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Survivability of recoated probiotic capsules in simulated gastrointestinal environment and Mao juice containing herbal extracts and antioxidants

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Abstract

This study aimed to examine the efficacy of alginate recoating on viability of *Lactobacillus casei* 01, *Lactobacillus acidophilus* LA5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 capsules in simulated gastrointestinal conditions and in Mao juice containing herbal extracts (i.e. cashew flower, roselle and green tea) or standard antioxidants (i.e ascorbic acid and α-tocopherol). It was found that the increasing of the survival rates of the cultures was observed at the recoated capsules with 0.3% alginate in the simulated gastrointestinal environments. This condition was chosen to recoat the probiotic beads for the storage assessment in Mao juice combining with herbal extracts and antioxidants. During refrigerated storage at 4°C, the results displayed that cashew flower extract, green tea extract and ascorbic acid noticeably improved the stability of free and entrapped probiotics in Mao juice as compared to the control sample, whereas roselle extract and α-tocopherol had no impact.

Introduction

Mao (*Antidesma bunius* L. Spreng), a Thai blueberry, is an economically fruit cultivated in Northeast Thailand, in particular Sakon Nakhon province. This fruit has a special sweet-sour taste and a unique flavor, and displays red-purple color when entirely ripe. It has been reported to have the antioxidant activity and capacity due to rich in polyphenols, specially anthocyanins, flavonoids and phenolic compounds, and vitamin C (Butkhup and Samappito, 2008; Samappito and Butkhup, 2010). Up to date, Mao products have been promoted as a recommended product of the One Tumbol One Product (OTOP) program by the Thai government. The development of new functional products from Mao juice containing some ingredients such as herbal-plant extracts is also very interesting, since the consumption of herbal products can prevent certain diseases, such as cancer and cardiovascular diseases, and also help to reduce blood pressure (Samappito and Butkhup, 2010; Chunthanom *et al*., 2013). To add value to this health beverage, the fortification of herbal-Mao juices with probiotics as producing the functional food is one of interest.

Probiotics are live microbes, which are able to improve the human intestinal microbial balance (Fuller, 1989). The numerous lactic acid bacteria (LAB) including *Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium animalis* subsp. *lactis* have been used in fruits and/or vegetable juices to produce probiotic beverages (Kim *et al*., 2012). The health-promoting effects of these probiotics are primarily reliant on their ability to achieve their suitable site of action in enough numbers, in particular the colon compartments (Kent and Doherty, 2014). Principally, the improvement of the immune system, reduction of lactose intolerance, reduction of blood cholesterol levels, anti-carcinogenic properties, resistance against pathogens and increasing nutritional values of foods can be enhanced by probiotic activities (Ogueke *et al*., 2010; Shah *et al*., 2010; Kent and Doherty, 2014). To perform the best function, they viability must be maintained during processing, storage and passage through the adverse environments of stomach and small intestine (da Silva *et al*., 2014; Tripathi and Giri, 2014).

Currently, encapsulation is an approach receiving considerable interest as a way to provide probiotic cells with some physical barriers, such as alginate, gelatin and chitosan, to protect them following processing, storage and ingestion in order to improve

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the probiotic load of a product (Kailasapathy, 2009; Chaikham *et al*., 2012; Cook *et al.*, 2012; Nualkaekul *et al*., 2012, 2013). The majority of encapsulation technique is based on immobilization of the cells into an enclosed matrix, which maintains structural integrity for a proper time until degrading and releasing the cells in the intestine (Kent and Doherty, 2014). Nualkaekul *et al.* (2012) investigated the effect of encapsulation on survivability of probiotic *Lactobacillus plantarum* NCIMB 8826 in gastric juice (pH 1.5) and found that the survivors were improved in the case of encapsulated cells by 8 log CFU/ml as compared to the free cells. Chaikham *et al.* (2013a) reported that the encapsulated *L. acidophilus* LA5 and *L. casei* 01 exhibited the highest viability in pressurized and pasteurized longan juice during storage at 4°C for 4 weeks. Additionally, Krasaekoopt and Watcharapoka (2014) found that the survival numbers of both *L. acidophilus* LA5 and *L. casei* 01 were maintained above the recommended therapeutic minimum (> 10^6 CFU/g or CFU/ml) throughout the refrigerated storage in yoghurt and fruit juices.

The objective of this work was to investigate the efficacy of alginate recoating on viability of probiotic capsules in the simulated gastrointestinal environments and in Mao juice containing different herbal extracts (i.e. cashew flower, roselle and green tea) or antioxidant reagents (i.e. ascorbic acid and α-tocopherol) during refrigerated storage.

Materials and Methods

Probiotic cultures

Probiotic microorganisms (*L. casei* 01, *L. acidophilus* LA5 and *B. animalis* subsp. *lactis* BB-12) were purchased from Chr. Hansen (Hørsholm, Denmark). Cell pellets of *L. acidophilus* LA5 and *L. casei* 01 were prepared according to the procedure of Chaikham *et al.* (2012a). *B. animalis* subsp. *lactis* was grown at 37°C for 20 h in de Man Rogosa and Sharp (MRS) broth (HI-Media, India) and supplemented with filter sterilized 0.05% (w/v) *L*-cysteine hydrochloride (Sigma, Australia) at 37°C for 20 h. After incubation, all cultures were harvested and washed twice with 0.85% (w/v) sterile saline water before centrifugation at 3,000 rpm for 15-20 min. All cell pallets were diluted to provide a bacterial concentration of 10^{11} CFU/ml by sterile saline water prior to use.

