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Survival of *Lactobacillus acidophilus* TISTR1338 and *Lactobacillus casei* TISTR390 in probiotic Gac ice cream

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Abstract

Ice cream is a kind of dairy products consumed all over the world and most popular among children and adults. However, ice cream available commercially is generally poor in natural antioxidants and vitamins. Thus, it is of interest to improve the nutritional values of ice cream using spiny bitter cucumber (Gac) which is a good source of lycopene and β-carotene along with probiotic bacteria. First, the survival of two different strains of probiotic bacteria including *L. acidophilus* TISTR1338 and *L. casei* TISTR390 in Gac ice cream was determined during the freezing and hardening steps and it was found that there was no significant difference in % survival of both probiotic bacteria (P>0.05) as *L. acidophilus* TISTR1338 and *L. casei* TISTR390 had % survival of 95.77 and 94.90 respectively. Then, survival promoting substances for probiotic bacteria in Gac ice cream including cryoprotectant as UnipectineTM RS 150 at 0.5%, 1.5%, and 2.5% and cells encapsulation with sodium alginate at 1%, 2%, and 3% on % survival of probiotic bacteria i.e. *L. casei* TISTR390 were investigated. It was found that cryoprotectant as Unipectine™ RS 150 at 0.5%, 1.5%, and 2.5% contributed % survival of 100.42, 99.94, and 100.37 respectively which were not significantly different from control (0%) with % survival of 99.95 (P>0.05). For cells encapsulation with sodium alginate at 1%, 2%, and 3%, it was found that the probiotic survival was 99.25, 99.06, and 99.11% respectively which were not significantly different from control (0%) with the % survival of 99.54 (P>0.05). Also, all five formulas of probiotic Gac ice cream were sensory evaluated for liking scores using nine points hedonic scales (1-9) with 50 untrained panelists and it was found that the attributes of appearance, color, odor, taste, texture, and overall liking had the significantly different scores (P≤0.05). Moreover, the probiotic Gac ice cream with cryoprotectant had overall liking score of 6.08 which was higher than the other formulas but was not significantly different from Gac ice cream (P>0.05). Furthermore, the survival of probiotic bacteria in Gac ice cream during storage at -20°C for 8 weeks was determined and it was found that when storage time increased, the numbers of probiotic bacteria and pH tended downwards while % acidity tended upwards. Finally, by the end of 8th week, the probiotic survival numbers in Gac ice cream were significantly different (P≤0.05) as probiotic Gac ice cream with cryoprotectant had the highest survival of probiotic *L. casei* TISTR 390 with the number of 8.40 log cfu/g.

Introduction

The markets for foods that provide nutritional function and new eating experiences for consumers have grown rapidly in recent years. Ice cream is one of the most consumed dairy products in the world but the ice cream available commercially is generally poor in antioxidants and vitamins (Sun-Waterhouse *et al.*, 2013). Thus, it is of interest to explore the possibility of improving the nutritional attributes of ice cream using ingredients with health benefits, focusing on lycopene from Gac (Spiny Bitter Cucumber) and probiotic bacteria.

Probiotics are defined as live microorganisms which, when administered in adequate amounts confer a health benefit to the consumers (Homayouni *et al.*, 2008a). Ice cream could be an alternate food vehicle to deliver probiotics to consumers. A minimum of 10^7 probiotic bacterial cells per gram of the product should be alive at the time of consumption according to the standard (Sultana *et al.*, 2000; Homayouni *et al.*, 2008a).

