Advanced Journal of Microbiology Research Vol. 2017
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Combined effects of dry matter content, incubation temperature and final pH of fermentation on biochemical and microbiological characteristics of probiotic fermented milk

Gelareh Shafiee[1], Amir Mohammad Mortazavian[2*], Mohammad Amin Mohammadifar[1], Mohammad Reza Koushki[3], Abdorreza Mohammadi[4] and Reza Mohammadi[2]

[1]Department of Food Science and Technology, Faculty of Nutrition Sciences, Food Science and Technology, The International and Virtual Branch of shahid Beheshti University of Mrdial Science, P. O. Box 19395-4741, Tehran, Iran. [2]Department of Food Science and Technology, Faculty of Nutrition Sciences, Food Science and Technology/National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, P. O. Box 19395-4741, Tehran, Iran.

[3]Department of Food Technology Reseach, National Nutrition and Food Technology Research Institute, Shahid Beheshti University (M. C.), P. O. Box 19395-4741, Tehran, Iran. [4]Payame Noor University, Arak, Iran.

Accepted 19 May, 2016

Combined effects of milk non-fat dry matter content (4, 8 or 12%), incubation temperature (37, 40 or 44°C) and final pH of fermentation (4.2 or 4.5) on biochemical and microbiological characteristics of probiotic fermented milk (*Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12, and yogurt bacteria) during and immediately after fermentation were investigated. Treatments containing higher dry matter contents had slower pH and redox potential decline rates, faster acidity increase rate, longer incubation time and greater final titrable acidity. Treatments fermented at higher temperatures with lower final pH displayed longer fermentation time and greater final titrable acidity. Treatments with higher dry matter non-fat contents, lower incubation temperatures and higher final pH of fermentation possessed greater viability of probiotic bacteria. The concentration of lactic acid in treatments increased in parallel with the titrable acidity and the concentration of acetic acid were proportional to the viability of bifidobacteria.

Key words: Bifidobacteria*,* fermented milk, *Lactobacillus acidophilus,* probiotic, viability.

INTRODUCTION

Nowadays, manufacture of fermented milks containing probiotic microorganisms is a common and popular issue with a commercial significance and many products of this kind are available in markets of different countries (Holzaspfel and Schillinger, 2001; Korbekandi et al., 2009; Shah, 2001; Tamime et al., 2005). Presently, the species *Lactobacillus* and *Bifidobacterium* are frequently used in production of probiotic fermented milks. Among bifidobacteria, the species *Bifidobacterium lactis* (*B. animalis* ssp. *lactis*) is preferred to be used by manufacturers because of its good tolerance to detrimental environmental factors of fermented milks such as acid, low pH and molecular oxygen (Korbekandi et al., 2009; Mortazavian and Sohrabvandi, 2006a; Tamime et al., 2005).

Viability of probiotic microorganisms in the final product until the time of consumption is their most important qualitative parameter. Although there is no world-wide agreement on the minimum of viable probiotic cells per gram or milliliter of probiotic product until the time of consumption, generally, the values of 10^6 and 10^7-10^8 cfu mL^{-1} or cfu g^{-1} have been accepted as the minimum and

*Corresponding author. E- mail: mortazvn@sbmu.ac.ir/ mortazvn@yahoo.com. Tel: + 98-912-7114977. Fax: + 98-21-22360657

satisfactory levels, respectively (Korbekandi et al., 2009; Lourens-Hattingh and Viljoen, 2001; Tamime et al., 2005; Shah, 2000). In Japan, the "fermented milks and lactic acid bacteria association" have developed a standard which requires a minimum of 10^7 cfu mL^{-1} viable probiotic cells to be present in dairy products (Tamime et al., 2005). In Iran, National standard requires minimums of 10^6 cfu mL^{-1} and 10^5 cfu mL^{-1} viable probiotic cells in yogurt and Doogh (typical Iranian drink based on fermented milk), respectively (Anon, 2008a-c). It has also stated that probiotic products should be consumed regularly with an approximate amount of 100 g d^{-1} in order to deliver about 10^9 viable cells into the intestine (Korbekandi et al., 2009; Lourens-Hattingh and Viljoen, 2001; Shah, 2000; Tamime et al., 2005) . Reaching these standards is generally a difficult issue due to the poor viability of probiotic microorganisms during the fermentation and storage periods (Holzaspfel and Schillinger, 2001; Korbekandi et al., 2009; Shah, 2000; Tamime et al., 2005).

Various compositional and process factors significantly affect the viability of probiotic microorganisms in fermented milks including pH, titrable acidity, molecular oxygen, redox potential, hydrogen peroxide, bacteriocins, short chain fatty acids, flavoring agents, microbial competitions, packaging materials and packaging conditions, rate and proportion of inoculation, step-wise/stage-wise fermentation, micro-encapsulation, milk solid non-fat content, supplementation of milk with nutrients, heat treatment of milk, incubation temperature, storage temperature, carbonation, addition of salt, sugar and sweeteners, cooling rate of the product and scale of production (Champagne and Rastall, 2009; Mortazavian and Sohrabvandi, 2006a; Ranadheera et al., 2009; Tamime et al., 2005). Among mentioned, milk solid non-fat content, incubation temperature and final pH of fermentation possess remarkable impacts on viability of probiotics (Cruz et al., 2007; Korbekandi et al., 2009; Mortazavian and Sohrabvandi, 2006a, 2009). A narrow point regarding the effects of these variables on viability of probiotic organisms is that the simultaneous (combined) effects of these variables might leads to considerably more intense or even contrast results compared to their individual (single) influences. pH is of the most critical factors decreases the viability of probiotic organisms in fermented milks (Champagne and Rastall, 2009; Korbekandi et al., 2009; Tamime et al., 2005).

Although, the individual effects of pH (Donkor et al., 2006), incubation temperature (Fernandez, 1995; Mortazavian et al., 2006b; Singh, 1983) and milk solid-non fat (Mortazavian et al., 2009) on viability of probiotics in fermented milks has been the subject of several studies, in none of them their detailed combined impact has been studied. Therefore, the aim of this study was to investigate the interactive (inter-related) effects of milk dry matter non-fat content, incubation temperature and final pH of fermentation on biochemical characteristics of fermented milk and viability of probiotic bacteria (ABY-type culture composition) during fermentation and immediately after fermentation.

MATERIALS AND METHODS

Starter culture

Fifty-unit pouches of commercial lyophilized ABY culture (containing *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) that are known as 'FD-DVS ABY- 1' were supplied by Chr-Hansen (Horsholm, Denmark). This culture is currently used by dairy industry to produce probiotic dairy fermented products. The cultures were maintained according to manufacturer's instructions at -18°C until used.