Recoating of probiotic beads

Probiotic capsules or probiotic beads of *L. casei* 01, *L. acidophilus* LA5 or *B. animalis* subsp. *lactis* BB-12 were produced following Chaikham *et al.* (2013a). The encapsulated cells were then recoated with different concentrations of sterile alginate solution (0.1%, 0.2% and 0.3%, w/v). Thirty grams of probiotic beads were mixed with 100 ml of each sterile alginate solution before stirring at 100 rpm for 10 min. The recoated beads were washed with 0.85% sterile saline solution and then separated by filter through a sterile metal sieve and kept at 4°C.

Enumeration of immobilized probiotics

For releasing the entrapped cells, one gram of coated or recoated beads was mixed with 99 ml of 0.1 M sterile phosphate buffer (pH 7) (Merck, Germany) in a stomacher for 15 min (Chaikham *et al.*, 2013a). Afterward, the decimal dilutions were made with 0.1% (w/v) sterile peptone water (Hi-Media, India) before plating on MRS agar. All plates were then incubated under anaerobic environment at 37°C for 24-72 h.

Survival of free and encapsulated probiotics in gastrointestinal fluids

One gram of encapsulated probiotics was inoculated into 9 ml of 0.85% (w/v) sterile saline solution, which had been adjusted to pH 2.5 (simulated stomach environment) using 37% hydrochloric acid (Merck, Germany) and then anaerobically incubated at 37°C. During incubation in simulated gastric condition, the samples were taken at 0.5, 1, 2 and 3 h, and the viability of free and encapsulated probiotics were determined. The decimal dilutions were made before pouring on MRS agar plates. The colonies were then computed after incubating under anaerobic environment at 37°C for 24-72 h and expressed as colony forming units (log CFU/ml).

Bile fluid (simulated small intestinal environment) consisted of distilled water combined with 1.5% (w/v) bile salt (Sigma-Aldrich, USA). The experiment was initiated by transferring 1 g probiotic beads to 9 ml of bile fluid and then anaerobically incubated at 37°C. The samples were collected for 1.5, 3, 4.5 and 6 h and the survivors were then enumerated following the protocol similar to simulated gastric environment.

Enumerations of survival cells were used to calculate the destructive value (*D*-value), which is the time required to reduce the quantity of cells by 90%. The *D*-values were estimated from the absolute values of the inverses of slopes in linear-regression equations by plotting log numbers of survival cells against incubation times (Chaikham *et al*., 2013a).

Viability of free and encapsulated probiotics in Mao juice containing herbal extracts and antioxidants during refrigerated storage

Dried herbal plants including roselle and green

tea were purchased from local market in Ayutthaya province, Thailand. Cashew flower was freshly harvested from an orchard in Chiang Mai province, Thailand. To dry cashew flower, the flower was dehydrated using a tray dryer (Mammert, Germany) at 65°C for 3 h. All dried plants were powdered using a blender (National, Thailand) at moderate speed for 5-10 min. A 100-g of powder was mixed with 900 ml hot water (70±5°C) by stirring at 100 rpm for 1 h. The extracts were filtered through Whatman® No.1 filter paper (Whatman, Spain) and then lyophilized using a Christ alpha 1-2 LD plus freeze dryer (SciQuip, Germany) at -50°C for 12 h (Chunthanom *et al.*, 2013). Standard antioxidants including *L*-Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) were purchased from Sigma-Aldrich (USA).

To assay the stability of probiotics in Mao juices, 1 g of probiotic beads was transferred into 9 ml of pasteurized Mao juice (80°C/1 min) containing 0.5% (w/v) of the plant extracts or standard antioxidants and then stored at 4°C for 30 days. The samples were taken in every five day for quantifying the survivors in MRS agar. Changes of pH in all samples were also measured using a pH meter (Sartorius PB-20, Germany).

Statistical analysis

The results are presented as mean ± standard deviation of six replications (n = 6). Analysis of variance (ANOVA) was carried out using a statistic program. The determination of significant differences among treatment means was done by Duncan's multiple range tests ($P \leq 0.05$).

Results and Discussion

Viability of encapsulated probiotic beads in gastrointestinal environments

Encapsulation has been found to be a useful method for protection of probiotics during gastrointestinal transit (Chaikham *et al*., 2013a, b; Krasaekoopt and Watcharapoka, 2014; Sathyabama *et al*., 2014). Table 1 illustrates the outcomes of recoated alginate beads on the viability of probiotic bacteria, which were embedded in the gastric juice (pH 2.5) for 3 h. The results showed that free and encapsulated cells apparently diminished ($P \leq 0.05$) when incubation time of the simulated stomach increased. Considering the survival of free cells, *B. animalis* subsp. *lactis* BB-12 completely eliminated after incubation at 3 h, while the survivors of *L. casei* 01 and *L. acidophilus* LA5 were 2.5 and 3.1 log CFU/ml, respectively (Table 1). It was clearly seen that *L. acidophilus* LA5 had a resistance to the gastric environment over *L. casei* 01 and *B. animalis* subsp. *lactis* BB-12, respectively. Ding and Shah (2007) revealed that *L. acidophilus* NCFM was more tolerance to gastric condition (pH 2) than *Lactobacillus rhamnosus* Lr-32, *Lactobacillus salivarius* Ls-33, *L. plantarum* Lpc-37, *Lactobacillus paracasei* Lp-115, *Bifidobacterium longum* Bl-05 and *B. lactis* (Bl-04 and Bi-07). In addition, Mokarram *et al.* (2009) also found that the survival numbers of *L. acidophilus* PTCC1643 had a significantly higher than *L. rhamnosus* PTCC1637 after incubation in simulated gastric fluid (pH 1.55) for 2 h.

