Songklanakarin J. Sci. Technol.
37 (3), 265-270, May - Jun. 2015

http://www.sjst.psu.ac.th

Original Article

Survival of free and microencapsulated human-derived oral probiotic *Lactobacillus paracasei* SD1 in orange and aloe vera juices

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Received: 8 October 2014; Accepted: 15 March 2015

Abstract

Microencapsulation was evaluated as a means of preserving *Lactobacillus paracasei* SD1, a human-derived strain with probiotic potential, in orange and aloe vera juices. The microencapsulation parameters included alginate concentration, calcium chloride concentration and hardening-time, and the efficacy of microencapsulation to preserve the survival of microencapsulated bacteria compared to free cells during exposure in fruit juices were determined. The results revealed that the viable count of free-cell form markedly decreased compared to microencapsulated form. The microencapsulation of 2% alginate (w/v) and 0.05 M $CaCl_2$ gave the best result to preserve the probiotic. It was found that viability of microencapsulated probiotic bacteria was significantly higher than free-cell in fruit juices during 8 weeks of storage time in the refrigerator. The potential probiotic trait related to inhibitory effect was not affected after microencapsulation process. In summary, the microencapsulation method may be an alternative way of preserving the viability of probiotic *L. paracasei* SD1.

Keywords: microencapsulation, probiotic, *Lactobacillus paracasei* SD1, fruit juices

1. Introduction

There has been an increased interest in the role of probiotic bacteria for promoting and maintaining human health including oral health (Salminen *et al.*, 1998; Parvez *et al.,* 2006), and *Lactobacillus* has been proposed recently to promote oral health. Most studies have reported an inhibitory activity of oral *Lactobacillus* against cariogenic *Streptococcus* (Simark-Mattsson *et al.*, 2007; Teanpaisan *et al.*, 2011), and some also have demonstrated growth suppression of periodontal pathogens (Koll-Klais *et al.,* 2005; Teanpaisan *et al.,* 2011). Therefore, probiotic strains have been paid increased an attention to incorporate into a variety of food products including yoghurts, cheese, drinks and dietary products. Fruit juices may be alternative products for the incorporation of probiotic. Nowadays, most people consume fruit juices as daily soft drink due to their nutritional relevance e.g. organic acids, minerals, ber and a good source of antioxidant compound (vitamins A, C and E). It has been recommended that the probiotic bacteria should be alive and be present in an amount of at least 10^6-10^7 CFU/ml or g in the products (Rybka and Kailasapathy, 1995). However, it was reported that the free-cell probiotic bacteria may not survive in sufficient numbers when incorporated into food products. The viability of probiotic strains could be influenced by several factors of soft drinks e.g. acidic pH, oxygen level, antibacterial components and packaging conditions (Vinderola *et al.,* 2011).

Among the available techniques for immobilizing living cells, entrapment in calcium alginate beads has been proven to be useful for the immobilization of various probiotic strains including *Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus plantarum, L. acidophilus, L. paracasei, Bifidobacterium bifidum, Bifidobacterium infantis* and *Bifidobacterium lactis* (Khalil and Mansour, 1998;

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Chandramouli *et al*., 2004; Ding and Shah, 2008). Calcium alginate beads act as a physical barrier to protect probiotic living cells from adverse environments e.g. acidic pH. In addition, alginate has the benefit of being non-toxic to the cells being immobilized, and it is an accepted as food additive (Prevost and Divies, 1992). However, there are different conditions for the use of calcium alginate as a matrix for bacterial cells microencapsulation. The reported concentrations of sodium alginate vary widely from 0.5% to 4% (w/v). Also, the concentration of calcium chloride and hardening time of capsules in the calcium chloride solution for stabilization of the beads vary greatly from 0.05 to 0.2 M and 5 min to 2 h, respectively (Sheu and Marshall, 1993; Jankowski *et al.*, 1997; Khalil and Mansour, 1998; Truelstrup Hansen *et al.*, 2002). Therefore, the appropriate condition of calcium alginate bead for individual product need to be optimized.

