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Characterization of Probiotic Fermented Milk Prepared by Different Inoculation Size of Mesophilic and Thermophilic Lactic Acid Bacteria

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Abstract

Background and Objectives: Importance of development of novel probiotic fermented milk and challenge made for its acceptability is well known. In this research, the impact of different inoculation sizes of yogurt and DL-type starter culture (mesophilic and thermophilic LAB) on titratable acidity, viscosity, sensorial and microbial properties of fermented milk was investigated; and finally, probiotic Langfil was produced.

Materials and Methods: Fermented milk produced by 1, 2 and 3% v v^{-1} inocula consisting thermophilic: mesophilic starter cultures 10:90 *(Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris. Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus*) were analyzed for determination of titratable acidity, viscosity, viability of mesophilic starter cultures and sensory properties on days 5, 10, and 15 of storage at 4°C. Then, the most suitable treatments were selected for the producing probiotic Langfil, containing probiotic starter culture (2% v v^{-1} inoculums with equal ratio of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. *Lactococcus lactis* and *Lactococcus cremoris* were counted on M17 agar, while Leuconostoc and Lactobacillus were counted aerobically on tomato juice agar and MRS bile agar, respectively. Bifidobacterium was cultured anaerobically on MRS bile agar. Sensory evaluation was carried out by ten trained panelists, based on a nine-point hedonic scale during the cold storage.

Results and Conclusion: According to results, the best organoleptic properties were achieved in the product prepared with 2% the mesophilic and thermophilic starter cultures and 2% probiotic. This product had a high viscosity. An Iranian probiotic Langfil with desired properties was produced using the best treatment prepared.

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1. Introduction

The role of the microbiota is important in production of fermented dairy product [1,2].

Lactococci are mesophilic microorganisms applied for acid production in dairy fermentations and able to produce exopolysaccharide (EPS) and

proteolytic capacity which leads to gel structure formation and the viscosity of fermented milks.

Viscosity of stirred milk is due to interaction between the EPS and the casein-matrix [3] and/or absorption of ropy strains to the protein matrix [4]. Lactic acid bacteria (LAB) producing EPSs have potential application as viscosity enhancer, texturizers [5,6] and emulsifiers [7]. Modification of texture properties of fermented milks by EPSs leads to a higher viscosity and a lower degree of syneresis compared with products produced without EPS producing cultures [2]. Nordic fermented milks contain EPSs produced by *Lactococcus lactis* subsp. *cremoris* as a homofermentative LAB on milk [8].

Langfil is the modern variant of the traditional tätmjölk and is produced in Norway and Sweden [9] and is made with the inoculation of lactococci strains, primarily *L. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc (L.) mesenteroides* subsp. *cremoris*, which are able to produce ropiness (ropiness or mucoidness in Langfil and Viili is essential for producing the desired texture of this products). The fermented milks of Scandinavia include Viili, Ymer, Skyr, Langfil, Keldermilk, and several local products [10-13]. Langfil is a popular product in Sweden milk with a mild and slightly acidic taste, high viscosity and ropy consistency [9,14-17]. The milk is incubated in cups for 18-20 h at 18-20°C to obtain an acidity of about 0.86% lactic acid [18]. Nordic fermented milks have proved to be well suited to carrying probiotic bacteria. As the pH remains constant during the storage period, the survival rate of lacto-bacilli and bifidobacteria have been excellent. The texture of products such as Swedish Langfil and Finnish viili is less acceptable for Iranian consum-ers. Keeping in mind the significant impact of sensorial characteristics of a new product, the determination of the best inoculation size of ropy strains incorporated of LAB in mixed cultures for making probiotic fermented milk was investigated. Both yogurt bacteria are used to improve taste, aroma and texture of the final product.

