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Survival of *Lactobacillus acidophilus* and *Bifidobacterium infantis* in yogurts manufactured from cowmilk and soymilk during storage at two temperatures

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Abstract - The survival of two microbial probiotics, *Lactobacillus acidophilus* and *Bifidobacterium infantis*, after inoculation into yogurts manufactured from cowmilk and soymilk during storage for 45 days at 4 and 12 °C was investigated. The sensory panel test carried out before the microbiological analyses showed that the flavour of soy yogurts made with cocoa powder or malt did not have the beany taste of soy beans. The survival of *L. acidophilus* in yogurts was significantly greater than the survival of *B. infantis* during storage at 4 and 12 °C. At 4 °C, the population remained between 10^7-10^8 CFU /ml for the former organism throughout the 45 day experiment. *B. infantis* reached a population of over 10^7 CFU /ml during the first 24 days at both temperatures but then it showed a marked decrease. The survival of *B. infantis* decreased substantially during storage at 12 °C, when no viable cells were found at day 31. In soy yogurts the survival of both probiotics was quite remarkable; *B. infantis* showed a significant increase of survival in plain- and flavoured-yogurts and more than 10^6 CFU/ml were detected in cocoa-flavoured yogurts after 38 days of storage.

Key words: probiotics, yogurt, survival.

INTRODUCTION

The consumption of yogurt and acidophilic milk-products in Italy is still below its potential (D'Amicis, 1996; Bottazzi, 1997). Moreover, the official standard of identity is lacking on important parameters of yogurt such as the expiration time, the date of production, the threshold values of microbial contaminants and the required number of viable lactic bacteria at the time of consumption (Robba, 1985; Vigano', 1996).

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Unclear is also the number of microorganisms required in a dairy product in order to be claimed as a probiotic food. Recently some values were published (Merlo, 1999) referring to the following bacteria: *Lactobacillus acidophilus* NCFB 1748 (3×10^{11} CFU/die), *Lactobacillus johnsonii* LA1 (1×10^{10} CFU/die), *Lactobacillus rhamnosus* GG ($10^9 – 10^{10}$ CFU/*die*).

Throughout the World, yogurt has got a primary position among dairy foods, primarily for women, children, and teenagers. In North Europe this is particularly true: individual consumption per year is as high as 18 kg in The Netherlands and Germany. In Italy, despite a significant increase of 1.4% during the last 10 years, only 3.6 kg are consumed (Bottazzi, 1997; Hekmat and McMahon, 1997). Besides the development of new marketing strategies, the dairy industry has recently focused on the production of new products, containing probiotic microorganisms, based on fresh milk, fermented milk, soft cheese and ice-cream (Biavati *et al*., 1992a; Bottazzi, 1993, 1997; Sanders *et al*., 1996 and 1999; Stefanelli, 1992; Vesa *et al*., 1996).

Yogurts are usually considered a selective environment for the growth of some contaminating microorganisms but, despite the low pH and the presence of probiotic bacteria, the spoilage of yogurts by fungi and yeasts has been widely described in the literature (Canganella *et al*., 1992; Deak, 1991; Rasic and Kurmann, 1978; Suriyarachchi and Fleet, 1981). Moreover, the introduction of fruit, sugar, and additional flavours into yogurts has largely expanded its marketing potential but the increased risk of spoilage, due to additional fermentable substrates, may diminish the beneficial effects of lactic acid bacteria (Bernet *et al*., 1994; Jin *et al*., 1996; Link-Amster *et al*., 1994; Link *et al*., 1995; Rafter, 1995). Further addition to yogurt of selected probiotics besides *Lactobacillus delbrueckii* and *Streptococcus thermophilus*, before or after fermentation, may reduce the risk of spoilage and also enhance the commercial value of the product (Bottazzi, 1997; Stefanelli, 1992; Vigano', 1996).

In Europe, soymilk products are becoming common alternatives to cowmilk products. In the last decades, the beneficial effects of soy-based products in the prevention and treatment of chronic deseases were recognized. In addition, further benefits have been demonstrated with respect to osteoporosis and kidney diseases (Lonnerdal, 1994; Lusas and Rhee, 1995; Messina, 1995). Thus, due to larger numbers of people with vegetarian attitudes, the consumption of soymilk has increased considerably.