Cryoprotectants can be added to maintain the viability of probiotic organisms during freeze-drying. Compatible cryoprotectants may be added to media or into the yoghurt mix prior to fermentation to assist in the adaptation of probiotics to the environment. As compatible cryoprotectants accumulate within

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the cells, the osmotic difference with their external environment is reduced (Capela *et al*., 2006). The cryoprotectants can reduce the bacterial cell damage during freeze drying and promote the formation of an amorphous state in cells rather than ice crystals during cooling cryostorage-warming cycle (Kanmani *et al*., 2011)

Microencapsulation of probiotics in hydrocolloid beads has been tested for improving their viability in food products and the gastrointestinal tract (Krasaekoopt *et al*., 2003; Mandal *et al*., 2006). Microencapsulation is a process where the cells are retained within the encapsulating material in order to reduce cell injury or cell loss. Encapsulation of lactic starter cultures is of interest to the dairy industry since it improves the control of the fermentation process. The advantage of encapsulation using natural polymers is that the reagents are non-toxic and the matrices formed are gentle to the microorganisms (Shah and Ravula, 2000). Alginate beads have been found to increase the survival of probiotics to 80–95% (Krasaekoopt *et al*., 2003) and alginate was the most widely used matrix for microencapsulation (Kailasapathy, 2002). Encapsulated probiotic bacteria can be used in many dairy products such as yoghurt, cheese, cultured cream and frozen dairy desserts (Shah and Ravula, 2000). The ice cream could be used as a good source for delivering these probiotic bacteria to the consumers (Hekmat and Mcmahon, 1992; Akin *et al*., 2007; Homayouni *et al*., 2008a).

Gac (Spiny Bitter Cucumber) with a scientific name as *Momordica cochinchinensis* Spreng is a tropical plant grown in many countries in tropical regions. Gac aril has been shown to be especially high in lycopene and beta carotene content (Aoki *et al*., 2002) which are antioxidants (Mai *et al*., 2013). They have been linked with a lower risk of prostate cancer, coronary heart disease and eye diseases (Kha *et al*., 2010; Nhung *et al*., 2010; Phan-Thi and Wache, 2014) Thus, the production of ice cream supplemented with a Gac aril and probiotics to increase the products nutritional value is explored. The objectives of this study were to investigate the survival of probiotic bacteria during the production of Gac ice cream. Also, the effects of cryoprotectant and microencapsulation technique on survival enhancement of probiotic bacteria in Gac ice cream were monitored during production and storage at -20°C.

Materials and Methods

Preparation of activated probiotic cultures

Pure freeze-dried probiotic cultures of *L. acidophilus* TISTR 1338 and *L. casei* TISTR390 were obtained from Mircen (Bangkok, Thailand) and activated by inoculating in MRS broth and incubating at 37°C for 48 h. Then they were subcultured into MRS broth for 2-3 times prior to use.

Ice cream production

Batches of Gac ice cream were produced following the method reported by Nousia *et al.* (2011) with slight modifications. Milk (23.33%) and fresh cream (6.22%) were mixed and temperature was increased to 50°C. For each mix, 2.07% skim milk powder, 0.1% xanthan gum (stabilizer), 0.16% tween 80 (emulsifier), 20.74% roselle juice and 47.37% Gac aril separated from clean Gac fruit were added. The mixes were pasteurized at 76°C for 20 min (Soukoulis *et al*., 2010) and after that, they were cooled to 4°C and then divided into five portions (A, B, C, D, and E). Portion A as Gac ice cream, portion B as Gac ice cream supplemented with probiotics, portion C as Gac ice cream supplemented with probiotics and cryoprotectant, portion D as Gac ice cream supplemented with encapsulated probiotics and portion E as Gac ice cream supplemented with cryoprotectant and encapsulated probiotics. Then they were aged at 4°C for 24 h. After that, they were iced in ice cream machinery (-7 to -8°C) (Gelato Pro300 NEMOX Co., Italy), hardened at -20°C for 24 h and subsequently stored at -20°C for 8 weeks.

The survival of different strains of probiotic bacteria during the production of Gac ice cream

Probiotic cultures of *L. acidophilus* TISTR1338 and *L. casei* TISTR390 in stationary phase of growth were harvested by centrifugation at 10,000 x g for 5 min at 4°C and washed twice with sterile 0.9% saline under the same centrifugation conditions. Then, 10 ml of milk were added. The milk suspended probiotic bacteria were added to the ice cream mix after aging for 24 h at 4°C. Then, the counts of viable *L. acidophilus* TISTR1338 and *L. casei* TISTR390 were enumerated before and after freezing/hardening steps.

