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Effect of Storage and Heat Treatment of Milk Destined for Cheese Production on Its Oxidative Characteristics

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Abstract: The oxidative stability of milk and dairy products is a very interesting topic for the dairy industry due to the growing demand for foods containing bioactive compounds with positive health effects. The aim was to evaluate the oxidative stability of milk intended for cheese production. The effect of storage time, heat pre-treatment, and milk pasteurization temperature on the characteristics of milk and cheese was investigated. The cheese samples were produced with pasteurized milk at both 72 and 77 °C for a time of 15 s using three types of milk: raw fresh milk processed within 48 h of milking, raw stored milk processed within 96 h, and thermized milk that was heat-treated upon arrival at the dairy and processed within 96 h of milking. In total, three repetitions were carried out for each type of milk and pasteurization. Samples of milk before and after pasteurization and cheese at 14 days of storage were analyzed. Antioxidant activity decreased from starting milk to milk after pasteurization to final cheese. The longer storage time of the milk had significant effects on the antioxidant stability of the cheese (64.95 vs. 59.05% of antioxidant activity). Thermization of the milk further reduced the stability of the cheese (54.05% of antioxidant activity). The greater antioxidant stability of fresh milk and cheeses produced with fresh milk is the first result that encourages the production of cheese from a milk that best preserves its original characteristics.

Keywords: fresh milk; antioxidant activity; pasteurized milk; milk oxidation; cheese production

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1. Introduction

In high-income countries, especially European ones, some dairy products may have characteristics designed to satisfy the demands of the consumer who is increasingly inclined to choose products with certain sensory or nutritional characteristics or made with methods that respect the environment and animal welfare. The European provisions on drinking milk establish rules aimed at safeguarding the nutritional value of heat-treated milk (EC Regulation 2597/1997) [1]. As part of its quality policy, European legislation has protected the names of foods with unique characteristics linked to their geographical origin (starting from EEC Regulation 2081/92) [2] through the Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI). Recently the obligation on the label of the origin of the product has been extended to all dairy products (EU Regulation No. 1169/2011) [3]. Some of these measures have helped to increase the added value of some dairy products.

In Italy, 52% of national milk used for cheese production is destined to become PDO cheeses [4]. For the other cheeses, except for the so-called "traditional" cheeses, for which the process is defined, there are no specifications to be respected. The Italian Ministry of Agriculture financed a project on the effects of the use of "fresh" milk on the quality of cheese. By "fresh" milk, we mean raw milk processed within 48 h of milking, as it is practiced in drinking milk called "fresh pasteurized milk" and "high-quality fresh

pasteurized milk." To evaluate the effects of the use of "fresh" milk on some specific characteristics of final cheese, we focused on the oxidative stability of milk processed at different times from milking. The effect of a thermal pre-treatment carried out in the case of longer storage of the milk, in addition to that of temperature of the pasteurization, was also evaluated.

The oxidative stability of milk and dairy products is a very interesting topic for the dairy industry. In recent years there has been an increasing demand for foods containing bioactive compounds that have positive effects on health. Milk also contains antioxidant compounds that reduce or prevent the risk of various diseases. Antioxidant activity in milk is due to many bioactive compounds such as proteins, peptides, vitamins E and C, retinol, β-carotene, glutathione (GSH), and glutathione peroxidase [5–7].

The main antioxidants present in milk can be grouped into lipophilic and hydrophilic antioxidants. Carotenoids, retinol, and α-tocopherol are lipid-soluble antioxidants present in milk fat globule and they have properties such as preventative, chain-breaking antioxidant, and quencher of singlet oxygen in milk, protecting milk fat against autoxidation [6,8,9]. In addition to highly active and more abundant lipophilic antioxidants, milk is also a source of hydrophilic antioxidants constituted by ascorbic acid and a large group of nitrogen compounds, such as casein fractions, whey proteins (in particular lactoferrin and β-lactoglobulin), bioactive peptides, low molecular weight nitrogen compounds and uric acid [10]. Another important group of antioxidant compounds are enzymes that counteract free radicals in milk, including superoxide dismutase, catalase, lactoperoxidase, and glutathione peroxidase. Those enzymes form a synergistic system that increases the antioxidant potential of milk. Antioxidant systems in milk can inhibit the free radical mechanism by donating the proton and thus inhibit the onset of autoxidation [9].

In particular, the presence and concentration of antioxidants such as vitamin E, polyphenols, and β-carotene, strongly depend on feeding management [11]. The intake of vitamins and antioxidants within the diet is transferred to milk [12,13]. Higher levels of antioxidants (tocopherol, β-carotene, and retinol) have been reported in milk from cows that consume fresh grass compared with diets rich in concentrate or silage [11,14]. The vitamin E content of milk is determined by tocopherol levels in feed while vitamin A and β-carotene concentrations are determined by carotenoids supplied with bovine feed [9].

In milk, as in dairy products, oxidative reactions can develop off-flavor and loss of nutritional quality [15]. Spontaneous oxidation in milk may progress as a result of external factors during storage and processing such as photo-oxidation or heat treatments. However, it depends on inherent factors in the milk itself which include fatty acid composition, content of antioxidant molecules, antioxidative enzyme systems, and transition metal ion content [6]. Moreover, external factors such as preservation, handling, agitation, temperature, exposure to light, and contamination by metals and microorganisms can induce additional oxidative reactions in milk [10,16,17].

The oxidative stability of milk and dairy products in function of time can be monitored by measuring lipid oxidation, protein oxidation, content of vitamins (riboflavin, tocopherols, carotenoids), or the use of a sensory panel. Since oxidation occurs in case of an imbalance between the presence of reactive oxidants and the antioxidant defense mechanism, sensitivity to oxidation can also be monitored by measuring the antioxidative capacity of a product [9]. Considering the heterogeneity of antioxidant molecules in milk, different methods have been developed to investigate total milk antioxidant capacity without distinguishing the contributions from individual compounds [18].

The general objective of the project is to evaluate the qualitative characteristics of cheese produced with fresh milk and to identify the most suitable markers to identify this type of cheese. The project activity includes innovative determinations as sensory analyses of cheese and the in vitro digestion tests of milk and cheese, near-infrared spectroscopy (NIR) and electronic nose analyses of cheese.

In this work, the oxidative stability of milk destined to produce fresh milk cheese was evaluated. The effects of milk storage time and heat treatments were investigated. The

latter include the thermal pretreatment practiced in the case of prolonged milk storage and pasteurization temperature.

