Original scientific paper - Izvorni znanstveni rad UDK: 637.112

Effect of nitrogen gas flushing treatments on total antioxidant capacity and ascorbic acid content in raw bovine milk during cold storage

doi: 10.15567/mljekarstvo.2017.0208

Oguz Gursoy[1], Patricia Munsch-Alatossava[2], Kubra Ertan[1], Yusuf Yilmaz[1], Tapani Alatossava[2]*

[1]Mehmet Akif Ersoy University, Faculty of Engineering and Architecture, Department of Food Engineering, TR-15030 Burdur, Turkey
[2]University of Helsinki, Faculty of Agriculture and Forestry, Department of Food and Environmental Sciences, FI-00014 Helsinki, Finland

Received - Prispjelo: 16.08.2016.
Accepted - Prihvaćeno: 06.04.2017.

Abstract

Continuous nitrogen gas (N_2) flushing extends the shelf life of raw milk (RM) during cold storage. The effect of N_2 treatment on the total antioxidant capacity (TAC) and ascorbic acid (AA) content of RM was determined during cold storage. TAC of RM or deproteinized RM was determined by ABTS and DPPH methods, while L(+)-AA content of RM was determined chromatographically on days 0, 4 and 7 during storage at 6±1 °C. With the ABTS method, the TAC of RM decreased from 472.33±16.70 to 369.47±62.06 μM TEAC while it reduced from 13.30±0.84 to 8.20±0.66 μM TEAC with DPPH method during cold storage. TAC of RM determined with ABTS method decreased after 4 day-storage; however, they remained statistically similar for N_2-treated samples during 7 day-storage. The AA content of RM ranged from 14.06 to 10.76 mg/L during storage but N_2-treatment did not influence AA content significantly. Deproteinization reduced TAC values of milk samples significantly, and the reduction with the ABTS method was about 47.50 % for control samples cold-stored for four days, while it was 11.67 % for N_2-treated deproteinized RM. In conclusion, N_2-flushing through the headspace of milk containing vessels showed a significant protective effect on the antioxidant components of RM during cold storage.

Key words: raw milk, nitrogen gas (N_2), shelf-life, antioxidant, ascorbic acid

Introduction

Cellular formation of free radicals and other reactive oxygen species such as superoxide, hydroxyl, peroxyl and alkoxy radicals takes place continuously by several mechanisms as a part of normal cellular functions (Young and Woodside, 2001). There is a sensitive balance between oxidizing agents, which are produced in living systems in excessive amounts for various reasons (i.e. chemical oxidants, UV and air pollution), and antioxidants from endogenous and exogenous sources. Disruption of this balance towards oxidants (oxidative stress) can play an important role in etiopathogenesis of several diseases (Buyuktuncel, 2013). Antioxidants interact with free radicals and cease the chain reaction before cellular components are damaged (Oroian and Escriche, 2015). Antioxidants can demonstrate their function by different mechanisms such as (i) scavenging free radicals, (ii) binding of metal ions, (iii) scavenging oxygen, (iv) converting hydroperoxides to non-radical species, (v) absorbing UV radiation and (vi) deactivating singlet oxygen (Gordon, 2010). The intake of antioxidants can prevent oxidative stress formation in living organisms. Several epidemiological studies have shown that dietary intake of foods containing natural antioxidants is highly associated with the decreased risk of coronary heart disease (Young and Woodside, 2001; Virgili et al., 2010), tumor development (Johnson, 2010),

*Corresponding author/Dopisni autor: Phone/Tel:+90 248 213 27 23, E-mail: ogursoy@yahoo.com, ogursoy@mehmetakif.edu.tr

and lowering incidence of different diseases and/or complications such as diabetes (Baynes, 1991) and cataract (Spector, 1995). Thus, the prevention of the losses of individual antioxidant compounds together with the protection of the total antioxidant capacities of foods throughout food production chains become critically important in providing high dietary intake of antioxidants in human nutrition.

Milk and dairy products contain several compounds having antioxidant properties. Main antioxidant compounds in milk are proteins (α-, β- and κ-caseins and lactoferrin), enzymes (superoxide dismutase, catalase and glutathione peroxidase) (Cervato et al., 1999; Lindmark-Mansson and Akesson, 2000; Pravst et al., 2000), vitamins (vitamin E, C and A), coenzyme Q10, carotenoids (mainly β-carotene) (Lindmark-Mansson and Akesson, 2000), phenolic compounds (Vazquez et al., 2015) and organic acids (uric acid) (Zulueta et al., 2009). The concentration of antioxidant compounds in milk is affected by both feeding rations of animals and milk storage conditions (Lindmark-Mansson and Akesson, 2000). Since there are numerous antioxidant components in milk and dairy products, the total antioxidant capacity measurement may be a useful method for detecting the sum of the antioxidant role of each component in milk (Gjorgievski et al., 2014).

Numerous bacterial genera successfully survive and grow despite the cold chain conditions during raw milk storage and transportation. More precisely cold storage does not prevent the growth of psychrotrophic bacteria which can constitute more than 90 % of the total bacterial population in cooled raw milk (Cousin, 1981; Samaržija et al., 2012). Psychrotrophs are usually able to form extracellular, heat-stable enzymes (proteases and lipases), which are mainly responsible for spoilage of milk and dairy products (Cousin, 1981). Some psychrotrophic bacterial species (a majority are Gram-negative representatives) have also shown to exhibit multiple antibiotic resistance features (Munsch-Alatossava and Alatossava, 2007). Thus, controlling psychrotrophic bacterial growth during cold storage of raw milk is important for both - technological and human health aspects. Several studies have indicated that the combination of modified atmosphere treatments (based on the use of CO_2, N_2 or mixtures of both) combined with cold storage may have a significant potential to extend shelf life of raw milk (Martin et al., 2003; Dechemi et al., 2005; Rajagopal et al., 2005). Previously, our research group showed that continuous N_2 gas flushing when applied in a so-called "open system" improved the microbiological quality and shelf life of raw and pasteurized milk samples during cold storage (Munsch-Alatossava et al., 2010a,b; Munsch-Alatossava et al., 2011; Munsch-Alatossava et al., 2013). Although, there are some evidences regarding improved microbiological quality of raw milk by batch or continuously treated modified atmospheres, to the best of our knowledge, no study that considered the effect of N_2 treatment on the total antioxidant capacity of raw milk has been published so far. Therefore, the objective of this research was to determine the effect of continuous N_2 flushing on the total antioxidant capacity and ascorbic acid content of raw milk samples during cold storage, by considering the same experimental setting as for previous investigations.