Study design and sample preparation

Eighteen yogurt treatments with different milk solid non-fat (4, 8 or 12%), different incubation temperature (37, 40 or 44°C), and two final pH of fermentation (4.5 or 4.2±0.02) were produced using reconstituted skim milk powder and sterilized potable water. Reconstituted milk samples containing different dry matter contents (4, 8 or 12%) were heat treated at 90°C–15 min. Fermentation was carried out at different temperatures (37, 40 or 44°C) until pH reached 4.5±0.02 or 4.2±0.02. Biochemical parameters including changes in pH drop, acidity increase and redox potential increase were measured during fermentation period. These parameters were recorded per 30 min time intervals. Parameters of pH fermentation/ incubation time, final titrable acidity, mean pH drop, mean acidity increase and mean redox potential increase rates were determined at the end of fermentation. The final samples were cooled down and kept at 5°C until the probiotic organisms were enumerated and the concentrations of lactic and acetic acids were determined. The study design of present study is shown in Figure 1.

Microbiological analysis

MRS-bile agar medium (MRS agar by Merck, Darmstadt, Germany and bile by Sigma-Aldrich, Inc., Reyde, USA) was used for the selective enumeration of *L. acidophilus* and bifidobacteria in ABY culture composition according to Mortazavian et al. (2007a). The plates were incubated aerobically and anaerobically at 37°C for at least 72 h. Anaerobic conditions were produced using the GasPac system (Merck, Darmstadt, Germany).

Growth proportion index (GPI) of probiotic microorganism at the end of fermentation was calculated as following (Mortazavian et al., 2009):

GPI = Final cell population (cfu mL^{-1})/initial cell population (cfu mL^{-1})

Chemical analysis

pH values and redox potential of the samples were measured at room temperature using a pH meter (MA235, Mettler, Toledo, Switzerland).

The titrable acidity was determined after mixing 10 mL of sample with 10 mL of distilled water and titrating with 0.1 N NaOH using 0.5% phenolphthalein according to Dave and Shah (1997).

Parameters of pH mean drop rate, mean acidity increase rate, and mean redox potential increase rate were calculated as following (Mortazavian et al., 2009):

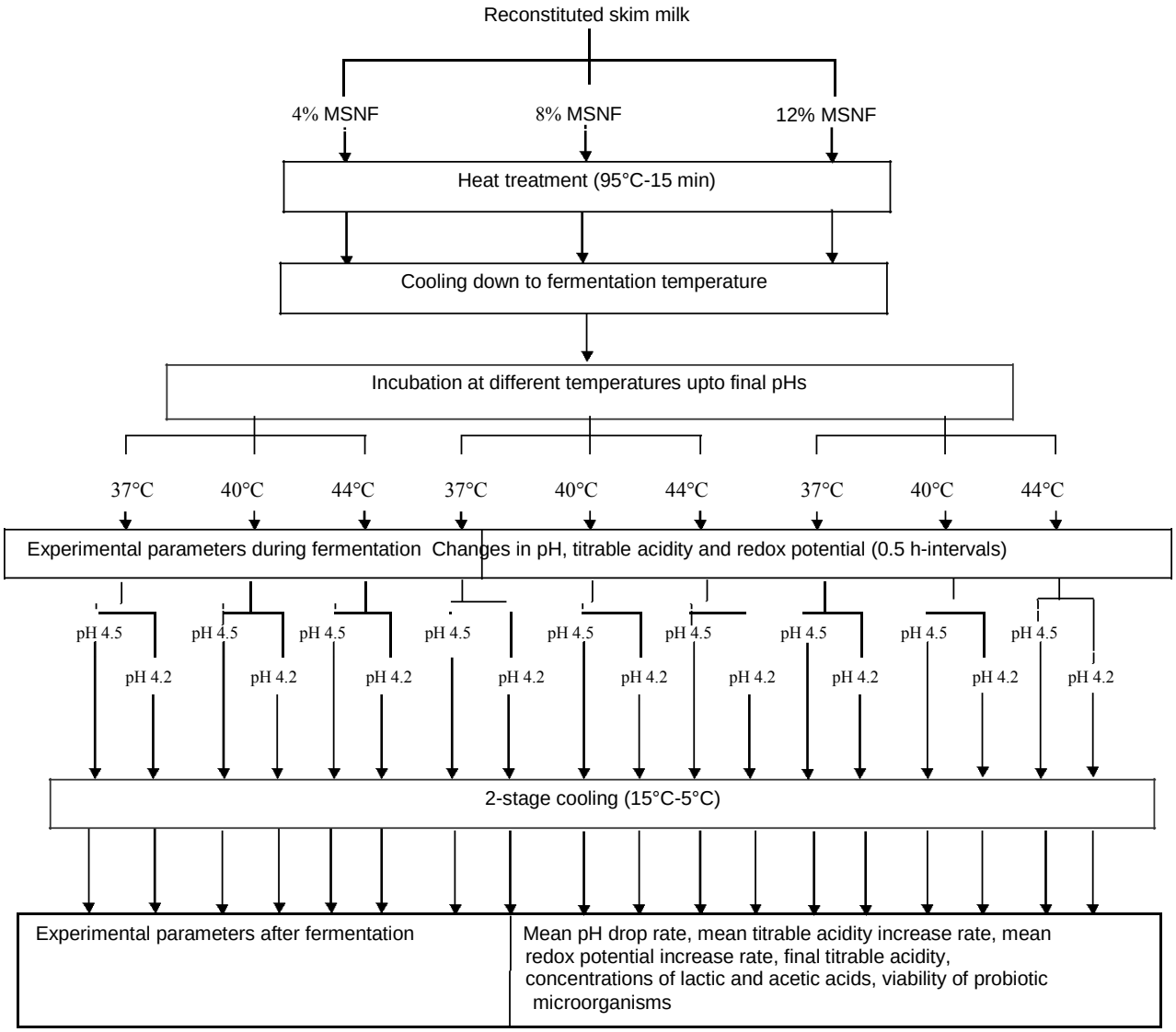

Figure 1. Study design of present study for single replication.

- pH drop rate = (final pH value – initial pH value) / incubation time pH value/min].
- Acidity increase rate = (final acidity value – initial acidity value) / incubation time [Dornic degree/min].
- Redox potential increase rate = (final value – initial value) / incubation time [mV/min].

