
Effects of different sources of probiotic encapsulation on some physico-chemical characteristics, viability, acceptability and its microstructure in probiotic salak juice

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Chanawanno, T. and Mongkontanawat, N. (2020). Effects of different source of probiotic encapsulation on some physico-chemical characteristics, viability and acceptability and its microstructure in probiotic salak juice. *International Journal of Agricultural Technology* 16(6):1331-1348.

Abstract The results displayed that salak juice fermented with soygurt (richesse) had high acceptability with a score of 7.33 (moderately like). As a result of the living probiotic located in the capsules, there were little changes in the pH and titrable acidity which slightly decreased and then increased until fermentation reached at 30 °C in 72 hours. Interestingly, the level of the living cells of all probiotic salak juices was at the standard level by the FAO/WHO (over 6 log CFU/ml) in all treatments. This encapsulation method protected the probiotic cells; therefore, the viability of the salak juice was slightly stable. However, the addition of the probiotic calcium alginate capsules did not affect the vitamin C concentration. For the microstructure of the capsules, the SEM showed that all probiotic calcium alginate capsules were round and oval, and the probiotic cells were located in the capsules. Overall, these results could be an alternative healthy non-dairy probiotic source for vegetarians and milk-allergic consumers. Moreover, the highlight of research finding is the first novel non-dairy probiotic salak juice, which could provide health benefits from the fruit and living probiotic.

Keywords: Probiotic, Encapsulation, Salak juice

Introduction

To date, probiotics have concerned several beneficial effects for human health. Lactic acid bacteria have been proven to exert health promoting activities; such as, the adjustment of the immune response to a desired level, enhancement of resistance against pathogens, and reduction of blood cholesterol levels (Herich and Levkut, 2002; Ljungh and Wadstrom, 2006). Usually, available probiotic products on the market nowadays are dairy-based. Consequently, they cannot be consumed by individuals who suffer lactose intolerance and milk protein allergies. Moreover, the number of vegan

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consumers is increasing, as well as the demand for vegan probiotic products (Nematollahi *et al.*, 2016; Sharma and Misha, 2008). In addition, fruit juice is reported to be rich in nutrients, has a high amount of minerals, vitamins, dietary fibre and antioxidants, which could encourage probiotic growth, and is beneficial for health (Columbo *et al.*, 2019; Ding and Shan, 2008). With regards to the term of probiotics, the Food and Agriculture Organisation/World Health Organisation (FAO/WHO) have defined this as “live microorganisms” which, when administered in adequate amounts, confer a health benefit on the host (Reddy *et al.*, 2018). Thus, this would provide beneficial effects to the host when administered in adequate amounts ($>10^6$ CFU/ml) (Columbo *et al.*, 2019). From a previous report, probiotic encapsulation increased the number of live probiotics when compared with free cells (Zhao *et al.*, 2008). In recent years, encapsulation in an alginate extrusion method has greatly enhanced the survival of probiotic bacteria against an artificial gastric digestive system (Ortakci and Sert, 2012). This technique was a method for the encapsulation of probiotic bacteria. Additionally, alginate is a natural heteropolysaccharide composed of D-mannuronic and L-guluronic acid residues joined linearly by (1-4) glycosides linkages, which are popularly used and safe for this technique (Mokarram *et al.*, 2009).

Salak (snake fruit), *Salacca zalacca*, is a unique tropical palm fruit in Southeast Asia, especially found in Thailand, Indonesia and Malaysia. For its nutritional profile, this fruit is higher in antioxidants, phenolics, vitamins and minerals than mango, kiwi fruit, and apple (Mazumdar *et al.*, 2019). Furthermore, the fruit’s flesh and peel have shown to exhibit some tremendous antioxidant, anti-inflammatory, anticancer and antidiabetic potential (Saleh *et al.*, 2018). For the interesting flavour, salak has become widely studied because of its complex aroma with hints of pineapple, citrus and honey, and sweet taste. From this point, salak was chosen to produce fruit tea by mixing it with pineapple (*Ananas comosus*) and longan (*Dimocarpus longan* Lour.) in the ratio of 20:20:60 and then adding 20% w/v of medicinal mushroom (*Schizophyllum commune*) in order to increase the health benefits in the researcher’s previous study (Mongkontanawat, 2013). Moreover, Zubaidah *et al.* (2018) found that salak Kombucha fermentation enhanced antioxidant activity, which was consistent with an increase in phenolics, tannins and flavonoids. In this fermentation, acetic acid was the major organic acid of this fermented product and showed an enhanced antibacterial activity by inhibiting Gram-positive and Gram-negative pathogens.

On the other hand, there was no reported in the investigation of the effect of different sources of probiotic encapsulation on some physico-chemical characteristics, viability, acceptability and its microstructure in probiotic salak

juice. To fill this gap, the aim of this study was to evaluate changes in some physico-chemical characteristics, viability, and sensory properties of probiotic salak juice supplemented with four sources of probiotic alginate encapsulated capsules consisting of salak juice (control), germinated native black rice milk probiotic (fermented with *L. casei* TISTR 390), germinated native black rice yogurt and soygurt (richesse). The microstructure of the probiotic alginate encapsulated beads was observed and photographed with scanning electron microscopy (SEM) (JEOL, model JSM-MEDEL jsm-5410LV, Japan) for the capsule's appearance, surface and cross section.

