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Original article

Production of functional dairy products low of phenylalanine content and supplemented with probiotics bacteria

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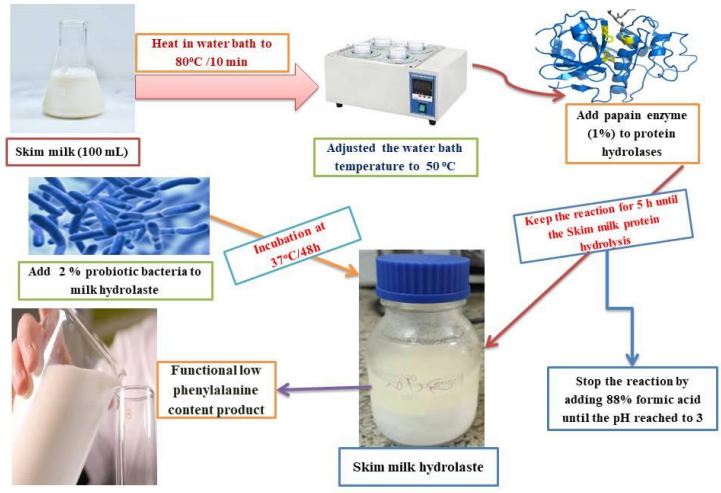
ABSTRACT

The present study was designed to produce new functional dairy products fortified with probiotics for phenylketonuria (PKU). The first step was to determine the phenylalanine content in skim milk and whole milk by HPLC as the base constituent for the product. The different enzymes to hydrolyze the protein of skim milk and whole milk were detected. Next, the usage of barium sulfate or mixed probiotics to lower the release of phenylalanine from hydrolysate protein was evaluated. Finally, the functional low phenylalanine dairy product was prepared by adding 2% probiotics to the skim milk hydrolysate. HPLC determined the phenylalanine content of the product to confirm that the final product was free from phenylalanine. The product was chemically and microbiologically analyzed. The significant findings showed that the concentrations of phenylalanine in skim milk and whole milk were 2.14 and 2.55 mg/mL, respectively. The amount of phenylalanine in skim and whole milk could be eliminated 100% by using either papain or papain combined with protease enzymes, followed by the adsorption agent barium sulphate. The data showed that after treating skim milk, the probiotic bacteria could consume the hydrolyzed protein. The percentages of elimination for skim milk hydrolysates containing papain alone and papain with protease were 100 and 75.23 %, respectively. The chemical composition of functional products had percentages of moisture, ash, protein, and fat as 84.2,8,5, 2.23, and 0.5%, respectively, and free from phenylalanine. The final products contained probiotics (different strains of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*) with a high level during storage.

Graphical abstract



Production of the fermented functional low phenylalanine content product

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1. Introduction

Phenylketonuria (PKU) is an inherited condition that affects about 1 in 10,000 babies born globally [1]. It is characterized by a disrupted metabolism of the essential amino acid phenylalanine (Phe) [2]. Phenylalanine hydroxylase (PAH) malfunction or a shortage of its cofactor tetrahydrobiopterin (BH4) can be the result of an autosomal recessive mutation in the PAH gene, which can disrupt phenylalanine metabolism [3]. The gut microbiome, a group of microorganisms primarily found in the colon, has been linked to conditions like type 2 diabetes, inflammatory bowel disease, and asthma [4]. Psychiatric conditions like anxiety, schizophrenia, and autism are linked to gut microbiome activity, which may alter blood-brain barrier permeability [5]. The gut microbiome's composition is influenced by host genetics, birth mode, and drugs, with nutrition playing a significant role [6]. Previous studies on children's gut microbial ecology reveal significant changes due to PKU treatment diet and Phe restriction compared to non-PKU controls [6].

Dietary treatment can partially prevent phenylketonuria (PKU) by influencing the composition of the gut microbiota and potentially impacting the behavioral outcome of PKU [7]. PKU patients' gut microbiota changes necessitate dietary treatment from newborn to life to prevent neurological damage from excess blood and brain Phe [8]. PKU patients must limit protein intake from high-protein items like meats, fish, eggs, and dairy products. They can consume low-protein, starch-heavy natural foods like potatoes and peas in small quantities, supplemented with medical food substitutes [9].