Materials and methods

Chemicals

2,2'-azinobis(3-ethylbenzothiazoline-6- sulfonic acid) (ABTS), ethanol (≥99.9 %), potassium persulfate and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). Methanol was obtained from Sigma Chemical Co. (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®, water-soluble vitamin E analogue) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Fluka (St. Louis, MO, USA). All other chemicals used were of analytical or HPLC grade. L(+)-ascorbic acid was purchased from PanReac AppliChem (Darmstadt, Germany).

Nitrogen gas treatment and microbiological analyses of raw milk samples

Three raw milk samples, representing lorry tank milk from June and August 2014, provided by the Helsingin Meijeriliike Ltd. (Helsinki, Finland) were considered. Experimental setup previously described by Munsch-Alatossava et al. (2010a) was used for the continuous N_2 flushing treatments of raw milk (Figure 1). Briefly, experiments were conducted in a cold room at a constant temperature of 6 °C. For nitrogen treatments, a glass bottle was connected to a flowmeter (Brooks Instruments B.V., Veenendaal, the Netherlands), via 0.2-μm sterile filters (Schleicher & Schuell GmbH, Dassel, Germany). The purity of nitrogen gas (AGA Ltd, Riihimäki, Finland) was 99.999 %. For nitrogen treatments, the headspace of a bottle was continuously purged by fresh nitrogen gas at a flow rate of 120 mL/min. Raw milk samples (100 mL) were continuously mixed with a magnetic stirrer (Variomag, Oberschleißheim, Germany) for both the control (C) and the N_2-treated milk, while cold stored at 6 °C in a refrigerated water bath (MGW Lauda MS/2, Lauda-Königshofen, Germany) during the course of the experiments. At the given sampling times, the gas flow was shortly interrupted and 0.5 mL of raw milk samples was serially diluted and cultured on PCA agar for 3 d at 30 °C in aerobic conditions. Simultaneously, milk fractions were collected and frozen (-20 °C) until subjected to chemical analyses.

Milk deproteinization

The procedure described by Zulueta et al. (2009) was used for the deproteinization of milk samples. Each of the raw milk sample (N_2 flushed or untreated) (5 mL) was deproteinized by the addition of trichloroacetic acid solution (5 mL, 20 % w/v). Subsequently, the milk samples were incubated for 10 min at 42 °C to remove all milk proteins, then cooled to room temperature and centrifuged for 10 min at 9500 g at room temperature. The obtained supernatants were collected from protein precipitates, and then promptly analyzed.

ABTS Radical Scavenging Assay

Total antioxidant capacity of raw and deproteinized raw milk samples corresponding to TCA supernatant fractions obtained from the corresponding raw milk was determined by the ABTS method described by Re et al. (1999), with slight modifications. Stock solution of ABTS radicals were prepared by the addition of potassium persulfate (2.6 mM) into aqueous solution of ABTS (7 mM), and the mixture was stored at room temperature for 12-16 h in dark place. The working solution was prepared by diluting the stock solution with methanol (final absorbance was about 1.1±0.02 at 734 nm). Then, 0.3 mL raw milk or TCA supernatants (deproteinized raw milk) were transferred into the working solution (2.7 mL). After incubating the mixture at room temperature for 30 min, the samples were centrifuged at 12,000 g for 2 min at room temperature. Decrease in absorbance values was measured at 734 nm against methanol as a reference. Results were expressed as Trolox® equivalent antioxidant capacity (TEAC).

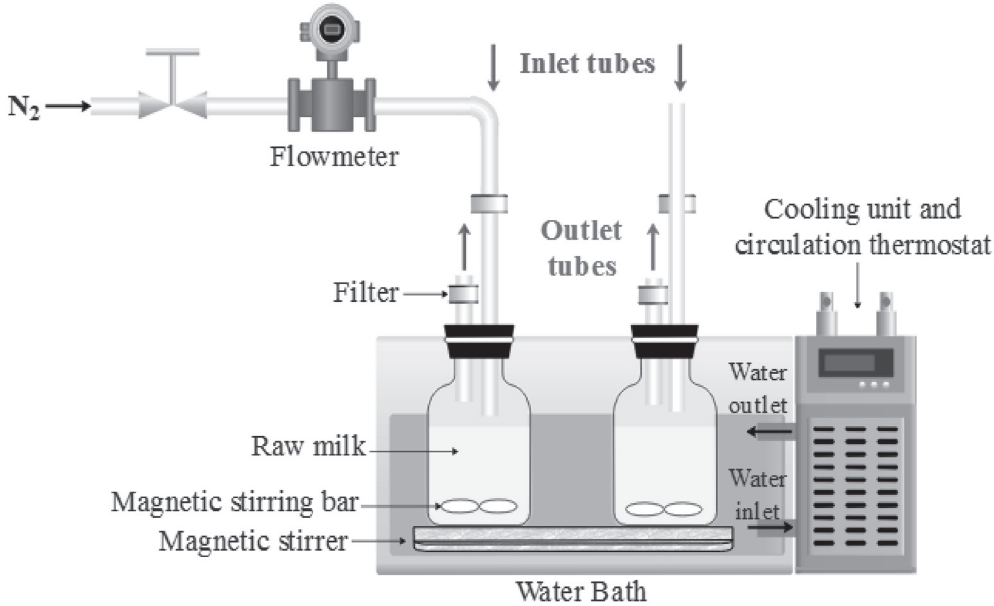


Figure 1. Experimental setup used for nitrogen treatments of raw milk in glass bottles at a flow rate of 120 mL/min

