International Food Research Journal 15(2): 219-232 (2008)

Survival of Free and Microencapsulated Probiotic Bacteria in Orange and Apple Juices

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Abstract: This study investigated the survival of free and microencapsulated probiotic bacteria in orange and apple juices. Eight different strains of probiotic bacteria were used in this study including *Lactobacillus rhamnosus*, *Bifidobacterium longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. paracasei*, *B. lactis* type Bi-04 and *B. lactis* type Bi-07. The free or microencapsulated probiotic bacteria were inoculated into orange and apple juices and their viability was assessed on a weekly basis for up to six weeks. °Brix, malic acid concentration, and pH were also monitored. Encapsulated probiotc bacteria survived in fruit juices throughout the six weeks of storage, whereas free probiotic bacteria lost their viability within five weeks. In general, fruit juices containing microencapsulated probiotic bacteria were more stable than those containing free probiotic organisms.

Keywords: Microencapsulation, probiotic bacteria, fruit juice

INTRODUCTION

Fruit juices may be an alternative vehicle for the incorporation of probiotics because they are rich in nutrients and do not contain starter cultures that compete for nutrients with probiotics. Furthermore, fruit juices are often supplemented with oxygen scavenging ingredients such as ascorbic acid, thus promoting anaerobic conditions. Fruit juices contain high amounts of sugars which could encourage probiotic growth and could easily be monitored using a refractometer. The food industry refers to foods supplemented with probiotics as 'functional foods'. Several health benefits associated with the consumption of live probiotic bacteria have been reported (Mital and Garg, 1995; Bolognani *et al.,* 1997; Gomes and Malcata, 1999). These benefits include controlling intestinal infections, improving lactose utilization, and lowering blood ammonia levels (Molder, 1990; Pool-Zobel *et al.,* 1993; Ebringer *et al.,* 1995). More recently researchers have found that probiotic bacteria can beneficially influence the immune system, and lower serum cholesterol levels (Ouwehand *et al.,* 2002; Liong and Shah, 2005). Due to the wide range of therapeutic benefits there has been an increase in the incorporation of probiotic bacteria in a wider variety of food products including yoghurts, cheese, drinks and dietary supplements (Lourens-Hattingh and Viljoen, 2001; Ong *et al.,* 2006).

A daily consumption of high levels of probiotic bacteria is required to confer health benefits. It is important that the organisms remain viable in the food product until the time of consumption and be present in significant numbers (at least 10^7 cfu/mL) in order to confer benefits to the consumer (Ishibashiand Shimamura, 1993). Despite the importance of viability of these beneficial bacteria, studies conducted have shown poor viability of probiotic bacteria, especially *Bifidobacterium,* in functional foods (Shah and Lankaputhra, 1997; Kailasapathy and Rybka, 1997; Schillinger, 1999; Talwalkar and Kailasapathy, 2004). It would be interesting to study the changes in the number of viable probiotic bacteria during storage of functional foods more extensively. Several factors have been claimed to affect the viability of probiotic bacteria in functional foods, including acid and hydrogen peroxide produced by starter cultures, high oxygen content and oxygen permeability through the packaging materials (Shah and Jelen, 1990; Shah and Lankaputhra, 1997).

Protection of probiotics by microencapsulation in alginate capsules is a method of improving their viability in functional foods. Alginate is often used as an encapsulating material because it has the benefits of being non-toxic and being readily available. Microencapsulation using the emulsion method is a gentle process that does not stress or harm the bacterial cells and can be applied easily

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to large volumes (Sultana *et al.,* 2000; Capela *et al.,* 2004).

The primary objective of this study was to determine the survival of probiotic bacteria in orange and apple juices during six weeks of storage in order to assess the suitability of a fruit juice as a new alternative functional food. The secondary objective was to assess microencapsulation techniques for improving the survival of probiotic bacteria in fruit juices. The physical changes to the fruit juice before and after the addition of probiotic organisms were also monitored by assessing changes in pH, °Brix, and organic acid content.

MATERIALS AND METHODS

Bacterial Strains and Their Inoculation

L. rhamnosus, *B. longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. paracasei*, *B. lactis* type Bi-04 and *B. lactis* type Bi-07 were obtained from Danisco (Copenhagen, Denmark). The identity of all probiotic bacteria was confirmed using biochemical methods as described by Shah and Lankaputhra (1997). The probiotic organisms were grown individually in MRS broth (Oxoid Ltd., Hampshire, United Kingdom) at 37°C using a 1% inoculum. MRS broths for growing *Bifidobacterium* spp were supplemented with filter sterilized 0.05% (w/v) L-cysteine hydrochloride (Sigma Chemical Co., Castle Hill, Sydney, Australia) in order to create an anaerobic environment. The probiotic organisms were activated by growing three times successively at 37°C for 18 h. Before inoculation into fruit juices, the bacterial cells were washed in sterile saline to remove any residual MRS.

Harvesting of Probiotic Cultures Before Inoculation

The eight probiotic organisms were grown separately in 500 mL of MRS broths for 18 h at 37°C to allow the cells to reach early log phase. To ensure a similar concentration of cells were used for both free and microencapsulated experiments, the cells were harvested with the same method. In brief, probiotic bacteria were concentrated by centrifuging at 15,000 x g for 30 min at 4°C for both free cells and cells to be microencapsulated. Before the probiotic cells were added to fruit juices or prior to encapsulation, they were washed with 25 mL of sterile phosphate buffered saline (PBS, pH 7.0). To ensure that approximately the same concentration of cells was used for each strain tested. The washed and concentrated cells were enumerated using the pour plate method on MRS agar prior to use. Microencapsulation was carried out as described below.

Microencapsulation of Probiotics

Probiotic organisms were microencapsulated using a modified method of Ravula and Shah (2000). Briefly, 100 mL of sterile 3% v/w sodium alginate (D3247 AJAX Chemicals Ltd., Melbourne, Australia) was mixed with 25 mL of washed probiotic organisms. The alginate and bacteria suspension was slowly dispensed using a pipette into a beaker containing 600 mL of vegetable oil and 1 g of Tween 80. This emulsion was mixed thoroughly at 200 rpm, with a magnetic stirrer. A solution of calcium chloride (0.01 M) was gently added to the side of the beaker until the emulsion was broken. After 30 min, the calcium alginate beads were removed from the aqueous phase and were refrigerated at 4°C for 10 h to allow the beads to fully harden.

Selection of Fruit Juice

Commercially available orange and apple juices (National Foods Limited, Melbourne, Australia) were used for all experiments. Fruit juices with no added preservatives and a long shelf life were obtained. Both the orange and apple juices were stored at 4°C overnight before inoculation.

Enumeration of Bacteria

The enumeration of free probiotic cells was performed using methods described by Shah and Lankaputhra (1997). In brief, for the enumeration of microencapsulated probiotic organisms, the bacteria were released from the capsules by sequestering calcium ions with a phosphate buffer at pH 7.0. Once liberated, the probiotic organisms were enumerated using the methods of Shah and Lankaputhra (1997) and Tharmaraj and Shah (2003). Enumeration of the probiotic bacteria in fruit juices was performed on a weekly basis over a period of 6 weeks, using MRS agar and incubation at 37°C for 72 h under anaerobic conditions.

pH and °Brix Measurements

The pH and °Brix were measured at weekly intervals in probiotic juices. A refractometer (Atago Bellevue,Washington, USA) was used to measure the Brix. A pH meter (Hanna Instruments, Singapore) was used to measure the pH of juices containing free and encapsulated bacteria at weekly intervals during a storage period of six weeks. All experiments were performed in triplicate to determine an average and standard error of the mean.