The aim of the present investigation was to develop the novel probiotic fermented milk manufactured with mesophilic and thermophilic LAB in Iran. In the current study, the impact of different inoculation size of starter cultures consisting of yogurt starter culture and DL-type starter culture on titratable acidity, viscosity, sens-orial properties and culture viability of fermented milk obtained.

2. Material and methods

2.1. Starter cultures

Starter cultures (*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis, L. lactis* subsp. *lactis biovar. diacetylactis* and *L. mesenteroides* subsp. *cremoris Streptococcus (S.) thermophilus* and *Lactobacillus (L.) delbrueckii* subsp. *bulgaricus*) were purchased from Chr. Hansen, Denmark. DL-type yogurt starter culture (YF-L811) and probiotic (*L. acidophilus* and *Bifidobacterium (B.) bifidum* were used. Characteristics of cultures and treatments used in the research are described in Table 1.

2.2. Sample preparation

Fat content of milk was standardized to 3.43%w w^{-1} and skim milk powder was added to milk (1.5-1.7%) and the milk was homogenized; then, it was heated at 95°C for 5 minutes. After cooling to 31°C, milk was inoculated with 1 (treatment A), 2 (treatment B) and 3%v v^{-1} (treatment C) starter cultures (Table 1) consisting of yogurt starter culture and DL-type starter culture (inoculum sizes were selected based on the results of pre-tests), and poured into 200-g plastic cups and incubated at 31°C to reach the acidity of 86°D. In fact, temper-ature of 31°C was the best temperature for incub-ation in producing Langfil based on the results of pre-tests. The cups were refrigerated at 4°C, for 15 days. Fermented milks were analyzed for determination of titratable acidity, viscosity, viability of mesophilic starter cultures and sensory properties in 5 days intervals. Then the most suitable treatments (treatment B, due to desired viscosity, and treatment C, due to high scores in sensory properties) were selected for the production of probiotic Langfil which were defined as treatments PB, PC, respectively (Table 1). For production of the product, after cooling of milk it was also inoculated with probiotic starter (2% v $v^{-1)}$ inoculums with equal ratio of *L. acidophilus* and *B. bifidum* (pretest showed better sensorial properties in such inocula).

2.3. Titratable acidity

Titratable acidity, as percent lactic acid, was measured for all treatments on days 5, 10, and 15 of storage using 0.1 N NaOH and 1% phenolphthalein (Sigma chemical Co.) solution in 95% ethanol as an endpoint indicator [19].

2.4. Viscosity measurement

Viscosity of fermented milks was measured at 10±1°C using a Brookfield DV-II+Pro viscometer (Brookfield Engineering Laboratories, USA). Viscometer was operated at 50 rpm with spindle number 3 after 15 s [20].

2.5. Microbiological analysis of fermented milks

L. lactis subsp. *lactis* and *L. lactis* subsp. *cremoris* were counted on M17 agar incubated aerobically at 25°C for 72 h. Viable cell numbers of Leuconostoc bacteria in the samples was cultured on tomato juice agar aerobically at 30°C for 72 h using pour plate method.

Table 1. Treatments used in fermented milk samples and probiotic fermented milk samples.

Fermented milk samples		Probiotic fermented milk samples		
Treatment	% Starter cultures (CHN-22[1] + YF-L811)[2, 3]	Treatment	% Starter cultures (CHN-22[1] + YF-L811)[2, 3]	% Probiotic starter cultures[4, 5]
A	1	PB†	1	2
B	2	PC††	2	2
C	3			

[1] CHN-22, DL-type starter culture, high flavor/ medium texture: *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis, L. lactis* subsp. *lactis biovar. diacetylactis* and *L. mesenteroides* subsp. *cremoris*.
[2] YF-L811, yogurt starter culture, very high EPS-producing/ very mild flavor ability: *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*.
[3] YF-L811:CHN22=10:90.
[4] LA-5 & BB-12: *L. acidophilus* and *B. bifidum*
[5] LA-5: BB-12 =1:1.
† Treatment B containing probiotics
†† Treatment C containing probiotics

Table 2. Viscosity and titratable acidity of fermented milks (treatments A, B, and C) during cold storage.