The range of soy-based products is still very limited, mostly because many consumers do not like the characteristic "beany" flavour; nevertheless, it is highly probable that, thanks to their chemical and nutritional features, flavoured soymilk and novel soy-containing foods will become more popular in the future. A significant improvement in the marketing of soy-foods could certainly develop from the optimization of industrial processing and the introduction of products with similar organoleptic and nutritional traits to cowmilk (Lusas and Rhee, 1995; Wilson, 1995).

Marketing of probiotic dairy products has increased worldwide and in Italy novel products were introduced (Bottazzi, 1993, 1997). The microbial species utilized for these applications are usually *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, *Bifidobacterium bifidum*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* and *Bifidobacterium infantis* (Bottazzi 1993, 1997; Macedo *et al*., 1999).

In this study, we evaluated the persistance of *Lactobacillus acidophilus* and *Bifidobacterium infantis* in yogurts manufactured with cowmilk and soymilk, during storage at two different temperatures for 45 days. Both species are valuable probiotic microorganisms as demonstrated by numerous studies which focused on their therapeutic effects or the prevention against the potential invasion of undesired microorganisms and alterations of intestinal microflora (Bernet *et al*., 1994; Link *et al*., 1995; Link-Amster *et al*., 1994; Rafter, 1995; Sanders *et al*., 1996; Vesa *et al*., 1996).

MATERIALS AND METHODS

Yogurt production. Yogurt samples were manufactured in the laboratory of the Microbiology Unit of the Department of Agrobiology and Agrochemistry, University of Tuscia. For the production of yogurts, UHT full-fat cowmilk (Scalda Sole, Italy) and soymilk (Valsoia, Italy) were used. The inoculum was composed by three strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and two strains of *Streptococcus thermophilus* isolated from commercially available plain yogurts (Scalda Sole and Danone) and identified by the API system (bioMérieux, Italy). No characterization of such strains was carried out before inoculum because our purpose was just to use common marketed cultures and not to investigate the physiological properties (production of α and β-galactosidase, etc.) of these isolates.

The mixed starter culture was pregrown in either cowmilk or soymilk at 37 °C before inoculation.

In order to evaluate the optimal conditions for the yogurt manufacture, a sensory analysis was conducted using 10 untrained voluntary evaluators prior to the microbiological work. These volunteers were given a set of yogurt samples in 50 ml Falcon tubes and the scoring system reported by Rasic and Kurmann (1978) was used.

The following yogurts were prepared: cowmilk yogurts (natural and raspberry flavours), soymilk yogurts (natural and cocoa flavours). Pasteurized raspberry cream was added as 2 g per 50 ml cowmilk (in Falcon tubes). Cocoa and malt powders were diluted (100 g/l) in pasteurized cowmilk and soymilk, respectively, before the addition of sugar (50 g/l), whereas black coffee was diluted (200 ml/l) in either cowmilk or soymilk with sugar (50 g/l). After the starter culture was added, the milk was dispensed in 50 ml Falcon tubes and incubated at 42 °C for 4-6 hours. The initial pH was 4.5.

Inoculation of yogurt. *Lactobacillus acidophilus* and *Bifidobacterium infantis* strains were chosen from our culture collection and used to inoculate the yogurt samples. After cultivation for 48 h in Peptone Tryptone Glucose (PTG) medium, cell pellets were collected by centrifugation at 7000 rpm (4 °C) for 30 min and resuspended in 50 ml sterile cowmilk or soymilk. PTG agar was composed of (g/l): tryptone, 10.0; phytone, 5.0; glucose, 15.0; yeast extract, 2.5; K_2HPO_4, 1.5; $MgCl_2$, 0.5; cysteine-HCl, 0.5; final pH 6.8.

Probiotic cultures were then inoculated (5% vol/vol) with a sterile pipette into 50 ml Falcon tubes and mixed vigorously before sealing. Two yogurts for each group were then analyzed to determine the yogurt microflora and the inoculated

probiotics at time zero (t_0). All other samples were divided into two lots; one stored at 4 °C and the other stored at 12 °C.

