ORIGINAL RESEARCH

Preliminary investigation of the combined effect of heat treatment and incubation temperature on the viability of the probiotic micro-organisms in freshly made yogurt

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*The combined effects of heat treatment and incubation temperature were studied on the viability and fermentation time of probiotics in ABY 1 probiotic yogurt (*Lactobacillus acidophilus*, Bifidobacterium spp. and yogurt bacteria). Three levels of heat treatment (85°C for 30 min, 95°C for 5 min and 95°C for 15 min) and three levels of incubation temperature (37, 40 and 44°C) were selected. At the end of fermentation, the maximum viability of probiotics (for both* L. acidophilus *and* Bifidobacterium *spp.) was observed when the milk was heated at 95°C for 15 min and incubated at 37°C. Incubation time was only affected by the incubation temperature and, at 37°C, the longest incubation time of about 6 h was needed to achieve the highest viable counts of* L. acidophilus *and* Bifidobacterium *spp.*

Keywords *Bifidobacterium* spp., Incubation time, *Lactobacillus acidophilus*, Probiotic, Viability, Yogurt.

INTRODUCTION

According to various definitions, 'probiotics' can be defined as live micro-organisms (bacteria and/or yeasts) that can bring health benefits to human/animals body mainly due to the maintenance and improvement of the microbial balance of the intestine environment (Fuller 1989, 1991; Gismondo *et al*. 1999; Holzapfel and Schillinger 2001; Shah 2001). Nowadays, the importance of probiotic products is well understood, and as a result, many products of this kind are available for the consumption of humans, farm animals and pets (Sanders 1999). More than 90 '*Acidophilus–Bifidobacterium*' products are produced worldwide. In Japan alone, over 53 dairy probiotic products are marketed. Furthermore, most of the yogurts produced in the US markets contain *Lactobacillus acidophilus* and in Europe more than 45 dairy plants produce only '*Acidophilus–Bifidobacterium*' products (Helferich and Westhoff 1980; Klupsch 1983; Hoier 1992; Shah 2001).

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 (Lourens-Hattingh and Viljoen 2001). During the manufacture, probiotic fermented dairy products, three main aspects, which have been identified that can affect the quality of the product, are: **1**, loss of viability of probiotic micro-organisms during the fermentation period and refrigerated storage; **2**, relatively long incubation time is required when compared to traditional yogurt; and **3**, unsatisfactory organoleptic properties of the final product. To improve the fermentation time and organoleptic properties of probiotic yogurt, coculturing of traditional yogurt bacteria with probiotic micro-organisms is a common practice and therefore various culture combinations are used, such as BY (*Bifidobacterium* spp. and yogurt bacteria), AY (*L. acidophilus* and yogurt bacteria), ABT (*L. acidophilus*, *Bifidobacterium* spp. and *Streptococcus thermophilus*) and ABY (*L. acidophilus*, *Bifidobacteria* and yogurt bacteria), and are commercially available on the market (Robinson 1990; Hoier 1992; Kurmann *et al*. 1992; Varnam and Sutherland 1994; Tamime *et al*. 1995). However, yogurt produced with mixed cultures including the degree of the heat treatment of the milk base and the incubation temperature used can play major roles in the quality of final product especially on the viability of probiotic micro-organisms (Singh 1983; Fernandez 1995). The objectives of this work were to study the interactive effect of different heat treatments of the milk base and different incubation temperatures on the viability of probiotic micro-organisms in ABY-1 yogurt, and

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also to consider whether there were interactive effects of these variables on the incubation time.

MATERIALS AND METHODS

Starter culture

Fifty unit pouches of commercial lyophilized (i.e. FD-DVS) ABY-1 starter culture (containing *L. acidophilus*, *Bifidobacterium* BB-12 and yogurt bacteria) were supplied by Chr. Hansen Company (Horsholm, Denmark). This culture is widely used by dairy industry to produce probiotic yogurt. The cultures were maintained according to manufacturer's instructions at −18°C until used.

