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Evaluation of human origin *Lactobacillus* isolates for the production of probiotic fermented milk

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ABSTRACT

In the present study, *L. rhamnosus* IF7, *L. paracasei* ssp. *paracasei* IF10, *L. fermentum* IF14 and *L. fermentum* IF15, isolated from infant faeces, were combined with *Streptococcus thermophilus* and examined to be used as starters to produce probiotic fermented milk (PFM). The experiments were stored at 4±2°C for 21 days. Lactic acid, pH, viscosity, serum separation, viability and sensory properties were assayed as quality criteria at 1, 7, 14, and 21st day of storage. Lactic acid contents of the experiments increased and pH decreased gradually during fermentation and storage. Viscosity of PFM experiments ranged between 9.10 and 19.60 cp. Viable counts of experimental *Lactobacillus* strains were found to be above the threshold for the therapeutic minimum (10^6-10^7cfu/g) and maintained during storage period. Panelists gave desirable organoleptic attitudes for all final products.

Keywords: *probiotic*, *human origin isolates*, *fermented milk*, *Lactobacillus*.

1. INTRODUCTION

Functional dairy products have recently become the focus of attention of consumers due to being supported by research findings about positive effects on health. Therefore, production of this kind of dairy products has gained momentum. Probiotics are defined as a mono-or mixed cultures of living microorganisms applied to animal or man affecting beneficially the host by improving the properties of the indigenous microflora [1]. Yoghurt is the most popular fermented dairy product. However, conventional yoghurt cultures, *Streptococcus salivarius* ssp. *thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), are not usually a part of the native microbiota of mammal intestine [2], lacking the ability to pass alive through the intestinal tract [3], showing limited survival after oral ingestion [4] and, thus they do not play a role in the human gut [3]. Therefore, it recently seems to be a new approach to develop novel fermented dairy products such as probiotic fermented milk that contain selected lactic acid bacteria (LAB) isolated from the human intestine [5]. The term "probiotic fermented milk" (PFM) is defined as alternative culture yoghurt when *L. bulgaricus* is substituted by other *Lactobacillus* species for the fermentation of milk or probiotic bacteria are added to the yoghurt cultures [6].

Selected strains of LAB belonging to *Lactobacillus* genus, present in the human gastrointestinal tract as its natural inhabitants, can be beneficial for the host and therefore are regarded as probiotics [7]. According to Gilliand [8], the culture, to be considered a valuable candidate for use as a probiotic, must be a normal inhabitant of the human intestinal tract. New probiotic strains should be screened by evaluating not only their potential beneficial outcomes, but also for their technological performances, such as growth rate, acidification ability and stability in milk and favorable organoleptic characteristics of final product [9]. In this study, *Lactobacillus* strains combined with *S. thermophilus* were investigated for their technological performances, such as growth rate and viability at cold storage in milk, acidification capacity, and suitable organoleptic properties of the final product. For this aim, the strains of *L. rhamnosus* IF7, *L. paracasei* ssp. *paracasei* IF10, *L. fermentum* IF14 and *L. fermentum* IF15 were evaluated as a potential strain in PFM products.

2. EXPERIMENTAL SECTION

2.1. Bacterial cultures.

L. rhamnosus IF7, *L. paracasei* ssp. *paracasei* IF10, *L. fermentum* IF14 and *L. fermentum* IF15 were obtained from culture collection of Department of Food Engineering, Namik Kemal University (Turkey). They had been isolated from the infant faeces and characterized previously such as acid and hydrogen sulphide (H_2S) production ability, antibiotic resistance and antibacterial activities against some pathogenic bacteria [10]. *S. thermophilus* and *L. bulgaricus* were supplied by Chr. Hansen-Peyma, Istanbul (Turkey). For mother culture, each strain was inoculated into pasteurized skim milk and incubated until the appearance of the coagulum (pH under 4.7). Laboring cultures were propagated successively three times before use for activation.

