MICROENCAPSULATION OF PROBIOTICS IN HERBAL DRINKS

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Abstract

Herbal beverages are nutritious beverages that contain various bioactive compounds with antimicrobial and antioxidant properties. Due to the present of antimicrobial properties, microbial such as probiotics can undergo deterioration. In this regard, microencapsulation process is used to enhance the survivability of probiotics. This experiment investigated the effect of microencapsulation of *Lactobacillus casei* 01 with calcium alginate on cell survival in Thai herbal drinks including pandan juice and chrysanthemum juice during storage at 4°C for 4 weeks. On day 0, the viability of free cells and probiotics beads in both drinks were not significantly different ($p<0.05$). However, upon storage from week 1 onwards, the viability of free cells was noticeably decreased ending the storage of week 4 with 5.34±017 log CFU/g for pandan juice and 5.37±0.17 log CFU/g for chrysanthemum juice, whereas probiotics beads in pandan juice and chrysanthemum juice were 6.17±0.02 and 6.18±0.02 log CFU/g, respectively. Overall, the viability of probiotics beads in products throughout 4-week storage reduced less than 0.5 log as compared to the controls. Microencapsulated probiotics in both products indicated better survival.

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1. Introduction

Nowadays, attention of healthcare and self-care are rising and stronger, leading to the rapid expanding of functional food market. Probiotic products show a strong growth area within the functional foods and many researchers are developing products into which probiotic organisms like *Lactobacillus* and *Bifidobacterium* species are incorporated (Stanton et al.,2001).

Probiotics are defined as "live micro- organisms which when administered in adequate amounts confer a health benefit on the host" (Agriculture Organization of the United Nations and World Health Organization,2002). They have been used for thousands of years to ferment foods and prepare alcoholic beverages. Food and beverage additives such as probiotics have gained an importance for functional foods. Probiotic foods may regulate gut microbial, improve overall gut health, resist to the pathogenic bacteria in the gut and prevent gastrointestinal infections (Shiby and Mishra ,2013). Products containing probiotics are mainly dairy-based food, such as yogurts, cheese, and kefir. However, drawbacks of dairy products have a negative effect for lactose intolerance persons and the cholesterol content. Non-dairy drinks such as herbal and botanical beverages can be alternative means of probiotics to the consumer by incorporating probiotic strains into such products.

Herbal beverages are commonly brewed from several parts of plants. They are well-known for rich sources of natural bioactive compounds, such as carotenoids, phenolic acids, flavonoids, coumarins, alkaloids, polyacetylenes, saponins and terpenoids. These bioactive compounds have several biological effects, such as antioxidant, antibacterial and anti-inflammatory (Craig,1999). With these reasons, probiotics viability is limited when added to herbal drinks. They need protection in order to maintain their viability.

Microencapsulation is the technique to protect probiotics from surrounding environment by entrapping the material within a carrier that creates micro-environment for bacteria to survive during processing and storage (Chaikham et al., 2013). Therefore, for these reasons, the survival of microencapsulated probiotic beads will be determined and compared with free cells to evaluate the efficiency of microencapsulation that preserves the probiotic bacteria.

2. Objective

To evaluate and compare the survival of free cells and encapsulated probiotics in herbal drinks.

3. Literature review

3.1 Probiotics

Probiotics are defined as live bacteria. According to Food and Agriculture Organization of the United Nation's World Health Organization, probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO,2001). The common probiotics genera are *Lactobacillus, Escherichia, Enterococcus, Bifidobacterium, or Bacillus, Streptococcus* and the fungal genus *Saccharomyces* (Andrew M. Abe et al., 2013). Almost all of the probiotics on the market contain *lactobacilli* and/or *streptococci*. Probiotics preparation can consist one strain or up to eight strains (Fuller R, 1989). Most probiotic belong to the group of Lactic Acid Bacteria (LAB). *Lactobacillus* species are member of LAB, group of gram-positive bacteria non-sporulating, non-respiring cocci or rods. During fermentation of carbohydrates, it produces lactic acid as the major product (Seppo Salminen et al., 2004).

Probiotics provide beneficial health effects when consumed. Overall, they can improve gastrointestinal health and balance the gut microbiome. Microbiome diversity is related to overall health and refers to different strains of bacteria in intestinal tract. The more different probiotic strains lead to better health (Tuddenham S and Sears, 2015).

Lactose intolerance are caused by the lack of enzyme β-D-galactosidase that digests lactose and some people with lactose intolerance may experience abdominal symptoms such as diarrhea, bloating, and flatulence (John R Saltzman et al.,1999). Lactic acid bacteria produce β-D-galactosidase helps to increase the digestion of lactose as well as improve abdominal pain and discomfort when consumed.

