Hindering milk quality storage deterioration by mild thermization combined with methylated chickpea protein

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Abstract

Methylated chickpea protein (MCP) or native chickpea protein (NCP) were supplemented to milk (0.5%) and combined with a mild thermization (65ºC/ 5 min) treatment before storing at 4ºC for 30 days. The influence of these combined treatments was assessed on milk physicochemical, nutritional and sensorial quality during storage. Supplementation of milk samples with MCP (0.5% w/v) significantly (p < 0.05) and considerably reduced the levels of the bacterial counts; i.e. total bacterial, psychrotrophic and *Pseudomonas* spp. counts by about 1.6-1.9 log CFU ml^{-1} after 16 days of storage at 4ºC. Within the same period, it could control the development of titratable acidity, limit lipolysis, attenuate proteolysis, maintain most of the vitamin contents, keep considerable heat stability, oxidative stability, rennetablity and sensorial properties.

Introduction

The traditional pasteurization is destined to eliminate potential spoilage and pathogenic microbes from raw milk while keeping the least possible changes in physicochemical and organoleptic quality. Heat treatments may be associated with negative effects on valuable milk bio-constituents particularly vitamins, so in most industrial practices short time heat treatment is preferred (Raynal-Ljutovac *et al.*, 2004). Pasteurization practices may lead to the destruction of important vitamins in milk, e.g. vitamin B1, B2, B12 and vitamin E (Macdonald *et al.*, 2011). At the same time, pasteurized milk may be still liable to microbial recontamination during subsequent handling and storage at refrigeration conditions, favoring the proliferation of many members of contaminating psychrotrophs (Dogan and Boor, 2003), whose biochemical activity may further affect milk nutritional, biochemical quality and shelf life (Fox *et al.*, 1989).

In the present research, a mild thermization treatment (65ºC / 5 min) is selected to avoid potential deleterious effects of higher heat treatments (72 - 96ºC) (Macdonald *et al.*, 2011) on the physicochemical quality as much as possible. However, this mild thermization may not be sufficiently adequate to control the bacterial contamination of milk and the associated biochemical changes during subsequent storage. This negative potentiality is compensated by the addition of methylated chickpea protein (0.5%)

which is an effective antimicrobial agent against both Gram negative and Gram positive bacteria (Sitohy and Osman, 2010). Recently, esterified legume proteins supplemented to raw milk were found to considerably reduce its levels of total bacterial count and psychrotrophic bacteria count during cold storage delaying the spoilage onset from 2 to 6 days (Sitohy *et al.*, 2011) and to counteract the potential spontaneous or artificial post-pasteurization contamination of milk during a 30-day cold storage (4ºC) (Mahgoub *et al.*, 2013).

The reflection of this complex situation on milk quality is still lacking. As a consequence, the objective of the present work was focused on the physicochemical, sensorial and nutritional quality of the stored milk when treated with such mild heat treatment (65ºC/ 5 min) combined with low supplementation (0.5%) of methylated chickpea protein. Based on the finding that chickpea protein possesses considerable antioxidant activity (Arcan and Yemeniciog˘Lu, 2007) supplementation of its esterified form to mildly heated milk may additionally exert some antioxidant activity counteracting the possible oxidative deterioration during storage.

Materials and Methods

Materials

Chickpea (*Cicer arietinum* L.) seeds were purchased from local market, Zagazig city, Sharkia, Egypt. Raw buffalo's milk was collected in a closed

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plastic container from a private farm located in Sharkia governorate, Egypt and was experimentally handled within one hour after milking.

Protein extraction and esterification

Chickpea seeds were ground to pass through a 1 mm^2 sieve and the resulting powder was defatted using a mixed solvent of chloroform: methanol (3:1 v/v) for 8 h. Dispersions of 5% (w/v) defatted chickpea flour in water were adjusted to pH 9 with 0.1 N NaOH at room temperature, shaken for 1 h and centrifuged for 15 min at 2000 x *g*. The extraction and centrifugation steps were repeated 3 times. The combined extracts were adjusted to pH 4.5 with 1 N HCl to precipitate chickpea protein which was subsequeny isolated by centrifugation at 2000 x *g* for 15 min. Crude protein was washed with distilled water, dispersed in a limited volume of distilled water, adjusted to pH 7.5, dialyzed for 72 h against distilled water and lyophilized (Johnson and Brekke, 1983). The total nitrogen was determined in native chickpea protein isolates (NCP) using micro Kjeldahl method according to AACC (2000) and converted to total protein content using the conversion factor 6.25.

