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Alteration of Antioxidant Vitamins and Ghrelin Hormones Amounts in Pasteurized Dairy Products Depend on the Shelf Life

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Abstract In this study, the amount of antioxidant vitamins and ghrelin hormones alteration in the packaged milk of five different brands commonly consumed were investigated depending on the shelf life. Besides the amounts of antioxidant vitamins and ghrelin hormones in fresh uncooked milk and boiled milk were determined. The milk samples were collected for a total period of 6 months with one-month periods beginning from date of manufacture. Then, the amounts of vitamins A, E and C with acylated and desacylated forms of ghrelin hormone were analyzed by using HPLC. Highest the amounts of vitamins A, E and C with acylated and desacylated forms of ghrelin hormone were found in the first month of production while the lowest amounts of A, E and C vitamins with acylated and desacylated forms of ghrelin hormone were found in the sixth month of after production ($p<0.001$). The amounts of vitamins A, E C and acylated and desacylated forms of ghrelin in fresh uncooked milk found to be higher than that of the boiled milk ($p<0.01$). The amounts of vitamins A, E, C and acylated as well as desacylated forms of ghrelin hormone in the packaged milk of all brands decrease over time depending on the shelf life of milk ($p<0.001$). Consumption of long time stored milk should be discouraged due to decrement of vitamins and ghrelin hormones.

Keywords: *milk, ghrelin hormone, vitamin A, vitamin E, vitamin C*

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1. Introduction

Milk is an essential nutrient for all stages of human life [1]. For healthy growth and development of new-born, should be fed with an adequate amount of milk [2]. Because milk contents provide a remarkable tool for management of infant feding and protect high risk infants against infection diseases [3].

In addition to breast milk, buffalo, sheep, goat, cow, camel etc. milk occupy an important place in human nutrition. The content of nutrients in milk varies according to source of species which obtained. The around 200 substances have been identified in breast milk [4]. The main Constituents of milk are lactose, fat and the other minor such as vitamins and minerals [5,6]. The milk proteins provide growth and development. The ghrelin is a growth hormone found to be very low concentration in milk [7,8]. Ghrelin is a peptide hormone of 28 amino acids and plays an important role in the regulating the food intake and releasing of the growth hormone [9]. The ghrelin hormone has two different forms depending on fatty acid attachment. The one which is the acylated (octanylated) form is the active form, and the other one is the desacylated form that is also known as the inactive form of ghrelin. In terms of ghrelin in milk, the active form is physiologically more important than that of inactive form for infant development [9,10].

If milk stored and processed in unhygienic conditions might turn to be harmful to human health [11]. To able to destroy the likely microorganisms and bacteria's in raw milk without losing its nutritional value, it is subjected to standards thermal process, such as pasteurization and UHT technique and after this process the milk maintain the nutritional values [12]. The Milk prepared in this manner is called ultra-high temperature processing or ultra-heat treatment (UHT). The milks produced by UHT technique were stored in plastic bottles or cardboard packages combined of the LDPE (low density polyethylene), cardboard / aluminum foil [11]. The other type of heat treatment applied to the raw milk at our homes was boiling. Boiling process of raw milk for 10-15 minutes kills microorganisms in the milk. The length of boiling time as well as continuous contact with the air results in the significant amount loss of nutritional value

of the milk [13]. It is known that vitamins, especially the water soluble vitamins are oxidized by oxygen, light and high temperature [14]. It has been reported that the loss of vitamin C increase with increment of boiling time and contact with oxygen of air [15]. Also**, t**he loss of vitamins (vitamin B derivatives, vitamin C) in the process of boiling in open containers at home is greater than pasteurization process [16,17].

