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DETERMINATION OF THE BEST RATIO OF STARTER CULTURE FOR MAKING MILK CO-FERMENTED WITH MESOPHILIC AND THERMOPHILIC LACTIC ACID BACTERIA AND MOULD *GEOTRICHUM CANDIDUM*

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ABSTRACT

The objectives of this research were:1) to produce milk co-fermented with 80:20 (treatment A), 70:30 (treatment B) and 60:40 (treatment C) ratios of the mesophilic and thermophilic starter cultures: mould *Geotrichum candidum*, 2) to test the influence of different ratios of starter cultures:mould on titratable acidity, viscosity, sensory properties and viability of mesophilic starter cultures and mould in the experimental samples produced, 3) to make a choice of the best treatment on basis of Iranian acceptance; and therefore,4) to develop the novel functional fermented milk manufactured with mesophilic and thermophilic lactic acid bacteria (LAB) and mould in Iran. According to results of ranking tests done by sensory panelists, optimum organoleptic properties were achieved in the product prepared with the mixed culture of 80:20 ratio of the mesophilic and thermophilic starter cultures: mould (treatment A).

Keywords: *Geotrichum candidum*, Mesophilic starter culture, Yoghurt culture, Fermented milk, viili

INTRODUCTION

A very large variety of fermented milks are offered to the consumer. They vary with the type of milk used, the bacterial starter culture used and the process. Correct appearance and texture are important parameters for fermented dairy products and vary from region to rejoin throughout the world. Viili (Finnish viili, Swedish fil) is a type of yoghurt, a mesophilic fermented milk, that originated in the Nordic countries. It has a ropy, gelatinous consistency and a sour taste resulting from lactic acid and is eaten fresh and chilled, usually for breakfast or as a snack; also, it may be eaten plain or sprinkled with various flavorings. This cultured milk product is the results of microbial action of LAB and mould *Geotrichum candidum*. The LAB identified in viili including *Lactococcus lactis* subsp. *Cremoris*, *Lactococcus lactis* subsp.*lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*. Among those mesophilic LAB strains, the slime-forming *Lactococcus lactis* subsp. *cremoris* produce a phosphate-containing heteropolysaccharide, named viilain. Viilian is similar to kefiran produced by kefir grains. The production of exopolysaccharides (EPSs) by the strain forms the consistency character of viili and it has been claimed to have various functional benefits toward the rheological properties of milk products and the health improving potential (Ruas-Madiedo *et al.*, 2006; Oba *et al.*,1999).

Different EPS-producing cultures produced fermented milks with distinct rheological and sensorial characteristics. Variations among EPS-producing cultures may be due to differences in EPS and their interaction with the protein network (Purohit *et al.*, 2009).

EPSs, a diverse group of polysaccharides, synthesized by LAB play a major role in the manufacturing of fermented dairy products. They may act both as texturizers and stabilizers (Doboc and Mollet, 2001); so that, can be considered as natural biothickeners (Ruas-Madiedo *et al.*, 2002). These polysaccharides reduce the rigidity of the protein network and increase viscosity of the serum phase (Hassan, 2008), They have found their most valuable application in the improvement of the rheology, texture and mouth feel of fermented milk products (Welman and Maddox, 2003;Yang *et al.*, 1999). They also possess antitumoral, immunostimulatory (Lin and Chang Chien, 2007; Welman and Maddox, 2003; Ruas-Madiedo *et al.*, 2002), macrophage and lymphocyte activities (Lin and Chang Chien, 2007) and have been claimed to lower blood cholesterol. EPSs will remain for longer in the gasterointestinal tract, thus enhancing colonization by probiotic bacteria (Welman and Maddox, 2003). So, a prebiotic role has been suggested (Ruas-Madiedo *et al.*, 2002). EPS produced by *L.lactis* ssp. *cremoris* did not have any positive influence on the survival of the bacteria when exposed to increased temperatures, freezing, freeze-drying, penicillin and vancomycin (Looijesteijn *et al.*, 2001).