Native chickpea protein (NCP) was esterified according to (Sitohy *et al.*, 2000) by dispersing a suitable amount (5% w/v) in concentrated methanol (> 99.5%). Amounts of hydrochloric acid equivalent to 50 Molar ratio (mole acid/mole carboxyl group) were added drop-wise at the start of the reaction. The reaction mixture was kept at 4°C under continuous stirring for a total period of 10 h, at the end of which the precipitate was isolated by centrifugation (10000 x *g* for 10 min at 4°C) and washed three times with an amount of methanol equivalent to the discarded supernatant. The final obtained precipitate was dissolved in an appropriate amount of distilled water and adjusted to pH 7.5 by 1 N NaOH. The obtained solution was dialyzed for 3 days against distilled water, lyophilized and kept at -20°C until analysis. The resultant methylated chickpea protein (MCP) was colorimetrically quantified for the esterification extent by the reaction with hydroxylamine hydrochloride using the procedure of (Halpin and Richardson, 1985) as modified by (Bertrand–Harb *et al.*, 1991).

Experimental milk storage

The delivered raw milk, almost obtained after milking, was maintained at 4°C for 1 h before dividing into nine equal portions of milk (200 mL) in 9 similar sterile screw-capped bottles. All milk portions were thermized at 65°C for 5 min. Three bottles were kept as such without any additions and served as control (single treatment). The second three bottles were

supplemented separately with NCP at 0.5% (w/v). The third three bottles were separately supplemented with MCP at 0.5% (w/v) (combined treatment). Samples were withdrawn from all bottles under aseptic conditions after 0, 2, 4, 6, 8, 10, 16, 24 and 30 days for the microbiological, physical and chemical analysis. The whole storage experiment was exactly repeated using the same design one month after the first one and the final recorded results were the mean of the two experiments.

Microbiological analysis

Milk samples (10 mL) were homogenized in 90 mL peptone solution (0.1% w/v) for 2 min and decimal dilutions were prepared with the same solution. Aliquots of the dilutions were plated onto Plate count agar (PCA, Merck, 1.05463), incubated for 2 or 10 days at 30 or 7°C for the recovery of total bacterial count (TBC) or psychrotrophic bacterial count (PBC) (Frank *et al.*, 1992), respectively. For Pseudomonas spp. count (PSC) dilutions of milk samples were plated onto Pseudomonas agar base (PAB) supplemented with 10 mg mL^{-1} cetrimide fucidin (Oxoid), incubated aerobically at 25°C for 2 days. For psychrotrophic proteolytic bacteria count (PPB), milk samples were plated on Milk agar (Oxoid, CM0021) incubated for 10 days at 7°C and for the psychrotrophic lypolytic bacteria count (PLB) Tributrin agar was used with 10 day incubation at 7°C. After the incubation period, the plates with 30–300 colony forming units (CFU) were enumerated and the results were expressed as CFU mL^{-1}.

Lipolytic activity

Lipolytic activity in milk was measured as a function of the changes in the levels of free fatty acids after solvent extraction with chloroform: methanol (2:1 v/v) followed by titration with an alkaline solution (Deeth *et al.*, 1975).

SDS-PAGE

SDS-PAGE was performed on a discontinuous buffered system according to (Laemmli, 1970). Stacking and separation gels were 3% and 10%, respectively. The electrode buffer (pH 8.3) contained 0.025 mol Tris, 0.192 mol glycine, and 0.1% SDS. Milk samples were centrifuged at 400 x *g* for 5 min and the fat layer was decanted. An aliquot of the defatted milk (20 μL) was mixed with 20 μL of SDS-sample buffer (4% SDS, 3% β-mercapto-ethanol, 20% glycerol, 50 mM Tris-HCl, pH 6.8 and traces of Bromo-phenol blue), heated at 96°C for 3 min and 10 μL aliquots from the final mixture were electrophoresed. Running on the stacking and separation gel was conducted at

10 and 20 mA, respectively. Staining was performed with Coomassie Brilliant Blue R-250 dye.