Two samples were used for each set of yogurts and for each temperature/flavour; all graphs represent the arithmetic average of microbial counts.

Microbiological analyses. Analyses were carried out every 3-4 days (first day indicated as t_0) for a total storage time of 45 days. From each sample, 1 ml was aseptically withdrawn, dispensed into 1 g/l sterile peptone solution and serially diluted for plating. Aliquots were plated using the appropriate microbiological media.

Microbiological media. The enumeration of *L. delbrueckii* subsp. *bulgaricus, S. thermophilus, L. acidophilus* and *B. infantis* in the yogurt was carried out after anaerobic incubation at 37 °C for 48 h using the following media: MRS agar (Biolife Italiana, Milano, Italy) and PTG agar for lactobacilli and bifidobacteria, and M17 agar (Biolife) for lactic streptococci.

After incubation, colonies and cells of bifidobacteria on MRS agar and PTG agar were morphologically distinguished using a microscope, and the same was done to distinghish *L. delbrueckii* and *L. acidophilus* on MRS agar. Raffinose Bifidobacterium (RB) agar was also used for the elective cultivation and enumeration of *B. infantis* after anaerobic incubation at 37 °C for 48 h (Hartemink and Rombouts, 1999).

Preparation of liquid media and plating was carried out following the standard microbiological procedures. Anaerobic incubations were performed in a glove box (COY Scientific, Grass Lake, MI, USA), flushed with N_2 (95%) and H_2 (5%).

pH measurement. The pH of each sample and liquid cultures was determined by a Hanna Instruments (Ronchi di Villafranca, Italy) pH meter, model 8417.

RESULTS

According to panel tests carried out with yogurts manufactured from cowmilk and soymilk, the raspberry flavour was more acceptable than the malt flavour. The panel test of the yogurts manufactured with soymilk was designed to determine which component would overcome the soymilk beany taste. The addition of sweetened cocoa gave a better flavoured soymilk yogurt than either coffee or malt flavours. For these reasons, malt and coffee flavours were not chosen for further experiments.

The pH values of yogurt samples slightly increased during the whole experiments, from 4.5 to 5.0-5.5.

Figures show the survival of experimentally inoculated probiotic microorganisms as well as *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in cowmilk and soymilk yogurts.

Viable counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, between 10^7-10^8 CFU/ml were detected at 4 °C (Fig. 1a, 2a, 3a, 4a). A remarkable decrease of viability was observed at 12 °C for *S. thermophilus* after day 25 in plain- and in raspberry-flavoured yogurt (Fig. 1b, 2b), showing a 100% mor-