The aim of this work was to investigate the amounts of vitamins A, E, C and ghrelin hormones (acylated and desacylated forms) of fresh raw and boiled milk and dairy product from different companies sold in markets and also how these parameters have been changed depend on the shelf life

2. Material and Methods

2.1. Materials

The milk samples of five different brands commonly consumed were collected for a total period of 6 months with a-monthly periods beginning from date of manufacture. The fresh raw cow's milk is locally obtained in Elazig city of Turkey and boiled and stored in room temperature.

2.2. Determination of Milk Vitamin A and E Concentration by HPLC

The one mL per sample milk was taken for the extraction of vitamins A and E. Three milliliter of ethanol (70 %) was added into the homogenates samples and vortexed, then the mixture was centrifuged at 3500 rpm for 5 min at 4°C. The supernatant was filtered through Whatman No. 1 paper (Whatman Limited, UK) and 0.5 mL, *n*-hexane (95 %) was added to the filtrate then the filtrates were mixed to extract vitamins A and E in *n*-hexane phase twice. The collected extract was dried under a stream of liquid nitrogen and the dried extracts were solubilized in 0.15 mL methanol (99.5 %) for analysis by the HPLC. Quantification of vitamins A and E was carried out according to Miller et al. [18]. The HPLC separation was carried out at room temperature on a Cecil liquid chromatography system (Series 1100, Cambridge, UK) consisting of a sample injection valve (Cotati 7125) with a 20 µL sample loop, a UV spectrophotometric detector (Cecil 68174), an integrator (HP 3395) and a Techsphere ODS-2 column (Mundells Industrial Centre, Welwyn Garden City, Hertfordshire, UK; 5mm particle size, 80A pore size, 250mm, 4.6mm ID) with 99.5 % methanol/99.9% acetonitrile/99.4 % chloroform (47:42:11 by vol.) as the mobile phase at a flow rate of 1.0 mL/min. Vitamins A and E were detected at 326 and 296 nm, respectively.

2.3. Determination of Milk Vitamin-C Concentration by HPLC

One mL per sample of milk was taken for the extraction of vitamin C. 1.0 mL of 0.5 mol/L $HClO_4$ was added to the homogenate, to precipitate the proteins [19]. Total volumes were made up to 4.0 mL with adding distilled water. The mixture was centrifuged at 3500 rpm for 8 min at 4°C. The supernatant was filtered by Whatman No. 1 paper (Whatman Limited, UK) and vitamin C level was determined with the method proposed by Tavazzi et al. [20] in HPLC on Supelcosil LC-18-DB HPLC reversed-phase column (3 µm particle size and 250×3.9 ID) was utilized for the detection of vitamin C levels. For the analysis of vitamin C, 3.7 mM phosphate buffer at pH 4.0 with the flow rate of 1.0 mL/min. was used as mobile phase and detected at 254 nm.

2.4. Determination of Milk Acylated and Desacylated Ghrelin Concentration by HPLC

Two mL of milk sample was taken for extraction of ghrelin and 1.0 mL of 0.5 mol/L $HClO_4$ was added to the homogenate, to precipitate the proteins (Cerhata et al. [19]. Total volumes were made up to 4.0 mL with addition of distilled water. The mixture was centrifuged at 3500 rpm for 8 min at 4°C. The acylated and desacylated ghrelin in supernatants was analyzed by a reverse-phase HPLC a SGE Walkosil 11 5Cl8 RS (5 mm particle and 120 Å pore size) column (150×4.6 mm ID) column (SGE Deutschland GmbH) Dawes and Dawes [20]**.** The mobile phase (pH 4.0) consisted of 50mM $NaClO_4$ and 1% H_3PO_4 with a flow rate of 1.0 mL/min at 215 nm [21,22].

The chemicals and reagents used in the present study were of analytical grade and purchased from Sigma Chemical Co. All glassware was acid washed and rinsed with doubly distilled deionized water (ddH2O). Analysis were carried out in parallel on three different samples, the standard errors of the data are calculated by the arithmetic mean.

