

Evaluation of Probiotic Survivability in Yogurt Exposed To Cold Chain Interruption

Rohollah Ferdousi^a, Millad Rouhi^b, Reza Mohammadi^c, Amir Mohamad Mortazavian^{d*},
Kianosh Khosravi-Darani^a and Aziz Homayouni Rad^e

^aDepartment of Food Technology Research, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ^bDepartment of Food Science and Technology, Faculty of Agriculture, University of Tehran, Karaj, Iran. ^cStudents Research Committee, Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences, Food Science and Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ^dStudents' Research Committee, Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences, Food Science and Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ^eDepartment of Food Science and Technology, Faculty of Nutrition, Tabriz University of Medical Sciences Tabriz, Iran.

Abstract

In this research, the survival of probiotic microorganisms in yogurts stored at room temperature (cold chain interruption conditions) was studied. Milk inoculated with yogurt bacteria (mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) and a single probiotic culture (*L. acidophilus* LA-5 or *Bifidobacterium lactis* Bb-12 or *L. rhamnosus* HN001 or *L. paracasei* Lpc-37) were incubated till pH of 4.5 was reached. Probiotic yogurts were stored at two different temperatures including cold (control) and room temperatures (5 and 20°C, respectively). Changes in pH decrease, titratable acidity increase and redox potential increase as well as the viability of probiotics per 6 h intervals during an assumptive interrupted cold storage (24 h) were monitored. The survival of probiotics was strongly dependent on the storage temperature and remarkable viability loss occurred in room temperature compared to refrigerated storage. In addition, the survivability was dependent on probiotic strain. Among our experimental strains, *B. lactis* Bb-12 showed the less resistance to be stored at 20°C (24 h) and referring to the recommended minimum numbers of 10⁷ cfu mL⁻¹, *L. rhamnosus* HN001 was the most suitable probiotic strain to be used in probiotic yogurts especially in countries having high possibility of cold chain interruption during storage.

Keywords: Bifidobacteria; *Lactobacillus*; Probiotic; Room temperature; Survival; Viability.

Introduction

Food industry companies have rather high expectations in food products that meet the

consumers' demand for a healthy life style. In this context, 'functional foods' play a specific role. These foods are not intended only to satisfy hunger and provide humans with necessary nutrients, but also to prevent nutrition-related diseases and increase physical and mental well-being of consumers (1-3). One of the most

* Corresponding author:

E-mail: mortazvn@yahoo.com

promising areas for the development of functional foods lies in the modification of gastrointestinal tract activity by the use of probiotics, prebiotics and synbiotics.

Probiotics are special types of live healthful bacteria or yeast which possess favorable impacts on animal and human host mainly via maintaining and/or improving microbial balance between harmful and beneficial microflora, especially in the intestine (4-6). They have an established role in reducing human illnesses, particularly gastrointestinal infections caused by deficient or compromised gut microflora. Other therapeutic functions are attributed to probiotics such as anti-cholesterol activity, alleviation of lactose intolerance symptoms, promotion of beneficial immune responses, antimicrobial impact, anti-high blood pressure effect and anticarcinogenic and anti-mutagenic activities (7-15). Recently, there have also been recent reports on the potential benefit of probiotics for human skin (16) and against colds and flu (17).

Probiotic microorganisms are common to be ingested through dairy products, mainly fermented milk products (5, 6). *Bifidobacterium* spp. and *Lactobacillus acidophilus* are by far the most important probiotics regularly added to the fermented milks (18). Among the dairy-fermented products, yogurt is the most popular one, and in Europe, the highest consumption of probiotic products is associated with probiotic yogurt (19).

Probiotics should be alive to an adequate number in order to exert their positive effects on the health of the host. This attribute is known as 'viability', namely the adequate number of live probiotic cells in a food product at the time of consumption (5). No general agreement has been reached on the recommended levels and the suggested levels ranged from 10^6 cfu mL⁻¹ (20) to over 10^7 and 10^8 cfu mL⁻¹ (5). However, it is generally recommended that the probiotic culture must be present in the product at minimum numbers of 10^7 cfu mL⁻¹ (21). These suggestions have been made to compensate for the possible decline in the concentration of the probiotic organisms during processing and storage of a probiotic product as well as passage through the upper and lower parts of the gastrointestinal tract. Numerous studies have demonstrated that

probiotic strains grow poorly in milk, resulting in low final concentrations in yogurt and even the loss of the viability during prolonged and/or inappropriate storage conditions. Survival of these bacteria during shelf life and until consumption is therefore an important issue.