HPLC Sample Preparation for Analysis of Malic Acid

For the detection of malic acid 5 ml aliquots of probiotic fruit juice were taken on a weekly basis and frozen in 50 mL Falcon tubes (Becton Dickinson, California, USA) until analysis. The samples were diluted with 70 µL of 15.5 N HNO_3 and 4930 µL of 0.009% H_2S0_4, and then mixed gently by inversion. The samples were then centrifuged at 14,000 x g for 10 min using an Eppendorf 5415C centrifuge (Hamburg, Germany). The supernatant was removed using a 3 mL sterile syringe and then filtered into HPLC vials using a 0.2 mm membrane filter.

HPLC Equipment and Operating Conditions for Analysis of Malic Acid

The analysis of organic acid concentration was performed using methods described by Ong *et al.* (2006). The HPLC apparatus used consisted of a Varian 9100 autosampler, Varian 9010 solvent delivery system, Varian 9050 variable wavelength ultra-violet - visible detector, Altech 330 column heater and Varian software. The column was an Aminex HPX-87H 300 mm x 7.8 mm (Bio-Rad Laboratories) with an attached guard column (Bio-Rad Laboratories, California, USA) and was heated to 65°C when used. Mobile phase consisted 0.009% H_2S0_4 which was filtered through a 0.45 mm filter and degassed using nitrogen. The mobile phase was set at a flow rate of 0.6 mL/min. A sample volume of 25 mL was used for both standards and samples, and detection was achieved at 220 nm. All samples were run for 40 min and all analyses were carried out in triplicate.

Buffering Fruit Juice Using Phosphate Buffer

Both apple and orange fruit juices were buffered to pH 3.50 using phosphate buffer. Phosphate buffer (pH 7.2) was prepared using phosphate monohydrate (4.4% w/v) and disodium phosphate (18.32 w/v) (Sigma Chemical Co., Castle Hill, Sydney, Australia). The buffered juices were inoculated with each probiotic strain as mentioned earlier and their viability was determined for a period of six weeks.

Statistical Analysis

Results from three individual experiments were used to calculate the mean of cell counts. A single factor ANOVA was used to calculate the SEM of the cell counts. Significant differences between the means of cell counts were determined using Tukey's HSD test. All statistical analysis was carried out using SPSS 15.0® for Windows XP (SPSS Programming and Data Management Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Survival of Free and Encapsulated Probiotic Bacteria in Fruit Juices

The survival of free and microencapsulated probiotic bacteria inoculated into orange juice is shown in Figure 1. There was a rapid loss of free probiotic bacteria in the orange juice within the four weeks period and by the fifth week there were no viable bacteria remaining (Figure 1A). All eight strains of probiotic bacteria showed a similar decline in viability. *L. acidophilus* had greater viable numbers than all the other organisms possibly due to its higher tolerance to acids. The encapsulated probiotic bacteria which were protected from the acidic environment of the orange juice did not lose their viability as rapidly as the free probiotic bacteria and > 10^5 CFU/mL were still present after six weeks of storage (Figure 1B).

Interestingly similar results were found for survival of free and microencapsulated probiotic bacteria inoculated into apple juice, exhibited in Figure 2. Free probiotic bacteria rapidly lost their viability in the apple juice within the four weeks period and by the fifth week there were no viable bacteria (Figure 2A). Results indicate that both orange juice and apple juice are both too acidic for probiotic growth due to the similar decline in viability. However, *L. acidophilus* and *B. longum* exhibited higher survival than all the other strains, possibly due to their higher tolerance level to acid. There was a >10^5 CFU/mL of probiotic bacteria still present after six weeks of storage (Figure 2B). Several reports have indicated differences among strains of probiotic bacteria with respect to their survival in acidic environment (Shah and Jelen, 1990; Kailasapathy, 2005). Studies have shown that only the microencapsulated probiotics were able to maintain viability in the acidic fruit juice in high numbers for almost two weeks (Champagne *et al.,* 1993; Adhikari *et al.,* 2000; Cui *et al.,* 2000; Lee and Heo, 2000; Picot and Lacroix, 2004; Chandramouli *et al.,* 2004; Saarela *et al.,* 2006). Microcapsules may provide a more favorable anaerobic environment for the sensitive probiotic bacteria, as well as a physical barrier from the harsh acidic conditions of the fruit juice.

Effect of Buffering Fruit Juices on Stability of Probiotic Bacteria

Adjusting the pH of both the apple juice and orange juice to 3.50 using phosphate buffer showed no major difference in survival compared to un-buffered orange juice with an initial pH of 2.81 and 2.97, respectively (results not shown). The reason for similar results despite the difference in

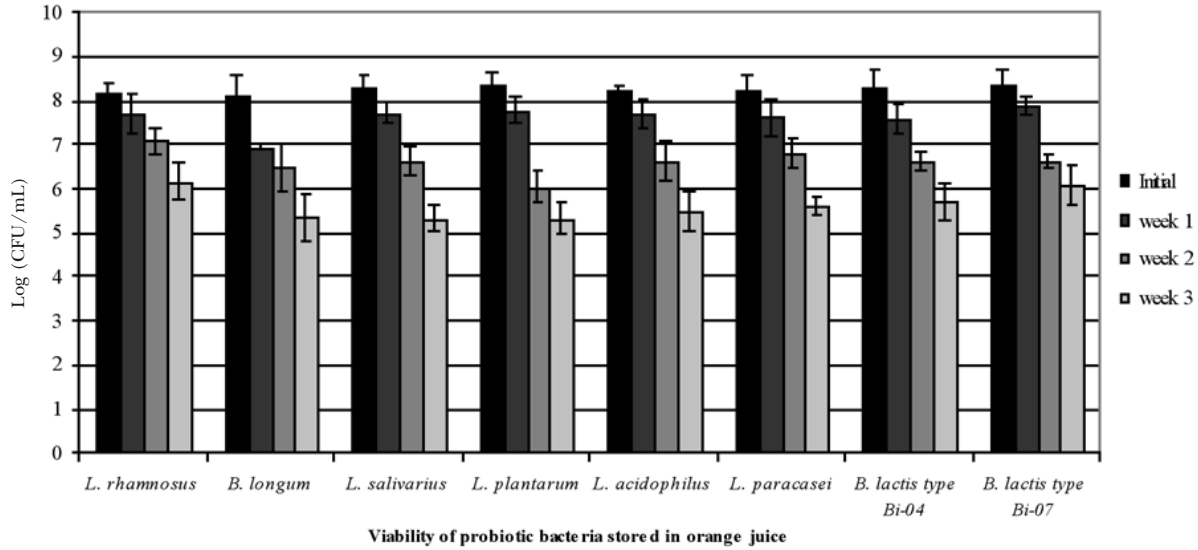

Figure 1A: Survival of free probiotic bacteria in orange juice stored at 4°C over a period of 5 weeks