2. Materials and Methods

2.1. Raw Milk

The cow's milk used in the trial to produce cheese samples was collected between October 2019 and March 2020 from local farms and arrived in the experimental dairy plant of the Council for Agricultural Research and Economics (CREA), Research Centre for Animal Production and Aquaculture of Bella Muro, Potenza, Italy, within 24 h of the first milking. Samples of raw milk upon arrival at dairy were collected and transferred to laboratory for routine analyses.

2.2. Milk Treatments

The treatments undergone by the milk before the production of the experimental cheese samples were as follows:

- raw fresh milk: pasteurization within 48 h of the first milking (F);
- raw stored milk: pasteurization within 96 h of the first milking (S);
- thermized and stored milk: thermization process (65 °C for 15 s) immediately upon arrival at the dairy and cooling, pasteurization within 96 h of the first milking (T).

The storage of milk was performed in tank at 4 °C. Each batch of milk was heat-treated at 72 °C for 15 s, the minimum recommended for milk pasteurization and called low temperature (L), and at 77 °C for 15 s called high temperature (H). A plate exchanger was used (Comat Dairy Equipment, Bellizzi, Salerno, Italy) equipped with software for the management and control of the pasteurization temperature.

Samples of raw milk immediately before pasteurization and pasteurized milk were collected and frozen to −80 °C for further analysis. Second the experimental design, six different treated milks were produced for three repetitions (3 treatments × 2 pasteurizations × 3 repetitions).

2.3. Cheese Production

Six tests were carried out to produce cheese samples. In each test, the three experimental milks (F, P, T) were submitted to the same type of pasteurization, for a total of three repetitions for each type of milk and pasteurization. The cheeses were produced artisanally in the small experimental dairy plant of CREA (Bella Muro, Potenza, Italy). Cheese manufacture was performed using a protocol detailed in Figure 1. A starter containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* was used. After coagulation, two curd cuts were made, and the final size of the curd grain was similar to a hazelnut. Once it reached pH 5.5–5.6, the cheese was transferred from the hot room to the cold room (10 °C). The cheeses were salted when the temperature of the cheese reached about 15 °C. Dry salting was practiced and about 15 g of salt/kg of cheese was used. The next day, the cheese was transferred to the cold room at 4 °C.

For each production, six shapes per group were collected and carried to the laboratory of CREA localized in Monterotondo, Rome, Italy. The analyses were carried out on the cheese after 14 days of storage at refrigeration temperature (4 °C). The cheeses were sampled by homogenizing the whole shape as they were fresh soft cheeses without rind.

2.4. Milk Analysis

On the batches of raw milk, sampled the day of being carried into dairy plant, the following routine analyses were carried out. pH values were determined using Metrohm mobile 826 pHmeter (Metrohm Ltd., Herisau, Switzerland); fat, protein, casein, and lactose levels (expressed as % of fresh weight), were determined using a MilkoScan™ 7 RM (Foss Electric A/S, Hillerød, Denmark) calibrated according to ISO-IDF references (ISO 9622:2013; IDF 141:2013) [19]. These analyses were carried out on fresh milk.

Moreover, to test the changes due to storage and heat treatments, each type of milk (F, S, T) was analyzed both before and after pasteurization determining lactoperoxidase activity, tryptophane, advanced Maillard's products, and antioxidant activity according to the methods specified below. These analyses were carried out on frozen at −80 °C samples.

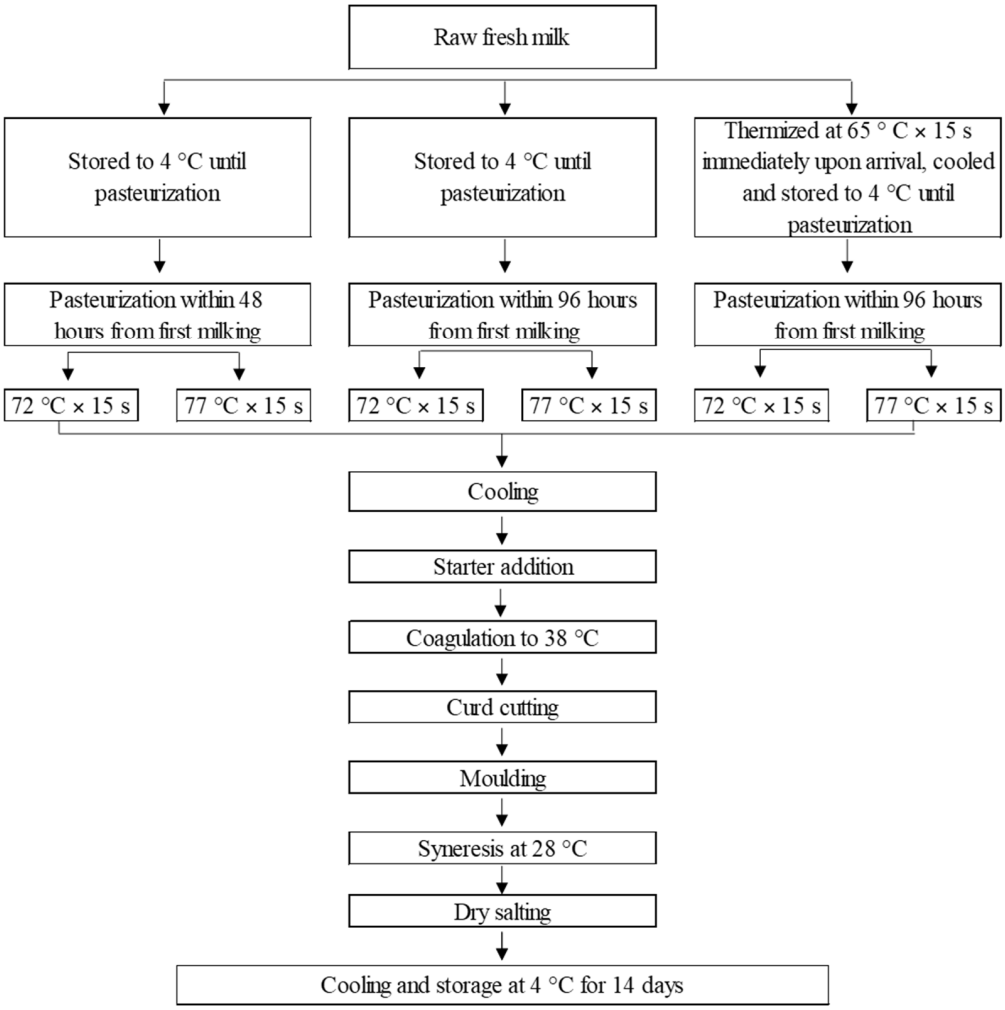

Figure 1. Flow chart of production of cheese samples.

