

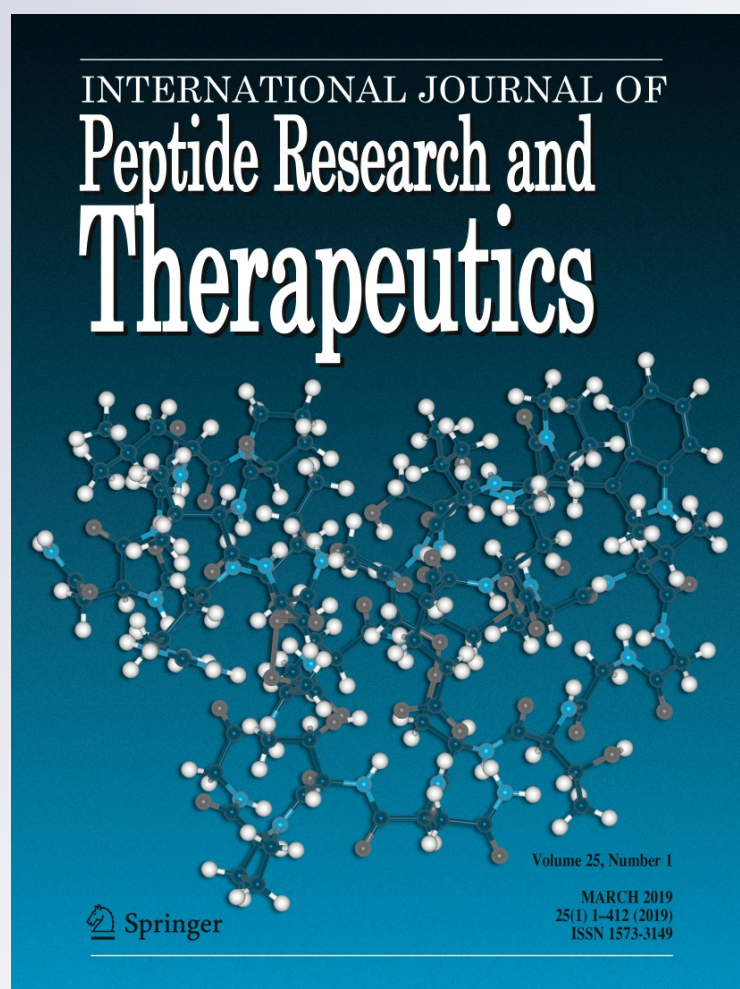
Release of Proteolysis Products with ACE-Inhibitory and Antioxidant Activities in Probiotic Yogurt Containing Different Levels of Fat and Prebiotics

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International Journal of Peptide Research and Therapeutics
formerly known as "Letters in Peptide Science"

ISSN 1573-3149
Volume 25
Number 1

Int J Pept Res Ther (2019) 25:367-377
DOI 10.1007/s10989-018-9679-8



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Release of Proteolysis Products with ACE-Inhibitory and Antioxidant Activities in Probiotic Yogurt Containing Different Levels of Fat and Prebiotics

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Accepted: 28 January 2018 / Published online: 12 February 2018
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Abstract

In this study, the effects of prebiotic ingredients (inulin and wheat fiber) and fat percentage (0, 2 and 3.5%) on physicochemical properties, degree of proteolysis, antioxidant and ACE-inhibitory activity within 21 days storage of yogurt containing probiotic *Lactobacillus casei* at 5 ± 1 °C were evaluated. The results of statistical analysis showed that the application of prebiotics led to a significant increase in water-holding capacity, proteolysis and ACE-inhibitory activity of samples. The degree of proteolysis in yogurt increased as storage time elapsed ($P < 0.05$) but when proteolysis exceeded a certain threshold, this trend began to decline. Also, during storage time, water holding capacity reduced initially but increased thereafter. Moreover, based on our findings, the survival of *L. casei*, in samples treated with inulin and wheat fiber, increased significantly as compared to the control sample ($P < 0.05$) whereas the fat percentage did not reflect significant ($P < 0.05$) effect on the survival of probiotic bacteria. Furthermore, prebiotic ingredients and the presence of probiotic cultures had significant ($P < 0.05$) effect on the antioxidant activity of samples.