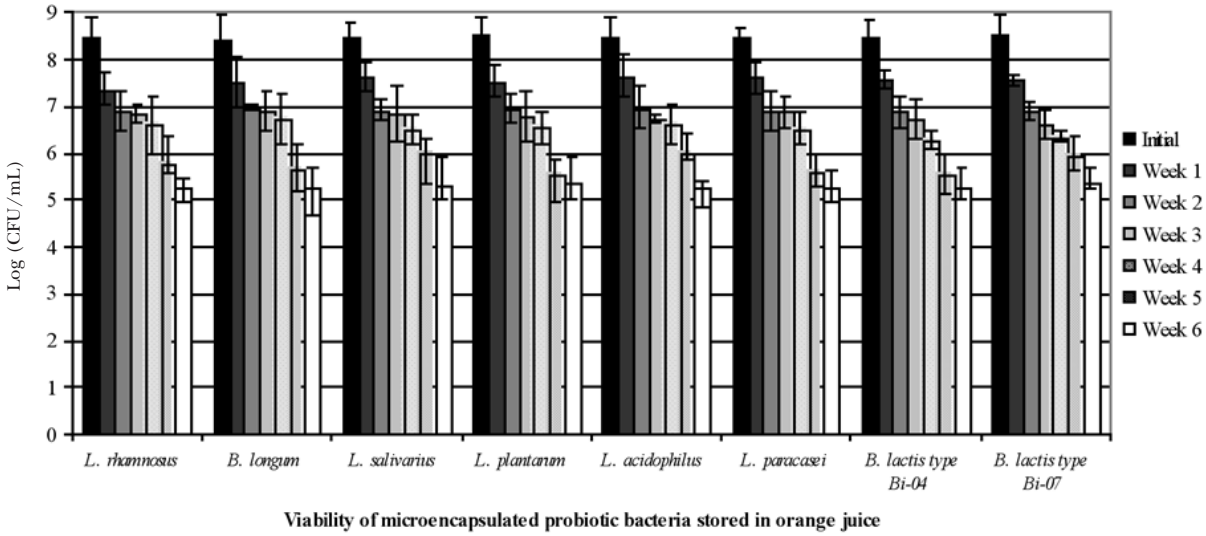

Figure 1B: Survival of encapsulated probiotic bacteria in orange juice stored at 4°C over a period of 6 weeks

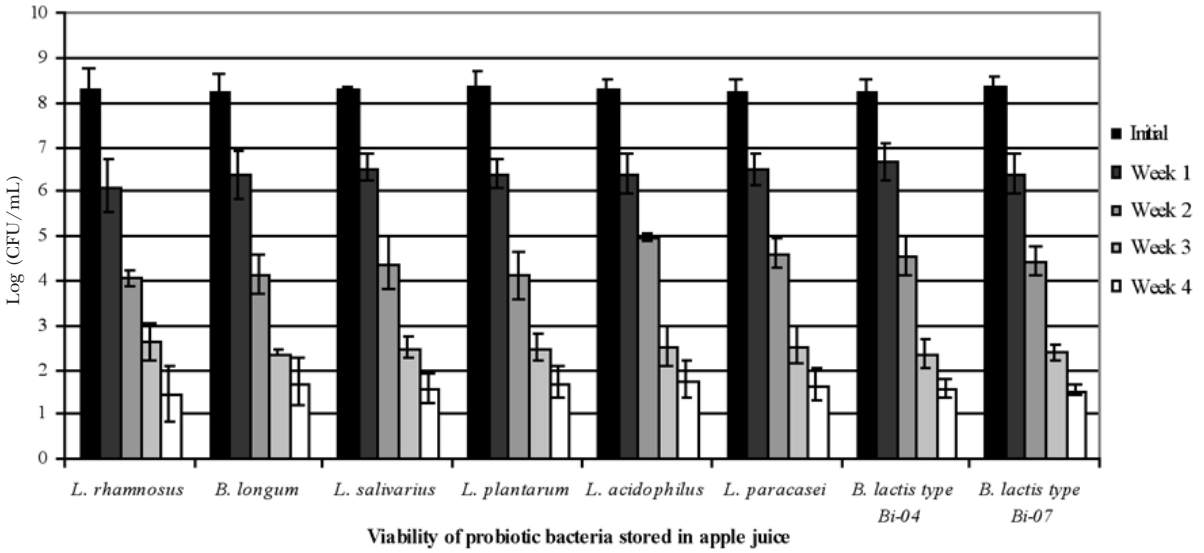

Figure 2A: Survival of free probiotic bacteria in apple juice stored at 4°C over a period of 5 weeks

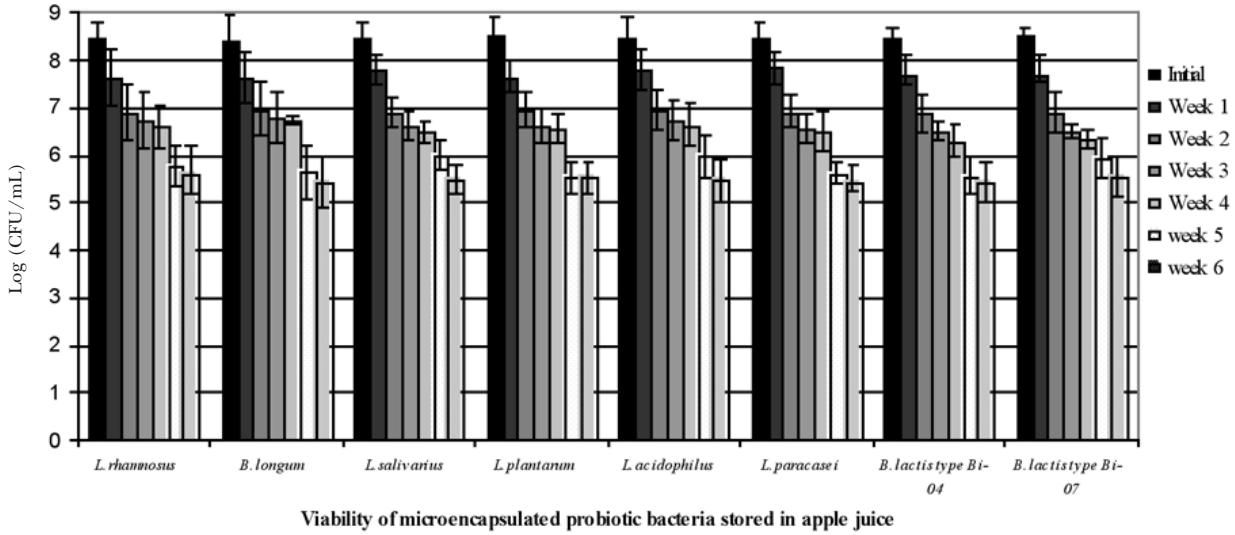

Figure 2B: Survival of encapsulated probiotic bacteria in apple juice stored at 4°C over a period of 6 weeks

a higher starting pH could be attributed to the low acid tolerance of the probiotic organisms that were used in this study. The optimum pH for growth of *Bifidobacterium* is 6.0 to 7.0, with virtually no growth at pH 4.5 to 5.0 or below (Gomes and Malcata, 1999). Below pH 4.1, most probiotic species of *Bifidobacterium* lose their viability within a week even at 4°C, and below pH 2.5 most species lose their viability within 3 h (Shah and Dave, 2002).

pH Changes During the Storage of Probiotic Fruit Juices

pH changes in orange juice containing free and microencapsulated probiotic bacteria during a storage period of six weeks are shown in Figure 3. A similar trend in the decline in pH was seen in both free and microencapsulated orange juice with all eight probiotic organisms tested. The final pH at the end of the six week storage period of orange juice with encapsulated probiotic bacteria was higher than that inoculated with free probiotic bacteria. The average pH decreased from 2.81 to 2.57 in orange juice containing free probiotic bacteria after six weeks of storage (Figure 3A), whereas the pH declined to only 2.81 in the juice containing encapsulated probiotics after the storage period (Figure 3B). This result suggests that probiotic bacteria in an immobilized microencapsulated state have a more stable environment.

