Combined effects of temperature-related variables on the viability of probiotic micro-organisms in yogurt

The authors

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Abstract

Combined effects of temperature-related variables including heat treatment, incubation temperature and refrigerated storage temperature on the viability of probiotic micro-organisms in ABY-type probiotic yogurt (*Lactobacillus acidophilus*, bifidobacteria and yogurt bacteria) were studied. Three heat treatments (85°C for 30 min, 95°C for 5 min and 95°C for 15 min), three incubation temperatures (37°C, 40°C and 44°C) and three refrigerated storage temperatures (2°C, 5°C and 8°C) were selected. Interactive effects of heat treatment and incubation temperature on the viability of probiotics were considered at the end of fermentation. Then, the highest viability of probiotics was investigated as a function of refrigerated storage temperature during refrigerated storage for 20 days. The viability change of the probiotic micro-organisms was analysed at five-day intervals throughout the refrigerated storage period. The final objective was to find the best combination of (heat treatment/incubation temperature/refrigerated storage temperature) for the viabilities of the probiotics. At the end of fermentation, the maximum viability of probiotics (for both *L. acidophilus* and bifidobacteria) was observed when the yogurt milk was heated at 95°C for 15 min and incubated at 37°C. Storage at 2°C for 20 days resulted in the highest viability of *L. acidophilus*, while for bifidobacteria the highest viability was obtained when yogurt was stored at 8°C.

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Introduction

Probiotics can be defined as live micro-organisms that bring health benefits to the host mainly by maintaining and/or improving the microbial balance of the intestine medium (Fuller 1989; Gismondo *et al.* 1999; Holzaspfel and Schillinger 2001; Shah 2001). Several health benefits have been attributed to probiotics including antimutagenic and anticarcinogenic effects, immune system stimulation or immunomodulation, anti-infection properties, reduction in serum cholestrol, alleviation of lactose maldigestion and nutritional enhancement (Saarela *et al.* 2000; Sanders 1999; Shah 2001). The species of *Lactobacillus* and *Bifidobacterium* are the most important probiotics used in probiotic products. Today, many products of this kind are available for consumption by humans, farm animals and pets (Hoier 1992; Holzaspfel and Schillinger 2001; Sanders 1999 and Shah 2001).

Probiotic products can be divided into unfermented and fermented (including yogurt) types. Among dairy fermented products, yogurt is the most popular, and in Europe, the highest consumption of probiotic products is associated with probiotic yogurt (Lourens-Hattingh and Viljoen 2001). Therefore, producing probiotic yogurt with optimum viability (as the critical value of the product) and suitable sensory properties is very important. The viability of probiotic micro-organisms in the final product until the point of consumption, which has been proposed to be specified by the parameter of minimum biovalue (MBV); i.e. the minimum count of viable probiotic cells per g or mL of probiotic product (Mortazavian and Sohrabvandi 2006a), is the most important parameter of probiotic products, since this determines their pharmaceutical effectiveness. Many kinds of probiotic yogurt with various types of cultures are being produced world-wide. However, loss of viability of probiotics in yogurt during fermentation and refrigerated storage is a major issue in the production of probiotic yogurt. Temperature-related variables (heat treatment, incubation temperature and refrigerated storage temperature) are important factors that can have significant effects on the viability of probiotic cultures in yogurt. Although the effect of each such variable on the viability of probiotics in fermented milks has been the subject of various studies (Bolin *et al.* 1998; Centeno-de-Lara 1987; Fernandez 1995; Han-Seung *et al.* 2000; Nighswonger *et al.* 1996; Schilliger 1999; Shah et al. 1995; Singh 1983 and Vinderola *et al.* 2000), to the best of authors' knowledge, only a few have been carried out with ABY-culture (as the most current and popular culture). Also, the combined effects of temperature-related variables on the viability of probiotic micro-organisms have not been investigated. The main objective of this work was to study the interactive effects of these variables relevant to the yogurt mix and freshly made yogurt on the viability of probiotic micro-organisms made using ABY culture.