Quantification of lactic and acetic acids was carried out by High Performance Liquid Chromatography (CE 4200- Instrument, Cecil, Milton Technical Center, Cambridge CB46AZ, UK) according to modified method of Akalin et al. (2004). Briefly, for extraction of acids, 4.0 g of sample was diluted to 25 mL with 0.1 N H_2SO_4, homogenized and centrifuged at 5000 g for 10 min. The supernatant was filtered through Whatman #1 filter paper and through a 0.20 µm membrane filter, and was immediately analyzed. A Jasco UV-980 detector and a Nucleosil 100- $5C_{18}$ column (Macherey Nagel, Duren, Germany) were used. The mobile phase was 0.009 N H_2SO_4 at a flow rate of 0.5 mL min^{-1}. The wavelength of detection was optimized at 210 nm. The standard solutions of lactic and acetic acids (Merck, Darmstadt, Germany) were prepared in distilled water. The retention times for lactic and acetic acids were 3.45 and 3.58 min and the standard curve regression coefficients were 0.989 and 0.991, respectively.

Statistical analysis

Experiments were performed in triplicate and the significant differences among means (p<0.05) were analyzed using the ANOVA test from Minitab software.

RESULTS AND DISCUSSION

Biochemical characteristics of treatments during fermentation and at the end of fermentation

Figures 2a-c show changes in pH drop, acidity increase and redox potential increase during fermentation. Table 1

Table 1. Mean pH drop rate, mean acidity increase rate, mean redox potential increase rate, incubation time, final acidity, and lactic and acetic acid contents in different treatments throughout the fermentation or at the end of fermentation (final pHs of 4.5 and 4.2)*.

Treatment						Parameters			
MSNF (%)	T (°C)	pH	pH-DR** (pH/min)	A-IR (°D/min)	RP-IR (mV/min)	Incubation time (min)	Final acidity (°D)	Lactic acid percentage	Acetic acid percentage
4	44	4.5	0.008^{a}	0.15^{er}	0.46^{a}	270^{l}	44.0^{k}	0.38^{l}	0.05^{c}
4	44	4.2	0.008^{a}	0.14^{f}	0.43^{ab}	330^{f}	51.2^{j}	0.43^{h}	0.07^{bc}
4	40	4.5	0.008^{a}	0.11^{g}	0.47^{a}	290^{h}	37.1^{m}	0.30^{j}	0.06^{c}
4	40	4.2	0.007^{b}	0.08^{h}	0.41^{b}	360^{d}	37.9^{m}	0.31^{j}	0.05^{c}
4	37	4.5	0.007^{b}	0.11^{g}	0.42^{b}	330^{f}	42.9^{kl}	0.36^{l}	0.05^{c}
4	37	4.2	0.007^{b}	0.10^{gh}	0.37^{bc}	390^{c}	45.6^{k}	0.39^{hi}	0.05^{c}
8	44	4.5	0.007^{b}	0.21^{cd}	0.41^{b}	300^{g}	71.3^{h}	0.60^{f}	0.10^{b}
8	44	4.2	0.007^{b}	0.20^{d}	0.38^{b}	360^{d}	79.8^{g}	0.68^{e}	0.10^{b}
8	40	4.5	0.007^{b}	0.16^{e}	0.39^{b}	330^{f}	65.1^{i}	0.56^{fg}	0.08^{bc}
8	40	4.2	0.006^{c}	0.14^{f}	0.38^{b}	390^{c}	67.3^{i}	0.58^{f}	0.08^{bc}
8	37	4.5	0.006^{c}	0.22^{c}	0.34^{c}	350^{e}	85.0^{f}	0.72^{cd}	0.12^{ab}
8	37	4.2	0.006^{c}	0.20^{d}	0.33^{c}	420^{b}	90.9^{e}	0.77^{c}	0.12^{ab}
12	44	4.5	0.007^{b}	0.29^{a}	0.41^{b}	300^{g}	111.1^{c}	1.00^{b}	0.10^{b}
12	44	4.2	0.006^{c}	0.29^{a}	0.39^{b}	360^{d}	122.4^{a}	1.09^{a}	0.11^{b}
12	40	4.5	0.006^{c}	0.28^{ab}	0.34^{c}	330^{f}	108.2^{d}	0.94^{b}	0.13^{ab}
12	40	4.2	0.006^{c}	0.26^{b}	0.33^{c}	390^{c}	120.2^{a}	1.06^{a}	0.13^{ab}
12	37	4.5	0.006^{c}	0.26^{b}	0.34^{c}	360^{d}	113.0^{c}	0.97^{b}	0.15^{a}
12	37	4.2	0.005^{d}	0.22^{c}	0.29^{d}	450^{a}	118.0^{ab}	1.05^{a}	0.12^{ab}

*Means in the same column shown with different letters are significantly different (p<0.05).
**pH-DR = pH drop rate, A-IR = acidity increase rate, RP-IR = redox potential increase rate.

presents mean pH drop rate, mean acidity increase rate, mean redox potential increase rate, incubation time, final titrable acidity, and lactic and acetic acid contents (%) in different treatments throughout the fermentation or at the end of this period. As shown in Figures 2a-c, for all treatments, three distinguished phases could be observed, namely, lag and pre-log phases (initial part of the charts with relatively low slopes), log phase (with considering higher slope), and late log and stationary phases (with significantly decrease in chart slop compared to previous phase). All charts follow this similar pattern. As can be seen in Figures 2a-c, at pH 4.5, the last parts of the charts substantially represent early stationary or mid-stationary state of starter bacterial growth phase. At pH 4.2, mentioned parts indicate mid-stationary or late stationary state of bacterial growth phase. It seems that lower than about pH 4.2, starter bacteria enter late stationary or death phase. These observations are in agreement with those reported by Mortazavian et al. (2009) for ABY type culture compositions. For all treatments, the minimum decrease rate of pH as well as the minimum increase rates of acidity and redox potential were observed within the initial steps of fermentation which represent late lag/early log phase of bacterial growth. Another reason for considerably slow decline in pH at the start of fermentation is buffering capacity of the product.