Figure 4 shows the pH changes in apple juice containing free and microencapsulated probiotic bacteria and their effect on the pH in apple juice during a storage period of six weeks. All probiotic organisms tested reduced the pH of the apple juice regardless of whether they were in a free or encapsulated state. However, the final pH at the end of the six weeks storage period of apple juice with encapsulated probiotic bacteria was higher than that inoculated with free probiotic bacteria. The pH in apple juice containing free probiotic bacteria changed from 2.81 to the final pH was 2.58 during storage (Figure 4A). Apple juice with the encapsulated probiotic bacteria had an initial pH of 2.81 and changed to a final pH of 2.74 at the end of the storage period (Figure 4B).

Free probiotic bacteria may have utilized carbohydrates and produced small amounts of organic acids thus lowering the pH of the product during storage. Many of the free bacteria were not viable at later stages of storage; although the dead probiotic cells could release enzymes for hydrolyzing sugars in the fruit juice, thus lowering the pH. These results demonstrated that microencapsulation of probiotic bacteria would make a more stable product over a longer storage period. Studies have shown that encapsulated probiotic bacteria make more stable functional food products (Kailasapathy, 2005; Saarela *et al.*, 2005).

Changes in °Brix During the Storage of Free and Microencapsulated Probiotic Fruit Juice

The changes in °Brix in apple juice containing free and microencapsulated probiotic bacteria during storage over six weeks are shown in Figure 5A. On average, the final °Brix at the end of the six weeks storage period of apple juice with encapsulated probiotic bacteria was higher than for the orange juice inoculated with free probiotic bacteria. On average the °Brix concentration in apple juice decreased by 2.6% after six weeks of storage. However there was only a 1.1% decrease in °Brix in orange juice containing microencapsulated probiotic bacteria during the same storage period (Figure 5B). This difference in the changed °Brix concentration would indicate that free probiotic bacteria could more readily utilize the sugars in the orange juice compared to probiotics which were trapped inside microcapsules. Apple juice without probiotics had the least change in °Brix with an initial value of 12.0% that decreased to 11.9% during storage.

The changes in °Brix in orange juice containing free and microencapsulated probiotic during storage for six weeks are shown in Figure 6. Initially orange juice had a lower °Brix value at 10.0%. The final °Brix at the end of the six weeks storage period of orange juice with encapsulated probiotic bacteria was greater than the orange juice inoculated with free probiotic bacteria. Free probiotic bacteria reduced the °Brix concentration by an average of 2.2% °Brix (Figure 6A), whereas encapsulated probiotic bacteria reduced the °Brix concentration by an average of 0.91% (Figure 6B). This suggests that free probiotic bacteria could more readily utilize some of the sugars in the orange juice compared to microencapsulated probiotics. Orange juice which had no added probiotics showed the least change in °Brix with only a shift of 0.1% °Brix and this shift occurred in the late stages of storage.

Malic Acid Profile of Apple and Orange Juices During Storage Containing Free and Microencapsulated Probiotic Bacteria

Figure 7 illustrates the malic acid concentration in apple juice containing free and encapsulated probiotic bacteria. The initial malic acid concentration of apple juice without probiotics was 4.12 mg/L. All eight probiotic bacteria used produced similar quantities of malic acid in the apple juice. On average there was a 0.15 mg/L increase in malic acid concentration in apple juice with free probiotic bacteria, during storage (Figure

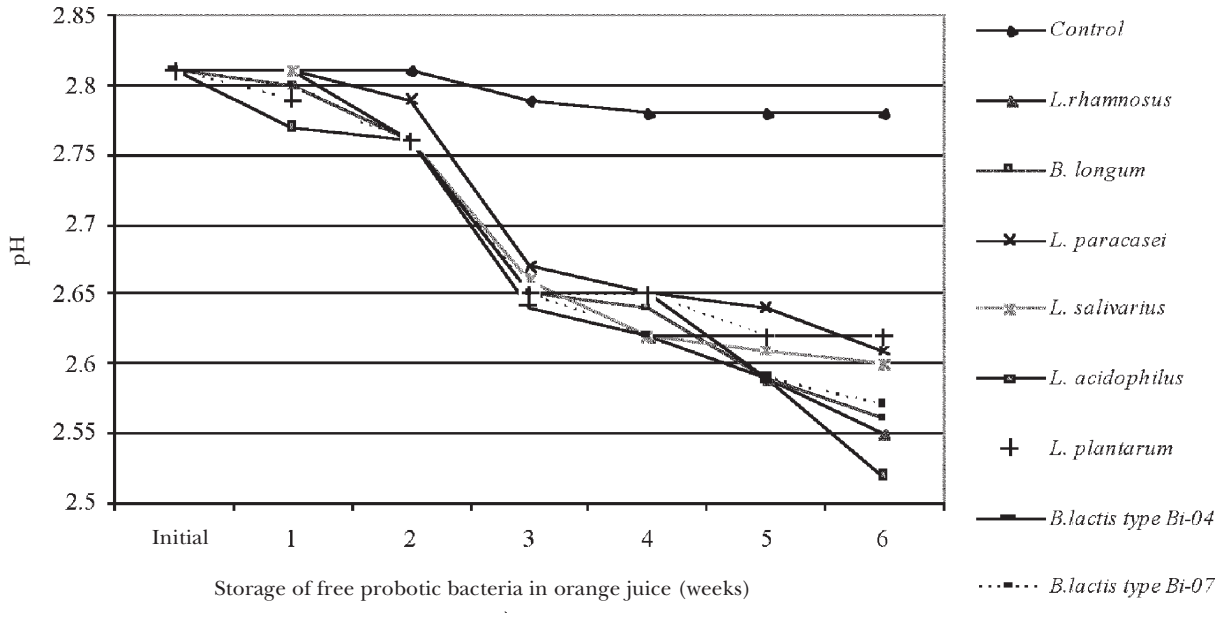

Figure 3A: Changes in pH in orange juice containing free probiotic bacteria over a period of 6 weeks