Effects of cryoprotectants as UnipectineTM RS 150 on survival of probiotic bacteria in Gac ice cream

The cryoprotectants including UnipectineTM RS 150 (Ingredient center Co, Thailand) with levels of 0%, 0.5%, 1.5%, and 2.5% (w/w) were added to the ice cream mixes (Capela et al., 2006). The mixes were then pasteurized at 76°C for 20 min (Soukoulis *et al*., 2010) and they were cooled to 4°C and aged for 24 h. After that, the milk suspended probiotic bacteria were added before freezing step. Eventually, the counts of viable probiotic bacteria were enumerated before and after freezing/hardening steps.

Effects of cells encapsulation on survival of probiotic bacteria in Gac ice cream

All glasswares and solutions used in the experiment were sterilized at 121°C for 15 min. The extrusion technique of microencapsulation was modified from a method reported by Krasaekoopt *et al*. (2004). Solutions of sodium alginate at 1%, 2%, and 3% (w/v) in distilled water were prepared. Then, the probiotic cells were harvested, washed twice with sterile 0.9% saline and mixed with sodium alginate solutions.

The probiotic cells suspensions were injected through a 0.11 mm. needle into sterile 0.1 M $CaCl_2$. The beads were allowed to stand for 30 min for gelification, and then washed with sterile 0.9% saline containing 5% glycerol and stored at 4°C.

The beads or encapsulated probiotic bacteria were added to the ice cream mix after aging for 24 h at 4°C. Then, the counts of viable probiotic bacteria were enumerated before and after freezing/hardening steps.

Enumeration of free and encapsulated probiotic bacteria

The viability of the probiotic bacteria was determined before and after freezing and hardening steps by pour plating. Twenty five grams of ice cream were diluted in 225 ml sterile peptone water (0.1%) and serial dilutions were made to the appropriate ones. Then, 1 ml aliquot dilutions were pipetted onto plates and MRS agar was poured in duplicate. All plates of probiotic bacteria were incubated at 37°C for 48 h under aerobic conditions (Christiansen *et al*., 1996 cited in Akalin and Erisir, 2008). The averages of all results were expressed as colony-forming units per gram of sample (CFU/g) and then percentages of probiotic bacteria survival in Gac ice cream during freezing and hardening were calculated.

To count the encapsulated bacteria in ice cream, the entrapped bacteria were released from the calcium alginate beads by sequestering calcium ions with phosphate buffer at neutral pH following the method reported by Shah and Ravula (2000).

Sensory analysis

The Ice cream samples were Gac ice cream treatment A, B, C, D, and E. Ice cream samples were sensory evaluated using preference test by a panel of 50 panelists. The tests were conducted according to Nousia *et al*. (2011) for evaluations of appearance, color, odor, taste, texture and overall liking. A 9-Point hedonic scale was used, with 1 as 'not like most' and 9 as 'like most'.

Physical analysis

The % overrun of ice cream was determined using the following formula reported by Nousia *et al.* (2011):

$$\%\text{overrun} = 100 \times (\text{ice cream volume} - \text{mix volume}) \times (\text{mix volume})^{-1}$$

Meltdown was determined at 25±2°C according to Santana *et al.* (2011) as 25-30 grams of sample were placed on a sieve with 2 mm. openings, suspended over a balance and then the mass of the drained ice cream was recorded as a function of time.

Survival of probiotic bacteria in Gac ice cream during storage at –20°C for 8 weeks

The Ice cream samples including Gac ice cream treatment B, C, D, and E were stored at -20°C for 8 weeks. Meanwhile, they were sampled to determine percent survival every week. Probiotics were enumerated on plates containing 25–250 colonies. The averages of all results were expressed as colony-forming units per gram of sample (CFU/g) and percent survival of probiotic bacteria in Gac ice cream was determined during storage at -20°C for 8 weeks. Also, the pH of ice cream was measured using a digital pH-meter (FE20/FG2, Mettler Toledo, Switzerland) and % titratable acidity expressed as lactic acid was determined according to AOAC (2000).

