Viability of lactic acid bacteria in polyphenol-enriched fermented milks

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Abstract. The enrichment of probiotic dairy products with polyphenol extracts from different plant origins is a good approach to enhance the beneficial health effects of functional foods. The aim of the present study was to evaluate the effect of rose (*Rosa damascena* Mill.) petals polyphenol extract (RPPE) fortification on the lactic acid production and the viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* S19 (*Lb. bulgaricus*), *Lactobacillus rhamnosus* YW (*Lb. rhamnosus*) and *Streptococcus thermophilus* S13 (*S. thermophilus*) in fermented milk samples during refrigerated storage. The fermented milk samples with RPPE (samples R1 and R2) and without RPPE (samples K1 and K2) were stored at 4 ± 2°C for 15 d. The results for physicochemical parameters showed a constant decrease in the pH values from 4.35 ± 0.04 to 3.99 ± 0.03 and an increase in the lactic acid concentration from 8.68 ± 0.17 g/L to 11.45 ± 0.26 g/L, respectively. The residual lactose concentration in the controls (K1 and K2) and supplemented samples (R1 and R2) at the end of the refrigerated storage was about 32.0 ± 0.24 g/L. Good survival of probiotic strains of lactic acid bacteria was observed. The results show that on the 15th day of refrigerated storage, the total count of lactic acid bacteria remains high - about 2.8×10^8 CFU/ml. Thus, RPPE could be used as a functional ingredient in fermented milk production.

1 Introduction

Probiotics are well known to have health benefits for human organisms when consumed in sufficient amounts. Thus, in recent decades, there has been a rapid increase in the production of functional foods, particularly probiotic fermented milk. In order to exhibit the optimal probiotic effects, the number of viable probiotic cells must be around 10^6–10^7 cfu/g or ml at the end of the product's shelf life [1, 2]. However, their slow growth in milk and loss of viability during storage are the main issues with adding probiotic bacteria to fermented milk formulas [3].

Polyphenols possess high antioxidant activity that has a positive effect on the prevention of oxidation processes [4]. In addition to being advantageous for human health because of their antioxidant activity, polyphenols are helpful for milk products during their shelf life [5]. The incorporation of phenolic-rich ingredients in milk products has been shown to improve the survival of probiotic cultures [6]. In a recent study, it was established that polyphenol extracts from industrial plant by-products stimulated the growth of lactic acid bacteria strains [7].

Bulgaria is one of the main rose-processing countries in the world, together with Turkey, which extracts rose oil from *Rosa damascena* Mill. petals using water-steam distillation. Several thousand tons of waste material are produced annually from the distilleries in Bulgaria alone since it takes more than 3,000 kg of petals to produce 1 kg of rose oil and 1 kilogram of the fresh raw material produces up to 2 kg of residue on a wet weight basis. With the use of water-steam distillation, the phenolic compounds are retained in the waste material as a result of the selective rose oil recovery without solvent extraction [8].

Fermented milks enriched with polyphenol extracts from plant by-products may be an alternative dairy product to deliver probiotic bacteria and have created great interest in the development of functional foods and nutraceuticals. Therefore, the objectives of this study were to evaluate the effect of rose (*Rosa damascena* Mill.) petal polyphenol extract (RPPE) fortification on the lactic acid production and the viability of *Lactobacillus delbrueckii subsp. bulgaricus* S19, *Lactobacillus rhamnosus* YW, and *Streptococcus thermophilus* S13 in fermented milk during 15 days of refrigerated storage.

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2 Materials and methods

2.1. Polyphenol extract

Rose (*Rosa damascena* Mill.) petals were supplied by Ecomaat Ltd. (Mirkovo, Bulgaria). The petals were dried in a thin layer at room temperature (25 - 27°C) for one week before final hot air drying (60°C, 1 h). Dried rose petals were stored in a desiccator in the dark until used to produce polyphenol extract. Rose (*Rosa damascena* Mill.) petals polyphenol extract (RPPE) was obtained by adsorption technology following the method described by [9]. Rose petal polyphenols were extracted with 30% aqueous ethanol using approximately 350 g of finely milled pomace (particle size < 4 mm) at a liquid to solid ratio of 20:1 (v/w). After 1 h of stirring at ambient temperature, the extraction mixture was filtered using a paper filter, and the organic solvent was evaporated under vacuum (40°C). The extract obtained was either purified on a column (465 × 30 mm) filled with Amberlite XAD 16 HP. Prior to sample application, the resin was conditioned and equilibrated as described above. Then 250 ml of the extract was applied and the column subsequently rinsed with 1000 ml of acidified water (TFA, pH 2). For the elution of the rose petal phenolic 500 ml of a mixture of ethanol and acidified water (pH 2) (95: 5, v/v) was applied to the column. After evaporation and concentration under vacuum (30°C), the polyphenols were lyophilized for 48 h.