2.2. Preparation of probiotic fermented milks (PFM).

It was used raw bovine milk containing 8.4% non-fat dry matter, 3.16% protein, 3.45% fat and 6.68 pH. Milk was tempered to 65 °C and homogenized at 150 bar. Than milk was batch pasteurized at 90 °C for 15 min and immediately cooled at 40 °C. After pasteurization, milk was divided into five experiments and transferred into 500 ml plastic cups. One experiment was the

control (C) inoculated with yogurt culture (2% inoculums) and, the other four experiments (PFM) were inoculated with different combination of bacterial cultures (5% inoculums) one by one as shown in Table 1. Then, all experiments were incubated at 40 °C in incubator until coagulated. All experiments were stored for 21 days at 4±2 °C after production.

Table 1. Product code and ratio of the culture combinations (% v/v) for PFM experiments.

Experiments Code	Strains and Ratio (% v/v)
C (Control)	*L. bulgaricus* + *S. thermophilus*(2/1)
PFM1	*L. rhamnosus* IF7 + *S. thermophilus*(2/2)
PFM2	*L. paracasei* ssp. *paracasei* IF10 + *S. thermophilus* (2/2)
PFM3	*L. fermentum* IF 14 + *S. thermophilus*(2/2)
PFM4	*L. fermentum* IF 15 + *S. thermophilus (*2/2)

2.3. Analyses.

Titratable acidity (%), pH, serum separation, viscosity, sensory characterization and viability of starter bacteria were analyzed at days 1, 7, 14 and 21 of storage. Fat, pH and protein were also assayed as quality criteria of milk. All experiments and analyses were replicated three times. The presented results are averages of all replicates.

2.3.1. Microbiological analyses.

For the enumeration of lactic acid bacteria (LAB), 1 g experiment was decimally diluted in sterile peptone water (0.1%) and 0.1 mL aliquot dilution plated over the appropriate media. *Lactobacillus* spp. and *S. thermophilus* were enumerated according to the method of Dave and Shah [11]. *Lactobacillus* spp. was enumerated on MRS agar adjusted to pH 5.2 and anaerobic incubation (Gas Pak System-Oxoid) at 43 °C for 72 h ST agar and aerobic incubation at 37 °C were used selective enumeration of *S. thermophilus.* Results were expressed as colony forming unit (cfu).

2.3.2. Physico-chemical analysis.

Dry matter, fat and titratable acidity were determined according to the standard methods of AOAC procedure [12]. pH measurements were carried out by a digital pH-meter (WTW 330). Viscosity was measured with digital AND SV10 vibro viscometer read in centipoises (cp). In serum separation analysis, 25 g experiment was put in the normal filter paper at +4 °C and the amount of separated serum was calculated after 2 hours as mL/25 g [13].

2.3.3. Sensory analysis.

The sensory panel comprised of 10 trained members familiar with dairy products. Panel members evaluated experiments for (1) colour, (2) consistency, (3) taste and (4) flavour and generally acceptability. Ten-point scale was used for all characteristics with 1-2 being poor, 3-4 fair, 5-6 good, 7-8 very good, and 9-10 excellent. Panelists also informed to report any defects in examined characteristics such as fat separation or lack of uniformity in appearance, granular or slimy in texture and off flavour or excess acid in flavour.

2.3.4. Statistical analysis.

The data were analyzed with one-way analysis of variance with SPSS version 10.0 for Windows (SPSS Inc., NY, USA). The comparison between means of data was carried out using the Tukey honestly significant difference test.