Materials and methods

Materials

Mature salak fruits were purchased from a local orchardist in Chanthaburi province, Thailand, which was harvested in June, the full maturity season, and transported to the laboratory. For native black rice, Maepayatong dum rice was purchased from a local farmer in Khao Khitchakut district, also in Chanthaburi province. This rice was prepared according to modified methods described by Panyanak *et al.* (2010). Briefly, the samples were selected and soaked in water at the ratio of rice and water 1:10 at 40 °C for six hours in a tray. Then, the water was drained. The rice sample was germinated for 48 hours at room temperature (37 °C) and then the germination was stopped by drying using a hot air oven, at 55 °C for 4.5 hours. Then, the germinated native black rice was stored at room temperature (37 °C). The germinated native black rice milk probiotic fermentation was proceeded in 100 ml glass bottles and then sterilised using an autoclave at 121 °C for 15 minutes. Then, the rice milk was inoculated with a 24-hour-old culture of *Lactobacillus casei* TISTR 390, which was purchased from the Microbiological Resources Centre at the Thailand Institute of Scientific and Technological Research (TISTR) in Pathum Thani province, Thailand ($>10^6$ CFU/ml) and fermented at 30 °C for 72 hours. For the germinated native black rice yogurt production, black rice yogurt was produced according to the method of the our's previous study (Mongkontanawat *et al.*, 2018). Briefly, the germinated native black rice was cooked in a rice cooker with a ratio of 1:2 (rice and water). Then the cooked rice was blended with water using a blender with a ratio of 1:2 (rice and water) and filtered by a straining cloth. The germinated native black rice milk was then added with 3% (w/v) sugar and 5% (w/v) lactose. The rice media were prepared in the small polypropylene container (50 ml) which were pasteurized at 80 °C for 10 minutes. The Revon starter culture (10 % w/w) was added and fermented at 42

°C for 8 hours. Original flavoured soygurt (richesse) was brought from a local supermarket and stored in the refrigerator before the experiment.

Encapsulated preparation

Four sources of probiotic calcium alginate capsule comprising salak juice (control), germinated local black rice milk probiotic (fermented with *L. casei* TISTR 390), and germinated local black rice yogurt were made. All samples were encapsulated in sodium alginate by the modified extrusion method of Muthukumarasamy *et al.* (2006). All glasswares and solutions used in the protocols were sterilised at 121 °C for 15 minutes. Briefly, 10 g of the samples (~10⁶ CFU/ml) were mixed with 20 g of a 2% w/v of sodium alginate solution with continuous gentle stirring to immobilise the samples. The alginate samples mixture was then added using a sterile plastic syringe into 500 ml of 0.5 M CaCl₂ with stirring at 300 rpm. Then, the calcium-alginate capsules were retained in the calcium solution for 10 minutes, and then washed with 0.85% of NaCl two times. The solidified capsules were collected by filtration through some white cloth and washed again with sterile water.

Salak juice preparation

The salak juice was produced as follows: the salak fruit was cleaned with distilled water and the juice was prepared. First, the fruit was separated from its pulp (aril) and pericarp. Then, the pulp was cut with a knife and then extracted by a hydraulic press (Thai sakaya-A2). The obtained juice was made by mixing with distilled water with a ratio of salak juice to water of 30:70. Second, the juice was added to 11% w/v of refined sugar and 0.30% w/v of salt. It was sampled and poured into small glass bottles (50 ml) and pasteurised at 80 °C for 15 minutes.

Change in salak juice supplemented with different sources of probiotic calcium alginate capsules during fermentation

Different sources of calcium alginate capsules (5% w/v) were mixed into the juice and then the solutions were incubated at 30 °C for 72 hours. The changes in some physico-chemical characteristics, viability and sensory properties of the probiotic salak juice were determined by using an experimental design of a completely randomised design (CRD) for the determination of the properties. Randomized Completely Block Design (RCBD) was used for sensory evaluation. The changes in the physical, chemical and microbiological properties were monitored at 24, 48 and 72

hours. For the sensory properties, the 72-hour fermentation samples were assessed. For the physical property evaluation, a solution of probiotic salak juice was measured for colour by using a colour metre (Nippon Denshoku, ZE-2000, Japan). The equipment was calibrated with a standard plate. The colour measurement was expressed in L*, indicating the lightness on a 0 to 100 scale from black to white and a* (+,-) indicated the redness or greenness whereas b* (+,-) indicated yellowness and blueness. For the chemical property examination, the samples were taken as mentioned above for determining the pH, total acidity (lactic acid), total soluble solid, and vitamin C. The pH was measured with a pH metre (Subtex, Taiwan). Total acidity expressed as percent lactic acid was determined by titrating with 0.02 N NaOH to pH 8.2. The total soluble solid was analysed by a hand refractometer (Atago, Japan). The determination of the vitamin C content was performed according to the method of AOAC International (2000). For the determination of the microbiological properties, the viable cell counts (log CFU/ml) were assessed by the standard plate count method with a lactobacilli MRS medium after 48 hours of inoculation at 37 °C and expressed as a colony forming unit (CFU/ml).

