

Effect of Fat Percentage and Prebiotic Composition on Proteolysis, ACE-Inhibitory and Antioxidant Activity of Probiotic Yogurt

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Abstract—In recent years, the consumption of functional foods, including foods containing probiotic bacteria, has come to notice. Milk proteins have been identified as a source of angiotensin-I-converting enzyme (ACE) inhibitory peptides and are currently the best-known class of bioactive peptides. In this study, the effects of adding prebiotic ingredients (inulin and wheat fiber) and fat percentage (0%, 2% and 3.5%) in yogurt containing probiotic *Lactobacillus casei* on physicochemical properties, degree of proteolysis, antioxidant and ACE-inhibitory activity within 21 days of storage at 5 ± 1 °C were evaluated. The results of statistical analysis showed that the application of prebiotic compounds led to a significant increase in water holding capacity, proteolysis and ACE-inhibitory of samples. The degree of proteolysis in yogurt increases as storage time elapses ($P < 0.05$) but when proteolysis exceeds a certain threshold, this trend begins to decline. Also, during storage time, water holding capacity reduced initially but increased thereafter. Moreover, based on our findings, the survival of *Lactobacillus casei* in samples treated with inulin and wheat fiber increased significantly in comparison to the control sample ($P < 0.05$) whereas the effect of fat percentage on the survival of probiotic bacteria was not significant ($P = 0.095$). Furthermore, the effect of prebiotic ingredients and the presence of probiotic cultures on the antioxidant activity of samples was significant ($P < 0.05$).

Keywords—Yogurt, proteolysis, ACE-inhibitory, antioxidant activity.

I. INTRODUCTION

RECENT growth in consumer awareness of the close connection between diet and health has caused an increased trend toward and attention to certain foods with desirable properties. General 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. In recent years, the consumption of nutraceuticals, including foods containing probiotic bacteria, has come to notice [1]. Probiotics have functional characteristics, and their ingestion is a way to restore and rehabilitate the gut microbiota [2]. Beneficial effects of probiotics include control of lactose intolerance symptom, reduction in the adverse side effects of

antibiotics, prevention of intestinal infection by producing organic acids and antibacterial agents, suppression and treatment of cancer, enhancement of the immune system and decline in serum cholesterol level [3]. Probiotics are “live microorganisms which when consumed in adequate amounts as part of food confer a health benefit on the host” [4]. For this purpose, the the number of viable probiotics must be at least 10^7 to 10^8 cfu g⁻¹ in the product upon consumption [2], [5]. 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, it is not enough to prove the health benefits of the product. 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 [6], [7]. Therefore, metabolites produced by probiotic microorganisms during the fermentation process should be subjected to scrutiny, which helps 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 [8]. *Lactobacillus casei* has been added to milk and as a probiotic and starter, improving nutritional and technological properties of the final fermented product [9].

Cardiovascular diseases are chronic stress-related disorders [10] and adequate intake of antioxidants presents a feasible solution to control further harmful effects of such diseases [11]. What is more, aging gradually reduces cellular and plasma antioxidant capacity, as well as the absorption of nutrients and antioxidants, leading to increased vulnerability of proteins to free radical attack. 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. It has been found that following a diet containing antioxidant compounds, which are able to increase antioxidant capacity in the human body, is a practical and effective measure in order to maintain physical health [12], [13].

By definition, bioactive compounds include nutritional compounds that occur naturally in food in small quantities or are produced in different amounts during various processes such as fermentation [14], [15].

Proteolysis happens as a result of proteolytic activity of lactic acid bacteria in yogurt. Bacterial cell-wall-bound

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proteinases and their intracellular peptidases are responsible for the hydrolysis of proteins. Proteolysis of milk proteins increases the release rate of amino acids and small peptides [16].

Bioactive peptides are studied as protein components which are in inactive state within the structure of the protein and exert several physicochemical functions after release by enzymatic hydrolysis. Peptides consist of 2 to 22 amino acids [17] and have molecular weights of less than 6000 daltons are believed to possess such bioactivity [18]. Amino acid structures and their sequences influence biological activity. 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. The presence of bioactive peptides has been studied in fermented milk products such as yogurt, sour milk and Dahi. ACE-inhibitory, immune regulatory and opioid peptides have been observed in yogurt and fermented milk containing the starter *Lactobacillus casei* subsp *rhamnosus*. However, several peptides have been found that manifest multiple functions [17]. The antioxidant capacity of these protein hydrolysates 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 [19]. Therefore, considering the importance of health 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 proteolysis, antioxidant and ACE-inhibitory activity in probiotic yogurt. This study can be enlightening about another aspect of the health-promoting effects of probiotic dairy products and a step toward improving public health and food safety.

