures of such milks (i.e., with cow and either goat or sheep milk; with cow and buffalo milk; with goat and sheep milk) are commercially produce (Reinbold, 1963).

Any Italian hard cheese made from ewes' milk is known as 'Pecorino'. Pecorino cheese is produced throughout Italy and the name often indicates the geographical origin. Outside the national borders, the most known Italian Pecorino cheese is 'Pecorino Romano', but there are other Pecorino cheeses, which also enjoy a protected designation of origin (PDO) status, that are being appreciated by foreigner consumers, in particular, 'Pecorino Sardo', 'Pecorino Siciliano' and 'Pecorino Toscano'. In general, Pecorino cheeses are characterized by a certain salty taste (Todaro et al., 2011).

Pecorino Siciliano cheese is, probably, the oldest European cheese (Betta et al., 2002), the PDO disciplinary goes back to 1955 (GURI n. 295 of 12-22-1955) and provides the use of entire ewe's raw milk produced in Sicily (southern Italy), the use of traditional

wooden equipment, the application of dry salting and a ripening period of at least 4 months. The dry salting technology is performed by manual aspersion of salt onto the cheese surface. However, this dry salting method produces high variability on cheese salt content (Todaro et al., 2011).

In Sicily, traditional cheese production from raw milk is often carried out in wooden vats without the inoculation of starter cultures (Settanni et al., 2014), but animal rennet paste is added for curdling (Cruciata et al., 2014). Under these conditions, the desirable lactic acid bacteria (LAB) that transform curd into cheese (Settanni et al., 2010) are provided only by the raw materials and/or the vat surfaces (Lortal et al., 2009). The wooden vat surfaces host microbial biofilms that include dairy LAB (Lortal et al., 2009; Licitra et al., 2007; Didienne et al., 2012; Settanni et al., 2012; Scatassa et al., 2015). PDO Pecorino Siciliano cheese is obtained without the addition of bacterial starters as mentioned. Thus, the microflora acting during cheese making and ripening is indigenous, deriving from milk or the transformation environment and, for this reason, it may be considered autochthonous. The presence of indigenous microorganisms provides characteristic features that link their presence to the typicality of a given cheese (Moreno et al., 2006). This is particularly true for the artisanal cheeses, which are produced in restricted areas, whose territory and habits, as well as the pedoclimatic conditions and anthropogenic activities (not reproducible elsewhere), are the expression of history and tradition (Todaro et al., 2011). It is important to underline the fact that raw milk microflora, in particular non-starter lactic acid bacteria, (NSLAB) increases the diversity of flavour in PDO cheeses, and is involved in the production of the typical characteristics of cheese (Steele and Ünlu, 1992). The manufacturers of the PDO cheeses often highlight this diversity in flavour and it is considered as an important

feature of European traditional cheeses (Grappin and Beuvier, 1997). The industrial Pecorino Siciliano cheese manufactured from thermized ewes' milk and inoculated with industrial starter cultures, can be sold immediately after the production. Generally the starter cultures used in the Pecorino Siciliano cheese-making are mesophilic and thermophilic starters, with mixed strains of Lactococcus lactis, Streptococcus thermophilus and Leuconostoc mesenteroides . The starters grow in high number during the first days and then decrease throughout ripening, when a secondary microflora, constituted by NSLAB become dominant. This population presumably originated from milk, ingredients used for cheese-making and dairy environment, contributes to flavour development (Martley and Crow, 1993). Flavor is defined as the combination of taste and aroma (Urbach, 1997) and the biochemistry of cheese flavour are inseparable from the biochemistry of cheese. The development of unique flavours in cheese is the result of a complex reactions (Dahl et al., 2000) such as lipolysis, proteolysis and metabolism of residual lactose, lactate and citrate (McSweeney and Sousa, 2000), mainly due to enzymes from milk, rennet and microrganisms (Fox et al., 1993).

## 1.2. Chocolate

The Sicilian food is well-known for numerous unique specialties, for its large use of important agricultural typical products that are like D.O.P. extra-vergine olive oils, D.O.C. wines and the Modican Chocolate is one of the best appreciated products of the Sicilian pastry. It has a rectangular shape, 15 cm long, and can be divided into four smaller bars. It is prepared according to a traditional recipe dating back to the Aztec ancient civilization and handed down to us by the Spanish. The production is made by

hand and with low temperatures, this avoids the loss of nutritive substances. In this chocolate it is possible to distinguish the three elements that composes the product: cacao, sugar and spices. Without added fats or emulsifiers. In 2003, the Consortium for the Protection of Handmade Chocolate of Modica (CTCM) was created. Composed of twenty local producers, to establish production rules and regolations, to earn recognition from the PGI (Protected Geographical Indication), and to protect and promote this product (Catania, 2008).

Chocolate originates from Mexico where the Mayas, Incas and Aztecs cultivated the cacao tree (Theobroma cacao) preparing this product only on special occasions (Ceo and Ceo, 1996). While we eat chocolate the pleasure centers of our brain are activated. One typical quality of chocolate is its melt point; it is solid at ambient temperature but it melts in the mouth and it is dissolved in saliva allowing a clear final assessment of its texture. Particle size distribution and ingredient composition of chocolate (sugar and cocoa about 70% total in a continuous fat phase) play an important role in shaping its rheological behavior and sensory perception (Afoakwa et al., 2007). In the past chocolate was seen only as a food of sensual pleasure with negative effects on health, however, today chocolate has been revaluated positively, thanks to greater nutritional information that discredits many fallacies. Despite high lipid and sugar content, its consumption has some beneficial effects on the human diet; cocoa is rich in antioxidants, above all polyphenols and minerals such as potassium, magnesium, copper and iron, so its intake may be useful in dietary deficiencies or may balance low levels of neurotransmitters involved in the regulation of food intake (serotonin and dopamine) (Bruinsma, 1999). Thus it is not surprising that chocolate has always been the most commonly and intensely craved food in western cultures (Weingarten and Elston, 1990;

Osman and Sobal, 2006). Switzerland ranks first among the world's consumers of chocolate, it is a "chocolate heavy user country" followed by Belgium and Denmark; Italy only ranks 12th place with a constant growing trend, in fact, the consumption per capita passed from 3.2 kg in 1997 to 4.3 kg in 2006 (+36.4%) (Bommezzadri, 2007). In the choices of the consumer, tradition has prevailed: as for bars, milk chocolate is always the best seller, and there is a continuing rise in dark chocolate sales, while white chocolate sales are declining (Bommezzadri, 2007).

Differences in the sensory characters of chocolate can be ascribed to the use of different cocoa types, i.e. flavor quality of chocolate usually depends on the origin of the cocoa beans (Jinap et al., 1995), variations in ingredient proportions and in the processing methods (Jackson, 1999) that differ in relation to national consumer preferences and producer company practices (Beckett, 2000; Whitefield, 2005). In this panorama there are niche chocolates that offer variety in sensory characteristics. For these products it is useful to define the standard of sensory identity through analyzing the characteristics of locally crafted chocolates. During the Spanish domination (1516-1713), an Aztec recipe to prepare the "xocoatl", cocoa mass mixed with vanilla or cinnamon and sugar, was transferred to the inhabitants of the county of Modica (Sicily) as a gift of fidelity. The formula of this chocolate was lost and only through historical sources the original recipe has been recovered. Until 1992 the chocolate of Modica was quite unknown, it was mainly consumed at a local level with a production of a few thousand bars/year. Unlike other chocolate commercial products, this type of chocolate has often been considered as a new product (Ciuffoletti and Cresti, 2004). Today some techniques and ingredients are employed to prepare a primitive type of chocolate bar whose ancient formula may be exploited to put on the market as a new product. Winning strategies of correct

visibility regarding marketing and packaging gave a new status to the product. In fact, 300.000 bars/year of production have entered into national distribution only in specialized shops. One of the greatest producers of chocolate of Modica has had a 25% growth trend in 2006 and the chocolate spiced with cinnamon/vanilla has been the most sold with a 10% growth trend (Bommezzadri, 2007).

With reference to the chocolate production technology (roasting of cocoa beans, mixing, conching, and tempering) the chocolate of Modica processing method is very simple. For the production of chocolate of Modica, the Disciplinary (2003) compiled by the Consortium of Guardianship foresees that a mass of cocoa heated to 45°C and mixed with sugar and spices (vanilla, cinnamon, chilli) without addition of emulsifiers is manually worked with stone tools at a constant temperature that does not allow the melting of sugar crystals. By subsequent cold tempering the cocoa butter consolidates and the product is ready to be formed in rectangular shape (www.cioccolatomodica.it).

Worldwide consumption of chocolate and cocoa-containing products increased by 10% from 2002 to 2010, which might be attributed to consumer economic enhancement and increasing knowledge of potential health benefits derived from cocoa constituents. Chocolate and cocoa-containing products are a good source of non-nutrient bioactive polyphenols with potential health benefits including reduced risk of cardiovascular disease and prebiotic activity (Hu et al., 2016).

Chocolate and cocoa products are a rich source of flavonoids. Flavanoids, naturally occurring polyphenolic compounds present in plant-based foods, represent up to 20% of the compounds present in cocoa beans. Flavanols, and in particular epicatechin, are a subgroup of flavonoids, and are the most common cocoa flavonoids. High levels of flavanols are also found in tea, red wine, and fruits such as grapes and apples. In

addition to cocoa flavonols, other psychoactive components of chocolate include the methylxanthines (caffeine and theobromine), both of which have been associated with improving alertness and cognitive function. One hundred grams of dark chocolate contain approximately 100 mg of flavanols, while 100 g of unsweetened cocoa powder without meythlxanthines can contain up to 250 mg of flavanols (Crichton et al., 2016).

Dark chocolate can be considered as a product with an important nutritional density, because of its richness in carbohydrates and fats. Cocoa butter is considered the most important cocoa by-product, due to its physical (rheology and texture) and chemical characteristics, and also organoleptic qualities. The prevalence of saturated fatty acids over unsaturated fatty acids is considered to be negative from the nutritional point of view. For many years, saturated fatty acids whose chain length is C12:0 – C16:0 have been considered to provoke atherosclerosis, and to be associated with cardiovascular disease. Thus, because of its high SFA content, chocolate is often postulated as having a hypercholesterolemia effect. However, it has been suggested in recent clinical trials that stearic acid (C18:0), a non-cholesterolemic and atherogenic type of dietary saturated fat, is neutral. These trials have shown that chocolate consumption has neutral effects on serum total cholesterol and LDL-cholesterol, as neither lowers HDL-cholesterol (Torres-Moreno et al., 2015).

Although evidence in the literature suggests that chocolate consumption may have beneficial effects on health, it must be noted that chocolate has a high total fat and sugar content; in consequence, daily consumption of large amounts of chocolate may increase weight in the long term. That is why scientific evidence suggests that chocolate consumption should be considered in the context of a healthy diet, and dark chocolate

must be consumed in moderate amounts (20 – 25 g per day) (Machálková et al., 2016; Torres-Moreno et al., 2015).

Calado et al. (2015) showed that bitter chocolate can have higher flavonoid content compared to some kinds of cooked vegetables and advises, that people who don't like foods like broccoli or eggplant (daily dose between 20 and 26 g) could eat more chocolate (daily dose only 8 g).

## 1.3. Importance and impacts of food product innovation

The progressive growth in private label and market share system represents a major competitive threat for many food manufacturers. However, product sectors dominated by strong manufacturer brands and unique product lines act as a deterrent to own label developments due to high cost of entry (McMaster, 1987), and the coercive sources of power of suppliers.

In the current increasingly globalizing food market, innovation is an essential strategic tool for SMEs (small and medium sized enterprises) to achieve competitive advantage (Avermaete et al., 2004; Gellynck et al., 2007; Murphy, 2002). This also applies for traditional food products, despite the seeming controversy between innovation and tradition (EC, 2007; Jordana, 2000) and the challenge this controversy involves (Amilien et al., 2005; Gellynck and Kühne, 2008; Jordana, 2000).

Traditional food products are closely linked to a system of historic and cultural traditions and connected to a specific geographical area and have an important role in maintaining and supporting agro-biodiversity as well as sustainability. Traditional food products are a good source of nutrients, and the levels of the entitled nutrients can be

influenced by a range of parameters related to the environment, as well as to food preparation and processing (Mattera et al., 2016). This study focuses on Pecorino cheese, which is a very famous traditional food of Italy, especially Sicily. We decided to join this product with another more popular product Modica chocolate. This study is a survey through innovation of new food product.

Innovation is a broad and multi-dimensional concept, and can be defined as the capacity to innovate, also in the future, along the whole innovation process of ongoing learning, searching and exploring, resulting in new products (Lundvall, 1995). On the other hand an important part of productive growth in advanced nations, as measured in terms of Gross Domestic Product, corresponds to innovation (Freeman, 1994).

So far, only few studies are published that focus particularly on innovations in traditional food products (Jordana, 2000). Innovations in the traditional food sector strengthen and widen the market for traditional food products in accordance to the emerging problems, such as poor imitations and changing preferences and eating patterns towards more manufactured foods and convenience (Trichopoulou et al., 2006). Innovations in traditional food mainly pertain to product innovations, such as packaging innovations and changes in product composition, product size and form or new ways of using the product (Gellynck and Kühne, 2008). Our product was chocolate cheese and we studied the textural, chemical, microbiological and sensory that support the possibility of massive production and the possibility of consumption in accordance to our population's taste. There are also other aspects in food innovation. Process innovations are less common, given their impact on the authentic identity of the product and its production process. Feasible applications relate to improving the production process in order to assure quality and traceability. Finally, the implementation of market

and organizational innovations can be valuable for traditional food products but their potential is not yet realized or recognized by all chain members in the traditional food sector (Gellynck & Kühne, 2008).

In recent years, the food culture manifests behaviors that tend to escape the standardization of consumption and, at the same time, to both recover/maintain the old culinary traditions, and to require innovative products and services in line with modern lifestyles (Torre and Wallet, 2015). There is an increased search for a food product that meets the tastes and preferences of the consumer, because it is now common the image of a consumer more aware and more attentive to the quality of food (Boccia, 2016).

Beside all these the base of our product, which is pecorino cheese, goes under the category of Italy's traditional food products. Our reason for choosing this specific product was the consumer as a target. Traditional food products as a food category are not yet extensively analysed from the chain perspective, with some notable exceptions (Gellynck and Kühne, 2008; Gellynck et al., 2008; Raynaud et al., 2005). The integration of chain partners in the innovation process enhances the capacity to innovate and reduces the risks involved in implementing innovation (Earle, 1997; Gellynck and Kühne, 2008; Omta, 2002; Pittaway et al., 2004). From this perspective, incorporating the opinion of customers and consumers further supports the realization of competitive advantage through innovation implementation (Dougherty, 1992; Earle, 1997).

Italy is one of the European countries that have always supported a recognition and institutionalization policy of geographical designations for food products. It has a significant heritage of traditional products: it has a high mass of resources to invest in, which represents the roots of the great Italian traditions. The dairy supply industry, in the Italian context, may be the one that conjures the tradition and the vocation of typical

products and, at the same time, seize the economic opportunities, especially if it can control the entire production chain and transformation of cheese in association with chocolate.

We did the combination of traditional food production with innovative food production in our chocolate cheese model for the first time. As previously described, this can affect the improvement of the organizational efficiency of the business and will definitely permit a better penetration of the typical regional products of quality on international markets. It also has the potential of targeting larger groups of consumers since it is new in material as well as known due to being traditional.

## 1.4. Chocolate cheese & Children and consumer

Food preference has a fundamental role in driving consumer's choices and habits, especially in children (Cooke, 2007; Nicklaus et al., 2004, Laureati et al., 2015a). Therefore, the number of food products specifically developed for children is growing and this market is acquiring increasing importance (Issanchou, 2015).

Consequently, the youngest consumers are frequently involved in research and development programs, since their food habits will influence choices as they grow older and because their preferences, even if partly driven by advertisements (Ustjanauskas et al., 2014), seem to be strictly related to the sensory aspects of food (Pagliarini et al., 2005; Mustonen and Tuorila, 2010).

In 1994, Moskowitz highlighted how a visually pleasing product tends to be more appreciated by children, as visual attributes seem to be, among different sensory characteristics, those that mainly determine its success (Kildegaard et al., 2011; Topcu,

2015). Indeed, children tend to create an “ideal picture” of each food product that can be related to their own idea of “good”; this picture represents a sort of reference point that can be used to dislike products whose appearance is not close to their expectation (Mustonen and Tuorila, 2009).

In an interesting study Tesini et al. (2015) worked on children preferences of colored fresh cheese prepared during an educational laboratory. The results of this study highlighted how visual preference for a food, in terms of color, changes during different stages of life. Indeed, the data demonstrates how children can be influenced by food appearance and how the aspect of a product can be related to its acceptance, especially among younger individuals; in fact, the youngest participants tended to prefer intensively colored cheese vs. the white and green version.

Many industries in the food sector would like to be able to take into account the opinions of children, in order to test products intended for them as consumers. The fact is that the world, which has been created for children, continues to grow and in most cases there are the experts (ISO, 1992) who evaluate products for them.

Children have financial autonomy, special tastes and money to spend. They are more informed than in the past and styles of consumerism are now evolving (McNeal, 1992). Various forms of research concerning child consumerism reveal a similarity between developed countries. Children from different cultures (Hong Kong, Taiwan and New Zealand) differ little from American children in the way they spend their pocket money (Gunter and Furnham, 1998). It would appear that one of the things which children between 7 and 11 spend their money on is sweets, chocolates and cereal bars.

Chocolate is a frequently and intensely craved food among women, and to a lesser extent among men (Hill and Heaton-Brown, 1994; Weingarten and Elston, 1991).

Chocolate contain relatively high amounts of inhibitors of iron absorption, such as polyphenols of the catechin type. These compounds are similar to the polyphenols present in red wine, which have been shown to inhibit iron absorption in human subjects (Cook et al., 1995). In addition, cheese contains the iron absorption inhibitors such as calcium (Hallberg et al., 1991). Therefore, chocolate would not be expected to be a good vehicle for iron fortification unless the iron used for fortification was in some way protected from the absorption inhibitors. The classic way to counteract inhibitors of iron absorption in industrially produced foods is to add ascorbic acid (Davidsson et al., 1994; Davidsson et al., 1997). Davidsson et al. (1998) studied about measuring iron absorption from an iron fortified, malted, chocolate-flavored milk drink in 6–7-y-old Jamaican children by using a stable-isotope technique and to evaluate the addition of ascorbic acid as an absorption enhancer. They found that the negative effect of phytic acid and polyphenols in the test meal was overcome by adding ascorbic acid, which resulted in a threefold increase in fractional iron absorption. There is no information about the existence of ascorbic acid in Pecorino cheese or sheep milk cheese, while Park et al. (2007) reported that vitamin contents, in particular vitamin C, in sheep milk are mostly higher than in cow and goat milk, however, research data on vitamins in sheep milk are scares.

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# 2. CHAPTER I

Determination of polyphenol content, oxidative stability and fatty acids in Pecorino chocolate cheese as an innovative food product

## 2.1 ABSTRACT

In this project we used two specific food products, which are typically Sicilian, Pecorino Siciliano cheese and Modica chocolate, that have unique texture and taste. “Chocolate cheese” is produced with the aim of increasing the consumption of cheese especially among children, targeting the purpose of improving public health. In this research we investigated chocolate cheese including solid and molten chocolate in three levels of 5%, 10% and 15% at four ripening times (T0 = fresh, T1 = 2 weeks, T2 = 4 weeks and T3 = 6 weeks). The results showed that the addition of chocolate significantly ($p \leq 0.05$) decreased the pH, ash and protein content of the Pecorino Siciliano cheese, whereas it had no significant ($p > 0.05$) effect on other cheese composition (DM, fat and NaCl).