3. RESULTS SECTION

3.1. Bacterial counts.

Table 2 showed microbiological values in PFM samples during 21 days storage duration. As can be seen, viable cell count of all strains were higher the critical level of 6 log cfu/g [14,15]. Therefore, high symbiotic relationship was observed between *Lactobacillus* used in this study. *S. thermophiles* and *Lactobacillus* strains which reached cell count of 7-9 log cfu/g at the end of fermentation period and were able to maintain their vitality 21 days storage duration. At the beginning of storage L. *paracasei* ssp. *paracasei* IF10 reached the highest number of 9.4 log cfu/g, whereas, *L. fermentum* IF14 gave the lowest value of 7.5 log cfu/g. Although *L. fermentum* IF14 was the lowest in the beginning, viable cell count of this strain reached the highest value of 9.9 log cfu/g at the 7th day of storage among other strains. After the 21 days of storage period, highest count number of 8.9 log cfu/g was observed in the control sample which was *L. bulgaricus* followed by *L. fermentum* IF15, *L. paracasei* ssp. *paracasei* IF10 and *L. fermentum* IF14 with the corresponding values of 8.8, 8.6 and 8.4 log cfu/g, respectively and *L. rhamnosus* IF7 had the lowest value of 7.5 log cfu/g. In a similar study, Hekmat and Reid [16] determined viable cell count of 8 log cfu/g for *L. rhamnosus* in milk into which any prebiotic was not added and protect this number during 1 month of storage period in refrigerator. Also, Schillinger [5] found cell counts of 8, 4-5 and 6-7 log cfu/g for *L. rhamnosus*, *L. casei* and *L. paracasei,* respectively in mild and probiotic yoghurt samples. Sodini et al. [17] observed the number of 7.75 log cfu/g for *L. rhamnosus* when it is mixed with *S. thermophilus* and after 4 weeks of storage duration at 4 °C, the number reached the level of 8 log cfu/g.

Table 2. Viable counts of *Lactobacillus* spp. and *S. thermophiles* in the experiments during the storage period.

	***Lactobacillus* spp. (log cfu/g)**			
Exp.*	**Day 1**	**Day 7**	**Day 14**	**Day 21**
C	9.1±0.1^{bc}[1]	9.8±0.1^{c}	8.9±0.1^{b}	8.9±0.1^{b}
PFM1	8.3±0.6^{ab}	7.7±0.3^{a}	7.5±0.4^{a}	7.5±0.4^{a}
PFM2	9.4±0.4^{c}	9.7±0.3^{c}	8.6±0.1^{b}	8.6±0.1^{b}
PFM3	7.5±0.3^{a}	9.9±0.3^{c}	8.4±0.2^{b}	8.4±0.2^{b}
PFM4	9.2±0.6^{c}	8.7±0.3^{b}	8.8±0.6^{b}	8.8±0.6^{b}
	S. thermophilus (log cfu/g)			
Exp.*	Day 1	Day 7	Day 14	Day 21
C	8.2±0.5^{a}	8.9±0.6^{a}	9.9±0.9^{b}	7.0±0.5^{a}
PFM1	9.1±0.4^{b}	9.7±0.1^{b}	9.6±0.6^{ab}	9.7±0.1^{c}
PFM2	9.2±0.1^{b}	9.7±0.2^{b}	9.8±0.2^{b}	9.8±0.5^{c}
PFM3	8.7±0.2^{ab}	9.5±0.2^{b}	9.3±0.3^{a}	9.2±0.4^{bc}
PFM4	9.1±0.3^{b}	9.3±0.1^{ab}	9.9±0.5^{b}	8.9±0.5^{b}

*:C (control): *L. bulgaricus*+ *S. thermophilus* PFM1: *L. rhamnosus* IF7 + *S. thermophilus* PFM2: *L. paracasei* ssp. *paracasei* IF10 + *S. thermophilus* PFM3: *L. fermentum* IF14 + *S. thermophilus* PFM4: *L. fermentum* IF15 + *S. thermophilus.*

[1]Different small letters in same column indicate the significant differences (*P<0.05*).

Concerning the viable cell counts for *S. thermophiles* in PFM samples, 8-9 log cfu/g values were obtained in all samples. At the first day of storage, *S. thermophilus* showed the highest value of 9.2 log cfu/g in PFM2, whereas, control sample gave the lowest value of 8.2 log cfu/g. Count number of *S. thermophiles* in all samples increased during 7th and 14th days of storage and maintained at the level between 7 and 9 log cfu/g at the 21th day. Similarly, count number of 8 log cfu/g for *S. thermophilus* was observed in Martin-Diana et al. [18] who studied PFM samples in milk and goat milk at the 1st and 21st days and in Dave and Shah [19] who studied yoghurt samples included probiotic bacteria at the 1st and 35th days of storage time.