Titratable acidity and pH

Titratable acidity was determined in milk samples stored at 4°C during 30 days and expressed as lactic acid (%) according to the standard methods (AOAC, 1997) and pH was assessed in the same samples by pH meter (pH 211 HANNA instruments Inc. Woonsocket- USA made in Romania).

Vitamins content

According to the procedures outlined in (AOAC, 1997), vitamins B1, B2, B6, B12, Nicotinic acid and folic acid were determined by HPLC (Shimadzu, Japan) and vitamin C was determined by titration with 2, 6-dichlorophenol-indophenol.

Mineral content

Treated milk samples were centrifuged at 400 x *g* for 5 min and the fat layer was decanted before re-centrifuging at 12000 x *g* for 15 min for separating whey from caseins. The two fractions were digested in concentrated HNO_3 (AOAC, 1997), quantitatively transferred to a 25 mL volumetric flask and made up to volume with deionized water. Calcium and phosphorus were determined using atomic absorption spectrometer (Varian 220FS Spectr AA, Les Ulis, France).

Milk rennetability

Milk rennetability test was conducted according to Gallagher and Mulvihill (1997). Milk samples were adjusted to pH 6.7 and equilibrated at 30°C for 10 min, rennet solution (1 % w/v) was added (10 µL mL^{-1}) and left to stand at 30°C until the coagulation is visually observed. The time of the rennet coagulation was recorded and taken as an index of rennetability.

Heat stability

Heat coagulation time (HCT) at 100°C was determined according to (Davies and White, 1966) with some modifications. A representative volume (5 mL) of milk from each sample was placed in test tube at 100°C and heat coagulation time was recorded and taken as an index of heat stability.

Antioxidant activity

All samples were subjected to antioxidant scavenging activity test using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by (Blois, 1958) with some modifications. Briefly, 500 µL of samples were mixed with 3 mL of 0.2 mM methanolic-DPPH solution, incubated for 0, 15, 30 and 60 min at room temperature and the absorbance

was measured at 520 nm. The wavelength of maximum absorbance of DPPH was recorded as A(sample), using a spectrophotometer (JENWAY, 6405 UV/Vis, U.K.). A blank experiment (without the test material) was carried out applying the same procedure and the absorbance was recorded as A (blank). The free radical scavenging activity of was calculated as percent inhibition according to the following equation:

$$\% \text{ inhibition} = ([A(blank) - A(sample)]/A(blank)) \times 100$$

All measurements were performed in triplicate. Free radical scavenging activity of NCP and MCP in aqueous solutions (0.5%, w/v) was assessed according to the same method.

Sensorial evaluation

All milk samples were subjected to sensory evaluation (color, taste and odor) by a trained panelist group of 10 members. Every attribute was given grade10 as the best quality while lower ones were given relatively lower grades as judged by the panelist group.

Statistical analysis

All experiments were performed in triplicates and results of the storage experiments were expressed as the mean plus the standard error. Data were statistically analyzed using ANOVA variance analysis through the general linear models (GLM) procedure of the statistical analysis system software (SAS version 9.1, SAS Institute, Inc., 2003). Least significant differences were used to separate means at $p < 0.05$.

Results and Discussion

Chemical characterization of methylated chickpea protein

Protein content was 91% in both NCP and MCP. The product MCP attained 80% esterification extent, i.e. 80% of the carboxyl groups were blocked by transformation into carboxylate methyl resulting in a positively charged protein with a more basic isoelectric point of ca. pH 8 (Sitohy and Osman, 2010). Some acute and sub-acute toxicological studies using Albino rats have shown that MCP is free from toxicological hazards (data will be published elsewhere). Accordingly, possible applications of this protein in milk treatments may not be precluded by potential toxic hazards.