Moreover, after encapsulation, the results exhibited that alginate matrix could improve the survivability of probiotic cells, indicating by the increasing of *D*-value (Table 1). Our results were similar to the findings of Favaro-Trindade and Grosso (2002) and Krasaekoopt *et al*. (2004) with probiotics *L. acidophilus, L. casei, B. bifidum* and *B. lactis.* Several literatures suggested that the recoating of probiotic beads with such polymers, for instance, alginate, gelatin, poly-*L*-lysine and chitosan, could fill the porous matrix of the beads and improve the survivability of probiotic cells in the simulated gastrointestinal conditions (Nualkaekul *et al*., 2012; Chaikham *et al*., 2013a; Krasaekoopt *et al*., 2004). In this study, after recoating with various levels of alginate solution, the probiotic beads recoated with 0.3% alginate displayed a significantly higher ($P \leq 0.05$) in the survival numbers than the other treatments, indicating by the highest *D*-values (Table 1). Moreover, Mokarram *et al.* (2009) exhibited that a multilayer of alginate coating prevented acid-induced reduction of probiotics *L. acidophilus* and *L. rhamnosus* in simulated gastric environment (pH 1.5). Krasaekoopt *et al.* (2004) also found that double-coated probiotic beads with poly-*L*-lysine plus alginate could also improve the survivability of the cells as compared to uncoated beads.

Similar to the gastric experiment, *L. acidophilus* LA5 was a resistant stain under bile salt solution, following by *L. casei* 01 and *B. animalis* subsp. *lactis* BB-12 respectively under the same condition (Table 2). Buck and Gilliland (1994) reported that *L. acidophilus* was significantly better with regard to bile tolerance than other cultures such *L. casei, L. plantarum* and *Bifidobacterium* ssp. In addition, recoated probiotic beads with 0.3% alginate gave rise to significantly the highest survivability ($P \leq 0.05$) in 1.5% bile juice, following by recoating with 0.2% and 0.1%, respectively. These results were confirmed by the *D*-values of each condition (Table 2). Kim *et al.* (2008) demonstrated that alginate beads could successfully increase the survival of *L. acidophilus*

Table 1. Survival of free and encapsulated probiotic cells in gastric solution

Coating conditions	Numbers of survival cells (log CFU/ml)					*D*-value (min)
	Initial stage	0.5 h	1 h	2 h	3 h	
	Lactobacillus casei 01					
Free cells	10.8 ±0.3^{a}	8.3±0.3^{b}	5.9±0.1^{c}	4.0±0.3^{d}	2.5±0.2^{e}	21.6±0.3^{J}
Uncoated beads	9.9±0.2^{a}	8.8±0.3^{b}	7.3±0.2^{c}	5.7±0.1^{d}	5.5±0.3^{e}	39.6±1.3^{G}
Alginate 0.1%	9.7±0.1^{a}	8.7±0.1^{b}	7.7±0.1^{c}	7.2±0.1^{d}	5.8±0.0^{e}	50.5±1.6^{D}
Alginate 0.2%	9.7±0.0^{a}	8.8±0.0^{b}	7.8±0.4^{c}	7.7±0.5^{d}	5.9±0.2^{e}	53.4±1.1^{C}
Alginate 0.3%	9.7±0.2^{a}	9.5±0.1^{b}	7.9±0.2^{c}	7.5±0.2^{d}	6.8±0.4^{e}	60.3±4.8^{B}
	Lactobacillus acidophilus LA5					
Free cells	9.8±0.3^{a}	8.1±0.4^{b}	6.3±0.3^{c}	4.2±0.3^{d}	3.1±0.2^{e}	25.9±1.7^{I}
Uncoated beads	9.6±0.0^{a}	8.8±0.1^{b}	7.1±0.1^{c}	6.2±0.2^{d}	5.4±0.2^{e}	42.5±0.5^{F}
Alginate 0.1%	9.6±0.2^{a}	8.8±0.0^{b}	8.0±0.5^{c}	7.4±0.5^{d}	5.9±0.3^{e}	51.9±1.0^{CD}
Alginate 0.2%	9.6±0.1^{a}	9.3±0.3^{b}	8.3±0.2^{c}	8.1±0.0^{d}	6.1±0.2^{e}	56.9±0.4^{B}
Alginate 0.3%	9.7±0.1^{a}	9.5±0.1^{b}	8.3±0.1^{c}	7.8±0.1^{d}	7.3±0.1^{e}	73.0±3.5^{A}
	Bifidobacterium animalis subsp. *lactis* BB-12					
Free cells	11.5±0.1^{a}	8.9±0.1^{b}	4.4±0.2^{c}	1.9±0.3^{d}	nd^{e}	15.8±0.1^{K}
Uncoated beads	10.0±0.3^{a}	8.6±0.1^{b}	6.9±0.1^{c}	5.4±0.3^{d}	4.8±0.0^{e}	35.3±0.6^{H}
Alginate 0.1%	9.7±0.0^{a}	8.7±0.2^{b}	7.9±0.1^{c}	7.1±0.1^{d}	5.6±0.2^{e}	46.8±0.8^{E}
Alginate 0.2%	9.6±0.1^{a}	8.9±0.0^{b}	8.0±0.4^{c}	7.8±0.0^{d}	5.7±0.1^{e}	51.9±0.7^{CD}
Alginate 0.3%	9.7±0.5^{a}	8.9±0.0^{b}	7.9±0.5^{c}	7.7±0.1^{d}	6.2±0.4^{e}	56.5±1.3^{B}

Means in the same row followed by the same lowercase letter or means in the same column by the same capital letter indicate an insignificant difference ($P > 0.05$). nd = not detected. Each data point is the average of six replications

ATCC43121 in 0.3-0.5% bile fluids. Sultana *et al.* (2000) found alginate-encapsulated *L. acidophilus* and *L. casei* declined by 2 logs after incubation in 1-2% bile solution. The efficacy of coating materials including alginate, gelatin and chitosan on the survival of various probiotics suspended in different concentrations of bile salt was previously reported by Annan *et al.* (2008), Chávarri *et al.* (2010), Chaikham *et al.* (2013a) and Krasaekoopt and Watcharapoka (2014).