Culture media and media performance

MRS–bile agar medium (MRS agar: Merck, Darmstadp, Germany; bile: Sigma, Reyde, USA) was used for the selective enumeration of *L. acidophilus* in ABY-1 culture as recommended by Vinderola and Reinheimer (1999). The plates were aerobically incubated at 37°C for 3 days. Based on direct microscopic observation (data not shown) that there was no growth of *L. acidophilus* under anaerobic incubation, the same agar medium was also used for the selective enumeration of *Bifidobacterium* spp. The plates were incubated at 37°C for 3 days under anaerobic conditions using a GasPak system (Merck, Darmstadt, Germany). The suitability of the media was tested by plating the serial decimal dilutions of the yogurt samples with Ringer solution and using the pour plate technique.

Yogurt preparation

For each experimental stage and based on the recommendations received from Chr. Hansen procedure, a 50-unit pouch of ABY-1 starter culture was dissolved in 1 l sterilized milk, and then an inoculum of 12.0 ml was used to inoculate 3 l of reconstituted skimmed milk that had been heat-treated (85°C for 30 min, 95°C for 5 min or 95°C for 15 min and cooled to the fermentation temperature (37, 40 or 44°C). The incubation was then carried out and the incubation time was measured when the pH reached 4.5. At the end of the fermentation period, samples were cooled and kept at 5°C until the probitic organisms were enumerated.

Statistical analysis

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

RESULTS AND DISCUSSION

Single effects of heat treatment and incubation temperature on the viability of probiotic micro-organisms

The results for single effects of heat treatment on the viability of *L. acidophilus* and *Bifidobacterium* spp. are shown in Table 1. There are no significant differences ($P > 0.05$) between the treatments at 85°C/30 min and 95°C/5 min on the viabilitie of both *L. acidophilus* and *Bifidobacterium* spp. Whereas the heat treatment of the milk base at 95°C for 15 min is 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 improvement of the nutritional quality of milk due to the liberation of small peptides and free amino acids (Tamime and Robinson 1999). Furthermore, in the case of *Bifidobacteria*, using proper heat treatments has two other advantages: first, more effective omission of dissolved oxygen, which may have an injurious effect on *Bifidobacteria*; and second, more reduction in the redox potential due to the depletion of molecular oxygen and the liberation of sulphur-containing amino acids. Reduction of dissolved oxygen and redox potential enhances the growth of *Bifidobacterium* spp. (Dave and Shah 1996, 1997).

The incubation temperature also had a significant effect ($P < 0.05$) on the viability of both probiotics (Table 1) and as a result the highest viable counts were obtained at 37°C; the lowest count was, however, observed at 44°C. These results were similar to those reported by Gomes and Malcata (1999) and Kneifel *et al*. (1993): all these researchers reported that the optimum growth temperature of probiotics is at approximately 37°C. Furthermore, higher temperatures enhance the antagonistic effect of

Table 1 Single effect of heat treatment (a) and incubation temperature (b) on the viable counts (log cfu/ml) of probiotic micro-organisms

	(a) Heat treatment (°C/min)			*(b) Incubation temperature (°C)*		
Probiotic	95/15	95/5	85/30	37	40	44
Lactobacillus acidophilus	7.29[a]	7.15[b]	7.09[b]	7.39[a]	7.23[b]	6.67[c]
Bifidobacterium spp.	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 effect of heat treatment and incubation temperature on the viable counts of probiotic micro-organisms

Heat treatment (°C/min)	*Incubation temperature (°C)*	*Viable counts of* L. acidophilus *(log cfu/ml)*	*Viable counts of* Bifidobacterium *spp. (log cfu/ml)*
85/30	37	7.3424[b]	7.49[a]
	40	7.1028[c]	7.27[c]
	44	6.4771[d]	6.63[d]
95/5	37	7.3617[b]	7.53[b]
	40	7.1358[c]	7.27[c]
	44	6.7782[d]	7.13[c]
95/15	37	7.4723[a]	7.53[a]
	40	7.3922[ab]	7.39[b]
	44	6.7267[d]	7.18[c]