II. MATERIALS AND METHODS

A. Materials and Equipment

In order to do this study, skimmed milk powder (Golshad Mashhad Food Industries Co.), commercial yogurt starter (mixed culture of *Streptococcus thermophilus* and *Lactobacillus Delbrueckii* subspecies *bulgaricus* (DSM, Australia), probiotic starter *Lactobacillus 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), wheat fiber (DMV, the Netherlands), analytical microbalance (0.0001 g, Sartorius, Germany), incubator (Mettler, Germany), autoclave (GMBH, Germany), refrigerated centrifuge (Sigma, Germany), spectrophotometer (Agilent, USA), pH meter (Metrohm, Switzerland), colony counter (Japan) and water bath (Mettler, Germany) were prepared and used.

B. Methods

Preparation of Probiotic Yogurt

Reconstituted milk was used for the preparation of yogurt. Milk powder was dissolved in water and the solids-non-fat was set on 12%. Then, inulin and wheat fiber were 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 and the probiotic bacterium *Lactobacillus casei*. 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 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 semi-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:

1. The first step (preparation of yogurt extract): Firstly, 10 g of yogurt samples were homogenized with 2.5 ml of distilled water; the pH was adjusted to 4 using hydrochloric acid 0.1 M, and incubated in a water bath at 45 °C for 10 min. The samples were then centrifuged at 4 °C in 5000×g for 10 min. pH of the isolated serum was adjusted to 7 with 0.1 M NaOH, and centrifuged again under the same conditions. The separated serum was stored at -20 °C [20].
2. The second step (preparation of OPA reagent): 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 μ l of beta-mercaptoethanol were mixed and diluted to a final volume of 50 ml with distilled water. The solutions were prepared using ultrasonic. OPA reagent should be prepared on test day and used before up to 2 hours [21].
3. The third step (measurement of absorbance): 150 μ l of yogurt extract was added to 3 ml of OPA reagent in 5 ml quartz cells and incubated at ambient temperature 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 sample proteolysis was measured based on the amount of peptide and free amino acid absorbance measured at 340 nm [16], [21], [22].

Measurement of Antioxidant Activity

20 g of yogurt sample was mixed with 5 ml of distilled water and centrifuged at 5000 × g. pH of the supernatant was adjusted to 4, 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).

Determination of ACE-Inhibitory Activity

According to the method described by [26], 25 g of yogurt sample was centrifuged at $4000 \times g$ for 15 min and the pH of the supernatant was then adjusted to 8.3 with 10 N NaOH. Afterwards, ACE-inhibitory activity of the obtained peptides was measured by spectrophotometer [25].

Syneresis

In order to determine the syneresis of the samples, about 30 g of yogurt was weighed in centrifuge tubes and centrifuged at $222 \times g$ at $4^\circ C$ for 10 min. The layer on the top was removed and weighed. Syneresis was reported as the ratio of the top layer to the initial yogurt weight [23].

Probiotic *Lactobacillus casei* Count

Five grams of yogurt samples was weighed in sterile conditions and homogenized with 45 ml of sterile peptone water 0.1%. 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 vancomycin. Incubation was performed in anaerobic conditions at $37^\circ C$ for 72 hours and the plates containing 30-300 colonies were counted [24].

Statistical Analysis

In order to evaluate the effect of the added variables on the properties under study, the research was conducted in a

completely randomized factorial design in duplicate. ANOVA and mean comparison was done with Duncan's multiple range test at a significant level of 0.05 using the software SPSS-19 and the graphs were drawn by the software EXCEL.

III. RESULTS AND DISCUSSION

A. Changes in pH

Table I indicates the changes in pH of yogurt samples in different formulae and at different storage times. As expected, pH of the samples decreased during storage. pH reduction resulted from the activity of probiotic bacteria in yogurt due to the production of lactic acid. *Lactobacillus casei* and yogurt starter bacteria have proteolytic features [25], [26]. According to other studies, when the amount of free amino acids and peptides is low, lactic acid bacteria, which are dependent on proteolytic systems, fulfill this requirement through sufficient hydrolysis of milk proteins. This causes a sharp increase in bacterial growth, resulting in a rise in acid concentration and a decrease in pH. 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 [27]. pH changes in yogurt samples with an increase in fat percentage 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 scanty growth of probiotic and yogurt starter bacteria in the samples containing higher fat percentage.