PKU patients frequently experience nutritional shortages as a result of strict dietary therapy, particularly if they do not completely ingest the recommended medical food alternatives. PKU patients may also be susceptible to deficits in calcium, iron, vitamin B12, vitamin D, and unsaturated long-chain fatty acids [10]. In PKU patients, these deficiencies may worsen neurological issues and lead to decreased bone density. PKU patients and their caregivers face significant financial challenges due to the high expense of specialized medical diets and formulas, as well as the need for regular medical appointments [11]. Special formula foods and amino acid supplements are the primary sources of PKU treatment costs. The patient's age primarily determines the cost of PKU therapy and includes specific formula foods and amino acid supplements [12].

Probiotics are described as "live microorganisms which confer a health benefit on the host when administered in adequate amounts." Bifidobacteria and lactic acid bacteria (LAB) are the two groups of lactic acid-producing microorganisms that are most frequently utilized as probiotics [13]. Fermented foods, such as fermented milk, have been consumed by humans for thousands of years without any noticeable negative effects, demonstrating a stellar safety record [14]. For instance, the combination probiotic formulation is used in ulcerative colitis remission maintenance therapy. In patients suffering from irritable bowel syndrome, *Lactobacillus plantarum* reduces pain and flatulence, while *Lactobacillus* GG considerably alleviates dermatitis in infants with cow's milk allergy and atopic eczema [15, 16]. Additional benefits that have been documented include lowering cholesterol, reducing small bowel bacterial overgrowth in cases of renal failure, and serving as a vehicle for the oral delivery of vaccines [17].

From the previous information, this study was designed to produce a new functional product with probiotic bacteria for people who had Phenylketonuria. It was the first study to use probiotic bacteria as an absorption agent after skim milk protein hydrolysis with different enzymes. In the beginning step of this study to determine the phenylalanine content in skim milk and whole milk, it was detected by HPLC analysis. After that, the more active and economical enzymes for protein hydrolases were evaluated. The study also evaluated the effect of mixed probiotic strains on treating the hydrolase skim milk protein and producing a functional product free from phenylalanine.

2. Materials and Methods

2.1. Materials

The skim milk and whole milk were purchased from a local market (Juhayna Company). Two enzymes were used in the enzymatic proteolytic process; the 1st was papain (Sigma Chemical Co., USA), and the 2nd was protease from Aspergillus oryzae (Sigma Chemical Co., USA). Adsorption material barium sulphate powder is obtained from Modern Chemicals Company (MCC) and used to remove phenylalanine from the hydrolysate. A functional dairy product was obtained from NRC dairy microbiological lab. Supplements of amino acids according to the FAO/WHO provisional pattern (supplied by Sigma Chemical Co., USA).

2.2. Microbial strains

Microorganisms (Bifidobacterium, Lactobacillus, and Streptococcus) were obtained from a capsule of the probiotic "GNC Ultra Probiotic Complex 100," which contains a mix of different probiotic strains. The capsules contained different probiotic strains of Bifidobacterium, Lactobacillus, and Streptococcus species, more than 10^{10} CFU/g.

2.3. Methods

2.3.1. Determination of phenylalanine content of skim milk and whole milk using High-performance Liquid Chromatography (HPLC)

The concentration of phenylalanine in skim milk and whole milk was determined using an HPLC system (Waters, USA). About 0.5 g of each protein source was separately mixed with 8 mL HCL acid (6 M). All samples were hydrolyzed in a water bath and placed in an incubator at 110 °C for 22–24h [18]. The supernatant was obtained after incubation time by centrifugation at 5000 rpm for 10 min. About 20 mL of each sample was injected into the RPC18 column (3.9 9 150 mm) for 26 min. Mobile phase speed was 0.8 mL/min under 107–109 kg/cm^2 pres-

sure. In the mobile phase, a mixture of acetonitrile (2:98) and phosphate buffer (pH = 3.5) was used, and UV detection at 214 nm at 25–30 °C [19].

2.3.2. Suitable enzymes for the hydrolysis of protein found in skim milk and whole milk

Firstly**,** four hydrolysates were prepared using only papain (PA) or papain (PA) with a protease from *Aspergillus oryzae* (AO). The skim milk or whole milk solutions at 0.35 g/100 mL (w/v), corresponding to a protein concentration of 0.125 g/100 mL, were prepared in 0.01 mol/L phosphate buffer, pH 6.0. All solutions were heated in a water bath at 80 °C for 10 min [20]. Then, the temperature was raised until it was adjusted to 50 °C. After that, the enzymes PA and protease of AO were added in such a concentration to attain the desired enzyme: substrate ratio (**Table 1**). The hydrolytic reactions were stopped by lowering the temperature to 10 °C in an ice bath (H1 and H2) and reducing the pH to 3.0 with 88% formic acid PA (H3 and H4). The hydrolysates were, finally, freeze-dried (Labconco freeze dryer, 77500 model, Kansas City, MI, USA). For all hydrolysates, the total time of hydrolysis was 5 h [20].