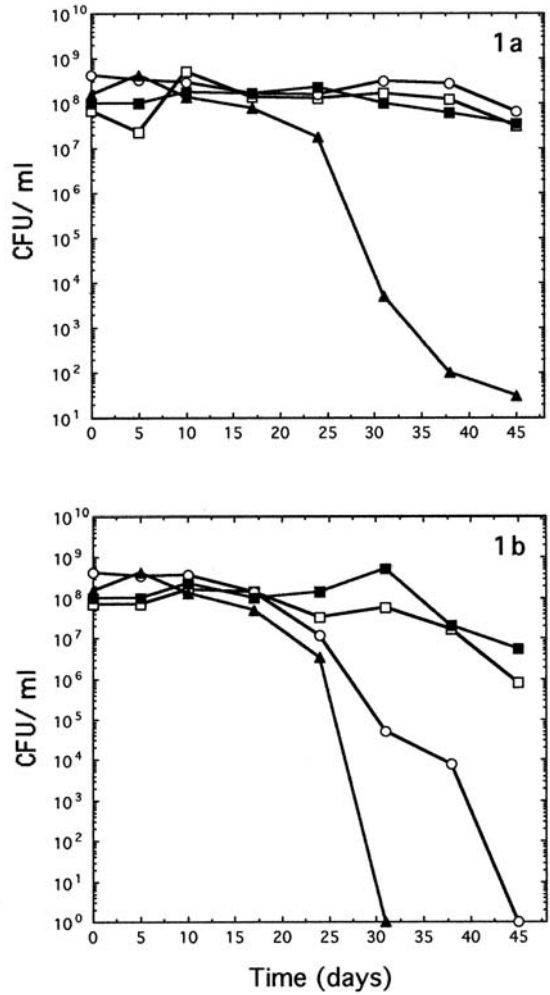

FIG. 1 – Microbiological counts of lactic bacteria and probiotic bacteria in plain cowmilk yogurts stored at 4 °C (**1a**) and 12 °C (**1b**). - ❑ - *L. delbrueckii* subsp. *bulgaricus*; - ❍ - *S. thermophilus*; - ■ - *L. acidophilus*; - ▲ - *B. infantis*.

tality at the end of trial (day 45), whereas a much higher survival was exhibited in soymilk yogurt (Fig. 3b, 4b) at the same temperature.

In plain cowmilk yogurts, *L. acidophilus* showed an elevated tolerance to the storage at 4 °C. Microbial counts remained very stable with values around 10^8 CFU/ml throughout the whole trial (Fig. 1a). Under the same conditions, counts of *B. infantis* decreased significantly after storage for 24 days, and a population of 2×10^1 CFU/ml was detected at the end of the experiment. The survival of both

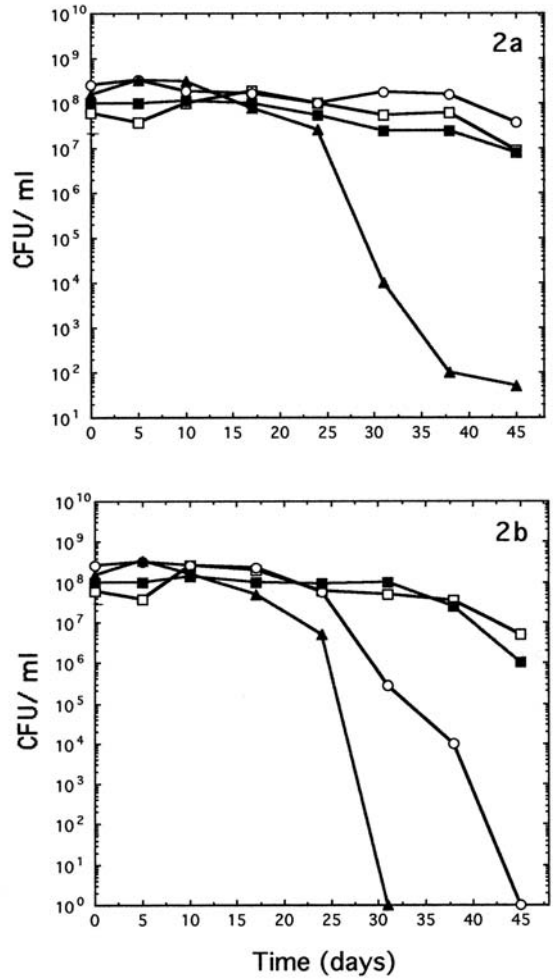

FIG. 2 – Microbiological counts of lactic bacteria and probiotic bacteria in raspberry-flavoured cowmilk yogurts stored at 4 °C (**2a**) and 12 °C (**2b**). - ❑ - *L. delbrueckii* subsp. *bulgaricus*; - ❍ - *S. thermophilus*; - ■ - *L. acidophilus*; - ▲ - *B. infantis*.

probiotics was affected when samples were stored at 12 °C, but more dramatically only for *B. infantis* (Fig. 1b). In this case, many viable cells were still observed at day 25 but after 31 days of storage no survivors were detected. The mortality of *L. acidophilus* was slightly enhanced at 12 °C compared to 4 °C and viable counts decreased to values around 10^7 CFU/ml during the last 5 days of storage.