Materials and methods

Starter culture

Fifty-unit pouches of commercial lyophilised ABY culture (containing *L. acidophilus*, *Bifidobacterium lactis* BB-12, *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus*) known as 'FD-DVS ABY-1' were supplied by Chr. Hansen (Horsholm, Denmark). This culture is currently used by

the dairy industry to produce yogurt. The cultures were maintained at -18°C according to manufacturer's instructions until used.

Culture media for enumeration

MRS-bile agar medium (MRS agar by Merck, Darmstadp, Germany and bile by Sigma-Aldrich, Inc., Reyde, USA) was used for the selective enumeration of *L. acidophilus* in the ABY culture according to Vinderola and Reinheimer (1999). The plates were incubated aerobically at 37°C for three days. Bifidobacteria were enumerated by the same medium using the 'subtractive enumeration method' (SEM) proposed by Mortazavian et al. (2006b) based on the subtraction of the colonies grown under aerobic conditions (only *L. acidophilus*) from the colonies grown under anaerobic conditions (both *L. acidophilus* and bifidobacteria). The plates were incubated at 37°C for three days under anaerobic conditions using the GasPac system (Merck, Darmstadp, Germany).

Experimental design

The study was performed in two stages: investigation of the combined effects of heat treatment and incubation temperature; and refrigerated storage temperature, respectively. For each experimental unit, according to Chr. Hansen's recommended procedure, a 50-unit pouch of FD-DVS ABY-1 starter culture was dispersed in 1 L sterilised milk and then 12 mL of this inoculum was inoculated into 3 L of reconstituted skimmed milk that had been heat-treated (85°C for 30 min, 95°C for 15 min or 95°C for 5 min) and cooled to the fermentation temperature (37°C, 40°C or 44°C). The incubation time was measured when the pH reached 4.50 ± 0.02. At the end of the fermentation period, samples were cooled and kept at 5°C until the probiotic organisms were enumerated. At the end of first stage, as mentioned above, the best combination of heat treatment conditions and incubation temperature was determined. In order to accomplish the second stage, samples prepared by this best combination (heat treatment of 95°C/15 min and incubation at 37°C) were cooled and stored at different refrigeration temperatures (2°C, 5°C or 8°C) for 20 days and their viable probiotic cell population enumerated at five-day intervals throughout the refrigerated storage period.

Statistical analysis

Experiments were performed in triplicate and the interactive effects of the variables and ranked orders of means were analysed using Factorial and Duncan's tests (on the basis of complete randomised design) using MSTATC software (Pussell D. Freed, Crop and Soil Science Department, Michigan State University, Version 2.10).

Results and discussion

Effects of heat treatment and incubation temperature on the viability of probiotics

Single effects of heat treatment and incubation temperature

The effects of heat treatment on the viability of L. acidophilus and bifidobacteria are shown in Table 1. There were no significant differences ($p<0.05$) between the treatments of 85°C/30 min and 95°C/5 min on the viabilities of both *L. acidophilus* and bifidobacteria, whereas the heat treatment of 95°C for 15 min was statistically different from the other two heat treatments. This can be attributed to the more appropriate elimination of competitive micro-organisms in the milk base and improving the nutritional quality of milk due to the liberation of small peptides and free amino acids (Tamime and Robinson 1999). In the case of bifidobacteria, proper heating of the milk base has further advantages, including effective omission of dissolved oxygen, which has an injurious effect on bifidobacteria, and a reduction in the redox potential due to both depletion of molecular oxygen and liberation of sulphur-containing amino acids. Reduction in dissolved oxygen and redox potential significantly enhance the growth of bifidobacteria (Dave and Shah 1997a, b).