In this study POV and TBARs ranged from 0.65 to 1.35 meq O2 $kg^{-1}$ fat and 0.006 to 0.027 mg MDA $kg^{-1}$ cheese respectively, which are in the range reported. There were significant differences ($p \leq 0.05$) in polyphenols between control samples and chocolate cheese among treatments and also during ripening time. In all samples 43 fatty acids (FA) were detected; the most abundant FA were saturated FA (SFA, 70%), followed by monounsaturated FA (MUFA, 22%) and finally, the least abundant were polyunsaturated FA (PUFA, 8%). Individually, C16:0 (palmitic acid) was the most abundant molecule (23%), followed by C18:1 (oleic acid) and C14:0 (myristic) that were approximately 15% and 11% respectively.

## 2.2. INTRODUCTION

The introduction of innovation and development of new foodstuffs is always welcomed in the food industry, mostly if these innovations bring bioactive properties to traditional foodstuffs.

In recent decades, nutrition research has focused on the investigation of bioactive dietary flavonoids, widely found in many plant-based foods and beverages, in order to elucidate their beneficial properties to human health. Cocoa (*Theobroma cacao* L.) and chocolate products appear to be one of the most promising foods due to their high polyphenol content, which evidently highlights the link with health-promoting properties (Alañón et al., 2016). Main groups of polyphenols are the catechins (37%), procyanidins (58%) and anthocyanins (4%). The polyphenols in cocoa beans contribute to about 12 – 18% of the dry weight of the whole bean (Bordiga et al., 2015).

Catechins are a class of flavonoids with demonstrated health benefits attributed to antioxidant activity (AA). These benefits include anti-allergic, anti-microbial and anti-carcinogenic effects (Yamamoto and Gaynor, 2001) and mitigation of heart disease (Hertog et al., 1993). The efficacy of catechins is associated with the dietary quantity. Consumption of five cups or more of green tea is required to show the beneficial effect of catechins (Kuriyama, 2008). In practice, most people do not drink such a large volume of green tea on a daily basis due to reasons such as the high caffeine content (Chu & Juneja, 1997), therefore, catechins are packaged in different dietary forms, such as supplements, and incorporated into functional foods to increase the amount consumed (Ferruzzi and Green, 2006; Najgebauer-Lejko et al., 2011; O'Connell et al., 1998; Pattono et al., 2009; Wegrzyn et al., 2008). As an example, Arts et al., (2001)

reported the mean daily catechin intake from a normal diet in the Dutch population was 50 mg, Tea was the main source of catechin in all age groups. Chocolate was the secondary source for children whereas apples and pears were the secondary source for adults and the elderly. In a population where tea consumption is low, consuming this amount of catechin from a delivery vehicle, such as cheese and chocolate, would be easily achievable without overconsumption. Concentrations of catechin 1-2 g $kg^{-1}$ could be added to cheese such that even a small portion consumed daily (25-50 g) should be able to provide a typical amount of catechin for an adult. Catechins are unstable at high pH values, high temperatures and over long time storage (Lun Su et al., 2003).

Pecorino Siciliano is a typical Sicilian hard cheese, produced in Sicily according to ancient manufacturing techniques. It is a Protected Designation of Origin (PDO) cheese made from raw ewe's milk without the addition of starter cultures.

Ewe milk is almost exclusively used to be processed into cheese. Ewe milk cheeses are strongly appreciated by consumers at large, owing to the variety of sensory attributes and excellent source of high quality protein, Ca, P and bioactive lipids. Although the cholesterol content of ewe milk is typically lower than in other animal foodstuffs, the figures for some manufactured products like cheese are relatively high and very variable (Güler and Park, 2010). For instance, Mele et al. (2011) found levels of cholesterol between 210 and 250 mg per 100 g of fat in Pecorino. An excessive amount of cholesterol in the human diet is regarded a risk factor with respect to cardiovascular diseases, and the emphasis today remains in favor of low cholesterol foods or dietary changes that may lower cholesterol.

Cheese is a good source of minerals like Ca, Mg, P and fat-soluble vitamins (Henning et al., 2006) and also has anticaries functions (Buttris, 2003). Besides, cheese has

functional ingredients like bioactive peptides or conjugated linoleic acid. Conjugated linoleic acid is associated with anti-cancer, anti-diabetic and anti-obesity potentials (Henning et al., 2006). Cheese contains a small amount of phenols (O'Connell and Fox, 2001) but lacks vitamin C and many important antioxidants (Buttris, 2003). Therefore, the main aim of this study was to develop a novel product for better nutrition and health promoting principles like antioxidants and other beneficial phytochemicals.

Chemical parameters of cheese quality change during storage. Chemical indicators of quality like peroxide values increase with storage time. In previous studies, it was observed that oxidation indicators in fat milk increase during storage (Pettersen et al., 2008; Wold et al., 2006).

Cheese contains a small amount of phenolic compounds of limited importance (O'Connell and Fox, 2001) due to low antioxidant activity (Han et al., 2011; Huvaere et al., 2011). Therefore, adding chocolate as a source of polyphenol could be a good way for increasing nutritional value.

# 2.3. MATERIALS AND METHODS

## 2.3.1. Cheese making

Briefly, the raw sheep milk is heated to 35–37℃ and rennet paste is added. Coagulation was is allowed to proceed for 45–60 min, after which the coagulum is cut into pea-sized pieces and then the curd is covered slowly with water at 75℃, to facilitate the release of whey. Subsequently, the curd is placed in a mould and in this step solid and molten Modica chocolate are added and then pressed by hand and turned in order to eliminate the residual whey. The cheeses were transferred to a cellar under a temperature of 16°C and a relative humidity of 80%.

## 2.3.2. Chemical analyses

The cheese samples were freeze-dried and DM, fat, protein (N × 6.38), and ash content were determined according to International Dairy Federation (IDF) standards [4A:1982 (IDF.,1982), 5B:1986 (IDF, 1986), 25:1964 (IDF, 1964a), and 27:1964 (IDF, 1964b) respectively]. Sample pH was measured directly with a pH-meter equipment with a spear electrode FC 200 (HI 9025 pH meter; Hanna Instruments Inc, Ann Arbor, MI) and NaCl according to the IDF procedure (17A:1972; IDF, 1972).

## 2.3.3. Oxidative product

The oxidation status of cheese fat was assessed on freeze-dried samples by determination of peroxide value (POV, mEq $O_2$/kg fat), as index of primary lipid

oxidation (74A; IDF 1991). In addition, thiobarbituric acid-reactive substances (TBARs), expressed as µg malonylaldehyde (MDA)/kg DM), used as a measure of the secondary lipid oxidation products, was determined according to the method proposed by Tarladgis et al. (1960) and modified by Mele et al. (2011). Briefly, 8 ml of phosphate buffer aqueous solution at pH 7 were added to 4 g of cheese in a 25 ml Sovirel tube and the mixture was homogenized using a high-speed homogeniser (Art-Miccra D-8, Moderne Labortechnik, Müllheim, Germany). Two millilitres of a 30% (v/v) trichloroacetic acid aqueous solution were then added and sample was vortex-mixed for a few seconds, followed by filtration through Whatman No.1 filter paper. Five millilitres of 0.02 M aqueous solution of thiobarbituric acid were added to 5 ml of the filtrate. The solution was placed in hot water bath at 90°C for 20 min, followed by refrigeration. After centrifugation at 4500 rpm for 5 min, the absorbance of the supernatant was read at 530 nm using an HACH DR/4000U spectrophotometer (HACH, Loveland, CO, USA). For the quantitative determination of TBARs, 1.1.3.3-tetramethoxypropane solutions with a concentration range from 0.016 to 0.165 µg/ml were used for the calibration curve ($R^2$ = 0.99). For both POV and TBARs determination, three replicates were run *per* sample.

### 2.3.4. Fatty acid composition

The fatty acid (FA) composition of freeze-dried samples of chocolate (50 mg) was determined using the one-step extraction and trans esterification procedure (Sukhija and Palmquist, 1988), with C23:0 as the internal standard (Sigma-Aldrich, Milano, Italy).

Cheese FA were determined using samples collected at the four ripening times; these were stored at −20°C and then freeze-dried. FA in freeze-dried cheese samples (100 mg) were directly methylated in 1 ml of hexane with 2 ml of 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, based on the bimethylation procedure described by Lee and Tweed (2008). Fatty acid methyl esters (FAME) were recovered in hexane (1.5 ml), One microliter of each sample was injected by auto sampler into an HP 6890 gas chromatography system equipped with a flame-ionisation detector (Agilent Technologies, Santa Clara, CA, USA). FAME from all samples were separated using a capillary column 100 m in length with an internal diameter of 0.25 mm and film thickness of 0.25 μm (CP-Sil 88, Chrompack, Middelburg, The Netherlands). The injector temperature was kept at 255°C and the detector temperature was kept at 250°C, with an $H_2$ flow of 40 ml/min, an airflow of 400 ml/min, and a constant helium flow of 45 ml/min. The initial oven temperature was held at 70°C for 1 min, increased by 5°C/min to 100°C, held for 2 min, increased by 10°C/min to 175°C, held for 40 min, then finally increased by 5°C/min to a final temperature of 225°C and held for 45 min. Helium, with a head pressure of 23 psi and a flow rate of 0.7 ml/min (linear velocity of 14 cm/s), was used as the carrier gas. A FAME hexane mix solution (Nu-Check-Prep, Elysian, MN, USA) was used to identify each FA. Individual standards (Larodan Fine Chemicals AB, Malmö, Sweden) were used to identify C15:0 *iso*, C15:0 *anteiso*, C17:0 *iso*, and C17:0 *anteiso*. A standard mixture of methyl esters of C18: 2 *c9 t11* and C18:2 *c10 t12* (Sigma, Milano, Italy) with published isomeric profiles (Kramer et al., 2004; Luna et al., 2005) was used to help identify the conjugated linoleic acid (CLA) isomers.

### 2.3.5. Polyphenol content

The cheese samples extracts were prepared to measure their total content of phenolic compounds, performed on three replicates per sample. Cheese extracts were prepared according to the method of Rashidinejad et al. (2013) with slight modifications. To prepare these extracts, milled and freeze-dried cheese samples (0.5 g) were homogenized for 30 s and then extracted for 30 min with 25 ml of methanol (95% aqueous solution) containing 1% HCl at 50°C on an orbital shaker at 200 rpm. The mixture was cooled and filtered with cheesecloth, and the residues were washed with 1 ml of the same solvent (95% methanol aqueous solution with 1% HCl) and then centrifuged at 7000 rpm for 10 min at 9°C.

The total concentration of polyphenols in the samples extracts was measured using the Folin–Ciocalteau colorimetric method, as described by López-Andrés et al. (2014). Briefly, 100 μl of the sample extract was transferred into a 15-ml centrifuge tube; then, 900 μl of distilled water and 500 μl of the Folin–Ciocalteau reagent, diluted to a concentration of 1 N with distilled water, were added. After 1 min, 2.5 ml of 20% (w/v) sodium carbonate was added; the mixture was vortex-mixed for 30 s and incubated for 40 min in the dark at room temperature. The absorbance of the samples was read at 725 nm using an HACH DR/4000U spectrophotometer (HACH, Loveland, CO, USA) against a blank containing all of the reagents except the sample extract. Gallic acid aqueous solutions of different concentrations (0 to 1 mg/ml) were used for the calibration curve ($R^2$ = 0.99). The results were expressed as grams of gallic acid equivalent (GAE) per kilogram of sample DM.

### 2.3.5. Statistical analysis

Data were analysed statistically by the GLM procedures in SAS 9.2 software (2010). The GLM procedure was used for all parameters (chemical properties, polyphenol content, oxidative product and FA composition) with a model including the effects of cheese-making day (three levels) treatment (TR) (seven levels), ripening time (RT) (four times, T0, T1, T2 and T3) and the interaction TR*RT. Then Tukey test was performed in case of variances were equal to identify the significant differences at 95% confidence level.

# 2.4. RESULTS AND DISCUSSION

## 2.4.1 Chemical Analysis

The average values of DM, protein, fat, pH, ash and NaCl of control cheese were 56.77 g/100 g, 48.18 g/100 g, 41.56 g/100 g, 6.21, 6.36 and 0.76, respectively. As expected, almost chemical parameters were changed during the ripening time and between each sample. The effect of the addition of different levels of chocolate on DM, protein, fat content, pH, ash and NaCl is presented in Table 1.

### 2.4.1.1. Dry matter

The results showed that there are no significant differences between samples in different levels of both type of chocolates and control sample, whereas significant differences were observed during ripening. In fact, a decrease in the dry matter was found during ripening. This finding is in agreement with the results of Barac et al. (2016) who studied the water-soluble and water-insoluble fractions of white-brined goat cheese at different stages of ripening. It is noteworthy that during cheese making, the curd is covered slowly with water at 75℃, to facilitate the release of whey. Macej et al. (2007) claimed that significant reduction of DM could be explained by the presence of hydrophilic cheese gel network caused by incorporation of denatured whey proteins, especially molecules of $\alpha$-lactalbumin after thermal treatment.

### 2.4.1.2. Fat

The results showed that there are no significant differences between samples and also during ripening time. Over this period of ripening, fat content was slightly decreased by 1.67% (from 40.58 g/100 g fresh cheese to 39.90 g/100 g cheese samples after 6 weeks) but the observed changes were not statistically different. This result is in agreement with Branciari et al. (2014), Gabr et al. (2015) and Hefnawy et al. (1984). Gabr et al. (2015) reported that there are no changes in fat content during ripening time (60 days) in control sample and cheese samples with different level of black rice powder.

### 2.4.1.3. Protein

Modica chocolate has 10% protein whereas control samples have around 48% protein. Therefore, by increasing amount of chocolate, sample protein content decreased. Therefore, observed significant differences between treatments and samples protein content ranged from 48.18 g/100 g to 40.27 g/100 g. The results showed that control sample contained the highest value and the lowest value was found in sample containing 15% molten chocolate. On other hand, there was no significant difference during ripening time. This result is in agreement with Branciari et al. (2014) who reported that there are no significant differences between pecorino cheese and pecorino cheese made from the raw milk of ewes fed Rosmarinus officinalis L, leaves during ripening.

### 2.4.1.4. pH

The results showed that addition of chocolate significantly ($p < 0.05$) decreased the pH. The treatments ranged from 6.21 to 5.89 and there are significant differences between them. The highest one is related to the control cheese and the lowest one was the chocolate cheese containing 10% solid chocolate. Generally adding the chocolate to cheese sample leads to the reduction of pH. These observations are in agreement with those seen for the effect of catechin on the pH of low-fat cheese (Rashidinejad et al., 2013), and also confirm the results reported by Giroux et al. (2013), and Gad and El-Salam (2010).

One of the possible mechanisms for decreasing pH by phenolic compounds, such as those from chocolate, could be a reaction of sodium ions that exist in the cheese matrix with the OH groups of the added phenolic compound. The findings of the present study also confirm the previous results (Rashidinejad et al., 2013) on the effect of catechin on the pH of low fat, and the decrease in the pH of the control cheese over the ripening period. The decrease in pH of the control cheese during ripening is comparable with the values found by other researchers (Foster et al., 1958; Fox, 2004; Johnson, 2002). Although in the present study, pH significantly decreased during ripening time. Marrone et al. (2014) claimed that in Pecorino Carmasciano pH decreases during ripening time.

Ruben et al. (2013) reported that the addition of oregano and rosemary essential oils in cream cheese show an effect decreasing acidity and pH. Marchiani et al. (2016) showed that Grape pomace powder addition in semi hard cheeses produced a significant pH

decrease ($p < 0.001$) due to the presence of organic acids (tartaric, malic and citric acids) in grape powder.

Obtaining a complete opposite result, Caturla et al. (2003) and Hara-Kudo et al. (2005) reported that since polyphenols have antibacterial ability, one might assume that these compounds could reduce the number of lactic acid bacteria when they are added to a fermented dairy food product. Najgebauer-Lejko et al. (2011) and Sun-Waterhouse et al. (2012) however, claimed that polyphenols from tea and apple could support the growth of lactic acid bacteria and thus cause the pH of yoghurt to decrease through production of lactic acid.

#### 2.4.1.5. Salt

As it shown in table 1, NaCl of samples ranged from 0.55 to 0.76 without significant differences between control sample and chocolate cheeses samples.

Salt content was slightly decreased by 27.63% (from 0.76 in control sample to 0.55 in cheese sample with 15% solid chocolate) but the observed changes were not statistically significant. On the other hand, salt content was slightly increased by 10.93% (from 0.57 in fresh cheese to 0.64 cheese samples after 6 weeks).

Todaro et al. (2017) reported that salt (NaCl) determined on Vastedda della valle del Belice cheeses made from ewes' raw milk showed an increase during storage especially after 60 days. Gaucheron et al. (1999) found a slightly increase during 14 days of storage; moreover, these authors established a migration of sodium, potassium and chloride ions from the outer layer versus the core of cheese which ended after 5 days. Other authors, on Minas cheese (Felicio et al., 2016), reported a significant decrease in

sodium during the first 14 days of storage, while no significant variation was reported from 25 to 360 days of storage by Pappa et al. (2016) for Urda whey cheese.

#### 2.4.1.6. Ash

As it shown in table 1 ash content was ranged from 6.36 (control sample) to 5.41 (cheese with 15% solid chocolate). Generally, as we raised the amount of chocolate in cheese, the ash of samples was significantly decreasing whereas, with the passage of time, the ash content significantly increased from 5.67 (T0) to 6.03 (T3).

**Table 1.** Average value of chemical analysis (% DM) of the different treatments during ripening time.

| Treatment | TRT | | | | | | | SEM | Time | | | | SEM | Significant (p<) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | | T0 | T1 | T2 | T3 | | TRT | Time | TRT*Time |
| **DM** | 56.77 | 55.35 | 55.19 | 56.37 | 54.98 | 55.20 | 55.59 | 0.5207 | 57.15 | 55.53 | 56.66 | 53.21 | 0.3936 | 0.1466 | <0.0001 | 0.6862 |
| **Protein** | 48.18 | 45.40 | 43.29 | 40.53 | 45.77 | 43.36 | 40.27 | 0.4370 | 43.65 | 43.59 | 44.01 | 44.06 | 0.3303 | <0.0001 | 0.6605 | 0.9985 |
| **Fat** | 41.56 | 41.14 | 40.39 | 39.50 | 40.88 | 40.19 | 39.32 | 0.5866 | 40.58 | 40.54 | 40.67 | 39.90 | 0.4434 | 0.0811 | 0.5978 | 1 |
| **pH** | 6.21 | 5.93 | 5.89 | 5.96 | 5.98 | 5.92 | 6.00 | 0.0685 | 6.21 | 6.12 | 5.84 | 5.76 | 0.0518 | 0.0429 | <0.0001 | 0.9999 |
| **Ash** | 6.36 | 6.01 | 5.71 | 5.41 | 6.03 | 5.81 | 5.57 | 0.0615 | 5.67 | 5.72 | 6.02 | 6.03 | 0.0465 | <0.0001 | <0.0001 | 0.8532 |
| **NaCl** | 0.76 | 0.62 | 0.56 | 0.55 | 0.61 | 0.60 | 0.56 | 0.0512 | 0.57 | 0.61 | 0.61 | 0.64 | 0.0387 | 0.095 | 0.5832 | 0.9988 |

Abbreviation: TRT: Treatment, CTL: Control sample, SC: Solid Chocolate, MC: Molten Chocolate, T0: fresh, T1: two weeks, T2: four weeks, T3: six weeks.