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of samples was measured by the modified method of McCue and Shetty (2005). DPPH stock solution was prepared by dissolving DPPH (24 mg) in ethanol (100 mL). Working solution was prepared by diluting the stock solution with ethanol (final absorbance was about 1.1±0.02 at 517 nm). Ethanolic DPPH solution (2.7 mL) was mixed with 0.3 mL of raw milk or TCA supernatant (deproteinized raw milk) and the mixture was vortexed well. After incubating the samples at room temperature for 30 min, they were centrifuged at 12,000 g for 2 min at room temperature. Decrease in absorbance values was measured at 517 nm against ethanol, and results were expressed as TEAC.

Determination of ascorbic acid content

Sample preparation for ascorbic acid determination was performed according to Kondyli et al. (2007). Metaphosphoric acid (1.12 %) was mixed with raw milk at a ratio of 1:1 (v/v). After centrifugation at 12,000 g for 30 min, the supernatant was filtered and analyzed immediately by the HPLC system. HPLC conditions described by Aktas et al. (2005) were used to determine the ascorbic acid content of raw milk samples. HPLC analyses were carried out by Shimadzu Prominence HPLC System (Shimadzu Corporation, Kyoto, Japan) equipped with a diode-array detector (SPD-M20A), a pump (LC20 AT), an auto sampler (SIL 20ACHT) and a column oven (CTO-10ASVp). The column used was ODS-4 (250 mm x 4.6 mm I.D., 5 μm) (GP Sciences, Intersil ODS-4, Japan). The mobile phase was ultrapure water adjusted to pH 3 with orthophosphoric acid. A flow rate of 1 mL/min was used for chromatographic separations.

Statistical analyses

Analysis of variance (ANOVA) was used to determine statistically significant differences by means of the SAS software program (The SAS System for Windows 9.0, Chicago, USA). Separation of means for significant differences was conducted using the Duncan's multiple-range test at α=0.01 level. Data were presented as means of three replicates (±standard deviation).

Results and discussion

Microbiological results

Total bacterial counts at initial conditions (day 0) ranged from 3.6 to 4.2 log-units, indicative of a good bacteriological quality (Figure 2). After 4 days of cold storage, counts from the controls (C1, C2, C3) increased by over 10 times for C3 and up to 1000 times for C1 during the 4 days cold storage, and exceeded the threshold value of 3.10^5 cfu/mL (5.5 log-units) of bacteriological acceptance; counts still increased between days 4 and 7 for the controls until 8.7-8.8 log units.

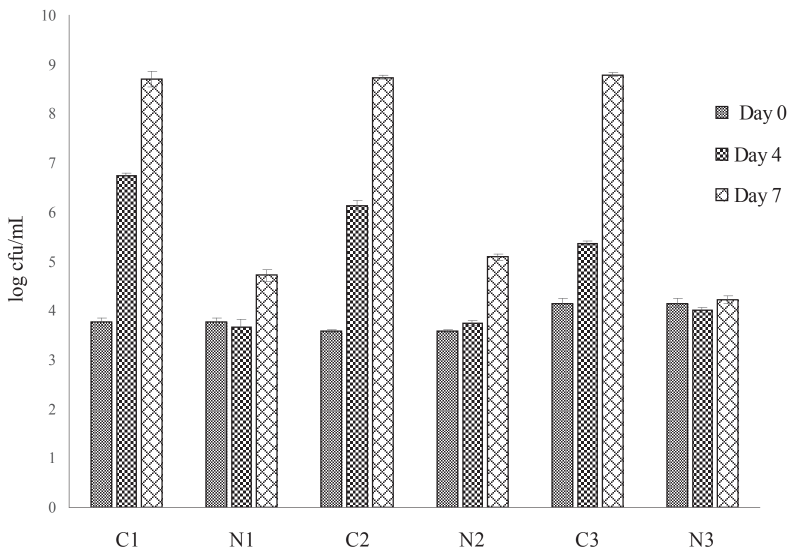


Figure 2. Total bacterial counts of control and N_2-treated raw milk samples during 7 days of storage at 6 °C (C1, C2 and C3: Control raw milk samples, N1, N2 and N3: N_2-treated raw milk samples). Error bars indicate standard deviations

Under the N_2 flushing, in contrary, the bacterial growth could be halted for 4 days (Figure 2); in following, after 7 days, counts had increased by about 10 times for N1 and N2, but remained unchanged for N3; however, the flushing treatment kept counts in all three experiments below the limit of 3.10^5 cfu/mL, which is in agreement with previous results (Munsch-Alatossava et al., 2010a).

Total antioxidant capacity of raw milk samples

Values of the total antioxidant capacity (TAC) of raw milk samples determined by both ABTS and DPPH methods are presented in Figures 3 and 4, respectively. TEAC values, for the controls, obtained by the ABTS method during storage ranged from 369.47±62.06 to 472.33±16.70 μM while those determined by the DPPH method ranged from 8.09±0.49 to 13.30±0.84 μM_TEAC. In this present study, the antioxidant capacity values of raw milk samples determined by the ABTS method were considerably higher than those determined by the DPPH method. Similar results were previously reported by Martysiak-Zurowska and Wenta (2012).