Milk microbiological status
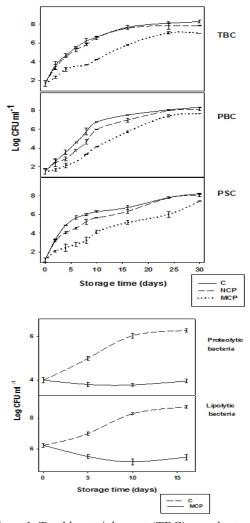
The results presented in Figure 1 show the

Figure1. Total bacterial count (TBC), psychrotrophic bacterial count (PBC), *pseudomonas* spp. Count (PSC), in thermized buffalo milk as supplemented with native (NCP) or methylated chickpea protein (MCP) at 0.5% (w/v) during 30 day storage as well as proteolytic and lipolytic bacteria during 16 day storage at 4°C.
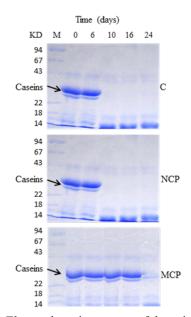

Figure 2. Electrophoretic patterns of thermized buffalo milk (C) as supplemented with native (NCP) or methylated (MCP) chickpea protein (0.5% w/v) during 24 day storage at 4°C.

microbiological status of the levels of total bacterial count (TBC), psychrotrophic bacteria count (PBC) and *Pseudomonas* count (PSC) in milk thermized at 65°C/5 min, supplemented with NCP or MCP (0.5%, w/v), and stored at 4°C for 30 days. It can be observed that TBC, PBC and PSC grew in the un-supplemented thermized milk to reach maximum levels after 16 days of storage at 4°C (log 7.68, log 7.51 and log 6.74 CFU mL^{-1}, respectively). This parallel increases in the three bacterial counts are due to the fact that *Pseudomonas* spp. (PSC) constitute the most common organisms in milk at the spoilage time (Mc phee and Griffiths, 2002) and represent the major component of PBC and TBC (Fox *et al.*, 1989 ; Uraz and Çitak, 1998). Supplementation of thermized milk with NCP (0.5% w/v) did not significantly ($p < 0.05$) affect the three bacterial counts (TBC, PBC and PSC) compared to the control (thermized

un-supplemented milk) but supplementation with 0.5% MCP significantly ($p < 0.05$) and considerably reduced these counts. After 16 days of storage at 4°C, the MCP-supplemented thermized milk reached bacterial counts comparatively 1.6-1.9 log lower than the un-supplemented thermized milk. These inhibitions in the bacterial load may restrict the potential associated physicochemical changes and extend milk shelf life. MCP-supplemented thermized milk reached a pre-spoilage TBC level of log 5 after a longer storage period (16 days) than in the case of the un-supplemented thermized milk (6 days). The antimicrobial action of the methylated protein (MCP) is mainly due to its acquired cationic character enabling to disrupt the bacterial cell membranes through the interactions with their negatively charged components. Thus, it may probably exert some protective action on milk constituents against the microbial attack.

Proteolytic and lipolytic changes

The lipolytic and proteolytic bacterial counts increased in the thermized un-supplemented milk proportionally with the storage time indicating that most of the detected psychrotrophic bacteria may have proteolytic and lipolytic activities (Figure 1) in accordance with Matta and Punj (1999). Milk receiving the combined treatment (thermized and supplemented with 0.5% MCP) evidently and significantly ($p < 0.05$) produced lower levels of lipolytic and proteolytic bacterial counts after 10 and 16 days of storage at 4°C, which were even lower

Table 1. Titratable acidity and pH of mildly thermized (65°C/ 5 min) milk as supplemented with methylated chickpea protein (0.5%) after different intervals of storage at 4°C

Preservation time (days)	Titratable acidity		pH	
	Control	MCP	Control	MCP
0	0.15±0.00	0.15±0.00	6.86±0.00	6.86±0.00
2	0.18±0.01	0.17±0.00	6.59±0.00	6.67±0.01
4	0.19±0.00	0.17±0.02	6.45±0.01	6.56±0.02
6	0.2±0.01	0.18±0.03	6.23±0.02	6.52±0.01
8	0.22±0.00	0.19±0.00	6.13±0.11	6.41±0.00
10	0.25±0.02	0.19±0.01	6.02±0.02	6.38±0.00
16	0.31±0.01	0.20±0.02	5.85±0.01	6.33±0.01
24	0.43±0.00	0.22±0.00	5.43±0.00	6.1±0.02
30	0.55±0.01	0.25±0.00	4.86±0.03	5.76±0.00

than the initial counts referring to nearly complete absence of degrading activities. This result was supported by SDS electrophoretic pattern showing completely intact casein bands in the combined treatment milk after 16 days of storage at 4°C (Figure 2), contrasted to complete casein proteolysis in the un-supplemented thermized milk after only 10 days of storage. The observed strong matching between casein degradation and the *Pseudomonas* count may be due to its considerable extracellular proteolytic activity (Hantsis-Zacharov and Halpern, 2007) and production of thermostable casein hydrolyzing proteases (Dogan and Boor, 2003). Keeping casein in the intact form in case of the combined treatment refers to good biochemical quality of milk and good preservative status (Fox *et al.*, 1989).

