Effect of ultrafiltered milk permeate and non-dairy creamer powder concentration on low phenylalanine yoghurt's physicochemical properties during storage

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Abstract Phenylketonuria (PKU) is a genetic disorder that has no cure and all patients with this disorder must adhere to a special diet to prevent the onset of symptoms and mental retardation in children. In this study, analog yoghurt with good and acceptable nutrition benefit for PKU patients was produced. Accordingly, ultrafiltered milk permeate was added at two different concentrations of 4 and 5 w/w % and non-dairy creamer at two different concentrations of 1.5 and 2 w/w %. Subsequently, pH, acidity, protein, fat, dry matter, humidity, syneresis and Phe of the yogurts were determined. Protein content, dry matter, pH, acidity and Phe in the analog yogurt with 5 % permeate and 2 % non-dairy creamer were higher than other yoghurt samples, significantly ($P < 0.05$). During the storage, pH and syneresis of samples reduced, while acidity significantly increased. Phe content of analog yoghurt was low (274 mg/kg).

Keywords Phenylketonuria · Analog yogurt · Permeate · Syneresis · Glycomacropetide

Introduction

Chemical method in which enzymes break down organic materials into smaller compounds is called fermentation process. Fermentation is a method that has been used for

thousands of years to increase the flavor and aroma of food products and provide longer shelf life for perishable foods. The most common examples of fermented foods are wine, bread, cheese, yogurt and other fermented milk products (e.g. kefir and kumis). Finally, with fermentation process, enhanced nutritional value, more digestible, stable and flavored foods with longer shelf life are produced (Hashemi Gahruie et al. 2015). Yogurt is the most consumed fermentative product of milk in the world. The production of fermented milk (yogurt) was started in the Middle East and developed all over the world. (Khurana and Kanawjia-Shurts 2007). In comparison with milk, yogurt contains higher concentration of conjugated linoleic acids (CLA). Additionally, CLA was evaluated to show anti-carcinogenic properties against breast and colon cancer cells. Moreover, it induced expression of the tumor inhibitor. When compared with milk, yogurt is more nutritious and is a great source of protein, calcium, phosphorus, riboflavin (B_2), thiamine (B_1), vitamin B_{12} , folate (B_9), niacin (B_3), magnesium and zinc. Also, it has therapeutic properties, it strengthens the immune system, has anti-cancer activity and serum cholesterol reduction properties. Yogurt is a coagulated milk product that results from the fermentation of *Streptococcus thermophilus* and *Lactobacillus delbrueckii bulgaricus* bacteria in milk (Hashemi Gahruie et al. 2015). People with lactose intolerance can consume yogurt without any harmful effect, because lactose in milk is transformed to lactic acid due to the presence of lactose fermenting bacteria in yogurt during fermentation.

There are three industrially available methods to extract transglutaminase (TGase; protein-glutamine γ -glutamyl-transferase, EC 2.3.2.13). First, from the tissues or body fluids of food animals to extract and purify the enzyme, second, by using host microorganisms such as *E.coli*, *Bacillus*, yeast, or *Aspergillus* with the means of genetic

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manipulation and third, with appropriate microorganism the mass production of TGase by traditional fermentation technology is possible (Yokoyama et al. 2004). MTGase catalyzes the reactions which causes the formation of cross-links in food proteins. In this way, intermolecular cross-linking of proteins (catalyzing the acyl transfer reaction between the γ -carboxamide group of peptide or protein-bound glutamyl) results in high molecular weight polymers which have different functional properties that improve the techno-functional properties of foods. Fit substrates for cross-linking with mTGase are milk proteins (especially caseins). The effect of cross-linking of milk proteins on different functional properties has been considered. In some types of yogurts, cross-linking of the proteins in milk improves gels firmness and reduces serum separation of acid-induced milk gel. Also, mTGase added to milk for the production of nonfat yogurt may be an alternative method instead of addition of extra protein and stabilizer (Sanli 2015).