2.5. Statistical Analysis

Statistical analysis was performed using SPSS 17.0 for Windows software (SPSS Inc. Chicago, IL, USA). Results for the groups are expressed as means ± SD. Differences between the groups' means were analyzed for significance using the Mann-Whitney U-test and Duncan's multiple range test. Statistical significance was defined as $p<0.01$.

3. Results and Discussion

To determine the recovery of acylated and desacylated ghrelin; known amounts (25, 50, 100, 200 and 300 pg mL^{-1}) of pure acylated or desacylated ghrelin were added to milk samples. The ghrelin concentrations of the samples with and without added ghrelin were analyzed in triplicate. The recovery of ghrelin in milk was found to be 95% for acylated and 106% desacylated form. The lowest detectable levels for acylated and desacylated ghrelin with this method were 1.0 (±0.2) and 5.4 (±0.8) pg/mL, respectively. The linear working ranges for acylated ghrelin and desacylated ghrelin were determine as 1.0-75 and 1-200 pg/mL, respectively. The equations of graphs for acylated ghrelin and desacylated ghrelin were found to be $y = 0.135x + 0.0484$ ($R^2 = 0.9996$) and $y = 0.0403x + 0.0177$ ($R^2 = 0.9993$), respectively.

Table 1. Time dependence of the amounts of vitamins A, E, C and acylated and desacylated ghrelin in milks with time

Months	Brands	Vitamin A (µg/mL)	Vitamin E (µg/mL)	Vitamin C (µg/mL)	Acylated Ghrelin (pg/mL)	Desacylated ghrelin (pg/mL)
1	1st	1.71 ±0.16	1.99±0.22	19.39±2.14	5.14±0.69	216.55±8.10
1	2nd	1.86±0.14	2.03±0.23	19.48±1.38	5.54±0.57	218.80±6.30
1	3rd	1.85±0.13	2.07±0.25	21.00±2.45	4.95±0.26	219.60±6.60
1	4th	1.83±0.11	2.14±0.20	19.2±2.38	5.76±0.60	220.27±4.60
1	5th	1.81±0.16	2.06±0.19	21.66±2.25	5.84±0.46	220.00±10.00
2	1st	1.44±0.21	1.71±0.20	18.22±1.08	4.86±0.27	213.07±5.00
2	2nd	1.69±0.17	1.68±0.16	18.42±2.24	4.41±0.51	213.31±5.70
2	3rd	1.70±0.11	1.86±0.20	20.54±1.59	4.28±0.62	214.02±6.10
2	4th	1.64±0.12	1.93±0.15	18.16±1.48	4.70±0.24	217.98±5.20
2	5th	1.57±0.09	1.81±0.20	21.00±1.35	4.94±0.55	216.41±6.30
3	1st	1.1±0.17	1.44±0.19	17.72±2.04	3.85±0.65	208.22±6.20
3	2nd	1.40±0.14	1.39±0.18	17.59±1.58	3.49±0.25	209.95±5.90
3	3rd	1.44±0.13	1.68±0.16	19.34±2.06	3.69±0.52	210.00±5.90
3	4th	1.45±0.14	1.70±0.12	17.70±1.41	3.80±0.45	215.04±4.80
3	5th	1.45±0.10	1.64±0.18	19.39±2.06	3.83±0.41	211.98±4.00
4	1st	0.82±0.15	1.12±0.15	16.29±1.62	2.57±0.26	203.69±3.90
4	2nd	1.18±0.08	1.17±0.19	17.05±2.05	2.76±0.27	206.44±9.40
4	3rd	1.19±0.10	1.38±0.15	17.79±1.72	2.58±0.27	206.44±8.50
4	4th	1.14±0.06	1.44±0.12	16.48±2.38	2.75±0.26	212.66±4.80
4	5th	1.17±0.10	1.20±0.14	18.78±1.84	2.92±0.26	206.77±4.50
5	1st	0.66±0.13	0.92±0.18	14.89±1.21	1.87±0.52	196.97±5.40
5	2nd	0.92±0.12	0.98±0.12	16.40±2.12	1.93±0.42	202.35±4.80
5	3rd	1.06±0.15	1.04±0.11	16.54±0.55	1.38±0.39	200.44±5.00
5	4th	0.87±0.11	1.14±0.10	15.81±1.10	1.76±0.24	209.31±6.20
5	5th	0.93±0.12	0.85±0.12	16.37±1.21	1.77±0.25	200.57±6.70
6	1st	0.48±0.10	0.60±0.10	13.12±1.45	0.88±0.14	190.28±4.80
6	2nd	0.67±0.11	0.64±0.09	15.22±1.53	1.23±0.20	198.45±7.00
6	3rd	0.86±0.12	0.68±0.09	15.05±1.41	1.01±0.39	196.79±5.20
6	4th	0.68±0.10	0.79±0.16	13.84±1.38	1.20±0.16	207.08±4.40
6	5th	0.58±0.10	0.65±0.08	15.43±1.58	1.15±0.20	196.79±4.20