The free fatty acid content of the un-supplemented milk increased from 3.76 meq L^{-1} at zero time to 5.64 and 8.46 meq L^{-1} after 10 and 16 days of storage at 4°C, respectively while it remained nearly at the same initial level (3.76-3.96 meq L^{-1}) in the combined treatment milk referring to the absence of the lipolytic activity (data not shown). This absence of both proteolytic and lipolytic activities in the combined treatment milk will most probably maintain the biochemical and sensorial quality of milk extending its shelf life. The increase in free fatty acids (FFA) in stored thermized milk is in accordance with previous findings (Antonelli *et al.*, 2002 ; Zygoura *et al.*, 2004) and may be caused by the elevated level of psychrotrophic count (> 7 log CFU mL^{-1}) triggering the activity of bacterial lipase (Law, 1979). The observed casein proteolysis in stored thermized milk agrees with (Zygoura *et al.*, 2004) and may be ascribed to the microbial proteases rather than to plasmin (Guinot-Thomas *et al.*, 1995). The absence of serum protein proteolysis indicated by SDS-electrophoresis in case of all milk samples during the whole storage period (30 days at 4°C) may be due to their native resistance to proteolytic degradation (Mc phee and Griffiths, 2002).
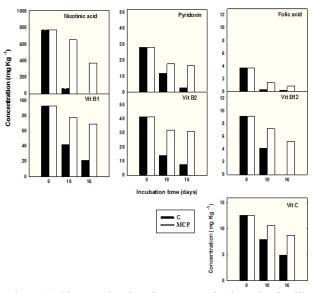

Figure 3. Changes in vitamin contents in thermized milk as upplemented with MCP (0.5% w/v) during 16 day storage at 4°C.

Milk acidity

Milk receiving the combined treatment (thermization and MCP-supplementation) showed much slighter changes in pH and titratable acidity than the solely thermized milk during 30 days storage at 4°C storage period (Table 1). Differences between the two groups of milk samples were statistically significant (p < 0.05) while NCP Supplemented thermized milk did not produce significant changes (p < 0.05) from the solely thermized milk in these two parameters (data not show).

MCP-supplemented thermized milk maintained an acceptable acidity level (0.20 ± 0.02) coupled with an acceptable pH 6.33 ± 0.01 after 16 days of storage at 4°C while the un-supplemented milk went down to unacceptable levels. This attenuating effect on acidity development is apparently due to the antimicrobial action of MCP (Sitohy and Osman, 2010; Mahgoub *et al.*, 2013) and is supported by the previously shown microbiological analytical results (Figure 1). A high rate of acidity development in solely thermized milk is apparently quicker and is apparently due to uncontrolled microbial contamination provoking higher extents of acid production. So, it can be inferred that the combination between mild thermization and low MCP supplementation (0.5%) can control the development of titratable acidity maintaining it within acceptable levels in milk during cold storage.

Milk vitamins

The data in Figure 3 show generally that vitamins contents in the solely thermized milk decreased gradually with the time of storage at 4°C. It could be observed that the initial mild thermization treatment

could avoid some vitamin destruction normally caused by traditional pasteurization (Macdonald, 2011). However, this treatment could not protect vitamins from the bacterial destruction during subsequent cold storage. The most storage affected vitamins were nicotinic acid and folic acid which went down to 8%, 0% and 8%, 6% from their original levels after 10 and 16 days of storage at 4°C, respectively. Vitamin B12 disappeared completely after 16 days of storage at 4°C in the solely thermized milk. Generally most vitamins were decreased to more than 50% after 10 days of storage except vitamin C which retained 63 % of its original level in accordance with (Andersson and Oste, 1994). The losses recorded in vitamin B2 are comparable to those reported by (Zygoura *et al.*, 2004).