Day	5	10	15
viscoity (cp)			
A**	13.79 ± 0.45^{abc}	13.62 ± 0.36^{abc}	13.34 ± 0.35^{abc}
B	13.89 ± 0.21^{bc}	14.39 ± 0.12^{c}	14.40 ± 0.24^{c}
C	12.95 ± 0.80^{ab}	12.76 ± 0.41^{a}	14.00 ± 0.04^{bc}
Acidity (D)			
A	89.67 ± 0.58^{b}	90.67 ± 1.15^{ab}	92.00 ± 2.00^{ab}
B	91.00 ± 1.00^{ab}	91.00 ± 1.00^{ab}	93.33 ± 1.53^{a}
C	91.00 ± 1.00^{ab}	91.33 ± 0.58^{ab}	91.67 ± 1.53^{ab}

*Mean± SD by the same superscripts in the row are not significant different.
**Inoculation sizes of 1% (A), 2% (B) and 3% (C) starter cultures.

Serial dilutions of samples were made in strength Ringer solution and spread plated on their special media. *L. acidophilus* was counted on MRS bile agar incubated aerobiocally at 37°C for 72 h. Viable cell numbers of *B. bifidum* was determined on MRS bile agar anaerobically at 37°C for 72 h [21].

2.6. Sensory evaluation

Samples were evaluated using nine-point Hedonic scale. A panel of 10 trained judges evaluated fermented milks for flavor, odor, texture, color characteristics and overall acceptability.

2.7. Statistical analysis

Data were submitted to ANOVA procedure using SAS software (Version 9.1; Statistical Analysis System Institute Inc., Cary, NC, USA) and General linear model (GLM) procedure. Duncan's multiple range test was used for comparison of means. Duncan's multiple range tests were used to compare means at the significant level of 0.05. All experiments were replicated three times. Kruskal-Wallis nonparametric test used to analyze the data obtained from sensory tests.

3. Results and discussion

3.1. Fermented milk

3.2. Acidity of fermented milks

Table 2 shows the acidity of samples during storage at 4°C. The results showed a significant difference in acidity between samples on days 5 and 15 of storage. As shown in Table 2, after 5 days storage, the highest acidity was achieved in the products containing 2 and 3% starter cultures, (treatments B and C). This factor increased in all treatments during storage time. It was worthy to mention that *L. lactis* subsp. *lactis* and subsp. *cremoris* are the main acid-producing strains in the starter culture, while *L. lactis* subsp. *lactis* biovar *diacetylactis* and *L. mesenteroides* subsp. *cremoris* ferment the citric acid present in the milk (9). However, the acidity of the three other treatments did not differ significantly at the end of the 15-day storage period. So, such observation confirms that acidity of samples was not affected by inoculation rate.

3.3. Viscosity of fermented milks

The results showed that viscosity value was significantly different among the samples. As shown in Table 2, the product containing 2% starter culture (treatment B) had the highest viscosity during cold storage.

Viscosity and the structure of the gel are influenced by several factors, including incubation temperature, casein concentration, heat treatment of the milk, acidity, and type of starter culture [22]. As it was mentioned Langfil has a very mild and slightly acidic taste, high viscosity and ropy consistency. Due to the production of EPS, the product is very stable and has a low tendency to syneresis [9].