### 2.4.2. Polyphenol content

The results of Total Phenolic Content (TPC) analysis for cheese samples can be seen in Fig.1. As expected, generally as we raised the amount of chocolate in cheese. the polyphenol content of samples was significantly increasing. These observations are in agreement with those seen for the effect of adding polyphenol-rich dietary sources such as cocoa, tea, wine, soy products and fruits on the polyphenol content of product such as cheese. Rashidinejad et al. (2016) reported that the addition of different concentrations of free green tea extract increased the TPC of full-fat cheese, but the increase was not proportional. As mentioned above, the TPC of chocolate cheeses was always higher ($P < 0.05$) than that of control cheeses, with highest values at T3. The rate of TPC rise in chocolate cheese samples, compared to the control samples was 14.08%, 26.59%, 36.26%, 16.81%, 37.26% and 42.74% for cheese with 5%, 10%, 15% of solid chocolate and 5%, 10%, 15% of molten chocolate, respectively. As is clear, there are slightly increases in samples containing molten chocolate in comparison with cheese samples with solid chocolate. The probable reason mainly can be putative interaction between chocolate polyphenols and cheese components (such as protein and fat) particularly in molten chocolate. This however is explained by the increase in contact surface while we use this kind of chocolate compared to solid one.

Our observations also defined that TPC significantly increase over the ripening period. Considering values at all ripening time, all cheeses with chocolate had higher TPC values compared to the control sample. A portion of the TPC values may be derived from proteins (such as tyrosine residues) and sugar components (oligosaccharide and

lactose) present in the milk (Lowry et al., 1951; Singleton et al., 1999). Complexation between phenolics and proteins can reversibly occur via hydrophobic association and/or hydrogen bonding between hydroxyl groups of polyphenolics and the undissociated carboxyl groups in the peptide residues of proteins (Rawel et al., 2001; Perez-Jimenez and Saura-Calixto, 2006). Some simple polyphenols not only bind onto different sites of proteins at low phenolic concentrations, but may also cross-link protein molecules at high phenolic concentrations (Haslam, 1974; Oh et al., 1980; McManus et al., 1981). Free thiol groups can form thiyl radicals that interact with catechin (Fujimoto and Masuda, 2012), and this cannot be excluded as a potential type of interaction between polyphenols and residual whey proteins. It is also possible that catabolism and changes in protein structure (Ha and Lindsay, 1991) may release bound phenolic compounds from milk macro components that are then detected by the Folin– Ciocalteu assay. This test is sensitive to ions, salts and other interfering agents (Gmeiner and Seelos, 1994). and some compounds such as uric acid; guanine, xanthine and thymol can also potentially react with the Folin–Ciocalteu reagent (Lowry et al., 1951).

Cheese contains a small amount of phenolic compounds of limited importance (O'Connell and Fox, 2001) due to low antioxidant activity (Han et al., 2011; Huvaere et al., 2011). Catechins are unstable at high pH values, high temperatures and over long time storage (Lun Su et al., 2003). The relatively high levels of protein and calcium in cheese, the low pH and storage temperature, along with widespread consumption, suggest that this would be a good vehicle for the incorporation of antioxidant compounds into a food product with high nutritional value.

Since chocolate is a natural source of polyphenol and specially catechin, and as a matter of fact that cheese mostly consists of protein and fat, putting chocolate inside cheese

causes an increase in the polyphenol absorbance during digestion. Therefore, as a result the mixture of these two leads to a reduction of cancer and cardiovascular diseases conclusively.

It has become clear that nutrients like proteins, carbohydrates and lipids that surround polyphenols inside the gastrointestinal tract, have a great impact on the bioaccessibility and bioavailability of polyphenols. Indeed, many such nutrients have a very complex, porous structure, which trap polyphenols and, as a consequence, change their availability for absorption. Studies conducted in recent years have shown the importance of these interactions (Chanteranne et al., 2008; Schramm et al., 2003). Moreover, these interactions with nutrients could give polyphenols a very different role. They could protect polyphenols from oxidation during their passage through the gastrointestinal tract and deliver them to the colon more intact. Here they can be metabolized under the influence of microflora. Recent studies describe the role of dietary bioactive compounds, intestinal microbiota and polyphenol metabolites (MacDonald and Wagner, 2012; Tuohy et al., 2012).

It has become increasingly clear that polyphenols have many potential bioactivities in the human body, which are affected by interactions of polyphenols with other macromolecules (Le Bourvellec and Renard, 2012).

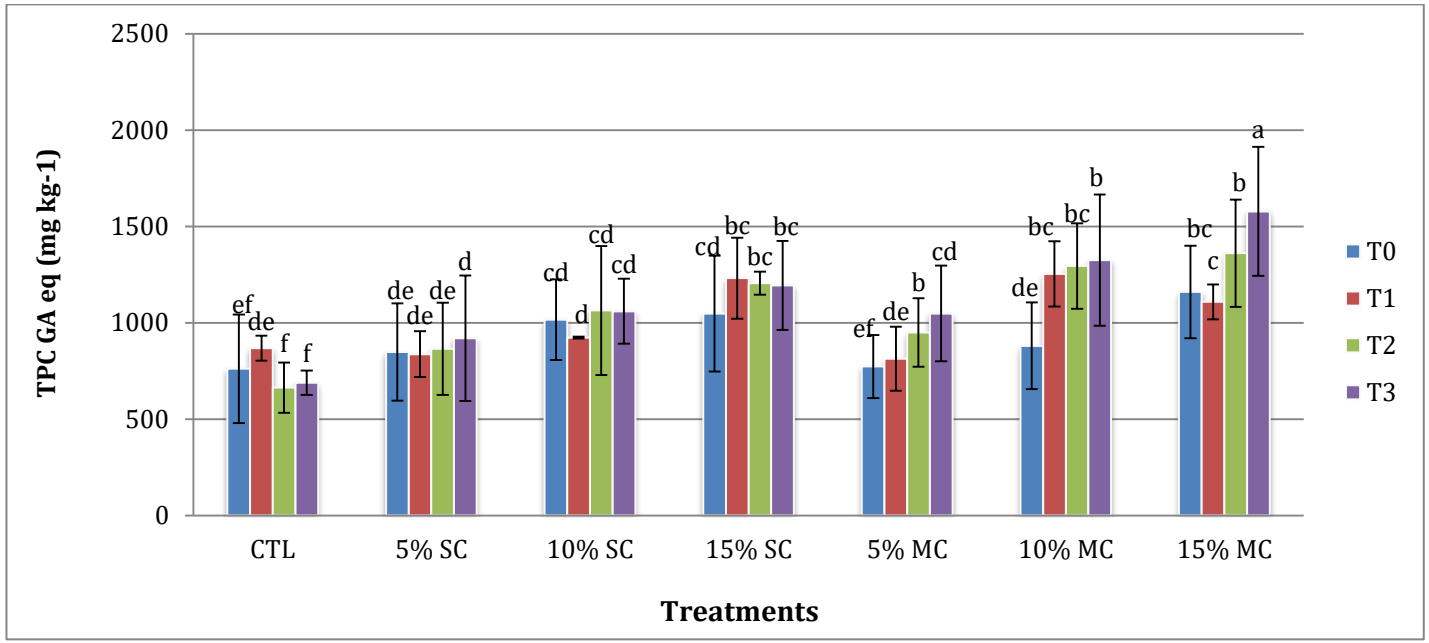



**Fig 1.** Effect of addition of different amount and type of chocolate in Pecorino cheese on polyphenol content.

**Table 2.** Polyphenol content of treatments during ripening.

| | TRT | | | | | | | SEM | Time | | | | SEM | Significant (p<) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | | T0 | T1 | T2 | T3 | | TRT | Time | TRT*Time |
| **Polyphenol (mg kg-1)** | 745.45 | 867.66 | 1015.49 | 1169.57 | 896.11 | 1188.24 | 1301.9 | 106.06 | 926.70 | 1005.08 | 1057.49 | 1116.12 | 80.18 | <. 0001 | <. 0001 | 0.0472 |

Abbreviation: TRT: Treatment, CTL: Control sample, SC: Solid Chocolate, MC: Molten Chocolate, T0: fresh, T1: two weeks, T2: four weeks, T3: six weeks.

## 2.4.3. Oxidative products

### *2.4.3.1. Peroxidase value*

The influence of ripening time and chocolate on the development in the POV of the lipid extracted from cheese samples are reported in Table 2.

The POV is an indicator of the initial stages in lipid oxidation, monitored by analysis of lipid peroxides. In this study POV ranged from 0.65 to 1.75-meq O2 $kg^{-1}$ fat and the highest value was detected in cheese sample including 10% solid chocolate after 42 days of ripening (fig 2). These values are in the range reported by Kristensen et al. (2001) and Mele et al. (2011).

The lowest value was found in fresh sample cheese. It is interesting emphasized that there were no significant difference in POV of every single sample at the time point T0 and as well in this time we had lowest POV value. When it comes to control samples, during ripening time, we had the highest POV value in T1 and the lowest POV value in T3 in control samples. Besides in the time point T1, samples with 5% of molten chocolate had similar values to the control samples.

### *2.4.3.2. TBARS*

Thiobarbituric reactive substances (TBARS), which are secondary lipid oxidation products, were quantified using the thiobarbituric acid method (TBARS). Table 2 reports the TBARs analysis of the samples during ripening time. In this study, TBARs content ranged from 0.006 to 0.027 (mg MDA $kg^{-1}$ cheese). The highest value was detected in cheese samples including 15% chocolate in fresh and also during ripening time. Control samples were the lowest value in all time points.

Our observation defined that TBARS decrease over the ripening period. The lower content of PUFA probably induced a decrease of TBARS.

The TBARs content in all cheeses was lower than that reported for other kind of cheese (Kristensen et al., 2001; Severini et al., 1998) and lower than 2, the value that is proposed as threshold to consider oxidized a fatty food (Hamilton and Rossel, 1986).

As an oxidative marker, the TBAR level may be important in cheese because lipid oxidation leads to the formation of various by-products that may result in flavor defects, loss of nutritional quality and food safety concerns (Botsoglou et al., 1994; Fox et al., 2000).

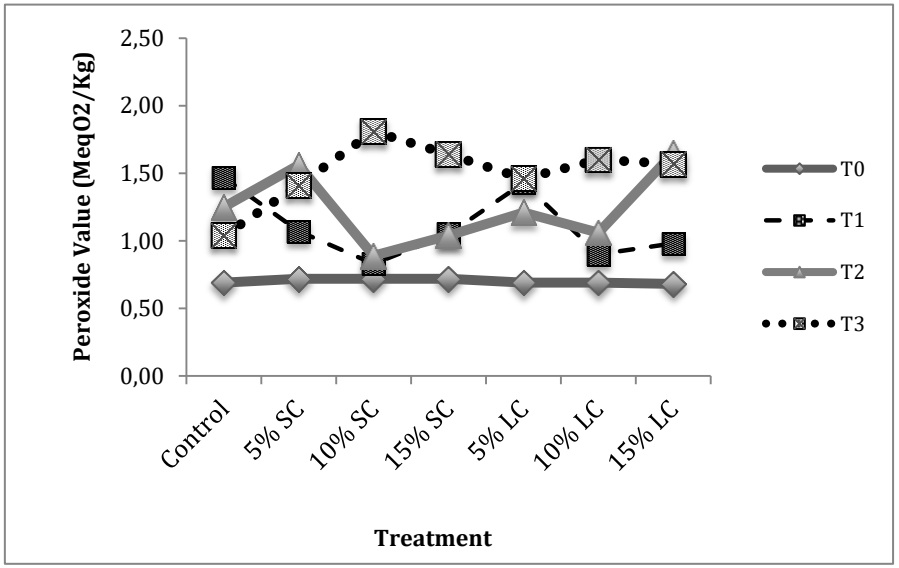



**Fig 2.** Peroxide value (meq O2 kg-1 fat) in cheese with chocolate during ripening time.

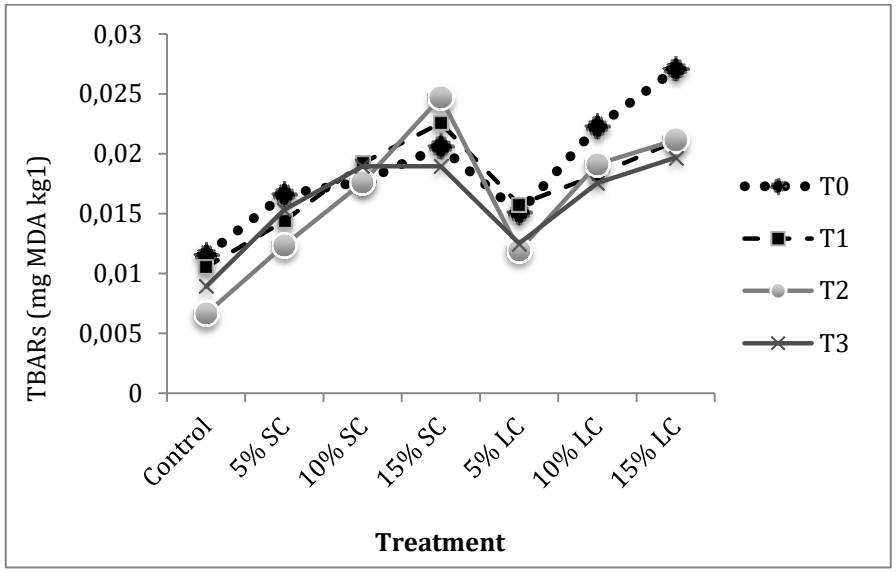



**Fig 3.** TBARS (MDA mg/kg cheese) in different treatments during ripening time.

**Table 2.** Average value of lipid oxidative markers in Pecorino cheeses with chocolate after 42 days of ripening.

| Treatment | TRT | | | | | | | SEM | Time | | | | SEM | Significant (p<) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | | T0 | T1 | T2 | T3 | | TRT | Time | T*T |
| **POV (meq O2 kg$^{-1}$ fat)** | 1.11 | 1.19 | 1.06 | 1.11 | 1.20 | 1.06 | 1.22 | 0.2378 | 0.70 | 1.10 | 1.24 | 1.50 | 0.1798 | <.0001 | <.0001 | 0.9995 |
| **TBARs (MDA mg/kg cheese)** | 0.009 | 0.015 | 0.018 | 0.022 | 0.014 | 0.019 | 0.022 | 0.0008 | 0.019 | 0.017 | 0.016 | 0.016 | 0.0006 | <.0001 | 0.0111 | 0.1631 |
| **TBARs (MDA mg/100 g fat)** | 0.004 | 0.006 | 0.008 | 0.010 | 0.006 | 0.009 | 0.010 | 0.0005 | 0.008 | 0.008 | 0.007 | 0.008 | 0.0003 | <.0001 | 0.0339 | 0.6405 |

Abbreviation: TRT: Treatment, CTL: Control sample, SC: Solid Chocolate, MC: Molten Chocolate, T0: fresh, T1: two weeks, T2: four weeks, T3: six weeks.

### 2.4.4. Analysis of cheese fatty acids

Tables 4, 5 and 6 report the effect of ripening time and different treatments on cheese fatty acid composition. In all samples 43 fatty acids were detected that can be depicted in tables mentioned. The most abundant fatty acids were saturated fatty acid (SFA, 70%), followed by monounsaturated fatty acid (MUFA, 22%) and finally, the least abundant were polyunsaturated fatty acids (PUFA, 8%). In comparison to plant oils, milk fat contains a low amount of polyunsaturated fatty acids (PUFA) (Fox, 2000) and monounsaturated fatty acids (MUFAs) (Smith et al., 1978).

Individually, C16:0 (palmitic acid) was the most abundant molecule (23%), followed by C18:1 (oleic acid) and C14:0 (myristic) that were approximately 15% and 11% respectively. Other fatty acids were represented with a share less than 10 %. According to Addis et al. (2005) the most abundant FAs in Pecorino cheese are palmitic acid (C16:0. 31%), oleic acid (C18:1, 23%), myristic acid (C14:0, 13%), stearic acid (C18:0, 10%) and lauric acid (C12:0, 6%). To aid the understanding of results, an ANOVA was carried out. The highest content of SFA and unsaturated fatty acid is due to the palmitic acid, which accounts for 23.34 % in cheese containing 15% solid chocolate and oleic acid, which accounts for 16.10% also in cheese with 15% solid chocolate, respectively.

It is also recognized that cheeses from cattle, goats and sheep on green forage have greater amounts of unsaturated long-chain fatty acids and lesser amounts of saturated short-chain fatty acids (Brun Bellut et al., 1985; Colombari and Zapparoli, 1989; Henninger and Ulberth, 1994; Innocente et al., 2002; Kelly et al., 1998; Wolff et al., 1995; Zegarska et al., 1996).

Fatty acid chains differ by length, often categorized as short to very long. Short-chain fatty acids (SCFA) are fatty acids with aliphatic tails of five or fewer carbons (e.g, butyric acid). Medium-chain fatty acids (MCFA) are fatty acids with aliphatic tails of 6 to 12 carbons, which can form medium-chain triglycerides. Long-chain fatty acids (LCFA) are fatty acids with aliphatic tails of 13 to 21 carbons. Very long chain fatty acids (VLCFA) are fatty acids with aliphatic tails of 22 or more carbons. In this study, Butyric acid (C4:0) is the most prevalent of the short-chain fatty acids and often identified with butter due to its characteristic impact on flavor (Morrison, 2004).

Butyric acid is generally a major aroma compound with cheesy sharp aroma and plays an important role in the flavor of many cheese types as Camembert, Cheddar, Grana Padano Pecorino, Ragusano and Roncal cheese (Curioni and Bosset, 2002).

Generally, as we raised the amount of chocolate in cheese, the C4:0 of samples was significantly decreasing because there is no butyric acid in chocolate, considering butyric has lower concentration than caproic acid. Previous works in Italian grana cheeses reported a role as key odorants for butyric and caproic acids (Moio et al., 1998). According to McSweeney and Sousa (2000) butyric acid has a rancid taste and it is mainly formed by lipolysis.

As was predictable by adding the amount of chocolate caproic, caprylic, capric, lauric and myristic acids roughly decreases regularly because there is no caprylic acid in chocolate.

In general, since chocolate does not contain SCFA and MCFA, when we add chocolate to cheese, we gain the final product with less amount of the entitled.

Among LCFAs in particular, our results elucidated four main fatty acids namely; palmitic acid > oleic acid> stearic acid > linoleic acid to be significantly increased in

our chocolate cheese products. There is however no change in the amount of these six LCFAs during the ripening period.

The intake of dietary fats through food is necessary for optimal functioning and balance of fats in the organism (Ribeiro et al., 2007; Ergnül et al., 2013). Unsaturated fatty acids are essential for our health, having a strong beneficial effect in the prevention and management of cardiovascular diseases, triglyceride level, blood pressure etc., whereas saturated fatty acids, which are present in higher amounts in food of animal origin, are associated with increased levels of triglycerides in the blood and commonly are associated with hypertension etc., (Simpoulos, 1999).