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One of the major sensory attributes important for consumer preference of dairy product is firmness and creaminess (Doboc and Mollet, 2001). In our previous work (Mortazavi *et al.*, 2012; Mortazavi *et al.*, 2011), improvement of texture and sensory properties of milk fermented with *mesophilic* starter cultures and mould *Geotrichum candidum* remained to be solved. Selection criteria for LAB starters are acidification rate, flavor and texture characteristics. So, the aim of the present research was to improve texture and sensory properties of the fermented milk. For this, milk is co-fermented with mesophilic and thermophilic lactic acid bacteria and mould *G.candidum.* The LAB used were *Lactococcus lactis* subsp.*cremoris*, *lactis* and *lactis* biovar *diacetylactis*; *Leuconostoc mesenteroides* subsp. *cremoris* for the mesophilic bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* for the thermophilic bacteria. The slime-forming thermophilic *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp.bulgaricus* strains, and mesophilic *Lactococcus lactis ssp.cremoris* strains have been widely used in dairy industry for making fermented milks, e.g. yoghurt, viili and langfil,etc. The production of EPSs by these lactic acid bacteria is essential for proper consistency of the products (Yang *et al.*, 1999).

Starter culture bacteria are important microorganism in the fermenting process. Indeed, by means of their metabolism, they have an effect on the flavor and the texture. We combined the mesophilic (90%) and thermophilic (10%) lactic acid bacteria to get the desired texture and flavor characteristics. Thermophilic yoghurt cultures are employed in the manufacture of the fermented milk to improve its texture and sensory properties. It is worth mentioning that the solids non fat and fat levels of raw milk were also increased.

In the present work, we have examined the production of fermented milk by 80:20 (treatment A), 70:30 (treatment B) and 60:40 (treatment C) ratios of the mesophilic and thermophilic bacteria starter cultures: mould *Geotrichum candidum* grown in milk; and the influence of different ratios of starter cultures on viscosity, acidity, sensory properties and mesophilic bacteria and mould viability of final products on the 5^{th}, 10^{th} and 15^{th} days of storage at 4°C.

MATERIALS AND METHODS

Starter cultures

Starter cultures were obtained from Chr. Hansen, Denmark. Treatments were fermented milks made with different ratios of mesophlic and thermophilic starter cultures and mould *Geotrichum candidum*; characteristics of cultures and treatments used in the study were described in Tables 1 and 2, respectively.

Fermented milk preparation

The following steps are involved in the production of fermented milk:

- Standardization of the fat and dry matter contents to 3.3 and 10.5%, respectively.
- Heat treatment at 95 °C for 5 minutes.
- Cooling to 30 °C.
- Inoculation with 80:20, 70:30, and 60:40 ratios of mesophilic and thermophilic starter cultures: mould *Geotrichum candidum* which were defined as treatments A, B, and C, respectively.
- Incubation at 30 °C for 18-24 h, to pH 4.3 in 200-g plastic cups.
- Cooling to the storage temperature, 4 °C.

Culture media for determination of culture viability

The pour plate technique was used to determine viable microbial cell counts. *Lactococci* were cultured in M17 agar and incubated at 25 °C for 72h. Tomato juice agar was used for the enumeration of *Leuconostoc* and incubated at 30 °C for 72h . *Geotrichum candidum* was counted on Potato dextrose agar and incubated at 25 °C for 72h. The cell count were performed 5, 10, and 15 days of storage at 4 °C.

Viscosity measurement

Viscosity of fermented milks was measured at 10±1°C using a Brookfield DV-II+Pro viscometer (Brookfield Engineering Laboratories, USA). The Viscometer was operated at 20 rpm with spindle number 4 after 15 s (Cinbas and Yazici, 2008).The measurements were carried out 5, 10, and 15 days of storage at 4°C.