The ABTS radical is soluble in both aqueous and organic solvents, and the ABTS method can be used to determine both hydrophilic and lipophilic antioxidant capacities of different samples like food extracts and body fluids. DPPH, a long-lived nitrogen radical, has been widely used to determine antioxidant potential of different compounds (Villaňo et al., 2007). However, this radical does not have any similarity with the highly reactive and transient peroxyl radicals involved in lipid peroxidation (Huang et al., 2005). DPPH radical dissolves in polar organic matrices like ethanol and methanol, and the DPPH assay is based mainly on the electron transfer reaction, while hydrogen-atom transfer pathway is marginal in this assay (Huang et al., 2005; Prior et al. 2005). Unlike α-tocopherol, BHA and BHT, carotenes and xanthophylls have no DPPH radical scavenging activity (Müller et al., 2011). Conformational inaccessibility of DPPH radicals with antioxidants and reduced reactivity of some compounds with DPPH radicals are other disadvantages of this method (Huang et al., 2005; Prior et al. 2005). Moreover, particular components (i.e. carotenoids) present in a reaction medium may interfere with the DPPH assay because their spectra may overlap with the spectra of DPPH radical itself (Prior et al., 2005). The advantages and disadvantages of these two methods have been reviewed extensively in literature (Prior et al., 2005; Cloetens et al., 2013).

Results from both methods tested to determine the antioxidant capacity indicated that N_2-treated raw milk samples exhibited higher TAC values than untreated samples (Figures 3 and 4). While the TAC values of the control samples decreased after 4 days of storage ($p<0.01$), the values for N_2-treated samples were found statistically similar during 7 days of storage ($p>0.01$) (Figure 3). According to the results of the ABTS assay, the antioxidant activity of control raw milk samples decreased about 22 % during 7 days of storage. Results of the DPPH method indicated that at the end of the storage period, the

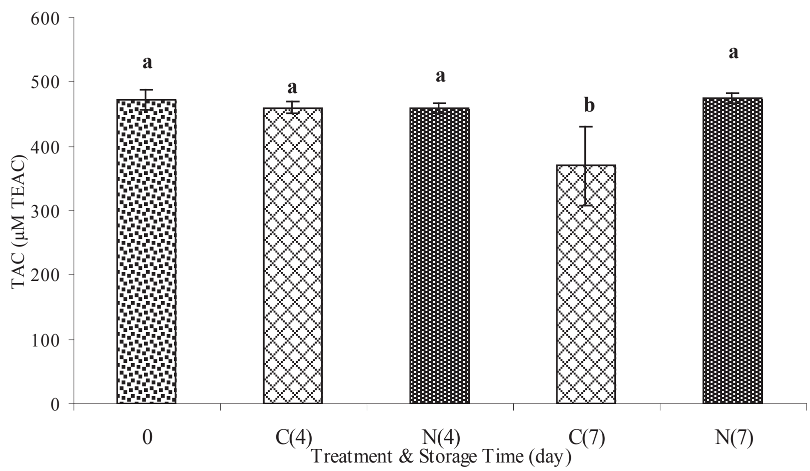


Figure 3. Total antioxidant capacity of raw milk samples assayed by the ABTS method during storage at 6 °C (C: Control, N: N_2-treated; different letters above the columns indicate statistical significant differences at the $p<0.01$ level)

mean TAC values of N_2-treated samples decreased about 20 %, whereas this decrease was about 38 % in untreated samples (Figure 4). Therefore, N_2 treatment could be effective in preventing loss of antioxidant components in raw milk. Moreover, in a previous research we observed that a 30-minute N_2 flushing of the headspace of raw milk containing flasks, at a flow rate of 120 mL/min, is effective in decreasing dissolved oxygen content of raw milk samples from 10.5 mg/L to 0.1 mg/L (Munsch-Alatossava et al., 2010a). The retained antioxidant capacity of N_2-treated raw milk samples was most likely due to the replacement of dissolved O_2 with N_2 in milk.

Total antioxidant capacity of deproteinized milk samples

The total antioxidant capacity (TAC) values of deproteinized raw milk samples determined by the ABTS method were noticeably lower than those obtained for the original raw milk samples (Figure 5). At the beginning of the storage, the TAC value of control raw milk sample was 472.33±16.70 μM TEAC (Figure 3) and decreased to 5.74±0.27 μM TEAC after milk protein removal (Figure 5). Deproteinization of milk samples with trichloroacetic acid may be responsible for the reduced TAC values since the deproteinization step could exclude

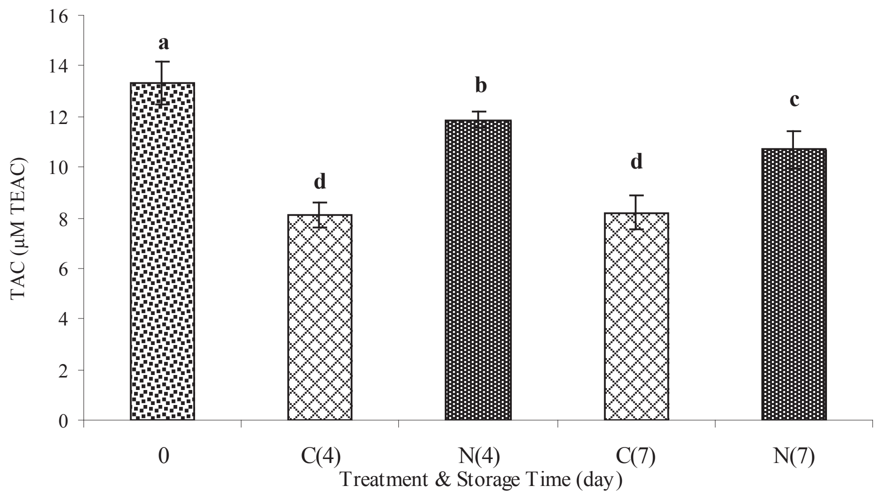


Figure 4. Total antioxidant capacity of raw milk samples assayed by the DPPH method during storage at 6 °C (C: Control, N: N_2-treated, different letters above the columns indicate statistical significant differences at the p<0.01 level)