Probiotics regulate an important part of immune system and contribute to balance the gut microbial that can reduce the risk of chronic diseases such as inflammatory bowel disease, obesity, type 2 diabetes, and cancer. Cancer-causing chemicals or carcinogens are caused by cells mutation. It has been hypothesized that carcinogens can be generated by microbes which live in digestive system. Probiotics will reduce the metabolic activities of bacteria that generate carcinogenic compounds and inhibit the growth of tumor cells (S. Parvez et al.,2006).

It also has been found that probiotics create a microbiome ecosystem that inhibit the growth of pathogens that causes diarrhea (Singh et al.,2017) by competing with pathogens for nutrients in the gut and taking up space in the intestine (Shori et al.,2018). Rotavirus, for example, is the most common cause of diarrhea in the world. Clinical studies have shown that probiotics can block the receptor site signals that produce the secretion, defense motility of the virus and produce substances that inactivate the viral particles (Kechagia, M. et al.,2013). A recent research has shown that a healthy gut is associated with psychological. Probiotics may have a significant improvement in mood state as well as overall psychological well-being such as sleep quality (Angela Marotta et al., 2019).

Since probiotics have proven plenty of beneficial health effects, specifically to the human digestive system, consumers awareness of nutritional in food content is increasing and therefore the growing interest about health diets of the marketplace for functional foods as well as demand for probiotics food and beverages is rising. The global market of probiotic product is predicted to grow stronger. In 2018, probiotics market is estimated to achieve USD 49.4 billion and is estimated to reach USD 69.3 billion by 2023 growing at a compound annual growth rate (CAGR) of 7%, where probiotics-fortified food and beverages constitute the largest share. Nevertheless, interestingly, liquid probiotics, for instance, kefir, yogurt drinks and probiotics juices are more popular than dry probiotics as they are inexpensive (Markets and Market,

2019). Thus, food industries are developing new food products containing probiotics (Espitia et al., 2015).

3.2 Herbal beverages

Herbal extracts and beverages have been used for thousands of years since the ancient period because of their bioactive compounds, such as polyphenols, carotenoids, flavonoids, coumarins, alkaloids, polyacetylenes, saponins and terpenoids which related to antioxidant activity (Skrajda-Brdak,2018). Herbs are usable parts from buds, bark, root, berries, aromatic seeds of plants or can come from stigma of flowers. Herbs are long well known for their health benefits and as well as preservatives (Tapsell et al., 2006). Herbal or botanical infusions can be made with dried or fresh flowers by boiling or steeping (Cabrera et al., 2006).

Herbs and botanicals are a medicinal plant that has microbial activity against several microorganisms (Juliano et al.,2000). Plants and their essential oils are useful for antimicrobial compounds that can go against microbes or food-borne pathogens. The study showed that there is a relationship between phenol components and antimicrobial activity. For example, essential oils extracted from oregano-type oils have higher percentage of phenol components and tend to have higher antimicrobial activity. This is because carvacrol, a monoterpenic phenol, has ability to inhibit bacterial growth by interfering cell membrane of bacteria as well as α- and β- glucanases of fungi. Antibacterial properties of essential oils have better activity with gram-positive than gram-negative.

3.2.1 Chrysanthemum

Chrysanthemum was first cultivated in the 15th century in China as a herd before introduced into European culture within the 17th century (Anderson,2006). Scientific name of Chrysanthemum is *Chrysanthemum morifolium* Ramat. The name chrysanthemum (*Chrysanthemum*) is from the Greek "chryos" meaning gold and "anthemon" meaning flower. It belongs to the Asteraceae family, which is one of the biggest families within the botanical world. All chrysanthemum flowers are edible, but chrysanthemum tea is often made up from yellow or white flowers of *Chrysanthemum morifolium* or *Chrysanthemum indicum*.

Figure 3.1: Left side: *Chrysanthemum morifolium*. Right side: *Chrysanthemum indicum*.
Source: http://foodforhealthguide.blogspot.com/2011/12/chrysanthemum.html

Chrysanthemum tea is an herbal infusion made from the dried flowers of the chrysanthemum plant and caffeine-free which makes it a great alternative to drinks containing caffeine like black tea and coffee. It has been reported that *Chrysanthemum morifolium* has many antioxidant activities that promote the function of cardiovascular system, and reduce the levels of lipid (Yu et al., 2013).

Chrysanthemum flowers contain nutrients and biologically active components. The chemical extracts of chrysanthemum include flavonoids, betaine, choline, and vitamin B1 (Shahrajabian et al.,2019). Flavonoids and other active compounds, including C-glycosylated flavones and aliphatic acid-containing caffeoylquinic acids were reported to be found in chrysanthemum flower. These phenolic compounds are believed to have healing benefits that act as antibiotic against pathogens (Marongiu et al., 2019). In addition, phenolic compounds act as antioxidants, which prevent chronic disease by fighting with free radicals and preventing cellular mutations.