TABLE I
PH VALUES OF YOGURT SAMPLES DURING STORAGE TIME

Item	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.

B. 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 II. 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 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 [28]. However, protein denaturation may affect water holding capacity inversely [29] or positively [30].

Survival of *Lactobacillus casei*

Figs. 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 *Lactobacillus casei* until 14th day of storage. The decreasing trend in all the samples showed a significant difference at alpha level of 5% within 14 days ($P < 0.05$). Nevertheless, from day 14 to 21 of storage, a slight increase was observed in the number of probiotic *Lactobacillus casei*. Yogurt starter cultures and probiotic *Lactobacillus casei* produce extra- and intra-cellular enzymes that are able to hydrolyze biologically active peptides and

bradykinin. This enzyme is one of the most important bacterial enzymes used in the dairy industry due to its high proline content [27]. Therefore, it seems that *Lactobacillus casei* uses prebiotic substances, and by turning them into new peptides

[27], particularly bioactive compounds, facilitates the availability of nutrients for growth, leading to an increased growth of *Lactobacillus casei* [31].

TABLE II
 WATER HOLDING CAPACITY VALUES OF YOGURT SAMPLES DURING STORAGE TIME

Item	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.

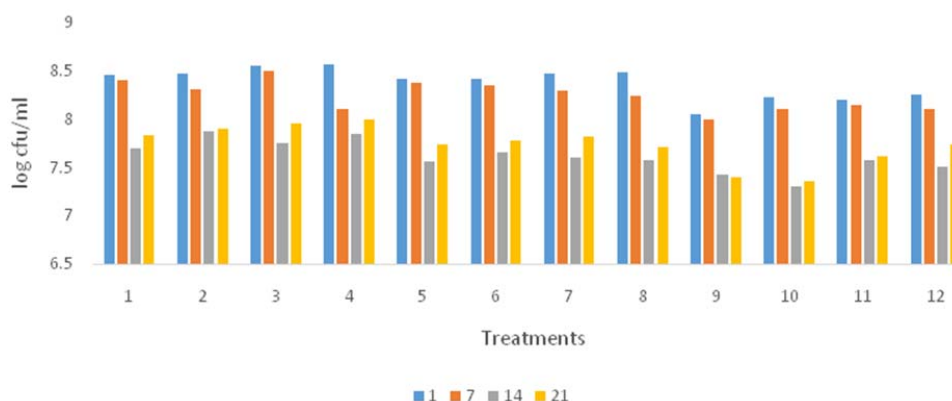


Fig 1 Total count of probiotic *Lactobacillus casei* in samples containing inulin during storage time

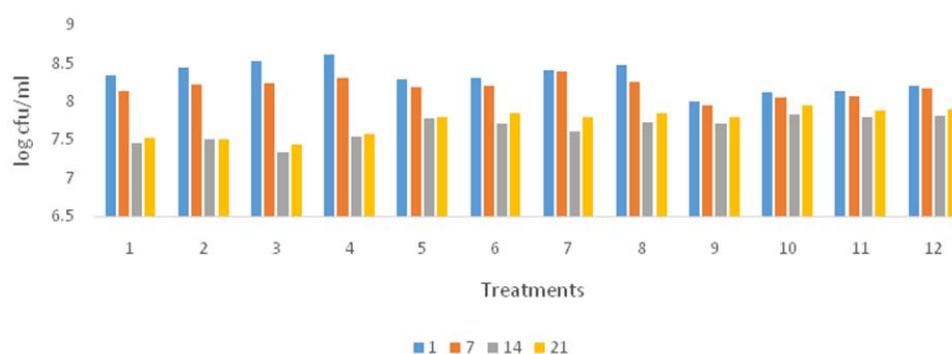


Fig. 2 Total count of probiotic *Lactobacillus casei* in samples containing wheat fiber during storage time

The highest growth of probiotic bacteria was observed 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 [32]. Nielsen et al. also demonstrated that proteases are active during refrigerated storage [33]. Ramachandran et al. reported that yogurt containing *Lactobacillus casei* and inulin achieved a high degree of proteolysis [32].

Proteolysis of Samples during Storage Time

Measuring the relative content of peptides by the OPA method as a criterion of proteolysis suggests that the levels of free amino groups formed in probiotic yogurt during fermentation depend on fat content and the amount of probiotic compounds. Fuglsang et al. obtained similar results investigating the fermentation process in yogurt production [34]. Figs. 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 [35]. Probiotic yogurt containing a higher

percentage of prebiotics compared to other samples had higher levels of free amino acids.