2.2 Bacterial strains

Pure strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* S19 (*Lb. bulgaricus*), *Lactobacillus rhamnosus* YW (*Lb. rhamnosus*) and *Streptococcus thermophilus* S13 (*Str. thermophilus*) were used as starter culture. They were provided by Department of Microbiology at the UFT, Plovdiv.

2.3 Fermented milk samples

The study used two the control and experimental batches of fermented milk were manufactured in laboratory conditions according to the following procedure: Cow milk was heated to 95°C for 15 min, cooled to 45 ± 1°C and separated into two lots: one experimental lot (samples R1 and R2) fortified with polyphenols to 0.39 mg/100 g by using of RPPE and an unfortified control lot (samples K1 and K2). The experimental and control samples of milk were inoculated with 2% Bulgarian yogurt starter culture, consisting of *Lb. bulgaricus*: *Str. thermophilus* in a ratio of 1: 5 (samples K1 and R1) and probiotic starter culture, consisting of *Lb. bulgaricus*: *Lb. rhamnosus*: *Str. thermophilus* in a ratio of 0.5: 0.5: 5 (samples K2 and R2). All samples were packaged in containers and incubated at 44 ± 1°C until pH 4.6 ± 0.1 was reached. Then, the fermented milk samples were cooled down to approximately 4°C and stored at the same temperature for 15 d.

2.4 Microbiological analysis

In order to investigate the effects of RPPE on the viable bacteria of enriched fermented milk, the enumeration of characteristic microorganisms was performed by means of the colony-count technique. The samples were prepared according to [10]. For enumeration of *Lb. bulgaricus* and *Lb. rhamnosus*, De Man rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany) was used. Plates were incubated at 43°C for 72 h. M17 agar (Merck, Darmstadt, Germany) was used for enumeration of *Str. thermophilus*. Plates cultured in aerobic conditions were incubated at 37°C for 48 h. The number of viable bacteria in each sample was counted after 1, 3, 6, 9, 12, and 15 days of cold storage at 4°C. The results were expressed based on log colony-forming units per millilitre (log cfu/ml). The total viable count (TVC) was determined as sum of count of lactobacilli and streptococci.

2.5 Physicochemical analysis

The pH values of the samples were determined using a digital MS 2011 pH meter (Microsyst, Plovdiv, Bulgaria) equipped with a Sensorex pH electrode (Garden Grove, CA, USA). The titratable acidity was measured by the titration method [11]. The residual lactose content was calculated on the basis of the results from initial lactose content in milk and lactic acid formation during the storage period.

Total polyphenols and antioxidant capacity were determined spectrophotometrically. Before the analysis, 20 ml of fermented milk samples were dissolved in 50 ml of pure methanol. For a better extraction of the polyphenolic compounds, the samples were left under refrigeration conditions (4 ± 1°C) for 12 h. Then they were tempered at room temperature and filtered through a paper filter.

The total polyphenol (TPP) content was determined by the method of [12]. The results were expressed as mg gallic acid equivalents (GAE) per 100 ml of sample.

The total antioxidant capacities were determined by the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and FRAP (ferric reducing antioxidant power) assays. The results of both tests were expressed as µmol Trolox equivalents (TE) per 100 ml of sample.

2.6 Statistical analysis

The results reported in the present study are presented as the means with standard deviations (SD) from three determinations. Data were analysed using one-way ANOVA performed with Microsoft Excel and the significance level was set at $P < 0.05$, followed by the Turkey test.