Coming back to our results, most importantly is the frequency of different fatty acids in accordance with saturation. In our products we have saturated and unsaturated (in mono and poly forms) fatty acids. In comparison with cheese, chocolate cheese had a higher amount of unsaturated fatty acids. Interestingly deep analysis showed that there is a raise in monounsaturated FA while polyunsaturated ones are reduced in the product with chocolate. Generally, the fatty acid composition of the samples changed during ripening. Among the saturated fatty acids, C16:0 and C18:0 fatty acid content slightly increased during ripening time.

**Table 3.** Average value of saturated fatty acids.

| Treatment | TRT | | | | | | | SEM | Time | | | | SEM | Significant (p<) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | | T0 | T1 | T2 | T3 | | TRT | Time | TRT*Time |
| **C4:0** | 2.606 | 2.575 | 2.381 | 2.176 | 2.477 | 2.385 | 2.310 | 0.0482 | 2.50 | 2.40 | 2.43 | 2.32 | 0.0364 | <.0001 | 0.0095 | 0.2365 |
| **C6:0** | 3.037 | 2.981 | 2.787 | 2.585 | 2.887 | 2.784 | 2.665 | 0.0418 | 2.89 | 2.81 | 2.82 | 2.75 | 0.0316 | <.0001 | 0.0315 | 0.3842 |
| **C7:0** | 0.064 | 0.064 | 0.060 | 0.058 | 0.061 | 0.062 | 0.059 | 0.0010 | 0.06 | 0.06 | 0.06 | 0.06 | 0.0008 | <.0001 | 0.0173 | 0.7113 |
| **C8:0** | 3.207 | 3.144 | 2.969 | 2.792 | 3.059 | 2.943 | 2.823 | 0.0329 | 3.04 | 2.98 | 2.99 | 2.95 | 0.0249 | <.0001 | 0.1394 | 0.6245 |
| **C9:0** | 0.100 | 0.097 | 0.093 | 0.087 | 0.096 | 0.093 | 0.088 | 0.0011 | 0.09 | 0.09 | 0.09 | 0.09 | 0.0008 | <.0001 | 0.0314 | 0.6717 |
| **C10:0** | 9.658 | 9.414 | 8.921 | 8.407 | 9.187 | 8.835 | 8.482 | 0.0856 | 9.09 | 9.00 | 8.98 | 8.87 | 0.0647 | <.0001 | 0.1306 | 0.6473 |
| **C11:0** | 0.139 | 0.136 | 0.129 | 0.121 | 0.132 | 0.127 | 0.123 | 0.0016 | 0.13 | 0.13 | 0.13 | 0.13 | 0.0012 | <.0001 | 0.0052 | 0.8299 |
| **C12:0** | 5.291 | 5.140 | 4.900 | 4.620 | 5.048 | 4.848 | 4.660 | 0.0407 | 4.97 | 4.95 | 4.93 | 4.87 | 0.0308 | <.0001 | 0.1618 | 0.7257 |
| **C13:0** | 0.207 | 0.201 | 0.193 | 0.182 | 0.199 | 0.191 | 0.184 | 0.0020 | 0.20 | 0.19 | 0.19 | 0.19 | 0.0015 | <.0001 | 0.2871 | 0.5114 |
| **C14:0** | 11.489 | 11.085 | 10.639 | 10.041 | 10.980 | 10.511 | 10.129 | 0.0740 | 10.76 | 10.66 | 10.73 | 10.64 | 0.0559 | <.0001 | 0.3853 | 0.7586 |
| **C15:0** | 1.164 | 1.120 | 1.080 | 1.023 | 1.116 | 1.067 | 1.028 | 0.0103 | 1.10 | 1.05 | 1.10 | 1.09 | 0.0078 | <.0001 | 0.0003 | 0.9967 |
| **C16:0** | 22.678 | 22.820 | 23.055 | 23.334 | 22.928 | 23.075 | 23.263 | 0.0554 | 22.98 | 23.01 | 23.01 | 23.08 | 0.0419 | <.0001 | 0.3601 | 0.4921 |
| **C17:0** | 0.712 | 0.663 | 0.675 | 0.648 | 0.665 | 0.669 | 0.655 | 0.0104 | 0.67 | 0.64 | 0.68 | 0.68 | 0.0078 | 0.0019 | 0.0002 | 0.3545 |
| **C18:0** | 6.775 | 7.873 | 8.958 | 10.508 | 8.069 | 9.258 | 10.197 | 0.1789 | 8.74 | 8.76 | 8.76 | 8.97 | 0.1352 | <.0001 | 0.5847 | 0.5729 |
| **C19:0** | 0.169 | 0.156 | 0.161 | 0.164 | 0.150 | 0.160 | 0.157 | 0.0079 | 0.15 | 0.19 | 0.15 | 0.15 | 0.0060 | 0.7356 | <.0001 | 0.4939 |
| **C20:0** | 0.234 | 0.265 | 0.296 | 0.340 | 0.272 | 0.305 | 0.333 | 0.0052 | 0.29 | 0.29 | 0.29 | 0.30 | 0.0039 | <.0001 | 0.2079 | 0.661 |
| **C22:0** | 0.121 | 0.117 | 0.112 | 0.107 | 0.116 | 0.112 | 0.107 | 0.0039 | 0.11 | 0.13 | 0.11 | 0.11 | 0.0029 | 0.1218 | 0.0003 | 0.9999 |

Abbreviation: TRT: Treatment, CTL: Control sample, SC: Solid Chocolate, MC: Molten Chocolate, T0: fresh, T1: two weeks, T2: four weeks, T3: six weeks

**Table 4.** Average value of unsaturated fatty acids.

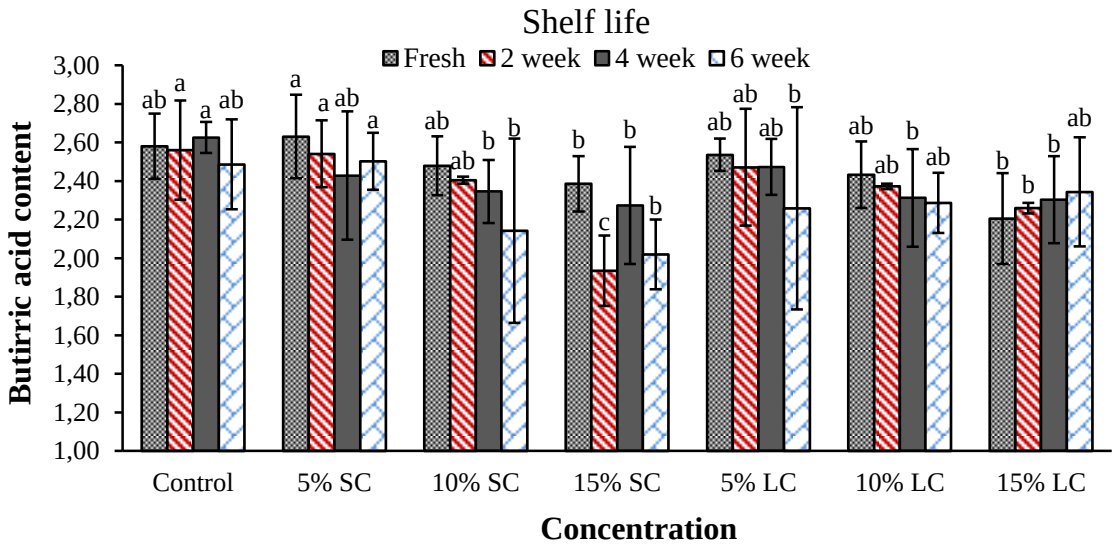
| Treatment | TRT | | | | | | | SEM | Time | | | | SEM | Significant (p<) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | | T0 | T1 | T2 | T3 | | TRT | Time | TRT*Time |
| **C10: 1** | 0.295 | 0.290 | 0.273 | 0.256 | 0.281 | 0.270 | 0.258 | 0.0031 | 0.28 | 0.27 | 0.28 | 0.27 | 0.0023 | <.0001 | 0.1699 | 0.7825 |
| **C13anteiso** | 0.046 | 0.047 | 0.044 | 0.031 | 0.047 | 0.037 | 0.040 | 0.0032 | 0.04 | 0.05 | 0.04 | 0.04 | 0.0024 | 0.0047 | 0.0859 | 0.265 |
| **C14iso** | 0.119 | 0.115 | 0.111 | 0.106 | 0.114 | 0.108 | 0.105 | 0.0023 | 0.11 | 0.10 | 0.11 | 0.11 | 0.0018 | 0.0006 | <.0001 | 0.9995 |
| **C15iso** | 0.209 | 0.203 | 0.193 | 0.186 | 0.202 | 0.190 | 0.187 | 0.0028 | 0.20 | 0.19 | 0.20 | 0.20 | 0.0021 | <.0001 | <.0001 | 0.9966 |
| **C15anteiso** | 0.502 | 0.482 | 0.462 | 0.438 | 0.480 | 0.458 | 0.442 | 0.0050 | 0.47 | 0.45 | 0.47 | 0.47 | 0.0038 | <.0001 | 0.0004 | 0.9948 |
| **C14: 1c9** | 0.207 | 0.198 | 0.190 | 0.179 | 0.196 | 0.188 | 0.183 | 0.0016 | 0.19 | 0.19 | 0.19 | 0.19 | 0.0012 | <.0001 | 0.9494 | 0.777 |
| **C16iso** | 0.367 | 0.351 | 0.348 | 0.316 | 0.348 | 0.328 | 0.318 | 0.0054 | 0.34 | 0.32 | 0.34 | 0.35 | 0.0041 | <.0001 | 0.0004 | 0.93 |
| **C17iso** | 0.726 | 0.692 | 0.674 | 0.631 | 0.691 | 0.656 | 0.639 | 0.0066 | 0.67 | 0.68 | 0.67 | 0.67 | 0.0050 | <.0001 | 0.4866 | 0.4053 |
| **C17anteiso** | 0.313 | 0.306 | 0.303 | 0.277 | 0.300 | 0.293 | 0.282 | 0.0067 | 0.29 | 0.30 | 0.30 | 0.29 | 0.0050 | 0.0027 | 0.2349 | 0.5175 |
| **C16: 1** | 1.121 | 1.082 | 1.059 | 1.014 | 1.084 | 1.044 | 1.017 | 0.0064 | 1.06 | 1.05 | 1.07 | 1.06 | 0.0048 | <.0001 | 0.0509 | 0.9352 |
| **C18: iso** | 0.070 | 0.070 | 0.071 | 0.065 | 0.071 | 0.065 | 0.062 | 0.0032 | 0.07 | 0.06 | 0.07 | 0.07 | 0.0024 | 0.2694 | 0.0156 | 0.9 |
| **C17: 1** | 0.193 | 0.183 | 0.180 | 0.170 | 0.186 | 0.177 | 0.176 | 0.0022 | 0.18 | 0.17 | 0.18 | 0.18 | 0.0017 | <.0001 | 0.0011 | 0.8342 |
| **C18: 1t11VA** | 4.357 | 4.128 | 3.995 | 3.809 | 4.213 | 3.947 | 3.851 | 0.0615 | 3.95 | 4.20 | 4.02 | 4.00 | 0.0465 | <.0001 | 0.0021 | 0.99 |
| **TOTC181CIS** | 2.490 | 2.344 | 2.290 | 2.157 | 2.362 | 2.295 | 3.377 | 0.4414 | 2.28 | 2.34 | 2.30 | 2.98 | 0.3336 | 0.5256 | 0.3932 | 0.4973 |
| **C18: 1c9** | 13.539 | 14.219 | 15.026 | 16.101 | 14.428 | 15.212 | 14.679 | 0.4833 | 14.88 | 14.67 | 14.96 | 14.47 | 0.3653 | 0.018 | 0.7848 | 0.4102 |
| **TOTC182n6NOCLA** | 2.415 | 2.195 | 2.158 | 1.984 | 2.247 | 2.150 | 2.022 | 0.0462 | 2.11 | 2.26 | 2.14 | 2.16 | 0.0349 | <.0001 | 0.0152 | 0.614 |
| **C18: 2LA** | 2.072 | 2.081 | 2.146 | 2.198 | 2.113 | 2.130 | 2.184 | 0.0143 | 2.12 | 2.12 | 2.13 | 2.16 | 0.0108 | <.0001 | 0.06 | 0.1726 |
| **C18: 3n6GAMMA** | 0.021 | 0.013 | 0.012 | 0.006 | 0.024 | 0.016 | 0.139 | 0.0283 | 0.01 | 0.09 | 0.02 | 0.01 | 0.0214 | 0.0201 | 0.0213 | 0.0004 |
| **C18: 3n3ALA** | 1.118 | 1.075 | 1.057 | 1.007 | 1.082 | 1.041 | 0.884 | 0.0341 | 1.03 | 1.03 | 1.05 | 1.04 | 0.0258 | 0.0004 | 0.9584 | 0.0216 |
| **C18: 2c9t11CLARA** | 1.504 | 1.432 | 1.396 | 1.319 | 1.444 | 1.377 | 1.324 | 0.0231 | 1.37 | 1.46 | 1.39 | 1.39 | 0.0175 | <.0001 | 0.0026 | 0.9999 |
| **ALTRICLA** | 0.227 | 0.214 | 0.195 | 0.175 | 0.212 | 0.189 | 0.169 | 0.0112 | 0.18 | 0.22 | 0.20 | 0.20 | 0.0085 | 0.0039 | 0.0129 | 0.7714 |
| **C20: 2n6** | 0.044 | 0.035 | 0.030 | 0.026 | 0.046 | 0.025 | 0.031 | 0.0071 | 0.03 | 0.04 | 0.03 | 0.04 | 0.0053 | 0.2111 | 0.0389 | 0.4759 |
| **C20: 3n6** | 0.089 | 0.093 | 0.096 | 0.099 | 0.093 | 0.098 | 0.102 | 0.0018 | 0.10 | 0.09 | 0.10 | 0.10 | 0.0013 | <.0001 | 0.0637 | 0.9717 |
| **C20: 4n6 AA** | 0.114 | 0.114 | 0.106 | 0.099 | 0.111 | 0.107 | 0.103 | 0.0016 | 0.11 | 0.11 | 0.11 | 0.11 | 0.0012 | <.0001 | 0.077 | 0.7061 |
| **C20: 5n3EPA** | 0.070 | 0.069 | 0.065 | 0.055 | 0.067 | 0.064 | 0.062 | 0.0023 | 0.06 | 0.07 | 0.07 | 0.07 | 0.0018 | 0.0009 | 0.4306 | 0.6314 |
| **C22: 5n3DPA** | 0.120 | 0.116 | 0.108 | 0.105 | 0.116 | 0.111 | 0.103 | 0.0017 | 0.11 | 0.11 | 0.11 | 0.11 | 0.0013 | <.0001 | 0.0017 | 0.6919 |

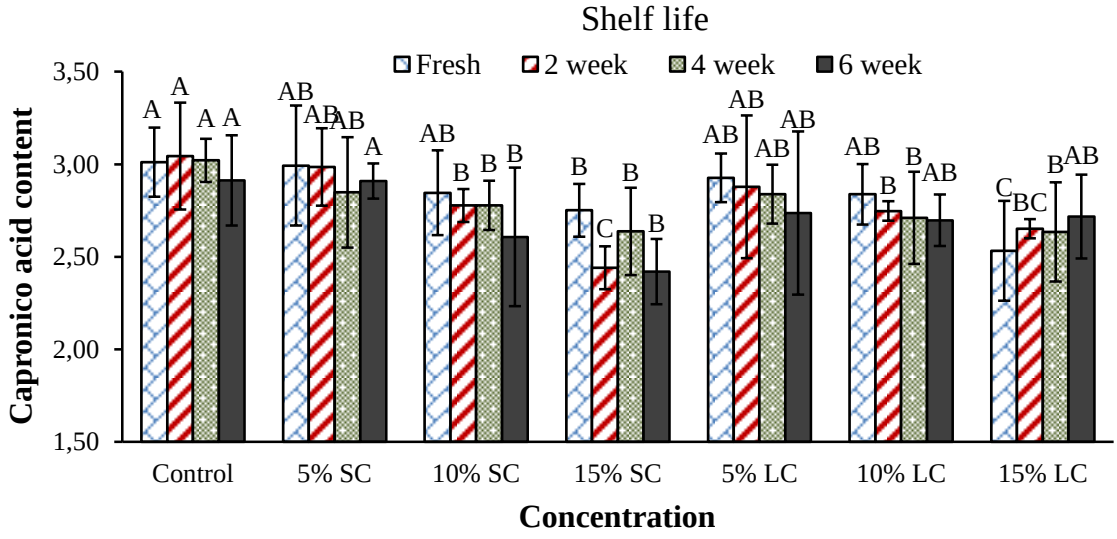
**Table 5.** Average and proportions of fatty acids.