In the milk receiving the combined treatment most vitamins were maintained to levels higher than 70% of the original level (nicotinic acid, vitamin B1, vitamin B2, vitamin B12 and Vitamin C). Some vitamins (B1, B2, C) retained more than 70% of their original levels after 16 days of storage at 4°C. This protective effect against vitamin degradation may partially be ascribed to two factors; the antimicrobial activity of the modified protein limiting the microbial degradation and consumption and its antioxidative activity protecting some vitamins, e.g. riboflavin and ascorbic acid from oxidation. So, the addition of MCP to the thermized milk may represent a good physicochemical combined treatment.

Mineral distribution and milk rennetability

The data in Table 2 trace the changes in diffusible and casein-bound calcium in milk samples stored at 4°C. Calcium moved into diffusible status accompanied by a parallel increase in diffusible phosphorus in the solely thermized milk with the advance of the storage period. This is apparently a result of the observed storage-driven pH drop. On the other hand, the combined treatment milk exhibited only minor changes in these two biochemical indicators probably due to limited pH drop. As a result, the rennet coagulation time (RCT) of the solely thermized milk significantly increased ($p < 0.05$) by about 20 and 30 times that of the fresh sample after 10 and 16 days cold storage, respectively. This considerable deterioration in milk rennetability may result from two factors; the release of calcium form casein micelles and the nearly complete casein degradation after 10-16 days of storage at 4°C. The disturbance in calcium distribution between whey and casein micelles may provide an explanation for the failure of rennet coagulation (Dalgleish, 1992). Since most of the casein is non-specifically hydrolyzed by
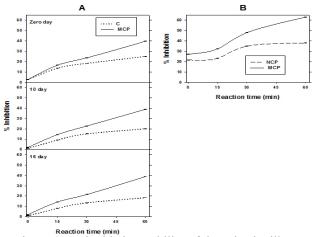

Figure 4. Antioxidative stability of thermized milk as supplemented with MCP (0.5%) during 16 day storage at 4°C (A) and DPPH scavenging activity of MCP and NCP solution (0.5%, w/v) (B).

the bacterial proteinases in the solely thermized milk during storage as observed in the SDS electrophoretic pattern (Figure 2), both stages of rennet coagulation (Davies and White, 1966) (specific hydrolysis of κ-casein at the phe-met and aggregation of casein micelles) are hindered.

On the other hand, only slight increases in RCT were observed in the combined treatment after corresponding storage periods probably due to maintained casein fractions and normal levels of bound calcium. Since milk rennetability forms the basis of cheese manufacture, it can be concluded that the combined treatment milk is quite appropriate for application even if stored for as long as 16 days at 4°C. The protective effect of MCP will assure good milk rennetability since it maintains the intact casein as well as the bound calcium essential for this process.

Heat stability

Solely thermized milk resisted heat coagulation at its fresh status (heat coagulation time at boiling > 60 min) but after storage for 10-16 days it coagulated after only 2 minutes (data not shown) in accordance with (Raynal-Ljutovac *et al.*, 2004) reporting a dramatic decrease in heat stability of goat milk during cold storage. On the other hand, combined treatment milk stored at 4°C for 10-16 days resisted heat coagulation for more than 60 min (data not shown).

The accelerated heat coagulation in the solely thermized milk may be due to different synergetic factors including pH drop (Table 1), casein hydrolysis (Figure 2) and the release of diffusible calcium (Table 2). The proteolytic action of plasmin on milk proteins was reported to reduce the heat stability of milk (Crudden *et al.*, 2005). Heat stability was also reported to be neutral pH-dependent (Gallagher and Mulvihill, 1997) and associated with high content

Table 2. Changes in calcium (mg 100 ml^{-1}) distribution between whey and casein during milk storage at 4°C and rennet coagulation time (RCT) at pH 6.7 and 30°C

Time of storage (days)	Control milk				MCP milk			
	Milk pH	Whey Ca	Casein Ca	RCT (min)	Milk pH	Whey Ca	Casein Ca	RCT (min)
0.0	6.86 ±0.00	6.41 ±0.12	31.86 ±0.12	2	6.86 ±0.02	6.41 ± 0.11	31.86 0.13	3
10	6.02 ±0.02	11.2 ±0.11	27.07 ±0.21	39	6.38 ±0.03	6.52 ± 0.06	31.86 0.14	3.5
16	5.85 ±0.01	16.89 ±0.08	21.41 ±0.17	>60	6.33 ±0.01	6.83 ± 0.11	31.27 0.16	4

of bound calcium (Montilla and Calvo, 1997). So keeping milk heat stability by supplementing thermized milk with MCP is apparently due to its pH maintaining action, bound calcium and casein during cold storage (10-16 days).