3 Results and discussion

3.1 Quality characteristics of polyphenol-enriched fermented milk

The main physicochemical and microbiological characteristics of control and polyphenol-enriched fermented milk samples are presented in Table 1. It could be seen that the pH values, lactic acid and residual lactose contents in the control samples (K1 and K2) and polyphenol-enriched samples (R1 and R2) did not differ significantly ($P < 0.05$). These results are in agreement with data obtained by other authors. Karaaslan et al. [13] found that the addition of red grape polyphenolic extracts had no effect on the active acidity (pH) of fermented milk samples compared to control samples.

The viable cells counts of probiotic lactic acid bacteria *Lb. bulgaricus* S19, *Lb. rhamnosus* and *Str. thermophilus* S13 in the experimental samples were above 10^8 CFU/g. An important prerequisite for the functional characteristics of fermented milk is that it should contain a high count of viable probiotic bacteria [14].

Table 1 Physicochemical and microbiological characteristics of yogurts enriched with polyphenol extracts

Parameters*	Samples			
	K1	K2	R1	R2
pH	4.34±0.04	4.35±0.05	4.42±0.04	4.41±0.03
Lactic acid, g/l	8.37±0.02	8.19±0.03	8.55±0.05	8.64±0.02
Lactose, g/l	34.65±0.2	34.82±0.3	34.48±0.20	34.39±0.4
TVC, cfu/ml	3.6×10^8	3.8×10^8	3.6×10^8	2.4×10^8
TPP, mg GAE/100 ml	0	0	55.8±2.9	54.2±2.5
DPPH, µmol TE/100 ml	32±3.5	35±1.5	167±0.5	169±3.0
FRAP, µmol TE/100 ml	41±4.0	44±2.0	197±5.5	199±6.0

*mean value ± SD (n=3)

The results show that the content of total polyphenols in the polyphenol-enriched fermented milk samples (R1 and R2) is significantly higher ($P < 0.05$) compared to the corresponding control samples (K1 and K2). The data on the content of total polyphenols in the studied samples directly correlates with their antioxidant activity. These dependencies support the statement that higher values of total polyphenols cause higher antioxidant activity. The experimental samples of fermented milk with added extracts of distilled rose petals were characterized by significantly higher values of radical scavenging (DPPH-test) and metal-reducing (FRAP-test) activities compared to the corresponding control samples.

3.2 Study of the growth and activity of the probiotic lactic acid microflora

During cold storage of fermented milk samples (Fig. 1 and Fig. 2) form the 1st to the 15th d, a constant decrease in the pH values from 4.35 ± 0.04 to 3.99 ± 0.03 and an increase in the lactic acid concentrations from 8.68 ± 0.17 g/L to 11.45 ± 0.26 g/L, was observed.

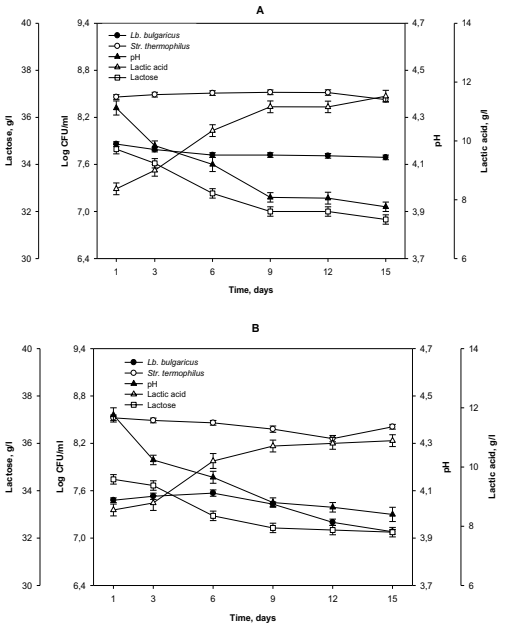

Fig. 1. Growth and acidification activity of starter culture in fermented milks during refrigerated storage: (A) control sample – K1; (B) polyphenol-enriched sample – R1

The residual lactose concentration in the controls (K1 and K2) and enriched samples (R1 and R2) at the end of the refrigerated storage was about 32.0 ± 0.24 g/L. No significant ($P < 0.05$) differences in the pH values or the lactic acid concentrations between control and enriched samples were observed. This shows that the post-acidification process of fermented milk during refrigeration storage was unaffected by the enrichment of the fermented milk with RPPE. These results are in accordance with the data obtained by [15], which reported that polyphenol addition did not affect the dynamic of the fermentation process during storage of yogurt. In this study (Fig. 2), the incorporation of *Lactobacillus rhamnosus* YW into the starter culture did not have a significant ($P < 0.05$) effect on the acidification of the starter microflora during the storage process.