Keywords Probiotic yogurt · Proteolysis · Antioxidant activity · ACE-inhibitory

Introduction

Functional foods have recently attracted special attention due to the increased awareness of the health benefits attributed to these products (Kumar et al. 2009; Peshev and van den Ende 2014). Tendency to consume functional foods does not only raise the level of public health but it also facilitates the development and expansion of the food industry (Burgain et al. 2011). In recent years, the consumption of nutraceuticals, that includes foods containing probiotic bacteria, has come up (Martin 2001; Rajasekaran et al. 2008). Probiotics have functional characteristics, and their ingestion is a way to restore and rehabilitate the gut microbiota (Aureli et al. 2011; Kumar et al. 2016). Beneficial effects of probiotics include control of lactose intolerance symptom (EFSA 2010; Vonk et al. 2012), suppression and treatment of

cancer (Kumar et al. 2010; Serban 2013), decline in serum cholesterol level (Mohania et al. 2013), reduction in the adverse effects of antibiotics (Rastall et al. 2005), prevention of intestinal infection by producing organic acids and antibacterial agents (Bron et al. 2017), and enhancement of the immune system (de Moraes 2016). To be termed as probiotic food, the number of viable probiotics must be at least about 10^6 (Shah 2007), 10^7 or 10^8 cfu g^{-1} in the product at the time of consumption (Lourens-Hattingh and Viljoen 2001). Nevertheless, several studies suggest that although the presence of a certain number of live bacteria during storage and consumption of a foodstuff is a necessary condition (FAO/WHO 2002), it is not enough to prove the health benefits of the product (Bertazzoni et al. 2013). Probiotic effects attributed to lactic acid bacteria and fermented dairy products develop not only from the whole of microorganisms and their cell wall components, but also from such metabolites as peptides and extracellular polysaccharides produced during fermentation (Stanton et al. 2005; Gallego and Salminen 2016; Villena and Kitazawa 2017). Therefore, metabolites produced by probiotic microorganisms during the fermentation process should be subjected to scrutiny, which helps

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verify the health benefits of the product and also select the probiotic strain.

Among probiotics, lactobacilli have been widely used in dairy products and are considered noteworthy because of their health benefits (Evivie et al. 2017; Markowiak and Śliżewska 2017). *Lactobacillus casei* has been added to milk as a probiotic and starter, improving nutritional and technological properties of the final fermented product (Matsuzaki 2003; Minelli et al. 2004). Adequate intake of antioxidants presents a feasible solution to control further harmful effects of cardiovascular diseases (Amiri and Amiri 2017; Varadharaj et al. 2017). Aging too, gradually reduces cellular and plasma antioxidant capacity (Okoduwa et al. 2015), as well as the absorption of nutrients and antioxidants, leading to increased vulnerability of proteins to free radical attack (Pandey et al. 2010). In addition, factors such as environmental pollutants, fatigue, extra calories and fatty diets can weaken the immune system, leaving the body vulnerable to oxidative attack (Gamon and Wille 2016). It has been found that following a diet containing antioxidant compounds is a practical and effective measure in order to maintain physical health (Elmadfa and Meyer 2008; Power-Grant et al. 2016). Furthermore, hypertension is a major risk factor for cardiovascular diseases (Franklin and Wong 2013). In most cases, inhibitors of the angiotensin-I-converting enzyme (ACE; EC 3.4.15.1) are used to control blood pressure in the renin-angiotensin system (Ribeill 2013). ACE is a multifunctional enzyme that also catalyzes the degradation of bradykinin, a vasodilator (Gobbetti et al. 2004; Murray and FitzGerald 2007). Because ACE has crucial functions in the regulation of blood pressure, the inhibition of this enzyme has been used to treat hypertension (Coppey et al. 2006). In fact; synthetic ACE inhibitors are broadly used for the treatment of cardiovascular and renal diseases (Pfeffer and Frohlich 2006). However, due to the side effects caused by the clinical use of ACE inhibitors (Sica 2004; Flattery and Sica 2007; Thompson et al. 2011), research on novel, natural product-based ACE inhibitors could noticeably improve the health condition of hypertensive patients.

Proteolysis happens as a result of proteolytic activity of lactic acid bacteria in yogurt (Hati et al. 2017). Bacterial cell-wall-bound proteinases and their intracellular peptidases are responsible for the hydrolysis of proteins (Liu et al. 2010; Mann et al. 2017). Proteolysis of milk proteins increases the release rate of amino acids and small peptides (Gonzalez-Gonzalez et al. 2011). Studies that investigate the health effects of biologically active peptides apply them in two different forms: either as hydrolysates of precursor proteins or as bioactive peptides (Li-Chan 2015). Bioactive peptides are studied as protein components that are in inactive state within the structure of the protein and exert several physico-chemical functions after release by suitable release mechanism, for instance during gastrointestinal digestion or during