Statistical analysis

The data were analyzed statistically using SPSS statistical software program version 17. Analysis of variance (ANOVA) and Duncan's New Multiple Range Test were used to determine significant differences among results and P values of ≤0.05 were regarded as significant.

Results and Discussion

Survival of different strains of probiotic bacteria during Gac ice cream production

Table 1 shows percent survival of probiotic bacteria during Gac ice cream production. It was found that percent survival of *L. acidophilus* TISTR1338 and *L. casei* TISTR390 was not significantly different (P>0.05) as 95.77 and 94.90 % respectively. Thus, *L. casei* was considered suitable for Gac ice cream production as it was more resistant to low temperature than *L. acidophilus* (Homayouni *et al*., 2008a). Therefore, it is likely for *L. casei* to

Table 1. Percent survival of *L. acidophilus* TISTR1338 and *L. casei* TISTR390 during Gac ice cream production

Probiotic bacteria	Survival (%)
L. acidophilus TISTR1338	95.77[a]±1.12
L. casei TISTR390	94.90[a]±1.76

[a]Means in the same column followed by the same letters are not significantly different *(P>0.05)*

survive better in Gac ice cream during storage at -20°C for 8 weeks.

Homayouni *et al.* (2008b) have shown that *L. casei* was the most resistant strain in simulated ice cream conditions. Their results showed the survival of bacteria against unfavorable conditions in ice cream such as oxygen toxicity or freezing and storage at lower temperature (-20°C). The stress factors investigated such as sucrose concentrations, oxygen, low temperatures and type of dairy foods and presence of air could affect the growth and survival of probiotic bacteria (Nighswonger *et al.*, 1996; Desai *et al*., 2004).

Effects of cryoprotectants as UnipectineTM RS 150 on survival of probiotic bacteria during Gac ice cream production

L. casei TISTR390 was chosen for Gac ice cream production as it was more resistant to low temperature. Table 2 shows the effects of cryoprotectant, UnipectineTM RS 150 on the survival of *L. casei* TISTR390 during Gac ice cream production.

It was found that Gac ice cream supplemented with UnipectineTM RS 150 at 0%, 0.5%, 1.5%, and 2.5% were not significantly different in percent survivals of probiotic bacteria which were 99.95, 100.42, 99.94, and 100.37% respectively (P>0.05). Therefore, the minimal concentration of UnipectineTM RS 150 at 0.5% was considered appropriate for Gac ice cream since it may enhance the probiotic survival during longer storage at -20°C.

Capela *et al.* (2006) had also found UnipectineTM RS150 to be a superior cryoprotectant as viable counts of *L. rhamnosus* and *L. casei* 1520 were improved by 28% and 40% respectively and the cryoprotectant could inhibit intracellular or extracellular ice formation by binding to the water. The cryoprotectants can reduce the bacterial cell damage owing to freeze drying and promote the formation of an amorphous state in cells rather than ice crystals during cooling-cryostorage-warming cycle (Son *et al*., 2004).

Survivability of free and microencapsulated probiotic bacteria during Gac ice cream production

Table 3 shows percent survival of microencapsulated *L. casei* TISTR390 with 1%, 2%, and 3% sodium alginate compared to free cells (control) during Gac ice cream production. It was found that the survival of microencapsulated *L. casei* TISTR390 was not significantly different from control (free cells) (P>0.05) with the percent survival of 99.25, 99.06, 99.11, and 99.54 % respectively.

Table 2. Percent survival of *L. casei* TISTR390 supplemented with cryoprotectant (UnipectineTM RS 150) at 0, 0.5, 1.5, and 2.5% during Gac ice cream production

Amount of Unipectine™ RS 150 (%)	Survival (%)
0 (control)	99.95 [a] ±1.18
0.5	100.42 [a] ±1.33
1.5	99.94 [a] ±1.28
2.5	100.37 [a] ±1.94

[a]Means in the same column followed by the same letters are not significantly different *(P>0.05)*

Thus, the minimal concentration of sodium alginate at 1% was considered suitable for Gac ice cream since it may subsequently enhance the probiotic survival during longer storage at -20°C. Homayouni *et al*. (2008a) had found that the survival of encapsulated probiotic bacteria (*L. casei* and *B. lactis*) with alginate in synbiotic ice cream had a reduction less than the free encapsulated cells. Shah and Ravula (2000) reported that microencapsulation improved the counts of *L. acidophilus* MJLA1 and *Bifidobacterium* spp. BDBB2 compared to free cells in frozen fermented dairy desserts stored for 12 weeks.