Table 1. Hydrolytic conditions are employed for preparing different hydrolysates.

Hydrolysates	Hydrolysis time (h)	E:S (g/100g)	
		AO	PA
H1	AO (1h) +PA (4h)	10	20
H2	AO (1h) +PA (4h)	10	20
H3	PA (5h)	-	1
H4	PA (5h)	-	1

H1 and H3 for skim milk; H2 and H4 for whole milk. E: S: enzyme: substrate ratio; PA: Papain; AO: protease from *Aspergillus oryzae*; Temperature: 50°C.

2.3.3. Methods for lowering the release of phenylalanine from hydrolysis

2.3.3.1. Barium sulphate for adsorption of phenylalanine according to Helbig [21].

The barium sulphate was put inside a syringe (20 mL) which contained glass wool and a filter of nylon. Then, the hydrolysate solution (80 mg/10 mL) was allowed to pass through the column and the elute was collected at 25 °C.

2.3.3.2. Reduce the release of phenylalanine from hydrolysis by probiotic bacteria

Firstly, 0.1g from probiotic strains capsule (which contain a mix of probiotic strains (Lactobacillus, Bifidobacterium and Streptococcus) was inoculated in sterilized 1000 mL MRS medium fortified with agents (0.2 g/L lithium chloride and 0.3 g/L sodium propionate) and incubated anaerobically for 72 hours at 37°C using Gas Generating Kit Anaerobic System, Oxoid, UK [22]. The probiotic bacteria cells were obtained by centrifugation at 5000 rpm for 20 minutes, and the cell pellets were then washed with saline for 5 minutes to remove any residuals of the broth medium by repeated centrifugation. One gram of these pellets was enumerated on LP-MRS agar using the colony counting method. After incubation at 37 °C for 48h, it was found that each 1 g of the previously prepared pellets contained 10 x 10^8 CFU/mL cells. Secondly, the skim milk hydrolysate, either with papain or papain and protease, was inoculated with 2% probiotics pellets, and after that, the inoculated samples were incubated for 48 h at 37°C.

2.3.4. Detection of phenylalanine content of different proteins after hydrolysis using HPLC

The concentration of phenylalanine in different samples after adsorption on barium sulphate or inoculation with probiotic bacteria was determined using an HPLC system (Waters, USA). 0.5 g of each sample source was separately mixed with 8 mL HCL acid (6 M). All samples were hydrolyzed at 110 °C for 22–24h, then placed in an incubator [18]. The supernatant was obtained by centrifugation at 5000 rpm for 10 min. 20 mL of each sample was injected into the RPC18 column (3.9 9 150 mm) for 26 min. The mobile phase speed was 0.8 mL/min under 107–109 kgf/cm2 pressure. In the mobile phase, a mixture of acetonitrile (2:98) and phosphate buffer (pH = 3.5) was used, and UV detection at 214 nm at 25–30 °C [19]. The efficiency of Phe removal was done according to Eq. (1), where the efficiency of phenylalanine (Phe) removal was calculated, where the initial amount of Phe = the amount of Phe present in different protein sources, and a final amount of Phe = the amount of Phe present in hydrolysates after adsorption on barium sulphate.

$$\text{Phe Removal (\%)} = \frac{\text{initial amount of Phe} - \text{final amount of Phe}}{\text{initial amount of Phe}} \text{X } 100 \quad (1)$$

2.3.5. Production of the fermented functional low phenylalanine content product

A functional low phenylalanine product containing probiotic bacteria was prepared in the NRC dairy microbiological lab. Probiotics bacteria, as mentioned before, were prepared from capsules that contained different strains of (Lactobacillus, Bifidobacterium, and Streptococcus), which were mixed and added at 2% to the skim milk hydrolysate by papain (H3) and incubated for 48 h at 37 °C. To confirm the functional final product was free from phenylalanine, the product was centrifuged at 5000 rpm for 20 minutes, then the supernatant was filtered at 0.45 µm before injection into the HPLC to determine the phenylalanine content if found. The final functional product was chemically and microbiologically analyzed.

2.3.5.1. Chemical analysis

The chemical analysis for moisture, ash, fiber, protein, and fat was determined according to [23]. Also, the amino acids of the final product were detected by HPLC as follows: 1 mL of the sample was mixed with 1.5 mL of H2O

and 2.5 mL of HCl (Note: final conc. of HCl is 6 M) and then heated at 100°C for 24 h and filtered. Finally, 1 mL of the filtrate was dried and re-suspended in 0.1 M HCl and injected into HPLC.