The incubation temperature also had a significant effect ($p<0.05$) on the viability of both probiotics (Table 1). The highest and lowest viable counts were obtained at 37°C and 44°C, respectively. These results were similar to those reported by Gomes and Malcata (1999) and Kneifel *et al.* (1993): These researchers reported that the optimum growth temperature of probiotics is approximately 37°C. Furthermore, higher temperatures enhance the antagonistic effect of *Lactobacillus delbrueckii ssp. bulgaricus* against probiotic micro-organisms when grown at 45°C (Varnam and Sutherland 1999) because this bacterium becomes the dominant species in yogurt and therefore produces a large amount of acid, hydrogen peroxide and, possibly, bacteriocins, resulting in the suppression of the probiotics. In the ABY culture, for example, the loss of viability of *L. acidophilus* has been reported to be mainly due to the hydrogen peroxide produced by *L. delbrueckii ssp. bulgaricus* (Dave and Shah 1997a; Shah *et al.* 1995). Furthermore, the optimum growth pH of L. acidophilus is in the range 5.5-6.0 (Gomes and Malcata 1999). Therefore, a rapid drop of pH below such a level due to the fast growth of *L. delbrueckii ssp. bulgaricus* leads to the lower growth rate of *L. acidophilus* and, as a result, lowers the viable count of such organisms after the fermentation period. Bifidobacteria are also sensitive to pH variations and their growth is restricted at pH<5.0 (Shah 1997; Gomes and Malcata 1999). As a consequence, higher fermentation temperatures can favour the growth of *L. delbrueckii ssp.*

Table 1: Individual effects of heat treatment (a) and incubation temperature (b) on the viable counts (log cfu/mL) of probiotics.

Probiotics	(a) Heat treatment (°C/min)			(b) Incubation temperature (°C)		
	95/15	95/5	85/30	37	40	44
L. acidophilus	7.29[a]	7.15[b]	7.09[b]	7.39[a]	7.23[b]	6.67[c]
bifidobacteria	7.39[a]	7.31[b]	7.25[b]	7.50[a]	7.31[b]	7.04[c]

** The means shown with different letters are significantly different (p<0.05).*

Table 2: Combined effects of heat treatment and incubation temperature on the viable counts of probiotics.

Heat treatment (°C/min)	Incubation temperature (°C)	Viable counts of *L. acidophilus* (log cfu/mL)	Viable counts of bifidobacteria (log cfu/mL)
85/30	37	7.34[b]	7.49[a]
	40	7.10[c]	7.27[c]
	44	6.478[d]	6.63[d]
95/5	37	7.36[b]	7.53[b]
	40	7.13[c]	7.27[c]
	44	6.778[d]	7.13[c]
95/15	37	7.47[a]	7.53[a]
	40	7.39[ab]	7.39[b]
	44	6.723[d]	7.18[c]

** The means shown with different letters are significantly different ($p<0.05$).*

Bulgaricus, which becomes the dominant organism. As a result, the sharp acidification will lead to suppression of the growth of bifidobacteria (i.e. lower viable counts after the fermentation period). It seems evident that by fermenting the yogurt mix at 37°C, apart from this being the optimum growth temperature of the probiotic micro-organisms, the synergistic effects between them and *L. delbrueckii ssp. bulgaricus* during fermentation are more prominent than antagonistic effects. The synergistic effects could be attributed to the partial digestion of casein to long-chain peptides by the yogurt Lactobacilli, followed by digestion of these components to small peptides and free amino acids by *L. acidophilus* and *Streptococcus thermophilus* (Gomes *et al.* 1998; Ishibashi and Shimamura 1993; Klaver *et al.* 1993; Tamime and Robinson 1999).

Combined effects of heat treatment and incubation temperature

The combined effects of heat treatment and incubation temperature of the milk base (yogurt mix) on the viability of probiotics are shown in Table 2. The highest viability for both probiotics was obtained when the milk base was heated at 95°C for 15 min and incubated at 37°C. As mentioned above, heat treatment at 95°C for 15 min (Table 1) results in the most nutritional enhancement of the milk base, due to the liberation of small peptides and free amino acids. Subsequently, by using lower incubation temperatures, i.e. around the optimum growth temperature of probiotic micro-organisms, nutrients are mainly used by these micro-organisms rather than by the yogurt bacteria. On the other hand, higher incubation temperatures (42-45°C) may lead to the consumption of these components by yogurt bacteria (Varnam and Sutherland 1999). This explains why heating at 95°C for 30 min, 95°C for 5 min or 95°C for 15 min, and incubating at 44°C results in the lowest viable counts of the probiotic micro-organisms compared with the highest counts when yogurt milk is incubated at 37°C.