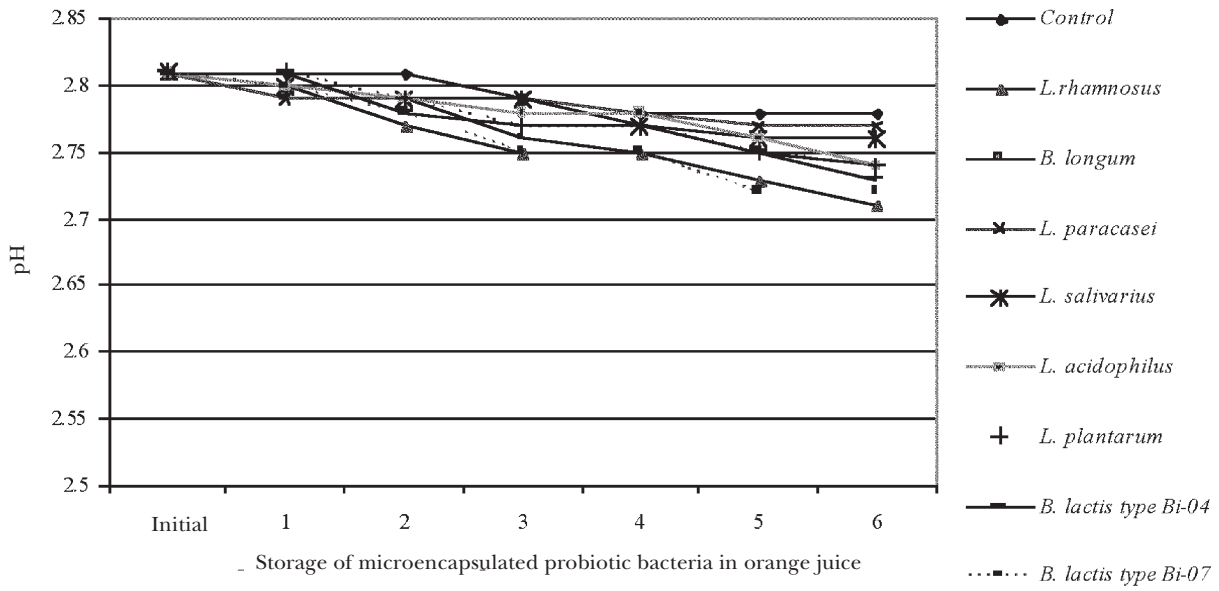

Figure 3B: Changes in pH in orange juice containing encapsulated probiotic bacteria over a period of six weeks

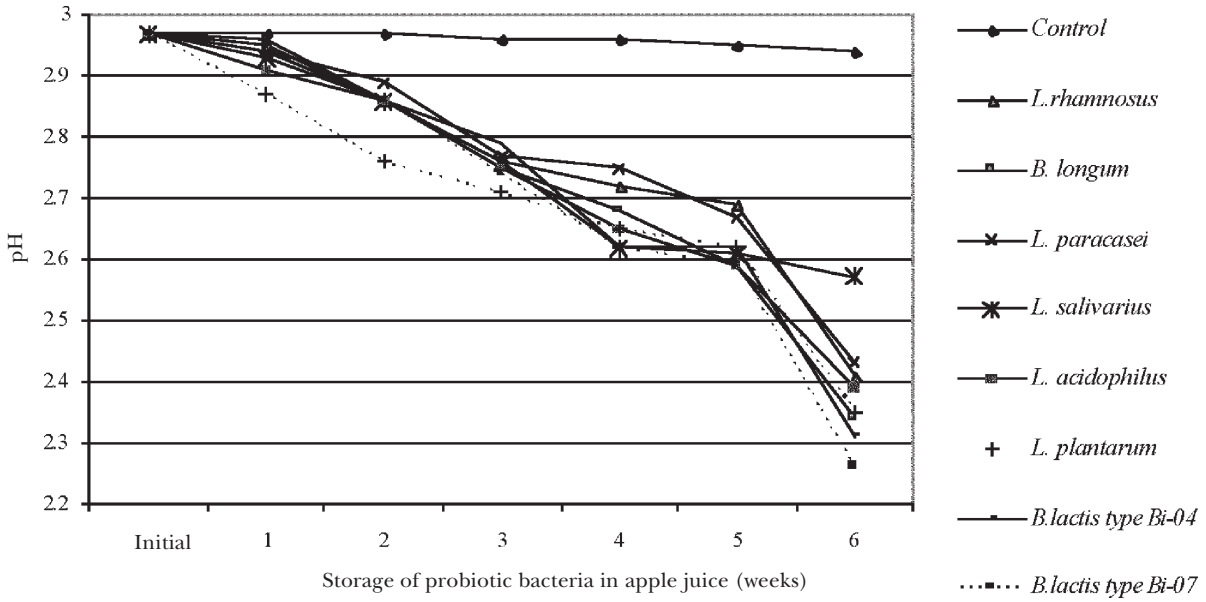

Figure 4A: Changes in pH in apple juice containing free probiotic bacteria over a period of 6 weeks

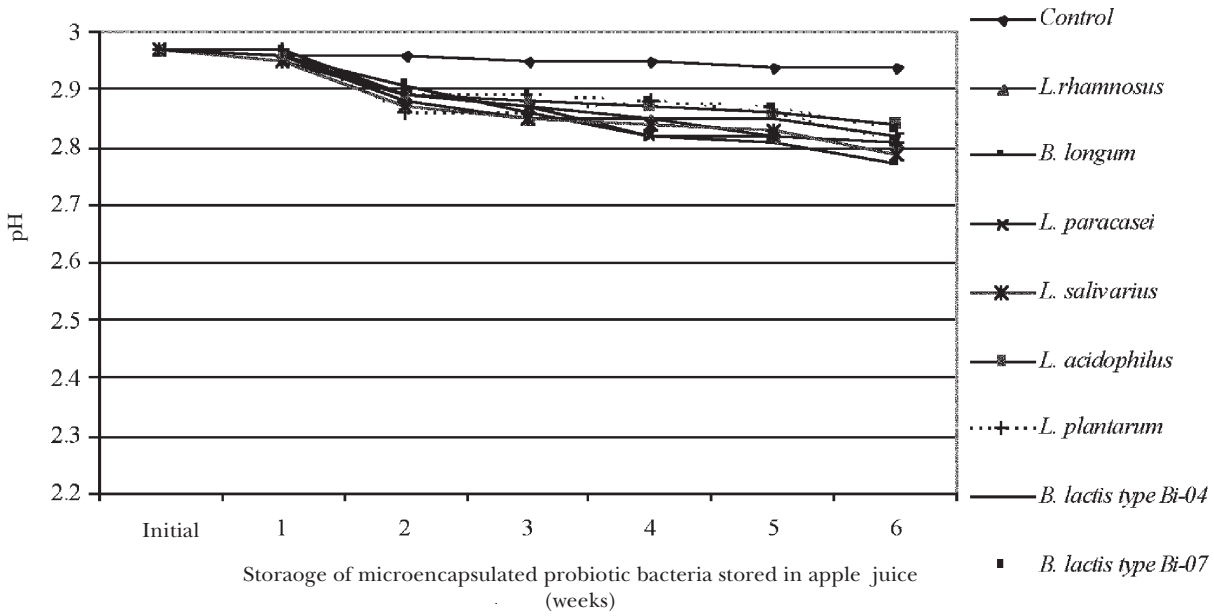

Figure 4B: Changes in pH in apple juice containing encapsulated probiotic bacteria over a period of 6 weeks