2.3.5.2. Microbiological analysis.

A microbiological assay of the product was performed to determine ???. The coliform groups were detected by lactose broth medium using an incubation time of 4h at 37°C [24]. Also, yeast and molds were detected using malt extract agar medium [25], and the medium was acidified to PH 3.5 using incubation temperature at 25°C for 3 days. The probiotics present in the fermented product were enumerated after 1,7,14, and 21 days of storage at 4°C, by LP-MRS agar. The plates were incubated anaerobically at 37°C for 72 h and an anaerobic condition was created using a Gas Generating Kit Anaerobic System (Oxoid, UK). All microbiological analysis was done during the storage period at 1,7,14, and 21 days of cold storage. Then the colonies were counted and expressed as Log CFU/mL.

2.3.6. Statistical analysis

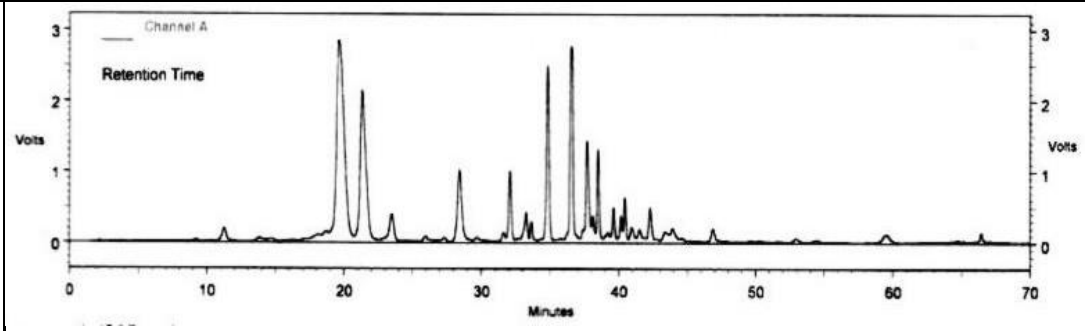
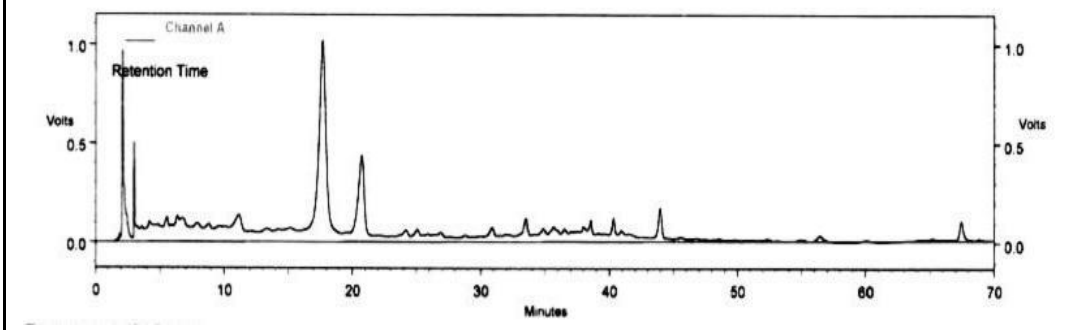
In this study, all tests were performed in triplicate. (ANOVA) the method was used for the analysis of variance, and the Duncan test was used for comparison [26].

3. Results and Discussions

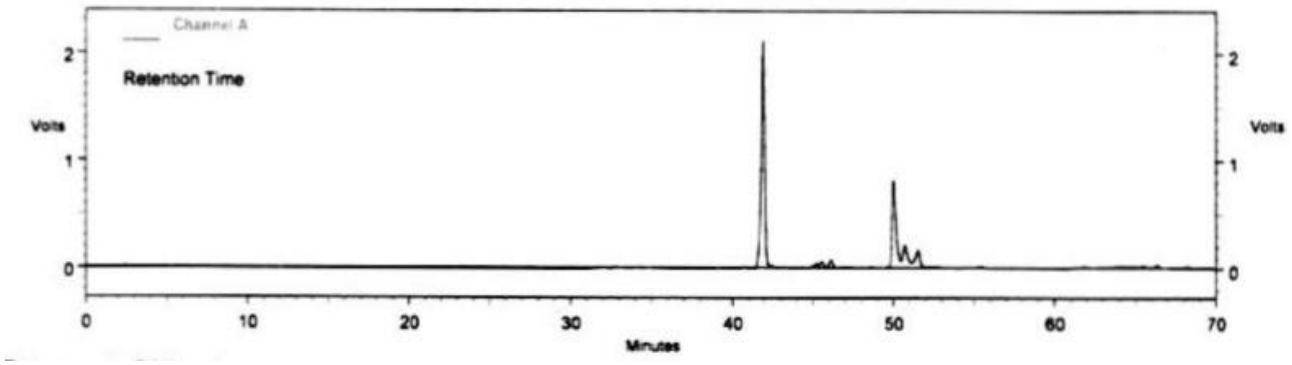
3.1. Detection of phenylalanine content in skim milk and whole milk