Sensory evaluation

The non-dairy probiotic fermented salak juice mixed with the four sources of probiotic calcium alginate capsules at 30 °C for 72 hours were sensory evaluated by 30 untrained panellists from the staff and students at Department of Product Development and Management Technology, Rajamangala University of Technology Tawan-ok, Chanthaburi campus, Chanthaburi province, Thailand. The panellists performed the samples using a nine-point hedonic scale ranging from 1 (extremely disliked) to 9 (extremely liked) (Watts *et al.*, 1989). Each panellist investigated the samples for the colour, aroma, taste, texture, and overall linking.

Microstructure of the capsules

After the salak juice was supplemented with the four sources of probiotic calcium alginate capsules and fermented at 30 °C for 72 hours, then the probiotic alginate encapsulation was sampled and prepared by mixing with a solution of 2.50% of glutaraldehyde in a 0.10 M phosphate buffer pH 7.20 for two hours and incubated overnight in a refrigerator. The samples were washed with the 0.10 M phosphate buffer pH 7.20 two times with distilled water for 15 minutes. The obtained samples were dehydrated with five steps of various ethanol concentrations of 30, 50, 70, 95 and 100% v/v, respectively for 10

minutes in each step. Finally, the dried samples were rehydrated by using a critical point dryer (Leica model EM CPD300, Austria). The final dried samples were fixed and coated with gold by using a sputter coater (Balzers model SCD 040, Germany). The samples were photographed with the SEM (JEOL, model JSM-MEDEL jsm-5410LV, Japan) at a magnification of 25x, 40x, 5,000x and 10,000x, respectively to capture the images of the capsule's appearance, surface and cross section condition.

Data analysis

Analysis of the above-mentioned properties data in three replicates was computed. Data were subjected to the analysis of variance (ANOVA) at $p \leq 0.05$. Means with significant differences were separated by Duncan's multiple range test (DMRT) using computer software.

Results

Change in salak juice supplemented with different sources of probiotic calcium alginate capsules during fermentation

Four sources of probiotic calcium alginate capsules composed of salak juice (control), germinated native black rice milk probiotic (fermented with *L. casei* TISTR 390), germinated native black rice yogurt, and soygurt (richesse) were mixed in salak juice and then incubated at 30 °C for 72 hours. The changes in the colour parameters, pH, tritatable acidity, total soluble solid, and vitamin C content was determined (Figures 1-4).

The changes in the colour parameters (L^* , a^* and b^*) of the salak juice were fermented with different sources of probiotic calcium alginate capsules for 0, 24, 48 and 72 hours, respectively at 30 °C. The lightness of all treatments unsteadily changed and tended to be significantly increased when the fermentation time was longer (Figure 1A). In case of the salak juice fermented with soygurt, it showed a significant increase when the fermentation time was increased. For a^* , the numbers of a^* in all treatments showed - , which indicated greenness. A significant high amount was revealed in the salak juice supplemented with soygurt and also showed a significant increase when the fermentation time was increased (Figure 1B). For b^* , the numbers of b^* found with + indicated yellowness in all treatments. All in all, the colour of all treatments tended to be a little green and yellowness to gold colour.

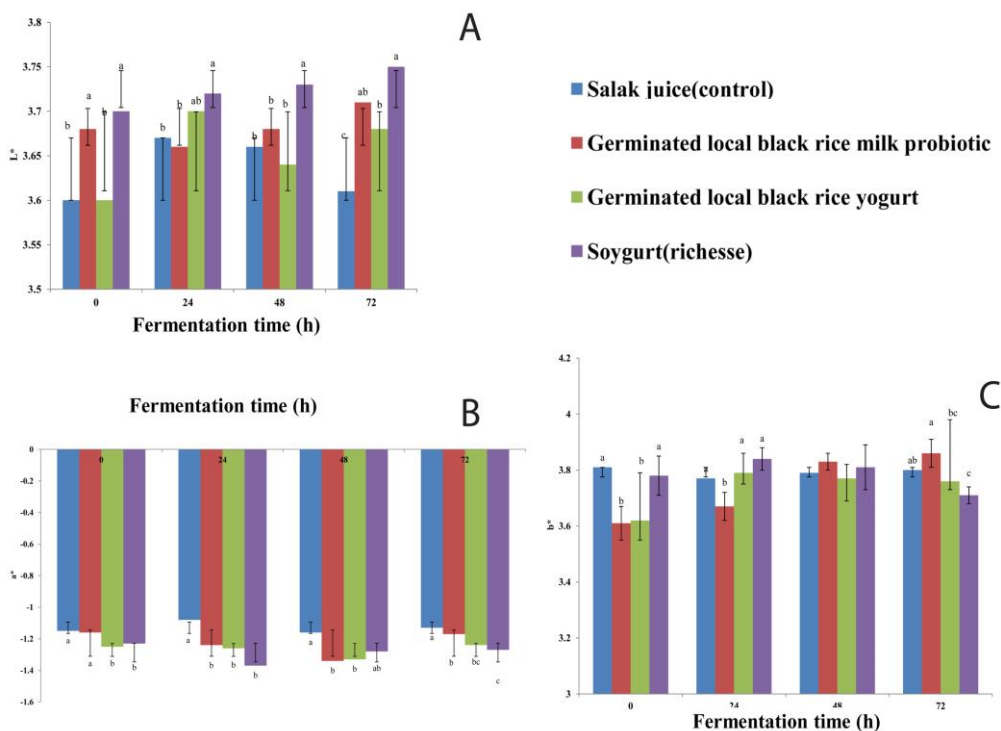


Figure 1. Change in the colour parameters (L*,a*and b*) of the salak juice fermented with different sources of probiotic calcium alginate capsules for 0, 24, 48 and 72 hours, respectively at 30 °C. The bars represent the standard deviation from the triplicate determination