3.2. Titratable acidity and pH.

The initial pH of milk (6.65) decreased under at 4.7-4.6 point, which shows the critical coagulation point of yoghurt, in all PFM experiments during fermentation (Table 3). While the PFM4 showed the lowest pH with 4.25, the highest pH value observed in PFM3 with 4.45. Xanthapoulos et al. [20] observed that human origin *L. rhamnosus* and *L. paracasei* subsp. *paracasei* strains acidified milk at 40 °C after 24 h of incubation as pH range from 3.40 to 3.51 and 4.71 to 5.01, respectively. After the fermentation, a gradual decrease in the pH was observed throughout the storage period in all experiments. The end of storage period, 21thday, the pH dropped to 3.85 for PFM1 and PFM3, 3.80 for PFM4 and to 3.59 for PFM2. The drop in the pH was almost the same for C, PFM1, PFM3 and PFM4. There were major differences in pH values between PFM2 and all other experiments ($P<0.05$). These values are similar to that found in strained yoghurts [21].

Table 3. pH and % lactic acid values of the experiments during the storage period.

	pH			
Exp.*	Day 1	Day 7	Day 14	Day 21
C	4.36±0.1^{ab1}	4.32±0.1^{a}	3.83±0.3^{a}	3.82±0.3^{b}
PFM1	4.43±0.1^{b}	4.25±0.3^{a}	3.89±0.6^{a}	3.85±0.5^{b}
PFM2	4.35±0.4^{ab}	4.32±0.7^{a}	3.85±0.2^{a}	3.59±0.1^{a}
PFM3	4.45±0.6^{b}	4.33±0.6^{a}	3.79±0.3^{a}	3.85±0.1^{b}
PFM4	4.25±0.2^{a}	4.31±0.2^{a}	3.78±0.4^{a}	3.80±0.2^{b}
	% lactic acid			
Exp.*	Day 1	Day 7	Day 14	Day 21
C	0.09±0.1^{a}	0.09±0.1^{a}	0.10±0.2^{a}	0.10±0.3^{a}
PFM1	0.09±0.6^{a}	0.08±0.4^{a}	0.11±0.9^{a}	0.12±0.4^{b}
PFM2	0.08±0.2^{a}	0.09±0.2^{a}	0.11±0.4^{a}	0.12±0.1^{b}
PFM3	0.08±0.8^{a}	0.09±0.6^{a}	0.11±0.4^{a}	0.12±0.7^{b}
PFM4	0.08±0.5^{a}	0.09±0.5^{a}	0.12±0.5^{b}	0.13±0.3^{b}

*:C (control): *L. bulgaricus*+ *S. thermophilus* PFM1: *L. rhamnosus* IF7 + *S. thermophilus* PFM2: *L. paracasei* ssp. *paracasei* IF10 + *S. thermophilus* PFM3: *L. fermentum* IF14 + *S. thermophilus* PFM4: *L. fermentum* IF15 + *S. thermophilus.*
[1]Different small letters in same column indicate the significant differences ($P<0.05$).

While the pH decreased progressively, the TA increased in all experiments during the storage period. Comparable results were reported by various researchers [19, 21]. There were no significant differences between the experiments at 1th and 7th day. On the other hand, there were a significant difference ($P<0.05$) between PFM4 and other experiments at 14th day. The TA of C statistically differed ($P< 0.05$) from PFM experiments at the end of the storage period (21th day). Similarly, Bonczar et al. [22] found that the concentration of acidity in cultures out of the two yoghurt strains is lower than in traditional yogurt cultures. It was seen that all PFM experiments produced reasonable acidity during fermentation. In another saying, TA increased above 20 percent from 1th to 21thday in PFM experiments (data not shown). Kim et al. [23] observed similar increase in acidity during cold storage of yoghurt made from commercial starter cultures that contained *L. acidophilus* and Bifidobacteria.