Sensory evaluations

The attributes mean scores of Gac ice cream were evaluated by 50 panelists (data not shown). The results indicated that the appearance, color, odor, taste, texture and overall liking were significantly different among Gac ice cream samples (P≤0.05). Probiotic Gac ice cream supplemented with cryoprotectant (treatment C) had highest overall liking scores which were not significantly different from Gac ice cream (treatment A). Moreover, Gac ice cream supplemented with microencapsulated probiotic bacteria plus cryoprotectant (treatment E) was not significantly different in overall liking from the ones supplemented with only microencapsulated probiotic (treatment D) and Gac ice cream (treatment A).

Physical properties

With regard to the physical properties of Gac

Table 3. Percent survival of microencapsulated *L. casei* TISTR390 with 1, 2 and 3% sodium alginate compared to free cells during Gac ice cream production

Amount of sodium alginate (%)	Survival (%)
0 (control)	99.54[a]±0.31
1	99.25[a]±0.57
2	99.06[a]±0.57
3	99.11[a]±0.64

[a]Means in the same column followed by the same letters are not significantly different (P>0.05)

ice cream including % overrun and % meltdown, it was shown that Gac ice cream supplemented with probiotics and cryoprotectant and Gac ice cream supplemented with microencapsulated probiotics plus cryoprotectant had highest overrun (P≤0.05) whereas Gac ice cream supplemented with probiotics and cryoprotectant had the lowest meltdown (P≤0.05) (data not shown) because cryoprotectant as UnipectineTM RS 150 was a kind of pectin which could act as stabilizer and then provide uniformity to the product and resistance to melting (Goff, 1997; Kurultay *et al*., 2010).

Muse and Hartel (2003) reported that the ice cream systems meltdown can be influenced by many factors like total solids, ice crystals size, fat globule size and low overrun. Sofjan and Hartel (2004) found that ice cream with a lower overrun value might possibly melt quicker than that made with higher overrun due to other factors, such as the difference in fat destabilization or air cell and ice crystal size.

An extremely low overrun indicating little air has been included, causing an excessively cold sensation in the mouth and lack of creaminess and smoothness. If overrun was too high, the ice cream would be frothy. % Overrun depended on the amount of fat, milk solid nonfat and solid (Sun-waterhouse *et al*., 2013).

The survival of L. casei *TISTR390 in Gac ice cream during storage at -20°C for 8 weeks*

The Ice cream samples including Gac ice cream supplemented with probiotics (control; treatment B), Gac ice cream supplemented with probiotic and cryoprotectant (treatment C), Gac ice cream supplemented with microencapsulated probiotics (treatment D) and Gac ice cream supplemented with microencapsulated probiotics plus cryoprotectant (treatment E) were stored at -20°C for 8 weeks. The survivals of *L. casei* TISTR390 in all samples were monitored during storage as shown in Figure. 1.

Table 4. Survival numbers of *L. casei* TISTR390 in Gac ice cream after 8 weeks of storage at -20°C

Samples	Log cfu/g
B	7.51[c]±0.04
C	8.40[a]±0.02
D	8.11[b]±0.07
E	8.20[b]±0.00

[a, b, c] Means in the same column followed by the same letters are not significantly different (P≤0.05)
B, Gac Ice cream supplemented with probiotics; C, Gac Ice cream supplemented with probiotics and cryoprotectant; D, Gac Ice cream supplemented with microencapsulated probiotics; E, Gac Ice cream supplemented with microencapsulated probiotics plus cryoprotectant