Table 2. The amounts of vitamins A, E, C and acylated and desacylated ghrelin in raw and boiled milks

Milks	Vitamin A (µg/mL)	Vitamin E (µg/mL)	Vitamin C (µg/mL)	Acylated Ghrelin (pg/mL)	Desacylated ghrelin (pg/mL)
Raw	1.94±0.22	2.58±0.24	26.13±1.40	5.06±0.76	226.1±12.00
Boiled	1.28±0.14	1.76±0.16	16.88±1.12	1.8±0.34	152±8.40

The amounts of vitamins A, E, C with acylated and desacylated forms of ghrelin hormone in raw and boiled milk with five different brands of packaged milk samples collected for a total period of 6 months with one-month periods beginning from date of manufacture are given in Table 1 and Table 2. Michlova et al. [23] suggested that temperature and waiting time caused the decrease in the amount of retinol in milk. It was found that the decrease of vitamin A level is the highest in the first brand (1.71±0.16 to 0.48±0.10 µg/mL, 71.93 %) and lowest in third brand (1.85±0.13 to 0.86±0.12 µg/mL; 53.51 %) (Table 1). In general, the amount of vitamin A in all brands of milk is regularly decrease (mean 64.73 %) with time. The amount of vitamin A in fresh and boiled cow's milk are 1.94±0.22 µg/mL and 1.28±0.14 µg/mL respectively. Loss of vitamin A in boiled milk was found to be average of 34.02 % less than that of the fresh milk (Table 2).

Saffert et al. [24] investigated the changes of vitamin A content in UHT milk in PET bottles exposed to light at 23°C, 0 th week until to 12th week. They observed the decrease of vitamin A level in a regular manner with the increment of shelf life. Present study show that the decrease of vitamin A concentration with the shelf life is consistent with the literature.

Vitamin E which is powerful than vitamin A; with regards to radical destruction, the chain breaking, pressing for repairing deteriorating structures of mechanisms such as using all antioxidant to fulfill the function of

antioxidant capacity [25]. Valverde et al. [26] are reported that the reduction in the amount of vitamin E in the milk is related to both self-life and temperature.

Our results show that the decrease in the amount of vitamin E is the most in 1st brand (1.99±0.22 to 0.60±0.10 µg/mL; 69.85 %), and the least in the 4th brand (2.14±0.20 to 0.79±0.15 µg/mL; 63.08 %) depending on shelf life (Table 1). In general, the vitamin E level of all brands regularly decreased with the shelf life (mean 68.02 %). The amount of vitamin E in fresh and boiled cow's milk are 2.58±0.24 µg/mL and 1.76±0.16 µg/mL, respectively. It was found that the average of 31.78 % loss of vitamin E in fresh milk by boiling (Table 2). The decrease of vitamin E with the shelf life is consistent with the findings of literature. Unlike vitamin E, vitamin C is water soluble and it has strong reducing activity and powerful antioxidant properties [27]. The loss of vitamin C in pasteurized milk is more than UHT milk depending on the shelf life [28].