The level of the total soluble solid of the fermented salak juice mixed with different source of probiotic calcium alginate capsules showed a slight significance ($p \leq 0.05$) lower than those of the control group; simultaneously, the pH also decreased slightly ($p \leq 0.05$) but was still significantly higher ($p \leq 0.05$) than those of the control group when the fermentation time was longer (Figures 2C and 2A). Furthermore, the number of titratable acidity of the fermented salak juice supplemented with the probiotic calcium alginate capsules was found to be significantly lower ($p \leq 0.05$) than those of the control group and then rose to be nearly equal to the control (salak juice) when the fermentation time was longer with the highest being at 72 hours of fermentation (approximately 0.49 % w/v) (Figure 2B).

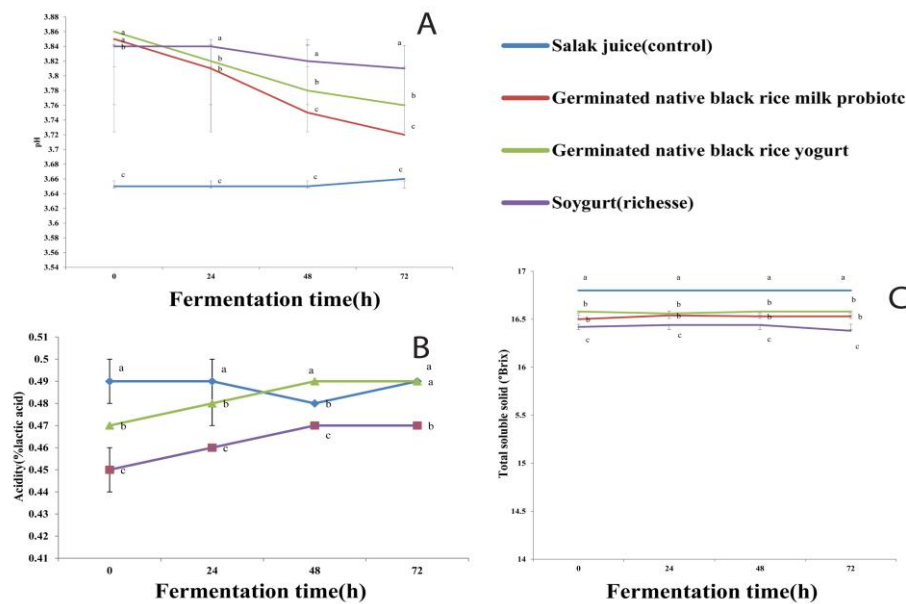


Figure 2. The change in the pH, acidity (lactic acid), and total soluble solid of the salak juice fermented with different sources of probiotic calcium alginate capsules for 0, 24, 48 and 72 hours, respectively at 30 °C. The bars represent the standard deviation from the triplicate determination

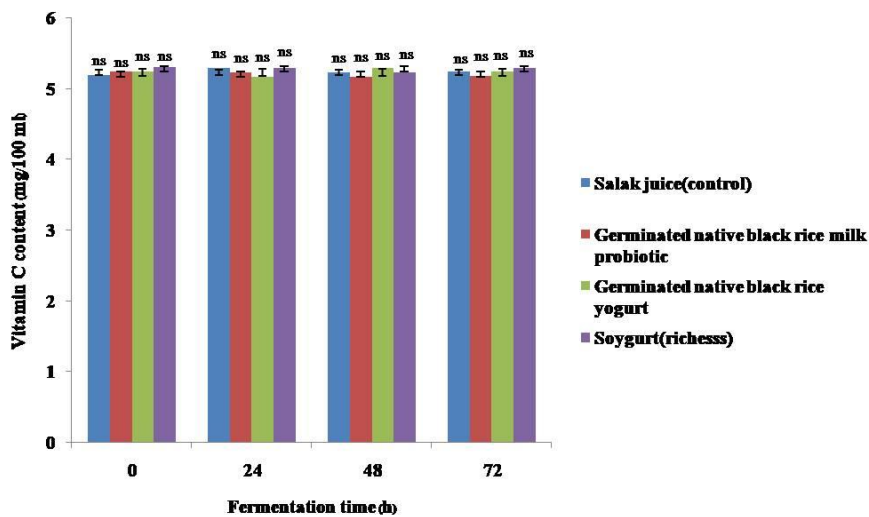


Figure 3. The change in the vitamin C of the salak juice fermented with different sources of probiotic calcium alginate capsules for 0, 24, 48 and 72 hours, respectively at 30 °C. The bars represent the standard deviation from the triplicate determination

The level of the vitamin C content of the salak juice fermented with different sources of probiotic calcium alginate capsules was not significant with the control group for the whole fermentation time (Figure 3). Thus, the addition of probiotic alginate encapsulated beads did not affect the ascorbic acid production in the salak juice in the control fermented condition.