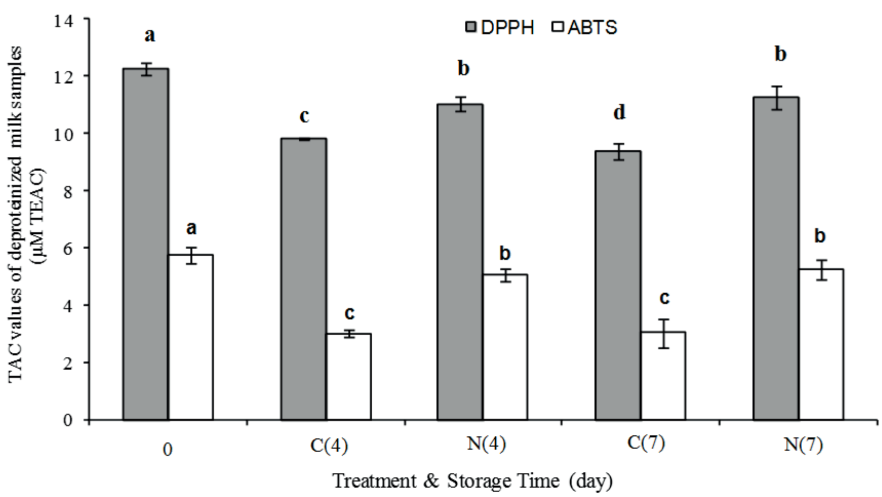


Figure 5. Total antioxidant capacity of deproteinized raw milk samples assayed by the ABTS and DPPH methods during storage at 6 °C (C: Control, N: N_2-treated, different letters above columns indicate statistical significant differences at the p<0.01 level)

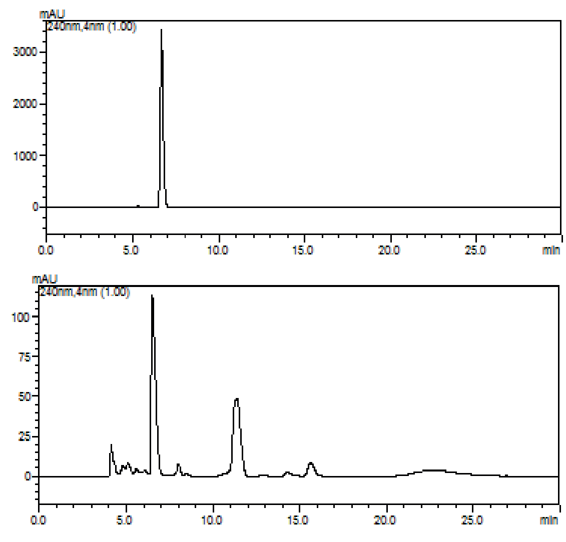
some antioxidant components like ascorbic and uric acids (Zulueta et al., 2009) as well as proteins with antioxidant properties. Interestingly, both N_2-treated raw milk samples and their deproteinized counterparts had higher TAC values compared to untreated deproteinized samples (Figures 3-5). While TAC values of control samples decreased by about 47.50 % after four days of storage ($p<0.01$), the reduction for N_2-treated deproteinized raw milk samples was of only 11.67 % ($p<0.01$) (Figure 5). After three additional days storage, the TAC values of both groups (either controls or treated milk) did not change ($p>0.01$) (Figure 5).

The total antioxidant capacity (TAC) values of deproteinized raw milk samples, determined by the DPPH method, are presented in Figure 5. The TAC value of control samples decreased from 12.26±0.22 to 9.40±0.27 μM TEAC during seven days of storage. Results with the DPPH method indicated that the TAC value of the control samples was 9.83±0.04 μM TEAC while the TAC value of N_2-treated samples was 11.04±0.25 μM TEAC after four days of storage. With respect to the initial TAC values, the TAC values of control and N_2-treated samples at the end of a four day storage reduced about 20 and 10 %, respectively. While the TAC values of N_2-treated samples remained similar for three additional days of storage ($p>0.01$), the TAC values of untreated samples still decreased significantly ($p<0.01$) (Figure 5). During the deproteinization step, trichloroacetic acid addition increases the acidity of the medium, which may also have an influence on the antioxidant activity values of deproteinized milk samples. He et al. (2015) reported that pH adjustment to either 3.7 or 6.8 in fruit juice-milk beverage models did not have any significant effect on the antioxidant capacity determined with the ABTS and FRAP (ferric reducing antioxidant power) assays. Chen et al. (2003) determined TAC values of milk samples with the ABTS and FRAP assays and found that acidity of medium had a significant effect on the antioxidant activity values. In our study, although a possible effect of acidity in the reaction medium of the ABTS and DPPH assays on the TAC values may be present, this effect remains similar for all samples. Therefore, differences in the TAC values of deproteinized milk samples (either control or N_2-treated) most likely reflected the effect of N_2-treatment on the antioxidant capacity of samples.