Sensory evaluation

A panel of trained judges evaluated fermented milks for scooping up, hardness, grittiness, stickiness, sourness, taste, after taste, ferment smell and scent characteristics and overall acceptability. A seven scale was used for sensory evaluation. Evaluations were performed 5, 10, and 15 days of storage at 4°C.

Statistical analysis

Each treatment was performed in three replications. All data were submitted to ANOVA procedure using SAS 9.1 software and General Linear Model (GLM) procedure. Duncan's multiple range test was used for comparison of means.

RESULTS AND DISCUSSION

Acidity changes in fermented milk by different ratios of mesophilic and thermophilic starter cultures:mould during cold storage

Values of lactic acid production at 5-day intervals during cold storage are shown in Table 3. During storage, the titratable acidity decreased significantly ($p<0.05$) in all treatments. Titratable acidity of the experimental samples varied from 86.0±1.00 to 81.6±1.15, 85.6±0.57 to 81.3±0.57 and 85.6±0.57 to 82.0±0.00 for treatments A, B and C, respectively. At the beginning of storage , Treatment A had higher acidity (86.0±1.00) than the other samples. In this experiment, no significant differences ($p>0.05$) in lactic acid development were found between the treatments. Reduction in acidity during cold storage could be due to presence of *Geotrichum candidum*; the mould lowers the acidity of the fermented milks (Wood, 1998).

Viscosity changes in fermented milk by different ratios of mesophilic and thermophilic starter cultures:mould during cold storage

The viscosity changes during cold storage of experimental samples are shown in Table 4. Treatment A showed higher viscosity than the other treatments. The end of cold storage, treatment A had the highest viscosity (10.14±2.22 cp) while treatment C had the lowest viscosity (7.92±1.05 cp); viscosity of treatment B was nearly close to that of treatment C.

The texture is obtained by aggregation of the casein particles in milk and takes place when the pH is lowered by the starter culture. The protein level of the milk as well as processing, addition of other ingredients and choice of starter cultures has significant impact on the texture obtained. Because of using yoghurt cultures which induces very high viscosity, the viscosity of the treatments improved in comparison to our previous work (Mortazavi *et al.*, 2011). EPSs have an important function as national biothickening agents to improve the rheology of a fermented product, and as physical stabilizers to bind water and limit syneresis (Duboc and Mollet, 2001). It is worth mentioning that the presence of polysaccharides which have specific interactions with the casein micelles gives a slimy consistency which is characteristic of milks fermented with ropy strains (Oba *et al.*, 1999).

Table 1. Cultures used in the study.

Cultures	Description	Source
GEO CB	cream white/ medium yeasty tendency	Chr. Hansen
YF-L811	Very mild flavor, very high viscosity(thermophilic yoghurt cultures)	Chr. Hansen
CHN-22	high flavor/ medium texture (mesophilic cultures)	Chr. Hansen

Changes in *Lactococcus* viability in fermented milk by different ratios of mesophilic and thermophilic starter cultures:mould during cold storage

Lactococcus count in treatment A was recorded the highest value on day of 5, then a down ward tendency was observed in all treatments during storage time (Table 5). After 15 days of storage at 4°C, the *lactococcus* counts were found to be within the range of 7.43±0.04, 7.40±0.11 and 7.30±0.12 log cfu ml^{-1} in treatments A, B and C, respectively. However, it should be noted that there were no significant differences between experimental treatments ($p>0.05$).

Changes in *Leuconostoc* viability in fermented milk by different ratios of mesophilic and thermophilic starter cultures:mould during cold storage

The effect of different ratios of lactic acid bacteria starter cultures:mould on viability of *Leuconostoc* was assessed during 21 days of refrigerated storage and presented in Table 6 . Similar to *Lactococcus* count, a gradual decrease was observed in viable *Leuconostoc* counts during storage time.

Table 2. Treatments used in the study.