As is evident in Table 1, the significantly (p<0.05) greater mean pH drop rates were observed for the treatments with 4% dry non-fat. The significantly slower mean pH drop rates were observed for the treatments constituting 12% dry non-fat. Therefore, increase in dry matter content of the treatments led to decrease in mean pH drop rate. Treatments with higher dry matter content showed significantly greater mean acidity increase rates (p<0.05) (Table 1). In contrast, treatments with lower dry matter content showed significantly lower mean acidity increase rates. The same characteristic was observed for final acidity of the treatments. For example, the greatest final titrable acidity was about 3.3 fold more than that of the lowest final titrable acidity (122.4 compared to 37.2°D). These characteristics can be attributed to the different buffering capacity of the treatments. Samples containing greater amounts of milk solid non-fat exhibit higher buffering capacity. Greater buffering capacity stimulates acidification rate by starter bacteria because they are inhibited significantly later during fermentation due to slower pH drop rate. Sharp decline in pH leads to pH drop shock to the starter bacteria, especially probiotic bacteria. This factor along with the falling pH values that is inhibitory (or even detrimental) to these bacteria results in lower acidification rate by them and lower viability of probiotics during fermentation.

According to that mentioned, treatments containing greater buffering capacity (higher dry matter content) had also greater final titrable acidity (Table 1). For instance,

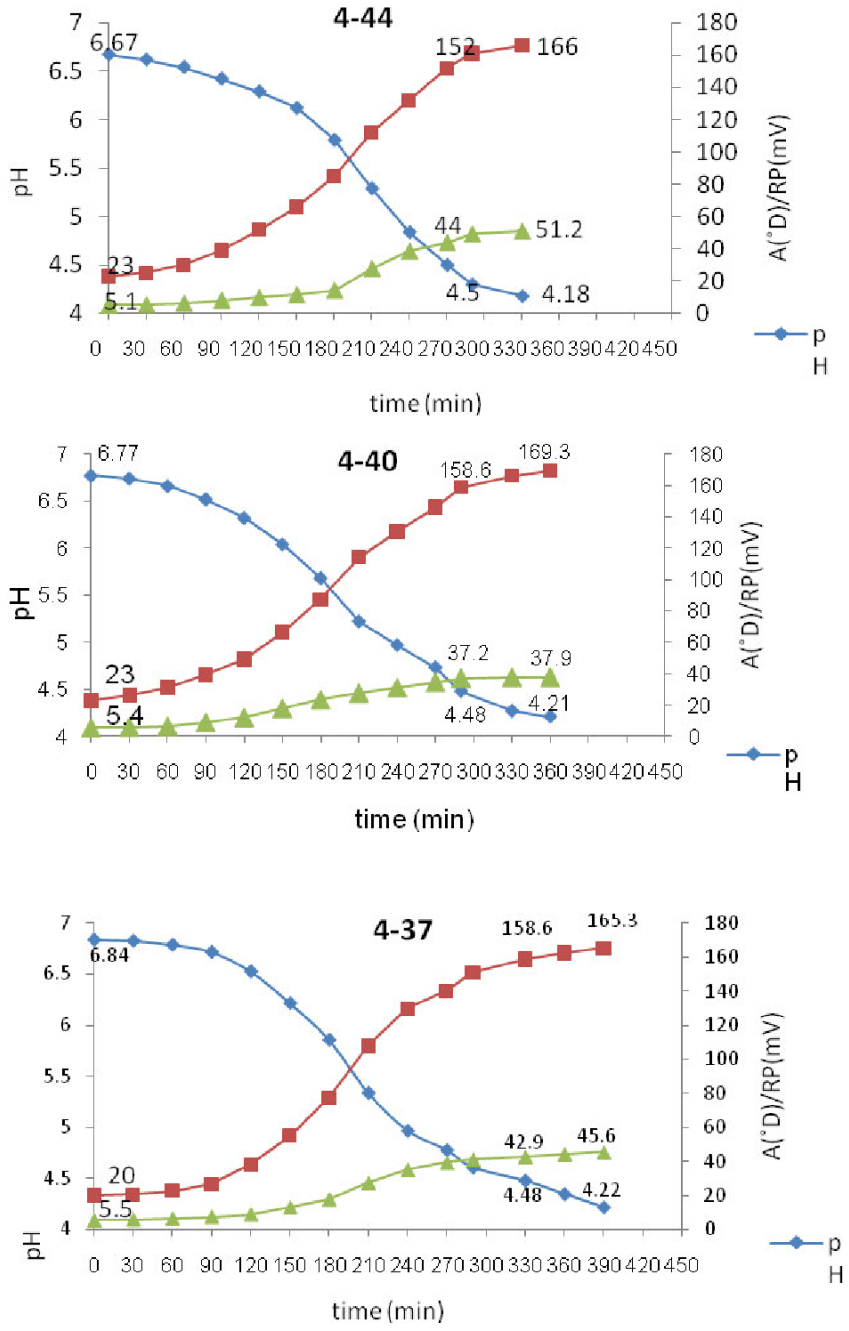

Figure 2a. Changes in pH drop, acidity increase and redox potential increase during fermentation period in treatments with 4% milk solid non-fat incubated at temperatures 44, 40 and 37°C (final pH 4.5 and 4.2).

the final titrable acidity in the treatment with 12% dry matter content/incubation temperature of 37°C/final fermentation pH 4.2 (12-37-4.2) was 72.4°D more than that of 4% dry matter content/incubation temperature of 37°C/final fermentation pH 4.2 (4-37-4.2) . The greatest final titrable acidity among the treatments was observed for those of 12-44 - 4.2°C and 12-40 - 4.2°C (Table 1). Considering Table 1, in treatments with final fermentation pH 4.5 and pH 4.2, the differences between the final titrable acidities were greater in treatments containing 12% dry non-fat solids compared to those containing lower dry matters (8 and 4%). For example, final titrable acidity in treatments 12-40-4.5 and 12-40-4.2 were 108.2 and 120.2, respectively; whilst these values for the treatments 4-40-4.5 and 4-40-4.2 were 37.2 and 37.9, respectively. The mean redox potential increase rate