The TAC values of raw and deproteinized milk samples obtained by both ABTS and DPPH methods showed that milk proteins were the major components responsible for the antioxidant capacity of raw milk. Similar results were also reported by Cervato et al. (1999) and Zulueta et al. (2009), who found that casein fractions of milk were responsible for the majority of the antioxidant capacity. Free radical quenching activity of caseins is due to the oxi dation of aminoacid residues of caseins themselves (Zulueta et al., 2009). Hydrolyzed casein fractions formed by the proteolytic activity of enzymes (Rival et al., 2001) or different microbial cultures (Gjorgievski et al., 2014) may also have antioxidative properties acting by different mechanisms.

Ascorbic acid contents of milk samples

Typical chromatograms of standard ascorbic acid (a) and ascorbic acid in a N_2-treated raw milk sample (b) are presented in Figure 6. Retention time, limit of detection, limit of quantification and coefficient of correlation were 6.7 min, 0.02 mg/L, 0.06 mg/L and 1.00, respectively. Recovery for ascorbic acid determination ranged from 91 to 98 %. Ascorbic acid contents of all raw milk samples ranged from 14.06 to 10.76 mg/L during cold storage. In previous studies, ascorbic acid contents between 10.20 and 27.00 mg/L were reported for raw milk (Woessner et al., 1939; Andersson and Ostłe, 1994; Lindmark-Mansson and Akesson, 2000). Our



(a)

(b)

Figure 6. Chromatogram of standard ascorbic acid (a) and chromatographic separation of ascorbic acid of a N_2-treated raw milk sample by the ODS 4 column

results were in good agreement with the results reported in the literature. The change in ascorbic acid contents of all samples was insignificant in the first four days of storage while ascorbic acid contents decreased significantly afterwards ($p<0.01$) (Figure 7). Ascorbic acid contents of both N_2-treated and control samples were statistically similar at the 4th and 7th day of storage ($p>0.01$). Some studies indicated that ascorbic acid content of milk and human milk can be influenced by several factors including storage time and conditions (Lindmark-Mansson and Akesson, 2000; Buss et al., 2001). In our study, ascorbic acid content of raw milk samples decreased about 23.5 % after seven days of storage. Buss et al. (2001) reported that refrigeration for 24 h decreased the total bioavailable vitamin C (ascorbic acid and dehydroascorbic acid) content of human milk samples by about 35 %. Possible mechanism for vitamin C losses was also indicated to be caused by lactoperoxidase activity in milk during cold storage (Buss et al., 2001). Although the dehydroascorbic acid form of vitamin C was not determined in this study, results of this present study showed that N_2-treatment did not have any protective effect on ascorbic acid contents of raw milk samples.

Conclusions

In this study, raw milk samples possessed higher antioxidant capacity when determined by the ABTS method compared to the DPPH method. Nitrogen gas treated raw milk samples had higher total antioxidant capacity (DPPH and ABTS) values than untreated samples when cold stored at 6 °C. With the ABTS method, deproteinized raw milk samples had particularly lower TAC values compared to their non-deproteinized counterparts. Reduced TAC values may have arisen from deproteinization which results in a reduction of antioxidant components. TAC values of raw and deproteinized milk samples determined by both, ABTS and DPPH methods suggest that the main components responsible for the antioxidant capacity of raw milk were proteins. Unlike the total antioxidant capacity values, N_2-treatment did not show any protective effect on L(+)-ascorbic acid contents of raw milk samples under the studied conditions. In conclusion, results of this study clearly indicated that N_2-flushing through the headspace of milk containing vessels to control microbiological growth and retard spoilage of raw milk during cold storage has also a significant protective effect on the antioxidant components of raw milk.

Acknowledgements

This study was partially supported by the Commission of Mehmet Akif Ersoy University Scientific Research Projects (Project No: 0158-KAYDEP-13) and the Support Program of Mehmet Akif Ersoy University for Academic Activities with Foreign Countries. The authors are most grateful to Damla Bayana for her technical assistance in laboratory studies. We gratefully acknowledge Antti Alavuotunki /Helsingin Meijeriliike Ltd. for the gift of the raw milk samples. Tapani Alatossava thanks the support of the Matti Sundberg quality research foundation, and the Finnish Cultural Foundation.

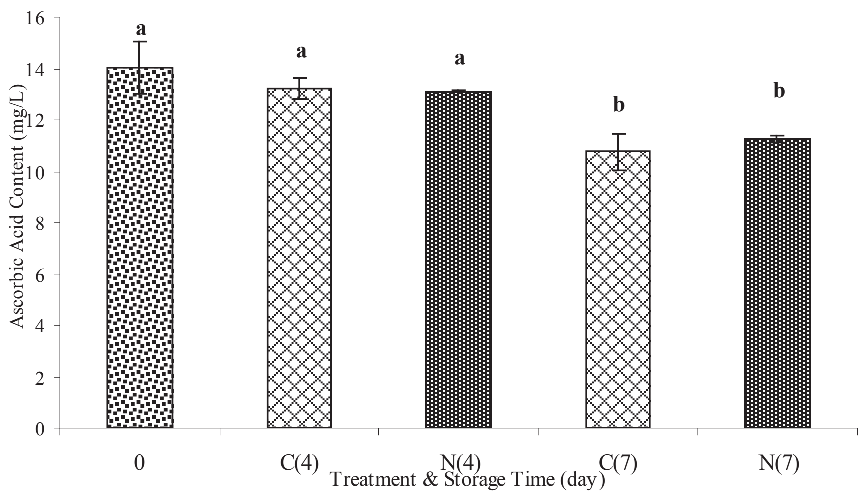


Figure 7. Ascorbic acid content of raw milk samples during storage at 6 °C (C: Control, N: N_2-treated, different letters above columns indicate statistical significant differences at the $p<0.01$ level).