Treatment	**Description (Ratio of mesophilic and thermophilic starter cultures:Geotrichum candidum)**	
	mesophilic and thermophilic starter cultures*	**Geotrichum candidum**
A	80	20
B	70	30
C	60	40

*YF-L811:CHN22=10:90

Table 3. Titratable acidity (%lactic acid) of fermented milks (trearments A,B and C) during storage*

Storage (day)	**Treatments**		
	A	**B**	**C**
5	86.0±1.00	85.6±0.57	85.6±0.57
10	83.6±1.52	83.0±2.00	82.6±0.57
15	81.6±1.15	81.3±0.57	82.0±0.00

* Mean ± SD values are not significantly different (*P* > 0.05).

Table 4. Viscosity (CP) of fermented milks (treatments A,B and C) during storage*

Storage (day)	**Treatments**		
	A	**B**	**C**
5	9.10±0.44	6.99±0.79	8.85±1.10
10	9.38±0.51	8.04±1.36	9.08±1.11
15	10.14±2.22	8.22±2.88	7.92±1.05

* Mean ± SD values are not significantly different (*P* > 0.05).

Table 5. *Lactococcus* count ($\log_{10}$ cfu ml^{-1}) of fermented milks (treatments A,B and C) during storage*

Storage (day)	**Treatments**		
	A	**B**	**C**
5	8.07±0.25	8.06±0.38	7.96±0.34
10	7.54±0.11	7.48±0.06	7.49±0.01
15	7.43±0.04	7.40±0.11	7.30±0.12

* Mean ± SD values are not significantly different (*P* > 0.05).

Table 6. *Leuconostoc* count (log_{10} cfu ml^{-1}) of fermented milks (treatments A,B and C) during storage*

Storage (day)	Treatments		
	A	B	C
5	7.48±0.13	7.62±0.47	7.64±0.38
10	7.34±0.04	7.18±0.11	7.15±0.09
15	7.16±0.08	6.93±0.04	6.76±0.04

* Mean ± SD values are not significantly different (*P* > 0.05).

Table 7. Mould count(log_{10} cfu ml^{-1}) of fermented milks (treatments A,B and C) during storage*

Storage (day)	Treatments		
	A	B	C
5	8.12±0.23	8.13±0.42	8.21±0.25
10	7.45±0.12	7.51±0.15	7.77±0.10
15	7.27±0.05	7.37±0.11	7.47±0.08

* Mean ± SD values are not significantly different (*P* > 0.05).

Table 8. Sensory properties of fermented milks (treatments A,B and C) during storage*