With regards to the viable plate count, the number of the viable cell count of the probiotic alginate encapsulated beads including the germinated native black rice milk probiotic, germinated native black rice yogurt, and soygurt (richesse) were slightly significantly higher ($p \leq 0.05$) than those of the control. The level of the living probiotic (approximately 6 log CFU/ml) in all probiotic alginate encapsulated beads had a longer fermentation time (Figure 4). Consequently, four treatments of fermented salak juice had a sensory evaluation by 30 untrained panellists from the researchers' department.

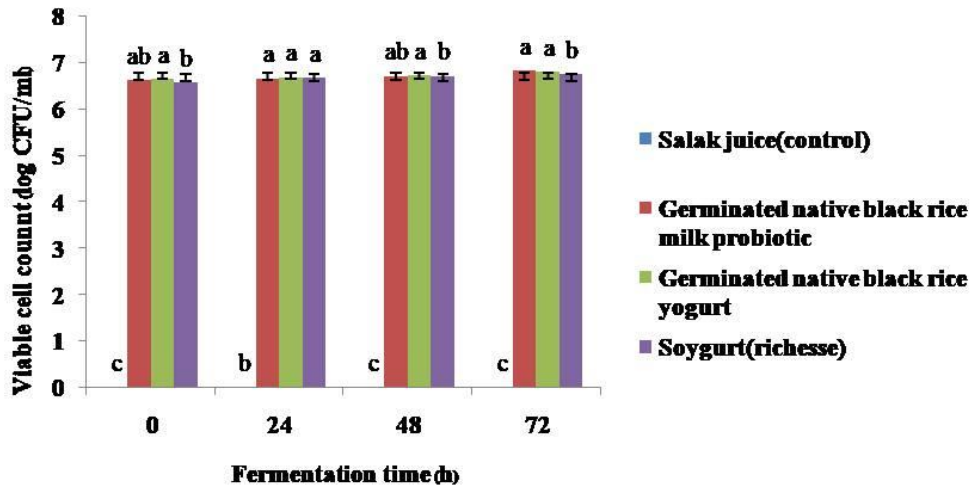


Figure 4. The change in the total viable cell count of the salak juice fermented with different sources of probiotic calcium alginate capsules for 0, 24, 48 and 72 hours, respectively at 30 °C. The bars represent the standard deviation from the triplicate determination

For the sensory evaluation, after the salak juice was fermented with different sources of probiotic calcium alginate capsules at 30 °C for 72 hours, the results revealed that the salak juice fermented with the soygurt (richesse) capsule had the highest acceptable score for the overall liking (Table 1) with a score of 7.33 ± 0.78 (moderately like). However, it was not significantly different ($p \leq 0.05$) from other samples in all the preference parameters. In

summary, the addition of different sources of probiotic calcium alginate capsules did not significantly affect the colour, aroma, taste, and overall acceptability.

Table 1. Mean sensory scores of the salak juice fermented with different sources of probiotic calcium alginate capsules at 30 °C for 72 hours

Type of Probiotic Calcium Alginate Capsules	Preference Scores				
	Colour ^{ns}	Aroma ^{ns}	Taste ^{ns}	Texture ^{ns}	Overall Linking ^{ns}
Salak juice (control)	7.07±0.87	6.90±0.76	7.30±0.92	7.00±0.91	7.13±0.68
Germinated native black rice milk probiotic	6.97±0.76	6.93±0.87	7.20±0.92	6.97±0.89	7.00±0.69
Germinated native black rice yogurt	7.10±0.80	7.20±0.89	7.30±0.79	7.07±0.87	7.13±0.78
Soygurt (richesse)	7.03±0.93	7.10±0.99	7.40±0.89	7.27±0.83	7.33±0.80

The microstructure of different sources of probiotic calcium alginate capsules

The four sources of calcium alginate capsules were prepared as previously mentioned, and the shapes were round and oval. The colour of the capsule showed clear, purple, and purple and white depending on the raw material of the capsule in the case of the salak juice (control), germinated native black rice milk probiotic, germinated native black rice yogurt, and soygurt (richesse), respectively (Figures 5A, 5B, 5C, 5D). In the present study, all capsule types were approximately 2.67±0.10 mm and 2.70 ± 0.11 mm in diameter. Non-significant differences were displayed in all capsules on both the x-axis and y-axis (Table 2). Then, the samples were collected and determined for their microstructure by being photographed with SEM as represented in Figures 6-9, respectively. The results showed that the shapes of all probiotic alginate capsules were also round and oval, and the probiotic cells were located in the microcapsule. The current results also concurred with Zhao *et al.* (2008) who found that the shapes of the microcapsule were round and oval, and the probiotic cells lived in the centre of the capsule.