As far as regard the experiments carried out with raspberry-flavoured yogurts (Fig. 2a,2b), differences were also found with regard to the survival of *B. infantis*

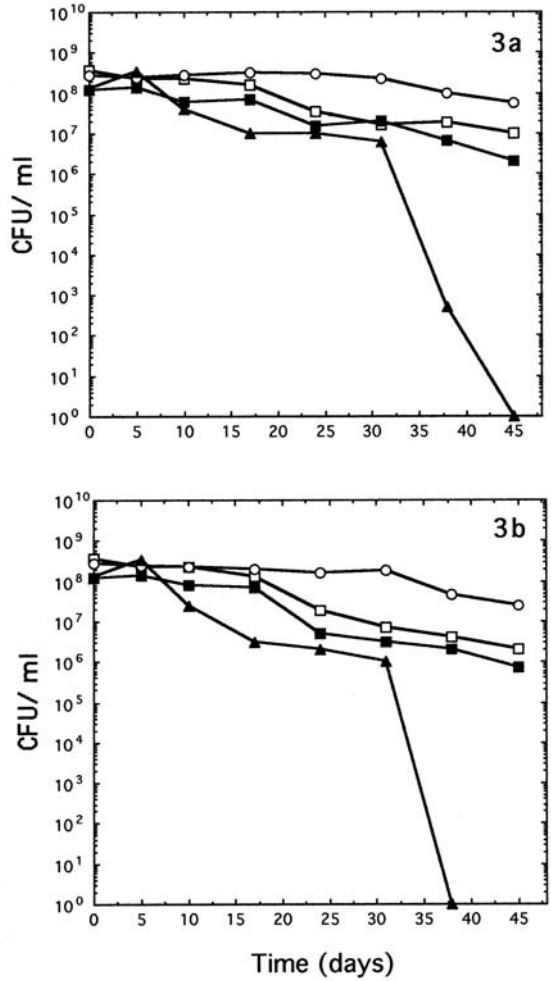

FIG. 3 – Microbiological counts of lactic bacteria and probiotic bacteria in plain soymilk yogurts stored at 4 °C (**3a**) and 12 °C (**3b**). - ❑ - *L. delbrueckii* subsp. *bulgaricus*; - ❍ - *S. thermophilus*; - ■ - *L. acidophilus*; - ▲ - *B. infantis*.

and *L. acidophilus* at 4 and 12 °C. *L. acidophilus* was affected by the flavouring of yogurts with the raspberry puree at 12 °C (Fig. 2b), when the cell mortality increased after day 31 and around $3x10^6$ CFU/ml were obtained at the end of experiment. The survival of *B. infantis* at 12 °C showed the same pattern observed in plain yogurt with a drastic enhancement of mortality after day 24.

When the trial was carried out with yogurts manufactured with soymilk, the tolerance of experimentally inoculated probiotic strains to long-term storage was

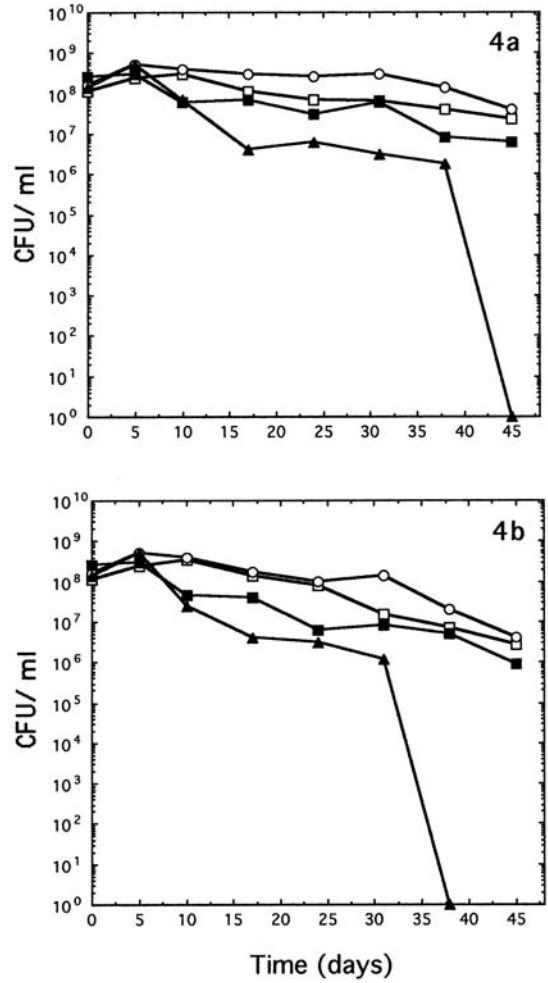

FIG. 4 – Microbiological counts of lactic bacteria and probiotic bacteria in cocoa-flavoured soymilk yogurts stored at 4 °C (**4a**) and 12 °C (**4b**). - ❑ - *L. delbrueckii* subsp. *bulgaricus*; - ❍ - *S. thermophilus*; - ■ - *L. acidophilus*; - ▲ - *B. infantis*.