We have previously reported the use of spray drying to preserve the probiotic *Lactobacillus paracasei* SD1 in milk power, and such produce has been proven to give a benefit in clinical trials of reducing the pathogenic agents in the oral cavity (Teanpaisan and Piwat, 2013; Ritthagol *et al.*, 2013). Thus, we now extend the use of calcium alginate as an alternative way to preserve human-derived *Lactobacillus paracasei* SD1. It hypothesized that calcium alginate could preserve the probiotic bacterial cells, therefore, the survival time of the probiotic bacteria in the microencapsulated form would be longer than that of the free cell form in fruit juices. The aims of the present study were to optimize the microencapsulation parameters including alginate concentration, calcium chloride concentration and hardening time, and to evaluate the efficacy of microencapsulation to preserve the survival of microencapsulated probiotic bacteria compared to free cells during exposure to fruit juices.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Probiotic strain *Lactobacillus paracasei* SD1 was isolated previously from the human oral cavity, and was identified as *L. paracasei* according to 16S-rRNA gene profiles by restriction fragment length polymorphism analysis (PCR-RFLP) and protein profiles by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Teanpaisan and Dahlén, 2006). The strain was stored at -80°C until used.

The probiotic strain was activated by growing twice on MRS agar at 37°C in anaerobic condition (80% N_2, 10% H_2, and 10% CO_2) for 24 h, and the strain was then propagated in 500 ml MRS broth in the same condition for 24 h. Cells were harvested by centrifugation at 3,000 × g for 10 min, and washed twice with 0.85% (w/v) normal saline. Cell count was determined by anaerobic on MRS agar after 48 h at 37°C in anaerobic condition. The cell suspension was subsequently used either directly (free cells) in assays or subjected to microencapsulation as described below.

2.2 Preparation of microencapsulation of probiotic

Initially, the microencapsulation parameters including alginate concentration (1.5%, 2% and 3% (w/v), calcium chloride concentration (0.01, 0.05 and 0.1 M) and hardening time (5 min, 30 min, and 1 h) were optimized for viability of microencapsulated bacteria.

The alginate and bacteria suspension with initial cell load approximately 10^9 CFU/ml was slowly dispensed using a pipette into a beaker containing a solution of calcium chloride with a magnetic stirrer at 200 rpm. After certain hardening time, the calcium alginate beads were removed and washed thoroughly with sterile distilled water. The calcium alginate beads were then kept at 4°C in 0.85% saline solution until further use.

2.3 Evaluation of bacterial viability in free-cell form and microencapsulated form

To determine the viable counts of *L. paracasei* SD1 in free-cell form and microencapsulated form, the initial cell loads of free-cell form (3×10^6 CFU/ml) and microencapsulated form ($1\text{-}5\times10^6$ CFU/ml) were monitored monthly over a period of 6 months of preserving at 4°C. For the enumeration of the microencapsulated cells, microcapsules were disrupted and counted using MRS agar and incubation at 37°C for 48 h in anaerobic condition. Also, free-cells were counted using the same procedure.

2.4 Evaluation of bacterial viability in fruit juices

Commercially sterilized (according to the manufacturing system) orange and aloe vera (TIPCO F&B Co., Ltd., Thailand) were used for all experiments. Fruit juices with no added preservatives and a long shelf life were obtained.

Probiotic free-cell form or microencapsulated form was added in 100 ml of each fruit juice at final 10^9 CFU/ml, and thoroughly mixed under sterilized process in a laminar flow cabinet. Fruit juices contained probiotic free-cell form or microencapsulated form were separated to a small portion in sterile bottles, and kept in room temperature (25°C) or refrigerator (4°C). For monitoring of probiotic viability, an enumeration of the probiotic cells was performed weekly over a period of 6 weeks and at 8 and at 12 weeks, using colony counting on MRS agar with incubation at 37°C for 48 h under anaerobic condition. The pH of juices contained free- or microencapsulated bacteria were also monitored using pH meter. All experiments were performed in triplicate to determine an average mean and standard deviation.

2.5 Inhibitory effect of *L. paracasei* SD1 before and after microencapsulation

Before and after microencapsulation, the inhibitory effect of *L. paracasei* SD1 against *Streptococcus mutans*

was assessed by an agar overlay method (Teanpaisan *et al.,* 2011). In brief, *L. paracasei* SD1 was inoculated on the surface of the brain heart infusion agar and incubated anaerobically (80% N_2, 10% H_2, and 10% CO_2) for 24-48 h at 37°C to develop visible macro-colonies.

Streptococcus mutans ATCC 25175 was precultivated in the brain heart infusion broth (BHI), and then the suspension of cells was adjusted to an optical density (OD) 0.25 at 600 nm. Thereafter, 5 ml of BHI soft agar (7 g/l agar) were seeded with 100 ml of an overnight culture of *S. mutans* ATCC 25175 strain and immediately poured over the macro-colonies of *L. paracasei* SD1. The plate was incubated anaerobically at 37°C for 24 h. The inhibitory zone, resulting in the releasing of inhibitory substance of *L. paracasei* SD1 against the growth of *S. mutans*, was observed.