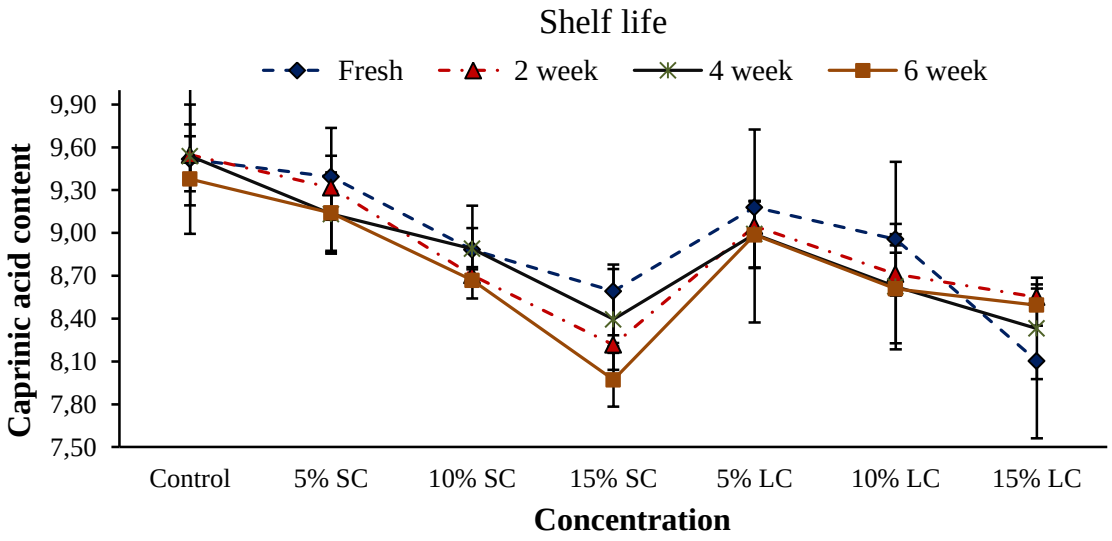
| Treatment | TRT | | | | | | | SEM | Time | | | | SEM | Significant (p<) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | | T0 | T1 | T2 | T3 | | TRT | Time | TRT*Time |
| **SFA** | 70.005 | 70.116 | 69.615 | 69.242 | 69.695 | 69.560 | 69.334 | 0.1341 | 69.96 | 69.50 | 69.69 | 69.46 | 0.1014 | 0.0001 | 0.0033 | 0.7738 |
| **Branched chain FA** | 2.352 | 2.265 | 2.206 | 2.049 | 2.253 | 2.134 | 2.075 | 0.0196 | 2.20 | 2.15 | 2.22 | 2.19 | 0.0148 | <.0001 | 0.0251 | 0.9374 |
| **MUFA** | 22.201 | 22.446 | 23.014 | 23.686 | 22.751 | 23.132 | 23.542 | 0.1049 | 22.83 | 22.90 | 22.99 | 23.15 | 0.0793 | <.0001 | 0.0319 | 0.4998 |
| **PUFA** | 7.795 | 7.438 | 7.371 | 7.072 | 7.555 | 7.308 | 7.124 | 0.0942 | 7.21 | 7.60 | 7.33 | 7.39 | 0.0712 | <.0001 | 0.0033 | 0.9834 |
| **USFA** | 29.995 | 29.884 | 30.385 | 30.758 | 30.305 | 30.440 | 30.666 | 0.1341 | 30.04 | 30.50 | 30.31 | 30.54 | 0.1014 | 0.0001 | 0.0033 | 0.7738 |
| **USFA/SFA** | 0.429 | 0.426 | 0.437 | 0.444 | 0.435 | 0.438 | 0.442 | 0.0028 | 0.43 | 0.44 | 0.44 | 0.44 | 0.0021 | 0.0001 | 0.0034 | 0.7583 |
| **PUFA/SFA** | 0.111 | 0.106 | 0.106 | 0.102 | 0.108 | 0.105 | 0.103 | 0.0015 | 0.10 | 0.11 | 0.11 | 0.11 | 0.0011 | 0.0008 | 0.0027 | 0.9815 |
| **n-6** | 4.755 | 4.531 | 4.549 | 4.411 | 4.634 | 4.526 | 4.581 | 0.0531 | 4.46 | 4.72 | 4.52 | 4.58 | 0.0401 | 0.0023 | 0.0003 | 0.003 |
| **n-3** | 1.309 | 1.260 | 1.230 | 1.167 | 1.264 | 1.217 | 1.049 | 0.0343 | 1.20 | 1.20 | 1.23 | 1.22 | 0.0259 | <.0001 | 0.8776 | 0.0272 |
| **n-6/n-3** | 3.641 | 3.603 | 3.707 | 3.786 | 3.675 | 3.732 | 4.810 | 0.0466 | 3.72 | 3.70 | 3.70 | 3.76 | 0.5892 | 0.0085 | 0.0476 | 0.0006 |
| **LA/ALA** | 1.853 | 1.936 | 2.031 | 2.183 | 1.953 | 2.046 | 2.470 | 0.0413 | 2.05 | 2.06 | 2.04 | 2.07 | 0.0157 | | | |
| **Thrombogenic index** | 10.267 | 10.046 | 9.943 | 9.677 | 10.074 | 9.886 | 9.376 | 0.1127 | 9.84 | 9.93 | 9.90 | 9.91 | 0.0852 | <.0001 | 0.8743 | 0.275 |
| **Atherogenic index** | 2.616 | 2.561 | 2.449 | 2.329 | 2.510 | 2.424 | 2.346 | 0.0181 | 2.49 | 2.45 | 2.47 | 2.44 | 0.0137 | <.0001 | 0.0334 | 0.6902 |
| **HPI** | 0.382 | 0.391 | 0.409 | 0.430 | 0.399 | 0.413 | 0.426 | 0.0031 | 0.40 | 0.41 | 0.41 | 0.41 | 0.0023 | <.0001 | 0.0404 | 0.6019 |
| **HSFA** | 73.927 | 72.299 | 70.510 | 68.117 | 71.895 | 69.968 | 68.437 | 0.3097 | 70.97 | 70.58 | 70.87 | 70.52 | 0.2341 | <.0001 | 0.4487 | 0.8559 |
| **HH** | 0.499 | 0.521 | 0.550 | 0.586 | 0.529 | 0.556 | 0.540 | 0.0148 | 0.54 | 0.54 | 0.55 | 0.53 | 0.0112 | 0.0048 | 0.8438 | 0.4084 |

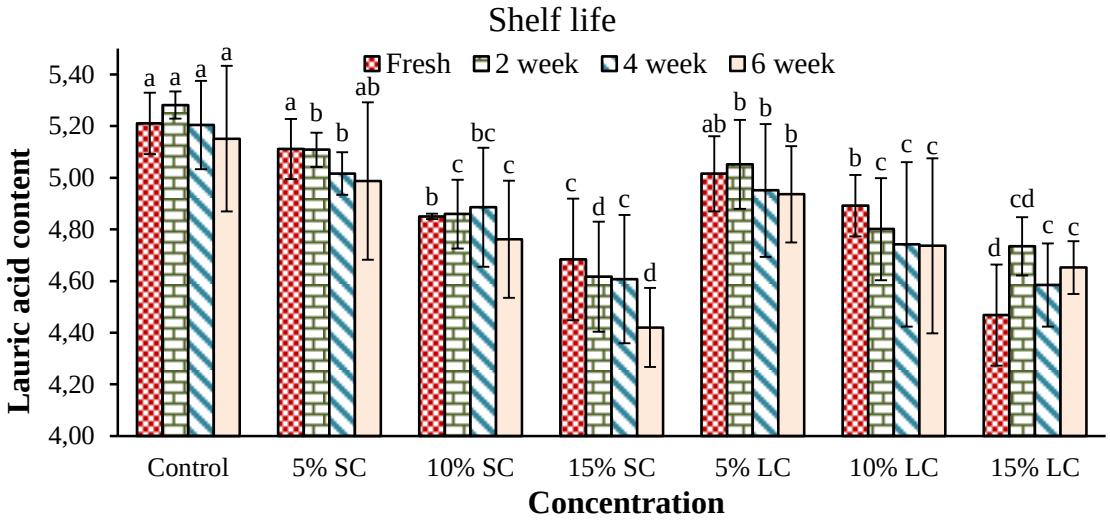
**Table 6.** Chocolate fatty acid composition

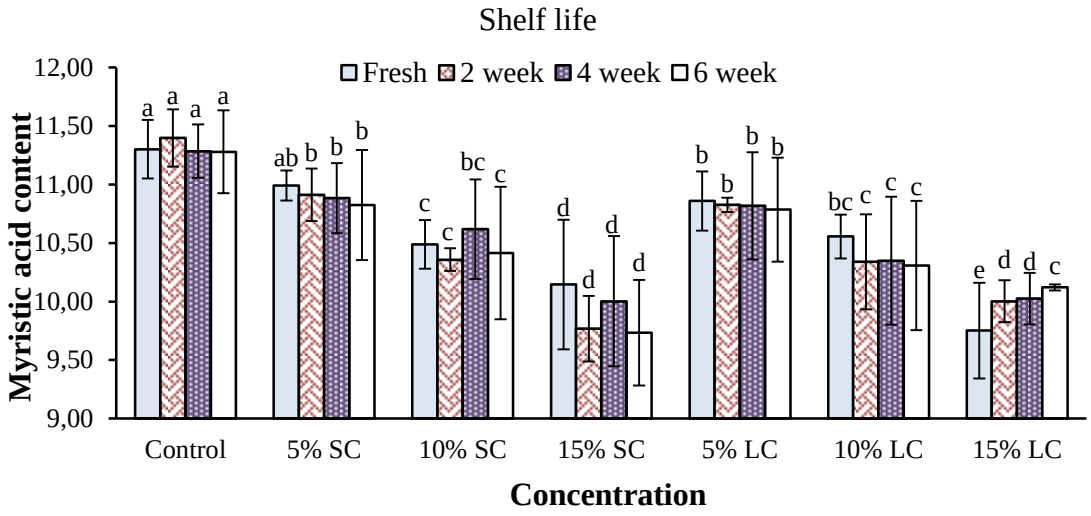
| Fatty acids | Chocolate |
|---|---|
| C12:0 | 0.082 |
| C14:0 | 0.127 |
| C16:0 | 26.813 |
| C16:1 | 0.242 |
| C17:0 | 0.233 |
| C18:0 | 34.649 |
| C18:1c9 | 32.138 |
| C18:1c11 | 0.474 |
| C18:2 LA | 2.954 |
| C20:0 | 1.087 |
| C18:3n3 ALA | 0.226 |
| C22:0 | 0.184 |
| Saturated FA | 63.180 |
| Unsaturated FA | 35.794 |

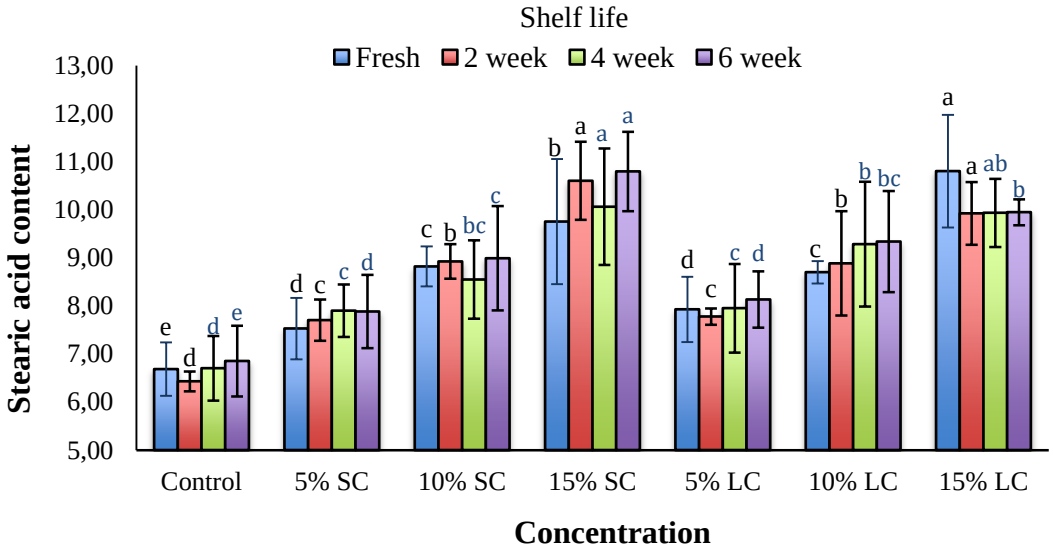

Shelf life
Fresh
2 week
4 week
6 week
Butirric acid content
3,00
2,80
2,60
2,40
2,20
2,00
1,80
1,60
1,40
1,20
1,00
Control
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
Concentration

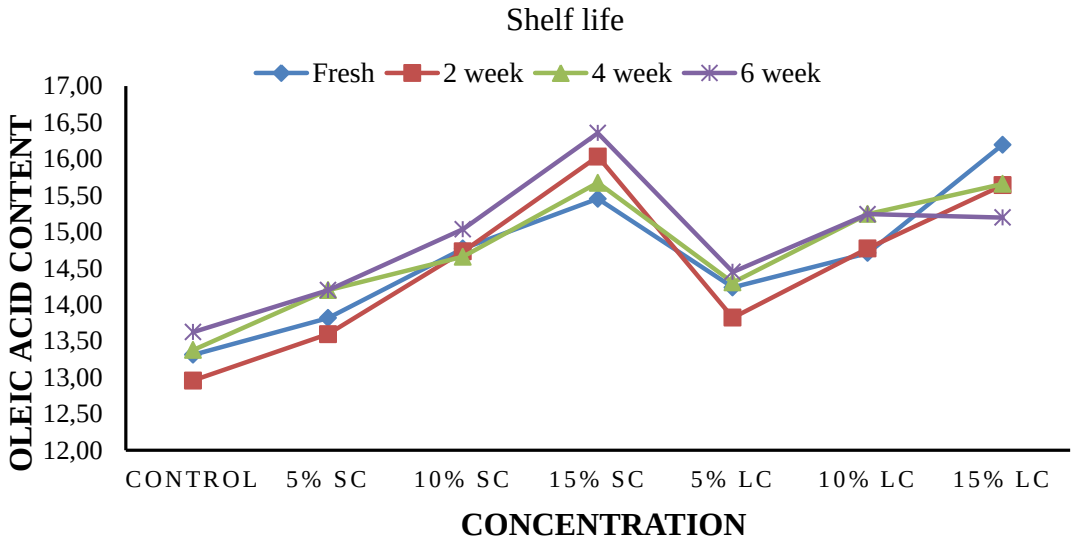

Shelf life
Fresh
2 week
4 week
6 week
Capronico acid content
3,50
3,00
2,50
2,00
1,50
Control
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
Concentration

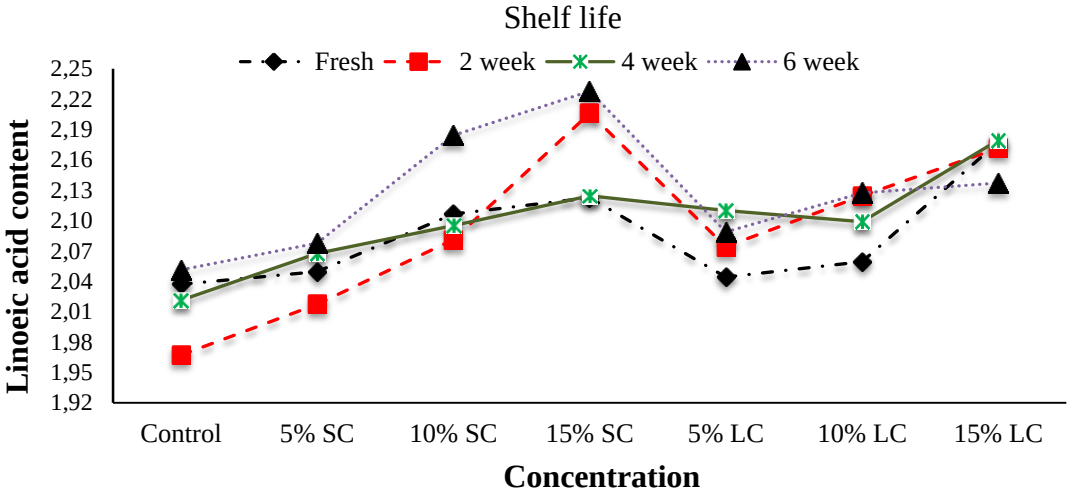

Shelf life
Fresh
2 week
4 week
6 week
Caprinic acid content
9,90
9,60
9,30
9,00
8,70
8,40
8,10
7,80
7,50
Control
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
Concentration


Shelf life
Fresh
2 week
4 week
6 week
Lauric acid content
5,40
5,20
5,00
4,80
4,60
4,40
4,20
4,00
Control
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
Concentration


Shelf life
Fresh
2 week
4 week
6 week
Myristic acid content
12,00
11,50
11,00
10,50
10,00
9,50
9,00
Control
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
Concentration


Shelf life
Fresh
2 week
4 week
6 week
Stearic acid content
13,00
12,00
11,00
10,00
9,00
8,00
7,00
6,00
5,00
Control
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
Concentration


Shelf life
Fresh
2 week
4 week
6 week
17,00
16,50
16,00
15,50
15,00
14,50
14,00
13,50
13,00
12,50
12,00
OLEIC ACID CONTENT
CONTROL
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
CONCENTRATION


Shelf life
Fresh
2 week
4 week
6 week
2,25
2,22
2,19
2,16
2,13
2,10
2,07
2,04
2,01
1,98
1,95
1,92
Linoeic acid content
Control
5% SC
10% SC
15% SC
5% LC
10% LC
15% LC
Concentration

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# 3. CHAPTER II

Elucidation of physicochemical, microbiological and sensorial characteristics of Pecorino chocolate cheese

## 3.1. ABSTRACT

The present work was carried out to evaluate the effect of addition of Modica chocolate to Pecorino Siciliano cheese on microbiological, textural and sensory properties. In this sudy we investigated chocolate cheese including solid and molten chocolate in three levels of 5%, 10% and 15% at four ripening times (T0 = fresh, T1 = 2 weeks, T2 = 4 weeks and T3 = 6 weeks). The results showed that mesophilic coccus LAB and total mesophilic count (TMC) were approximately at the same level (about $10^8$ cfu/g) and no statistically significant differences within cheese types were found during ripening whereas, remarkable differences among mesophilic rod LAB, Enterobacteria family, total yeast and fungi filamentous were observed. In this study, hardness ranged from 0.858 $N/mm^2$ to 1.229 $N/mm^2$. These results showed that there is a slight significant difference between control samples and chocolate cheese including solid chocolate, whereas a significant difference between chocolate cheese with molten chocolate and control sample was observed.

Sensorial analysis performed in two methods, hedonic and descriptive analysis. The results of sensory evaluation test showed that chocolate cheese including 5% chocolate after 14 days ripening time are the most proper product and this product was highly acceptable for the consumers.

## 3.2. INTRODUCTION

Cheese classification based on raw materials and microbial inocula includes six categories (Mucchetti and Neviani, 2006): pasteurized milk and selected starters; pasteurized milk and natural starters; thermal treated milk and natural starters; raw milk and selected starters; raw milk and natural starters; raw milk without starters. From a hygienic perspective, the latter cheese category is the one that deserves major attention, since final cheeses can become contaminated by pathogenic microorganisms as a result of their presence in raw milk and their subsequent survival during the cheese making process (Donnelly, 2004). Regarding pathogenic bacteria, the factors that mainly contribute to the safety of cheese are milk quality, starter cultures or native lactic acid bacteria (LAB), pH, salt, control of ripening conditions and chemical changes that occur in cheese during ripening (Johnson et al., 1990). Cheese cannot be made without the action of certain species of LAB (Parente and Cogan, 2004). Thus, cheese production performed with raw milk without starter addition relies on the presence of indigenous LAB in milk and/or those transferred by the equipment used for the processing and from the environment. However, this may also determine a great variability of the final characteristics of the cheese that cannot be easily controlled by the cheese maker (Franciosi, 2008). Considering that the microbiology of the cheeses produced with raw milk without starters can be unpredictable, the addition of selected LAB may drive the fermentation process in an appropriate direction (Caplice and Fitzgerald, 1999). The occurrence of bacterial population, especially of lactic acid bacteria (LAB), during manufacture and ripening of most cheese varieties is already well

documented (Beresford et al., 2001; Wouters et al., 2002). The origin of microorganisms may vary, entering from milk and/or with other ingredients used in cheese making, or adventitiously from the environment, and LAB are considered the microorganisms mainly involved in flavor formation of cheese variety (Fox et al., 1996). Several studies have demonstrated the occurrence of LAB species in several Italian cheeses like Canestrato Pugliese (Aquilanti et al., 2006), Parmigiano Reggiano (Gala et al., 2008), Pecorino (De Angelis et al., 2001; Randazzo et al., 2006, 2008), Ragusano (Randazzo et al., 2002), Raschera and Castelmagno (Dolci et al., 2008a,b), Provola dei Nebrodi (Cronin et al., 2007), Fontina (Giannino et al., 2009); in several Spanish artisanal starter-free cheese types (Oneca et al., 2003; Sánchez et al., 2006; Abriouel et al., 2008; Martín- Platero et al., 2008), and in French cheeses (Duthoit et al., 2003; Callon et al., 2004). Some other microbial groups, such as Enterobacteriaceae, have been reported in different Mediterranean cheeses made from ewes' raw milk and subjected to a short time of ripening, (Freitas, 2000; ,Macedo, 2004). The presence of Enterobacteriaceae is supposed to influence the final characteristics of cheese (Chatelain, 2003).