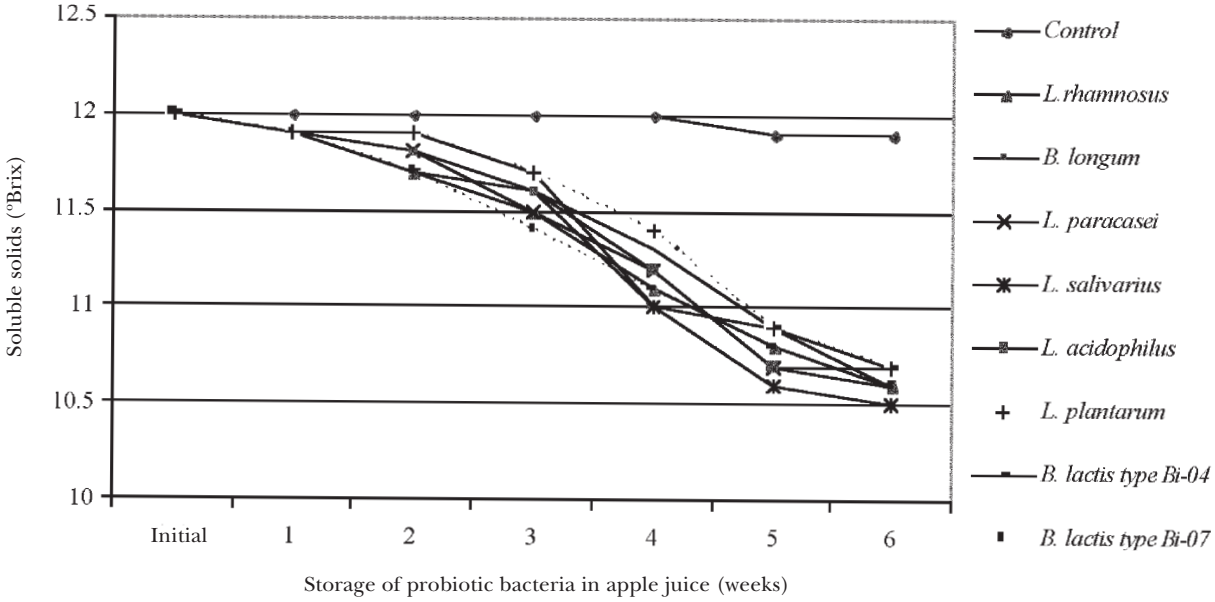

Figure 5A: Changes in °Brix in apple juice containing free probiotic bacteria. Apple over a period of 6 weeks

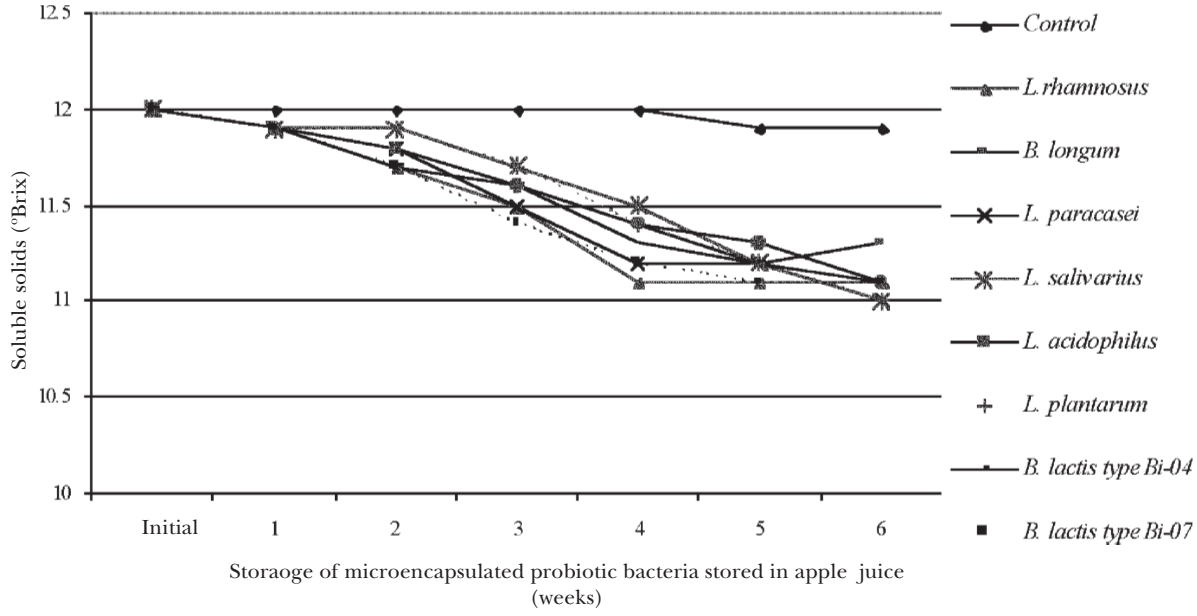

Figure 5B: Changes in °Brix in apple juice containing encapsulated probiotic bacteria over a period of 6 weeks

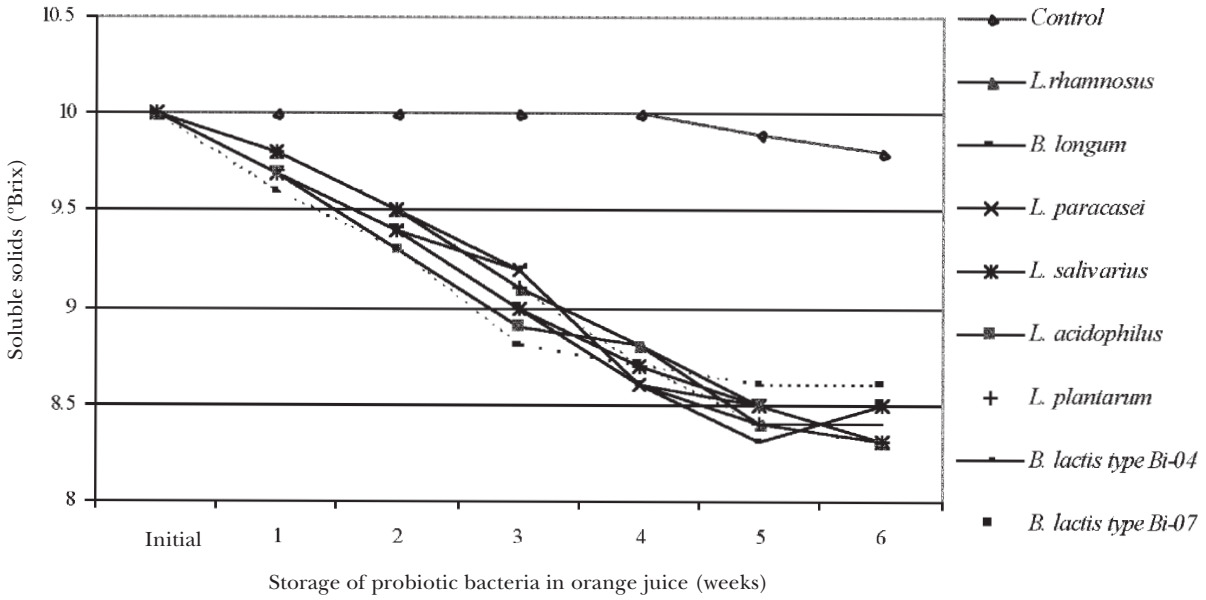

Figure 6A: Changes in °Brix in orange juice containing free probiotic bacteria over a period of 6 weeks