According to Traditional Chinese Medicine (TCM), chrysanthemum flowers are in the group of "Cool/Acrid" herbs and versatile herb that is able to treat the early stage of diseases by affecting the upper respiratory tract such as eyes, ears, nose, throat or skin and is believed to be able to cool down the body heat when consumed (Yang et al., 2019). The combination of chrysanthemum flowers with other herbs, fruits or flowers have also been using in TMC formulas. For example, chrysanthemum flowers with dandelions and honeysuckle flowers to reduce hypertension, chrysanthemum flowers with goji berries to relieve headache and innitus, chrysanthemum flowers with gambir stems and thorns, cassia seeds and white peony roots to reduce high blood pressure and chrysanthemum flowers with mulberry leaves, forsythia fruits, wild mint and platycodon roots to reduce body temperature that causes fever (Shahrajabian et al., 2019).

3.2.2 Pandanus

Pandan (*Pandanus amaryllifolius* Roxb.) is a tropical plant of the family Pandanaceae and screw pine genus, with the spiral arrangement of long, narrow, and strap-shaped leaves. This plant is found in Southern India, the Southeast Asia peninsular, Indonesia and Western New Guinea. Its delightful flavor and aroma of pandan leaves make them to become an important ingredient in

Asian cookery. The leaves are often applied into food and desserts as fresh leaf or juice.

Figure 3.2: *Pandanus amaryllifolius* Roxb leaves.
Source: http://www.thai-foodonline.co.uk/

The volatile compounds that gives pandanus a unique aroma consist of alcohols, aromatics, carboxylic acid, ketones, aldehydes, esters, hydrocarbons, furans, furanones and terpenoids. Furthermore, pandanus has a rich source of lipophilic antioxidants in the group of carotenoids such as vitamin E, neoxanthin, violaxanthin, α and β -carotene, lutein, and zeaxanthin (Lee et al., 2004). Esters non-specific lipid transfer protein is one of the essential oils extracted from this plant has shown a potential to promote insulin secretion which helps to reduce blood glucose (Chiabchalard et al., 2015).

Moreover, pandanus juice has a cooling effect as healing properties which is suitable for treating internal inflammations. The previous study indicated bioactive compounds in pandan such as catechin, gallic acid, kaempferol and naringin capable of inhibiting breast cancer cells (Ghasemzadeh et al., 2013).

3.3 Microencapsulation

Microencapsulation is a process where thin films or polymer coats are applied to small particles. Coating material acts as a shell or membrane wall. The range of coating can be used from natural or synthetic film-forming polymers. The main purpose of microencapsulation is to provide environmental protection as well as to stable the storage of starter cultures. The selection methods of microencapsulation depend on the physical and chemical properties of core and coating material and applications of the food.

Physical methods of microencapsulation are spray drying, extrusion, air suspension coating, spray cooling and chilling, co-crystallization and multi-orifice centrifugal extrusion (Jackson and Lee, 1991) However, techniques commonly applied for probiotic microencapsulation are emulsion, extrusion, spray drying, and adhesion to starch and bead can be improved by using different coating materials (Rokka and Rantamaki,2010).

Extrusion method is based on the gelation of an anionic polysaccharide, when in contact with calcium or any other multivalent ion, immobilizing microorganisms. This gel is stable in acidic medium (Cui et al.,2000). It is the oldest method of microencapsulation that involve a polymer solution and hardening solution. Various polymers can be used to obtain capsules by this method, but the most used agents are alginate, k-carrageenan and whey proteins (Rokka and Rantamaki,2010). Among these materials, alginate has been the most applied for extrusion (Favaro and Grosso, 2003).

Alginate is extracted from brown algae (*Phaeophyceae*), including *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis pyrifera* by treating with aqueous alkali solutions (Smidsrod and Skjak, 1990). Alginate a copolymer of glucuronic acid and mannuronica acid, composed of residues of D-mannuronic acid and L-guluronic acid are used in biomedical applications and are capable of being processed under mild conditions (Mattiason, 1983). Alginate is one of the polysaccharide-based film coatings which is edible and commonly used in food industries as gelling and thickening agents as well as encapsulating agents (Stephen and Churms,2006). It is the most polysaccharide used as encapsulating material, due to its non-toxic, and low cost and easy to handle. The properties of alginate include improving the quality of moisture retention by reducing dehydration (Jost et al.,2014). With the combination of extrusion and coating with an additional film can prevent their exposure to oxygen during storage as well as improving their stability at low pH.

The encapsulation in alginate has shown that it effects microorganism survival during storage. Also, probiotics that encapsulated in alginate alone and in a mixture with other compounds were found that more resistant to the acidic medium than the free cells (Liserre et al.,2007). Despite its ability to protect cells from surrounding factors, calcium alginate beads are porous. Therefore, they are coated with polycationic polymer to forms a membrane at the bead surface (Albarghouthi et al.,2000). The film of coating material must be cohesive with the core material to provide strength, impermeability, and stability.