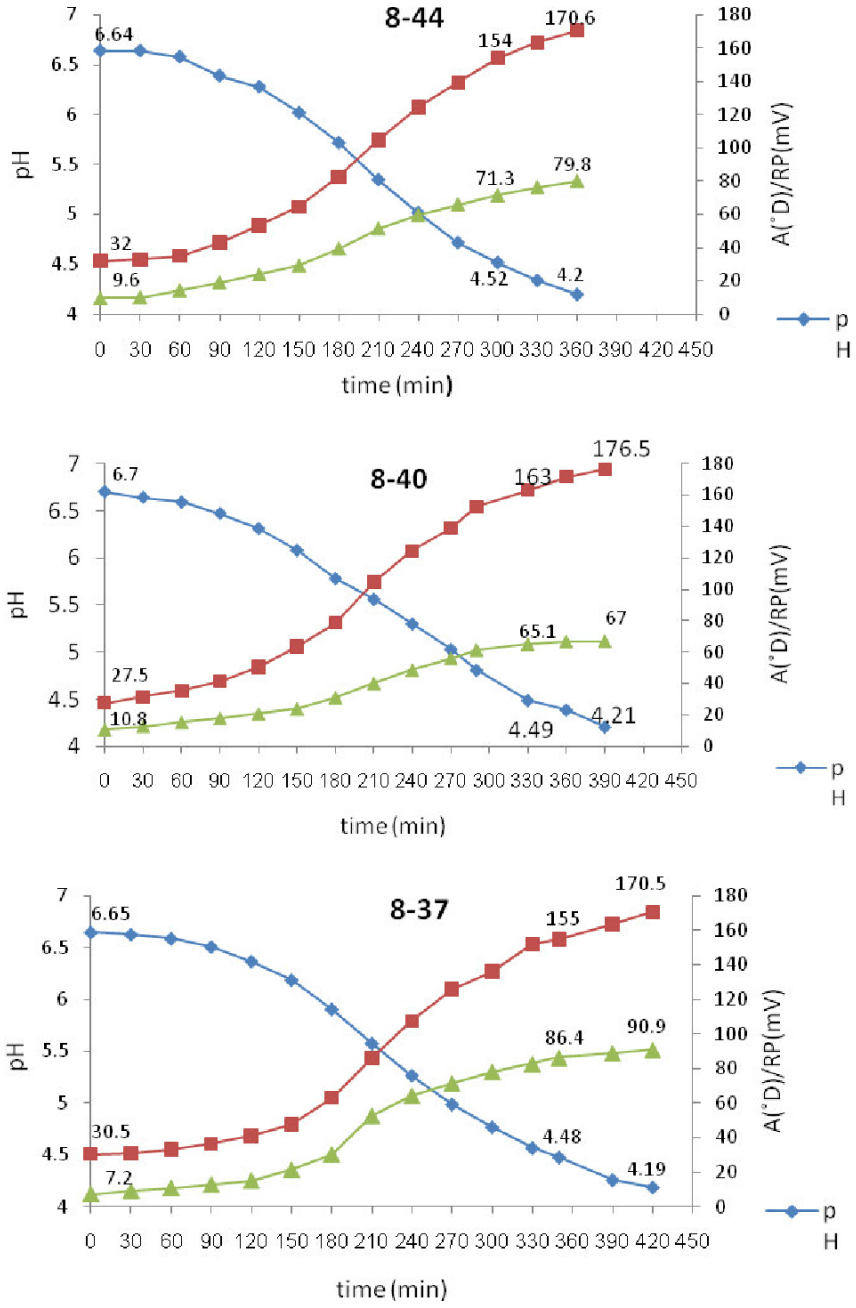

Figure 2b. Changes in pH drop, acidity increase and redox potential increase during fermentation period in treatments with 8% milk solid non-fat incubated at temperatures 44, 40 and 37°C (final pH 4.5 and 4.2).

showed converse behavior compared to the mean acidity increase rate.

On the other words, decrease in dry matter content is in parallel to increase in the mean redox potential increase rate. This phenomenon can be attributed to the noticeably richer media from reducing agents point of view in the treatments with greater dry matter contents. Milk proteins (especially sulfur-containing amino acids) efficiently decrease the redox potential of media after sufficient heat treatment of and keep it significantly lower during fermentation (Dave and Shah, 1997; Korbekandi et al., 2009; Shah, 2000; Singh, 1983; Tamime et al., 2005). This observation was consistent with that of Mortazavian et al. (2009).

According to Table 1, increase in dry matter non-fat content of fermented milk as well as decrease in fermentation temperature is directly and indirectly (respectively) proportional to the incubation time (upto a definite final

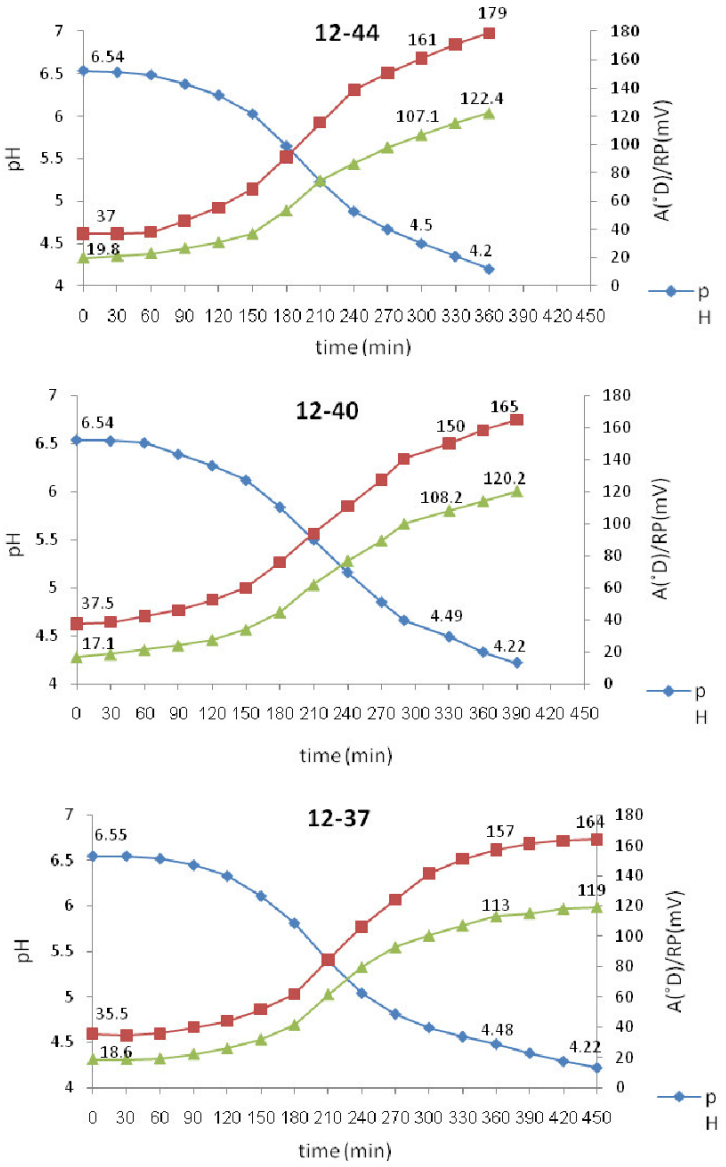

Figure 2c. Changes in pH drop, acidity increase and redox potential increase during fermentation period in treatments with 12% milk solid non-fat incubated at temperatures 44, 40 and 37°C (final pH 4.5 and 4.2).