Effects of refrigerated storage temperature on the viability of probiotics

Effects of refrigerated storage temperature on the viability of *L. acidophilus*

Table 3 shows the trend in the microbial cell counts at five-day intervals during the refrigerated storage period. After five days, storage at 2°C resulted in a significantly higher viability of *L. acidophilus* compared to storage at 5°C and 8°C. This can be explained by the antagonistic effects of *L. delbrueckii ssp. bulgaricus* against *L. acidophilus* (and also other probiotic bacteria), especially at higher temperatures, as mentioned above. At the higher storage temperatures (5°C and 8°C), *L. delbrueckii ssp. bulgaricus* grows faster; therefore, the amounts of lactic acid and hydrogen peroxide produced are increased. Besides, in general, with an increase in storage temperature and as a result, increased metabolic activities of bacterial cells, their death rate is increased. As shown in Table 3, storing yogurt at 8°C for 10 days resulted in the lowest viability of *L. acidophilus* compared to storage at 2°C or 5°C.

Tables 4 and 5 show the cumulative and stepwise loss per five-day interval, respectively, in the viability of *L. acidophilus* during 20 days of refrigerated storage. The maximum loss of viability for 5°C and 8°C are observed after five days of refrigerated storage; but for 2°C it is observed after 10 days. At 5°C and 8°C, higher activity of L. delbrueckii ssp. bulgaricus led to considerable loss of *L. acidophilus* viability after five days of storage. In addition to the gradual loss of *L. delbrueckii ssp. bulgaricus* activity and possibly the relatively greater adaptation of *L. acidophilus* to detrimental environmental conditions, viability loss did not increase. By storing yogurt at 2°C, all the phenomena discussed occur at a slower rate and, as a result, the maximum loss of viability appears at a later storage time (>10 days).

After 15 days of refrigerated storage, yogurt with the highest

Table 3: Variation in the viable cell counts of probiotics (*L. acidophilus* and bifidobacteria) during refrigerated storage (log cfu/mL).*

Probiotics	Storage temperature (°C)	Storage period (days)				
		0**	5	10	15	20
L. acidophilus	2	7.41	7.28[a]	7.04[a]	6.60[a]	6.47[a]
	5	7.41	7.14[b]	7.03[a]	6.27[b]	5.44[c]
	8	7.41	7.13[b]	6.77[b]	6.23[b]	5.77[b]
bifidobacteria	2	7.53	7.41[a]	6.87[b]	6.38[b]	5.80[b]
	5	7.53	7.42[a]	6.97[b]	6.15[c]	5.65[b]
	8	7.53	7.44[a]	7.17[a]	6.62[a]	6.15[a]

** The means shown with different letters are significantly different ($p<0.05$).*
*** 0 days = immediately after fermentation.*

Table 4: Loss in viability of *L. acidophilus* and bifidobacteria during refrigerated storage (compared to the initial viable cell counts immediately after fermentation).

Probiotics	Storage temperature (°C)	Storage period (days)			
		5	10	15	20
L. acidophilus	2	25.0%	57.7%	84.6%	88.5%
	5	46.2%	57.7%	92.7%	98.9%
	8	48.1%	88.4%	92.7	97.7%
bifidobacteria	2	24.6%	78.3%	92.7%	98.1%
	5	23.2%	72.5%	95.6%	98.7%
	8	18.9%	56.5%	86.9%	95.6%

viability of L. acidophilus was observed at 2°C compared with 5°C and 8°C (Table 3). The reason for this is similar to that mentioned for the first five days of storage.

According to Table 5, except for the 8°C data, a minimum increase in the loss of viability was observed after 20 days of refrigerated storage. This phenomenon can be attributed to the reduction in the activity of *L. delbrueckii ssp. bulgaricus* and the adaptation of *L. acidophilus* to the harsh conditions of the product. After 20 days of refrigerated storage, the highest, intermediate and lowest viabilities were obtained at 2°C, 8°C and 5°C, respectively (Table 3). The reason for higher viability after storage at 8°C, compared to that of 5°C, could not be explained. Therefore, among these three temperatures, 2°C can be considered the best temperature to maintain the viability of *L. acidophilus* during storage for20 days.