2.5. Cheese Analysis

Cheese samples were submitted to the following analyses: pH, moisture (IDF, 1986) [20], ashes (AOAC, 2000) [21], protein, and fat (ISO 21543:2006; IDF 201:2006) [22].

To value oxidation level in cheese after storage for 14 days, tryptophane, advanced Maillard's products and antioxidant activity were determined such as for milk using the same methods reported below. Moreover, Malondialdehyde (MDA) analysis was carried out as reported by Rinaldi et al. [23] to test fat oxidation in cheese samples. The analyses were carried out on clopped samples frozen at −80 °C. For each analysis, at least two repetitions of each sample were performed.

2.6. Lactoperoxidase Activity Method

In milk, enzymatic activity of lactoperoxidase was measured using 2,2′-Azinobis-3-ethylbenzothiazoline-sulfonic acid (ABTS) as substrate, following the spectrophotometric method of Marin et al. [24]. In the spectrophotometer cuvette, 50 µL milk was added with 1 mL 0.65 mM ABTS substrate prepared in 0.1 M sodium phosphate buffer (pH 6.0) and 50 µL of the same sodium phosphate buffer. The reaction was initiated by adding 1 mL of 0.1 mM hydrogen peroxide as a reaction activator. Increasing absorbance at 412 nm was

measured for 1 min at 20 °C by a double beam UV-VIS spectrophotometer (Lambda 25, PerkinElmer, Waltham, MA, USA).

The blank was prepared with 50 µL milk, 1 mL of substrate (0.65 mM ABTS), and 1050 µL of phosphate buffer, without hydrogen peroxide. The ratio between absorbance and time was linear, at least in the 1st 20 s of the reaction. Enzymatic activity was calculated as the slope of the curve in the linear part, considering the molar extinction coefficient of 32.4 mM^{-1} cm^{-1}, and it was expressed in units per mL, where 1 unit is the quantity of enzyme which catalyzes the oxidation of 1 micromole of substrate per min at 20 °C.

2.7. FAST Method

The FAST method (Fluorescence of Advanced Maillard products and Soluble Tryptophan) was used to evaluate the modifications induced in milk by heat-treatments analyzing tryptophane and advanced Maillard's products in samples [25,26]. This method has also been applied to Mozzarella cheese in a previous work [23].

Samples of milk (500 µL) or cheese (0.5 g) were mixed to 4.5 mL sodium acetate buffer (0.1M, pH 4.6) and shanked vigorously for 30 s. After centrifugation at 4000× *g* for 10 min at 20 °C, the supernatant was diluted (1:10), filtered through a 0.45 µm filter, and analyzed by Spectrofluorometer (FP-6300, Jasco, Tokyo, Japan).

Two fluorescence intensities were measured:

- fluorescence of tryptophan (Trp-F) at 290 nm excitation and 340 nm emission,
- fluorescence of advanced Maillard's products (AMP-F) at 350 nm excitation and 440 nm emission and expressed in arbitrary units (a.u.).

The FAST index was calculated as follows:

$$(\text{AMP-F}/\text{Trp-F}) \times 100$$

Tryptophan measurement is an estimate of soluble proteins content in solution. In the acidic conditions (pH 4.6) used in this analysis, caseins precipitate, so that Trp-F is positively related to whey protein soluble at pH 4.6. Denaturation and precipitation of proteins due to heat treatments may result in decrease of Trp-F.

AMP-F is related to the content of advanced Maillard's products, and generally, Maillard's reaction occurs in products subjected to high heat treatments. Milk thermized and/or exposed to high pasteurization could be characterized by higher values of FAST index.

2.8. Antioxidant Activity—DPPH Method

The antioxidant activity of the samples was analyzed by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method, as reported by Unal [27] and Rinaldi et al. [23]. The DPPH radical is reduced by the antioxidant compounds of the sample and the antioxidant activity expresses the capacity of the sample to inhibit the DPPH radical.

Milk samples (2 mL) or cheese samples (2 g) were mixed with 8 mL of 0.11 mM DPPH ethanolic solution. As control, 2 mL of ethanol, in place of the sample, were added to 8 mL of 0.11 mM DPPH solution. The mixtures were vortexed and incubated at room temperature for 20 min in the dark and centrifuged for 10 min at 9000× *g* at 22 °C.

Absorbance was recorded at 517 nm on the supernatant by a double beam UV-VIS spectrophotometer (Lambda 25, PerkinElmer, Waltham, MA, USA). The antioxidant activity was reported in percentage according to the following equation:

$$\%\ \text{Antioxidant activity} = (A_0 - As)/A_0 \times 100$$

where A_0 is the absorbance of the control (without sample) and As is the absorbance of the tested sample. Using Trolox as standard, antioxidant activity of the samples can be expressed in Trolox equivalents (mmol eq Trolox/100 mL of milk or 100 g of cheese).

2.9. Statistical Analysis

Data of characteristics of the milk before and after pasteurization (lactoperoxidase activity, FAST index, and antioxidant activity) were statistically analyzed by GLM procedure in the statistical software package SAS/STAT (version 9.4 SAS Institute Inc.) [28]. For both kinds of milk, the model included 3 levels of treatment of milk (fresh, stored, and thermized milk) as fixed effect. For pasteurized milk, the model also included two kinds of pasteurization (low and high) for each level, as fixed effect, for a total of 6 levels. The same 6 level model was used for the cheese data (pH, moisture, ashes, protein, fat, lactoperoxidase activity, oxidative characteristics, and FAST Index). Differences were considered significant at $p < 0.05$, in each model.

3. Results

3.1. Chemical Characteristics of Raw Milk

The chemical characteristics of raw milk used for cheese-making in this study are reported in Table 1. These are the average data of analyses carried out on raw milk sampled upon arrival at the dairy and then used in the six trials.

Table 1. Chemical characteristics of raw milk upon arrival at the dairy. Data are expressed as the mean of six repetitions. sd = standard deviation. For each parameter, maximum and minimum are reported.

Parameter	Average ± sd	Minimum	Maximum
Fat (%)	3.91 ± 0.08	3.78	4.01
Protein (%)	3.49 ± 0.04	3.43	3.54
Casein (%)	2.74 ± 0.03	2.69	2.77
Lactose (%)	4.80 ± 0.03	4.73	4.86
pH	6.68 ± 0.02	6.65	6.71

The determination of the characteristics of raw milk was carried out to evaluate the initial condition of used milk. The hygienic and healthy characteristics of the milk were within the limits established by current legislation. The rate fat/protein was 1.12. The results show that raw milk had the fat and protein content slightly higher than average data provided by the National Association of Italian Friesian and Jersey Breed (ANAFIJ) [29], respectively 3.79 and 3.35%, for the year 2020.