Various factors have been recognized to affect the viability of probiotic bacteria during storage of fermented dairy products such as pH, redox potential and acidity, buffering capacity, packaging, molecular oxygen and storage time and temperature (5, 22). Storage temperature has substantial impact on maintaining viability of probiotic bacteria and it is generally well-known that the probiotic yogurt must be kept under refrigerated storage (22- 24). However, mentioned product might be subjected to cold chain interruption for hours during industrial distribution, retailing and home storage. This leads to the question whether the number of probiotic bacteria declines to such an extent that there are not enough bacteria remaining in the product to be useful. Few studies have studied the effect of cold chain interruption during storage on the survivability of probiotics (25). In this study, the viability of probiotic bacteria in yogurts exposed to cold temperature interruption (stored at room temperature) was investigated.

Experimental

Starter cultures

The DVS pouches of commercial lyophilized cultures including Y-type/yogurt bacteria (mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) commercially known as YF-702 were supplied by Chr. Hansen (Horsholm, Denmark). Besides, a single probiotic culture containing *L. acidophilus* LA-5 (Chr. Hansen) or *Bifidobacterium lactis* Bb-12 (Chr. Hansen) or *L. rhamnosus* HN001 (Danisco, Copenhagen, Denmark) or *L. paracasei* Lpc-37 (Danisco) was used. These starter cultures are widely used by the dairy industry to produce fermented milk products. The cultures were maintained according to the manufacturer's instructions, until used.

Microbiological analysis

Lactobacilli and bifidobacteria were

enumerated selectively using MRS-bile agar (MRA agar: Merck, Darmstadt, Germany, and Bile: Sigma, Rede, USA) (Mortazavian *et al.* 2007a). The plates were incubated at 37°C for at least 72 h under both aerobiosis and anaerobiosis. Anaerobic condition was generated by using the Gas Pack system (Merck, Darmstadt, Germany).

Viability proportion index (VPI) of probiotic microorganism at the end of storage time were calculated as following (26, 27): $VPI = \text{Final cell population (cfu mL}^{-1}) / \text{initial cell population (cfu mL}^{-1})$.

Sample preparation

Yogurt milk with 12.0% dry matter was formulated using reconstituted skim milk powder. After heat treatment (90°C–15 min), cooling to inoculation temperature (40°C) and addition of yogurt starter culture, treatments were inoculated with different probiotic single strain cultures (10^6 cfu mL⁻¹). The treatments were incubated at 40°C until pH of 4.5 ± 0.02 was reached. The yogurts were stored for 24 h at two different temperatures including cold or room temperatures (5 or 20°C, respectively). Therefore, eight treatments were produced; namely yogurts containing *L. rhamnosus* HN001 stored at 5°C (RY-5) or 20°C (RY-20), *L. paracasei* Lpc-37 stored at 5°C (PY-5) or 20°C (PY-20), *L. acidophilus* La-5 stored at 5°C (AY-5) or 20°C (AY-20), and *B. Lactis* Bb-12 stored at 5°C (BY-5) or 20°C (BY-20). Changes in pH decrease, redox potential increase and titratable acidity increase as well as the viability of probiotics per 6-h intervals during 24 h of storage were monitored.

Chemical analysis

pH and redox potential values of the samples were measured at room temperature using a pH meter (MA235, Mettler, Toledo, Switzerland). The titratable 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 (26).

Statistical analysis

All results were an average of three replicate determinations and the significant differences among the means were analyzed using the

two-way ANOVA test (based on the complete randomized design (full Factorial test design) from Minitab software (Version 13, 2002).

Results and Discussion

Changes in pH, titratable acidity and redox potential

Figure 1 shows changes in pH, redox potential and acidity changes at every 6 h, among treatments stored at 20°C compared to the control (stored at 5°C). pH, titratable acidity and redox potential in treatments stored at 5°C were not significantly changed during 24 h of storage and were about 4.47, 110.8°D and 151 mV at the end of storage, respectively. Nevertheless, these values were approximately 4.25, 120.2°D and 164 mV in those stored at 20°C, respectively. As shown in Figure 1, pH decrease as well as titratable acidity and redox potential increase showed highest slopes at initial hours of storage than the later ones.