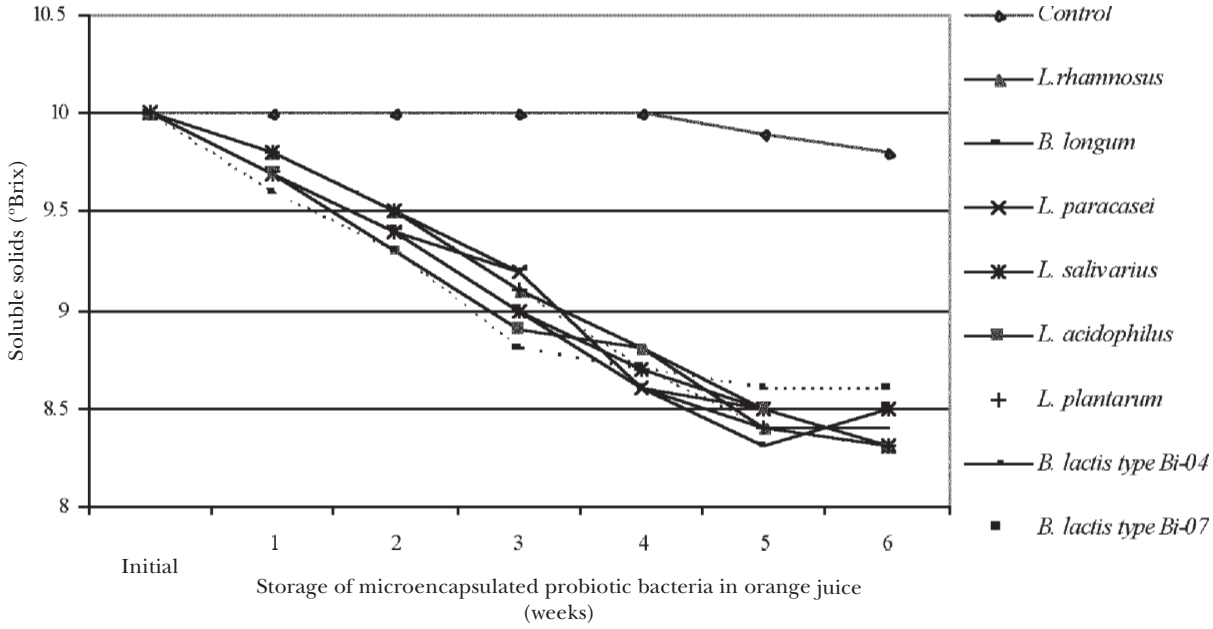

Figure 6B: Changes in °Brix in orange juice containing encapsulated probiotic bacteria over a period of 6 weeks

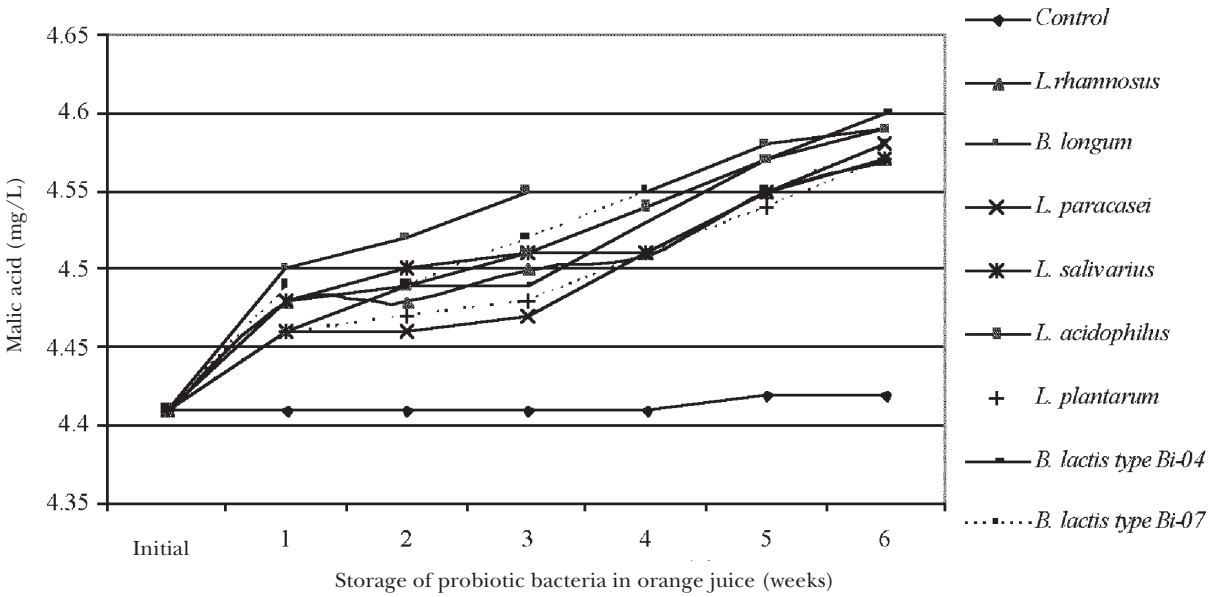

Figure 7A: Changes in malic acid concentration in orange juice containing free probiotic bacteria over a period of 6 weeks

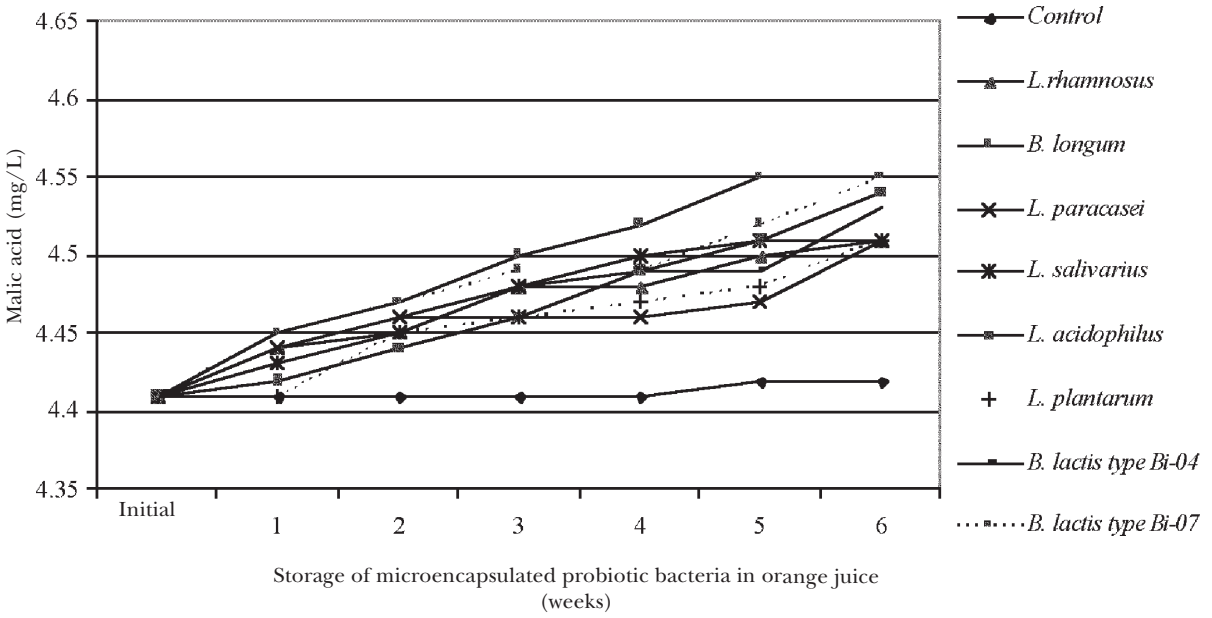

Figure 7B: Changes in malic acid concentration in orange juice containing encapsulated probiotic bacteria over a period of 6 weeks