3.3. Viscosity and serum separation.

Viscosity is the property of a material to resist deformation. The use of *S. thermophilus* and *L. bulgaricus* as culture is a common practice in the production of yoghurt and yoghurt-relate products to increase the viscosity of the product [24]. On the contrary, in this study, the PFM4, manufactured with *L. fermentum* IF15 + *S. thermophilus*, had the statistically highest viscosity ($P<0.05$) with 11.2 cp at 1th day (Table 4). These differences could be due to exopolysaccharides (EPS) production of LAB. Because, it has been known that different strains of *Lactobacillus* are able to secrete many different types of EPS into milk [25]. On the other hand, the lowest viscosity was obtained in PFM2 experiment with 9.10 cp at 1th day. Viscosity progressively increased in all experiments during the storage period. The end of the storage period, at 21th day, viscosity had statistically different from each other ($P<0.05$) in all experiments. As in the beginning of the storage period, the highest viscosity was observed in PFM4 and the lowest viscosity in PFM2 manufactured with *L. paracasei* ssp. *paracasei* IF10 + *S. thermophilus*.

Table 4. Viscosity and serum separation rates of the experiments during the storage period.

	Viscosity (cp)			
Exp.*	Day 1	Day 7	Day 14	Day 21
C	10.30±0.2^{b}	12.4±0.2^{e}	15.2±0.6^{d}	16.9±0.9^{c}
PFM1	10.30±0.5^{b}	12.0±0.5^{d}	15.3±0.3^{d}	16.2±0.4^{b}
PFM2	9.10±0.1^{a}	10.2±0.6^{a}	11.6±0.4^{a}	12.1±0.4^{a}
PFM3	10.4±0.6^{b}	11.8±0.5^{c}	13.5±0.6^{b}	17.1±0.5^{d}
PFM4	11.2±0.2^{c}	11.5±0.5^{b}	14.3±0.8^{c}	19.6±0.2^{e}
	Serum separation (mL/25 g)			
Exp.*	Day 1	Day 7	Day 14	Day 21
C	-	-	-	4.00±1.0
PFM1	-	-	-	7.00±0.3
PFM2	-	4.00±0.5	5.00±0.2	15.0±1.8
PFM3	-	-	3.00±0.6	10.0±0.6
PFM4	-	-	3.00±1.2	10.0±0.5

*:C (control): *L. bulgaricus*+ *S. thermophilus* PFM1: *L. rhamnosus* IF7 + *S. thermophilus* PFM2: *L. paracasei* ssp. *paracasei* IF10 + *S. thermophilus* PFM3: *L. fermentum* IF14 + *S. thermophilus* PFM4: *L. fermentum* IF15 + *S. thermophiles.*
[1]Different small letters in same column indicate the significant differences ($P<0.05$).

Serum separation is a common phenomenon and a major physical defect occurring in acidified dairy fermented milks. Gel structure deteriorates and consists serum separation with reorganization of the casein molecules without any external force in the gel structure [26]. While the earliest and highest serum separation was observed in PFM2, manufactured with *L. paracasei* ssp. *paracasei* IF10 + *S. thermophiles*, the lowest and latest serum separation was occurred in C (Table 4). It was started the serum separation in PFM2 with 4 ml at 14th day, continued with 5 mL at 14th day and reached level 15 mL at 21th day. It was observed the serum separation in PFM3 and PFM4 at 14th and 21th day, it was seen only at the end of storage (21th day) in C.

3.4. Sensory properties.

Sensory scores of the experiments during the storage period are seen in Table 5. In colour, generally similar results were obtained in all experiments including C. The results obtained

at the beginning of storage were remained the same level until the end of the storage with a slight decrease. In consistency, the values determined as 8 log cfu/g levels at 1th and 7th days were showed a decrease in the level of 1-1.5 log at the end of storage period. The highest decreases were observed with 1.7 and 1.6 log levels in PFM2 and PFM3 experiments. In taste and flavour, the most decisive of the sensory properties, it were obtained significantly low values ($P<0.05$) in all PFM experiments compared with C at initially and throughout the storage period. Similar results were obtained in overall acceptability. It was obtained the significantly highest values ($P<0.05$) in C at the beginning, during and the end of the storage period. The various probiotic strains tested in this study had very different metabolic profiles in PFM's with respect to known organoleptically important compounds. These differences in profiles would undoubtedly affect the sensory quality of products made using these different organisms. According to Saarela et al. [27], it is important that the probiotic culture used contributes to good sensory properties in fermented probiotic products. Therefore it is quite common to use probiotic bacteria mixed together with other types of bacteria suited for the fermentation of the specific product. For milk-based products the probiotic strains are often mixed with *S. thermophilus* and *L. delbrueckii* to achieve the desired flavour and texture.