2.6 Statistical analysis

All the results were expressed as mean±SD from three individual experiments. Differences among treatments were evaluated by one-way ANOVA and paired *t*-test. Significant differences between means within and among the treatment were determined using Tukey's HSD test. Results were considered statistically significant when $P<0.05$. All statistical analysis was carried out using SPSS (Chicago, Illinois, USA).

3. Results and Discussion

3.1 Effect of alginate concentration and $CaCl_2$ concentration on viability of *L. paracasei* SD1

In the present study, we investigated the use of micro-encapsulation to preserve viable cells of the oral human-derived *L. paracasei* SD1 strain.

The initial microencapsulated parameters were investigated to determine the conditions which were optimum for probiotic viability. There was a 2- to 3-log CFU decrease in bacterial cells count (from 10^9 CFU/ml) after microencapsulation. The data obtained in these trials indicated that the viability count of *L. paracasei* SD1 significantly decreased ($P<0.05$, one way ANOVA) when the $CaCl_2$ concentration was further increased to 0.10 M (Figure 1). An increased survival of probiotic was found in the microcapsules that had been hardened for 30 min compared with that in the microcapsules which had been hardened for only 5 min (data not shown).

3.2 Survival of free cells and microencapsulated *L. paracasei* SD1 during storage at refrigerator

There is no uniformity in the reported microencapsulation procedure for certain use, thus, the microencapsulation parameters, including alginate concentration, calcium chloride concentration and hardening time of capsules in calcium chloride, were quantitatively determined and optimized in this study. The survival time of probiotic bacteria in the microencapsulated form compared to the free-cell form was performed. The detail of result is given in Figure 2. The viable count of free-cell decreased markedly, and no viable cells were recovered after 3 months of refrigerated storage. It demonstrated that the survival time of probiotic bacteria in the microencapsulated form was longer than in free cell form. Generally, viability of microencapsulated *L. paracasei* SD1 in various concentrations of alginate and $CaCl_2$, gradually decreased from a 2-log to 4-log CFU of probiotic counts over 6 months of refrigerated storage. Results were similar to the previous reports (Khalil and Mansour, 1998; Chandramouli *et al*., 2004; Ding and Shah, 2008), which revealed that calcium alginate could preserve the bacterial cell viability. This is due to its ability of calcium alginate act as a physical barrier to protect probiotic cells from adverse effect.

There were probiotic count differences (1-log to 2-log CFU) among the various concentrations of calcium alginate. However, the microencapsulation of 2% alginate (w/v) and 0.05 M $CaCl_2$ gave the maximum viable cells count of probiotic and uniform spherical microcapsule formation. Thus, it was selected for further use in the experiment of fruit juices.

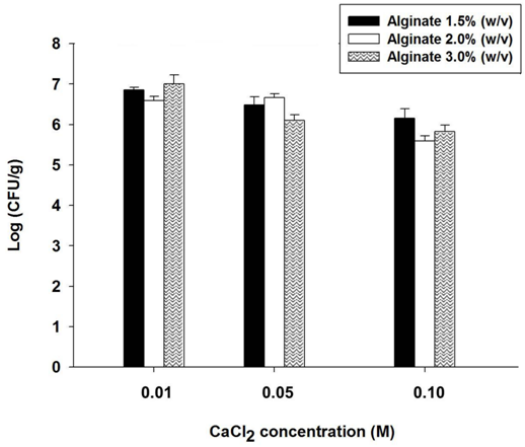

Figure 1. Effect of alginate concentration and $CaCl_2$ concentration on viability of *L. paracasei* SD1

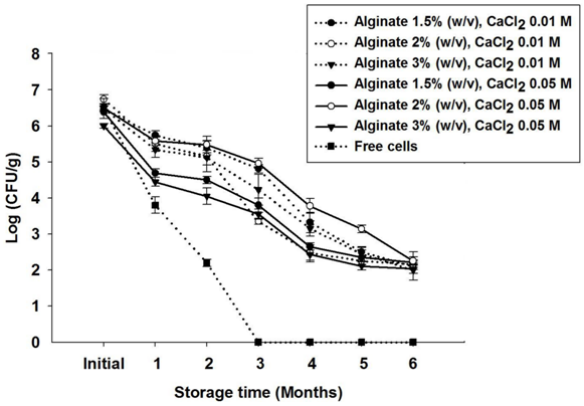

Figure 2. Survival of free-cell and microencapsulated *L. paracasei* SD1during storage in a refrigerator