still remarkable (Fig. 3, 4) and the survival of *B. infantis* seemed to enhance by the use of soymilk. At day 31, counts higher than 10^6 CFU/ml were detected regardless of the temperature of storage. Moreover, further improvement in the viability of *B. infantis* was observed during the storage of cocoa-flavoured yogurts at 4^o C when $3x10^6$ CFU/ml were detectable at day 38.

DISCUSSION

In Italy an increasing number of "novel" dairy products are being marketed, especially fresh preparations containing viable probiotic microorganisms. The benefits of probiotics for human health such as the production of lactic acid, antimicrobial compounds and the detoxification of noxious compounds are well known (Bottazzi, 1993; Fuller, 1986; Hammes and Tichaczek, 1994). Recently, the work of investigators focused on the promotion of local antigen-specific immune responses (Majamaa and Isolauri, 1997) and antimutagenic efficiency caused by whole cells or acidic metabolites (Lankaputhra and Shah, 1998). The addition of probiotic bacteria to yogurts before or after fermentation may be highly significant for human health due to a more effective action on the intestinal microflora, and to the higher acid tolerance of strains (Stefanelli, 1992). Moreover, novel fermented milk products may be introduced in the market by the use of animal protein substitutes like soybeans, cereals and legumes (Lonnerdal, 1994; Morales de Leon *et al.*, 1988; Nizami *et al*., 1996). The nutritional characteristics of soymilk, along with relatively simple and inexpensive production processes, make this aqueous suspension of great value as a source of proteins, vitamins and goitrogenic substances (Snyder and Kwon, 1987; Wilson, 1995; Xu *et al*., 1994). The potential role of soymilk in the western World as well as in developing countries has been stated (Lusas and Rhee, 1995).

According to the organoleptic test performed in this study, sweetened cocoa and soluble malt gave the best flavours for the preparation of soymilk yogurts. After the addition of *L. acidophilus* and *B. infantis* to yogurt samples, the storage at different temperatures for 45 days showed that the former organism was more resistant to the long-term storage, particularly in cowmilk yogurt at 12 °C. In this case 10^7 CFU/ml of *L. acidophilus* were observed at the end of storage in plain yogurts, whereas a slight decrease of viable cells was observed in yogurt samples supplemented with the raspberry puree. The viability of *B. infantis* was enhanced in soymilk yogurts regardless of the storage temperature. Counts over 10^6 CFU/ml were always obtained through 31 days and survival was particularly stimulated in cocoa-flavoured yogurts during storage at 4 °C.

We found particularly significant the higher survival of *S. thermophilus* observed in soymilk yogurts compared to cowmilk yogurts. The beneficial effect of soymilk on the viability of *S. thermophilus* was more remarkable during storage at 12 °C, whereas at 4 °C no significant improvement was detected.

In a previous investigation on the survival of undesirable microorganisms in fruit yogurts (Canganella *et al*., 1998), *L. delbrueckii* subsp. *bulgaricus* survived at 8 °C less than *S. thermophilus*. However, the cultures used in that study were physiologically and taxonomically different from that used in the present study.

The tolerance of strains to the acidic pH environment may represent an important parameter for the survival of probiotic microorganisms in dairy products but, as demonstrated by several authors, the survival of bifidobacteria in fermented and unfermented milk at low storage temperature is lower than that of *L. acidophilus* (Biavati *et al*., 1992b; Hughes and Hoover, 1995).

These results suggest that soymilk-based foods, particularly yogurts are suitable for the addition of probiotic microorganisms. Moreover, the survival of these organisms in such products may be higher than in cowmilk-derived products, providing significant benefits for both the manufacturer and the consumer.

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