Table 5. Sensory scores of the experiments during the storage period. Values are means and standard deviation for n = 10 panelists.

	Colour				Consistency			
Exp.*	Day 1	Day 7	Day 14	Day 2	Day 1	Day 7	Day 14	Day 21
C	8.5±0.15^{ab1}	8.0±0.10^{a}	8.8±0.20^{b}	8.2±0.25^{ab}	8.2±0.25^{ab}	8.4±0.10^{ab}	7.6±0.20^{bc}	7.2±0.15^{c}
PFM1	8.5±0.25^{ab}	8.2±0.10^{ab}	8.6±0.15^{ab}	8.0±0.10^{a}	8.0±0.10^{a}	8.4±0.10^{ab}	7.8±0.20^{cd}	7.4±0.30^{cd}
PFM2	8.5±0.20^{ab}	8.4±0.25^{b}	8.4±0.25^{a}	8.1±0.15^{a}	8.1±0.15^{a}	8.2±0.25^{a}	7.2±0.15^{b}	6.5±0.25^{a}
PFM3	8.3±0.10^{a}	8.4±0.15^{b}	8.8±0.10^{b}	8.3±0.30^{ab}	8.3±0.30^{b}	8.4±0.15^{ab}	7.0±0.25^{a}	6.8±0.15^{b}
PFM4	8.5±0.20^{ab}	8.4±0.30^{b}	8.8±0.30^{b}	8.0±0.10^{a}	8.0±0.10^{a}	8.2±0.20^{a}	7.8±0.10^{cd}	7.5±0.10^{cd}
	Taste and flavor				Overall acceptability			
Exp.*	Day 1	Day 7	Day 14	Day 21	Day 1	Day 7	Day 14	Day 21
C	7.0±0.20^{c}	6.6±0.10^{b}	6.2±0.10^{bc}	6.0±0.20^{c}	7.3±0.20^{c}	6.4±0.15^{b}	6.4±0.15^{bc}	6.2±0.20^{cd}
PFM1	7.8±0.15^{e}	6.4±0.25^{ab}	7.2±0.15^{c}	6.8±0.30^{d}	7.7±0.10^{d}	7.2±0.10^{d}	6.6±0.25^{c}	6.0±0.15^{c}
PFM2	6.8±0.30^{b}	6.2±0.15^{a}	4.0±0.20^{a}	3.8±0.10^{a}	6.4±0.15^{a}	6.0±0.10^{a}	5.8±0.10^{a}	5.5±0.15^{a}
PFM3	7.5±0.10^{d}	6.4±0.15^{ab}	6.0±0.15^{b}	5.8±0.25^{b}	6.5±0.25^{a}	6.5±0.15^{b}	6.4±0.10^{bc}	5.8±0.20^{b}
PFM4	6.3±0.20^{a}	6.4±0.10^{ab}	6.2±0.10^{bc}	6.0±0.10^{c}	7.0±0.10^{b}	6.7±0.25^{c}	6.2±0.15^{b}	6.0±0.15^{c}

*:C (control): *L. bulgaricus*+ *S. thermophilus* PFM1: *L. rhamnosus* IF7 + *S. thermophilus* PFM2: *L. paracasei* ssp. *paracasei* IF10 + *S. thermophilus* PFM3: *L. fermentum* IF14 + *S. thermophilus* PFM4: *L. fermentum* IF15 + *S. thermophilus*.
[1]Different small letters in same column indicate the significant differences ($P<0.05$).

4. CONCLUSIONS

In this investigation, good compatibility with *S. thermophilus* and fermentative performance were obtained for evaluated *L. rhamnosus* IF7, *L. paracasei* ssp. *paracasei* IF10, *L. fermentum* IF14 and *L. fermentum* IF15 which grew plentifully reaching counts of around 7.5-9.9 log cfu/g after incubation, and maintained these counts thorough the storage period. These strains also showed the adequate technological characteristics for production of PFM: acceptable acidification capacity final products reduced pH to 4.25-4.45 after fermentation at 40°C. In addition, it was observed good texture and adequate organoleptic properties.

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