Table 3. Mesophilic bacteria count (cfu ml^{-1}) of fermented mliks (treatments A, B, and C) and probiotic milk during cold storage (interaction between time and different surfaces).*

Treatment	Day	Bacteria	
		Lactococcus	Leuconostoc
A**	5	8.31 ± 0.19^{a}	8.39 ± 0.22^{b}
	10	7.59 ± 0.25^{de}	7.49 ± 0.12^{d}
	15	7.32 ± 0.03^{e}	7.39 ± 0.08^{d}
B	5	8.31 ± 0.12^{b}	8.28 ± 0.17^{abc}
	10	7.99 ± 0.20^{abcd}	7.70 ± 0.17^{d}
	15	7.62 ± 0.15^{de}	7.73 ± 0.15^{cd}
C	5	8.21 ± 0.13^{abc}	8.42 ± 0.11^{a}
	10	7.71 ± 0.36^{cde}	7.61 ± 0.39^{d}
	15	7.49 ± 0.13^{de}	7.35 ± 0.23^{d}

*Mean± SD by the same superscripts in the column are not significant different.
**Inoculation sizes of 1% (A), 2% (B) and 3% (C) starter cultures.

Table 4. Sensory properties of fermented milks (treatments A, B, and C) and probiotic milk during cold storage.*

		Sensory properties				
	Day	Flavor	Odor	Color	Texture	Overall acceptance
Fermented milks						
A**	5	6.2 ± 0.7^{abcd}	5.2 ± 1.0^{a}	6.1 ± 0.6^{a}	5.7 ± 0.8^{a}	5.5 ± 0.8^{bcd}
	10	5.8 ± 0.9^{bcd}	4.7 ± 0.5^{a}	5.8 ± 1.2^{a}	6.0 ± 0.7^{a}	5.0 ± 0.7^{d}
	15	5.4 ± 1.2^{d}	4.7 ± 1.1^{a}	5.9 ± 1.4^{a}	5.6 ± 1.3^{a}	5.2 ± 1.1^{cd}
B	5	6.6 ± 1.1^{abcd}	5.4 ± 1.1^{a}	6.4 ± 0.9^{a}	6.0 ± 0.7^{a}	6.2 ± 1.0^{abcd}
	10	6.4 ± 0.9^{abcd}	4.7 ± 0.5^{a}	6.6 ± 1.1^{a}	6.4 ± 0.5^{a}	6.2 ± 0.8^{abcd}
	15	6.6 ± 1.3^{abcd}	5.2 ± 0.8^{a}	6.6 ± 1.3^{a}	6.3 ± 1.0^{a}	5.9 ± 0.9^{abcd}
C	5	7.2 ± 0.8^{ab}	5.1 ± 0.9^{a}	6.0 ± 0.8^{a}	6.2 ± 0.7^{a}	6.5 ± 1.1^{abc}
	10	7.1 ± 1.2^{abc}	4.5 ± 1.2^{a}	6.3 ± 1.1^{a}	6.4 ± 1.0^{a}	6.6 ± 1.2^{ab}
	15	7.3 ± 1.3^{a}	5.2 ± 0.8^{a}	6.2 ± 1.3^{a}	5.7 ± 0.7^{a}	6.9 ± 1.1^{a}
Probiotic fermented milks						
PB***	5	6.7 ± 0.9^{a}	5.2 ± 1.3^{a}	7.3 ± 0.8^{a}	7.4 ± 1.0^{a}	6.9 ± 1.0^{a}
	10	6.5 ± 0.8^{a}	5.2 ± 0.7^{a}	7.0 ± 0.8^{a}	7.2 ± 1.0^{a}	6.7 ± 1.0^{ab}
	15	6.2 ± 1.5^{a}	4.9 ± 0.8^{a}	6.5 ± 1.3^{a}	6.8 ± 1.3^{a}	6.7 ± 1.1^{ab}
PC	5	6.0 ± 0.9^{a}	4.9 ± 1.2^{a}	6.6 ± 1.0^{a}	6.5 ± 0.6^{a}	5.8 ± 0.7^{b}
	10	6.0 ± 0.8^{a}	4.6 ± 1.2^{a}	6.8 ± 1.1^{a}	6.6 ± 0.6^{a}	6.2 ± 0.8^{ab}
	15	6.0 ± 0.9^{a}	4.9 ± 0.5^{a}	6.3 ± 0.8^{a}	6.4 ± 0.8^{a}	5.9 ± 0.6^{ab}

*Mean± SD by the same superscripts in the row are not significant different.
**Inoculation sizes of 1% (A), 2% (B) and 3% (C) starter cultures.
***PB: Treatment B containing probiotics; PC: Treatment C containing probiotics.