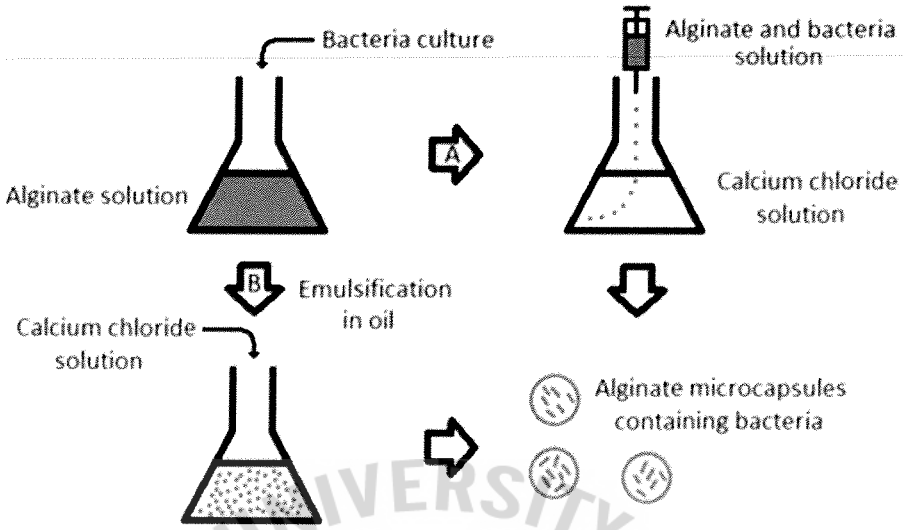

Figure 3.3: Schematic of extrusion encapsulation technique.

Source: http://www.semanticscholar.org/paper/Microencapsulation-ofprobiotics-for-delivery-cook-tzortzis

Microparticles of calcium alginate is formed by dropping a solution of sodium alginate into a solution of calcium salt such as calcium chloride. When carboxyl groups of the polymer chains of alginate encounters divalent cations, such as Ca^{2+} in calcium chloride solution, they will form a cross-linkage and hydrogel (Etchepare et al., 2015). Alginate has also been used with the combination of chitosan to form ionic complexes.

Chitosan is derived from chitin, cationic polymer and composes of poly β-(1-4) *N*-acetyl-d- glucosamine, a non-toxic polymer. It is soluble in acidic aqueous solution and can be used in many applications such as food, cosmetics, biomedical and pharmaceutical (Marguerite,2006). It is suitable for edible coatings to maintain the quality and extend the shelf-life (Tamer and Copur,2010). Chitosan membranes have been applied to alginate beads formed by droplet extrusion. It has strong ionic interactions between the carboxyl residues of the alginate and the amino of the chitosan to form a polyelectrolyte complex, resulting in a smoother surface with a reduced permeability to water soluble molecules, stabilizing the gel, reduce porosity of the alginate beads (Semidsrot and Skjak,1990).

4. Methodology

4.1 Preparation of Probiotics

Culture of *Lactobacillus casei* 01(LC 01) was prepared by inoculating in 10 ml of MRS broth at 37°C for 48 hours under aerobic conditions. Then the culture was transferred into 90 ml MRS broth and incubated at 37°C for 24 hours under aerobic conditions. Cells were harvested by centrifugation at 1500 rpm for 15 minutes before washing twice with sterile water.

4.2 Microencapsulation of Probiotics

The extrusion technique of encapsulation was derived from Krasaekoopt et al. (2004). The cells were suspended with 20 ml of 2% (w/v) sodium alginate solution. The suspension was transferred into sterile syringe with 0.11 mm needle before undergoing drop-wise extrusion into 0.05 M calcium chloride containing 0.1% tween 80. Beads were rinsed with and kept in sterile water at 4°C.

4.3 Coating Alginate Beads with Chitosan

Alginate beads were coated with chitosan using two-stage method (Krasaekoopt *et al.*,2004). Low molecular weight chitosan was prepared by dissolving 90 ml distilled water with 0.4ml glacial acid. pH was adjusted to 5.7 to 6 by adding standardized 1 M sodium hydroxide to naturalize the solution. The mixture was filtered through filter cloth before adjusting the volume to 100 ml using distilled water. Alginate beads were added into chitosan solution and shaken by an orbital shaker at 100 rpm for 30 minutes. The chitosan coated beans were washed and kept in sterile water at 4°C.

4.4 Evaluation of initial free cell and probiotic beads viability.

The initial viable counts of free cell and probiotic beads were performed before adding into herbal drinks. Enumeration of free cell and microencapsulated cells were counted using MRS agar and incubated at 37°C for 48 hours under anaerobic condition.

4.5 Selection of herbal drinks

A survey was conducted with 100 people for two most consumed herbal beverages from six commercial herbal beverages.