Table 2 shows the phenylalanine concentrations that were measured by HPLC. From the obtained results, the sources of proteins contained phenylalanine with concentrations of 2.14 and 2.55 mg/mL for the skim milk and whole milk, respectively. Also, the results did not detect a significant difference between samples in the phenylalanine content. The data by **Lopez-Bajonero & Reinmuth-Selzle** [27, 28] confirmed the results, which indicated that skim milk and others are whey proteins rich in their content of phenylalanine.

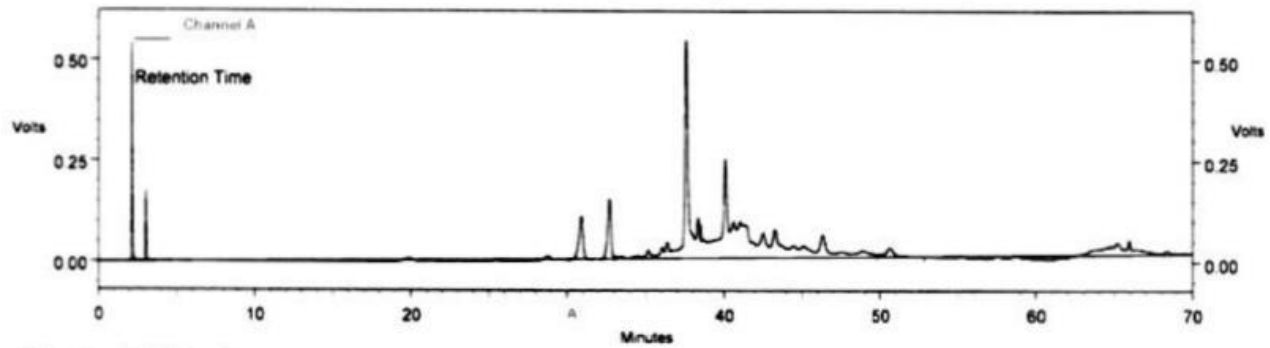
Table 2: The phenylalanine contents in skim milk and whole milk.

Protein sources	HPLC Graph	Ph content (mg\mL)
Skim milk		2.14 ± 0.018
Whole milk		2.55 ± 0.024

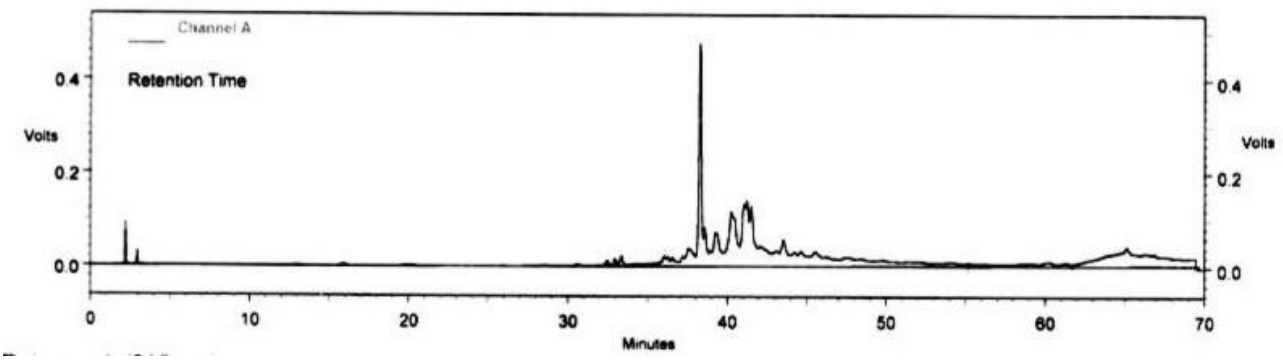
Data expressed as a mean of 3 replicates ± SD. Means with the same lower-case superscript in the same row are not significantly different (P > 0.05).