Duboc and Mollet [6] also reported that EPS's may act both as texturizers and stabil-izers, firstly increasing the viscosity of a final product, and secondly by binding hydration water interacting with other milk constituents, such as proteins and micelles, to strengthen the rigidity of the casein network; as a consequence EPS can decrease syneresis and improve product stability The results obtained are according to Ruas-Madiedo et al. [4], who showed that EPS production during milk-gel formation was the most important factor that influenced the structure of the milk gels and the viscosity of the stirred product. However, in this research a moderate EPS producing mesophilic starter culture was used, so that the texture of fermented milks was not as ropy as it should be but was accepted by panelists. It was found no correlation between viscosity and the inoculation size.

3.4. Viability of mesophilic LAB

The changes in the viable counts of mesophilic LAB in the fermented milk samples during refrigerated storage are reported in Table 3. There were significant differences in the viability of these bacteria between the samples. Data shows that by elongation of storage, viability of Lactococcus and Leuconostoc decreased. This observation is in line with the study of Varga et al. which reported the decreasing Lactococcus viability throughout the entire storage period in fermented milk prepared by mesophilic starters [23].

3.5. Sensory evaluation

Table 4 shows the sensorial evaluation ranks of samples during cold storage at 4°C. As shown in Table 4, the product containing 3% starter culture (treatment C) had the highest overall acceptability scores during cold storage.

Table 5. Titratable acidity and viscosity of probiotic fermented milk (trearments PB and PC) during cold storage.*

Treatment	5th day	10th day	15th day
acidity (D)			
PB	90.67±1.5 [a]	90.00±1.0 [a]	91.67±1.5 [a]
PC	91.33±1.5 [a]	89.67±1.5 [a]	89.67±0.6 [a]
viscoity (cp)			
PB	13.68±0.3 [a]	13.49±0.2 [a]	13.23±0.2 [a]
PC	13.48±0.3 [a]	13.56±0.3 [a]	13.33±0.1 [a]

*Mean± SD by the same superscripts in the row are not significant different.

Table 6. Mesophilic and probiotic bacteria counts (cfu ml^{-1}) of probiotic fermented mliks (treatments PB and PC) during cold storage.*

	Day	Lactococcus	Leuconostoc	*L. acidophilus*	*B. bifidum*
PB	5	8.63±0.17[a]	8.34±0.17[a]	8.21±0.06[a]	8.50±0.23[a]
	10	8.04±0.08[bc]	7.83±0.08[bc]	7.95±0.11[abc]	7.98±0.16[abc]
	15	7.76±0.25[bc]	7.47±0.16[cd]	7.33±0.04[d]	7.73±0.34[bcd]
PC	5	8.17±0.21[ab]	8.14±0.22[ab]	7.99±0.14[ab]	8.05±0.06[ab]
	10	7.67±0.18[c]	7.39±0.09[d]	7.24±0.05[d]	7.38±0.12[d]
	15	7.39±0.09[c]	7.24±0.08[d]	7.13±0.12[d]	7.24±0.13[d]

*Mean± SD by the same superscripts in the column are not significant different.