3.2. Characteristics of Pasteurized Milk

Characteristics of stored (S) and thermized (T) milk compared to fresh (F) milk subjected to low (L) or high (H) pasteurization are reported in Table 2. The results show that modifications occurred in some parameters of stored and thermized milk compared to those of fresh one, as discussed below.

Table 2. Lactoperoxidase activity, oxidative characteristics, and FAST Index of fresh (F), stored (S), and thermized (T) milk subjected to low (L) or high (H) pasteurization.

Milk		Lactoperoxidase U mL^{-1}	Trp-F a.u.	AMP-F a.u.	FAST Index	Antioxidant Activity %
Fresh	F-L	2.53 a	647 a	25.4 b	3.94 c	81.3 a
	F-H	0.64 b	650 a	26.5 ab	4.09 bc	78.9 b
Stored	S-L	2.37 a	633 a	26.1 ab	4.13 bc	76.8 c
	S-H	0.68 b	612 ab	27.5 a	4.38 ab	74.7 d
Thermized	T-L	2.10 a	594 b	25.7 b	4.33 abc	76.2 cd
	T-H	0.56 b	581 b	26.9 ab	4.65 a	74.9 cd

Data are expressed as mean of three repetitions. For each parameter, means followed by a different letter in the column are significantly different ($p < 0.05$). Trp-F = tryptophan fluorescence, AMP-F = Maillard's advanced products fluorescence; FAST = Fluorescence of advanced Maillard products and soluble tryptophan.

3.2.1. Lactoperoxidase Activity

The Lactoperoxidase (LPO) enzyme is recognized for its high heat stability and, due to this property, LPO is used as an index of pasteurization efficiency in milk.

Lactoperoxidase activity decreased significantly ($p < 0.001$) in milk subjected to high pasteurization compared to milk subjected to low pasteurization (on average from 2.33 to 0.63 U mL^{-1}) independently by preservation and thermization of the milk. The reduction of Lactoperoxidase activity in fresh low pasteurized milk (F-L) was about 7.3% compared to this activity in raw milk that was on average 2.73 U mL^{-1} in our samples. These data show that the parameters used in low pasteurization (72 °C for 15 s) were suitable to maintain good levels of LPO activity while the temperature of 77 °C for 15 s induced inactivation of about 73% of LPO activity compared to activity in low pasteurized milk.

Data for LPO activity in the literature vary widely because of the various chromogens used for its assay and the variability in the assay conditions (pH, temperature). The levels of LPO change in different types of milk as reported by Seifu et al. [30]. In bovine milk, LPO is the second most abundant enzyme after xanthine oxidase. A report indicated that the mean LPO activity in cow raw milk throughout lactation ranged from 1.5 to 2.7 U mL^{-1} with an overall mean of 2.3 U mL^{-1} [31] as reported even by Ozer [32]. However, other authors reported lower LPO activity, on average 1.42 U mL^{-1}, in bovine milk [30,33].

The LPO is active (able to catalyze the reaction between H_2O_2 and thiocyanate at a sufficient level) after heat treatment at 74 °C for a short time. Complete LPO deactivation occurred at 80 °C for 15 s, whereas at 72 °C for 15 s residual LPO activity was about 70% [34]. LPO activity is used together alkaline phosphatase, another original milk enzyme, to investigate the retrospective determination of the heat treatment undergone by milk. Alkaline phosphatase activity must be negative as assessment of correct pasteurization, and it is used as an index of adequate pasteurization of milk.

3.2.2. Protein Denaturation and the FAST Index

The FAST index (fluorescence of advanced Maillard products and soluble tryptophan) is based on the quantification of two complementary indicators, the fluorescence of tryptophan (Trp-F) and that of advanced Maillard products (AMP-F) in the milk protein fraction soluble at pH 4.6. This method has been used in other works to evaluate the changes due to heat treatments in milk [25,26].

Trp-F decreased significantly ($p < 0.01$) in thermized milk compared to fresh milk (from 648 to 587 a.u.), but no significant differences were found between milk subjected to high and low pasteurization (Table 2). There was also a tendency to reduction in Trp-F from 648 to 622 a.u. in stored milk compared to the fresh one.

The Trp fluorescence appears to be a good indicator of the protein concentration, as shown by the high correlation with colorimetric methods of protein determinations and even by a good correlation with the acid-soluble β-lactoglobulin content observed in milk in precedent works [25]. So, Trp fluorescence may be used to determine protein denaturation due to heat treatments. However, when high heat treatments are applied, the decrease of Trp fluorescence is due to both protein conformational changes and Trp degradation [25,35].

AMP-F was significantly higher ($p < 0.05$) in stored milk (S-H) than in T-L and F-L (ranging from 27.5 a.u. to 25.4 a.u.) while the other treated milk had middle levels of AMP-F (Table 2). AMP level usually increased in samples during high heat treatments, in fact, there was no effect on AMP-F due to thermization (65 °C) while a tendency of increasing AMP-F level was found due to high pasteurization (77 °C) compared to low pasteurization (72 °C). The fluorescence of AMP generally increases with prolonged heat treatment due to extensive conversion of the early Maillard product to AMP. Consequently, AMP should be a good indicator of nutritional damage to evaluate severe treatments (such as indirect UHT treatment and sterilization) where the advanced step of the Maillard reaction is reached [25,26,36].

The FAST index was significantly ($p < 0.05$) lower in F-L milk than in T-H and S-H (3.94 vs. 4.65 and 4.38) while all other milk samples showed middle values (4.33–4.09). The FAST index, depending on AMP-F/Trp-F ratio, generally increases with contemporaneous decrease in Trp-F and production of AMP due to heat-treatments. The relation between Trp-F and FAST index is reported in Figure 2, where each pasteurized milk was plotted.

