ORIGINAL RESEARCH

# Effect of refrigerated storage temperature on the viability of probiotic micro-organisms in yogurt

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*The effect of refrigerated storage temperature was studied at 2, 5 and 8°C on the viability of probiotics in ABY (* Lactobacillus acidophilus, Bifidobacterium lactis *BB-12 and yogurt bacteria.* Bulgaricus, *i.e.* Streptococcus thermophilus *and* Lactobacillus delbrueckii *ssp. Bulgaricus) probiotic yogurt. The study was carried out during a 20-day refrigerated storage period to identify the best storage temperature(s). Also, the viability change of the probiotic micro-organisms was analysed at 5-day intervals throughout the refrigerated storage period. After 20 days, storage at 2°C resulted in the highest viability of* L. acidophilus, *whereas for* Bifidobacterium lactis *the highest viability was obtained when yogurt was stored at 8°C.*

**Keywords** Bifidobacterium lactis, *L. acidophilus*, Probiotic, Refrigeration temperature, Viability, Yogurt.

## INTRODUCTION

As a general definition, probiotics can be described as live micro-organisms (bacteria or yeast) that bring health benefits to their host (human or animal) mainly by maintaining and/or improving the microbial balance of the intestinal medium (Fuller 1989; Gismondo *et al*. 1999; Holzaspfel and Schillinger 2001; Shah 2001). Several health benefits have been attributed to probiotics such as antimutagenic and anticarcinogenic effects, immune system stimulation or immunomodulation, anti-infection properties, reduction of serum cholesterol, alleviation of lactose intolerance/lactose maldigestion and nutritional enhancement (Sanders 1999; Saarela *et al*. 2000; Shah 2001). The species of lactobacilli and *bifidobacteria* are by far the most important probiotics used in probiotic products. Nowadays, many probiotic products are available for the consumption of humans, farm animals and pets (Hoier 1992; Sanders 1999; Shah 2001; Holzaspfel and Schillinger 2001).

Probiotic products can be divided into non-fermented and fermented. Probiotic yogurts are categorized in the second group. As yogurt is the most popular fermented milk product, producing probiotic yogurt with optimum viability (as the critical value of the product) and suitable sensory, as well as economic properties is very important. The viability of probiotic micro-organisms in the final product until the moment of consumption has been proposed with the descriptor minimum of biovalue (MBV). This is the minimum of viable probiotic cells per gram or millilitre of probiotic product (Mortazavian and Sohrabvandi 2006a), and is the most important qualitative parameter of probiotic products as it determines their pharmaceutical effectiveness. Many kinds of probiotic yogurt with various types of culture composition are being produced worldwide. However, loss of viability of probiotics during the fermentation process and refrigerated storage is a major issue in the production of probiotic yogurt. Although the effect of cold storage on the viability of probiotics in fermented milks has been the subject of various studies (Centeno-de-Laro 1987; Shah *et al*. 1995; Nighswonger *et al*. 1996; Bolin *et al*. 1998; Schilliger 1999; Han-Seung *et al*. 2000; Vinderola *et al*. 2000), to the best of the authors' knowledge, only a few have been carried out with the ABY culture. Also, in none of them the viability change of probiotic micro-organisms has been investigated throughout the refrigerated storage, as a function of different refrigeration temperatures. In our previous related study (Mortazavian *et al*. 2006b), the best conditions of two heat variables (heat treatment and incubation temperature) on the viability of probiotics in ABY probiotic yogurt were identified. Following the mentioned study, the effect of refrigerated storage temperature, as the third temperature variable, was investigated on the viability of probiotics in ABY (*L. acidophilus*, *B. lactis*, and *Streptococcus*

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*thermophilus* and *Lactobacillus delbrueckii* spp. *Bulgaricus*) probiotic yogurt in order to determine the viability change of probiotics during a 20-day refrigerated storage and identify the best refrigeration temperature(s).

## MATERIALS AND METHODS

### Starter culture

Fifty-unit pouches of commercial lyophilized ABY culture (containing *L. acidophilus*, *Bifidobacterium BB-12* and *S. thermophilus* and *L. delbrueckii* spp. *Bulgaricus*) that are known as 'FD-DVS ABY-1' were supplied by Chr-Hansen company (Horsholm, Denmark). This culture is currently used by the dairy industry to produce yogurt. The cultures were maintained according to the manufacturer's instructions at −18°C.

### Culture media for enumeration

MRS-bile agar medium (MRS agar: Merck, Darmstadt, Germany and bile: Sigma–Aldrich, Inc., Reyde, USA) was used for the selective enumeration of *L. acidophilus* and *B. lactis* in the ABY culture composition according to Mortazavian *et al.* (2006c), by applying the subtractive enumeration method (SEM). The plates were incubated at 37°C for 3 days under aerobiosis and anaerobiosis. Anaerobiotic conditions were produced using the GasPac system (Merck, Darmstadt, Germany).