food processing (Behera et al. 2013). The released peptides usually have 2–20 amino acids (Meisel and FitzGerald 2003) and molecular masses of less than 6000 Da (Sun et al. 2004). Amino acid structures of peptides and their sequences influence biological activity (Pihlanto-Leppälä 2001). Based on their structural characteristics and amino acid composition, bioactive peptides demonstrate numerous functions such as trace element inhibition, immune system enhancement, cholesterol level reduction, as well as antimicrobial, antioxidant, and antihypertensive activity (Power et al. 2012; Fekete et al. 2015; Hsieh et al. 2015; Nongonierma and FitzGerald 2015; Park and Nam 2015). The presence of bioactive peptides has been studied in fermented milk products such as yogurt (Papadimitriou et al. 2007; Kunda et al. 2012), sour milk (FitzGerald et al. 2004) and *Dahi* (Ashar and Chand 2004; Mohania et al. 2013). ACE-inhibitory, immune regulatory and opioid peptides have been observed in yogurt and fermented milk containing the starter *L. casei* subsp *rhammosus* (Rokka et al. 1997). Several peptides have been found that manifest multiple functions (Donkor et al. 2007a; Sarmadi and Ismail 2010). The antioxidant capacity of casein hydrolysates produced through protein hydrolysis by enzymes is attributed to multiple effects. Some of these features include the ability to eliminate free radicals, act as chelating agents, oxygen scavengers or hydrogen donors and prevent lipid oxidation initiators from penetrating by forming a layer around oil droplets (Hernandez-Ledesma et al. 2011; Zhao et al. 2014; Cheng et al. 2015; O'Loughlin et al. 2015).

Therefore, since proteolysis products in potential salubrious functional foods are considered as natural alternatives to synthetic ACE-inhibitor drugs, and in view of the special significance of dairy products, especially yogurt, in delivering probiotic bacteria, this study was conducted with the aim of investigating the effect of different treatments on the release of proteolysis products which represent antioxidant and ACE-inhibitory activities in probiotic yogurt.

Materials and Methods

Materials and Equipment

The following consumables were used in this study: skim milk powder (Golshad Mashhad Food Industries Co.), commercial yogurt starter (mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus*, DSM, Australia), probiotic adjunct starter *L. casei* LAFTI-L26 (DSM, Australia), MRS agar (Merck, Germany), vancomycin, DPPH and OPA reagent (Sigma-Aldrich, Australia), methanol (HPLC grade, Merck, Germany), sodium-tetraborate (Merck, Germany), sodium dodecyl sulfate (Merck, Germany), β -mercaptoethanol (Sigma-Aldrich, Australia), inulin (Orafti, Belgium) and

wheat fiber (DMV, The Netherlands). Furthermore, the following equipment was used: analytical microbalance (0.0001 g, Sartorius, Germany), incubator (Memmert, Germany), autoclave (GMBH, Germany), refrigerated centrifuge (Sigma, Germany), spectrophotometer (Agilent, USA), pH meter (Metrohm, Switzerland), colony counter (Japan) and water bath (Memmert, Germany).

Methods

Preparation of Probiotic Yogurt

Reconstituted milk was used for the preparation of yogurt. In order to produce reconstituted milk, calculations based on fat and protein contents of skim milk powder (SMP) were implemented and finally three types of milk with different fat percentages (non-fat, medium-fat, and high-fat) were prepared. Appropriate amounts of SMP were weighed and dissolved in deionized water, and the total solid was set on 12%. Table 1 shows the composition of the dried milk used for yogurt preparation.

Table 2 shows the characteristics of the prepared milk samples, which were analyzed by reference methods. The reconstitution ratios for yogurt preparation were as follows: 120 g of skim milk powder (0.05% fat) + 880 ml of water for non-fat milk, and 120 g of dried full fat milk (31% fat) + 880 ml of water for high-fat milk. As for medium-fat milk, 52 g of skim milk powder was mixed with 68 g of full fat milk powder, followed by the addition of 880 ml of distilled water. All these ratios were calculated using the Pearson's square method; since the protein content in both samples of full-fat and skim milk powder was the same (34%), the protein content of all three reconstituted milk samples are almost equal. Inulin and wheat fiber were then added to the reconstituted milk in the predetermined ratios. The mixture was pasteurized at 85 °C for 30 min. Pasteurized milk was cooled to 43–42 °C and the mixture was well stirred after the addition of yogurt starter (mixed culture

of *S. thermophilus* and *L. delbrueckii* subspecies *bulgaricus*) (CODEX standard for fermented milks 2010) and the probiotic bacterium *L. casei* as an adjunct culture. Finally, 100 ml sterilized cups were filled with the mix, incubated at 43–42 °C, and regularly controlled until reaching pH 4.6. After incubation, the samples were immediately cooled to 10 °C, transferred to cold storage at 4 °C, and stored at the mentioned temperature for 21 days. In general, 9 treatments were considered for each prebiotic ingredient wherein treatments 1, 2 and 3 refer to non-fat samples containing 0, 0.3 and 0.5% prebiotics, treatments 4, 5 and 6 pertain to medium-fat samples containing 0, 0.3 and 0.5% prebiotics, and treatments 7, 8 and 9 relate to high fat samples containing 0, 0.3 and 0.5% prebiotics.

Determination of Proteolytic Activity (Using the o-phthaldialdehyde Method)

Analysis of the proteolysis process was conducted in three stages.