3.3 Survival of free-cell form and microencapsulated form of *L. paracasei* SD1 in fruit juices during storage at either refrigerator or room temperature

The results of survival of probiotic bacteria in free-cell form and microencapsulated form in orange and aloe vera juices at a refrigerator and room temperature are shown in Figure 3. It revealed that viability of microencapsulated probiotic bacteria was significantly (P<0.05, paired t-test) higher than that of free probiotic bacteria in both orange and aloe vera juices during 12 weeks of storage time at either a refrigerator (Figure 3A) or room temperature (Figure 3B).

After 8 weeks, only the microencapsulated form could preserve probiotic bacteria at the level of 10^6 CFU/ml, which means numbers of $1.02 \pm 0.43 \times 10^6$ and $2.51 \pm 0.28 \times 10^6$ CFU/ml for orange and aloe vera juices respectively in a refrigerator (Figure 3A). As it has recommended that the probiotic bacteria should be alive and be present at amount of at least 10^6-10^7 CFU/ml or g in the products (Rybka and Kailasapathy, 1995). Therefore, results in this study have proven that microencapsulated probiotic in a refrigerated condition was able to maintain viability in the fruit juices in sufficient numbers (10^6 CFU/ml) for at least 8 weeks.

The loss of viability of probiotic bacteria seemed to be greater in the orange juice than in the aloe vera juice when it was compared in the same form (either encapsulated form or free cells form). However, the difference was not statistically significant (P>0.05, paired t-test). Some reports also showed that acid environment influenced the survival rate of probiotic bacteria (Kailasapathy and Rybka, 1997; Ding and Shah, 2008).

It is noted that the storage time in this study could be more prolonged than the others, in which storage time was only 2 week (Adhikari *et al.*, 2000; Saarela *et al.*, 2006). Microcapsules may provide a favorable environment for the probiotic strain, as well as a physical barrier from the harsh, e.g. acidic condition of the fruit juices.

3.4 pH changes in fruit juices containing free cells and microencapsulated probiotic bacteria during a storage at either a refrigerator or room temperature

The pH changes in the orange juice and the aloe vera juice containing free- and microencapsulated probiotic bacteria during a storage period of 12 weeks either in a refrigerator (Figure 4A) or at room temperature (Figure 4B) were moni-

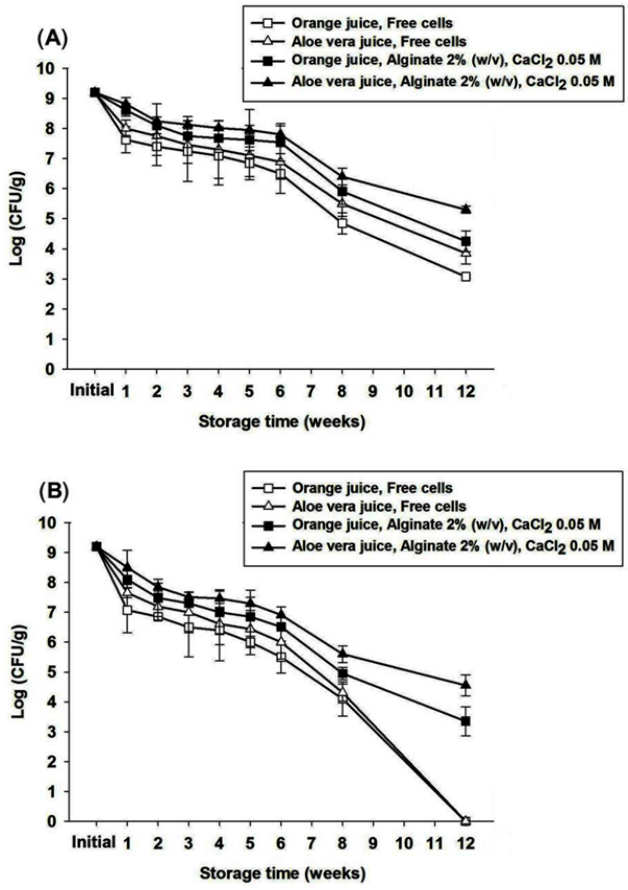

Figure 3. Survival of free-cell and microencapsulated *L. paracasei* SD1 in juices during storage in a refrigerator (A) and at room temperature (B)