4.6 Evaluation of free cell and probiotic beads in herbal drinks.

Ten percentage of encapsulated beads and probiotic freecell were added in 10 ml of commercially pasteurized pandan juice and chrysanthemum juice separately and kept in 4°C refrigerator. Enumeration of free-cell and probiotic bead were preformed over 4 weeks period on day 0, week 1, week 3 and week4. Serial dilutions were prepared from 10^{-2} to 10^{-7} before colonizing on MRS agar incubation at 37°C for 48 hours under anaerobic condition.

4.7 Statistical analysis

A Randomized Complete Block Design (RCBD) with 3 replications was used to analyzed and Duncan's Multiple Range Test at 95% confidence level to compare the significant differences of free-cell and microencapsulated beads.

4.8 Chemical analysis of herbal drinks

Determination of total titratable acidity (TTA) was done by titrating 10 ml of drinks with sodium hydroxide (NaOH) concentration of 0.1 M and phenolphthalein as an indicator. The data reported as %TTA (w/v) of citric acid. pH and °Brix of drinks were monitored using pH meter and refractometer, respectively.

5. Result and discussion

5.1 Survival of probiotics in pandan juice

The line graph explained the viability of free cell and encapsulated bead in pandan juice. The experiment lasted for four weeks and the results are shown in the Log CFU/g unit.

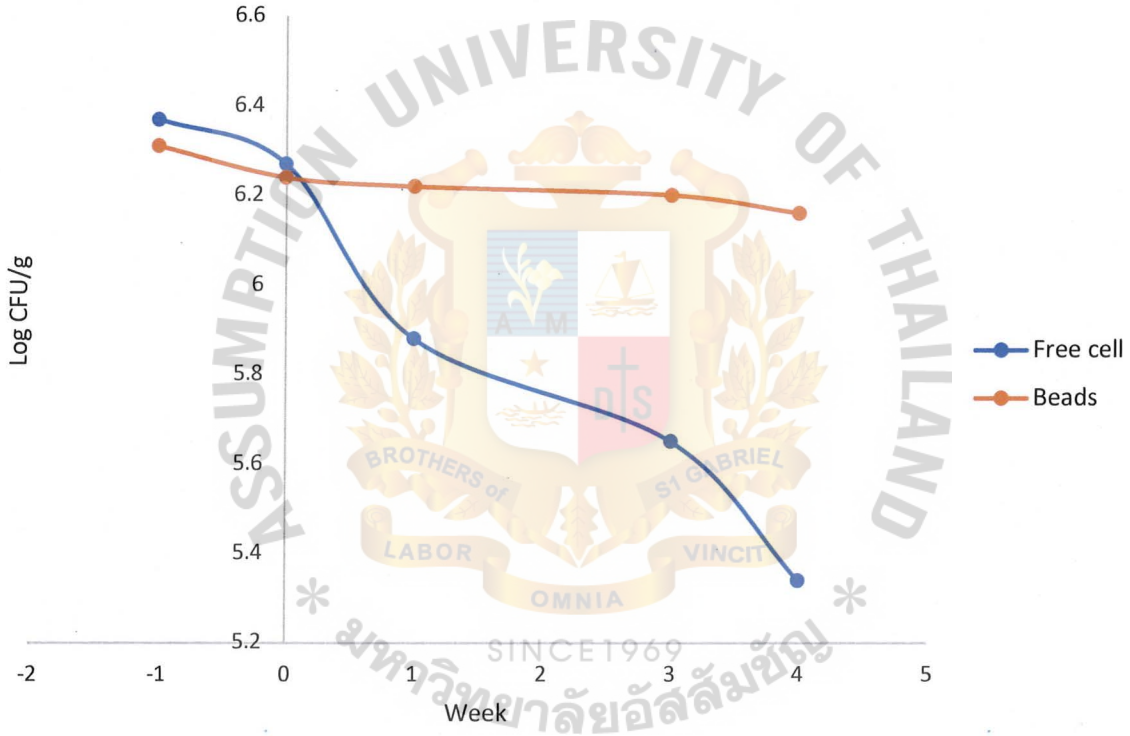

Figure 5.1: Survival of free cell and encapsulate *L.casei* in pandan juice during storage at 4°C during 4 weeks.

Beverage		Log CFU/g				
		Initial	Day 0	Week 1	Week 3	Week 4
Pandan	Free cell	6.27±0.03[a]	6.27±0.01[a]	5.88±0.03[b]	5.65±0.09[b]	5.34±017[b]
	Encapsulated	6.24±0.04[a]	6.24±0.06[a]	6.22±0.02[a]	6.20±0.03[a]	6.17±0.02[a]

Table 5.1: Means± standard deviation of three replications of free cell and encapsulated *L.casei* in pandan juice during storage at 4°C for 4 weeks. Same letter in the column is not significantly different ($p<0.05$).