In phenylketonuria patients (PKU; OMIM261600), the conversion of phenylalanine (Phe) to tyrosine is blocked, due to disruption of the activity phenylalanine hydroxylase enzyme (PAH; EC 1.14.16.1) in the liver (Wiig et al. 2012). Notwithstanding, Phe is an essential amino acid and is important to ensure normal anabolism. PAH enzyme functions in converting Phe to tyrosine in the body. Lack of this enzyme leads to high amounts of phenyl pyruvic acid in the blood, with its most severe clinical complication being irreversible mental retardation (Galvao et al. 2009). On the basis of blood Phe concentrations, PAH deficiency can be classified as classic PKU when blood Phe is raised to above more 1200 $\mu\text{mol/L}$; mild PKU when blood Phe is elevated to 600–1200 $\mu\text{mol/L}$ and mild hyperphenylalaninaemia (HPA), when blood Phe is elevated and is above upper reference limit, but <600 $\mu\text{mol/L}$ (Williams et al. 2008). Since most food contains Phe, the ones these patients can consume is very limited. These include food which can use industrial formulas without Phe, like milk powder and starch, especially manufactured low protein foods such as bread, pasta and biscuits, and natural foods such as fruits, vegetables, some cereals, sugar and butter which are low in Phe, are also necessary (Feillet and Agostoni 2010). These patients should avoid eating food that contains high amount of protein like red meat, fish, chicken, egg, dairy products, dried beans, peas, meat, standard bread, nuts, seeds, foods and drinks containing aspartame, flour, soya, beer, or cream liqueurs (Hainline 1999).

Milk has two main groups of protein; the caseins and whey proteins. Whey, a by-product of cheese making process, was earlier considered as a waste product. The components of whey include β -lactoglobulin, α -lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins,

lactoperoxidase enzymes, glycomacropeptides, lactose and minerals. Recent discoveries have shown whey to be a functional food with several nutritional properties. After the passage of milk through the ultrafiltration system (10^{-1} – 10^{-2} μm membrane), it is called permeate. Macromolecules like protein and fat, suspended solids and colloids are concentrated at the back side of the membrane and form retentate (Grish 1999).

In cheese production, as a result of chymosin enzyme effect on kappa-casein in 105–106 connection, kappa-casein is hydrolyzed and converted into two peptides. The larger peptides or amino terminal part includes amino acid sequences 1–105, which are called para kappa-casein and logs in cheese clots. The smaller peptide or carboxyl terminus part includes amino acid sequences 106–169 and due to its solution, it enters into whey. This peptide is called glycomacropeptide (GMP). GMP is also referred to as casein macropeptide (Hartmann and Meisel 2007). It is a bio-active peptide that has no aromatic amino acids such as Phe, tyrosine and tryptophan, and is used as a primary source of protein in PKU patient's diet. It forms 15–20 % of whey proteins and has 2.5–5 mg of Phe in each gram of protein (MacDonald et al. 2009). According to the study of Abdel-Salam and Effat (2010) on dairy production based on GMP and permeate, and its influence on PKU patients, after 3 days of consuming this product by PKU patients, a 30–80 % reduction of Phe in blood serum was observed. In the research of Shehata et al. (2008), low Phe formula preparation using enzymatic method was studied. *Aspergillus oryzae* and Papain enzyme were used in the enzymatic hydrolysis of defatted milk test. Phe in this examined milk was 6.34 % and after absorption by active carbon and barium sulfate, it reduced to 3.41 %. In the study of Silvestre et al. 2012, the effect of enzyme and temperature on the isolation of Phe was analyzed. The use of *Bacillus subtilis* protease at 50 °C and *Aspergillus sujae* protease at 30 °C showed the highest absorption of Phe.

The prevalence of PKU disease in various populations is different. The lowest incidence of PKU disease was in Finland (less than 1 in 200,000), Japan (1 in 125,000) and Philippines (1 in 102,000). Among the European countries, the prevalence of PKU disease is uncommonly high in Turkey (1 in 2600), Ireland (1 in 4500) and Scotland (1 in 5300) (Williams et al. 2008). In a documentary study, prevalence of PKU disease in Iran was evaluated to be 1.6 in 10,000 people (Moradi et al. 2012).

Unfortunately, very few researches have been conducted in this field and food products for PKU patients have not been produced in Iran. In this research, a low Phe yogurt with high nutritional property was produced and its physicochemical properties were compared with an acceptable level of international standards.

Materials and methods

Yogurt treatments

In this investigation, four yogurt treatments were made as follows: yogurt sample 1 with 4 % w/w permeate and 1.5 % w/w non-dairy creamer, yoghurt sample 2 with 4 % w/w permeate and 2 % w/w non-dairy creamer, yoghurt sample 3 with 5 % w/w permeate and 1.5 % w/w non-dairy creamer and yogurt sample 4 with 5 % w/w permeate and 2 % w/w non-dairy creamer were used; the sample formulations are tabulated in Table 1.