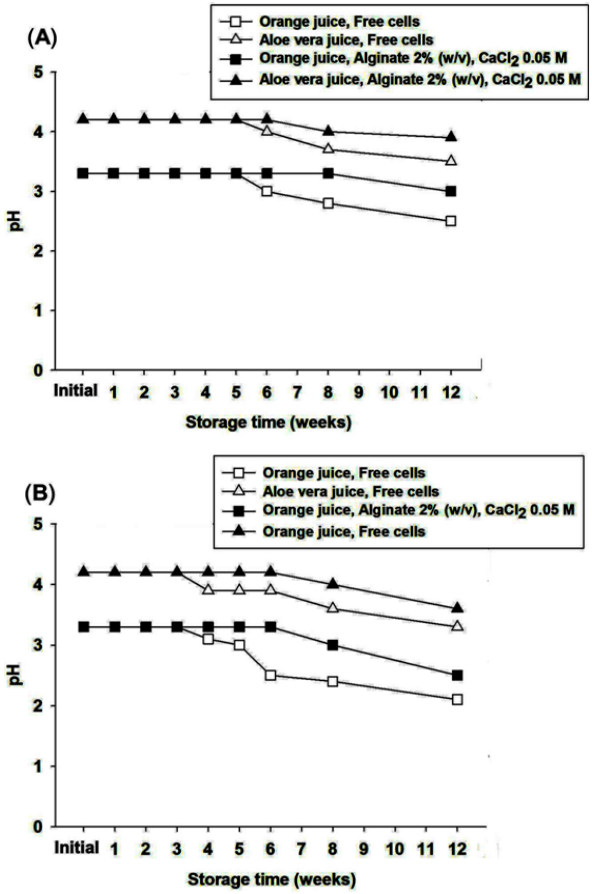

Figure 4. pH changes in fruit juices containing free-cell and microencapsulated probiotic bacteria during a storage in a refrigerator (A) and at room temperature (B)

tored. It was found that the pH of orange juice (3.3±0) and aloe vara juice (4.2±0) containing microencapsulated probiotic cells did not change over the whole period of 6 weeks of storage either in a refrigerator or at room temperature. The pH of juices with free-cell form started to drop after 3 and 5 weeks at room temperature and in a refrigerator, respectively. Free probiotic bacteria may have utilized sucrose in juices and produced small amounts of organic acids, lowering the pH of the product during storage. The results indicated that microencapsulation could maintain the pH of juices containing probiotic cells.

3.5 Inhibitory effect of *L. paracasei* SD1 before and after microencapsulation

L. paracasei SD has been reported as a novel probiotic strain that could reduce cariogenic bacteria in volunteers receiving milk powder containing *L. paracasei* SD1 (Teanpaisan and Piwat, 2013). It has been shown previously that *L. paracasei* SD1 produces a broad-spectrum antimicrobial activity that exhibits activity against oral pathogens such as *Streptococcus mutans, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Tanerella forsythia* (Teanpaisan *et al*., 2011). Antimicrobial activity is a desirable trait for probiotic cultures (Collins *et al*., 1998) and may be used to competitively exclude undesirable microorganisms in the oral cavity, thereby playing a role in probiotic persistence in the host. In this study, it was shown that the potential probiotic trait related to inhibitory activity was not affected after microencapsulation process compared to before microencapsulation process (Figure 5).

4. Conclusions

Our findings highlight the need to take into consideration the technological properties of probiotic strains, and emphasize the importance of strain selection with regard to processing, as well as health-promoting properties. In this study, it was shown that 2% alginate and 0.05 M $CaCl_2$ could provide a survival of *L. paracasei* SD1 up to 8 weeks. The fruit juices contained microencapsulated *L. paracasei* SD1 had high levels of viable probiotic ($\geq 10^6$ CFU/g) in the refrigerated storage. In addition, inhibitory effect of *L. paracasei* SD1 was not affected after the microencapsulation process. Thus, supplemented fruit juice by incorporating microencapsulated *L. paracasei* SD1 in calcium alginate beads might be an alternative source for providing a live beneficial organism to consumers. However, 8 weeks survival of *L. paracasei* SD1 in microencapsulated form may be too short for the manufacturing products, it will need to improve for a longer time.

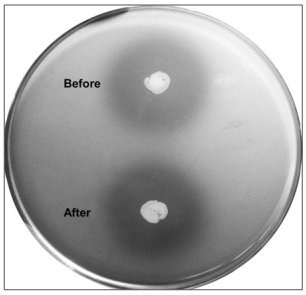

Figure 5. Inhibitory effect of *L. paracasei* SD1 against *S. mutans* ATCC 25175 tested before and after microencapsulation process.