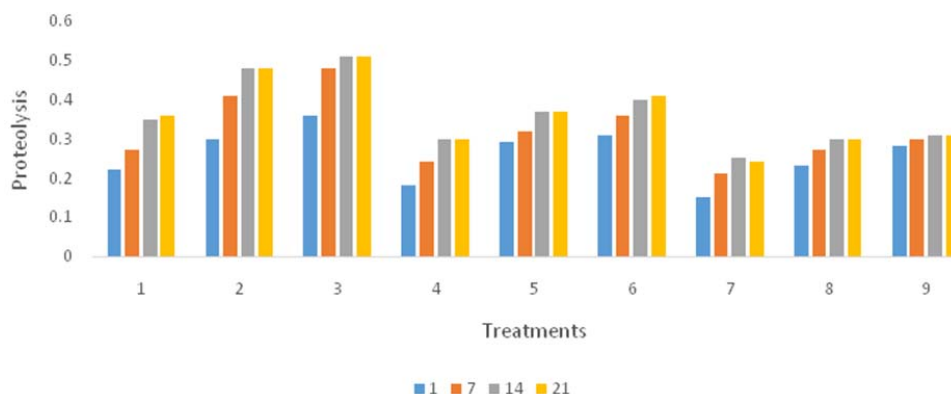


Fig. 3 Degree of proteolysis in samples containing inulin (absorption at 340 nanometers)

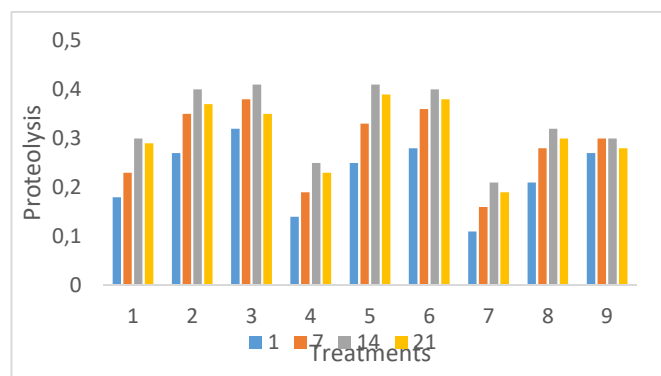


Fig. 4 Degree of proteolysis levels in samples containing wheat fiber (absorption at 340 nanometers)

The results were consistent with the findings of Nielsen et al. and Leclerc et al., reporting an increase in the amounts of free amino groups during fermentation time [22], [36]. Yuksel and Erdem and Donkor et al. also achieved similar results about the dependence of proteolysis levels on the nutrients available to proteolytic microorganisms [27], [35].

Evaluation of Antioxidant Activity of Peptides Derived from Proteolysis 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 Peptides Derived from Proteolysis in Yogurt Sample

Figs. 7 and 8 address the ACE-inhibitory 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 hydrolysis, a rising trend was initially observed in ACE-inhibitory activity, but over time and upon achieving maximum inhibitory ability (more than 45%), this activity decreased, which is attributed to the possibility of cleavage in the structure of peptides with ACE-inhibitory effect during long hydrolysis time periods, as a result of which the inhibitory activity decreased.

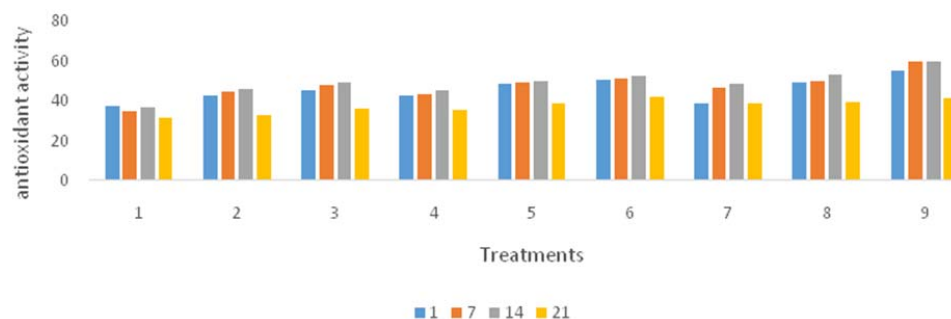


Fig. 5 Antioxidant activity of peptides derived from proteolysis in yogurt containing inulin