The addition of polyphenolic compounds from different plant by-products to probiotic fermented milk has been proposed as a promising strategy to enhance the beneficial health effects of functional dairy products [16]. Phenolic compounds are reported to inhibit the growth of pathogenic bacteria and fungi [17] as well as lactic acid bacteria [18]. Ahmad et al. [19] evaluated the effect of apple peel polyphenol extract (APPE) on the viable count of *L. bulgaricus*, *S. thermophilus, B. lactis*, and *L. acidophilus* in yoghurts during 21 d of refrigerated storage. The authors found that the survival of probiotics was enhanced with the addition of APPE.

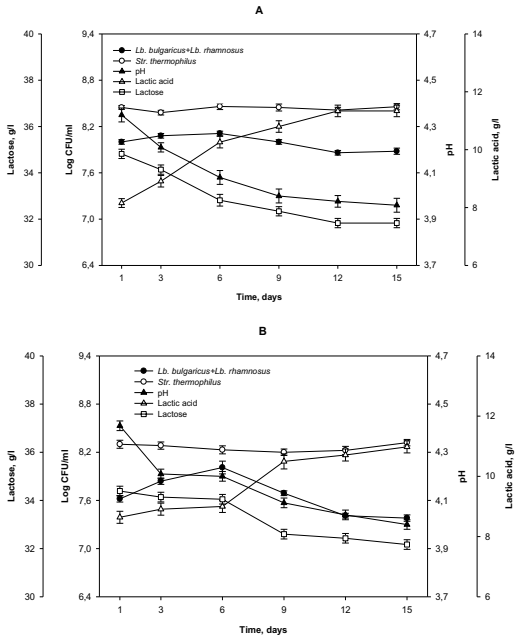

Fig. 2. Growth and acidification activity of starter culture in fermented milks during refrigerated storage: (A) control sample – K2; (B) polyphenol-enriched sample – R2

In the present study, the total viable count of lactobacilli and streptococci during 15 d of storage was investigated (Table 1). During the entire storage period, good survival of probiotic strains of lactic acid bacteria was observed. The count of *Streptococcus thermophilus* did not change significantly ($P < 0.05$) in all samples (Fig. 1 and Fig. 2). These results revealed that RPPE did not directly affect the viability of *Str. thermophilus*. A similar trend was observed by other authors [20]. A slight decrease (about 0.5 log) in the viable count of *Lactobacillus delbrueckii subsp. bulgaricus* S19 in all fermented milk samples was perceived. It was observed that the incorporation of *Lactobacillus rhamnosus* YW into the starter culture was accompanied by a slightly higher count of lactobacilli at the end of storage period (samples K2 and R2). This is probably due to its better survival under cold conditions than *Lactobacillus delbrueckii subsp. bulgaricus* S19. No significant changes in the viability of lactic acid bacteria in control samples and supplemented samples were observed. This showed that, adding RPPE to fermented milk had no effect on the viability of lactic acid bacteria after 15 days of refrigerated storage.

4 Conclusion

Rose (*Rosa damascena* Mill.) petal polyphenol extract (RPPE) is a good source of bioactive compounds. The use of RPPE increased the total phenolic content and antioxidant activity of probiotic fermented milk. Rose petal polyphenols, which are known for their beneficial effects on the modification of gut microbiota, may be added to probiotic foods to further increase the health advantages for customers. The addition of RPPE to fermented milk had no significant effect on the post-acidification during 15 d of refrigerated storage. All samples tested maintained a high level of total count of lactic acid bacteria (3.4×10^8) throughout the refrigerated storage period.

Funding for this study was provided by the Bulgarian Ministry of Education and Science through National Research Program "Healthy Foods for a Strong Bio-Economy and Quality of Life" approved by DCM # 577 / 17.08.2018".

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