Activity of microorganisms during the ripening process can also influence the nutritive value of cheeses. For example, research results have shown that the fermentation activity of lactic acid bacteria (LAB) present in milk increases the share of short-chain fatty acids (SCFAs) and MCFAs found in the final product (Slačanac et al., 2005).

Pecorino is a common name given to indicate Italian cheeses made exclusively from pure ewes' milk characterized by a high content of fat matter. However, fat content and FA profiles are greatly influenced by the pastures and seasons (Carta et al., 2008; Meluchová et

al., 2008). This type of cheese, having in most cases a Protected Denomination of Origin (PDO) status, is produced particularly in the middle and south of Italy by a traditional procedure from raw or thermized milk, as extra-hard varieties, with a ripening time ranging between 8 and 12 months (Di Cagno et al., 2003). Compositional and microbiological characteristics of Pecorino cheeses produced in different Italian geographical areas such as Pecorino Sardo (Cosentino et al., 2001; Manca et al., 2001; Mannu and Paba, 2002), Pecorino Siciliano (Randazzo et al., 2006), Pecorino del Salento (Cappello et al., 2001), Pecorino Crotonese (Gardini et al., 2006), Pecorino abruzzese (Chaves- Lopez et al., 2006), Pecorino Romano, Pecorino Toscano, Pecorino Umbro (De Angelis et al., 2001) and Pecorino marchigiano (Aquilanti et al., 2007). On the other hand, several typologies of Pecorino without PDO are produced in Italy, also characterized by a shorter ripening time (20– 40 days), semi-hard consistency, but low flavor and aroma (Caridi et al., 2003). For this reason, a differentiation as well as the reduction of the ripening time can positively influence the consumption of this product. More specifically, traditional products can take advantage from variants form specific attributes.

Pecorino Siciliano (PS) is a typical Sicilian hard cheese, produced in Sicily according to ancient manufacturing techniques. It is probably the oldest European cheese.

On the other hand, the Modica Chocolate is one of the best-appreciated products of the Sicilian pastry. Latest studies have shown that chocolate was not only a simple blend of fat and sugar, but also a rich source of flavonoids and polyphenols which shows high antioxidant activities (Erdem et al., 2014). Furthermore, these non-nutrient bioactive compounds have potential health benefits including reduced risk of cardiovascular disease

and prebiotic activity (Hu et al., 2016).

Texture analysis is primarily concerned with measurement of the mechanical properties of products, often a food product, as they relate to its sensory properties detected by humans via applying controlled forces to the product and recording its response in the form of force, deformation and time. Texture measurements can be very valuable for the quality control and process optimization as well as for the development of new products with desirable properties and characteristics (Abdel Satar et al, 2015).

Cheese is a viscoelastic material that is formed by a continuous network of casein in which fat globules and water are interspersed (Prentice 1987). Raw milk is generally used to produce extra-hard cheeses that are ripened for a long period.

Sensory and described properties are some of the most important factors on consumer attention and preference. Therefore, these properties are very important to determine factors affecting the acceptance of product especially for foods (Dos et al., 2005). Sensory evaluation is frequently utilized in the stages of formulation of new products and improvement of available products (Royer 2006).

Descriptive Sensory Analysis has been widely used to characterize in detail aroma, flavor, and oral texture attributes of food products. All descriptive analysis methods involve the objective detection, description and quantification of sensory attributes of a product by trained panelists (Meilgaard et al., 1999). The objective of descriptive methods is to characterize the sensory properties of a product, by panelists that evaluate the samples quantitatively and qualitatively (Murray et al., 2001). With PCA, a sensory space was created where samples were positioned in the attribute-sample space according to their

characteristic sensory attributes. The distance between a sample and an attribute indicated the extent to which the attribute can be used to describe such sample. Individual observations for product attributes were used to perform PCA. If two variables had high loadings along the same PC, it meant that the two variables were highly correlated. If both loadings had the same sign, the correlation was positive (when one variable increased, so did the other). Otherwise, it was negative (when one variable increased, the other decreased) (Pavon, 2003).

The aim of this study is to characterize the physicochemical, microbiological and sensorial properties of a new product called “Pecorino chocolate cheese”.

## 3.3. MATERIALS AND METHODS

### 3.3.1. Texture analysis

The resistance to compression was performed on Instron 5564 tester (Instron, Trezzano sul Naviglio, Milano, Italy). The samples were placed on the table of the texture analyzer at 20 ℃. The hardness ($N/mm^2$) is the maximum resistance to compression.

### 3.3.2. Microbiology

Microbiological analyses were carried out to evaluate the main microbial groups associated with cheese production and investigated for quality, hygiene, and safety aspects. Samples (15 g) were first homogenized in 135 ml of sodium citrate (2% [wt/vol]) solution in a stomacher (Bag- Mixer 400; Interscience, Saint Nom, France) for 2 min at the highest speed and then serially diluted. The inoculation, cultivation, and incubation of the different microbial groups were as follows. Total mesophilic microorganisms were spread plated on plate count agar (PCA) supplemented with 1 g/liter skimmed milk and incubated aerobically at 30°C for 72 h. Mesophilic coccus LAB were pour plated on M17 agar and incubated anaerobically at 30 °C for 48 h. Mesophilic rod LAB on Man Rogosa Sharpe (MRS) agar acidified at pH 5.4 with lactic acid (5 mol/L) and incubated anaerobically for 48 h at 30 °C; Members of the Enterobacteriaceae family were pour plated on double-layered violet red bile glucose agar (VRBGA) and incubated aerobically at 37°C for 24 h. Total yeast were spread

plated on YPDA (yeast peptone dextrose agar) and incubated aerobically at 30 °C for 48 h; fungi filamentous on potato dextrose agar (PDA) supplemented with chloramphenicol at 0.1% incubated aerobically at 25 °C for 7 d. Microbiological counts were carried out in duplicate. All media were purchased from Oxoid.

### 3.3.3. Sensory analysis

#### *3.3.3.1. Descriptive analysis*

Descriptive sensory analysis was carried out following the ISO (2003) indications. Seven panelists were selected by standard methods as assessors. They were informed that for all panel session, approximately 10 g of each sample were placed in randomly coded disposable container with a 3-digit number. All samples were left at ambient temperature (about 20 °C) for 30 min before administration. The panelists evaluated fourteen descriptors regarding the aspect (color and uniformity of structure), the smell (strength of odor, milk, butter and unpleasant smell), the taste (salty, sweet, acid, spicy and bitter taste) and the consistency (soft/hard, solubility and grittiness following mastication). In order to eliminate the taste from the previous sample, the evaluators were required to drink some water. The seven selected panelists were trained and after tasting, each panelist rated the overall acceptability of the product. Quality was scored using a line scale anchored on the left (visual analogue scale) with dislike/low quality and on the right with like/high quality. The hedonic scale results were converted as distance (cm) of mark from the left end of the line.

#### *3.3.3.2. Hedonic test*

The overall acceptance of the produced cheese samples as well as control sample were evaluated using the 9-point hedonic scale (9: like extremely; 1: dislike extremely). The panelists evaluated five attribute, taste, texture, mouthfeel, overall appearance and overall liking.

#### *3.3.3.3. Principal Component Analysis*

PCA method is a useful technique to analyze results and many researchers used this method in analyzing results of sensory evaluation. The method decrease amount of main variables and create a smaller number of artificial variable (as main components), which contain the most variance of observed variables. The main components are linear combination of main variables that are ordered from the largest to the smallest value of variance. This variance value is known specific value that is described through each main component (de Melo et al., 2009). In this study, PCA method was used to analyze sensory-described parameters and finding the main agents. All statistical analyses were carriedi out using SPSS Software.

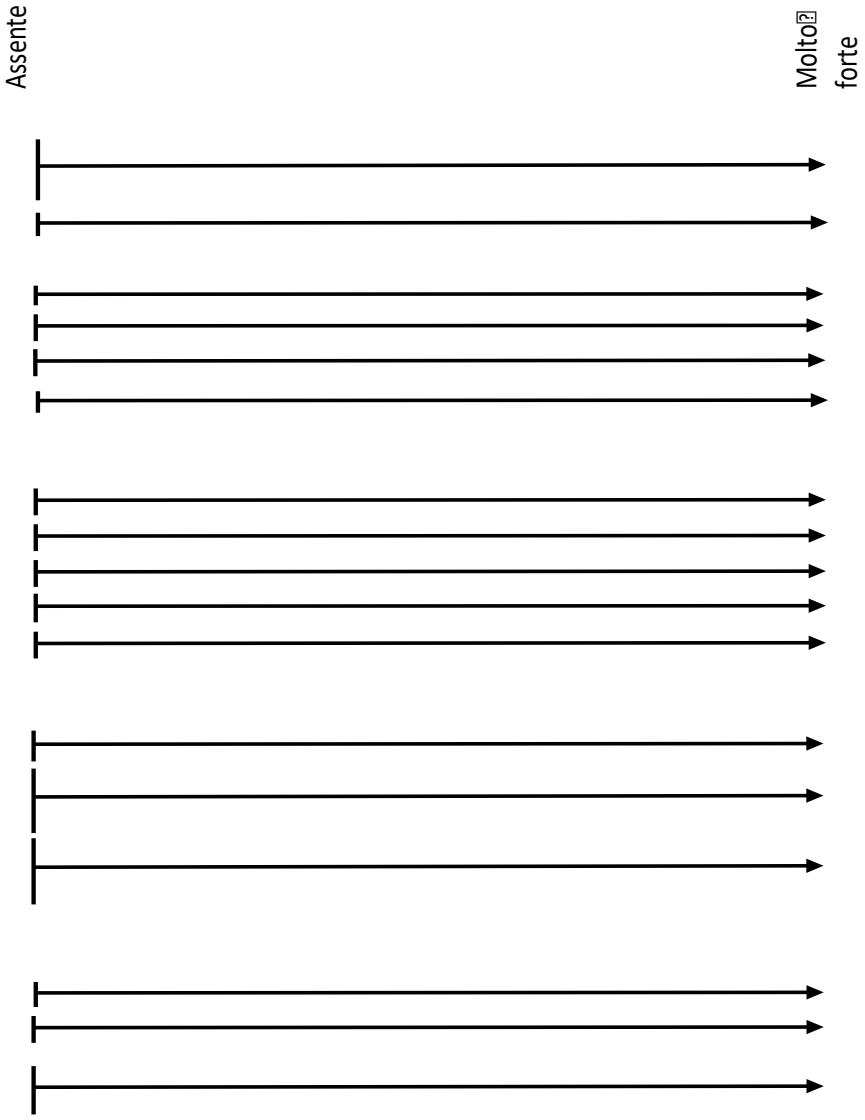
Età…… Data……

Nome…… N. Postazione……

Cognome…… Cod. Campione……

Gradimento formaggio nella dieta alimentare (da 1 a 10)……

**DESCRITTORI**




**Esprire comunque un giudizione per ciascun descrittore**

**Masticabilità:** Tempo necessario per masticare il campione pronto per la deglutizione

**Solubilità dopo masticazione:** dispesione nella bocca

**Granuli dopo masticazione:** presenza di granuli (come sabbia) nella bocca

**Record Sheet**
**Hedonic Rating Scale**

1. How would you rate the TASTE of this product?

☐ Like extremely
☐ Like very much
☐ Like moderately
☐ Like slightly
☐ Neither like nor dislike
☐ Dislike slightly
☐ Dislike moderately
☐ Dislike very much
☐ Dislike extremely

2. How would you rate the TEXTURE of this product?

☐ Like extremely
☐ Like very much
☐ Like moderately
☐ Like slightly
☐ Neither like nor dislike
☐ Dislike slightly
☐ Dislike moderately
☐ Dislike very much
☐ Dislike extremely

3. How would you rate the MOUTHFEEL of this product?

☐ Like extremely
☐ Like very much
☐ Like moderately
☐ Like slightly
☐ Neither like nor dislike
☐ Dislike slightly
☐ Dislike moderately
☐ Dislike very much
☐ Dislike extremely

4. How would you rate the OVERALL APPEARANCE / COLOR this product?

☐ Like extremely
☐ Like very much
☐ Like moderately
☐ Like slightly
☐ Neither like nor dislike
☐ Dislike slightly
☐ Dislike moderately
☐ Dislike very much
☐ Dislike extremely

5. How would you rate the OVERALL LIKING this product?

☐ Like extremely
☐ Like very much
☐ Like moderately
☐ Like slightly
☐ Neither like nor dislike
☐ Dislike slightly
☐ Dislike moderately
☐ Dislike very much
☐ Dislike extremely

### 3.3.4. Statistical analysis

Data were analysed statistically by the GLM procedures in SAS 9.2 software (2010). The GLM procedure was used for all parameters (texture and microbiology) except of sensory analysis with a model including the effects of cheese-making day (three levels) treatment (TR) (seven levels), ripening time (RT) (four times, T0, T1, T2 and T3) and the interaction TR*RT. Then Tukey test was performed in case of variances were equal to identify the significant differences at 95% confidence level.

# 3.4. RESULTS AND DISCOSION

## 3.4.1. Microbiological analyses

The mean of microbial counts and standard error of the mean (SEM) obtained by classical enumeration of bacterial population present in the samples in different formula and ripening time are shown in Table 6.

In comparison with other raw ewes' milk cheeses, our data showed lower levels of the unwanted bacteria, especially Enterobacteriaceae. A level in the range $10^5$–$10^6$ cfu/g is common for cheeses at around 2–3 months of ripening [Macedo, 2004; Prodromou, 2001; Tavaria, 2000) but the concentration of Enterbacteriaceae greatly decreases after that period (Dahl, 2000; Hatzikamari, 1999; Ortigosa, 2006). Our observation defined that concentration of Enterbacteriaceae ranged from $10^3$ to $10^4$ cfu/g in all samples and in samples containing chocolate significantly decreased. As well, over the ripening period, one log unit decrease from fresh samples to 42 days ripening time was observed. As a consequence, Enterobacteriaceae are not commonly detected in the final product due to their gradual decrease during cheese ripening (Medina et al., 1991; Hatzikamari et al., 1999; Dahl et al., 2000). However, high numbers of Enterobacteriaceae have been reported in different Mediterranean cheeses made from ewes' and goats' raw milk after 30 days of ripening (Nun˜ez and Martı´nez-Moreno 1976; Sa´nchez-Rey et al., 1993; Freitas et al., 1995,1996; Freitas and Malcata 2000; Psoni et al., 2003; Macedo et al., 2004). Although

large population of this microbial group may endanger the market of ripened cheese (Medina et al., 1991), the specific characteristics, such as taste, aroma and texture, of some artisanal cheeses have been correlated with Enterobacteriaceae counts (Dahl et al., 2000; Morales et al., 2004). In Pecorino Abruzzese, a traditional cheese produced in Central Italy, Enterobacteriaceae can be detected after 15 days of ripening at levels of $10^3$ and $10^5$ CFU $g^{-1}$ in spring and summer, respectively, and are still present (102 CFU $g^{-1}$) after 60 days (Serio et al., 2006). Chavez et al. (2006) also reported that many strains of Enterobacteriaceae isolated from Pecorino cheese proved to possess physiological features that could contribute to the sensory characterization of this product. The enzymes released by these microorganisms could influence cheese flavour and their action could also continue when cells are not viable. However, some of these gram-negative microorganisms are potential pathogens and their presence is of great concern in cheese making because of their public health significance.

TMC were detected in all samples reaching the maximum level (8.80 Log CFU/cm2) in the chocolate cheese with 10% solid chocolate and the lowest (8.52 Log CFU/cm2) in the control sample. All samples displayed the presence of both cocci and rods LAB. TMC were almost superimposable with the counts of coccus LAB. This data showed that the dominant microbial group of each cheese analysed was represented by coccus LAB.

Statistical differences were evidenced for mesophilic coccus LAB and fungi filamentous, which were more concentrated and less concentrated, respectively, in all treatments. No statistical differences were found for the other microbial groups with regard to the control samples with chocolate cheese samples (Table 7). Mesophilic LAB cocci clearly dominated

the microbial populations of all samples.

Yeast counts were ranged between 2.72 and 3.52 Log CFU/g. As we raised the amount of chocolate in cheese, the total yeast of samples was increasing but the observed changes were not statistically significant.

The microbiological results obtained in this work also showed that coccus LAB dominated any cheese samples analyzed, while the counts registered for rod LAB was slightly lower. This reduction in number could be due to the lower pH of MRS used in this study (final pH 5.4). This trend was observed in other similar Italian cheeses (Caridi et al., 2003) and the concentration levels of rod LAB was, on average, in the same order of magnitude of those reported by other authors (Gobbetti, 1999; Pisano, 2006). In a previous work carried out on the microbial ecology of Pecorino Siciliano cheese produced in winter and spring (Vemile, 2006) data, collected at latest at 90 days of ripening, showed that mesophilic rod and coccus LAB were approximately at the same level (about $10^7$ cfu/g). LAB cocci were one Log unit higher than LAB rods in all analyzed samples. To our interest, there were two unit of difference in fresh samples as though by the time passing and after 42 days both of these concentrations reach to a similar unit (about $10^8$ cfu/g).

Mesophilic LAB rod are important in the maturation of cheeses as they are able to ferment citrate and could be involved in proteolysis as well as in other enzymatic processes that occurred during cheese ripening (Crow et al., 2001).

With the exception of yeasts, whose levels were in the range of those known for similar aged products (Fadda, 2004; Pisano, 2006) but lower than those previously reported for

Pecorino Siciliano ripened for 3 months (Vemile, 2006), the other microbial populations, were detected at relatively high levels.

The results showed that mesophilic coccus LAB and TMC were approximately at the same level (about $10^8$ cfu/g ) and no statistically significant differences within cheese types were found during ripening whereas remarkable differences among mesophilic rod LAB, Enterobacteria family, total yeast and fungi filamentous were observed. Mesophilic rod LAB had an initial population of log 6.02 CFU $g^{-1}$ that increased by about 2 log cycle during ripening ($p < 0.001$). Enterobacteria and total yeast had an initial population of log 4 and 3 CFU $g^{-1}$ respectively, that both of them decreased by about 1 log cycle during ripening ($p < 0.001$) and finally the greatly increased obtained in fungi filamentous that statistically decreased from $10^3$ cfu/g to $10^1$ cfu/g.