pH). Also, incubation time was significantly longer ($p<0.05$) in treatments with final fermentation pH 4.2 compared to 4.5. Increasing dry matter non-fat content of fermented milks prolongs the incubation time (upto a definite final pH of fermentation) due to increase in buffering capacity of the media. The best growth and activity temperature for probiotic bacteria is significantly lower than that of yogurt bacteria. Although the growth of *L. acidophilus* may occur at temperatures as high as 45°C, its optimum growth occurs within 40-42°C. The optimum growth temperature for bifidobacteria is 37-40°C. This range for traditional yogurt bacteria is 42-45°C (Guler-Akin, 2005; Kneifel et al., 1993; Korbekandi et al., 2009; Lourens-Hattingh and Viljoen, 2001; Mortazavian and Sohrabvandi, 2006a).

Therefore, considering this point that yogurt bacteria possess noticeably higher -galactosidase and protease activities (Gomes and Malcata, 1999; Lourens-Hattingh and Viljoen, 2001), in the culture compositions containing both probiotic and yogurt bacteria (such as in present study), increasing fermentation temperature from 37 towards 45°C will leads to considerably higher acidification rate by yogurt bacteria (rather than probiotic bacteria) and as a result, significantly shorter incubation time. According to Table 1, the longest incubation time was observed for the treatment 12-37-4.2 (450 min) in com-

Table 2. Viability of probiotic microorganisms and the relevant growth proportion index in different treatments at the end of fermentation (final pHs 4.5 and 4.2)*.

Treatment			Initial population (log cfu/mL)			final population (log cfu/mL)			GPI**		
MSNF (%)	T (°C)	pH	A***	B	A+B	A	B	A+B	A	B	A+B
4	44	4.5	6.21	6.44	6.64	6.15^{lA}	5.60^{pB}	6.26^{m}	0.87	0.14	0.42
4	44	4.2	6.21	6.44	6.64	5.70^{nA}	5.30^{qB}	5.84^{p}	0.31	0.07	0.16
4	40	4.5	6.21	6.44	6.64	6.40^{jA}	6.11^{lB}	6.58^{k}	1.55	0.47	0.87
4	40	4.2	6.21	6.44	6.64	5.95^{mA}	5.70^{noAB}	6.15^{no}	0.55	0.18	0.32
4	37	4.5	6.21	6.44	6.64	6.76^{hA}	6.50^{lB}	6.95^{n}	3.55	1.15	2.04
4	37	4.2	6.21	6.44	6.64	6.46^{lA}	5.90^{mB}	6.57^{k}	1.78	0.29	0.85
8	44	4.5	6.21	6.44	6.64	6.23^{kB}	6.73^{gA}	6.30^{l}	1.05	1.10	0.46
8	44	4.2	6.21	6.44	6.64	5.95^{mA}	5.78^{nAB}	6.18^{n}	0.55	0.22	0.35
8	40	4.5	6.21	6.44	6.64	6.75^{hA}	6.40^{lB}	6.91^{hi}	3.47	0.91	1.86
8	40	4.2	6.21	6.44	6.64	6.48^{lA}	6.28^{kB}	6.69^{j}	1.86	0.69	1.12
8	37	4.5	6.21	6.44	6.64	6.98^{eA}	6.48^{lB}	7.17^{f}	5.89	1.95	3.39
8	37	4.2	6.21	6.44	6.64	6.86^{gA}	6.58^{hB}	7.04^{g}	4.47	1.38	2.51
12	44	4.5	6.21	6.44	6.64	7.03^{dAB}	7.06^{eA}	7.35^{d}	6.61	4.17	5.13
12	44	4.2	6.21	6.44	6.64	6.94^{efA}	6.97^{fA}	7.26^{e}	5.37	3.39	4.17
12	40	4.5	6.21	6.44	6.64	7.17^{cA}	7.18^{cdA}	7.48^{e}	9.12	5.50	6.92
12	40	4.2	6.21	6.44	6.64	7.00^{dB}	7.10^{cA}	7.36^{d}	6.17	4.57	5.25
12	37	4.5	6.21	6.44	6.64	7.31^{aAB}	7.33^{aA}	7.61^{a}	12.59	7.76	9.33
12	37	4.2	6.21	6.44	6.64	7.23^{bAB}	7.26^{bA}	7.54^{b}	10.47	6.61	4.17

*****Means shown with different small and capital letters represent significant differences (p <0.05) in the same columns (among the treatments) and rows (between the two probiotic bacteria in each treatment), respectively.
**GPI = Growth proportion index.
***A = *L. acidophilus*, B = bifidobacteria, A + B = total probiotics.

parison with 4-44- 4.5 (270 min), which exhibited the shortest incubation time. Considering data presented in Table 1, it seems that increase in incubation temperature have significantly higher impact on reduction of incubation time compared to increase in dry matter non-fat content of fermented milk. For example, the incubation time difference between the treatments 4-44-4.2 and 8-44-4.2 (change in dry matter content) was 30 min, whilst this difference between the treatments 4-44-4.2 and 4-37-4.2 (change in incubation temperature) was 60 min.

Similarly, there was no significant difference in incubation time between the treatments 8-44-4.2 and 12-44-4.2 or 8-44- 4.5 and 12-44-4.5 (change in dry matter content), whilst this difference between the treatments 8-44-4.2 and 8-37-4.2 or 8-44- 4.5 and 12-37-4.5 (change in incubation temperature) was 60 min.

With respect to Table 1, the greatest and lowest amounts of lactic and acetic acids were observed in treatments containing 12 and 4% dry matter non-fat, respectively. Acetic acid concentration in treatments was in the range of 0.05- 0.15%. Sum of other organic acids except lactic acid in treatments were less than 0.03%. Treatments with pH 4.2 had significantly higher (p<0.05) amount of lactic acid compared to those with pH 4.5. However, the percentage of acetic acid was not significantly different (p>0.05) between the treatments with pH 4.5 and 4.2. Bifidobacteria forms acetic acid during fermentation and its concentration is highly stain dependent (Gomes and Malcata, 1999). Therefore, the concentration of acetic acid in treatments at the end of fermentation should be proportional to the growth and activity rate of bifidobacteria (Mortazavian et al., 2009). The optimum pH for growth of bifidobacteria is 6.5-7.0 and the growth of this bacterium is significantly retarded below pH 5.5 (Gomes and Malcata, 1999; Klaver and Weerkamp, 1993; Shah, 1997; Tamime et al., 2005; Vuyst, 2000). This justifies why the concentration of acetic acid in treatments was not significantly changed (p>0.05) from pH 4.5 to 4.2 (Table 1).