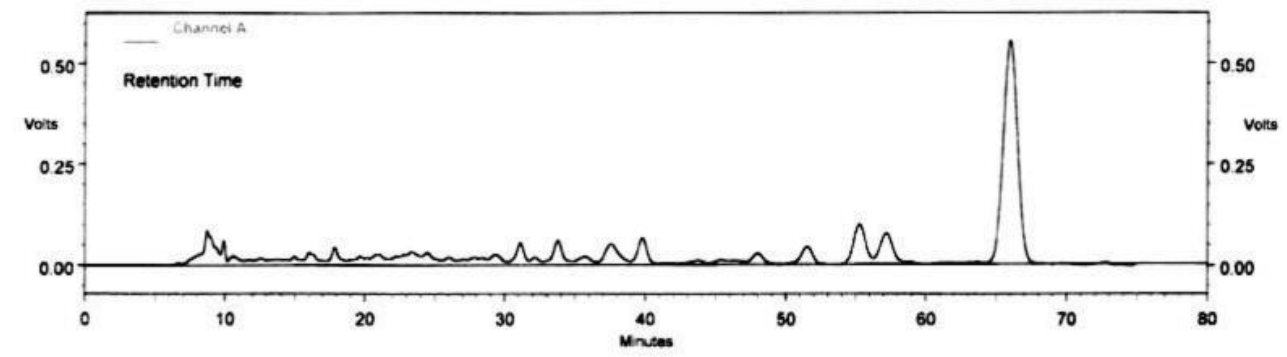
H1: Skim milk hydrolysate treated with papain and protease then treated with barium sulphate.



H2: Whole milk hydrolysate treated with papain and protease then treated with barium sulphate.



H3: Skim milk hydrolysate treated with papain then treated with barium sulphate.



H4: Whole milk hydrolysate treated with papain then treated with barium sulphate.

Figure 1: Efficiency of phenylalanine removal from different hydrolysates by barium sulphate.

3.2. Effect of enzymatic action on protein hydrolysis

The data found in **Table 3 and Figure 1** showed that when either papain or papain with protease was used, followed by adsorption agent barium sulphate, it had the ability to remove the phenylalanine content in skim milk and whole milk. The removal percentage reached 100% for all samples except for H4 (whole milk that was treated with papain), which reached 60.39%. Also, by using HPLC analysis, the phenylalanine content in different hydrolase samples was not detected (reached zero concentration) except for the H4 sample, where phenylalanine was detected at a concentration of about 1.01 mg/mL. The data in the same line as **Shehata** [20], who revealed that the hydrolyzed skim milk had 0.71 grams of phenylalanine per 100 grams of protein, while the skim milk contained 3.26 grams of amino acids per 100 grams of protein. After binding to barium sulfate, the amount of free phenylalanine in the skim milk dropped from 6.34% to 0%.

So, the next step was to evaluate the effect of probiotic bacteria instead of barium sulphate as an absorption agent on skim milk hydrolysate. In this study, the usage of probiotic bacteria was the first time their influence was studied.

Table 3: Efficiency of phenylalanine removal from different hydrolysates by barium sulphate.

Hydrolysates	Removal of Phe (%)	Final Phe content (mg\mL Hydrolysates)
H1	$100.0^{a} \pm 0.1$	0.00 ± 0.5
H2	$100.0^{a} \pm 0.3$	0.00 ± 1.2
H3	$100.0^{a} \pm 1.1$	0.00 ± 4.5
H4	$60.39^{b} \pm 0.1$	1.01 ± 0.5

Final Phe content = Concentration of phenylalanine after adsorption on barium sulphate. H1 and H3: hydrolase for skim milk. H2 and H4: hydrolase for whole milk. Data expressed as a mean of 3 replicates ± SD. Means with different lower-case superscripts in the same column are significantly different (P > 0.05).

3.3. Effect of probiotic bacteria action on protein hydrolysis of skim milk

The effect of probiotic bacteria on protein hydrolysis of skim milk was indicated in **Table 4 and Figure 2,** which demonstrated that the probiotic bacteria were able to consume the hydrolysate protein after treating skim milk with either papain or papain and protease. The percentage of removal after enzymes were treated reached 100 and 75.23 % for H1 and H3 (skim milk hydrolysates with papain only and papain with protease), respectively. Moreover, the HPLC analysis was used to determine the phenylalanine content after incubating the two hydrolysates, and data recorded that those probiotic bacteria consumed the hydrolysates and did not detect phenylalanine content in H1, and detected a low percentage, about 0.53 mg/mL, for H3. So, probiotic bacteria had effective and beneficial properties for treating skim milk for phenylalanine removal to be more suitable for patients with phenylketonuria. According to the study by **Kim** [29], *Lacticaseibacillus rhamnosus* IDCC 3201 actively secretes proteases that hydrolyze milk proteins. In addition, they demonstrated that in a mouse model fed a high-protein diet, co-administration of milk proteins and *L. rhamnosus* enhanced the digestibility and plasma concentrations of amino acids. Consequently, adding *L. rhamnosus* to food may be an alternate method of improving protein digestibility.