There were significant differences in flavor scores between fermented milks prepared with different inoculation rate. Different inoculum size leads to significant differences in flavor of samples. The highest scores of sensorial evaluation in the sample with inoculation of 3% starter can be interpreted by aroma production. In fact the starter culture used contains a blend of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. biovar *diacetylactis* and *L. mesenteroides* subsp. *cremoris*. The latter two organisms are the main aroma-forming bacteria in the product [24]. It seems that the improvement in flavor appears to be related to high inoculation rate. The citrate metabolism is very low by lactococci and Leuconostoc species, while thermophilic cultures are not citrate metabolizing LAB. Certain carbonyl/flavouring compounds, such as diacetyl, acetate, 2, 3-butanediol, acetoin and carbon dioxide are produced in milk through the metabolism of citrate. Although acetoin and butanediol are tasteless and not involved in flavor, diacetyl is an important flavor component. Mixed strains of mesophilic starter cultures produce much more acetoin than diacetyl from citrate [8].

No differences were observed in the odor scores of all fermented milks. Also data show that different amount of inoculum size not have a significant influence on the color and texture of fermented milks which can be related to constant ratio of ESP-producing thermophilic LAB in all treatments. In this study yogurt starter (YF-L811) with a very high EPS-Producing and very mild flavor ability, in addition to medium-EPS producing DL-type starter culture were used; so, the texture of fermented milk samples was not ropy as it must be. The highest overall acceptability scores of samples with 3% starter inoculation indicated that the high viscosity of treatment B was not important in determining desirability of the fermented milk.

According to above mention results, the best treatments (treatment B due to desired viscosity and texture as well as treatment C due to high overall acceptance) were selected for the production of probiotic Langfil.

3.6. Characterization of probiotic Langfil

The sensory scores of the samples are given in Table 4. No Significant difference was observed in the flavor, odor, color, texture and overall acceptability between treatments. In general, treatment PB (inoculating with 2% v v^{-1} mixed starter cultures containing yogurt starter culture and DL-type starter culture, in addition to 2% probiotic starter) was ranked higher scores than another treatment (treatment PC, inoculating with 3% v v^{-1} mixed starter cultures containing yogurt starter culture and DL-type starter culture, in addition to 2% probiotic starter). There was no significant difference between acidity of the probiotic samples. As shown in Table 5, the highest acidity value was measured on the day 15 in treatment PB. It is worth mentioning that, in contrast treatment PB, there was a slight decline in titratable acidity during storage for treatment PC that was no significant.

According to Table 5, there was no significant difference in viscosities of experimental treatments. This factor in treatment PB decreased during cold storage but in PC increased after 10 days of storage and declined thereafter. The survival of the characteristic microflora in fermented milk samples stored at 4°C is illustrated in Table 6. Significant decreases in the viability of probiotics, *L. acidophilus* and *B. bifidum*, were observed in both treatments during 15 days of storage. A similar trend was generally found in relation to the viable counts of mesophilic LAB, Lactococcus and

Leuconostoc, during cold storage. In both samples, the number of *B. bifidum* was higher than that of *L. acidophilus*. Loss of viability of probiotic bacteria in fermented milk products is reported to be due to the acid injury to the organisms [25]. In general, the concentration of probiotics in both fermented milks was more than recommended therapeutic level of 10^6 to 5×10^8 cfu g^{-1} at the end of storage [26]. Meanwhile, viability of probiotics in PB was retained higher in comparison to PC during cold storage condition.

4. Conclusion

Almost all dairy fermentation is done by LAB for acidification and flavor production. It is worthwhile to note that the understanding consumer needs and preferences are critical to successful marketing and enhancing marketing value of a product. In this study, we produced Langfil by the mixed culture of 1% (A), 2% (B) and 3% (C) the mesophilic and thermophilic starter cultures (thermophilic:mesophilic starter culture 10:90); the best treatments (treatment B due to desired viscosity and texture as well as treatment C due to high overall acceptance) were selected for the producing probiotic Langfil, containing 2% probiotic starter. From the overall results, it could be concluded that optimum organoleptic properties were achieved in the product formulation prepared with the mixed culture of 2% the mesophilic and thermophilic starter cultures and 2% probiotic starter, with equal ratio of *L. acidophilus* and *B. bifidum*.

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6. Conflict of Interest

There is no kind of interests.

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