Viability of probiotic bacteria at the end of fermentation

Table 2 shows viability of probiotic microorganisms as well as the relevant growth proportion index (GPI) in different treatments immediately after fermentation. As represented from this Table, the viability of both probiotic bacteria (*L. acidophilus* LA-5 and *Bifidobacterium lactis* BB-12) were significantly and markedly greater and lower in the treatments containing 12 and 4% solid non-fat contents, respectively. According to Table 2, the growth

proportion index (GPI) for the treatments containing 12% dry matte content is significantly greater ($p<0.05$) than the treatments with other dry matter contents (4 or 8%), especially for bifidobacteria. Significant greater viability of probiotics ($p<0.05$) in the treatments with higher dry matter content can be attributed to several reasons.

First, higher buffering capacity in treatments with higer milk solid non-fat content leads to longer fermentation time until reaching the final fermentation pHs (4.5 or 4.2) (Table 1) and as a result, longer time for multiplication of starter bacteria. Lower buffering capacity of treatments results in sharper decrease in pH during fermentation period (Table 1; greater mean pH drop rate) which causes pH drop shock to probiotic cells. This phenolmenon leads to decrease in viability of probiotic bacteria (Korbekandi et al., 2009; Mortazavian et al., 2009). In treatments with shorter fermentation periods, yogurt bacteria are more active compared to probiotics and become dominant species of the media. In such condition, pH is rapidly decreased below the inhibitory limits of probiotics before they can proliferate efficiently. With respect to GPI in Table 2, it could be understood that bifidobacteria cells were considerably more sensitive to sharp pH decline than *L. acidophilus* cells because in parallel with increase in dry matter content of the treatments, the GPI for the former bacteria increased noticeably greater than those of the latter (the proportion of "maximum GPI/minimum GPI" for bifidobacteria and *L. acidophilus* were 7.76/0.07 = 110.8 and 12.59/0.31 = 40.6, respectively).

According to Table 2, only for the treatments with 12% dry matter content, the viable counts of bifidobacteria and the relevant GPIs were statistically greater or equal to those of *L. acidophilus*. For the rest treatments, the viable populations of the latter bacteria were significantly higher than the former ($p<0.05$); with respect to the point that the initial number of bifidobacteria cells inoculated in milk was considerably greater than *L. acidophilus* cells (Table 2). Second, lower redox potential increase rate of the treatments containing higher solid non-fat (Table 1) enhances the growth and activity of probiotics, particularly bifidobacteria (Dave and Shah, 1997; Korbekandi et al., 2009). Third, richer fermenting media from nutritional point of view in treatments containing higher amounts of dry matter considerably reduces the bacterial competitions among starter cultures that can result in the loss of viability of probiotics (Mortazavian et al., 2009). Forth, media containing higher amounts of dry matter (e.g., 12% compared to 4%) increases the viability of probiotic bacteria due to possessing appropriate protective effect of the solid matrixes on these organisms against detrimental environmental factors such as molecular oxygen, hydrogen ions, hydrogen peroxide and organic acids (Mortazavian and Sohrabvandi, 2006a).

As indicated from Table 2, fermentation temperature of 37°C resulted in significantly greater viability for both probiotic bacteria ($p<0.05$) compared to 40 and 44°C (viable counts of probiotics as well as the relevant GPIs). This was in agreement with the related previous reports (Fernandez, 1995; Gomes and Malcata, 1999; Kneifel et al., 1993; Mortazavian et al., 2006b; Singh, 1983). In mixed probiotic cultures, particularly when yogurt starters are co- cultured with probiotic cultures (such as ABY-type culture compositions), changing fermentation temperature significantly affects viability of probiotics. As, mentioned in Section 3.2, the optimum growth of *L. acidophilus*, bifidobacteria and yogurt bacteria cells are within temperature ranges of 40-42°C, 37-40°C and 42-45°C, respectively. Therefore, employing higher incubation temperatures (e.g., 44°C in comparison with 37-40°C) leads to significantly lower viability of probiotics due to domination of yoghurt bacteria over probiotics (Korbekandi et al., 2009; Mortazavian et al., 2006b; Tamime et al., 2005). Yogurt bacteria, especially *L. delbrueckii* ssp. *bulgaricus* can suppress probiotics via sharp- and over-acidification, formation of hydrogen peroxide, and possibly, bacteriocins. It is well-known that in fermented milks with ABY culture composition, remarkable viability loss of *L. acidophilus* is mainly due to hydrogen peroxide produced by *L. delbrueckii* ssp. *bulgaricus* (Dave and Shah, 1997; Korbekandi et al., 2009; Mortazavian et al., 2006b; 2007b; Shah et al., 1995; Tamime et al., 2005).

According to Table 2, the final pH 4.5 resulted in the significantly greater viability of both probiotic microorganisms compared to the final pH 4.2. In treatments with similar dry matter content (4, 8 or 12%), those with incubation temperature of 37°C and final pH 4.5 led to the greatest viability of probiotic organisms. Conversely, treatments with incubation temperature of 44°C and final pH 4.2 led to the lowest viability of probiotics. As mentioned in Section 3.1 (Figures 2a-c), at pHs about 4.2, starter bacteria used in present study must be in late stationary phase of growth and less than that, in predeath or death phases, resulting in loss of viability of probiotics. These observations are consistent with the results reported by Mortazavian et al. (2009) . It can be observed in Table 2 that in some treatments containing 4% dry matter content, the GPI for both ptobiotics were less than 1, meaning that final populations of both probiotic bacteria were less than their viable cells initially inoculated into milk. With respect to Table 2, *Bifidobacterium* cells were significantly less tolerant to the lower pH values, that is, pH 4.2 compared to 4.5.

Generally, bifidobacteria are sensitive to low pH amounts and their growth and activity is restricted at pHs < 5.0. The optimum growth pH of *L. acidophilus* has been reported in the range 5.5 - 6 (Gomes and Malcata, 1999; Holzaspfel and Schillinger, 2001; Lourens-Hattingh and Viljoen, 2001). It can understood from Table 2 that in the treatments containing similar dry matter content, the impact of incubation temperature on viability of both probiotic bacteria was more prominent than the effect of final pH of fermentation. For example, the viability of probiotics in the treatment 12-37-4.2 was significantly greater than that of 12-44-4.5 or the viability of probiotics in the treatment 4-37-4.2 was significantly higher than that

of 4-44-4.5 ($p<0.05$). The treatment 12-37-4.5 resulted in the greatest viability of both probiotics among all treatments and the treatment 4-44-4.2 led to the lowest viability of them (Table 2).