Materials

Raw milk with 2.5 % w/w fat and pasteurized butter were purchased from Pegah dairy company of Tehran. Transglutaminase enzyme (BDF, Spain), permeate (composed of water, sugar, some minerals and non-protein nitrogen compounds) by product of the ultrafiltration of cow's skim milk using 10^{-1} µm membrane filter (Binarazan, Hamedan, Iran), non-dairy ordinary creamer, which is a mixture of fats or vegetable oils (30 % w/w), dietary protein (sodium caseinate) edible additives (Atie Pars, Iran), GMP (Alra Food, Denmark), modified cornstarch (National Starch, US), high performance inulin (BeneoTM HP) with an average degree of polymerization ≥ 23 was kindly gifted (DKSH, London, UK) and starter culture which was a blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* (1:1 ratio, code: Express, from Chr. Hansen Company, Denmark) were purchased and prepared.

Preparation of yogurt starter culture

The yogurt culture consisting of *L. delbrueckii Bulgaricus* and *S. thermophiles* was weighed (10 g) and added to 100 mL of sterile skim milk. Two mL of this mixture was used for inoculation of 1000 mL of yogurt mixture.

Preparation of yogurt mixtures

First, milk was diluted with water at a ratio of 1–15. The milk diluted with butter was pre-heated up to 60 °C for

better powder material resolving. Powder materials were mixed slowly using a mixer. Raw formulated milk was pre-heated to 65 °C and then homogenized at 180 bar pressure. Pasteurization was done at 90 °C for 20 min. Then temperature was reduced to 44 °C and starter and trans-glutaminase (EC 2.3.3.13) were added. After sealing, the samples were incubated for 4 h at 42 °C until their pH reached about 4.6. The samples were refrigerated (4 °C) and after 24 h, first step of the test was performed on them. To ensure uniformity, heating, incubation and cooling were done at the same time and condition for all the samples (Dello Staffolo et al. 2004; Tamime and Robinson 1999). Variables and levels used in the formulation samples are shown in Table 1.

Acidity and proximate composition

Evaluation of acidity, fat (Gerber method), protein (Kjeldahl method) and carbohydrate was performed following AOAC (2002) methods. Moisture content was instrumentally determined using an infrared moisture analyzer (Sartorius, Germany).

Phenylalanine content

Phenylalanine content of different yogurts was using an HPLC system (Waters, USA). Exactly 0.5 g yogurt was mixed with 8 mL hydrochloric acid (6 M). Samples were placed in an incubator after they were hydrolyzed at 110 °C for 22–24 h (Fountoulakis and Lahm 1998). The supernatant was centrifuged for 10 min at 5000 rpm. Injection volume was 20 µL on RPC18 column (3.9 × 150 mm). Duration of analyzing the samples with chromatography was 26 min. Mobile phase speed was 0.8 mL/min under 107–109 kgf/cm² pressure. In the mobile phase, a mixture of phosphate buffer (pH = 3.5) and acetonitrile (2:98) was used, and measurement of wavelength ($\lambda = 214$ nm) was performed by UV-2487 detector at 25–30 °C (Piecyk et al. 2007).

Syneresis

Set yogurt (30 g) was stirred using a glass rod and transferred to 50 mL tube. Then, it was centrifuged for 15 min

Table 1 Variables and levels used in the formulation samples (W/W %)

Materials	Milk	Water	Permeate	Non-dairy creamer	Starch	Inulin	Butter	Trans-glutaminase	GMP
Sample 1	5.0	81.9	4.0	1.5	2.0	2.0	3.5	0.1	3
Sample 2	5.0	81.4	4.0	2.0	2.0	2.0	3.5	0.1	3
Sample 3	5.0	80.9	5.0	1.5	2.0	2.0	3.5	0.1	3
Sample 4	5.0	80.4	5.0	2.0	2.0	2.0	3.5	0.1	3

at 10 °C and $3313 \times g$. The amount of serum released was measured and the result was expressed as percentage by weight (Amatayakul et al. 2006).

Syneresis (w/w %)

$$= (\text{weight of supernatant} / \text{weight of yogurt sample}) \times 100$$

Organoleptic evaluation

Sensory evaluation from color, flavor, taste, texture and overall acceptability point of views was done using hedonic grade of 1, 2, 3, 4 and 5, respectively, for very bad, bad, average, good and very good. It should be noted that these questionnaires were given to 10 panelists for sensory evaluation (Meilgaard et al. 1999).

Calorie content

A single factor was used for each of the energy-yielding substrates (protein, fat and carbohydrate). The energy factors were calculated: 4.0 kcal/g for protein, 9.0 kcal/g for fat, 4.0 kcal/g for carbohydrates and 2.0 kcal/g for dietary fiber (FAO 2003).