The amount of free cell and encapsulated bead at initial were not significantly different ($p<0.05$). In day 0, the viability of the probiotic remained stable and started at 6.27±0.01 and 6.24±0.06 for free cell and encapsulated bead, respectively. However, the viability of probiotic in encapsulated bead minimally fell to 6.17±0.02 at the end of week four. On the other hand, there was a dramatic reduction in the viability of probiotics in pandan juice in the first and last week. The reduction was from 6.27±0.01 to 5.88±0.03 and 5.65±0.09 to 5.34±0.17 during the first and last week, respectively. However, the number of free cells in week two and three were steadily decreased. At the end of the experiment, the rate of living probiotic was 5.34±0.17.

5.2 Survival of probiotics in chrysanthemum juice

The line graph illustrates the viability of probiotic contains in Chrysanthemum juice both free cell and encapsulated bead during the four-week period, and the graph shows the number of probiotic in Log CFU/g.

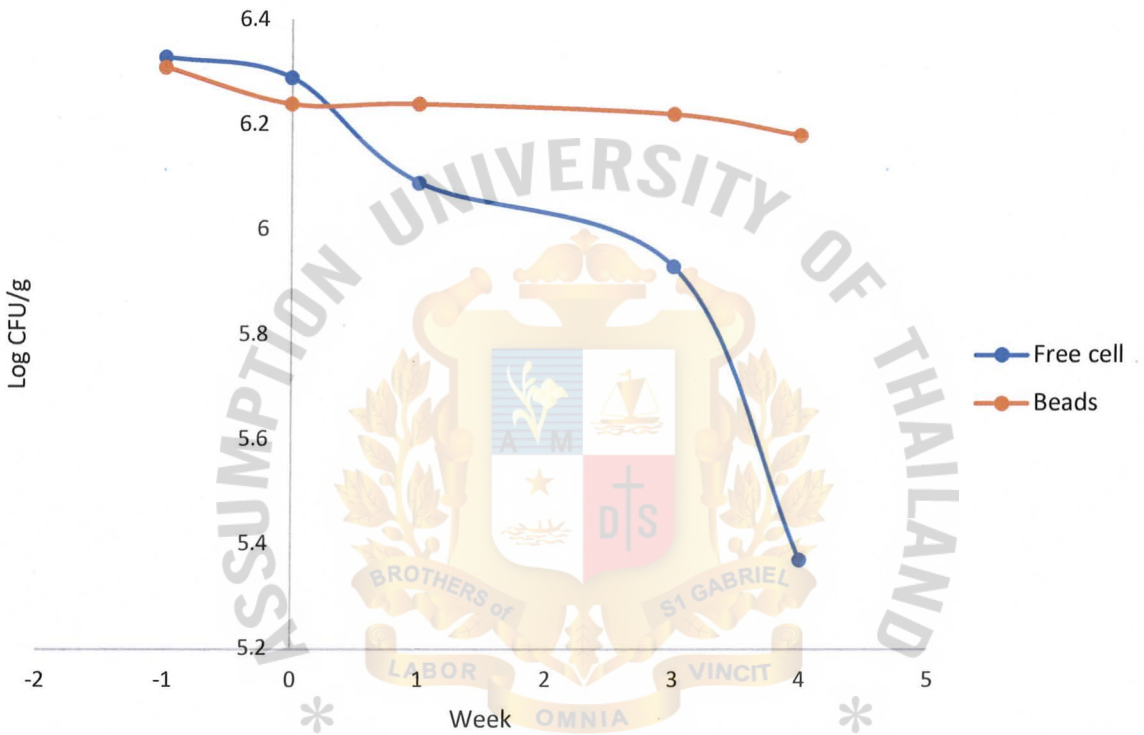

Figure 5.2: Survival of free cell and encapsulate *L.casei* in chrysanthemum juice during storage at 4°C during 4 weeks.

Beverage		Log CFU/g				
		Initial	Day 0	Week 1	Week 3	Week 4
Chrysanthemum	Free cell	6.28±0.06[a]	6.29±0.03[a]	6.09±0.02[b]	5.93±0.05[b]	5.37±0.17[b]
	Encapsulated	6.24±0.03[a]	6.24±0.09[a]	6.24±0.02[a]	6.22±0.02[a]	6.18±0.02[a]

Table 5.2: Means± standard deviation of three replications of free cell and encapsulated *L.casei* in chrysanthemum juice during storage at 4°C for 4 weeks. Same letter in the column is not significantly different ($p<0.05$).

From day 0, the viability was 6.28±0.06 and 6.24±0.03 for free cell and encapsulated bead methods, respectively. The viability of free cell was higher than encapsulated bead in the first place. However, encapsulated bead successfully conserved the lives of probiotics in the long term. There was a petite change in the number of probiotics in the four weeks therefore, at the end of the experiment, there were 6.18±0.02 probiotics alive. In contrast, the viability of free cell dropped significantly from 6.29±0.03 to 5.37±0.17. There was a slight decrease in the first to the third weeks, but the viability declined heavily during week three to four from 5.93±0.05 to 5.37±0.17.