Considering Table 1, the viability of bifidobacteria was directly proportional to the concentration of acetic acids in treatments; namely, their greatest viabilities were observed in treatments containing the highest amounts of this acid. This phenomenon was in agreement to the findings of Mortazavian et al. (2009).

Conclusions

Results of this study revealed that dry matter non-fat content, incubation temperature and final pH of fermentation significantly ($p<0.05$) affected viability of *L. acidophilus* and bifidobacteria and interactive relationships were observed among mentioned three variables. Treatments with higher amounts of dry matter non- fat, lower incubation temperatures and higher final pH of fermentation possessed greater viability of probiotic bacteria. The greatest viability of both probiotic microorganisms obtained in fermented milk with 12% dry matter, incubation temperature of 37°C and final pH 4.5. The lowest viability was observed in the treatment with 4% dry matter, incubation temperature of 44°C and final pH 4.2. Generally, bifidobacteria cells were considerably more sensitive to sharp pH decline than *L. acidophilus*. Treatments containing higher dry matter contents had slower pH decline, faster acidity increase, slower redox potential increase, longer incubation time and greater final titrable acidity. The concentration of lactic acid in treatments increased in parallel with the amounts of titrable acidity and the concentration of acetic acid was proportional to the viability of bifidobacteria.

REFERENCES

Akalin AS, Fenderya S, Akbulut N (2004). Viability and activity of bifidobacteria in yogurt containing fructooligosaccharide during refrigerated storage. Int. J. Food Sci. Tech., 39: 613-621.

Anon (2008a). Iran national standard for plain Doogh; No. 2453. Available on www.isiri.org [In Persian].

Anon (2008b). Iran national standard for probiotic Doogh; No. 11324. Available on www.isiri.org [In Persian].

Anon (2008c), Iran national standard for Doogh-code of practice; No. 10528. Available on www.isiri.org [In Persian].

Champagnem CP, Rastall RA (2009). Some Technological Challenges in the addition of probiotic bacteria to Foods. In: Charalampopoulos D, Rastall RA (eds) Prebiotics and Probiotics Science and Technology: Springer, UK, pp 763-806.

Cruz AG, Faria JAF, Van Dender AGF (2007). Packaging system and probiotic dairy foods. Food Res. Int., 40: 725-732.

Dave RI, Shah NP (1997). Viability of yogurt and probiotic bacteria in yoghurts made from commercial starter cultures. Int. Diary J., 7: 31-34.

Donkor ON, Henriksson A, Vasiljevic T, Shah NP (2006). Effect of acidification on the activity of probiotics in yoghurt during cold storage. Int. Dairy J., 16: 1181-1189.

Fernandez ML (1995). Influence of the incubation temperature on the autolytic activity of *L. acidophilus*. J. Appl. Bacteriol., 78: 426-429.

Gomes AMP, Malcata FX (1999). *Bifidobacterium* spp. and *Lactbacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends Food Sci. Technol. 10: 139-157.

Guler-Akin MB (2005). The effects of different incubation temperatures on the acetaldehyde content and viable bacteria counts of bio-yogurt made from ewe's milk. Int. J. Dairy Tech., 58: 174-179.

Holzaspfel WH, Schillinger U (2001). Introduction to pre- and probiotics. Food Res. Int., 35: 109-16.

Klaver FAM, Weerkamp AH (1993). Growth and survival of bifidobacteria in milk. Neth. Milk Diary J., 47: 151-164.

Kneifel W, Jaros D, Erhard F (1993). Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. Int. J. Food Microb., 18: 179-189.

Korbekandi H, Mortazavian AM, Iravani S (2009). Technology and stability of probiotic in fermented milks. In: Shah N (ed) Probiotic and Prebiotic Foods: Technology, Stability and Benefits to the human health, Blackwell Publishing Ltd, UK, (In press).

Lourens -Hattingh A, Viljoen B (2001). Yoghurt as a probiotic carrier food. Int. Dairy J., 11: 1-17.

Mortazavian AM, Khosrokhavar R, Rastgar H (2009). Effects of dry matter standardization order on biochemical and microbiological characteristics of Doogh (Iranian fermented milk drink). Ital. J. Food Sci., (In Press).

Mortazavian AM, Sohrabvandi S, Reinheimer JA (2007a). MRS-bile agar: Its suitability for the enumeration of mixed Probiotic cultures in cultured dairy products. Milchwissenschaft, 62: 270-272.

Mortazavian AM, Ehsani MR, Mousavi SM, Rezaei K, Sohrabvandi S, Reinheimer JA (2007b). Effect of storage temperature on the viability of probiotic microorganisms in yogurt. Int. J. Dairy Technol. 60: 123-127.

Mortazavian AM, Sohrabvandi S (2006a). Probiotics and Food Probiotic Products; based on dairy probiotic products. In: Mortazavian AM (ed) Technological aspects of probiotic fermented milk products, Eta Publication, Iran, pp. 220-228

Mortazavian AM, Ehsani MR, Mousavi SM, Reinheimer JA, Sohrabvandi S, Rezaei K (2006b). Preliminary investigation of the combined effect of heat treatment and incubation temperature on the viability of the probiotic microorganisms in freshly made yogurt. Int. J. Diary Technol. 59: 8-11.

Shah NP (2001). Functional foods from probiotics and prebiotics. Food Technol. 55: 46-53.

Shah NP (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci., 83: 894-907.

Shah NP (1997). Bifidobacteria: Characteristics and potential for application in fermented milk products. Milchwissenschaft 52: 16-21.

Singh J (1983). Influence of heat treatment of milk and incubation temperature on *S. thermophilus* and *L. acidophilus.* Milchwissenschaft, 38: 347-348.

Tamime AY, Saarela M, Korslund Sondergaard A, Mistry VV, Shah NP (2005). Production and maintenance of viability probiotics microorganism in dairy products. In: Tamime AY (ed) Probiotic Dairy Products, Blackwell Publishing Ltd, UK, pp. 39-97.

Vuyst LD (2000). Technology aspects related to the application of functional starter cultures. Food Technol. Biotechnol. 38: 105-12.