### Yogurt preparation and viability analysis

For each experimental stage, according to Chr-Hansen's recommended procedure, a 50-unit pouch of FD-DVS ABY-1 starter culture was dissolved in 1 L sterilized milk and then 12.0 mL of this inoculum was inoculated into 3 L of reconstituted heat-treated (95°C for 15 min) milk that had been cooled down to the fermentation temperature (37°C). The incubation was then carried out up to pH 4.50 ± 0.02. At the end of the fermentation stage, samples were cooled down and stored at different refrigeration temperatures (2, 5 or 8°C) for 20 days and their viable probiotic cell populations were enumerated throughout the refrigerated storage period, at 5-day intervals.

### Statistical analysis

Experiments were performed in triplicate and ranked orders of the means were analysed using Duncan's test (on the basis of complete randomized design) from MSTAT software (Pussell D. Freed, Crop and Soil Science Department, Michigan State University, Version 2.10).

## RESULTS AND DISCUSSION

### The effect of refrigerated storage temperature on the viability of *L. acidophilus*

Table 1 shows the variation in the microbial cell count during the refrigerated storage period at 5-day intervals. After 5 days, storage at 2°C resulted in significantly higher viability of *L. acidophilus* compared to the conditions of 5 and 8°C. This can be explained by the fact of one-way antagonistic effects of *L. delbruecki* ssp. *bulgaricus* against *L. acidophilus* (and also other probiotic bacteria), especially at higher temperatures (Shah *et al*. 1994; Dave and Shah 1997; Mortazavian and Sohrabvandi 2006a; Mortazavian *et al*. 2006b). At higher temperatures (5 and 8°C compared to 2°C) the *L. delbruecki* ssp. *bulgaricus* bacteria grow faster, therefore the amounts of produced lactic acid and hydrogen peroxide are increased. According to Dave and Shah (1996), hydrogen peroxide produced by *L. delbruecki* ssp. *bulgaricus* bacteria is the most important viability-reducing factor during refrigerated storage, which is in agreement with our results. Besides, in general, the increase in storage temperature resulted in the increase in metabolic activities of bacterial cells, thereby causing an increase in their death rate. As shown in Table 1, storing yogurt at 8°C for 10 days resulted in the lowest viability of *L. acidophilus* compared to that stored at 2 or 5°C.

**Table 1** Variation trend in the viable cell counts of probiotics (*Lactobacillus acidophilus* and *Bifidobacterium lactis*) during 20 days of refrigerated storage period, at 5-day intervals (log CFU/mL)*

| | | *Storage period (days)* | | | | |
|---|---|---|---|---|---|---|
| *Probiotics* | *Refrigeration temperature (°C)* | *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] |
| *B. lactis* | 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 2** Viability loss of *Lactobacillus acidophilus* and *Bifidobacterium lactis* during 20 days of refrigerated storage period, at 5-day intervals (compared to the reference point of initial viable cell counts after fermentation)

| *Probiotics* | *Refrigeration 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% |
| *B. lactis* | 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% |

Table 2 shows the variation trend of viability loss percentage and its stepwise increase for *L. acidophilus* and *B. lactis*, respectively. As shown in the Table, the maximum of viability loss percentage for 5 and 8°C is observed after 5 days of refrigerated storage; but for 2°C, it is observed after 10 days of storage. At 5 and 8°C, higher activity of *L. delbruecki* ssp. *bulgaricus* bacteria leads to considerable loss of *L. acidophilus* viability after 5 days of storage. Then, along with gradual loss of *L. delbruecki* ssp. *bulgaricus* activity and possibly relative rise in adaptation of *L. acidophilus* to detrimental environmental conditions, viability loss percentage is not increased as much as before. By storing yogurt at 2°C, all the mentioned phenomena occur at a slower rate and as a result, the maximum of viability loss percentage appears at a latter storage time (> 10 days).

After 15 days of refrigerated storage, yogurt had the highest viability of *L. acidophilus* was recognized at 2°C compared to the two other temperatures (Table 1). The reason for such phenomenon is similar to what was mentioned for the first 5 days of storage.

According to Table 2 except for the temperature of 8°C, a minimum increase in the viability loss percentage is observed after 20 days of refrigerated storage. This phenomenon can be attributed to the reduction in the activity of *L. delbruecki* ssp. *bulgaricus* bacteria and rising the adaptation of *L. acidophilus* to the harsh conditions of the product. After 20 days of refrigerated storage, the highest, intermediate and the lowest viabilities were obtained at 2, 8 and 5°C, respectively (Table 1). We could not think of any reason to explain why storage at 8°C led to a higher viability compared to that of 5°C. Therefore, among these three temperatures, 2°C can be selected as the best temperature to maintain the viability of *L. acidophilus* for a refrigerated storage of around 20 days.