Survival of probiotic microorganisms during storage

Table 1 indicates viable counts of probiotic bacteria in different treatments during the storage time. Table 2 represents viability proportion index (VPI) in different treatments during this time. According to Table 1, viable counts of probiotics in all treatments stored at 5°C had no significant changes during storage ($p < 0.05$). For example, the VPI (Table 2) for all strains at the end of storage ranged between 0.95-0.97. However, for treatments stored at 20°C, the viable counts of probiotics showed significant decrease during storage. This decline varied among probiotic species because of different sensitivity to environmental stresses such as low pH and high titratable acidity. Considering Table 2, the most survivability throughout the storage in treatments stored at 20°C belonged to *L. rhamnosus* HN001, *L. paracasei* Lpc-37, *L. acidophilus* LA-5 and *B. Lactis* Bb-12, respectively. *B. Lactis* maintained only 60% of its initial viable population at the end of storage, whilst this amount was 81% for *L. rhamnosus*. Scharl *et al.* (25) demonstrated that the number of living probiotic bacteria in yogurt decreased dramatically after exposure to room temperature.

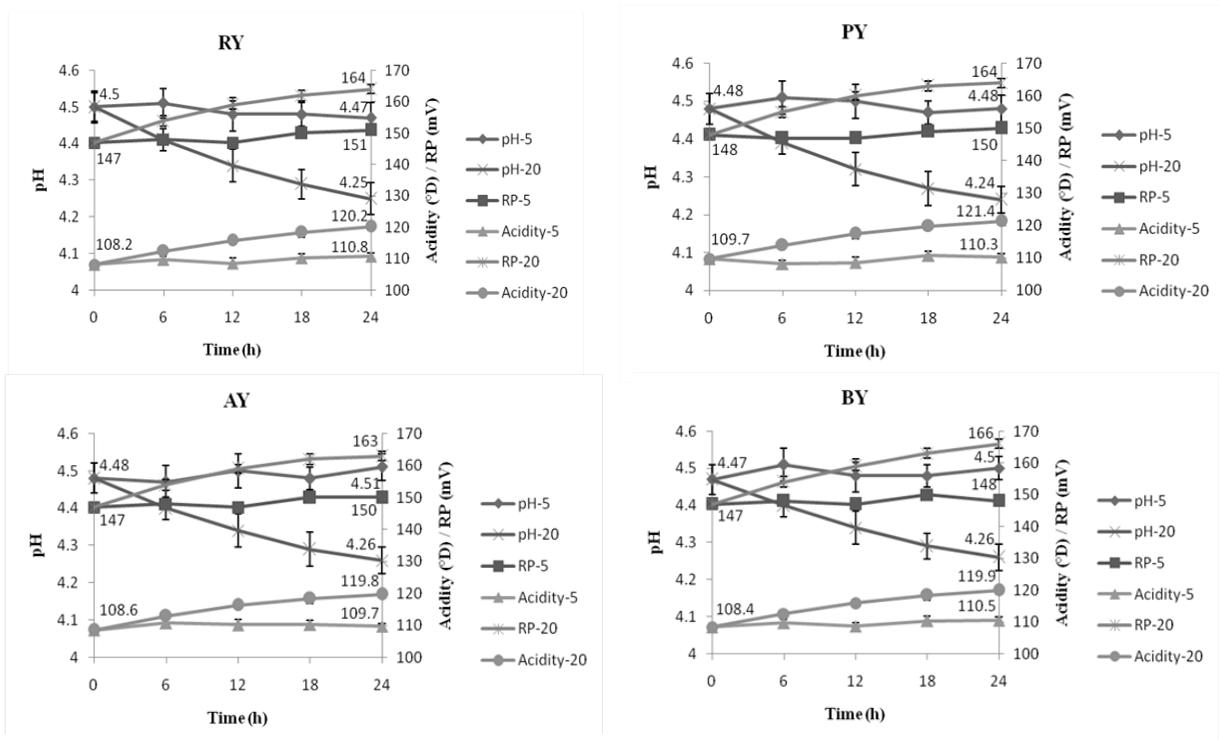


Figure 1. Changes in pH, titratable acidity and redox potential in treatments during storage (5 or 20°C). RY = *L. rhamnosus*, PY = *L. paracasei*, AY = *L. acidophilus*, BY = *B. Lactis*. The numbers '5' and '20' represent the storage temperature.