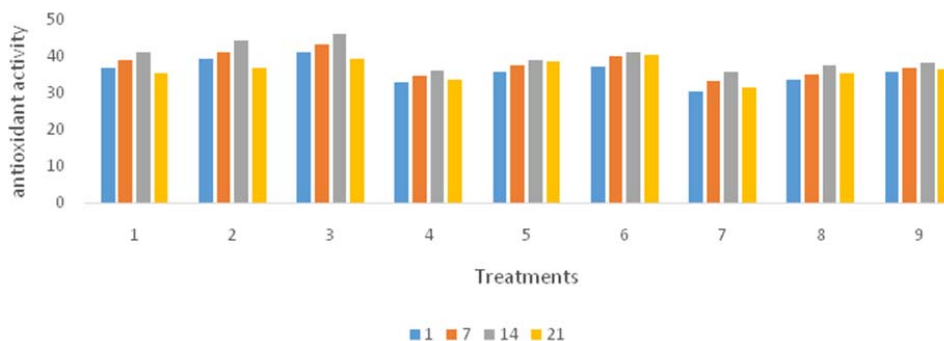


Fig. 6 Antioxidant activity of peptides derived from proteolysis in yogurt containing wheat fiber

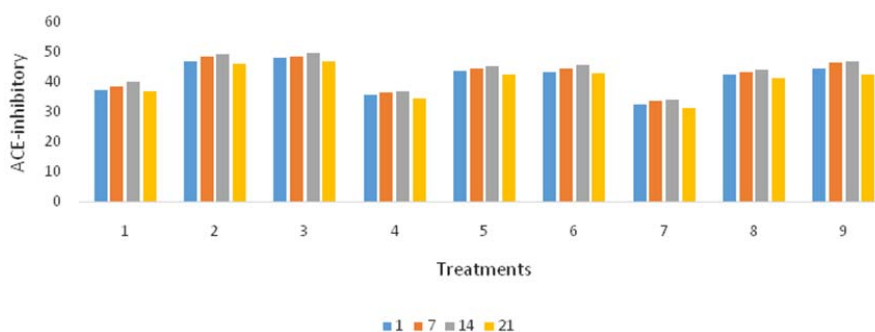


Fig. 7 ACE-inhibitory percentage of peptides derived from proteolysis in yogurt containing inulin

Hata et al. suggested that lactic acid-producing bacteria have the potential to prevent cardiovascular disease in individuals with high blood pressure [37]. Our findings indicate that mainly lactic acid-producing bacteria account for ACE inhibitory activity. Similarly, Pedroche et al. reported the ACE-inhibitory activity of chickpea protein hydrolysate to be 35.4% [38]. Suh et al. stated that the ACE-inhibitory activity of corn gluten hydrolysate was 83.4% [39]. Additionally, several studies have demonstrated soy protein hydrolysate potential to reduce high blood pressure in laboratory rats [40], [41]. Kuba et al. reported an ACE-inhibitory activity of 75.3% for peptides found in tofu produced from soy milk [42].

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, the concentration of bioactive peptides and consequently, antioxidant and ACE-inhibitory properties of yogurt ($P < 0.05$). Antioxidant and ACE-inhibitory activity levels of non-fat yogurt samples exceeded those of semi-fat and high fat samples. Also, according to our findings, although the viability of *Lactobacillus 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 ACE-inhibitory activity of yogurt reveals an aspect of the functionality of probiotic dairy products.

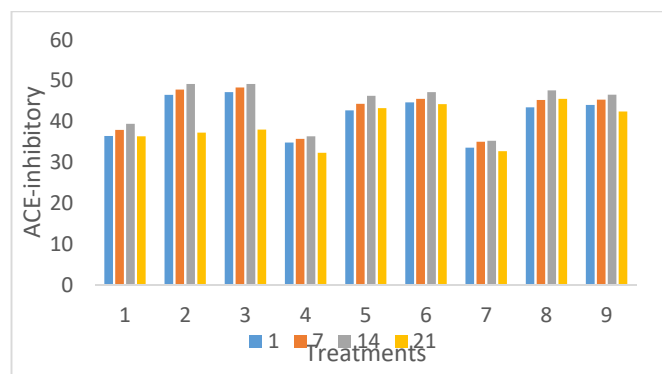


Fig. 8 ACE-inhibitory percentage of peptides derived from proteolysis in yogurt containing wheat fiber

IV. CONCLUSION

The findings of this study showed that there was a

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