The first step (preparation of yogurt extract): Firstly, 10 g of yogurt sample was mixed with 2.5 ml of distilled water. The pH was adjusted to 4.0 using 0.1 M hydrochloric acid, and incubated in a water bath at 45 °C for 10 min. The sample was then centrifuged at 4 °C in 5000×g for 10 min (Ultracentrifuge, Sigma, 3–30 K). pH of the isolated serum was adjusted to 7 with 0.1M NaOH, and centrifuged again under the same conditions. The separated serum was stored at –20 °C (Amirdivani and Salihin Baba 2011).

The second step (preparation of OPA reagent according to Church et al. 1983): 25 ml of 100 mM sodium-tetraborate, 2.5 ml of sodium dodecyl sulfate 20% (w/w), 40 mg of OPA (dissolved in 1 ml of methanol) and 100 µl of β-mercaptoethanol were mixed and diluted to a final volume of 50 ml with distilled water. The solutes were dissolved using an ultrasonic bath (50 Hz). OPA reagent should be prepared and used within 2 h (Church et al. 1983).

The third step (measurement of absorbance): 150 µl of yogurt extract was added to 3 ml of OPA reagent in 5 ml

Table 1 Composition of the dried milk used for yogurt preparation

	Fat (%)	Moisture (%)	Acidity (%)	Protein (%)	pH	Total plate count	Coliform
Full-fat dried milk	30	4.88	1.31	34.03	6.59	5000	< 10
Skim milk powder	0.5	4.75	1.44	34.03	6.52	5000	< 10

Table 2 Composition of reconstituted milk samples

Type of milk	Fat (%)	Protein (%)	Acidity (%)	pH	Total plate count	Coliform
Non-fat	0.5 ± 0.1	4.6	1.44	6.59	< 5000	< 10
Medium-fat	2 ± 0.2	4.61	1.5	6.5	< 5000	< 10
High-fat	3.5 ± 0.3	4.61	1.6	6.5	< 5000	< 10

SD ± standard deviation

quartz cells and incubated at ambient temperature (25 °C) for 2 min. Absorbance was measured with a spectrophotometer at 340 nm. As control, distilled water was used instead of the sample. The degree of proteolysis was measured based on the amount of peptide and free amino acid absorbance measured at 340 nm (Gonzalez-Gonzalez et al. 2011; Church et al. 1983; Nielsen et al. 2001).

Measurement of Antioxidant Activity

Twenty grams of yogurt sample was mixed with 5 ml of distilled water and centrifuged at 5000×g. pH of the supernatant was adjusted to 4.0, it was then centrifuged again, and the pH was adjusted to 7. This solution was used to determine the antioxidant activity according to free radical scavenging method (DPPH) (Blois 1958).

Determination of ACE-Inhibitory Activity

According to the method described by Cushman and Cheung (1971) with some modifications, 25 g of yogurt sample was centrifuged at 4000×g for 15 min at 4 °C. The pH of the supernatant was then adjusted to 8.3 with 10 M NaOH. Following the procedures explained by these authors, ACE-inhibitory activity of the obtained peptides was measured using a spectrophotometer (Cushman and Cheung 1971; Donkor et al. 2005).

Water Holding Capacity (WHC)

In order to determine the water holding capacity of the samples, 30 g of yogurt (Y) was weighed in centrifuge tubes and centrifuged at 222×g at 4 °C for 10 min. The layer on the top (W) was removed and weighed. Water holding capacity

was reported as the ratio of the top layer to the initial yogurt weight: (Donkor et al. 2007a).

$$\text{WHC} = (Y - W)/Y \times 100$$

Probiotic *Lactobacillus casei* Count

Five grams of yogurt sample was weighed in sterile conditions and mixed well with 45 ml of 0.1% sterile peptone water. Serial dilutions were prepared by adding 1 ml of each dilution to 9 ml of sterile peptone water, which were then cultured on pour plates in MRS agar containing 10 mg/l vancomycin. Incubation was performed in anaerobic conditions at 37 °C for 72 h and the plates containing 30–300 colonies were counted (Ravula and Shah 1998).

Statistical Analysis

In order to evaluate the effect of the added variables on the properties under study, experiments involving yogurt production were triplicate. The average rating of the three values was calculated for each sample (n = 3). One-way ANOVA was used to analyze the data following a general linear model in SPSS.14.0 (SPSS Inc., Chicago, IL, USA). Significance level was set at p < 0.05 to make comparisons between the means and obtain the standard deviation using Duncan test, and the graphs were drawn by the software Microsoft EXCEL (version 2013).