Considerable loss in viability of probiotics in room temperature could be attributed to increasing cell metabolism and death at higher temperatures (compared to refrigerated storage) as well as to the enhanced antagonistic impact of yogurt bacteria (especially *L. delbrueckii* ssp. *bulgaricus*) on probiotic bacteria. Yogurt bacteria can suppress probiotics during yogurt storage via 'post-acidification' process (28) which is noticeably intensified in temperatures of more than 5°C. Within aforementioned process, not only increasing titratable acidity and decreasing pH but also formation of some metabolites such as hydrogen peroxide, short-chain fatty acids and bacteriocins are highly detrimental to probiotic cells (5). In all treatments exposed to room temperature, an increase in titratable acidity to 120.2°D during storage is the main evidence for post-acidification. Mortazavian *et al.* (29) reported that storage of ABY-type fermented milks at temperatures of more than 5°C (8°C) led to a domination of *L. delbrueckii* ssp. *bulgaricus* and excessive post-acidification by this organism.

Considering Table 2, the rate of viability loss for each probiotic strain increased appreciably toward the end of storage time (hours 0, 6, 12 and 18 in VPI_{6, 12, 18 and 24} respectively; *e.g.*, 0.93, 0.90, 0.87 and 0.81 for RY-20). The viable counts of probiotics were decreased by < 1 log cycle in all treatments (Table 1) and the count of > log 7 cfu mL⁻¹ (minimum recommended level) in the treatments stored at room temperature was only observed for *L. rhamnosus* at the end of storage (24 h). *B. lactis* had the poorest viability and was able to maintain its survival higher than log 7 cfu mL⁻¹ only for 12 h (room temperature). This time for *L. acidophilus* and *L. paracasei* was 18 h. Therefore, *L. rhamnosus* HN001 was the most suitable probiotic strain to use in probiotic yogurts especially in countries having high possibility of cold chain interruption during storage (after industrial dispatching) or in those that refrigeration facilities are absent.

Conclusion

This study demonstrated that the survival of

Table 1. Viable counts (log cfu mL⁻¹) of probiotic bacteria in different treatments during storage time*.

Treatments	Storage time (h)				
	0	6	12	18	24
RY-5**	7.24 ^a	7.24 ^a	7.24 ^a	7.24 ^a	7.23 ^a
RY-20	7.24 ^a	7.21 ^{ab}	7.17 ^b	7.11 ^b	7.02 ^c
PY-5	7.20 ^a	7.19 ^a	7.20 ^a	7.19 ^a	7.19 ^a
PY-20	7.20 ^a	7.16 ^{ab}	7.11 ^b	7.02 ^c	6.90 ^d
AY-5	7.28 ^a	7.29 ^a	7.29 ^a	7.28 ^a	7.27 ^a
AY-20	7.28 ^a	7.22 ^a	7.13 ^b	7.03 ^c	6.88 ^d
BY-5	7.18 ^a	7.18 ^a	7.17 ^a	7.17 ^a	7.16 ^a
BY-20	7.18 ^a	7.11 ^b	7.00 ^c	6.84 ^d	6.63 ^e

* Means in the same row with different letters are significantly different ($p < 0.05$). ** Treatments stored at room temperature include: RY-20 = yogurts containing *L. rhamnosus*; PY-20 = yogurts containing *L. paracasei*; AY-20 = yogurts containing *L. acidophilus*; BY-20 = yogurts containing *B. Lactis*.

probiotic bacteria in commercially available yogurts is critically dependent on the conditions of how the products are stored. The survival of probiotics was strongly dependent on the storage temperature and remarkable viability loss occurred in room temperature. In addition, the decline in viability was dependent on the strain of probiotic. Among our experimental strains, *B. lactis* Bb-12 showed the less resistance to be stored at 20°C (24 h) and referring to the recommended minimum numbers of 10⁷ cfu mL⁻¹, *L. rhamnosus* HN001 was the most suitable probiotic strain to be used in probiotic yogurts especially in countries having high possibility of cold chain interruption during storage (after industrial dispatching) or in

those that refrigeration facilities are absent. However, the viability of *L. rhamnosus* HN001 for over 24 h storage of probiotic yogurt, utilization of other probiotic strains and effect of microencapsulation of probiotics on their viability during storage at room temperature should be investigated.