Sensory characteristics / Storage(day)	Treatments								
	A			B			C		
	5	10	15	5	10	15	5	10	15
Scooping up	3.6±1.04^{a}	3.9±0.79^{a}	4.1±1.33^{a}	3.5±0.99^{a}	4.3±0.81^{a}	4.1±1.16^{a}	3.9±0.70^{a}	4.1±0.96^{a}	4.1±1.09^{a}
Hardness	5.2±0.59^{d}	4.7±0.88^{bcd}	4.7±0.59^{bcd}	3.8±0.67^{a}	4.3±0.81^{ab}	4.6±1.05^{bc}	4.6±1.11^{bc}	4.6±1.11^{bc}	5.1±0.79^{cd}
Grittiness	5.5±0.63^{a}	5.4±1.24^{a}	6.0±1.00^{a}	4.6±1.29^{a}	5.1±1.18^{a}	5.6±1.11^{a}	5.1±1.06^{a}	5.1±1.27^{a}	5.8±0.12^{a}
Stickness	4.3±1.04^{a}	4.6±1.24^{a}	4.5±0.74^{a}	4.0±0.92^{a}	4.3±0.89^{a}	3.7±1.03^{a}	4.6±1.29^{a}	4.9±0.79^{a}	5.4±0.63^{a}
Sourness	4.1±0.74^{abc}	4.5±0.63^{bcd}	4.8±0.51^{d}	4.4±1.05^{abcd}	3.9±1.16^{a}	3.9±0.59^{a}	4.7±1.03^{d}	4.0±1.09^{ab}	4.3±0.81^{abcd}
Taste	5.4±0.73^{ef}	4.4±1.35^{abc}	6.0±0.53^{f}	4.0±0.92^{a}	4.2±0.96^{ab}	4.2±0.88^{ab}	4.4±0.73^{abc}	4.7±0.45^{bcd}	5.1±1.03^{de}
Aftertaste	5.1±0.83^{g}	4.4±1.68^{bcdef}	5.4±0.63^{g}	3.2±0.86^{a}	4.1±1.22^{bc}	4.2±0.94^{bcd}	4.3±0.59^{bcde}	4.0±1.19^{b}	4.1±0.83^{bc}
Ferment smell	3.8±1.14^{ab}	4.1±1.30^{abcd}	5.1±0.79^{g}	4.3±1.04^{abcdef}	3.6±1.11^{a}	4.2±1.08^{abcde}	4.4±0.99^{bcdefg}	3.9±1.06^{abc}	4.4±0.99^{bcdefg}
Scent	4.6±0.98^{a}	3.8±1.24^{a}	5.2±0.79^{a}	3.8±0.51^{a}	3.6±1.04^{a}	4.1±0.99^{a}	4.6±0.73^{a}	4.2±0.86^{a}	4.4±1.30^{a}
Overall acceptability	5.4±0.82^{gh}	4.9±1.20^{cde}	6.0±0.53^{i}	4.2±1.03^{ab}	4.4±0.99^{abc}	4.1±0.83^{a}	4.6±0.48^{bcd}	4.9±0.74^{cdef}	5.1±0.79^{defg}

*Mean ± SD values with different superscript letters differ significantly (P<0.05).

Changes in mould viability in fermented milk by different ratios of mesophilic and thermophilic starter cultures:mould during cold storage

The survival of the mould in fermented milk samples stored at 4°C is illustrated in Table 7. There was a significant decrease (p<0.0001) in viable numbers of *Geothricum* candidum during cold storage. The mould counts of the 3 treatments did not differ significantly (p>0.05) during the 15-day storage period. *Geotrichum candidum* consumes oxygen from airtight cup and produces carbon dioxide, which is partially dissolved in the milk; and under anaerobic conditions, the overgrowth of the mould is restricted (Wood, 1998).

Sensory properties of fermented milk by different ratios of mesophilic and thermophilic starter cultures:mould during cold storage

Panelists did not differentiate scooping up, grittiness, stickiness and scent characteristics ($p>0.05$) between the treatments (Table 8). Hardness and sourness of treatment B enhanced and decreased non-significantly ($p>0.05$) during storage time, respectively; the same trend was observed for those of treatment C. There was no definitive trend for ferment smell in treatments B and C, the same as aftertaste in treatments A and C. Optimum taste and overall acceptability appeared to be related to the product made using 80:20 ratio of mesophilic and thermophilic starter cultures:mould. As consumer acceptance will be the ultimate test of the application, we accept treatment A as preferred product.

Conclusion

Fermented milk plays an important role in the daily diet of Iranian people; for this, we decided to produce a fermented milk ,nearly similar to viili, basis on Iranian acceptance. We combined mesophilic and thermophilic LAB with mould *Geotrichum candidum* to get a novel product with the desired texture and flavor characteristics. The results indicated that using starter cultures consist of 10% thermophilic LAB (thermophilic yoghurt cultures with very mild flavor and very high viscosity) and 90% mesophilic LAB, with high flavor/ medium texture, co-cultured with mould *Geotrichum candidum* to a ratio of 80:20, respectively, is the best mixed culture for producing viili on basis of Iranian acceptance.

The use of EPS-producing LAB could result in the development of novel products with enhanced texture and improved stability. Results obtained in the present work could be considered in design of new dairy products on basis of consumer acceptance.

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