Results and Discussion

Changes in pH

Table 3 indicates the changes in pH of yogurt samples in different formulae and at different storage times. As expected,

Table 3 pH values of yogurt samples during storage time

Treatments	pH							
	Samples containing wheat fiber				Samples containing inulin			
	Day 1	Day 7	Day 14	Day 21	Day 1	Day 7	Day 14	Day 21
1	4.37 ± 0.01	4.3 ± 0.01	4.27 ± 0.00	4.28 ± 0.00	4.37 ± 0.01	4.34 ± 0.04	4.31 ± 0.08	4.3 ± 0.01
2	4.33 ± 0.01	4.36 ± 0.00	4.23 ± 0.01	4.23 ± 0.00	4.29 ± 0.01	4.25 ± 0.09	4.21 ± 0.07	4.2 ± 0.02
3	4.3 ± 0.00	4.29 ± 0.02	4.25 ± 0.01	4.24 ± 0.02	4.25 ± 0.05	4.2 ± 0.03	4.17 ± 0.01	4.18 ± 0.01
4	4.43 ± 0.05	4.4 ± 0.08	4.37 ± 0.01	4.36 ± 0.01	4.33 ± 0.02	4.3 ± 0.00	4.25 ± 0.02	4.25 ± 0.02
5	4.41 ± 0.01	4.38 ± 0.05	4.32 ± 0.02	4.31 ± 0.07	4.34 ± 0.01	4.3 ± 0.02	4.28 ± 0.00	4.27 ± 0.00
6	4.39 ± 0.00	4.35 ± 0.07	4.31 ± 0.06	4.3 ± 0.05	4.31 ± 0.00	4.28 ± 0.03	4.23 ± 0.01	4.21 ± 0.01
7	4.45 ± 0.07	4.41 ± 0.06	4.37 ± 0.05	4.36 ± 0.09	4.41 ± 0.00	4.37 ± 0.01	4.3 ± 0.05	4.28 ± 0.01
8	4.42 ± 0.05	4.39 ± 0.05	4.35 ± 0.03	4.34 ± 0.01	4.38 ± 0.01	4.34 ± 0.02	4.33 ± 0.01	4.3 ± 0.11
9	4.4 ± 0.01	4.36 ± 0.02	4.3 ± 0.06	4.31 ± 0.00	4.35 ± 0.00	4.31 ± 0.00	4.28 ± 0.01	4.29 ± 0.01

Mean ± standard deviation of duplicate tests are reported

pH of the samples decreased during storage due to post acidification. pH decrease can be attributed to the residual activity of microorganisms (Mani-López et al. 2014). *L. casei* and yogurt starter bacteria have proteolytic features (Shihata and Shah 2000; Donkor et al. 2007b). Donkor et al. (2006) reported that *L. casei* L26 showed the highest proteolytic activity compared to 6 other LAB species (Donkor et al. 2006).

According to Germani et al. (2014), the proteolytic activity of bacteria continues in yogurt for the whole duration of the storage (Germani et al. 2014). Lactic acid bacteria exhibit an enormous demand for peptides and amino acids (Shihata and Shah 2000; Velez-Ruiz et al. 2013). The metabolic activity of lactic mixed cultures caused the formation of lactic acid and pH reduction through the storage (Vinderola et al. 2000). Modest increase or no change in pH at the end of the storage period may be due to a rise in free amino acid groups (Donkor et al. 2006).

Changes in pH with an increase in fat content in yogurt samples were significantly different ($P < 0.05$) such that the pH of yogurt samples with a higher fat percentage showed slight change and declined less within 21 days, probably due to the higher inhibitory effect of yogurts with high-fat contents on probiotic cultures than those with reduced fat (Cumby et al. 2008).

Water Holding Capacity

The values obtained for water-holding capacity of different samples containing different percentages of fat content and prebiotic ingredients are reported in Table 4. According to Niamah et al. (2016), water holding capacity is among the most important parameters in physical analysis of yogurt, which has an inverse correlation with susceptibility to syneresis and whey drainage. Therefore, WHC was analyzed in

this study since it represents the physiochemical properties of the product. As expected, there was a significant increase in water-holding capacity of the samples with a rise in the percentage of fat content and prebiotic ingredients ($P < 0.05$). Also, the combination of prebiotic ingredients had a significant effect on water-holding capacity ($P < 0.05$) such that water-holding capacity of the samples containing wheat fiber was higher than that of inulin-containing samples.

Similar results in literature review were observed for the water-holding capacity. Over time, free amino acids and short-chain polypeptides, which are hydrophilic and enhance water-holding capacity, are produced with protein hydrolysis (Remeuf et al. 2003). However, protein denaturation may affect water-holding capacity positively (Sodini et al. 2006) or inversely (Parnell-Clunies et al. 1986). Remeuf et al. (2003) stated that milk heating had an effect on the WHC of yogurts; when milk was enriched with WPC, heating led to a high level of cross-linking within the gel network, which enhanced yogurt viscosity and water-holding capacity (Remeuf et al. 2003). According to Sodini et al. (2006), the lower the denaturation of protein in WPC, the higher the water-holding capacity of the WPC-fortified yogurt (Sodini et al. 2006). On the other hand, Parnell-Clunies et al. (1986) concluded that high levels of whey protein denaturation in milk were not necessarily associated with an improved water-holding capacity in yogurt (Parnell-Clunies et al. 1986; Donkor et al. 2007c).