Effect of refrigerated storage temperature on the viability of bifidobacteria

Table 4 shows the trend in the microbial cell counts of bifidobacteria during the refrigerated storage determined at five-day intervals. There was no significant difference between the trends at 2°C, 5°C and 8°C at five days' storage. This can be attributed to the relatively high tolerance of the strain of bifidobacteria used in this starter culture against the stress factors such as molecular oxygen, acidity, hydrogen peroxide and antagonistic effects of *L. delbrueckii ssp. bulgaricus* in comparison with other species/strains of bifidobacteria. Therefore, the adverse effects of the environmental conditions on the bifidobacteria strain used must not have been severe enough to significantly affect viability of the cells at different refrigeration temperatures.

Table 5 shows the stepwise loss of viability of bifidobacteria per five-day interval during 20 days of refrigerated storage. The maximum increase in the loss of viability for all the refrigeration temperatures was observed at 10 days. This can be associated with a sharp loss of viability of bifidobacteria at five days of storage due to the decrease in its tolerance against the stress factors mentioned earlier. Table 3 shows that for bifidobacteria, storage at 8°C resulted in the highest viability compared to storage at 2°C and 5°C over the 20 days of storage. The reason that storage at 8°C resulted in the highest viability may be due to the synergistic relationships between bifidobacteria, *L. acidophilus* and *L. delbrueckii ssp. bulgaricus* that are enhanced at higher storage temperatures as previously mentioned. Storage temperatures of 2°C and 5°C did not show significant differences in the viability of both probiotics.

According to Table 5, at 10 days of refrigerated storage, the highest increase in loss of viability was observed at 2°C, while at 15 days the highest increase was associated with storage at 8°C. This observation could not be explained.

The minimum increase in viability loss for all the refrigeration temperatures was observed at 20 days' refrigerated storage. This is possibly related to the adaptation of those bifidobacteria that survived the unfavourable environmental conditions and also the decrease in the antagonistic effect of *L. delbrueckii ssp. bulgaricus* due to the reduction of their activity. As a result, among the storage temperatures of 2°C, 5°C and 8°C, to obtain the highest viability of bifidobacteria for a storage period of around 20 days, a storage temperature of 8°C is recommended.

Conclusions

The results of this study demonstrated that all three temperature-related variables in yogurt processing (i.e. heat treatment, incubation temperature and refrigerated storage temperature) significantly affected the viability of probiotic organisms in yogurt. Heat treatment and the incubation temperature of the yogurt mix (milk base) showed interactive effects on the viability of both

Table 5: Stepwise increase in loss of viability of *L. acidophilus* and bifidobacteria per five-day interval during refrigerated storage.

Probiotics	Refrigeration temperature (°C)	Storage period (days)			
		5	10	15	20
L. acidophilus	2	25.00%	32.7%	27.3%	3.5%
	5	46.2%	11.5%	35.0%	6.2%
	8	48.1%	40.4%	3.8%	5.4%
bifidobacteria	2	24.6%	53.6%	14.5%	5.4%
	5	23.2%	49.3%	23.2%	3.1%
	8	18.9%	37.7%	30.4%	8.7%

L. acidophilus and bifidobacteria in ABY-type culture and heat treatment of the milk base at 95°C for 15 min and incubation at 37°C led to the highest viability of both probiotic organisms after fermentation period. A combined treatment at 95°C for 15 min and an incubation temperature of 37°C is recommended for the production of ABY-type probiotic yogurt in order to achieve the highest viable counts in the product at the end of the fermentation period. Subsequent storage at 2°C led to the highest viability of *L. acidophilus* throughout the 20 days of refrigerated storage, whereas the highest viability for bifidobacteria throughout the 20 days of storage was observed when yogurt was kept at 8°C.

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