Statistical analysis

Statistical package for social sciences (SPSS) software version 23 (IBM, New York, USA) was used for the statistical analysis. To determine the significance, one way analysis of variance (ANOVA) method was used, and for comparison, the average Duncan test at 95 % confidence level was used. The test for each sample was performed in three duplicate.

Results and discussion

As shown in Fig. 1, pH of all the samples during the storage reduced significantly. The reduction in pH can be attributed to activity of bacteria in yogurt and production of lactic acid from lactose in permeate. Cogan (1996) showed that yogurts bacteria are also active in refrigerator temperature and can cause a significant reduction in pH. Panesar and Shinde (2011) reported that activity of live bacteria is the cause of pH reduction during storage in activity of β -galactosidase enzyme. This enzyme is active in refrigerator temperature. Yeasts also use carbohydrates and organic acids, so pH value decreased (Kailasapathy 2006).

As shown in Fig. 2, samples acidity increased significantly during 14 days of storage. Together with pH reduction, acidity increased and the highest acidity was

Fig. 1 Changes in pH of different yogurts during storage of 14 days at 4 °C (data represent triplicate determinations) (sample 1 with 4 % w/w and 1.5 % w/w, sample 2 with 4 % w/w and 2 % w/w, sample 3 with 5 % w/w and 1.5 % w/w and sample 4 with 5 % w/w and 2 % w/w of permeate and non-dairy creamer, respectively)

Fig. 2 Changes in acidity of different yoghurts during storage of 14 days at 4 °C (data represent triplicate determinations) (sample 1 with 4 % w/w and 1.5 % w/w, sample 2 with 4 % w/w and 2 % w/w, sample 3 with 5 % w/w and 1.5 % w/w and sample 4 with 5 % w/w and 2 % w/w of permeate and non-dairy creamer, respectively)

observed on the fourteenth day. Increase in acidity was very slow and can be attributed to bacterial activity and lactose fermentation. According to Costa et al. (2015) *Lactobacillus delbrueckii bulgaricus* is responsible for post acidification (from lactic acid). Due to the absence of *L. delbrueckii bulgaricus* in the fermented milks, during refrigerated storage, increase in acidity was very slow. Some researchers attributed pH reduction and acidity increase to enzymes produced by starter (Christopher et al. 2009). Results of the samples acidity are not similar to codex standard (acidity >60°D). Low acidity in products probably with lactose mutarotation from α to β form is as a result of permeate powder. Starter cannot ferment β -lactose form. This finding was probably due to the absence of *L. delbrueckii bulgaricus* in the fermented milks, because this bacterium is responsible for post-acidification (from lactic acid) during refrigerated storage. In order to increase

Fig. 3 Changes in syneresis of different yoghurts during storage of 14 days at 4 °C (data represent triplicate determinations) (sample 1 with 4 % w/w and 1.5 % w/w, sample 2 with 4 % w/w and 2 % w/w, sample 3 with 5 % w/w and 1.5 % w/w and sample 4 with 5 % w/w and 2 % w/w of permeate and non-dairy creamer, respectively)

acidity up to 60°D, liquid permeate without mutarotation in lactose or acetic acid after fermentation can be used.

Syneresis of yogurts decreased during the storage period of 14 days (Fig. 3). The decrease in syneresis can be attributed to cornstarch and inulin used in the formulation. Aziznia et al. (2008) revealed that with increasing dry matter, syneresis was decreased. The reduction in syneresis can be related to the absorption of unbound water by carbohydrates (Panesar and Shinde 2011). Amatayakul et al. (2006) also reported prevention of syneresis with increase of milk dry matter. Syneresis during the 14 days storage

related directly to pH and inversely to acidity (Fox et al. (2000).

Protein content of yogurts was in accordance with international codex standard (>2.7 % w/w) (Table 2). Abdel-Salam and Effat (2010) reported 2.5 % proteins in drinks for PKU patients while Pimentel et al. (2014) reported 0.9 % proteins in low-protein homemade yogurts. According to *Codex Alimentarius* standard, Yoghurt with >3.0 % w/w milk fat content should be designated as high fat yoghurt, so the simulated yogurt were classified as high fat yogurt (Table 2). Also, carbohydrates of the samples were in the range of 5.55–6.15 g/100 g (Table 2). In the study by Pimentel et al. (2014), the amount of carbohydrate and fat in homemade low protein yogurt for PKU patients reported 12.9 % w/w and 2.4 % w/w, respectively. Dry matter is also in accordance with international codex standard. Phenylalanine content of different yogurts is shown in Table 2. The amount of Phe allowed for 1–10 years is 220–500 mg/day, for 11–18 years is 230–700 mg/day and for > 19 years is 220–700 mg/day (MacLeod and Ney 2010). Abdel-Salam and Effat (2010) reported 12 mg/100 g Phe in diet drinks containing 2.5 % GMP. Also, Van Calcar et al. (2009) focused on producing food specifically for PKU patients and stated that, food fortified with GMP contains 15–30 mg Phe in each serving. Moreover, Pimentel et al. (2014) estimated that the amount of 21.3 mL/g Phe in low protein homemade yogurt.