**Fig 4. PCA culture**

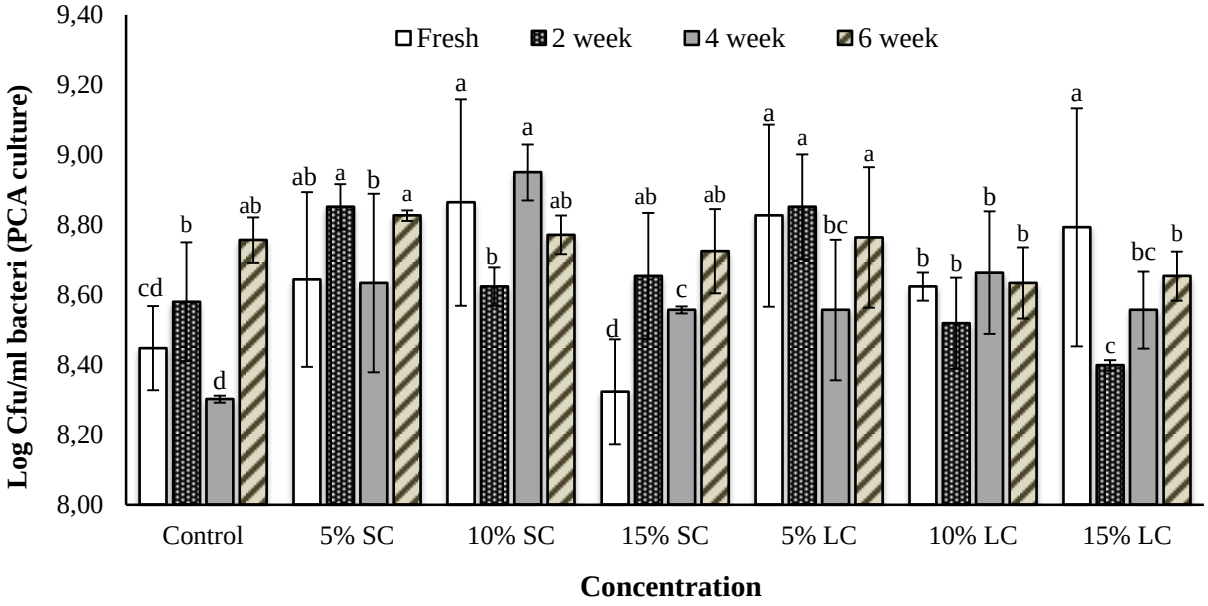



**Fig 5. M17 culture**

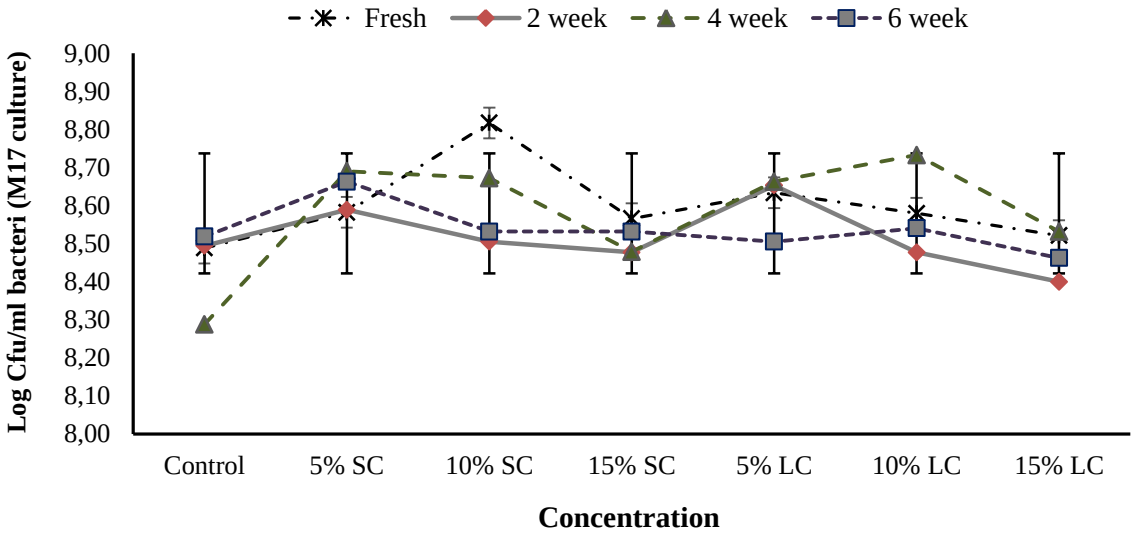


**Fig 6. MRS culture**

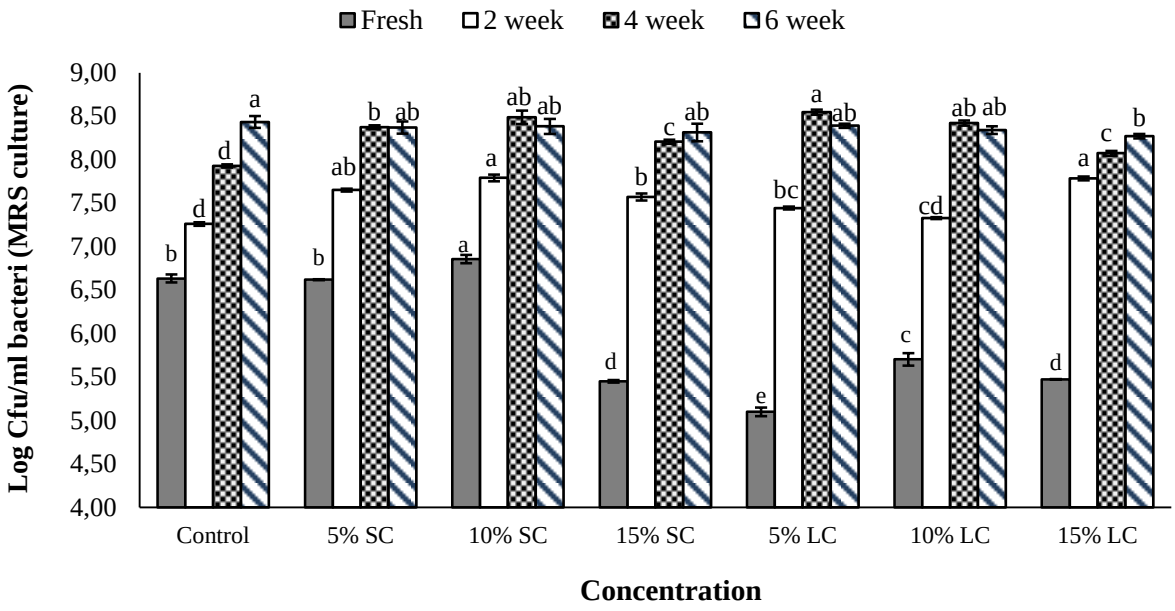



**Fig 7. VRBGA culture**

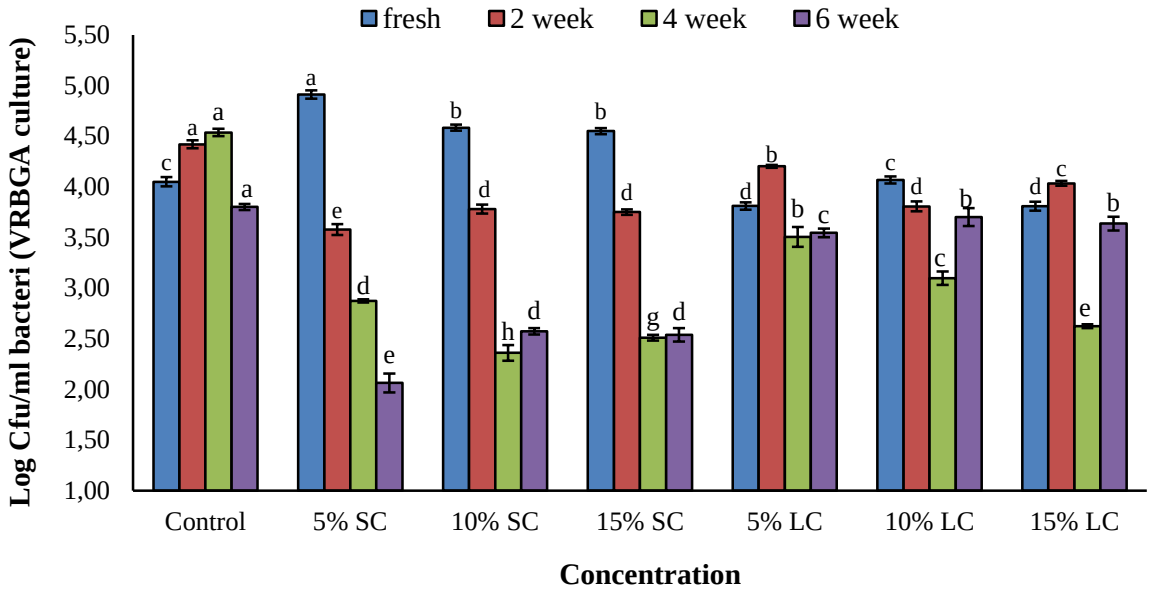


**Fig 8. YPDA culture**

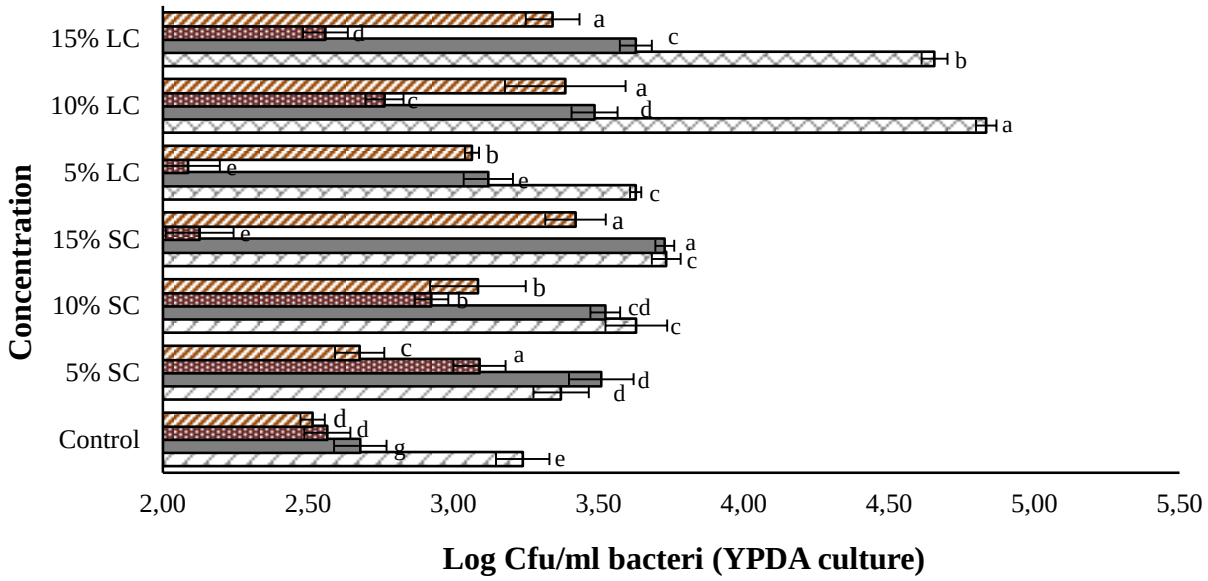



**Fig 9. PDA culture**

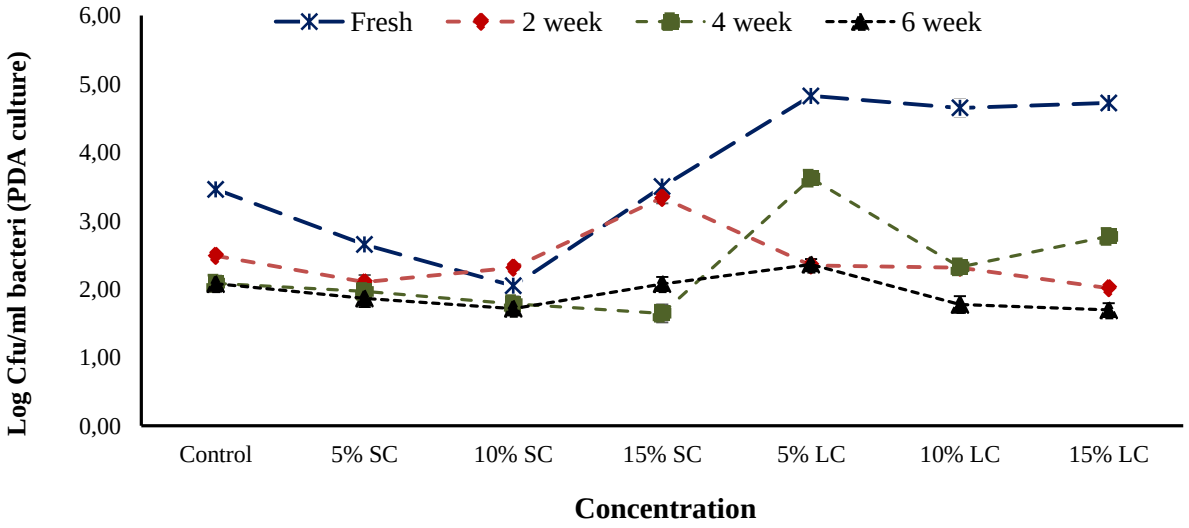


**Table 6.** Microbiological characteristics of samples during ripening (as Log CFU/cm2).

| Treatment | **TRT** (Plate count (log CFU/ml) | | | | | | | **SEM** | **Time** (Plate count (log CFU/ml) | | | | **SEM** | **Significant (p<)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | | T0 | T1 | T2 | T3 | | TRT | Time |
| **PCA** | 8.52 | 8.74 | 8.80 | 8.56 | 8.75 | 8.61 | 8.60 | 0.0733 | 8.65 | 8.64 | 8.60 | 8.73 | 0.0554 | 0.1004 | 0.4207 |
| **M17** | 8.45 | 8.63 | 8.63 | 8.51 | 8.61 | 8.58 | 8.48 | 0.0443 | 8.60 | 8.51 | 8.58 | 8.54 | 0.0335 | 0.0343 | 0.2923 |
| **MRS** | 7.58 | 7.83 | 8.01 | 7.48 | 7.36 | 7.51 | 7.41 | 0.2032 | 6.02 | 7.61 | 8.34 | 8.41 | 0.1536 | 0.2844 | <.0001 |
| **VRBGA** | 4.23 | 3.36 | 3.33 | 3.32 | 3.74 | 3.64 | 3.51 | 0.2804 | 4.28 | 3.96 | 3.04 | 3.09 | 0.2120 | 0.2926 | 0.0009 |
| **YPDA** | 2.72 | 3.09 | 3.24 | 3.20 | 2.92 | 3.52 | 3.52 | 0.1986 | 3.84 | 3.37 | 2.51 | 2.97 | 0.1501 | 0.0933 | <.0001 |
| **PDA** | 2.48 | 1.65 | 1.58 | 2.43 | 3.28 | 2.59 | 2.62 | 0.3547 | 3.69 | 2.38 | 1.96 | 1.47 | 0.2681 | 0.0430 | 0.0001 |

Abbreviations: PCA, plate count agar added with skimmed milk incubated at 30℃ for total mesophilic counts; VRBGA, violet red bile glucose agar for Enterobacteriaceae; MRS, de Mane Rogosae Sharpe agar for mesophilic rod LAB; M17 agar for mesophilic coccus LAB; YPDA, Yeast Peptone Dextrose Agar for total yeast and PDA, Potato Dextrose Agar for fungi filamentous

### 3.4.2. Texture analysis

Table 7 and fig10; show the evolution of texture analysis of cheese samples under different ripening time.

In this study, hardness ranged from 0.858 $Nmm^2$ to 1.229 N. These results showed that there is a slight significant difference between control samples and chocolate cheese including solid chocolate whereas observed significant differences among chocolate cheese including molten chocolate. Chocolate is polymorphic and can be made to crystallize in five to six different crystalline forms and reheating convert the stable crystals to unstable ones. In order to make liquid chocolate, we needed to melt the solid chocolate. Therefore with a change in temperature, there was a transformation in chocolate from stable form (melting point: ambient temperature) to unstable one (melting point: 21-22℃). As a result melting temperature of the chocolate declined and we got almost liquid chocolate present in our products in normal environment temperature. This however caused a decrease in hardness of sample cheeses. However, hardness showed remarkable increased with the highest level (15%) in both type of chocolate (solid and molten).

From the results it could be observed that all texture profile cheese with different levels of chocolate and both two types of chocolate decreased at time T1 compared with fresh samples and also all cheese samples hardness increased at time T2 compared with time T1.

Some authors claimed that the proteolysis is undoubtedly the most important biochemical process for flavour and texture properties of semi-hard and hard cheese types. Proteolytic enzymes from LAB play an important role in the degradation of casein and peptides leading to the production of free amino acids, which are rapidly converted into specific volatile compounds by nonstarter lactic acid bacteria (NSLAB) as well as by lactococci (Ayad et al., 2000; Amarita et al., 2001; Kieronczyk et al., 2003). The specific characteristics, such as taste, aroma and texture, of some artisanal cheeses have been correlated with Enterobacteriaceae counts (Dahl et al., 2000; Morales et al., 2004).

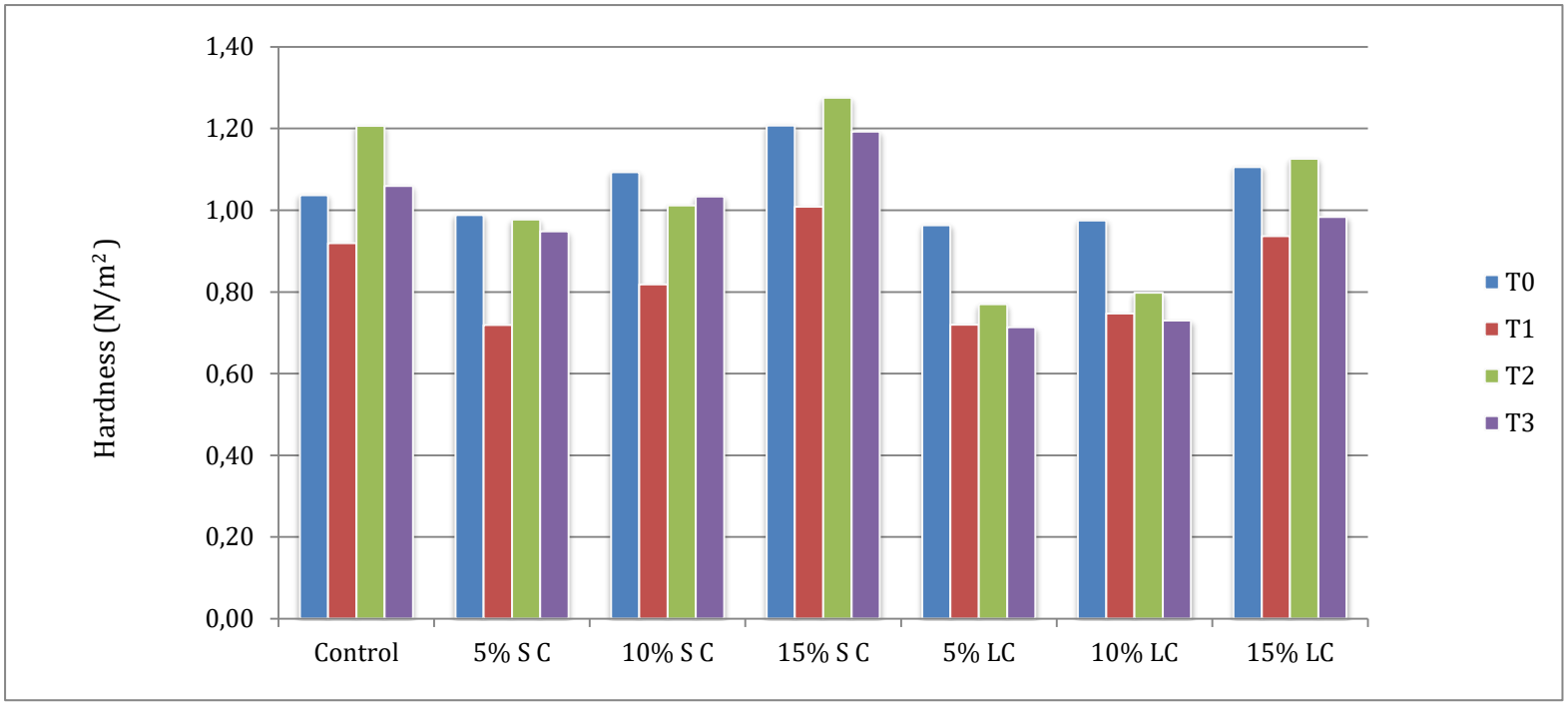



**Fig 10.** Texture analysis

**Table 7.** Average value of texture analysis among treatments during ripening.

| | TRT | | | | | | | | Time | | | | | Significant (p<) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC | SEM | T0 | T1 | T2 | T3 | SEM | TRT | Time | T*T |
| Texture | 1.159$^{ab}$ | 1.015$^{b}$ | 1.074$^{b}$ | 1.229$^{a}$ | 0.858$^{c}$ | 0.877$^{c}$ | 1.095$^{b}$ | 0.0395 | 1.086$^{a}$ | 1.002$^{b}$ | 1.092$^{a}$ | 0.994$^{b}$ | 0.0298 | <.0001 | 0.0336 | 0.4103 |

Abbreviation: TRT: Treatment, CTL: Control sample, SC: Solid Chocolate, MC: Molten Chocolate, T0: fresh, T1: two weeks, T2: four weeks, T3: six weeks.