Acknowledgements

This work was supported by the National Research Council of Thailand (DEN550005M), the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (AGR 540555M), and the National Science and Technology Development Agency (NSTDA) of Thailand.

References

Adhikari, K., Mustapha, A., Grum, I.U. and Fernando, L. 2000. Viability of microencapsulated bifidobacteria in set yoghurt during refrigerated storage. Journal of Dairy Science. 83, 1946-1951.

Chandramouli, V., Kailaspathy, K., Peiris, P. and Jones, M., 2004. An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. Journal of Microbiological Methods. 56, 27-35.

Collins, J.K., Thornton, G. and Sullivan, G.O. 1998. Selection of probiotic strains for human applications. International Dairy Journal. 8, 487-490.

Ding, W.K. and Shah, N.P. 2008. Survival of free and microencapsulated probiotic bacteria in orange and apple juices. International Food Research Journal. 15, 219-232.

Jankowski, T., Zielinska, M. and Wysakowska, A. 1997. Encapsulation of lactic acid bacteria with alginate/starch capsules. Biotechnology Techniques. 10, 31-34.

Kailasapathy, K. and Rybka, S. 1997. *Lactobacillus acidophilus* and *Bifidobacterium* spp. Their therapeutic potential and survival in yogurt. Australian Journal of Dairy Technology. 52, 28-35.

Khalil, ALI H. and Mansour, ESAM H. 1998. Alginate Encapsulated Bifidobacteria Survival in Mayonnaise. Journal of Food Science. 63, 702-705.

Koll-Klais, P., Mandar, R., Leibur, E., Marcotte, H., Hammarstrom, L. and Mikelsaar, M. 2005. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. Oral Microbiology and Immunology. 20, 354-361.

Parvez, S., Malik, K.A., Ah Kang, S. and Kim, H.Y. 2006. Probiotics and their fermented food products are beneficial for health. Journal of Applied Microbiology. 100, 1171-1185.

Prevost, H. and Divies, C. 1992. Cream fermentation by a mixed culture of Lactobacilli entrapped in two-layer calcium alginate beads. Biotechnology Letters. 14, 583-588.

Ritthagol, W., Saetang, C. and Teanpaisan, R. 2013. Effect of probiotics containing *Lactobacillus paracasei* SD1 on salivary mutans streptococci and Lactobacilli in orthodontic cleft patients: A double-blinded, randomized, placebo-controlled study. Cleft Palate-Craniofacial Journal. 51, 257-263.

Rybka, S. and Kailasapathy, K. 1995. The survival of culture bacteria in fresh and freeze-dried AB yoghurts. Australian Journal of Dairy Technology. 50, 51-57.

Saarela, M., Virkajarvi, I., Alakomi, H.L., Mattila, P. and Matto, J. 2006. Stability and functionality of freeze-dried probiotic *Bifidobacterium* cells during storage in juice and milk. International Dairy Journal. 16, 1477-1482.

Salminen, S., Ouwehand, A.C. and Isolauri, E. 1998. Clinical applications of probiotic bacteria. International Dairy Journal. 8, 563-572.

Sheu, T.Y. and Marshall, R.T. 1993. Micro-encapsulation of Lactobacilli in calcium alginate gels. Journal of Food Science. 54, 557-61.

Simark-Mattsson, C., Emilson, C.G., Hakansson, E.G., Jacobsson, C., Roos, K. and Holm, S. 2007. *Lactobacillus*-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. European Journal of Oral Sciences. 115, 308-314.

Teanpaisan, R. and Dahlen, G. 2006. Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral Lactobacillus species. Oral Microbiology and Immunology. 21, 79-83.

Teanpaisan, R., Piwat, S. and Dahlen, G. 2011. Inhibitory effect of oral *Lactobacillus* against oral pathogens. Letters in Applied Microbiology. 53, 452-59.

Teanpaisan, R. and Piwat, S. 2013. *Lactobacillus paracasei* SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. Clinical Oral Investigations. 18, 857-862.

Truelstrup Hansen, L., Allan-Wojtas, P. M., Jin, Y. L. and Paulson, A. T. 2002. Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. in milk and simulated conditions. Food Microbiology. 19, 35-45.

Vinderola, G., Binetti, A., Burns, P. and Reinheimer, J. 2011. Cell viability and functionality of probiotic bacteria in dairy products. Frontiers in Microbiology. 2, 1-5.