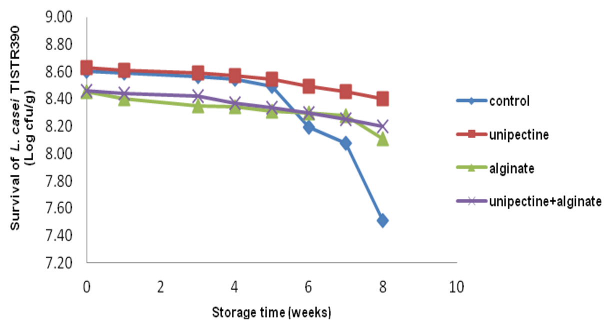

Figure 1. Survival of *L. casei* TISTR390 in Gac ice cream during 8 weeks of storage at -20°C

It was found that the bacterial counts of all treatments tended downwards with longer storage time. In case of free *L. casei* TISTR 390 (control), the cell numbers decreased about 1.09 log cfu/g after 8 weeks whereas those of probiotic Gac ice cream supplemented with cryoprotectant, Gac ice cream supplemented with microencapsulated probiotics and Gac ice cream supplemented with microencapsulated probiotics plus cryoprotectant decreased about 0.23, 0.34, and 0.26 log cfu/g respectively. In addition, the survival numbers in all samples after 8 weeks of storage were shown in Table 4. Interestingly, the samples with survival-promoting substances including cryoprotectant or cell encapsulation had survived better than free cells (control) after 8 weeks of storage (P≤0.05) and particularly, the one with only cryoprotectant had the highest survival numbers (P≤0.05).

The declines in bacterial counts, as a result of freezing, was likely due to the freeze injury of cells and mechanical stresses of the mixing and freezing process and also the incorporation of oxygen into the mixture may result in a further decrease in bacterial count (Homayouni *et al*., 2008a).

Capela *et al.* (2006) reported that the addition of cryoprotectant agent as UnipectineTM RS 150 improved the counts of *Bifidobacterium longum* and *Lactobacillus* spp. compared to the control which did not contain UnipectineTM RS 150 and the cryoprotectants can inhibit intracellular or extracellular ice formation by binding to the water. UnipectineTM RS 150 as pectin was a polysaccharide or carbohydrate which may be a food source for probiotic bacteria and enhance the survival of *L. casei* TISTR390.

Shah and Ravula (2000) reported that microencapsulation improved the counts of *L. acidophilus* MJLA1 and *Bifidobacterium* spp. BDBB2 compared to free cells in frozen fermented dairy desserts during 12 weeks storage.

Mandal *et al.* (2006) had found that the microencapsulation of *L. casei* NCDC-298 in alginate beads resulted in better survival than free cells at low pH, high bile salt concentration and heat treatment. Increasing alginate concentrations also had a positive effect on the survival of *L. casei* in simulated harsh conditions of gastrointestinal tract.

Homayouni *et al*. (2008a) had also found that the survival of encapsulated probiotic bacteria (*L. casei* and *B. lactis*) with alginate in synbiotic ice cream had a reduction less than the free encapsulated cells. Pavunc *et al.* (2011) reported the survival of encapsulated *Lactobacillus helveticus* M92 with sodium caseinate in set yoghurt. Better survival of microencapsulated than free probiotic bacteria was found in produced yoghurts during storage, as well as during exposure to simulated gastrointestinal conditions. Also, it was found that the numbers of probiotic bacteria in all samples of ice cream were above 10^7 cfu/g at the end of 8 weeks storage.

Conclusion

The survival promoting substances for probiotic bacteria (*L. casei* TISTR390) in Gac ice cream including cryoprotectant as UnipectineTM RS 150 and microencapsulation in sodium alginate could significantly improve the survival in Gac ice cream during storage. Also, the probiotic Gac ice cream with cryoprotectant did not have the effect on ice cream attributes when compared with the one without the substances. The numbers of viable *L. casei* TISTR390 remained above the recommended minimum level of 10^7 cfu/g at the end of storage.

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