To sum up, recoated probiotic beads with 0.3% alginate showed the highest survivability in both gastric and bile fluids. Thus, this condition was chosen to recoat probiotic beads for the further storage assessment in Mao juice containing herbal extracts and antioxidants.

Viability of recoated probiotic beads in Mao juice containing herbal extracts and antioxidants

Viability of free and immobilized probiotic cells were investigated after transferring into Mao juice containing herbal plant extracts and standard antioxidants, and subsequent storing at 4°C for 30 days (Table 3). It was found that the survival numbers of free and entrapped cells in all juices markedly declined ($P \leq 0.05$) throughout the entire storage because these probiotic microorganisms were inhibited at refrigerate temperature and/or at high acidity condition under long time storage. In general, the decreasing rates of free cells were gradually higher ($P \leq 0.05$) than that of immobilized cells in all treatments because cells were prevented to adverse condition by immobilization and/or recoating. It was noticeably observed that the alginate recoated probiotic beads was efficient technique for enhancing the survivability of microorganism probiotics in acid condition of Mao juice containing different herbal extracts or different antioxidant compounds during storage at 4°C for 30 days.

In addition, the results clearly indicated that the lost cells of *L. casei* 01 had lower than *L. acidophilus* LA5 and *B. animalis* subsp. *lactis* BB-12 respectively, as compared with the same treatment. In control juice, free cells of *B. animalis* subsp. *lactis* BB-12 were not detected in all treatment after day 20 onward, whereas the survival numbers of free cells of *L. casei* 01 and *L. acidophilus* LA5 were 2.4-6.2 and 2.2-4.2 log CFU/ml, respectively at 20 days of storage. In addition, no detectable of free cells of all stains in day 30 were observed, except free cells of *L. casei* 01 in Mao juice containing cashew flower, ascorbic acid or α-tocopherol and free cells of *L. acidophilus* LA5 in Mao juice containing cashew flower or ascorbic acid.

The numbers of recoated probiotic beads of *L. casei* 01, *L. acidophilus* LA5 and *B. animalis* subsp. *lactis* BB-12 in the juices were beyond the minimum dose (10^6 CFU/ml) in order to achieve the claimed health benefits at 20, 15 and 10 days of storage, respectively. This suggested that *L. casei* 01 was more tolerant to chilling stage and acidity of fruit juice than the other cultures. Previously, Chaikham and Apichartsrangkoon (2012) depicted that the numbers of free and coated *L. casei* 01 cells in

Table 2. Survival of free and encapsulated probiotic cells in bile salt solution

Coating conditions	Numbers of survival cells (log CFU/ml)					*D*-value (min)
	Initial stage	1.5 h	3 h	4.5 h	6 h	
	Lactobacillus casei 01					
Free cells	11.6±0.1^{a}	9.8±0.11^{b}	7.8±0.0^{c}	6.7±0.11^{d}	5.5±0.1^{e}	58.6±1.2^{I}
Uncoated beads	9.9±0.2^{a}	9.2±0.1^{b}	7.6±0.2^{c}	6.8±0.1^{d}	6.0±0.0^{e}	88.0±1.2^{F}
Alginate 0.1%	9.7±0.2^{a}	9.0±0.2^{b}	8.2±0.1^{c}	7.2±0.3^{d}	6.4±0.2^{e}	107.8±6.4^{D}
Alginate 0.2%	9.7±0.1^{a}	9.0±0.1^{b}	8.0±0.2^{c}	7.6±0.2^{d}	6.6±0.2^{e}	114.6±2.1^{BC}
Alginate 0.3%	9.8±0.0^{a}	9.2±0.3^{b}	9.0±0.3^{c}	7.7±0.6^{d}	6.6±0.4^{e}	118.0±5.5^{AB}
	Lactobacillus acidophilus LA5					
Free cells	9.8±0.2^{a}	8.6±0.0^{b}	7.9±0.2^{c}	6.8±0.2^{d}	5.7±0.2^{e}	90.6±5.0^{F}
Uncoated beads	9.6±0.1^{a}	9.2±0.3^{b}	8.0±0.0^{c}	7.1±0.1^{d}	6.3±0.2^{e}	101.6±3.1^{E}
Alginate 0.1%	9.7±0.1^{a}	9.0±0.1^{b}	8.2±0.1^{c}	7.3±0.4^{d}	6.5±0.1^{e}	112.0±3.1^{CD}
Alginate 0.2%	9.6±0.3^{a}	9.0±0.3^{b}	8.3±0.1^{c}	7.5±0.2^{d}	6.5±0.3^{e}	118.2±3.0^{AB}
Alginate 0.3%	9.8±0.0^{a}	9.1±0.2^{b}	8.4±0.2^{c}	7.5±0.1^{d}	6.8±0.4^{e}	120.8±3.2^{A}
	Bifidobacterium animalis subsp. *lactis* BB-12					
Free cells	10.1±0.1^{a}	9.0±0.0^{b}	6.3±0.2^{c}	5.1±0.4^{d}	3.2±0.5^{e}	50.8±1.6^{J}
Uncoated beads	10.0±0.4^{a}	7.7±0.1^{b}	6.2±0.3^{c}	5.6±0.2^{d}	5.2±0.1^{e}	62.9±2.8^{H}
Alginate 0.1%	9.7±0.0^{a}	9.0±0.2^{b}	8.5±0.2^{c}	7.1±0.2^{d}	5.1±0.2^{e}	80.1±2.9^{G}
Alginate 0.2%	9.8±0.3^{a}	9.2±0.1^{b}	8.7±0.5^{c}	7.2±0.1^{d}	6.2±0.2^{e}	91.6±3.6^{F}
Alginate 0.3%	9.7±0.1^{a}	9.2±0.3^{b}	8.7±0.1^{c}	7.2±0.2^{d}	6.3±0.4^{e}	108.8±2.8^{D}