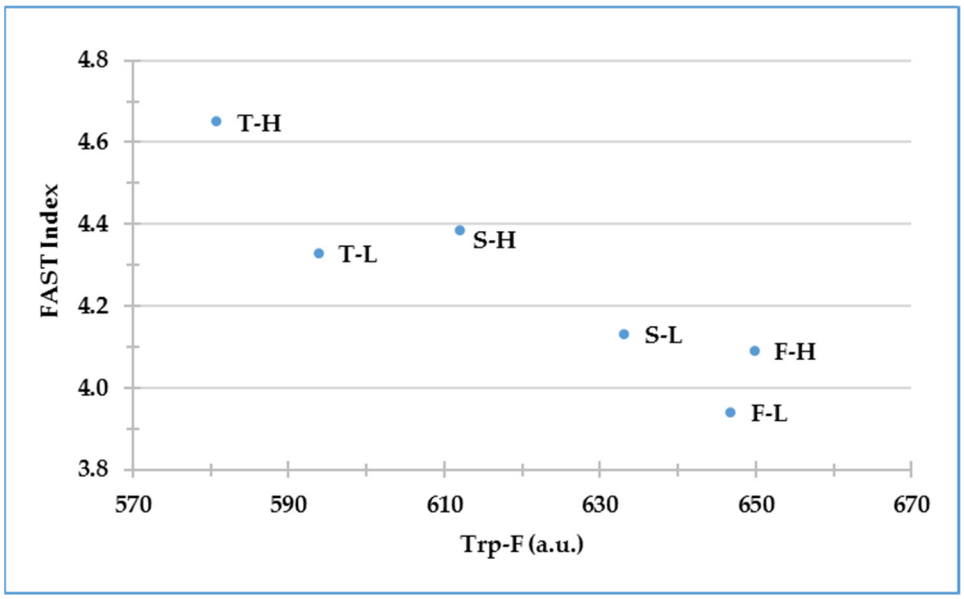

Figure 2. Relation between tryptophan fluorescence (Trp-F) and FAST index in fresh (F), stored (S), and thermized (T) milk subjected to low (L) or high (H) pasteurization.

The Trp-F decreased with heat treatment and in particular with thermization, so that samples of fresh milk could be discriminated easily by those of thermized milk both on the Trp-F and FAST Index axis. The main decrease in Trp-F was induced by the double heat-treatment, thermization and high pasteurization (T-H) and it was related to highest FAST index value. The highest Trp-F levels characterized fresh milk (F-L and F-H) and F-L was even characterized by the lowest FAST index value, while T-L and S-H milk had middle levels of Trp-F and FAST Index (Figure 2).

The values of the FAST index in our samples were lower than those reported in other works [26,37], demonstrating the good nutritional quality of the milk and in particular of the low-pasteurized fresh milk. However, the fast index rises more when higher temperatures are used and/or for longer times than those used in this trial, as in the case of UHT and sterilized milk due mostly to AMP production [36].

3.2.3. Antioxidant Activity

Antioxidant activity determination is a useful tool in estimating the total ability of milk to counteract the oxidation that normally occurs during storage and processing in milk [38,39]. The antioxidant activity of milk (Table 2), determined as DPPH free-radical scavenging activity, was significantly higher ($p < 0.01$) in fresh milk than in thermized and stored milk, ranging from 81.3% in F-L to 74.7% in S-H (corresponding to 17.2 and 15.8 mmol eq Trolox/100 mL, respectively). No significant differences in antioxidant activity were found between thermized and stored milk (non-thermized). Moreover, there were significant ($p < 0.05$) differences between high and low pasteurized milk both in fresh milk and stored milk.

These values could depend on the antioxidant activity in raw milk that decreased with the heat-treatment of thermization and mostly with high pasteurization, but even with preservation time. Milk is a complex system of pro and antioxidant components [6] and oxidation occurs in case of an imbalance between the presence of reactive oxidants and the antioxidant defense mechanism [7,40]. Among spectrophotometric methods, the DPPH

method is useful to measure the total antioxidant activity without giving any detailed information about individual antioxidant components [18]. The antioxidant components neutralized DPPH radical using both hydrogen atom transfer and single electron transfer mechanisms [39]. Antioxidant content in raw milk, in particular the content of lipophilic antioxidants, is largely determined by the composition of animal diets [9].

The storage time may affect the antioxidant activity of milk favoring better antioxidant stability in fresh milk. The thermal pre-treatment was finalized to reduce the microbial load, especially psychrotrophic bacteria, and to increase the keeping quality of raw milk until the final process [41,42], which did not affect antioxidant activity of the two milks stored for a longer time. During pasteurization, milk is exposed to heat treatment for a time performant to kill the pathogens in milk and this time and temperature combination may have an impact on the antioxidant characteristics of milk.

3.3. Chemical and Physical Characteristics of Cheese

Table 3 shows the chemical and physical characteristics of the cheese produced using fresh, stored, and thermized milk subjected to low and high pasteurization temperatures. The highest pH value ($p < 0.05$) was found in the S-L cheese, the lowest in the T-H cheese. The moisture content of cheese varied from 59.1% in S-H cheese to 56.9% content in T-L cheese.

Table 3. Chemical and physical characteristics of cheese produced using fresh (F), stored (S), and thermized (T) milk subjected to low (L) or high (H) pasteurization.

		pH	Moisture %	Ashes %	Protein %	Fat %
Fresh	F-L	5.32 ab	58.5	2.79	19.3 a	22.2 a
	F-H	5.17 abc	58.0	2.79	18.3 bc	20.9 ab
Stored	S-L	5.41 a	57.6	2.78	19.0 ab	21.2 ab
	S-H	5.12 abc	59.1	2.75	18.0 c	20.4 ab
Thermized	T-L	5.34 ab	56.9	2.69	19.6 a	22.0 a
	T-H	4.99 c	58.2	2.70	18.3 bc	19.4 b

Data are expressed as mean of three repetitions. For each parameter, means followed by a different letter in the column are significantly different ($p < 0.05$).

In Figure 3 we can observe that in cheese from stored and thermized milk the pH values were lower when the pasteurization temperature was higher. Furthermore, the pH trend was opposite to that of the moisture content of the cheese. Cheese made with high pasteurized milk had a lower protein and fat content. In particular, the lowest percentage of proteins was found in the S-H cheese ($p < 0.01$) and the lowest percentage of fat in the T-H one ($p < 0.05$). The highest protein and fat content was found in F-L and T-L cheeses (Table 3).

Similar results on the characteristics of cheese made with pasteurized milk at different temperatures were obtained from previous studies [43,44]. As the pasteurization temperature increased, the moisture content increased, the fat and protein content decreased, and the pH values also decreased. The lowering of the fat and protein content is due to the reduction of the dry matter of the cheese following the increase in moisture content. The reduction in pH is to be attributed to the higher moisture content (Figure 3) which leads to a greater presence of lactose and, consequently, of lactic acid [45].

3.4. Characteristics of Cheese in Comparison with Milk

The characteristics of cheese after 14 days of storage were compared with those of milk immediately before pasteurization and pasteurized milk with the different treatments. The following figures show the results of some characteristics in fresh, stored, and thermized milk immediately before pasteurization and after low and high pasteurization, and in the corresponding cheese made from these types of milk.