Survival of *Lactobacillus casei*

Figures 1 and 2 show the number of probiotic microorganisms during storage time. In all the cases, a decreasing trend emerged in the number of probiotic *L. casei* until day 14th of storage. The decreasing trend in all the samples

Table 4 Water-holding capacity values of yogurt samples during storage time

Treatments	WHC							
	Samples containing wheat fiber				Samples containing inulin			
	Day 1	Day 7	Day 14	Day 21	Day 1	Day 7	Day 14	Day 21
1	35.45 ± 0.21	37.64 ± 0.1	42.37 ± 0.14	40.17 ± 0.13	33.5 ± 0.41	34.5 ± 0.34	40.38 ± 0.25	38.71 ± 0.00
2	43.41 ± 0.23	44.5 ± 0.00	47.35 ± 0.11	44.3 ± 0.12	41.38 ± 0.00	42.51 ± 0.00	45.38 ± 0.17	42.31 ± 0.13
3	43.54 ± 0.00	43.35 ± 0.22	45.28 ± 0.00	42.2 ± 0.25	40.55 ± 0.27	41.38 ± 0.25	44.22 ± 0.14	41.2 ± 0.17
4	37.81 ± 0.25	51.25 ± 0.18	54.28 ± 0.41	52.23 ± 0.18	35.38 ± 0.00	50 ± 0.41	52.39 ± 0.32	50.25 ± 0.22
5	47.49 ± 0.26	55.32 ± 0.15	55.87 ± 0.13	51.24 ± 0.00	45.94 ± 0.00	54.63 ± 0.00	54.95 ± 0.14	52.58 ± 0.10
6	47.9 ± 0.10	55.34 ± 0.17	56.41 ± 0.06	54.28 ± 0.05	45.84 ± 0.20	54.68 ± 0.13	54.73 ± 0.00	52.43 ± 0.25
7	52.31 ± 0.07	60.31 ± 0.06	60.87 ± 0.05	57.38 ± 0.09	49.21 ± 0.00	57.23 ± 0.01	59.34 ± 0.05	57.24 ± 0.01
8	53.05 ± 0.35	61.54 ± 0.00	62.34 ± 0.13	60.18 ± 0.11	49.45 ± 0.13	58.53 ± 0.12	59.83 ± 0.43	58.34 ± 0.14
9	58.23 ± 0.23	61.4 ± 0.42	59.54 ± 0.46	52.85 ± 0.00	49.38 ± 0.10	58.28 ± 0.13	58.73 ± 0.25	58.18 ± 0.14

Mean ± standard deviation of duplicate tests are reported

showed a significant difference at alpha level of 5 percent within 14 days ($P < 0.05$). Nevertheless, from day 14 to 21 of storage, a slight increase was observed in the number of probiotic *L. casei*. Yogurt starter cultures and probiotic *L. casei* produce extra- and intracellular enzymes that are able to generate biologically active peptides (Farnworth and Champagne 2016) and hydrolyze bradykinin (Ramachandran and Shah 2010). Donkor et al. (2006) reported a significant improvement of proteolytic activity by probiotic organisms in the presence of selected prebiotics (Donkor et al. 2006). The growth of probiotic bacteria was observed highest in samples supplemented with a higher percentage of prebiotics and at the end of the storage period. Apparently, proteolysis provides the essential growth factors as peptides and amino acids for improved growth and survival of probiotic bacteria in products (Nielsen et al. 2009). Nielsen et al. (2009) also demonstrated that proteases are active during refrigerated storage (Nielsen et al. 2009). Ramachandran and Shah (2010) reported that yogurt containing *L. casei* and inulin achieved a high degree of proteolysis (Ramachandran et al. 2010).

Proteolysis of Samples During Storage Time

Figures 3 and 4 are concerned with the proteolysis of samples over storage time. As expected, proteolysis levels increased for all samples during the storage period, representing proteolytic activity of lactic acid bacteria during the storage period (Yuksel and Erdem 2010). Probiotic yogurt containing a higher percentage of prebiotics compared to other samples had higher levels of free amino acids. The results were consistent with the findings of Nielsen et al. (2001) and Leclerc et al. (2002) reporting an increase in the amounts of free amino groups during fermentation time (Nielsen et al. 2001; Leclerc et al. 2002). Donkor et al. (2006) and Yuksel and Erdem (2010) also achieved similar results about the dependence of proteolysis levels on the nutrients available to proteolytic microorganisms (Donkor et al. 2006; Yuksel and Erdem 2010). The elevated proteolysis may induce improved survival of probiotic microorganisms (Donkor et al. 2006).