5.3 Chemical analysis of drinks

Beverages	Determination		
	% TTA (w/v) (citric acid)	pH	°Brix
Pandan	0.016 ± 0.01	5.59 ± 0.03	2
Chrysanthemum	0.023 ± 0.006	5.30 ± 0.02	1.8

Table 5.3: Chemical analysis of drinks. Data of %TTA and pH are means± standard deviation of three replications.

Only trace amount of citric acid was found in both beverages. The equivalent weight of citric acid was 64.04, making the %TTA (w/v) of 0.016±0.01 and 0.023±0.006 with low acidic condition of 5.59±0.03 and 5.30±0.02 in pandan and chrysanthemum juice, respectively. Since total soluble solid is related to sugar content, both drinks pandan and chrysanthemum shown a low °Brix values of 2 and 1.8, respectively.

According to the experiment, even though, the maximum growth rate of lactic acid bacteria is between pH 5.5 to 5.8 (Robert et al., 1993), which fall on pH range of both drinks, free cells were unable to survive due to the lack of nutrients such as sugar content, and antimicrobial compounds that presence in herbal drinks. Encapsulated bead has shown is the most effective way to prolong the life span of probiotics in both beverages. Since the probiotics in encapsulated bead with pandan and chrysanthemum juices survived around 6.17 Log CFU/g after four weeks of the experiment. Even though there was no significant difference between pandan and chrysanthemum juice when the encapsulated bead was first used on Day 0, chrysanthemum had better performance in the first three weeks. The number of survive probiotics was 5.93±0.05 at the end of week three while pandan juice had 5.65±0.09 living probiotics. After week three, the results of both juices were identical as they both have remarkably decreased in the free cell moreover, chrysanthemum had sharper fall.

6. Conclusion

In conclusion, although the initial cell counts of free cell were higher than microencapsulated probiotic before applied to both drinks, microencapsulated of probiotic were more stable in compare to free probiotic cells in herbal drinks during 4-week refrigerated storage. Microencapsulation method is the best way to conserve living probiotic regardless of the types of these two beverages.

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8. Appendix

Viable cell count of LC beads in Pandan juice

Initial

Dilution	Replication			
	No. of colonies			
	1	2	3	Average
10^{-3}	210	224	189	208
10^{-4}	174	150	169	164
10^{-5}	127	102	133	121
10^{-6}	94	52	65	70

Day 0

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	180	211	204	198
10^{-4}	133	137	149	140
10^{-5}	102	89	81	91
10^{-6}	57	45	49	50

Week 1

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	174	159	163	165
10^{-4}	142	126	120	129
10^{-5}	97	80	93	90
10^{-6}	42	44	38	41

Week 3

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	159	165	150	158
10^{-4}	121	114	98	111
10^{-5}	75	69	82	75
10^{-6}	37	20	48	35

Week 4

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	169	153	142	155
10^{-4}	120	135	117	124
10^{-5}	65	82	66	71
10^{-6}	17	27	15	20

Viable cell count of free cell in Pandan juice

Initial

Dilution	Replication			
	No. of colonies			
	1	2	3	Average
10^{-3}	220	263	252	245
10^{-4}	197	169	177	181
10^{-5}	138	155	179	157
10^{-6}	95	89	104	96

Week 0

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	176	188	189	184
10^{-4}	147	154	163	155
10^{-5}	112	132	105	116
10^{-6}	74	61	49	61

Week 1

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	69	80	74	74
10^{-4}	42	31	38	37
10^{-5}	21	15	11	16
10^{-6}	0	2	0	1

Week 3

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	45	37	55	46
10^{-4}	21	16	43	27
10^{-5}	8	5	11	8
10^{-6}	5	1	0	2

Week 4

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	33	21	15	23
10^{-4}	6	11	4	7
10^{-5}	0	2	5	2
10^{-6}	0	0	1	0

Viable cell count of LC beads in Chrysanthemum juice

Initial

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	198	247	203	216
10^{-4}	166	152	186	168
10^{-5}	117	148	93	119
10^{-6}	82	64	68	71

Day 0

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	268	274	192	245
10^{-4}	169	164	170	168
10^{-5}	119	106	102	109
10^{-6}	58	36	32	42

Week 1

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	178	167	172	172
10^{-4}	138	117	145	133
10^{-5}	66	72	58	65
10^{-6}	29	32	55	39

Week 3

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	163	165	174	167
10^{-4}	132	127	139	133
10^{-5}	74	63	57	65
10^{-6}	34	29	42	35

Week 4

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	155	152	138	148
10^{-4}	129	112	124	122
10^{-5}	89	69	40	66
10^{-6}	15	22	18	18