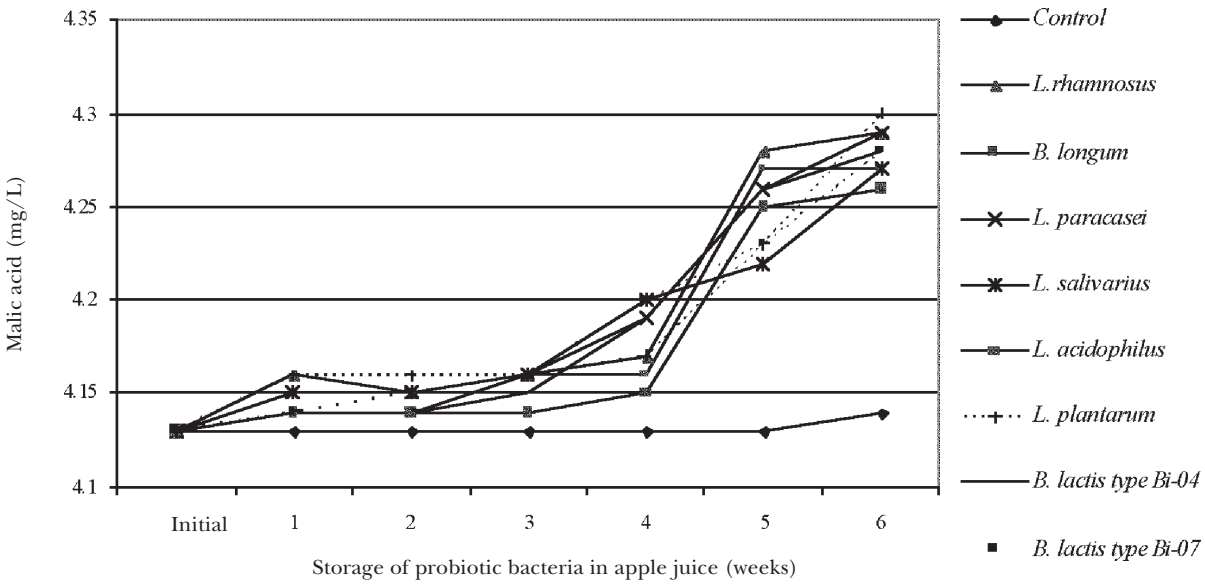

Figure 8A: Changes in malic acid concentration in apple juice containing free probiotic bacteria over a period of 6 weeks

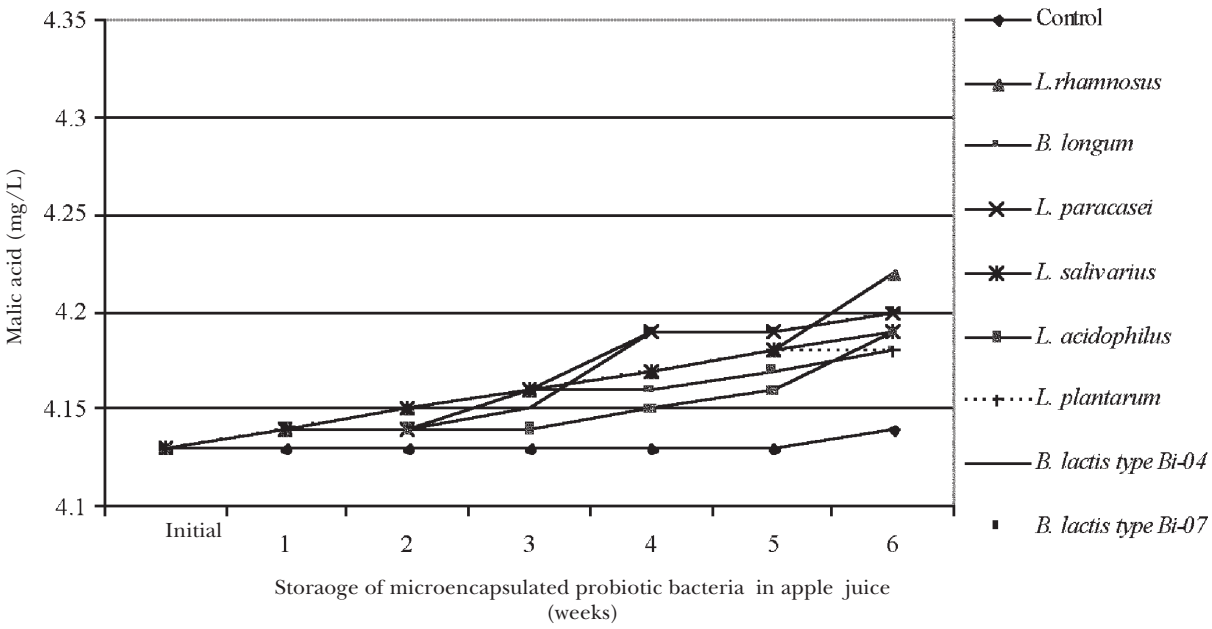

Figure 8B: Changes in malic acid concentration in apple juice containing encapsulated probiotic bacteria over a period of 6 weeks

7A). However, microencapsulated probiotic bacteria increased the average malic acid concentration by 0.07 mg/L during storage (Figure 7B). Overall, microencapsulated probiotic bacteria in apple juice produced less malic acid than free probiotic bacteria.

The malic acid concentration in orange juice containing free and encapsulated probiotic bacteria is shown in Figure 8. The initial malic acid concentration of orange juice without probiotic organisms was 4.41 mg/L. The control orange juice without probiotics had a slight increase in malic acid of 4.42 mg/L after six weeks of storage. Free probiotic orange juice had only a slight increase in the malic acid concentration, with an average increase of 0.16 mg/L during the six weeks of storage (Figure 8A). The malic acid concentration in orange juice containing microencapsulated probiotic bacteria increased by 0.11 mg/L in six weeks (Figure 8B). Overall, microencapsulated probiotic bacteria in orange juice produced less malic acid than free probiotic bacteria. Results also indicate that malic acid was produced in small quantities by each of the probiotic bacteria.

CONCLUSIONS

Microencapsulated probiotic organisms showed a much higher survival in fruit juices compared to fruit juices containing free probiotic bacteria. In general, probiotic fruit juices showed a decrease in °Brix concentration and an increase in pH during storage. Buffering fruit juices may enhance the survival of probiotic bacteria however, it reduces the shelf life of the product. Malic acid concentrations were higher in free probiotic fruit juices compared to microencapsulated probioitic fruit juices, suggesting that immobilized cells may make a more stable food product. The natural acidity and lack of nutrients may not make orange or apple juice a suitable functional food. Thus, further optimization of microencapsulation techniques is needed to make fruit juice a novel functional food.

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