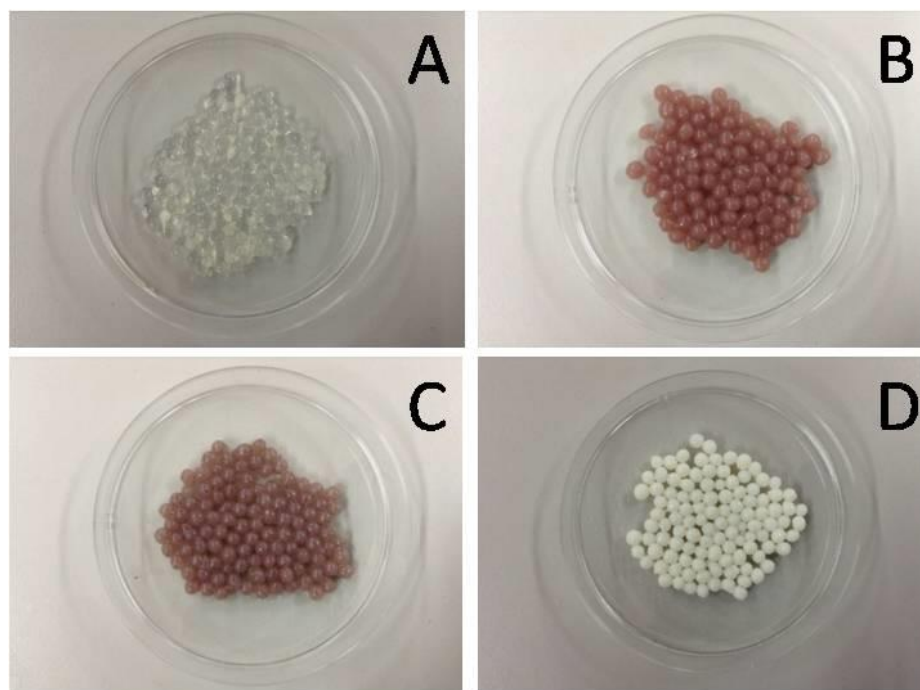


Figure 5. Calcium alginate capsules of the salak juice (control) (A), germinated native black rice milk probiotic c(B), germinated native black rice yogurt (C), and soygurt (richesse) (D)

Table 2. Size (x-axis vs y-axis) of different sources of probiotic calcium alginate capsules

Type of Probiotic Calcium	Diameter (mm)	
	x-axis ^{ns}	y-axis ^{ns}
Salak juice (control)	2.67 ±0.11	2.71 ±0.09
Germinated native black rice milk probiotic	2.68 ±0.12	2.71 ±0.12
Germinated native black rice yogurt	2.66 ±0.09	2.72 ±0.08
Soygurt (richesse)	2.67 ±0.10	2.70 ±0.11

The structure of the salak juice capsule (control) membrane was shown to be more smoothly compact and symmetrical, and some salak particles were found on the surface (Figures 6A and 6B). For the cross section, the structure of the capsule core was formed by cross linking between sodium alginate with calcium ion of CaCl_2 with a tight and regular network as exhibited in Figures 6C and 6D at a magnification of 5,000x and 10,000x, respectively. In addition, no living cells were located in the control capsule.

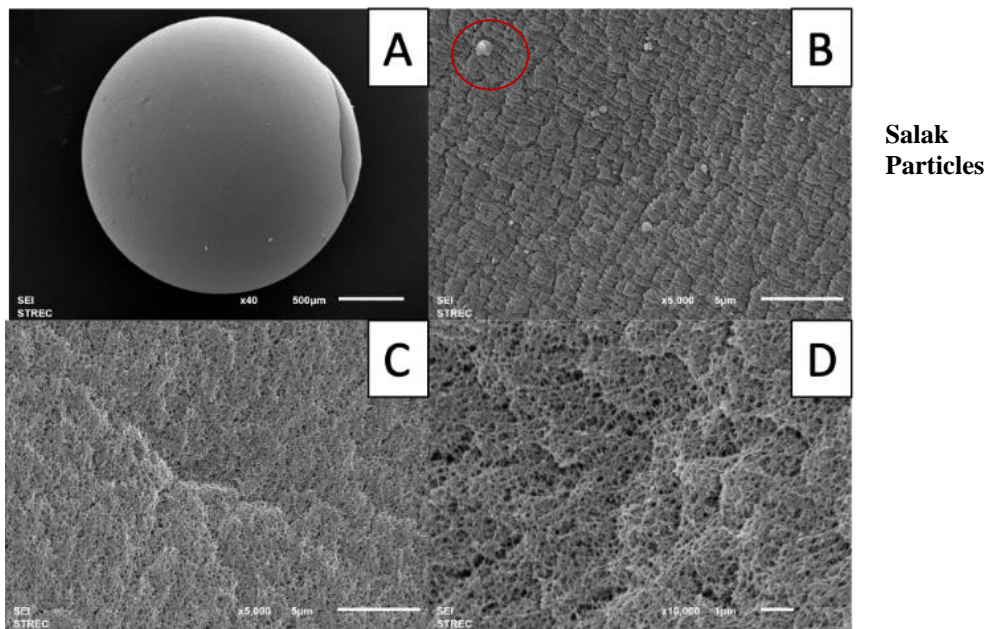


Figure 6. Scanning electron microscope photos of the salak juice calcium alginate capsule (control) (A: capsule appearance, 40x; B: surface structure, 5,000x; C and D: cross section of the capsule at a magnification of 5,000x and 10,000x)

The structure of the germinated native black rice milk probiotic capsule membrane showed that it was roughly compact, symmetrical, and a salak particle was found on the surface (Figures 7A and 7B). For the cross section, the structure of the capsule core was formed by cross linking between sodium alginate with calcium ion with a tight network and coated with *L. casei* TISTR 390 in the capsule (Figures 7C and 7D). For this case, the amylase and amylopectin from the black rice could interact with the network of the calcium-alginate. Therefore, the network tended to be tightly packed than those in the case of the control group (salak juice).