Acknowledgment

We are grateful to the Department of Food Science and Technology, National Nutrition and Food Technology Research Institute (Shahid Beheshti University of Medical Sciences) for support of this study. This paper has been resulted from the Student M.Sc. thesis.

Table 2. Viability proportion index (VPI) in different treatments during storage time (compared to the initial viable cell counts immediately after fermentation or the viable cell counts at the last hours of each 6-h storage interval).

Treatments	VPI ₆		VPI ₁₂		VPI ₁₈		VPI ₂₄	
	h 0	h 6	h 0	h 6	h 0	h 12	h 0	h 18
RY-5*	1.00	1.00	1.00	1.00	1.00	1.00	0.97	0.97
RY-20	0.93	0.85	0.90	0.74	0.87	0.60	0.81	0.81
PY-5	0.97	1.00	1.02	0.97	0.97	0.97	0.97	1.00
PY-20	0.91	0.81	0.88	0.65	0.81	0.50	0.76	0.76
AY-5	1.02	1.02	1.00	1.00	0.98	0.97	0.97	0.97
AY-20	0.86	0.70	0.81	0.56	0.79	0.39	0.70	0.70
BY-5	1.00	0.97	0.97	0.97	1.00	0.95	0.98	0.98
BY-20	0.84	0.66	0.78	0.45	0.69	0.27	0.60	0.60

* Treatments stored at room temperature: RY-20 = yogurts containing *L. rhamnosus*; PY-20 = yogurts containing *L. paracasei*; AY-20 = yogurts containing *L. acidophilus*; BY-20 = yogurts containing *B. Lactis*.