Figure 4 shows the results of the Trp-F level. In milk immediately before pasteurization, Trp content in F milk (810 a.u.) and S milk (787 a.u.) was significantly ($p < 0.05$) higher than that of T sample (709 a.u.). In the comparison between the three modes of milk storage, the Trp-F content was more sensitive to longer storage in presence of thermization treatment.

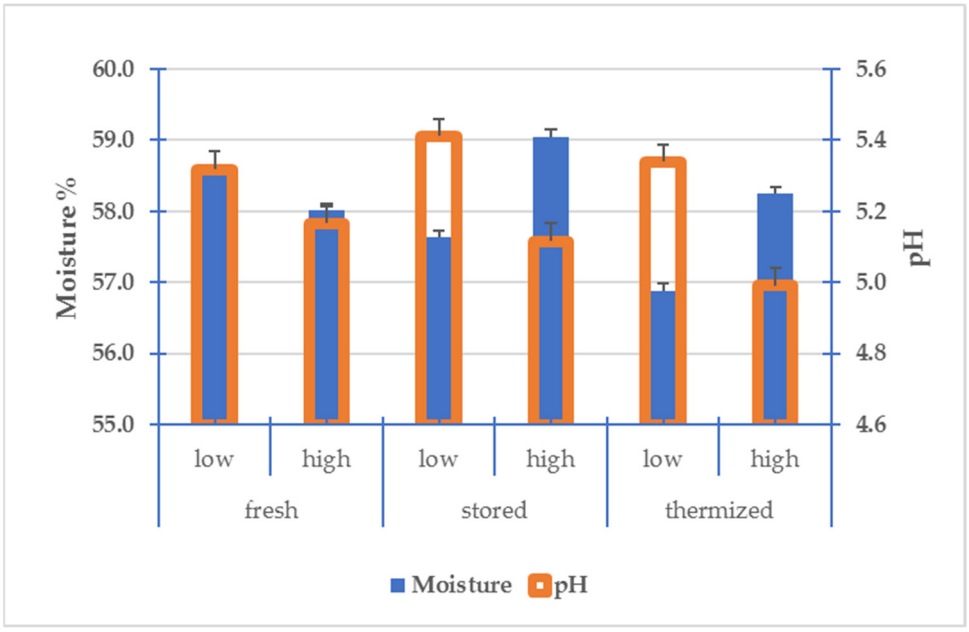

Figure 3. pH values and moisture content in cheese produced using fresh (F), stored (S), and thermized (T) milk subjected to low (L) or high (H) pasteurization.

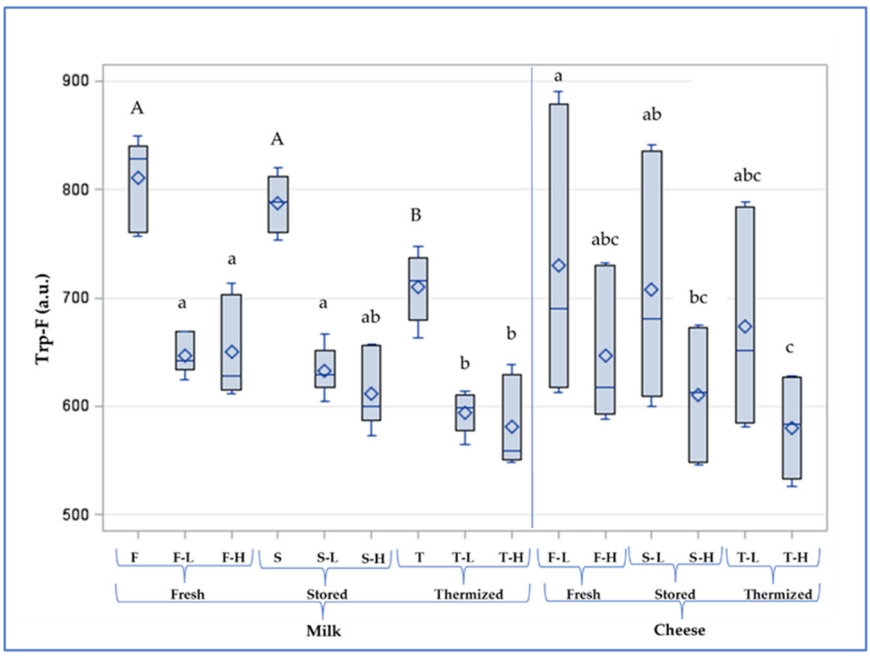

Figure 4. Comparison of Trp-F (Tryptophan fluorescence) in milk before pasteurization and after low and high pasteurization, and in the corresponding cheese made from each type of milk. Different letters indicate significant differences ($p < 0.05$) within the group of milk or cheese. In the milk group, capital letters were used for milk before pasteurization (F, S and T) while lowercase letters for milk after pasteurization (F-L, F-H, S-L, S-H, T-L, T-H). Milk before pasteurization: F = fresh milk, S = stored milk, T = thermized milk. Milk after pasteurization: L = low pasteurization (72 °C for 15 s), H = high pasteurization (77 °C for 15 s).

After pasteurization, T-L and T-H milk are confirmed to have the lowest Trp-F content, the highest values were obtained from F-L and F-H milk ($p < 0.05$) as detailed above (Table 2). Moreover, in all samples, Trp was not affected by the low and high pasteurization. The decrease in the Trp content found in milk before and after pasteurization (average value of low and high pasteurization) was about 17% for fresh milk and about 21% for the other two types of milk.

In opposite to milk, significant differences were found between the two types of pasteurization within F and T cheese groups (Figure 4). Among the groups, the value of F-L cheese was higher (730 a.u.; $p < 0.01$) compared to those of S-H (611 a.u.) and T-H cheeses (580 a.u.). Trp-F is an indicator of the whey protein denaturation [46] and therefore of the thermal damage that occurs in heat-treated milk. In our cheese samples, it was effective in discriminating cheese made with milk subjected to different treatments.

Figure 5 shows the results of the AMP content. The AMP-F values of the three milk samples before pasteurization were not significant and were similar to those found after pasteurization. The AMP level after pasteurization was highest in S-H milk ($p < 0.05$) and lowest in F-L and T-L milk, as reported above (Table 2), therefore it appears to be influenced mainly by the pasteurization temperature. In cheese the lowest values were obtained in F-L and F-H cheeses, respectively 50.6 and 51.2 a.u., significantly different ($p < 0.05$) from T-H cheese, 58.0 a.u. These results show that, by the AMP content of the cheese, one could discriminate fresh milk cheese from cheese whose milk has received the most intense heat treatment, i.e., thermization and high pasteurization.