Fig. 1 Total count of probiotic *L. casei* in samples containing inulin during storage time

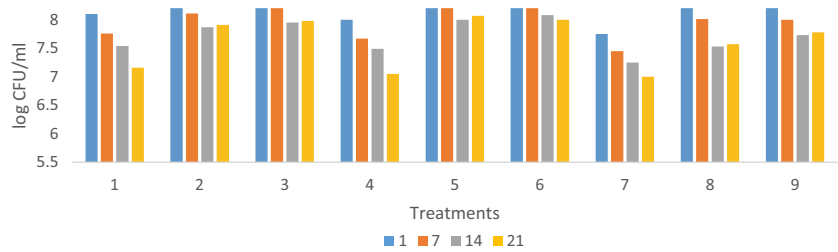


Fig. 2 Total count of probiotic *L. casei* in samples containing wheat fiber during storage time

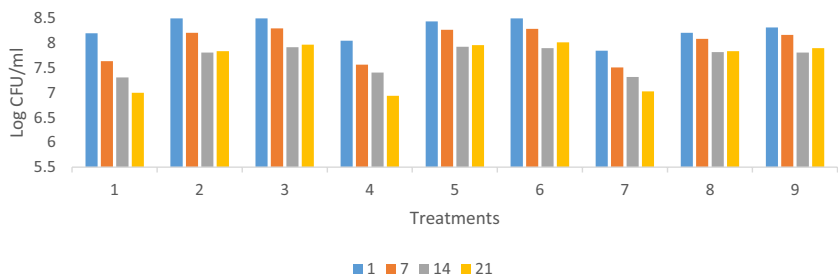


Fig. 3 Degree of proteolysis in samples containing inulin (absorption at 340 nm)

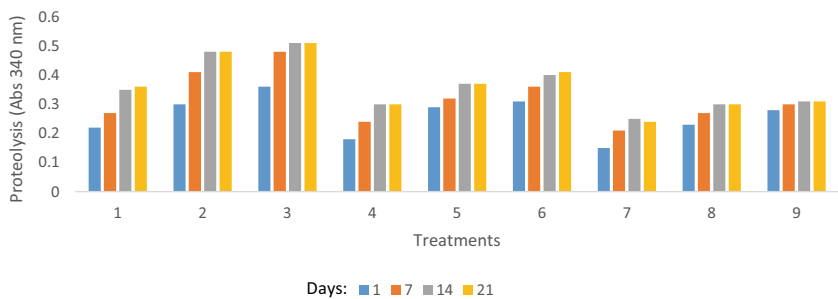


Fig. 4 Degree of proteolysis levels in samples containing wheat fiber (absorption at 340 nm)

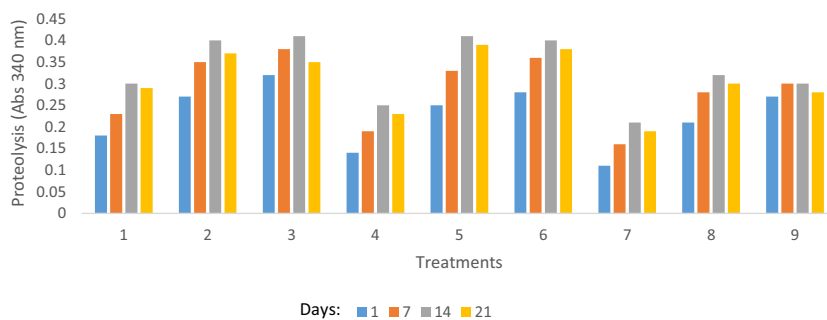


Fig. 5 Antioxidant activity of peptides derived from proteolysis in yogurt containing inulin

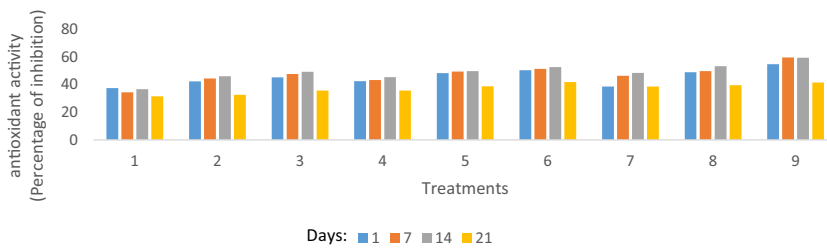


Fig. 6 Antioxidant activity of peptides derived from proteolysis in yogurt containing wheat fiber

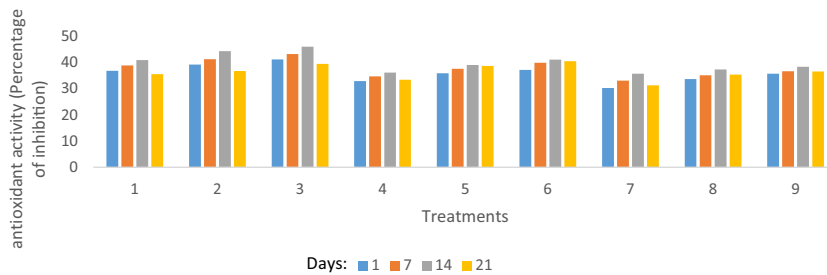
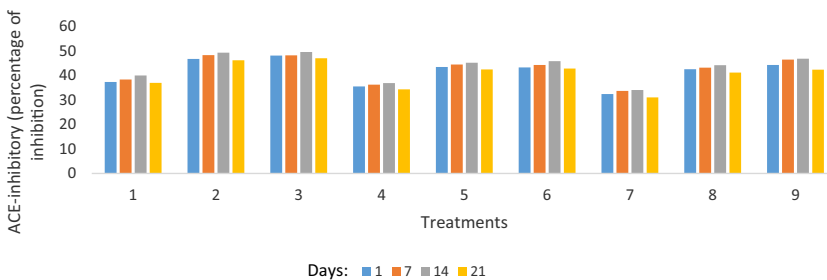