Generally, the study by **Al Hafid, & Christodoulou** [6] concludes that the change in gut microbiota by the probiotic strain supplementation had an appositive effect on PKU by an altered gut microbiome composition. This may suggest that the current Phe-restricted diet for PKU patients could be optimized by taking dietary effects on the microbiome into account. Therefore, the next step in this study was to produce functional products with probiotic bacteria and hydrolysate skim milk to be healthier for the patients.

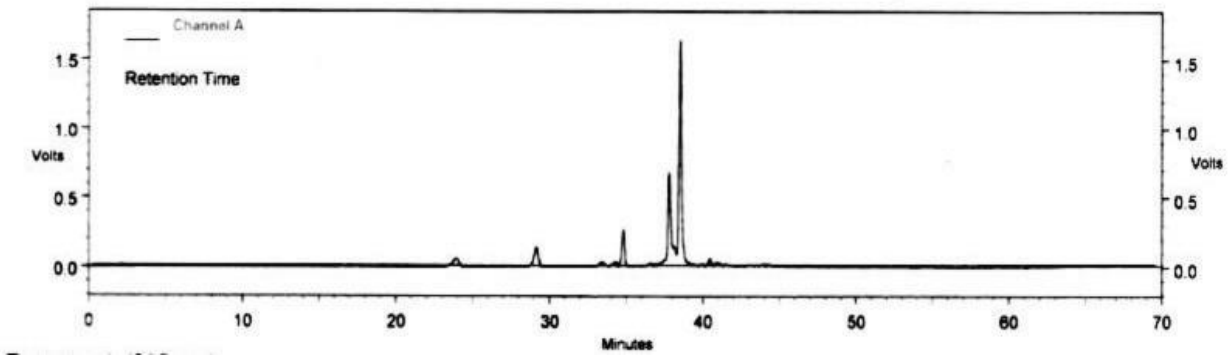
Table 4: Efficiency of phenylalanine removal from skim milk after inoculation with probiotic bacteria.

Hydrolysates	Removal of Phe (%)	Final Phe content (mg\mL Hydrolysates)
H1*	$100.0^{a} \pm 0.2$	0.00 ± 0.8
H3*	$75.23^{b} \pm 0.3$	0.53 ± 1.3

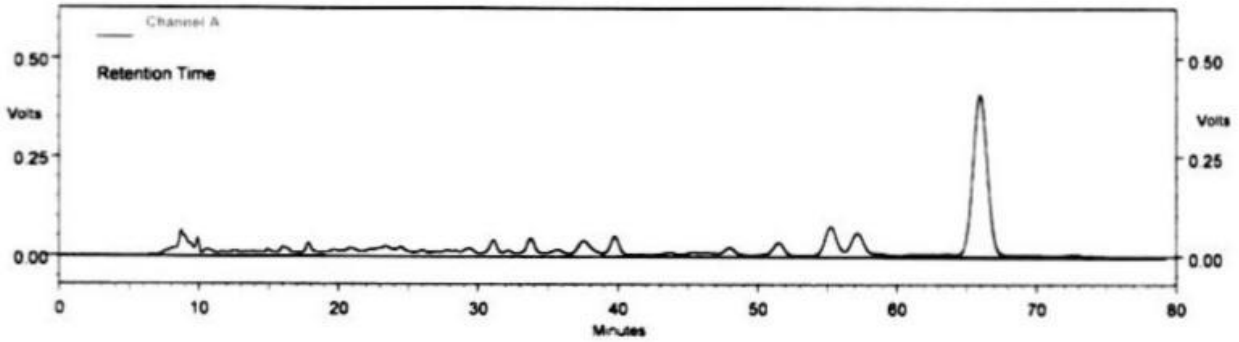
Data expressed as a mean of 3 replicates ± SD. Means with different lower-case superscripts in the same column are significantly different (P > 0.05). H1 and H3: hydrolase for skim milk.