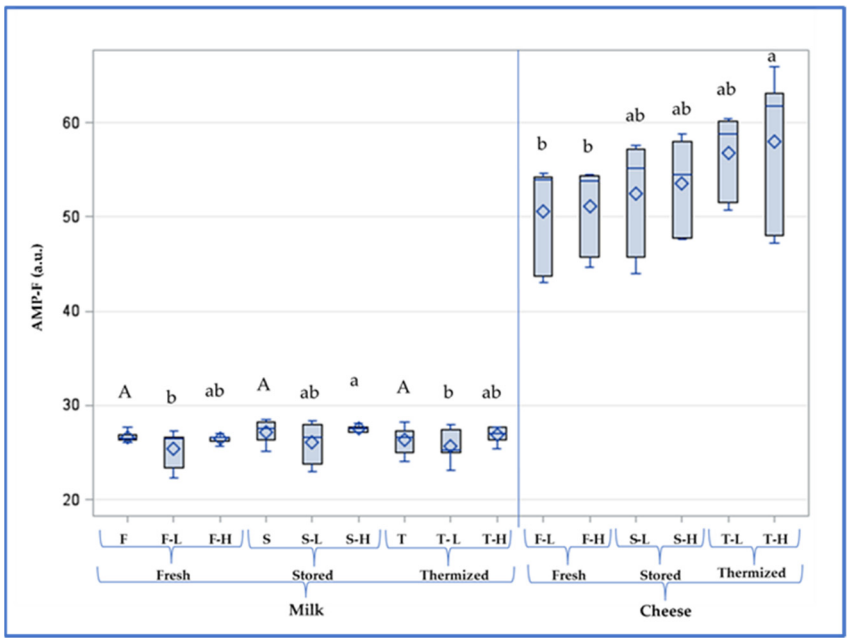

Figure 5. Comparison of AMP-F (advanced Maillard products fluorescence) in milk before pasteurization and after low and high pasteurization, and in the corresponding cheese made from each type of milk. Different letters indicate significant differences ($p < 0.05$) within the group of milk or cheese. For the milk group, different capital letters were used in milk before pasteurization (F, S and T) while lowercase letters in milk after pasteurization (F-L, F-H, S-L, S-H, T-L, T-H). Milk before pasteurization: F = fresh milk, S = stored milk, T = thermized milk. Milk after pasteurization: L = low pasteurization (72 °C for 15 s), H = high pasteurization (77 °C for 15 s).

The results about the antioxidant activity in milk and cheese are represented in Figure 6. The milk before pasteurization is more sensitive to long storage and thermization and the three values were significantly different from each other (F: 84.8% vs. S: 81.0% vs. T: 78.7%; $p < 0.05$). After pasteurization, the antioxidant activity was higher in milk F than in S and T milk samples and was affected by the pasteurization temperature, as

detailed above (Table 2). The decrease in antioxidant activity found in milk before and after pasteurization (average value of low and high pasteurization) was about 5% for all three types of milk.

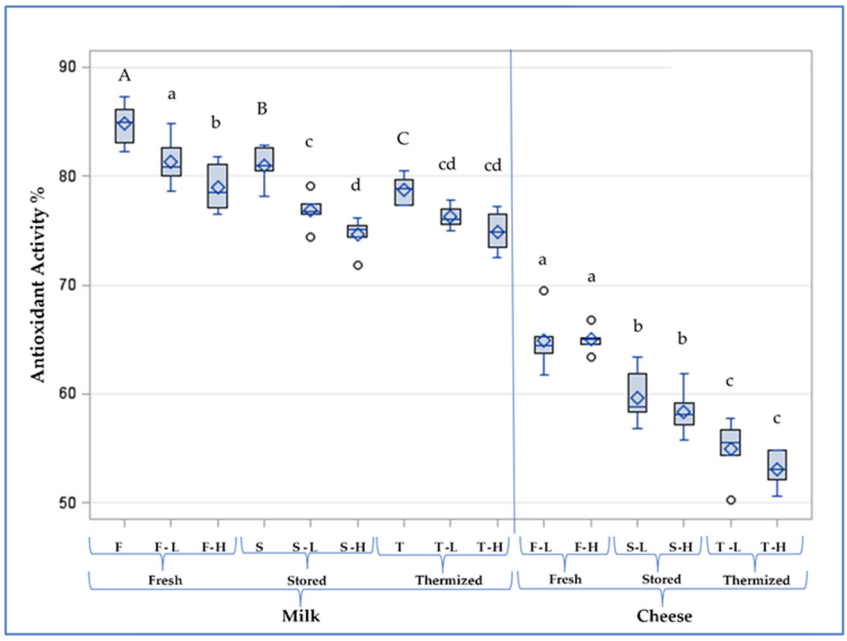

Figure 6. Comparison of antioxidant activity in milk before pasteurization and after low and high pasteurization, and in the corresponding cheese made from each type of milk. Different letters indicate significant differences ($p < 0.05$) within the group of milk or cheese. For the milk group, different capital letters were used in milk before pasteurization (F, S, and T) while lowercase letters in milk after pasteurization (F-L, F-H, S-L, S-H, T-L, T-H). Milk before pasteurization: F = fresh milk, S = stored milk, T = thermized milk. Milk after pasteurization: L = low pasteurization (72 °C for 15 s), H = high pasteurization (77 °C for 15 s).

It can also be observed that the reduction in antioxidant activity due to the cheese-making process is more marked than that due to the pasteurization treatment. No differences were found between the two pasteurization treatments within all three groups of cheese, while the differences between the three groups were significant ($p < 0.05$). Fresh milk cheeses had the greatest antioxidant activity, thermized milk cheeses had the worst (64.9 and 65.0 vs. 55.0 and 53.1%). From these results, it seems that it is possible to discriminate between the three cheese groups according to their antioxidant activity.

According to Lucas et al. [47], total antioxidant capacity varied mainly with the cheese-making process. During the cheese-making, a variable quantity of some compounds with antioxidant activity, such as β-carotene, vitamin A and vitamin E, is lost. This loss could be due to oxidative degradation by atmospheric oxygen and light and could be accelerated by increasing temperature and acidity [48,49]. In addition, some compounds are lost in the whey [47]. The DPPH values found in buffalo Ricotta cheese at 1 day (64.6%) and at 21 days (66.3%) of storage [50] were similar in comparison to those found in fresh milk cheese.