Means in the same row followed by the same lowercase letter or means in the same column by the same capital letter indicate an insignificant difference ($P > 0.05$). Each data point is the average of six replications.

pressurized and pasteurized longan juices (pH ~ 6.51) were > 10^6 CFU/ml during refrigerated storage at 4°C for 28 days. Krasaekoopt *et al*. (2008) noticed that encapsulated *L. casei* 01 displayed a better survival in fruit juices, such as grape, pineapple, apple and orange juices, than encapsulated *L. acidophilus* TISTR 450 and Champagne and Gardner (2008) also found that *L. rhamnosus* in a commercial fruit drink could more resist under cold storage for 80 days than *L. acidophilus*. Additionally, Nualkaekul *et al*. (2012, 2013) discovered that recoated probiotic capsules with chitosan, gelatin and glucomannan gave the increase of survival rates of *L. plantarum* and *B. longum* in fruit juices during keeping at 4°C for 6 weeks.

Survivability of free and entrapped probiotic bacteria suspended in Mao juice containing some herbal extracts and antioxidant reagents was determined. Table 3 depicts that cashew flower extract, green tea extract and ascorbic acid apparently improved ($P \leq 0.05$) the stability of all probiotic cultures in Mao juice as compared to the control, whereas roselle extract and α-tocopherol had no impact. The most effective herbal extracts in preserving probiotics were cashew flower extract followed by green tea extract, while roselle extract did show an inhibitory effect against to the probiotic cultures. According to Shah *et al*. (2010)'s study, which examined the survivability of probiotics including *L. rhamnosus* HN001, *B. lactis* HN001 and *L. paracasei* LPC 37 in a model fruit juice supplemented with white grape seed and green tea extracts during storage at 4°C for 6 weeks. They reported that green tea extract markedly enhanced the viability of those cultures throughout the entire storage, as compared to the rest. Similar finding was obtained by López de Lacey *et al.* (2014) with three probiotic strains of *L. paracasei* LAFTI-L26, *L. acidophilus* LAFTI-L10 and *B. animalis* ssp. *lactis* LAFTI-B94 during incubation in green tea extract for 72 h. Up to date, no literature has been published about the survival of probiotics in fruit juice containing cashew flower extract. Chunthanom *et al.* (2013) reported that cashew flower extract contained high amount of total phenolics and antioxidant capacity. Thus, cashew flower extract might be creates a more favorable anaerobic environment for probiotic bacteria due to its oxygen-scavenging and antioxidant properties (Shah *et al*., 2010). Tripathi and Giri (2014) established that oxygen content is an important factor affecting the viability of probiotics especially during the storage period. Oxygen affects probiotics in three ways; (i) it is directly toxic to some cells, (ii) certain cultures produce toxic peroxides in the presence of oxygen and (iii) free radicals produced from the oxidation of components are toxic to probiotic cells (Korbekandi *et al*., 2011).

Concerning the addition of vitamins, ascorbic acid (vitamin C) could maintain the viability of probiotic cells greater ($P \leq 0.05$) than α-tocopherol (vitamin E). Our results were in accordance with the findings of Shah *et al*. (2010) with *L. rhamnosus*, *B. lactis* and *L. paracasei* in the model fruit juices. Dave and Shah (1997) reported that an addition of ascorbic acid in yoghurt could have a protecting effect on probiotic cells during storage. They also explained that ascorbic acid is an oxygen scavenger, thus

Table 3. Survival of free and encapsulated probiotic cells in Mao juice containing herbal extracts and antioxidants during refrigerated storage at 4°C