Oxidative stability

The oxidative stability of single and combined treatment milk (solely thermized or thermized and MCP supplemented) stored at 4°C for 0-16 days is shown in Figure 4 (A). The single thermization treatment showed diminishing oxidative stability proportional to the time of storage; the capacity of oxidation inhibition capacity decreased from about 25 to 20 then 18% at storage periods of 0.0, 10 and 16 days, respectively. On the other hand, the combined treatment showed nearly stable oxidative stability; the capacity of oxidation inhibition was in the level of 40% for the three storage periods. The relative superiority in oxidative inhibition capacity of the combined treatment over the single one at zero time is apparently due to the added MCP whose action extended over the whole period of storage (16 days). To verify whether the oxidative inhibition is due to the original protein identity or the chemical modification, this parameter was measured in aqueous solutions (0.5%) (Figure 4 B) indicating that it was much higher in MCP than NCP albeit some activity in the native form in accordance with (Arcan and Yemeniciog˘Lu, 2007). So, esterification does not only endow protein with antibacterial activity but also with some antioxidative capacity. The relatively lower antioxidative capacity of MCP against DPPH in milk than in solution may be due to the reaction of DPPH with other oxidative species in milk. Native protein may initially have antioxidative activity accentuated by the chemical modification and associated conformational changes exposing some regions of the protein molecule to react with the free radicals. The presence of the ester bond in the MCP may scavenge the free radicals by virtue of its oxygen atoms lone pairs. The enhanced solvent accessibility to amino acid residues triggered by the modification and the increased hydrophobicity may enhance the protein scavenging reactivity in accordance with

(Elias *et al.*, 2008). Free radical scavenging will preclude the termination of lipid oxidation, since their formation constitutes the first step of lipid oxidation. This will in turn diminish the formation of lipid off-flavors and maintain milk acceptability, sensorial quality and shelf life.

Sensorial characteristics

Sensorial traits of single and combined treatment milk samples stored for 0-16 days at 4°C were assessed by a group of 10 well trained panelists (data not shown). It was apparently clear that sensorial traits undergone progressive deterioration with storage time for all samples. Color, taste and odor of single treatment milk were reduced by about 80, 70 and 80% of the initial level after 10 day storage at 4°C, respectively. Further deterioration was recorded when extending storage up to 16 days. Relatively much slower deterioration was noticed in case of the combined treatment (MCP-supplemented thermized milk) after 10-16 days of storage at 4°C, i.e. the allover sensorial quality was never below 90% of the original value up to the 16th day. So, supplementation of thermized milk with MCP can help maintain its sensorial quality for 10-16 days of storage at 4°C.

The deteriorated sensorial properties of control milk by storage are mostly due to bacterial proteolytic and lipolytic changes or oxidation products causing off-flavor products. The antibacterial potency of MCP limited these activities when supplemented to the mildly thermized milk. The previously recorded antioxidative activity of MPC may preclude the light or autoxidation occurring in pasteurized milk stored at cold conditions and thus hindering the sensorial deterioration which limits the milk shelf life (Karatapanis *et al.*, 2006). Proteolysis and lipolysis incurred in stored thermized milk may be associated with casein hydrolysis and fat lipolysis, releasing unsaturated fatty acids liable to oxidation giving rise to off-flavor and rancid odor (Deeth and Fitz-Gerald, 1994) deteriorating milk sensorial quality. Limiting the proteolytic and lipolytic activities by MCP-supplementation introduces another pathways of the mechanism maintaining milk sensorial quality.

It can be summed up that milk receiving mild thermization treatment combined with MCP supplementation (0.5%) formulated a process maintaining the sensorial, nutritional and physicochemical quality of milk during 16 days of cold storage, essentially due to the antimicrobial and antioxidant activity of the added MCP. The impact of these two activities were reflected on maintaining many quality aspects of the stored milk; acidity level, proteolysis and proteolytic changes, heat and

antioxidative stability, vitamin content as well as mineral distribution and rennetability. Maintaining the physicochemical quality of the stored milk for longer period will extend milk shelf life for direct consumers and the food technological applications.

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