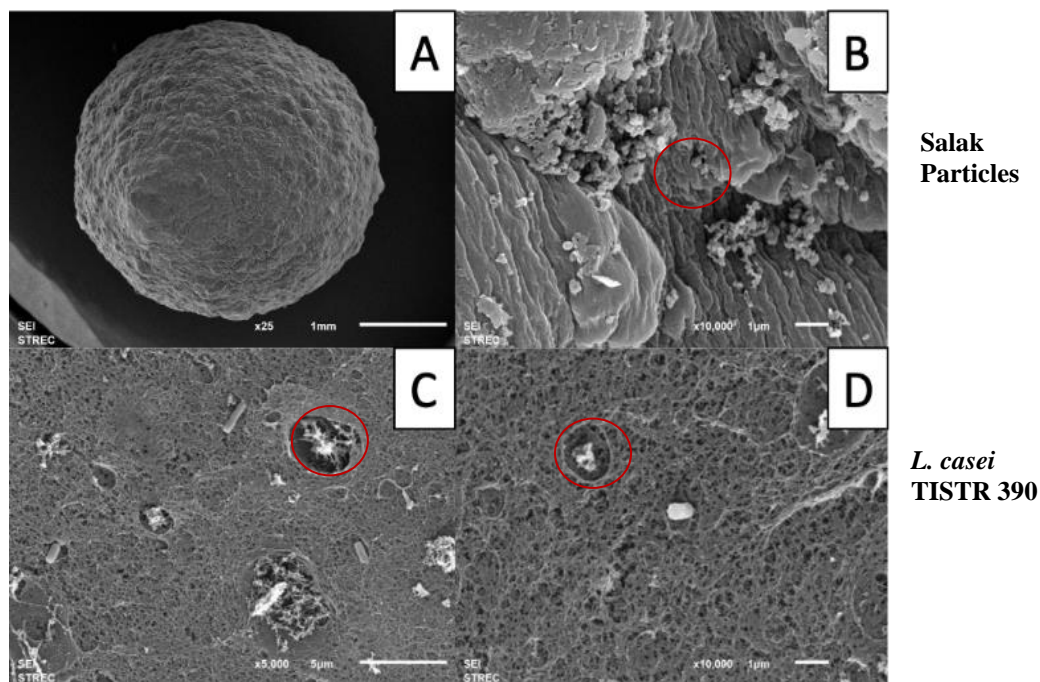


Figure 7. Scanning electron microscope photos of the germinated native black rice milk probiotic calcium-alginate bead (A: bead appearance, 25x; B: surface structure, 5,000x; C and D: cross section of the bead at a magnification of 5,000x and 10,000x)

The structure of the germinated native black rice yogurt capsule membrane was shown to be smoothly compact, symmetrical, and a salak particle was found on the surface (Figures 8A, 8B and 8C). For the cross section, the structure of the microcapsule core was formed by cross linking between sodium alginate, amylose, and amylopectin with calcium ion with a much tighter network and entrapped the probiotic cells inside the capsule (Figure 8D).

The structure of the soygurt (richesse) capsule membrane was shown to be slightly roughly compact, symmetrical, and also some salak particles were found on the surface (Figures 9A, 9B and 9C). For the cross section, the structure of the capsule core was formed by cross linking between sodium alginate with calcium ion and combined with the soygurt that formed a tight network with different shaped probiotic cells located inside the capsule in different magnifications (Figures 9D, 9E and 9F).

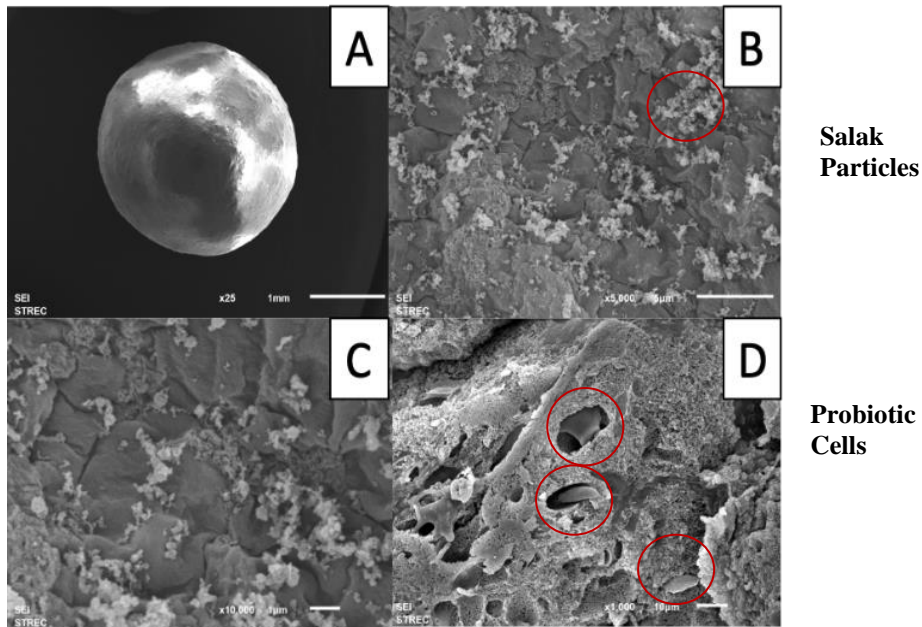


Figure 8. Scanning electron microscope photos of the germinated native black rice yogurt calcium alginate capsule (A: capsule appearance, 40x; B and C: surface structure of the capsule at a magnification 5,000x and 10,000x; D: cross section of the capsule, 1,000x)