Utjecaj tretmana protočnim plinovitim dušikom na ukupni antioksidativni kapacitet i sadržaj askorbinske kiseline u sirovom kravljem mlijeku tijekom hladnog skladištenja

Sažetak

Tretman kontinuiranim protokom plinovitog dušika (N_2) produžuje trajnost sirovog mlijeka tijekom hladnog skladištenja. Utjecaj tretmana dušikom na ukupni antioksidativni kapacitet (TAC) i sadržaj askorbinske kiseline (AA) u sirovom mlijeku određivan je tijekom hladnog skladištenja. TAC sirovog ili deproteiniziranog mlijeka određivan je pomoću ABTS i DPPH metode, dok je sadržaj L (+) - AA u sirovom mlijeku određen kromatografski i to nakon 0., 4. i 7. dana skladištenja na 6±1 °C. TAC utvrđen pomoću ABTS metode snizio se tijekom hladnog skladištenja s 472,33±16,70 na 369,47±62,06 μM TEAC, dok je TEAC izmjeren pomoću DPPH metode pao s 13,30±0,84 na 8,20±0,66 μM. TAC sirovog mlijeka određen ABTS metodom smanjio se nakon 4-dnevnog skladištenja, međutim, ostale izmjerene vrijednosti bile su statistički jednake za N_2-tretirane uzorke tijekom 7-dnevnog skladištenja. Sadržaj askorbinske kiseline (AA) sirovog mlijeka kretao se u rasponu od 14,06 do 10,76 mg/L tijekom skladištenja, te N_2 tretman nije značajno utjecao na sadržaj AA. Deproteiniziranjem je značajno smanjena TAC vrijednost uzoraka mlijeka, te u slučaju mjerenja pomoću ABTS metode iznosilo je oko 47,50 % za kontrolne uzorke hladno skladištenih tijekom četiri dana, a 11,67 % za deproteinizirano sirovo mlijeko tretirano N_2. Zaključno, tretman protočnim N_2 kroz gornji dio posude u kojoj se skladišti mlijeko također je pokazao značajan zaštitni učinak na antioksidativne sastojke sirovog mlijeka tijekom hladnog skladištenja.

Ključne riječi: sirovo mlijeko, plinoviti dušik (N2), rok trajanja, antioksidans, askorbinska kiselina

References

1. Aktas, A.H., Sen, S., Yilmazer, M., Cubuk, E. (2005): Determination of carboxylic acids in apple juice by RP HPLC. *Iranian Journal of Chemistry and Chemical Engineering 24*, 1-6.
2. Andersson, I., Oste, R. (1994): Nutritional quality of pasteurized milk. Vitamin B12, folacin and ascorbic acid content during storage. *International Dairy Journal* 4, 161-172. https://doi.org/10.1016/0958-6946(94)90066-3
3. Baynes, J.W. (1991): Role of oxidative stress in development of complications in diabetes. *Diabetes* 40, 405-412. https://doi.org/10.2337/diab.40.4.405
4. Buss, I.H., McGill, F., Darlow, B.A., Winterbourn, C.C. (2001): Vitamin C is reduced in human milk after storage. *Acta Paediatrica* 90, 813-815. https://doi.org/10.1080/080352501750315753
5. Buyuktuncel, E. (2013): Toplam fenolik içerik ve antioksidan kapasite tayininde kullanılan başlıca spektrofotometrik yöntemler, *Marmara Pharmaceutical Journal* 17, 93-1033. https://doi.org/10.12991/201317377
6. Cervato, G., Cazzola, R., Cestaro, B. (1999): Studies on the antioxidant activity of caseins. *International Journal of Food Science and Nutrition* 50, 291-296. https://doi.org/10.1080/096374899101175
7. Chen, J., Lindmark-Mansson, H., Gorton, L., Akesson, B. (2003): Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods. *International Dairy Journal* 13, 927-935. https://doi.org/10.1016/S0958-6946(03)00139-0
8. Cloetens, L., Panee, J., Akesson, B. (2013): The antioxidant capacity of milk - the application of different methods in vitro and in vivo. *Journal of Cell and Molecular Biology* 59, 43-57.
9. Cousin, M.A. (1981): Proteolytic activity of psychrotrophic microorganisms in milk and dairy products. In: Psychrotrophic microorganisms in spoilage and pathogenicity, Academic Press, London, pp 63-72.
10. Dechemi, S., Benjelloun, H., Lebeault, J.M. (2005): Effect of modified atmospheres on the growth and extracellular enzymes of psychrotrophs in raw milk. *Engineering in Life Sciences* 5, 350-356. https://doi.org/10.1002/elsc.200520082
11. Gjorgievski, N., Tomovska, J., Dimitrovska, G., Makarijoski, B., Shariati, M.A. (2014): Determination of the antioxidant activity in yogurt. *Journal of Hygienic Engineering and Design* 8, 88-92.
12. Gordon, M.H. (2010): Antioxidants and food stability. In: Antioxidants in food: practical applications, Woodhead Publishing Ltd., Cambridge, UK, pp 7-21.
13. He, Z., Yuan, B., Zeng, M., Tao, G., Chen, J. (2015): Effect of simulated processing on the antioxidant capacity and in vitro protein digestion of fruit juice-milk beverage model systems. *Food Chemistry* 175, 457-464. https://doi.org/10.1016/j.foodchem.2014.12.007
14. Huang, D., Ou, B., Prior, R.L. (2005): The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry* 53, 1841-1856 https://doi.org/10.1021/jf030723c