References

- (1) Baluchnejadmojarad T and Roghani M. Chronic Oral Epigallocatechin-gallate Alleviates Streptozotocin-induced Diabetic Neuropathic Hyperalgesia in Rat: Involvement of Oxidative Stress. *Iranian J. Pharm Res.* (2012) 11: 1243-1253.
- (2) Ghiassi Tarzi B, Gharachorloo M, Baharinia M and Mortazavi SA. The Effect of ermination on Phenolic Content and Antioxidant Activity of Chickpea. *Iranian J. Pharm Res.* (2012) 11:1237-1243.
- (3) Menrad M, Hüusing B, Menrad K, Reib T, Beer-Borst S and Zenger CA. Functional Food, TA 37/2000. Bern: Schweizerischer Wissenschafts und Technologierat (2000).
- (4) Dave RI, and Shah NP. Viability of yogurt and probiotic bacteria in yogurts made from commercial starter cultures. *Int. Dairy J.* (1997) 7: 31-41.
- (5) Korbekandi H, Mortazavian AM and Iravani S. Technology and stability of probiotic in fermented milks. In Shah NP (eds). *Probiotic and Prebiotic Foods: Technology, Stability and Benefits to the human health*, ed. Nova Science Publishing Ltd, USA, (2011) 121-146.
- (6) Tamime AY, Saarela M, Korslund sondergaard Mistry AV and Shah NP. Production and maintenance of viability of probiotic microorganisms in dairy products. In Tamime AY (ed). *Probiotic Dairy Products*, Blackwell Publishing Ltd, UK (2005) 39-72.
- (7) Agrawal R. Probiotics: An emerging food supplement with health benefits. *Food Biotechnol.* (2009) 19: 227-246.
- (8) Erkkilä S and Petäjä E. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Sci.* (2000) 55: 297-300.
- (9) Fuller R. Probiotics in man and animal. *J. Appl. Bacteriol.* (1989) 66: 365-378.
- (10) Jahreis G, Vogelsang H, Kiessling G, Schubert, R, Bunte C and Hammes WP. Influence of probiotic sausage (*Lactobacillus paracasei*) on blood lipids and immunological parameters of healthy volunteers. *Food Res. Int.* (2002) 35: 133-138.
- (11) Mogensen G. Realities and trends in probiotic attributes of lactic acid bacteria and their market impact. In *Lactic acid bacteria - Lactic*, Caen: Presses universitaires de Caen. (1995) 175-185.
- (12) O'Brien J, Crittenden R, Ouwehand AC and Salminen S. Safety evaluation of probiotics, *Trends Food Sci. Technol.* (1999) 10: 418-424.
- (13) Ouwehand AC, Kirjavainen PV, Shortt C and Salminen S. Probiotics: mechanisms and established effects, *Int. Dairy J.* (1999) 9: 43-52.
- (14) S. Salminen, M. Deighton, Y. Benno and S. Gorbach, Lactic acid bacteria in health and disease. In Salminen S. and vonWright (eds). *Lactic Acid Bacteria*, New York, (1998) 211-253.
- (15) Shah NP. Functional cultures and health benefits. *Int. Dairy J.* (2007) 17: 1262-1277.
- (16) Krutman J. Pre- and probiotics for human skin. *J. Dermatol. Sci* (2009) 54: 1-5.
- (17) Leyer GJ, Li S, Mubshaer ME, Reifer C and Ouwehand AC. Probiotics effects on cold and influenza-like incidence and duration in children. *Pediatrics.* (2009) 124: 172-178.
- (18) Mortazavian AM, Khosrokhavar R, Rastegar H and Mortazaei GR. Effects of dry matter standardization order on biochemical and microbiological characteristics of freshly made probiotic Doogh (Iranian fermented milk drink). *Ital. J. Food Sci.* (2010) 22: 98-104.
- (19) Lourens-Hattingh A and Viljoen CB. Yoghurt as a probiotic carrier food. *Int. Dairy J.* (2001) 11:1-17.
- (20) Kurman JA and Rasic JL. The health potential of products containing bifidobacteria. In Robinson RK (ed). *Therapeutic properties of fermented milks*. Elsevier Applied Food Sciences, London, UK, (1991) 117-158.
- (21) Ishibashi N and Shimamura S. Bifidobacteria: research and development in Japan. *Food Technol.* (1993) 47: 126-135.
- (22) Mohammadi R and Mortazavian AM. Review Article: Technological Aspects of Prebiotics in Probiotic Fermented Milks. *Food Rev. Int.* (2011) 27: 192-212.
- (23) Ahmadi E, Mortazavian AM, Fazeli MR, Ezzatpanah H and Mohammadi R. The effects of inoculant variables on the physicochemical and organoleptic properties of Doogh. *Int. J. Dairy Technol.* (2012) In press.
- (24) Mortazavian AM, Ehsani MR, Azizi A, Razavi SH, Mousavi SM and Sohrabvandi S. Viability of calcium alginate-microencapsulated probiotic bacteria in Iranian yogurt drink (Doogh) during the refrigerated storage period and under the simulated gastrointestinal conditions. *Aust. J. Dairy Tech.* (2008) 63:24-29.
- (25) Scharl M, Geisel S, Vavricka SR and Rogler G. Dying in Yoghurt: The Number of Living Bacteria in Probiotic Yoghurt Decreases under Exposure to Room Temperature. *Digestion.* (2011) 83:13-17.
- (26) Mortazavian AM, Khosrokhavar R and Rastgar H. Effects of dry matter standardization order on biochemical and microbiological characteristics of freshly made probiotic Doogh. *Ital. J. Food Sci.* (2010) 22:98-104.
- (27) Mortazavian AM, Ghorbanipour S, Mohammadifar MA and Mohammadi M. Biochemical properties and viable probiotic population of yogurt at different bacterial inoculation rates and incubation temperatures. *Philipp. Agric. Sci* (2011) 94: 111-116.
- (28) Shafiee G, Mortazavian AM, Mohammadifar MA, Koushki MR, Mohammadi AR and Mohammadi R. Combined effects of dry matter content, incubation temperature and final pH of fermentation on biochemical and microbiological characteristics of probiotic fermented milk. *Afr. J. Agr. Res.* (2010) 4: 1265-1274.
- (29) Mortazavian AM, Ehsani MR, Mousavi SM, Rezaei K, Sohrabvandi S and Reinheimer JA. Effect of refrigerated storage temperature on the viability of probiotic micro-organisms in yogurt. *Int. J. Dairy Technol.* (2007) 60: 123-127.