3.4. The chemical composition of the functional product

Table (5) shows the chemical composition of a functional product that is suitable for phenylketonuria patients. The percent of moisture, ash, protein, and fat in the final functional product was recorded as 84.2,8,5, 2.23, and 0.5 %, respectively, in comparison with skim milk. The chemical composition of skim milk was 90, 7.2, 3.6, and 0.2 % for moisture, ash, protein, and fat, respectively. The important difference was found in protein content. More protein was detected in skim milk (3.6%) than in the final product (2.23%). This result may be related to the hydrolase influence of papain and the action of probiotic bacteria on skim milk during production. So, this study gave an important final product for phenylketonuria patients fortified with probiotic bacteria that added other health benefits for the patients.



H1*: Skim milk hydrolysate treated with papain enzyme and then inoculated with probiotic bacteria.



H3*: Skim milk hydrolysate treated with papain and protease then inoculated with probiotic bacteria.

Figure 2: skim milk hydrolysates (H1*, H3*) and inoculated with probiotic bacteria.

Table 5: The chemical composition of the functional product

Sample	DH (%)	Ash	Protein	Fat
Skim milk	**90**	**7.2**	**3.6**	**0.2**
Product	**84.2**	**8.5**	**2.23**	**0.5**

3.5. The amino acid content of the functional product

The amino acid content in the functional final product was recorded in **Table 6.** The data showed that the amino acid content was different when compared with skim milk, which was related to the action of papain and the activity of probiotic bacteria. The high amino acid content for the final product was detected for glutamic acid (23.9), aspartic acid (9.2), and cystine (5.3) when compared with amino acids in skim milk. In contrast, the variation for the lysine, proline, phenylalanine, and valine, which are more detected in skim milk with percentages 8.5, 10.4, 4.5, and 6.5, respectively, but not detected phenylalanine in the final product and there was little content for proline (3.0), lysine (3.0) and valine (5.1). Moreover, slight differences in other amino acids were detected between the final product and skim milk content as alanine, glycine, isoleucine, serine, methionine, and tyrosine. The data by **Amiri-Rigi** [30] found that methionine, tyrosine, tryptophan, and histidine concentrations in skim milk were relatively greater than those in the skim milk hydrolysate (1.9, 2.5, 3.2, and 1.4 against 1, 1.9, 2.5, and 1.1 mg /100 mg protein, respectively).

Table 6: Amino acids content in skim milk and final product

Amino acid	Skim milk	Final product
Alanine	3.2	3.5
Arginine	3.4	3.4
Aspartic acid (ASP)	7.5	9.2
Cystine	0.7	5.3
Glutamic acid (GLU)	19	23.9
Glycine	1.5	1.9
Histidine	2.5	2.3
Isoleucine	6.1	6.0
Leucine	10.1	10.8
Lysine	8.5	6.0
Methionine	2.5	2.7
Phenylalanine	4.5	0.0
Proline	10.4	3.0
Serine	5.8	5.9
Threonine	4.0	5.6
Tryptophane	0.0	0.0
Tyrosine	5.0	4.9
Valine	6.5	5.1

3.6. The microbiological analysis of the functional product

The results found that during the storage period, the final functional product was free from coliform, mold, and yeast counts for 21 days. This is related to the hygienic and sterilized conditions during the manufacture of the final product, as detected by Khalil et al**.** [31]. Also, the count of probiotics was determined during the storage period. On day one, the count was 10.49 log CFU/mL. The viable count of probiotics was gradually decreased with the storage period to 8.52, 6.55, and 6.49 log CFU/mL at 7, 14, and 21 days of storage, respectively. Generally, the count of probiotics was more than 10^6 CFU/mL according to the regulation of FAO [32]. Therefore, the change in the microbiome by the consumption of food fortified with probiotic strains had a positive influence on human health by colonizing these strains inside the intestine and gave different healthy effects, such as type 2 diabetes, inflammatory bowel disease, lowering cholesterol, and asthma [33-35].

4. Summary

The goal of the study was to develop probiotic-fortified functional dairy products for people with phenylketonuria (PKU). The amount of phenylalanine in skim and whole milk was measured using HPLC analysis, and the effectiveness of using barium sulphate or a combination of probiotic bacteria to reduce phenylalanine release was assessed. Phenylalanine concentrations in skim milk and whole milk were 2.14 and 2.55 mg/mL, respectively. Probiotic microorganisms, a recent development in place of barium sulphate, have the capacity to absorb protein hydrolysate and eliminate phenylalanine content. Using papain, 2% mixed probiotic strains were added to skim milk hydrolysate to create the functional low phenylalanine dairy product. The functional products were free of phenylalanine, mold, yeast, and coliform levels throughout storage, and they had a higher proportion of moisture, ash, and fat than skim milk. Additionally, after 21 days of storage, the finished products were free of mold and yeast and supplemented with high probiotic bacterial counts.

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