Fig. 7 ACE-inhibitory percentage of peptides derived from proteolysis in yogurt containing inulin



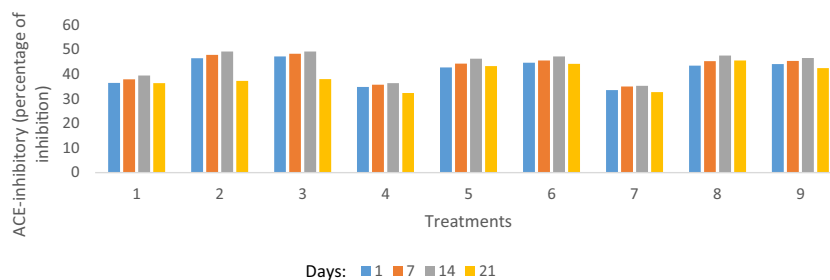
Evaluation of Antioxidant Activity of Proteolysis Products in Yogurt Sample

Examining the percentage of DPPH radical scavenging as a criterion of antioxidant activity of protein hydrolysates derived from bacterial activity in Figs. 5 and 6 reveals that antioxidant activity increased in all the samples during the first 14 days, whereas this property decreased by an increase in the rate of hydrolysis until day 21. This reduction is probably due to higher hydrolysis and cleavage in regions of bioactive peptides that have antioxidant activity.

Evaluation of ACE-Inhibitory Potential of Proteolysis Products in Yogurt Sample

Figures 7 and 8 address the percentage ACE-inhibition values of peptides derived from proteolysis in yogurt samples containing inulin and wheat fiber. As can be seen from the results, as a consequence of casein and whey protein hydrolysis (Kunji et al. 1996), a rising trend was initially observed in ACE-inhibitory activity, but over time and upon achieving maximum inhibition ability achieved in the current study (more than 45%), this activity decreased, which is attributed

Fig. 8 ACE-inhibitory percentage of peptides derived from proteolysis in yogurt containing wheat fiber



to the possibility of cleavage of N-terminal amino acids from a wide range of peptides with ACE-inhibitory effect during long hydrolysis time periods, as a result of which the inhibitory activity decreased (Hata et al. 1996).

Hata et al. (1996) suggested that lactic acid-producing bacteria have the potential to prevent cardiovascular disease in individuals with high blood pressure (Hata et al. 1996). Our findings indicate that lactic acid-producing bacteria account for ACE inhibitory activity. Similarly, according to data published by Donkor et al. (2007a), ACE inhibition reached a high on day 14, indicating further proteolytic modification of inactive peptides or release of new peptides from caseins (Donkor et al. 2007a). They also reported that ACE inhibition of peptides in probiotic yogurt declined in the last week of storage.

Our results suggested that ACE-inhibitory activities were influenced by fat content. Higher ACE inhibition was achieved from non-fat yogurt samples than from samples with higher fat contents, which were associated with a decrease in ACE-inhibitory activity. Such correlation was in agreement with the findings of a study on the effects of fat content on ACE inhibitory activity of probiotic yogurt (Shakerian et al. 2015).

Conclusion

The findings of this study showed that there was a significant difference in proteolysis levels between different probiotic yogurt samples containing prebiotic ingredients and the control sample ($P < 0.05$). Syneresis was lower in samples containing higher percentages of prebiotic compounds. Furthermore, during storage for 14 days, an increase occurred in proteolysis levels, and consequently in antioxidant and ACE-inhibitory properties of yogurts containing prebiotic ingredients compared to the control sample ($P < 0.05$). Antioxidant and ACE-inhibitory activity levels of non-fat yogurt samples exceeded those of medium-fat and high fat samples. Also, according to our findings, although the viability of *L. casei* decreased during storage time, it significantly increased in samples treated with inulin and wheat fiber compared to the control sample ($P \geq 0.05$). The effect of increased proteolysis on enhanced antioxidant and ACE-inhibitory activity of

yogurt reveals a new aspect of the functionality of probiotic dairy products.

Acknowledgements This work was financially supported by Ferdowsi University of Mashhad-Research Affairs (Contract No. 32051).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Human and Animal Participants This article does not contain any studies with human participants or animals performed by any of the authors.

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