## 3.4.3. Sensory analysis

### *3.4.3.1. Hedonic test*

The 7 panelists evaluated the control and chocolate cheese samples for liking of taste, texture, mouth feel, overall appearance and overall liking. Results showed that the chocolate cheese with 10% molten chocolate received most of the high hedonic scores after control sample and that most panelists like this sample because of the overall appearance. In general, assessors more preferred the control sample in all features and we compared other samples except of control samples.

The results for texture indicated that the cheese sample with 5% and 15% molten chocolate presented the highest score of evaluation but this samples did not differ significantly ($P \geq 0.05$) from the cheese sample with 10% molten chocolate. This feature showed that according to the hardness data, panelists preferred cheese samples with less hardness. The chocolate cheese with 5% solid chocolate presented the lowest score of evaluation in relation to the texture attribute. As mentioned in the previous section, chocolate cheeses with molten chocolate were almost liquid in ambient temperature, therefore it seems that this property have a strong relationship with mouthfeel as showed in Figures 11-14.

According to the hedonic test, assessors preferred all of attributes of cheese samples after 14 days ripening time. The results showed that there are significant differences between treatments at time T0, T1, T2 and T3. The highest score presented by T1 and followed by T0, T2 and T3, respectively.

Considering the degree of liking of appearance, taste, texture, mouthfeel and overall liking, the sample added with 10% molten chocolate after 14 days ripening time

presented the highest scores for all attributes, showing a higher acceptance in comparison to the other samples.

#### *3.4.3.2. Descriptive analysis*

Table 8 reports the changes in sensory scores attributed to the cheese descriptors registered over time. As expected, generally as we raised the amount of chocolate in cheese, the bitterness and sweetness of samples was significantly increasing whereas, salinity significantly decreased. Some authors claimed that among samples with the same level of sucrose, perceived sweetness increased with fat content. The same additive effect of sugar and fat was observed in previous studies conducted on ice cream (Guinard et al., 1997), yogurt (Tuorila et al., 1995) and oils (Lynch et al., 1993). In this study as mentioned in previous chapter, there are no significant differences between all the treatments for fat content, therefore it seems this results is due to the increase of chocolate carbohydrates. Bitterness in chocolate is mainly due to the caffeine and theobromine, which are naturally occurring in cocoa beans. We expected sugar to decrease the perception of bitterness by masking it, and cocoa butter to decrease bitterness by coating the bitter compounds. However this did not happened.

During ripening time perceived sweetness decreased whereas perception of acid increased. This results is entirely consistent our finding in terms of pH. Lowering the pH of the samples during ripening improved the perception of acid taste. Pettersen et al. (2005) observed an increase in acidulous flavour in cream cheese during 6 months of storage.

The results defined that odor of butter and milk significantly decreased in the presence of chocolate whereas unpleasant odor increased. As well, unpleasant odor sharply increased after 4 weeks.

Our observation showed that, cheese sample containing 10% molten chocolate after 14 days ripening time had highest score among all samples. This result is in agreement with hedonic test as mentioned above.

#### *3.4.3.3. Principal Component Analysis of sensory data*

In this study PCA method was used to analyze sensory described parameters and finding the main agents.

The first three main components constitute 74.16% of total variability of the results.

The first main component constitutes 39.26% of the total variance, which have variance of 6.28 (the largest specific value). As well as the second main component have variance of 3.38 and share of 21.14%. Moreover, the third main component has variance of 2.27 that constitutes 14.2% of the total variance.

The first three main components constitute 74.16% of total variability of the results, and so it seems that three components can generally show difference of data. Therefore the color, distribution, unpleasant smell, odor of milk and bitter were found as important effective component on the first agent; however, salinity, spicy, acid and solubility attributes were found as important effective component on the second agent; and finally sweetness, chewiness, uniformity of structure, strength of odor and odor of butter attributes were found as important effective component on the third agent. Fig. 15 shows the relation between sensory properties giving to position in space. As mentioned above, these three components are independent and have not correlation together.

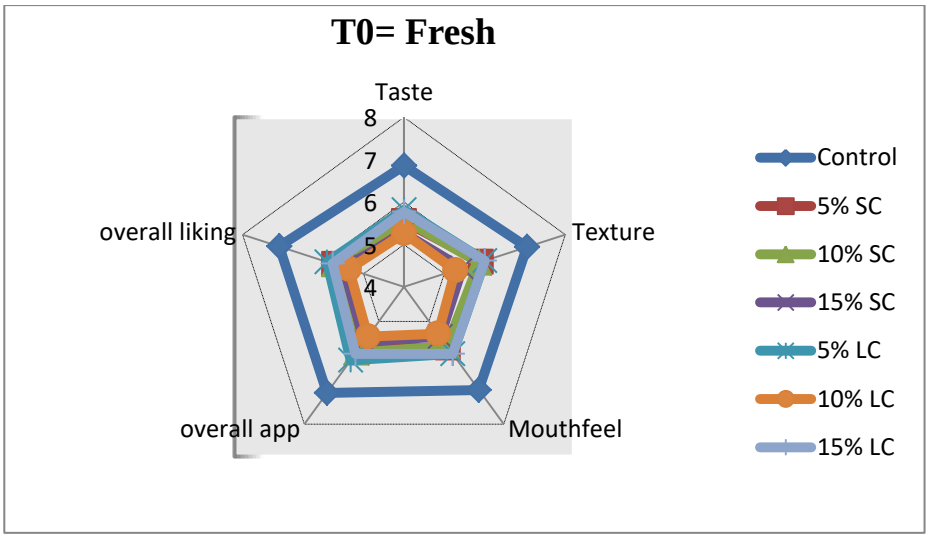



Fig 11. Mean value of hedonic test in fresh samples.

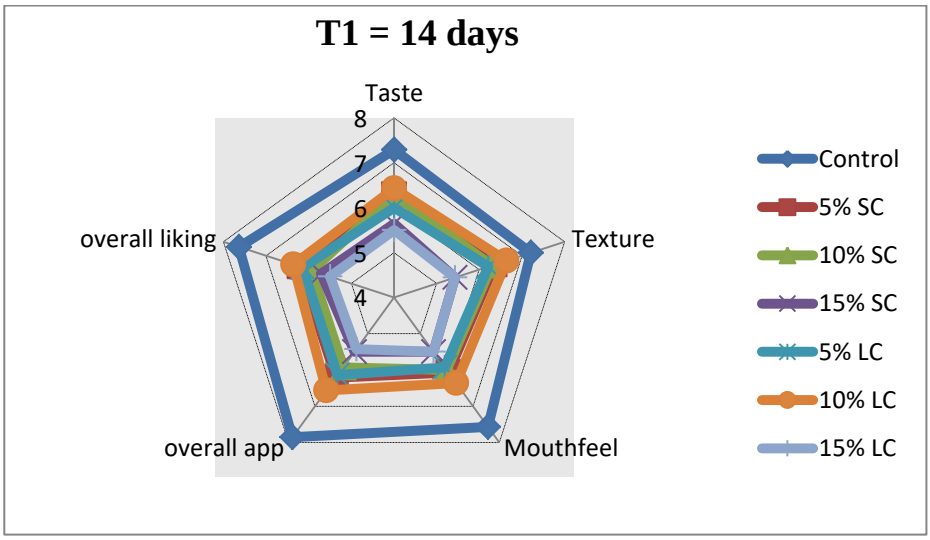



Fig 12. Mean value of hedonic test over the two weeks ripening.

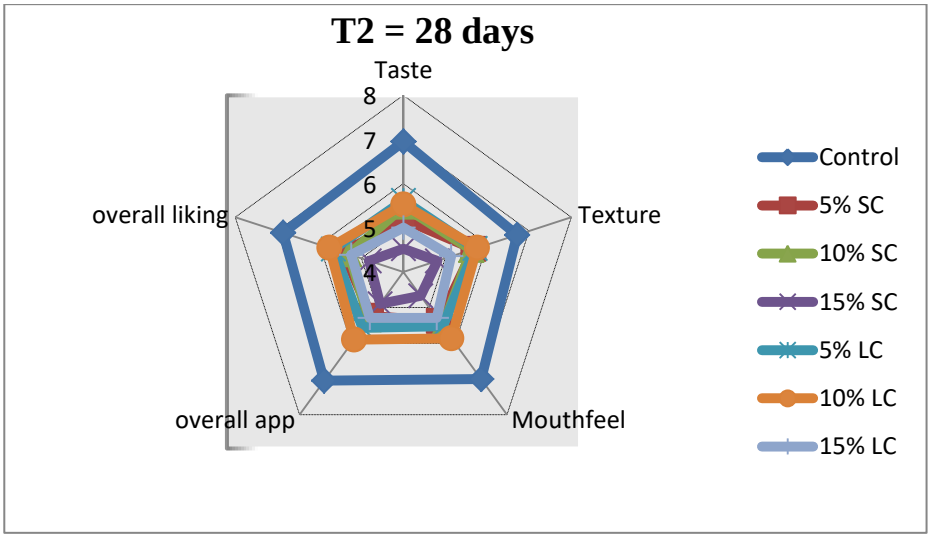



Fig 13. Mean value of hedonic test after 4 weeks.

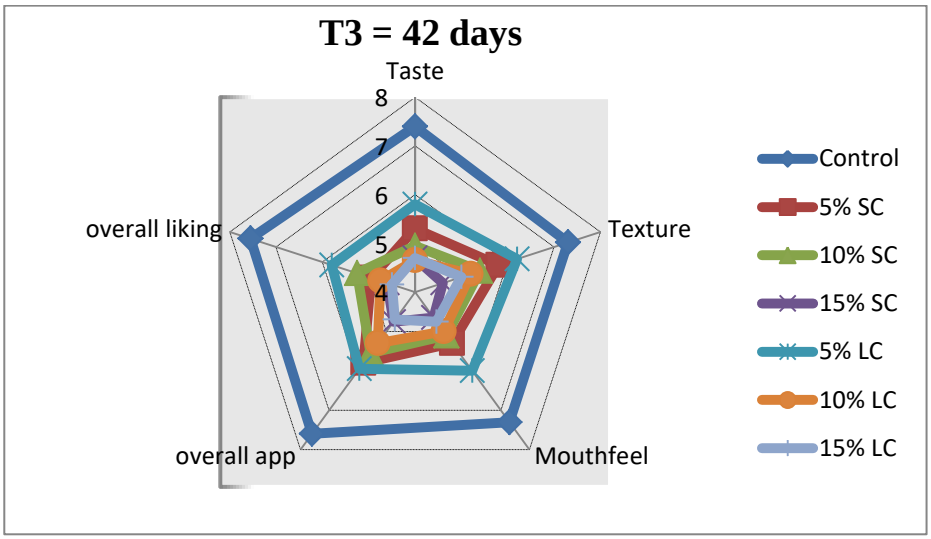



Fig 14. Mean value of hedonic test after 6 weeks.

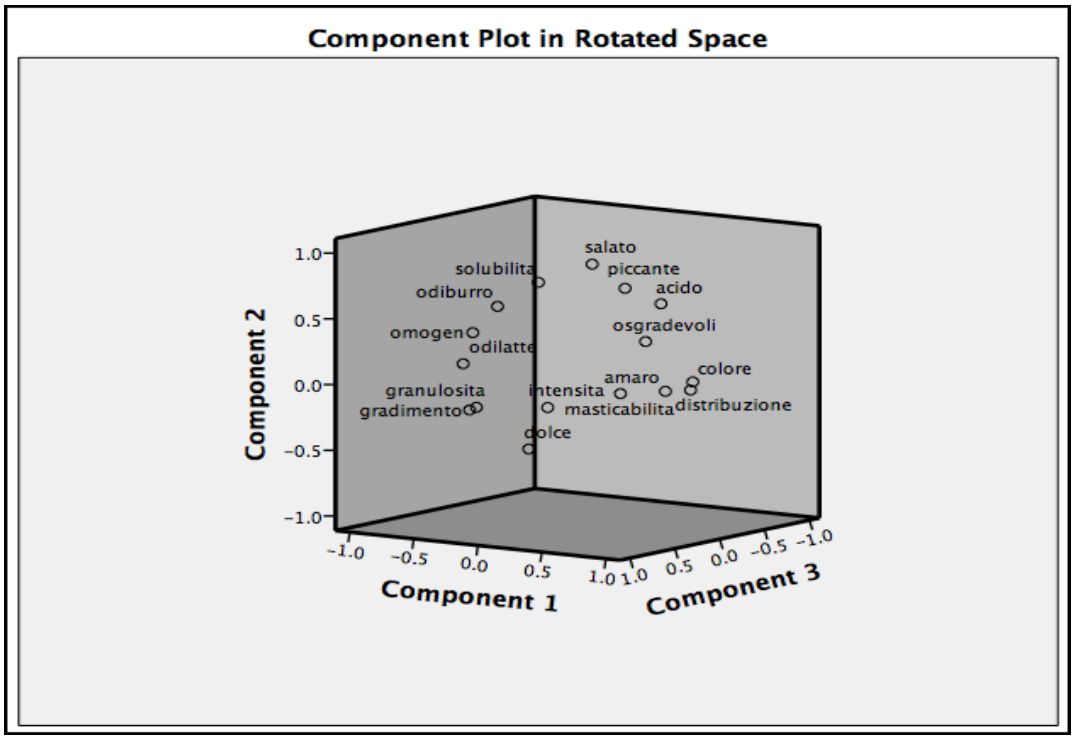



**Fig 15**. Principle component analysis for sensory attributes.

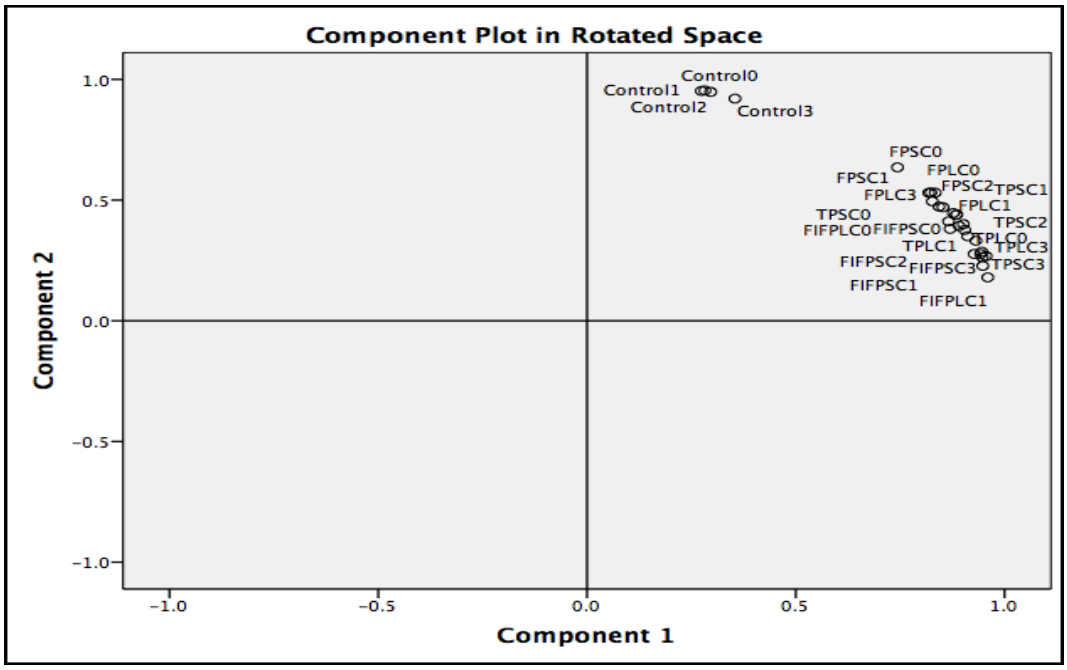



**Fig 16**. PCA analysis for panelist, 0 = fresh, 1 = 14 days, 2 = 28 days, 3 = 42 days; FP = Five Percent, TP = Ten Percent, FIFP = Fifteen Percent; SC = Solid Chocolate, LC = Liquid Chocolate.

**Table 8.** Mean values of sensorial parameters.

| Attribute | CTL | 5% SC | 10% SC | 15% SC | 5% MC | 10% MC | 15% MC |
|---|---|---|---|---|---|---|---|
| Color | 8.41 | 23.73 | 25.23 | 34.77 | 30.50 | 34.64 | 35.68 |
| Uniformity | 70.41 | 40.86 | 39.41 | 29.73 | 41.23 | 28.82 | 28.23 |
| Strength of odor | 46.91 | 34.09 | 44.45 | 49.18 | 41.86 | 44.91 | 47.91 |
| Odor of butter | 35.14 | 26.77 | 27.50 | 25.91 | 28.18 | 24.91 | 25.05 |
| Odor of milk | 20.91 | 17.55 | 12.09 | 14.27 | 15.73 | 13.23 | 11.55 |
| Unpleasant odor | 2.64 | 2.50 | 5.00 | 4.36 | 3.41 | 5.18 | 4.95 |
| Salty | 11.59 | 11.09 | 9.27 | 8.59 | 9.95 | 10.27 | 9.27 |
| Sweet | 14.55 | 17.82 | 29.73 | 32.82 | 20.32 | 23.73 | 29.55 |
| Acid | 3.68 | 4.59 | 3.41 | 3.27 | 4.50 | 5.55 | 4.36 |
| Bitter | 3.32 | 4.41 | 5.55 | 7.18 | 5.55 | 6.59 | 7.27 |
| Spicy | 1.86 | 2.55 | 2.05 | 2.00 | 1.91 | 4.09 | 2.14 |
| Chewiness | 34.32 | 34.14 | 30.82 | 33.73 | 32.36 | 27.95 | 32.68 |
| Solubility | 43.36 | 47.50 | 41.14 | 41.23 | 43.91 | 40.68 | 41.41 |
| Grittiness | 42.36 | 40.09 | 39.41 | 45.82 | 41.32 | 41.73 | 40.09 |
| Distribution | 2.00 | 34.09 | 43.45 | 40.45 | 42.95 | 41.27 | 37.95 |
| Overall satisfaction | 56.64 | 38.95 | 39.77 | 37.09 | 41.41 | 33.23 | 41.02 |

Abbreviation: TRT: Treatment, CTL: Control sample, SC: Solid Chocolate, MC: Molten Chocolate, T0: fresh, T1: two weeks, T2: four weeks, T3: six weeks.

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