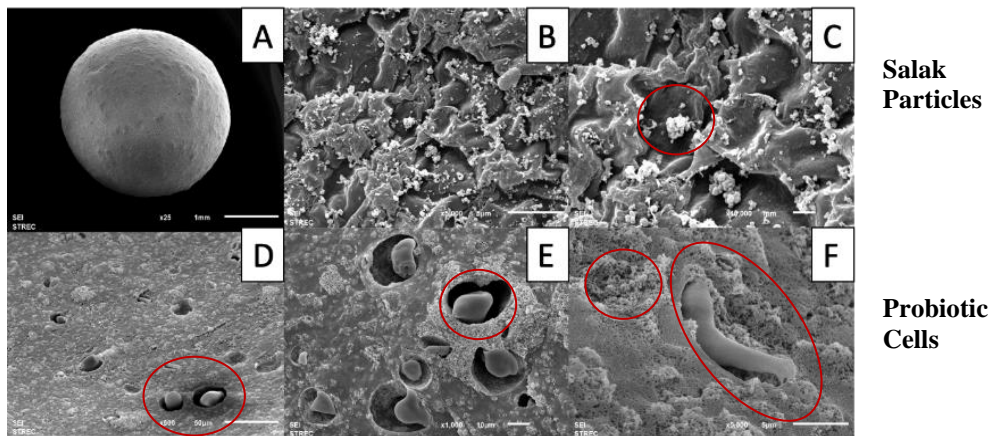


Figure 9. Scanning electron microscope photos of the soygurt (richesse) calcium alginate capsules (A: capsule appearance, 25x; B and C: surface structure of the capsule at a magnification of 5,000x and 10,000x; D, E and F: cross section of the capsule at a magnification of 500x, 1,000x and 5,000x)

Discussion

Regarding the change in the physio-chemical and microbiological analyses, because of the colour of the salak fruit, the colour parameter of all treatments tended to be slightly green, yellowness to a gold colour. Before fermentation, the addition of the probiotic alginate capsules increased the pH and decreased the acidity because of the interference of the black rice molecule and soygurt, which were higher than the salak juice. After fermentation, there were little changes in the pH, and the titrable acidity tended to slightly decrease and increase until fermentation at 30 °C for 72 hours. Because the probiotic cells were composed of slightly metabolised glucose to the lactic acid of the salak juice while the fermentation time increased, the pH, total soluble solid and titrable acidity displayed very little change. The most appropriate capsule cells were protected by living microorganisms, so they could not emerge from the capsule cells and still survived as agree with previously research (Zhao *et al.*, 2008; Ortakci and Sert, 2012).

In this work, the result did not agree with the our previous study in the case of the addition of a free cell probiotic (Mongkontanawat *et al.*, 2018). The current results showed a dramatic change in the pH levels and total soluble solid of the probiotic gac juice and was significantly decreased ($p \leq 0.05$) at 72 hours of fermentation. On the other hand, the amount of titratable acidity expressed as lactic acid was significantly increased ($p \leq 0.05$) at fermentation for 72 hours. Interestingly, the level of the living cells of all probiotic salak juices were exhibited in the standard level by the FAO/WHO (more than 6 log CFU/ml). This could be the probiotic cells located inside the encapsulated cells that still survived. Hence, the living cell was constantly exhibited. Additionally, this fermentation of probiotic calcium alginate capsules did not affect the vitamin C content when the fermentation was longer time. Moreover, the addition of different sources of probiotic capsules did not affect the sensory scores including the colour, odour, taste, texture and overall linking. This could be because the low amount of probiotic alginate beads were insufficient for consumers to detect the taste. The current results agreed with Ortakci and Sert (2012) who reported that the addition of probiotic culture in a free and alginate-encapsulated form in yogurt did not significantly affect the appearance of the colour and flavour of the yogurt. In contrast, salak juice supplemented with soygurt (richesse) had the highest score (7.33 out of 9; moderately like) of the overall acceptability.

For the structure of the capsules, the tight compact network of the capsules was shown in the germinated native black rice milk probiotic, germinated native black rice yogurt, and soygurt (richesse). It could be integrated structure with the amylose, amylopectin and soy molecule in the soygurt. Moreover, the capsules surface had some salak particles in all of the calcium alginate capsules.

In conclusion, the calcium alginate encapsulation could be protected of the living probiotic cells when fermented in salak juice. Interestingly, the level of the living cells of all the probiotic salak juices was in the standard level by the FAO/WHO. Therefore, this novel probiotic salak juice could be serve as healthy drink for health-conscious consumers in the future. However, further research is also needed to determine if the taste could be improved in order to increase the acceptability of the product, and the viability of the probiotic salak juice could be observed during cold storage. Likewise, the stability of the capsule in the salak juice could be investigated.

Acknowledgements

This research was financially supported by Rajamangala University of Technology Tawan-ok. The authors gratefully acknowledge the technical assistance of Ms. Sonsri Toikham. And finally the special thanks go to all of friend for their helpful.

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(Received: 15 April 2020, accepted: 29 October 2020)