Herbal extracts or antioxidants	Cell conditions	Numbers of survival cells (log CFU/ml)							Cell loss (log cycle)
		Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	
	Lactobacillus casei 01								
No added	Free cells	9.4±0.3^{a}	8.6±0.4^{b}	7.7±0.3^{c}	6.2±0.1^{d}	4.2±0.6^{e}	2.1±0.2^{f}	nd^{g}	9.4
	Encapsulated cells	9.6±0.2^{a}	9.0±0.0^{b}	8.3±0.3^{c}	7.9±0.2^{d}	6.2±0.4^{e}	4.8±0.2^{f}	4.2±0.5^{f}	5.4
Cashew flower	Free cells	9.5±0.1^{a}	8.4±0.2^{b}	8.3±0.2^{c}	7.1±0.2^{d}	6.2±0.4^{e}	3.7±0.4^{f}	1.8±0.4^{g}	7.7
	Encapsulated cells	9.6±0.1^{a}	9.0±0.2^{b}	8.6±0.4^{c}	8.1±0.3^{d}	6.9±0.3^{e}	6.2±0.2^{f}	4.9±0.5^{g}	4.7
Roselle	Free cells	9.5±0.1^{a}	6.6±1.1^{b}	6.5±1.0^{c}	5.2±0.2^{d}	2.4±0.4^{e}	1.0±0.3^{f}	nd^{g}	9.5
	Encapsulated cells	9.6±0.1^{a}	8.8±0.3^{b}	8.0±0.0^{c}	7.4±0.5^{d}	5.7±0.3^{e}	4.3±0.2^{f}	4.0±0.5^{f}	5.6
Green tea	Free cells	9.5±0.2^{a}	8.6±0.5^{b}	8.3±0.2^{c}	5.2±0.4^{d}	4.6±0.5^{e}	2.9±0.5^{f}	1.8±0.2^{g}	7.7
	Encapsulated cells	9.5±0.1^{a}	8.8±0.3^{b}	8.4±0.2^{c}	7.8±0.4^{d}	6.0±0.4^{e}	4.9±0.2^{f}	4.4±0.2^{g}	5.1
Ascorbic acid	Free cells	9.4±0.2^{a}	8.5±0.5^{b}	8.1±0.2^{c}	7.2±0.4^{d}	6.0±0.1^{e}	4.5±0.9^{f}	2.5±0.7^{g}	6.9
	Encapsulated cells	9.6±0.0^{a}	8.9±0.3^{b}	8.6±0.3^{c}	8.1±0.1^{d}	7.0±0.2^{e}	5.9±0.2^{f}	4.4±0.6^{g}	5.2
α-Tocopherol	Free cells	9.4±0.2^{a}	7.0±0.3^{b}	6.1±0.4^{c}	5.4±0.3^{d}	4.2±0.3^{e}	1.0±0.1^{f}	nd^{g}	9.4
	Encapsulated cells	9.6±0.2^{a}	8.2±0.2^{b}	7.6±0.2^{c}	6.4±0.4^{d}	5.7±0.3^{e}	4.5±0.4^{f}	4.0±0.2^{f}	5.6
	Lactobacillus acidophilus LA5								
No added	Free cells	9.6±0.1^{a}	7.5±0.4^{b}	7.0±0.0^{c}	4.7±0.4^{d}	3.1±0.1^{e}	1.8±0.2^{f}	nd^{g}	9.6
	Encapsulated cells	9.5±0.1^{a}	8.5±0.1^{b}	7.3±0.2c	6.5±0.2^{d}	5.4±0.3^{e}	4.3±0.1^{f}	3.1±0.1^{g}	6.4
Cashew flower	Free cells	9.5±0.2^{a}	7.7±0.2^{b}	7.2±0.0^{c}	5.8±0.3^{d}	4.1±0.1^{e}	2.2±0.2^{f}	1.2±0.4^{g}	8.3
	Encapsulated cells	9.6±0.1^{a}	8.4±0.2^{b}	7.5±0.3^{c}	6.9±0.3^{d}	6.2±0.2^{e}	5.9±0.4^{f}	4.4±0.2^{g}	5.2
Roselle	Free cells	9.5±0.2^{a}	6.5±0.4^{b}	5.8±0.1^{c}	4.4±0.3^{d}	2.2±0.2^{e}	1.4±0.1^{f}	nd^{g}	9.5
	Encapsulated cells	9.5±0.2^{a}	8.2±0.0^{b}	7.1±0.3^{c}	6.2±0.2^{d}	5.1±0.1^{e}	4.2±0.1^{f}	3.9±0.2^{f}	5.6
Green tea	Free cells	9.5±0.2^{a}	7.7±0.2^{b}	7.1±0.2^{c}	4.4±0.5^{d}	3.8±0.4^{e}	1.6±0.3^{f}	nd^{g}	9.5
	Encapsulated cells	9.6±0.2^{a}	8.5±0.4^{b}	7.5±0.0^{c}	6.6±0.1^{d}	5.9±0.2^{e}	4.8±0.1^{f}	4.1±0.5^{g}	5.5
Ascorbic acid	Free cells	9.5±0.0^{a}	8.1±0.2^{b}	7.3±0.1^{c}	5.4±0.5^{d}	4.2±0.2^{e}	2.3±0.2^{f}	1.0±0.2^{g}	8.5
	Encapsulated cells	9.6±0.1^{a}	8.4±0.2^{b}	7.6±0.2^{c}	7.1±0.2^{d}	6.1±0.2^{e}	4.7±0.1^{f}	3.6±0.3^{g}	6.0
α-Tocopherol	Free cells	9.6±0.0^{a}	6.8±0.2^{b}	5.9±0.1^{c}	4.9±0.2^{d}	3.7±0.3^{e}	2.2±0.2^{f}	nd^{g}	9.6
	Encapsulated cells	9.5±0.2^{a}	8.3±0.2^{b}	7.5±0.2^{c}	6.7±0.2^{d}	5.1±0.5^{e}	4.4±0.3^{f}	3.3±0.3^{g}	6.2
	Bifidobacterium animalis subsp. *lactis* BB-12								
No added	Free cells	9.5±0.1^{a}	6.8±0.6^{b}	3.8±0.2^{c}	1.3±0.2^{d}	nd^{e}	nd^{e}	nd^{e}	9.5
	Encapsulated cells	9.7±0.1^{a}	8.0±0.2^{b}	6.4±0.1^{c}	5.3±0.1^{d}	4.4±0.2^{e}	3.2±0.3^{f}	2.0±0.3^{g}	7.7
Cashew flower	Free cells	9.5±0.1^{a}	6.8±0.2^{b}	4.3±0.2^{c}	2.9±0.3^{d}	1.4±0.4^{e}	nd^{f}	nd^{f}	9.5
	Encapsulated cells	9.5±0.3^{a}	8.0±0.3^{b}	6.8±0.2^{c}	6.1±0.1^{d}	4.8±0.2^{e}	3.1±0.4^{f}	2.8±0.2^{f}	6.7
Roselle	Free cells	9.6±0.2^{a}	6.3±0.2^{b}	3.1±0.1^{c}	1.7±0.6^{d}	nd^{e}	nd^{e}	nd^{e}	9.6
	Encapsulated cells	9.6±0.3^{a}	7.4±0.2^{b}	6.6±0.5^{c}	5.1±0.8^{d}	4.7±0.3^{e}	3.3±0.2^{f}	2.1±0.3^{g}	7.5
Green tea	Free cells	9.6±0.3^{a}	6.6±0.2^{b}	4.0±0.4^{c}	2.4±0.1^{d}	0.4±0.2^{e}	nd^{f}	nd^{f}	9.6
	Encapsulated cells	9.5±0.2^{a}	7.7±0.6^{b}	6.1±0.2^{c}	5.4±0.4^{d}	4.4±0.1^{e}	3.6±0.4^{f}	2.4±0.1^{g}	7.1
Ascorbic acid	Free cells	9.5±0.1^{a}	6.7±0.3^{b}	4.4±0.2^{c}	2.8±0.1^{d}	0.6±0.3^{e}	nd^{f}	nd^{f}	9.5
	Encapsulated cells	9.5±0.1^{a}	8.1±0.2^{b}	6.6±0.3^{c}	6.1±0.2^{d}	4.8±0.2^{e}	3.8±0.2^{f}	2.6±0.2^{g}	6.9
α-Tocopherol	Free cells	9.6±0.2^{a}	5.5±0.0^{b}	4.1±0.2^{c}	2.6±0.4^{d}	nd^{e}	nd^{e}	nd^{e}	9.6
	Encapsulated cells	9.7±0.2^{a}	6.9±0.6^{b}	6.2±0.3^{c}	5.8±0.1^{d}	4.6±0.2^{e}	3.5±0.3^{f}	2.1±0.2^{g}	7.6