Furthermore, the reduction in antioxidant activity in S and T cheeses does not negatively affect the oxidation of fat. In fact, the MDA content is often used as a marker of oxidative damage in cheese, varied from 0.22 to 0.25 nmol g^{-1} and are not significantly different ($p = 0.4957$) in the three groups. These values were lower than average values found in buffalo Mozzarella cheese (0.36 nmol g^{-1}) [23] and in buffalo Ricotta cheese (1.51 nmol g^{-1}) [50]. The MDA content of our cheese samples was also lower than the data for other dairy products [51] and well below the threshold indicated by some authors for other foods [52].

4. Discussion

Oxidative processes are important factors regarding freshness and suitability of the milk for further processing and the oxidative stability of milk is the result of a delicate balance between the overall anti- and pro-oxidative processes. The imbalance of this equilibrium is an advantage of the prooxidative activity which induces a deterioration process that can decrease the technological quality of the milk and cause a lower sensory quality of the dairy products [10,15,53].

Our results showed that oxidation takes place throughout storage of milk with differences mainly between fresh milk and the two kinds of stored milk with the higher effect on thermized milk. There are a few investigations about the changes in antioxidant content during storage of milk [54]. Kant et al. [54] reported that up to 3 days of storage there are no significant changes in the antioxidant activity measured by DPPH assay in both raw and pasteurized milk while at 6 days of storage there is an increase in the level of oxidation. DPPH assay after 6 days of storage period indicated significant decline in antioxidant activity of both raw and heat-treated milk. The decrease in antioxidant activity is mostly depending on the degradation of antioxidant components, such as vitamins and enzymes. A prolonged period of storage could affect a decrease of vitamin C, A, and E content in milk.

Ascorbic acid (vitamin C) is the major water-soluble antioxidant in milk and can act as a strong free radical scavenger, but it is present in low concentration in milk, and it is very sensitive to treatment and storage period that considerably affect its content in milk. Vitamin C decreased in raw milk at 3 days storage and moreover, its content decreased in pasteurized milk compared to raw milk [54]. Loss of vitamin C in pasteurization of cow milk was 40%. With more intense heat treatments loss of vitamin C become even greater, at 82% [54].

Vitamin A and tocopherol up to 3 days of storage was maintained, while they decreased at 6 days. However, their concentration in pasteurized milk was not significantly different from their content in raw milk while when the milk is boiled their content decreased compared to raw milk [54].

Studies on human milk reported that glutathione peroxidase activity decreased while MDA increased in refrigerated milk even after 24 h confirm that storage of mother's milk results in a decrease of its antioxidant capacity associated with lipid peroxidation reactions [55]. However, contrasting data showed that refrigerated storage at 4 °C did not affect the oxidative status of human milk with no evidence of lipid peroxidation [56,57].

So, storage period and conditions could affect the antioxidant properties of milk, which could be related to the oxidative stability of pasteurized milk and dairy products. The same authors reported that the addition of ascorbic acid and tocopherol in milk enhanced the flavor and photooxidative stability during prolonged storage increasing its antioxidant activity as compared to non-supplemented milk [58].

Our results also show that the pasteurization treatment induced an evident protein denaturation which is more pronounced in thermized milk. In the same milk, no effect of the pasteurization temperature was found. Both results were most likely caused by the double heat treatment. The reduction of the antioxidant activity in the pasteurized milk compared to the starting milk was moderate. The effect of pasteurization on the antioxidant activity was less evident in F-milk which was influenced by the pasteurization temperature as was S-milk. Additionally, in this case, the milk subjected to thermization was not sensitive to the pasteurization temperature.

There are many studies on the effects of pasteurization and other heat treatments on antioxidants content in milk and the results showed that loss of natural antioxidants, protein unfolding, and formation of oxidation products or novel antioxidants greatly depends on the processing conditions [59]. In particular, heat treatment of milk can induce both the loss of natural antioxidants and the formation of novel oxidative molecules with a consequent decrease in the antioxidant activity of milk. However, milk antioxidant activity may increase due to protein unfolding and exposure of thiol groups, potentially

acting as hydrogen donors. Most of the consequences of milk heating are related to the development of the Maillard reaction but under the processing conditions usually carried out at industrial level to produce pasteurized milk, only the early phase of the Maillard reaction takes place [8]. These results showed a potential depletion in the overall antioxidant properties of milk depending on the time and temperature combinations of heat treatments and should be taken into consideration in the industrial processing of milk and milk products [16].

Although the effect of heat treatment on antioxidant activity and content of vitamins in milk was extensively studied, little information is available regarding the transition of antioxidant capacity during cheese-making with respect to alteration in vitamin A, E, and C [7,40]. Total antioxidant capacity was strongly correlated with vitamins content in milk, but chemical composition of cheese is considerably different from parent milk and changes in antioxidant components of cheese occurred during processes and ripening.

Lipophilic milk antioxidants are characterized by high levels of thermal stability, and they remain active in all dairy products regardless of the applied thermal processing method [9]. Results of an earlier investigation on migration of carotenoids from milk to cheese have shown that the content of retinol in cheese is influenced by heat treatment during manufacturing [60]. According to other authors [47,48], during the cheese-making process, an important amount of the lipophilic vitamins originally present in milk fat was lost: for vitamin A on average one-third, while the loss of vitamin E was around two-thirds.

Even the casein fractions are characterized by high levels of antioxidant activity [9]. Zulueta et al. [18] demonstrated that total antioxidant status of milk subjected to pasteurization and other processes was determined mostly by caseins which are characterized by greater thermal stability. Moreover, it is noted that production of bioactive peptides due to heat treatments and ripening can increase the antioxidant capacity of cheese [61].

Milk quality influences the quality of processed dairy products and offers a perspective on the merit of investing in quality. Dairy processors may incentive to use high-quality raw milk to improve processed product quality and manufacturing efficiencies. Using high-quality milk could increase cheese yields and improve texture and flavor characteristics in cheese and other products [54].

5. Conclusions

Antioxidant activity decreases from the starting milk to the milk after pasteurization to the final cheese. This reduction could be attributed to some factors (oxygen, light, temperature, acidity, whey loss) that affect the main compounds responsible for this activity, such as vitamins and enzymes. However, despite the losses that occurred during the milk treatments and in the cheese-making process, the residual antioxidant activity of the cheese was found to protect the fat from oxidation. Under our experimental conditions, the longer storage time of the milk, especially if preceded by heat pre-treatment, had a significant effect on the quality of milk and cheese. The greater antioxidant stability of cheeses made from fresh milk, closely linked to the greater presence of antioxidant compounds, is a valid reason to favors the production and consumption of these products.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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