## Effect of refrigerated storage temperature on the viability of *B. lactis*

Table 1 shows the variation trend in the microbial cell counts of *B. lactis* during the refrigerated storage period at 5-day intervals. There is no significant difference between the refrigeration temperatures of 2, 5 and 8°C after 5 days of refrigerated storage. This may be because of the relatively high tolerance of the strain of *B. lactis* used in this starter culture against the stress factors such as molecular oxygen, acidity, hydrogen peroxide and antagonistic effects of *L. delbruecki* ssp. *bulgaricus* bacteria in comparison with other species/strains of *bifidobacteria*. Therefore, the adverse effects of environmental conditions on the *B. lactis* strain used must not have been severe enough to significantly affect the viability of the cells at different refrigeration temperatures.

Table 3 shows the percentage viability loss of *B. lactis* and its stepwise increase, respectively. According to the Table, maximum increase in

**Table 3** Stepwise increase in viability loss of *Lactobacillus acidophilus* and *Bifidobacterium lactis* during 20 days of refrigerated storage period, at 5-day intervals

| *Probiotics* | *Refrigeration temperature (°C)* | *Storage period (days)* 5 | 10 | 15 | 20 |
|---|---|---|---|---|---|
| *L. acidophilus* | 2 | 25.0% | 32.7% | 27.3% | 3.5% |
| | 5 | 46.2% | 11.5% | 35.0% | 6.2% |
| | 8 | 48.1% | 40.4% | 3.8% | 5.4% |
| *B. lactis* | 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% |

 

viability loss percentage for all the refrigeration temperatures is observed after 10 days of refrigerated storage. This phenomenon can be associated with the sharp viability loss of *B. lactis* after 5 days of storage due to the decrease of its tolerance of the factors mentioned earlier. Table 1 shows that for *B. lactis*, storage at 8°C resulted in the highest viability compared with that of storage at 2 and 5°C over the 20-day storage. The reason that storage at 8°C in comparison with 5°C results in the higher viability can possibly be due to the early synergistic relationships between *B. lactis*, *L. acidophilus* and *L. delbruecki* ssp. *bulgaricus* that are enhanced at higher storage temperatures as previously reported by Gomes *et al*. (1998), Ishibashi and Shimamura (1993), and Klaver *et al*. (1993). Refrigeration temperatures of 2 and 5°C did not show significant differences in the viability of both probiotics.

According to Table 3, at 10 days of refrigerated storage, the highest increase in viability loss is observed at the refrigeration temperature of 2°C, whereas after 15 days of storage, the highest increase occurs at a storage temperature of 8°C. We could not think of any feasible reason for this observation.

The minimum increase in viability loss for all the refrigeration temperatures was observed after 20 days of refrigerated storage. This is possibly related to the adaptation of those *B. lactis* cells that survived the adverse environmental conditions; and also to the decrease in the antagonistic effect of *L. delbruecki* ssp. *bulgaricus* bacteria due to the reduction of their activity. In conclusion, of the storage temperatures of 2, 5 and 8°C used, obtaining the highest viability of *B. lactis* for a storage period of around 20 days requires a storage temperature of 8°C.

## CONCLUSIONS

The main purpose of the present study was to observe the effect of different refrigerated storage temperatures on the viability change of *L. acidophilus* and *B. lactis* in ABY probiotic yogurt during 20 days of refrigerated storage, in order to identify the best refrigeration temperatures. The results demonstrated that a refrigeration temperature of 2°C led to the highest viability of *L. acidophilus* throughout the 20 days of refrigerated storage, whereas the highest viability for *B. lactis* throughout the 20 days of storage time occurred when yogurt was kept at 8°C. In order to select the best refrigeration temperature(s) for both of the probiotics, economic aspects of storage, the number of initial viable cells of both probiotics after fermentation (before refrigerated storage), and the viability loss patterns of probiotics according to Tables 2 and 3 needed to be considered. As viability losses for *L. acidophilus* and *B. lactis* are different from each other and can also vary during different days of storage (5, 10, 15 and 20 days), the best refrigeration temperature from both the viability and economic points of view can be identified by considering expected viable cell counts of each probiotic micro-organism and the average time needed for refrigerated storage of yogurt prior to sale and consumption. Furthermore, the sensory acceptability of yogurts stored at different refrigeration temperatures also needs to be studied.

## ACKNOWLEDGEMENTS

This study was made possible by the collaborative support of Pak Dairy Co. (Tehran, Iran) and University of Tehran. Authors gratefully acknowledge Mr Venling-hilsen (technical sales manager, Chr-Hansen A/S- International Operations Dairy, Denmark) and Ms. Anne Soendergaard (development scientist, Chr-Hansen A/S, Denmark) for their useful and sincere guides and recommendations.

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