Means in the same row followed by the same lowercase letter indicate an insignificant difference ($P > 0.05$). nd = not detected. Each data point is the average of six replications

promoting a more favorable anaerobic environment. Shah *et al.* (2010) stated that α-tocopherol is a lipid soluble vitamin, which did not mix well with fruit juice; therefore, its antioxidant potential may not have been utilized by the cultures.

Changes of pH of herbal-Mao juices fortified with free and encapsulated probiotics

As presented in Table 4, pH of Mao juice containing roselle extract was lower than that of other juices. The pH values of all juices were trended apparently ($P \leq 0.05$) to decrease throughout the entire storage. This might be primarily due to lactic acid bacteria utilized sugars and released acids to produce ATP in maintenance cells, but not for growth. This result was previously confirmed by our report with pressurized and pasteurized longan juices combined with free and entrapped probiotic bacteria (Chaikham and Apichartsrangkoon, 2012). Moreover, Krasaekoopt and Watcharapoka (2014) found that total acidity of yoghurt and orange juice containing encapsulated probiotics slightly increased during storage at 4°C for 4 weeks. Vice versa, Nualkaekul *et al.* (2012) and Ying *et al.* (2013) elucidated that pH of pomegranate and apple juices fortified with encapsulated probiotics had no change.

Conclusion

The recoated capsules with 0.3% alginate showed the best protective effect for probiotics tested under the adverse environments of gastric and bile solutions. Thus, this condition was selected to recoat probiotic beads for the storage assessment in Mao juice combining with various herbal extracts or antioxidant reagents. Our results illustrated that cashew flower extract, green tea extract and ascorbic acid could improve the stability of probiotics in Mao

Table 4. Changes of pH in herbal-Mao juices fortified with free and encapsulated probiotics during refrigerated storage at 4°C