Viable cell count of free cell in Chrysanthemum juice

Initial

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	211	193	180	195
10^{-4}	142	157	163	154
10^{-5}	119	128	135	127
10^{-6}	99	75	83	86

Day 0

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	201	198	249	216
10^{-4}	192	174	163	176
10^{-5}	132	121	108	120
10^{-6}	80	76	78	78

Week 1

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	116	129	119	121
10^{-4}	55	48	68	57
10^{-5}	20	26	15	20
10^{-6}	9	8	2	6

Week 3

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	80	77	65	74
10^{-4}	37	59	20	39
10^{-5}	11	8	5	8
10^{-6}	0	3	4	2

Week 4

Dilution	Replications			
	No. of colonies			
	1	2	3	Average
10^{-3}	29	33	16	26
10^{-4}	12	9	13	11
10^{-5}	0	3	5	3
10^{-6}	0	1	0	0

Duncan's Multiple Range Test of Pandan juice

Day 0

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000667

Number of Means	2
Critical Range	.05853

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	6.27333	3	2**
A			
A	6.24000	3	1*

Note: * = Microencapsulated beads.
** = Free cells.

Week 1

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.00075

Number of Means	2
Critical Range	.06208

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	6.22000	3	1*
B	5.88000	3	2**

Note: * = Microencapsulated beads.
** = Free cells.

Week 3

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.003933

Number of Means	2
Critical Range	.1422

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	6.20333	3	1*
B	5.65333	3	2**

Note: * = Microencapsulated beads.
** = Free cells.

Initial

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000667

Number of Means	2
Critical Range	.05853

Means with the same letter
are not significantly different.

Duncan Grouping	Mean	N	treatment
A	6.27333	3	2**
A			
A	6.24000	3	1*

Note: * = Microencapsulated beads.
** = Free cells.

Duncan's Multiple Range Test of Chrysanthemum juice

Day 0

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000617

Number of Means	2
Critical Range	.05629

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	6.28667	3	2**
A			
A	6.24000	3	1*

Note: * = Microencapsulated beads.
** = Free cells.

Week 1

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.0003

Number of Means	2
Critical Range	.03926

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	6.24000	3	1*
B	6.09000	3	2**

Note: * = Microencapsulated beads.
** = Free cells.

Week 3

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.009767

Number of Means	2
Critical Range	.2240

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	6.22000	3	1*
B	5.92667	3	2**

Note: * = Microencapsulated beads.
** = Free cells.

Week 4

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.011383

Number of Means	2
Critical Range	.2419

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	6.18333	3	1*
B	5.37333	3	2**

Note: * = Microencapsulated beads.
** = Free cells.

Initial

The GLM Procedure

Duncan's Multiple Range Test for logcfu

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000617

Number of Means	2
Critical Range	.05629

Means with the same letter
are not significantly different.

Duncan Grouping	Mean	N	treatment
A	6.28667	3	2**
A			
A	6.24000	3	1*

Note: * = Microencapsulated beads.
** = Free cells.

Chemical analysis

1. Titratable acidity

$$\% \text{ Total acid (w/v)} = \frac{\text{volume of Sodium hydroxide used x conc. NaOH x equivalent weight of predominat acid x 100}}{\text{volume of sample used x 1000}}$$

Volume of Sodium hydroxide (NaOH) used.

Beverage	Rep #1	Rep #2	Rep #3
Pandan	0.1	0.2	0.5
Chrysanthemum	0.3	0.4	0.5

Volume of sample used = 10ml

Equivalent weight of Citric acid = 64.04

Concentration of NaOH = 0.0905

Pandan juice :

Rep #1: $\frac{0.1 \times 0.0905 \times 64.04 \times 100}{10 \times 1000} = 0.006\%$ (w/v)

Rep #2: $\frac{0.2 \times 0.0905 \times 64.04 \times 100}{10 \times 1000} = 0.012\%$ (w/v)

Rep#3: $\frac{0.5 \times 0.0905 \times 64.04 \times 100}{10 \times 1000} = 0.029\%$(w/v)

Average = 0.016±0.01% (w/v)

Chrysanthemum juice :

Rep #1: $\frac{0.3 \times 0.0905 \times 64.04 \times 100}{10 \times 1000} = 0.017\%$ (w/v)

Rep #2: $\frac{0.4 \times 0.0905 \times 64.04 \times 100}{10 \times 1000} = 0.023\%$ (w/v)

Rep#3: $\frac{0.5 \times 0.0905 \times 64.04 \times 100}{10 \times 1000} = 0.029\%$ (w/v)

Average = 0.023±0.006%(w/v)

Beverage	Rep #1	Rep #2	Rep #3	Average±SD
Pandan	5.59	5.62	5.57	5.59±0.03
Chrysanthemum	5.29	5.32	5.28	5.30±0.02