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

Lactobacillus delbrueckii ssp. *bulgaricus* against probiotic micro-organisms when grown at 45°C (Varnam and Sutherland 1994) because this bacterium becomes the dominant species in yogurt and therefore produces a large amount of acid (sharp acidification), hydrogen peroxide and possibly, bacteriocins, resulting in the suppression of the probiotic micro-organisms. In the ABY-1 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* (Shah *et al*. 1994). Furthermore, the optimum growth pH of *L. acidophilus* is at a range of 5.5–6.0 (Gomes and Malcata 1999). Therefore, a rapid drop of pH below such 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 the level of pH variations and their growth is restricted at pH < 5 (Shah 1997; Gomes and Malcata 1999). As a consequence, higher fermentation temperatures can favour the quick growth of *L. delbrueckii* ssp. *bulgaricus*, which becomes the more 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 milk base at 37°C, apart from being the optimum growth temperature of the probiotic micro-organisms, has the beneficial effect on the synergistic relationship between them and *L. delbrueckii* ssp. *bulgaricus* due to the reduced growth rate of yogurt bacteria. The synergistic effect between the probiotic micro-organisms and *L. delbrueckii* ssp. *bulgaricus* may be due to the partial digestion of casein to long-chain peptides by the yogurt lactobacilli, followed by the digestion of these components to small peptides and free amino acids by *L. acidophilus* and *Streptococcus salivarius* ssp. *thermophilus* (Tamime and Robinson 1999).

Combined effect of heat treatment and incubation temperature on the viability of probiotic micro-organisms

The combined effect of heat treatment and incubation temperature of the milk base on the viability of probiotic micro-organism is shown in Table 2. The highest viability for both probiotic micro-organisms was obtained when the milk base was heated at 95°C for 15 min and incubated at 37°C. As reported in the previous section, heat treatment at 95°C for 15 min (Table 1) results in the most effective nutritional enhancement in the milk base. Subsequently, by using lower incubation temperature*s*, i.e. around the optimum growth temperature of probiotic micro-organisms, nutrients are mainly used by these 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 1994). This explains why using heat treatments at 85°C for 30 min, 95°C for 5 min and 95°C for 15 min, and an incubation temperature of 44°C, result in the lowest viable counts of the probiotic micro-organisms compared with highest counts when grown at 37°C.

Effect of incubation temperature on the incubation time

The results of the effect of incubation time are shown in Table 3. Incubation temperature had a significant effect ($P < 0.05$) on the incubation time. Fermentation at 37°C needed the longest time (approximately 6.17 h), but those at 40 and 44°C needed about 5.26 and 4.39 h, respectively, which was due to the higher activity of the yogurt bacteria. Also, as yogurt bacteria have higher enzymatic activity than probiotic micro-organisms (Varnam and Sutherland 1994), their growth rate is increased at higher incubation temperatures, which results in the reduced fermentation time.

Table 3 Effect of incubation temperature on the incubation time

Incubation temperature (°C)	*Incubation time (h)*
37	6.29[a]
40	5.43[b]
44	4.65[c]

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

CONCLUSIONS

The results of this study demonstrate that heat treatment and incubation temperature of the milk base, i.e. the two variables in yogurt processing, have a noticeable effect on the viability of probiotic micro-organisms in the fresh product. Heat treatment and incubation temperature showed interactive effects on the viability of both *L. acidophilus* and *Bifidobacteria* in ABY-1 starter culture. Thus, heat treatment of the milk base at 95°C for 15 min and incubation at 37°C (single or combined effects) led to the highest viability of both probiotic organisms in the fresh product. However, the incubation time was only affected by the incubation temperature. In conclusion, the combined treatment at 95°C for 5 min and the incubation temperature of 37°C is recommended for the production of ABY-1 probiotic yogurt in order to achieve the highest viability counts in the product. Nevertheless, sensory profiling and rheological measurements and enumeration of the probiotic micro-organisms in the stored products should be studied for a comprehensive selection of the mentioned heat-related and incubation temperatures variables.

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