Herbal extracts or antioxidants	Cell conditions	Change of pH during storage at 4°C						
		Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
	Lactobacillus casei 01							
No added	Free cells	3.43±0.02^{a}	3.36±0.01^{b}	3.34±0.00^{c}	3.30±0.02^{d}	3.29±0.01^{d}	3.27±0.02^{de}	3.25±0.00^{e}
	Encapsulated cells	3.41±0.02^{a}	3.40±0.01^{a}	3.38±0.01^{ab}	3.36±0.02^{b}	3.34±0.03^{b}	3.30±0.00^{c}	3.30±0.00^{c}
Cashew flower	Free cells	3.40±0.02^{a}	3.35±0.02^{b}	3.32±0.03^{bc}	3.30±0.02^{c}	3.28±0.02^{c}	3.28±0.03^{cd}	3.24±0.01^{d}
	Encapsulated cells	3.40±0.01^{a}	3.38±0.03^{ab}	3.35±0.02^{b}	3.34±0.02^{b}	3.33±0.02^{b}	3.31±0.03^{bc}	3.30±0.00^{c}
Roselle	Free cells	3.25±0.01^{a}	3.20±0.02^{b}	3.19±0.02^{b}	3.19±0.01^{b}	3.16±0.01^{c}	3.15±0.01^{c}	3.15±0.01^{c}
	Encapsulated cells	3.26±0.03^{a}	3.24±0.01^{a}	3.22±0.04^{ab}	3.20±0.01^{b}	3.19±0.02^{bc}	3.17±0.01^{c}	3.16±0.02^{c}
Green tea	Free cells	3.39±0.01^{a}	3.39±0.00^{a}	3.34±0.02^{b}	3.30±0.02^{b}	3.25±0.02^{c}	3.20±0.01^{d}	3.17±0.01^{e}
	Encapsulated cells	3.42±0.01^{a}	3.39±0.02^{ab}	3.36±0.01^{b}	3.35±0.01^{b}	3.31±0.02^{c}	3.28±0.01^{d}	2.24±0.03^{d}
Ascorbic acid	Free cells	3.38±0.02^{a}	3.35±0.01^{b}	3.32±0.02^{b}	3.30±0.05^{bc}	3.28±0.04^{bc}	3.22±0.04^{cd}	3.18±0.02^{d}
	Encapsulated cells	3.36±0.03^{a}	3.34±0.01^{a}	3.33±0.03^{a}	3.33±0.02^{a}	3.30±0.03^{ab}	3.28±0.02^{b}	3.26±0.01^{b}
α-Tocopherol	Free cells	3.39±0.01^{a}	3.35±0.02^{b}	3.30±0.02^{c}	3.28±0.03^{c}	3.28±0.01^{c}	3.27±0.02^{c}	3.22±0.00^{d}
	Encapsulated cells	3.38±0.02^{a}	3.35±0.02^{ab}	3.34±0.01^{b}	3.33±0.02^{b}	3.32±0.01^{b}	3.30±0.03^{bc}	3.25±0.02^{c}
	Lactobacillus acidophilus LA5							
No added	Free cells	3.40±0.01^{a}	3.38±0.02^{ab}	3.36±0.02^{b}	3.32±0.01^{c}	3.30±0.02^{c}	3.25±0.02^{d}	3.23±0.04^{d}
	Encapsulated cells	3.41±0.01^{a}	3.40±0.00^{a}	3.36±0.01^{b}	3.34±0.01^{b}	3.32±0.01^{c}	3.30±0.02^{cd}	3.28±0.01^{d}
Cashew flower	Free cells	3.41±0.02^{a}	3.35±0.02^{b}	3.32±0.01^{b}	3.30±0.01^{c}	3.28±0.00^{d}	3.26±0.02^{e}	3.25±0.01^{e}
	Encapsulated cells	3.39±0.02^{a}	3.37±0.03^{ab}	3.35±0.01^{b}	3.33±0.03^{bc}	3.31±0.00^{c}	3.30±0.01^{c}	3.29±0.02^{c}
Roselle	Free cells	3.30±0.02^{a}	3.30±0.01^{a}	3.28±0.02^{a}	3.24±0.00^{b}	3.20±0.03^{c}	3.17±0.00^{c}	3.17±0.02^{c}
	Encapsulated cells	3.29±0.03^{ab}	3.30±0.01^{a}	3.30±0.02^{a}	3.26±0.02^{b}	3.24±0.03^{bc}	3.22±0.01^{c}	3.20±0.01^{c}
Green tea	Free cells	3.42±0.02^{a}	3.38±0.01^{b}	3.35±0.02^{bc}	3.32±0.02^{c}	3.28±0.01^{d}	3.25±0.01^{e}	3.24±0.03^{e}
	Encapsulated cells	3.41±0.02^{a}	3.41±0.03^{a}	3.35±0.01^{b}	3.34±0.01^{b}	3.30±0.02^{c}	3.29±0.04^{cd}	2.25±0.00^{d}
Ascorbic acid	Free cells	3.41±0.01^{a}	3.32±0.02^{b}	3.30±0.00^{b}	3.30±0.02^{b}	3.28±0.01^{c}	3.26±0.02^{cd}	3.25±0.01^{d}
	Encapsulated cells	3.41±0.03^{a}	3.38±0.02^{ab}	3.35±0.01^{b}	3.32±0.01^{c}	3.31±0.01^{cd}	3.29±0.01^{d}	3.28±0.03^{d}
α-Tocopherol	Free cells	3.38±0.01^{a}	3.34±0.02^{b}	3.31±0.00^{c}	3.30±0.04^{c}	3.29±0.01^{c}	3.25±0.02^{d}	3.22±0.02^{d}
	Encapsulated cells	3.40±0.03^{a}	3.35±0.01^{b}	3.32±0.00^{c}	3.31±0.02^{cd}	3.30±0.03^{cd}	3.30±0.00^{d}	3.26±0.02^{e}
	Bifidobacterium animalis subsp. *lactis* BB-12							
No added	Free cells	3.41±0.03^{a}	3.37±0.02^{ab}	3.34±0.02^{b}	3.30±0.00^{c}	3.28±0.01^{d}	3.28±0.02^{d}	3.26±0.01^{e}
	Encapsulated cells	3.42±0.01^{a}	3.40±0.02^{ab}	3.40±0.03^{ab}	3.38±0.01^{b}	3.35±0.01^{c}	3.32±0.00^{d}	3.32±0.01^{d}
Cashew flower	Free cells	3.40±0.02^{a}	3.36±0.01^{b}	3.35±0.02^{b}	3.32±0.01^{c}	3.30±0.03^{cd}	3.29±0.01^{d}	3.28±0.03^{d}
	Encapsulated cells	3.40±0.02^{a}	3.39±0.03^{a}	3.38±0.02^{ab}	3.35±0.02^{bc}	3.33±0.02^{c}	3.30±0.01^{d}	3.30±0.03^{d}
Roselle	Free cells	3.30±0.02^{a}	3.28±0.04^{ab}	3.25±0.02^{b}	3.24±0.01^{bc}	3.21±0.02^{c}	3.20±0.02^{d}	3.20±0.01^{d}
	Encapsulated cells	3.31±0.03^{a}	3.29±0.02^{a}	3.28±0.01^{ab}	3.24±0.04^{b}	3.22±0.03^{bc}	3.22±0.04^{bc}	3.20±0.02^{c}
Green tea	Free cells	3.39±0.02^{a}	3.35±0.02^{ab}	3.32±0.02^{b}	3.30±0.00^{c}	3.26±0.01^{d}	3.25±0.02^{de}	3.23±0.00^{e}
	Encapsulated cells	3.42±0.02^{a}	3.38±0.02^{ab}	3.35±0.01^{b}	3.34±0.02^{b}	3.30±0.00^{c}	3.28±0.03^{cd}	2.26±0.02^{d}
Ascorbic acid	Free cells	3.38±0.01^{a}	3.35±0.00^{b}	3.32±0.01^{c}	3.29±0.01^{d}	3.26±0.00^{e}	3.25±0.02^{ef}	3.24±0.01^{f}
	Encapsulated cells	3.40±0.03^{a}	3.39±0.01^{a}	3.38±0.03^{ab}	3.36±0.01^{b}	3.32±0.01^{c}	3.30±0.01^{cd}	3.28±0.02^{d}
α-Tocopherol	Free cells	3.39±0.02^{a}	3.35±0.02^{ab}	3.33±0.02^{b}	3.30±0.00^{c}	3.27±0.01^{d}	3.27±0.01^{d}	3.25±0.02^{d}
	Encapsulated cells	3.38±0.02^{a}	3.38±0.01^{a}	3.35±0.02^{ab}	3.32±0.03^{b}	3.30±0.02^{c}	3.29±0.04^{c}	3.29±0.01^{c}

Means in the same row followed by the same lowercase letter indicate an insignificant difference ($P > 0.05$). Each data point is the average of six replications.

juice, whilst roselle extract and α-tocopherol had no impact.

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