15. Johnson, I.T. (2010): Antioxidants and antitumour properties. In: Antioxidants in food: practical applications, Woodhead Publishing Ltd., Cambridge, UK, pp 100-123.
16. Kondyli, E., Katsiari, M.C., Voutsinas, L.P. (2007): Variations of vitamin and mineral contents in raw goat milk of the indigenous Greek breed during lactation. *Food Chemistry* 100, 226-230. https://doi.org/10.1016/j.foodchem.2005.09.038
17. Lindmark-Mansson, H., Akesson, B. (2000): Antioxidative factors in milk. *British Journal of Nutrition* 84 (Suppl. 1), 103-110. https://doi.org/10.1017/s0007114500002324
18. Martin, J.D., Werner, B.G., Hotchkiss, J.H. (2003): Effects of carbon dioxide on bacterial growth parameters in milk as measured by conductivity. *Journal of Dairy Science* 86, 1932-1940. https://doi.org/10.3168/jds.S0022-0302(03)73780-1
19. Martysiak-Zurowska, D., Wenta, W. (2012): A comparison of ABTS and DPPH methods for assessing the total antioxidant capacity of human milk. *ACTA Scientiarum Polonorum Technologia Alimentaria* 11, 83-89.
20. McCue, P.P., Shetty, K. (2005): Phenolic antioxidant mobilization during yogurt production from soymilk using Kefir cultures, *Process Biochemistry* 40, 1791-1797. https://doi.org/10.1016/j.procbio.2004.06.067
21. Munsch-Alatossava, P., Alatossava, T. (2007): Antibiotic resistance of raw milk associated psychrotrophic bacteria. *Microbiological Research* 162, 115-123. https://doi.org/10.1016/j.micres.2006.01.015
22. Munsch-Alatossava, P., Ghafar, A., Alatossava, T. (2013): Potential of nitrogen gas (N_2) flushing to extend the shelf life of cold stored pasteurised milk. *International Journal of Moleculer Sciences* 14, 5668-5685. https://doi.org/10.3390/ijms14035668
23. Munsch-Alatossava, P., Gursoy, O., Alatossava, T. (2010a): Potential of nitrogen gas (N_2) to control psychrotrophs and mesophiles in raw milk. *Microbiological Research* 165, 122-132. https://doi.org/10.1016/j.micres.2009.02.002
24. Munsch-Alatossava, P., Gursoy, O., Alatossava, T. (2010b): Exclusion of phospholipases (PLs)-producing bacteria in raw milk flushed with nitrogen gas (N_2). *Microbiological Research* 165, 61-65. https://doi.org/10.1016/j.micres.2008.07.001
25. Munsch-Alatossava, P., Gursoy, O., Alatossava, T. (2011): Improved storage of cold raw milk by continuous flushing of N_2 gas separated from compressed air: a pilot scale study. *Journal of Food Processing and Technology* 1, (101), 4.
26. Müller, L., Fröhlich, K., Böhm, V. (2011): Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chemistry* 129, 139-148. https://doi.org/10.1016/j.foodchem.2011.04.045
27. Oroian, M., Escriche, I. (2015): Antioxidants: characterization, natural sources, extraction and analysis. *Food Research International* 74, 10-36. https://doi.org/10.1016/j.foodres.2015.04.018
28. Pravst, I., Zmitek, K., Zmitek, J. (2000): Coenzyme Q10 contents in foods and fortification strategies. *Critical Reviews in Food Science and Nutrition* 50, 269-280. https://doi.org/10.1080/10408390902773037
29. Prior, R.I., Wu, X., Schaich, K. (2005): Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry* 53, 4290-4302. https://doi.org/10.1021/jf0502698
30. Rajagopal, M., Werner, B.G., Hotchkiss, J.H. (2005): Low pressure CO_2 storage of raw milk: microbiological effects. *Journal of Dairy Science* 88, 3130-3138. https://doi.org/10.3168/jds.S0022-0302(05)72995-7
31. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999): Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 26, 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3
32. Rival, S.G., Boeriu, C.G., Wichers, H.J. (2001): Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of Agricultural and Food Chemistry* 49, 295-302. https://doi.org/10.1021/jf0003911
33. Samaržija, D., Zamberlin, Š., Pogačić, T. (2012): Psychrotrophic bacteria and milk quality. *Mljekarstvo* 62, 77-95.
34. Spector, A. (1995): Oxidative stress-induced cataract: mechanism of action. *The FASEB Journal* 9, 1173-1182.
35. Vazquez, C.V., Rojas, M.G.V., Ramirez, C.A., Chavez-Servin, J.L., Garcia-Gasca, T., Martinez, R.A.F., Garcia, O.P., Rosado, J.L., Lopez-Sabater, C.M., Castellote, A.I., Montemayor, H.M.A., Carbot, K.T. (2015): Total phenolic compounds in milk from different species: design of an extraction technique for quantification using the Folin-Ciocalteu method. *Food Chemistry* 176, 480-486. https://doi.org/10.1016/j.foodchem.2014.12.050
36. Villaňo, D., Fernández-Pachón, M.S., Moyá, M.L., Troncoso, A.M., García-Parrilla, M.C. (2007): Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta* 71, 230-235. https://doi.org/10.1016/j.talanta.2006.03.050
37. Virgili, F., Scaccini, C., Packer, L., Rimbach, G., (2010): Cardiovascular disease and nutritional phenolics, In: Antioxidants in food: practical applications, Woodhead Publishing Ltd., Cambridge, UK, pp 87-99.
38. Woessner, W.W., Elvehjem, C.A., Schuette, H.A. (1939): The determination of ascorbic acid in commercial milks. *Journal of Nutrition* 18, 619-626.
39. Young, I.S., Woodside, J.V. (2001): Antioxidants in health and disease. *Journal of Clinical Pathology* 54, 176-186. https://doi.org/10.1136/jcp.54.3.176
40. Zulueta, A., Maurizi, A., Frigola, A., Esteve, M.J., Coli, R., Burini, G. (2009): Antioxidant capacity of cow milk, whey and deproteinized milk. *International Dairy Journal* 19, 380-385. https://doi.org/10.1016/j.idairyj.2009.02.003