Furthermore, in this method, the amount of Phe in samples, which was measured by HPLC were 24.09–

Table 2 Physicochemical properties of yogurts prepared with different permeates

Yogurt	Dry Matter (%)	Humidity (%)	Fat (%)	Protein (%)	Carbohydrates (g/100 g)	Phe (mg/100 g)	Kcal/100 g
1	16.30 ± 0.03 ^b	83.70 ± 0.04 ^a	3.03 ± 0.58 ^a	2.87 ± 0.01 ^a	5.55 ± 0.03 ^a	24.09 ± 0.15 ^c	64.95
2	14.80 ± 0.06 ^a	85.23 ± 0.03 ^b	3.33 ± 0.58 ^b	2.92 ± 0.01 ^b	5.95 ± 0.01 ^b	25.21 ± 0.07 ^b	69.45
3	16.27 ± 0.02 ^b	83.73 ± 0.02 ^a	3.03 ± 0.58 ^a	2.92 ± 0.01 ^b	5.78 ± 0.01 ^c	27.28 ± .028 ^a	66.09
4	17.00 ± 0.00 ^c	83.00 ± 0.00 ^c	3.27 ± 0.12 ^c	2.99 ± 0.02 ^c	6.15 ± 0.02 ^d	27.64 ± 0.64 ^d	69.99

a,b,c,d Mean ± SD values in the same columns having different superscripts are significantly different ($p < 0.05$) (Sample 1 with 4 % w/w and 1.5 % w/w, sample 2 with 4 % w/w and 2 % w/w, sample 3 with 5 % w/w and 1.5 % w/w and sample 4 with 5 % w/w and 2 % w/w of permeate and non-dairy creamer, respectively)

Table 3 Mean scores for sensory properties of yoghurts prepared with different permeates

Yogurt	color	Flavor	Taste	Texture	Overall acceptability
1	4.50 ± 0.53 ^a	4.00 ± 0.00 ^b	3.10 ± 0.74 ^b	3.40 ± 0.99 ^b	3.70 ± 0.48 ^{bc}
2	3.50 ± 0.42 ^b	3.60 ± 0.52 ^c	2.30 ± 0.48 ^a	2.50 ± 0.71 ^a	2.80 ± 0.42 ^a
3	4.80 ± 0.52 ^c	3.60 ± 0.52 ^c	3.50 ± 0.94 ^{bc}	2.60 ± 0.71 ^a	3.50 ± 0.71 ^b
4	4.90 ± 0.42 ^c	5.00 ± 0.00 ^a	4.40 ± 0.52 ^d	3.80 ± 0.32 ^b	4.00 ± 0.32 ^c

a,b,c,d Mean ± SD values in the same columns having different superscripts are significantly different ($p < 0.05$) (Sample 1 with 4 % w/w and 1.5 % w/w, sample 2 with 4 % w/w and 2 % w/w, sample 3 with 5 % w/w and 1.5 % w/w and sample 4 with 5 % w/w and 2 % w/w of permeate and non-dairy creamer, respectively)

27.64 mL/100 g (Table 2). Consequently, this product will be safe for PKU patients' consumption.

The results of sensory evaluation of different yogurts are shown in Table 3. Sample 4 had the highest average value from color, flavor, taste, texture and overall acceptability point of views; subsequently sample 1, 3 and 2 gained points, respectively. Caloric values of different yogurts ranged from 64.95–69.99 kcal/100 g (Table 2). In the drinks and low protein homemade yogurt for PKU patients amount of calories reported individually were 53.8 and 137 kcal/100 g (Abdel-Salam and Effat 2010; Pimentel et al. 2014).

Conclusion

pH and syneresis of all yogurts decreased with storage duration and with increase in the amount of permeate. However, the amount of protein and Phe increased with increased incorporation of permeate. Non-dairy creamer powder had insignificant effect on the same. Sensory evaluation showed that all the yogurts were appropriate. Also, producing yogurt with this method is feasible for PKU patients that cannot consume dairy product.

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Compliance with ethical standards

Conflict of interest None.

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