The decrease of vitamin C while is the most in 1st brand (19.39±2.14 to 13.12±1.45 µg/mL; 32.34 %), the least in 2nd brand (19.45±1.38 to 15.22±1.53 µg/mL, 21.87 %) (Table 1). On the other hand the amount of vitamin C in fresh and boiled cow's milk 26.13±1.40 and 16.88±1.12 µg/mL was found respectively. The fresh milk, lose its 35.40 % of vitamin C level by boiling (Table 1).

In general, the amounts of vitamin C in the milk of all brands steadily decrease (average 28.37 %) according to the shelf life.

The loss of vitamin C has been increased by increasing temperature *and* increasing exposure to *oxygen* [17]. Depending on the shelf life of vitamin C losses are seem to be compatible with the literature. The ghrelin hormone found in milk plays an important role in the regulation of appetite in humans and feeding behavior of animals. Therefore, the abnormal activity of ghrelin hormone may cause excessive weight or weight lost. Thus, the amount of ghrelin intake taken by foods has a great importance nowadays [4]. Yesim Ozarda and Hizli [29] have reported to increase in the amounts of ghrelin activity and total ghrelin depend on the lactation period in breast milk.

Here it was investigated the change of ghrelin hormone content belong to different brands depend on the shelf life. It was observed that, the highest amount of ghrelin found in 4th and 5th brand. The loss of acylated ghrelin in milks with the shelf life is the most in the 1st brand (5.14±0.69 to 0.88±0.14 pg/mL; 82.88 %) and least in the 2nd brand (5.54±0.57 to 1.23±0.20 pg/mL; 77.80 %) (Table 1).

Generally, steady decline of acylated ghrelin in the all brands approximately 79.69 % depend on the shelf life was observed. The amount of the acylated ghrelin in fresh and boiled cow's milk were obtained to be 5.06±0.76 pg/mL and 1.80±0.34 pg/mL respectively. The amount of acylated ghrelin hormone decrease by boiling of fresh cow milk appears to be a in the average of 64.43 %. (Table 2)

The reduction of the active acylated ghrelin hormone can be explained by the antioxidant effect of acylated from of ghrelin. The decrease of desacylated form of ghrelin is maximum in the 1st brand (216.55±8.10 to 190.28±4.80 pg/mL; 12.13 %), while least in 4th brand (220.27±4.60 to 207.08±4.40 pg/mL; 5.99 %) (Table 1). The acylated and desacylated ghrelin hormone content found to decrease about 9.97 % in a month for all brands of milks. While the amount of desacylated ghrelin in fresh cow milk found to be 226.10±12.0 pg/mL and 152.00±8.40 pg/mL in boiled milk. When fresh milk is boiled about 32.77 % of desacylated ghrelin hormone is lost (Table 2). The chemical composition of milk varies based on used technological processes and shelf life. The difference of vitamins A, E and C with ghrelin hormone content can be also explained by the animal feed, lactation period and seasons in packet milks of various companies [30].

Decrement of the amounts of vitamin C and desacylated ghrelin is less than that of the amount of antioxidant vitamins A, E and acylated ghrelin at the base of shelf life. This might be partly explained by hydrolysis of vitamins A and E or degradation of acylated ghrelin. The decrement of vitamins A, E, C and both forms of the ghrelin hormone (acylated and desacylated forms) content in boiled milk might be also explained by the influence of heat, boiling time and light.

All in all it was concluded that the amounts of vitamins and ghrelin hormone in all brands of milk decreased with the shelf life and boiling. Therefore, the dairy product consumers should pay attention to date of production of packaged milk and stored condition before buying them and also the fresh milk should not to be boiled prolong time.

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