การเพิ่มความอยู่รอดได้ของโพรไบโอติกแบคทีเรียที่ถูกห่อหุ้มร่วมกับพรีไบโอติกสำหรับการต้านทานโรคในกุ้งขาว *Litopenaeus vannamei*

นางสาวศุภรดา จันทรทิณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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INCREASING VIABILITY OF PROBIOTIC BACTERIA CO-ENCAPSULATED WITH PREBIOTIC FOR DISEASE RESISTANCE IN WHITE SHRIMP *Litopenaeus vannamei*

Miss Suppharada Jantarathin

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By	Miss Suppharada Jantarathin
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Romanee Sanguandeekul, Ph.D.
Thesis Co-Advisor	Assistant Professor Chaleeda Borompichaichartkul, Ph.D.
	Associate Professor Somkiat Piyatiratitivorakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Kanitha Tananuwong, Ph.D.)

.....Thesis Advisor
(Assistant Professor Romanee Sanguandeekul, Ph.D.)

.....Thesis Co-Advisor
(Assistant Professor Chaleeda Borompichaichartkul, Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Somkiat Piyatiratitivorakul, Ph.D.)

.....Examiner
(Assistant Professor Sanit Piyapattanakorn, Ph.D.)

.....Examiner
(Associate Professor Cheunjit Prakitchaiwattana, Ph.D.)

.....External Examiner
(Assistant Professor Anadi Nitithamyong, Ph.D.)

ศุภรดา จันทรทิณ : การเพิ่มความอยู่รอดได้ของโพรไบโอติกแบคทีเรียที่ถูกห่อหุ้มร่วมกับพรีไบโอติกสำหรับการต้านทานโรคในกุ้งขาว *Litopenaeus vannamei* (INCREASING VIABILITY OF PROBIOTIC BACTERIA CO-ENCAPSULATED WITH PREBIOTIC FOR DISEASE RESISTANCE IN WHITE SHRIMP *Litopenaeus vannamei*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. รมณี สงวนดีกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ชาลีดา บรมพิชัยชาติกุล, รศ. ดร. สมเกียรติ ปิยะธีรธิติวรกุล, 90 หน้า.

ปัญหาหลักของโพรไบโอติกก็คือเซลล์มีความไวต่อการเปลี่ยนแปลงสภาวะแวดล้อมและอัตรารอดต่ำทั้งในระหว่างกระบวนการผลิต การเก็บรักษาและในระบบทางเดินอาหาร ดังนั้นเทคโนโลยีที่เหมาะสมในการห่อหุ้มเซลล์และปกป้องเซลล์โพรไบโอติกเพื่อให้เซลล์มีความสามารถอยู่รอดได้คือเทคนิคไมโครเอนแคปซูเลชัน การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของการรอดชีวิตของจุลินทรีย์โพรไบโอติกแลคโตบาซิลลัส แอซิโดฟิลัส หลังจากห่อหุ้มด้วยอัลจิเนตร่วมกับพรีไบโอติก โดยใช้อินูลินและแก่นตะวันและเคลือบผิวไมโครแคปซูลด้วยสารละลายไคโตซาน จากนั้นทำการทดสอบประสิทธิภาพการอยู่รอดของโพรไบโอติกที่ถูกห่อหุ้มหลังจากทำแห้งแบบแช่เยือกแข็งและการทนต่อความร้อนที่อุณหภูมิ 70 องศาเซลเซียสเป็นเวลา 60 นาที จากการศึกษาพบว่าการห่อหุ้มเซลล์แลคโตบาซิลลัส แอซิโดฟิลัสด้วยอัลจิเนตความเข้มข้น 3% ร่วมกับอินูลินหรือแก่นตะวันความเข้มข้น 3% และเคลือบผิวด้วยไคโตซานความเข้มข้น 0.8% มีอัตราการรอดหลังจากทำแห้งแบบแช่เยือกแข็งสูงที่สุด คือ 84.2% และ 85.3% ตามลำดับ และมีอัตรารอดจากการทดลองที่อุณภูมิสูง คือ 62.8% และ 68.1% ตามลำดับ ซึ่งมีอัตรารอดสูงกว่ากลุ่มควบคุมที่ไม่ได้ผ่านการห่อหุ้มอย่างมีนัยสำคัญ จากผลดังกล่าวจึงนำเซลล์โพรไบโอติกที่ผ่านการห่อหุ้มร่วมกับแก่นตะวันมาทดลองผ่านขั้นตอนการผลิตอาหารกุ้งขาวและนำมาทดลองเลี้ยงกุ้งขาวเป็นเวลา 60 วัน พบว่ากุ้งขาวกลุ่มที่ได้รับอาหารผสมเซลล์โพรไบโอติกที่ห่อหุ้มร่วมกับแก่นตะวันมีอัตรารอดและน้ำหนักสุดท้ายสูงกว่ากุ้งกลุ่มควบคุมที่ไม่ได้รับอาหารเสริมโพรไบโอติก นอกจากนี้กุ้งกลุ่มทดลองที่ได้รับอาหารเสริมโพรไบโอติกยังมีความต้านทานต่อเชื้อก่อโรควิบริโอ ฮาร์วิอายสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ จากการศึกษานี้ สรุปได้ว่าการปกป้องเซลล์แลคโตบาซิลลัส แอซิโดฟิลัสด้วยเทคนิคไมโครเอนแคปซูเลชันสามารถนำไปพัฒนาและประยุกต์ใช้ในอุตสาหกรรมอาหารสัตว์ต่อไปเพื่อยืดอายุและความคงทนของเซลล์โพรไบโอติก

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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SUPPHARADA JANTARATHIN: INCREASING VIABILITY OF PROBIOTIC BACTERIA CO-ENCAPSULATED WITH PREBIOTIC FOR DISEASE RESISTANCE IN WHITE SHRIMP *Litopenaeus vannamei*. ADVISOR: ASST. PROF. ROMANEE SANGUANDEEKUL, Ph.D., CO-ADVISOR: ASST. PROF. CHALEEDA BOROMPICHAICHARTKUL, Ph.D., ASSOC. PROF. SOMKIAT PIYATIRATITIVORAKUL, Ph.D., 90 pp.

The main problem of probiotics is the low survival of these microorganisms in food products and sensitive to harsh conditions during food processing, storage and in the gastrointestinal tract. The suitable technology for protecting probiotics to maintain the high number of probiotic cells in product and survived within the host which is microencapsulation technique. The aim of this work was to investigate the effect of prebiotic which is inulin or Jerusalem artichoke on the survival of encapsulated *Lactobacillus acidophilus* TISTR 1338 within alginate matrix and double-coated with chitosan after freeze-drying process and heat processing at 70°C for 60 min. Furthermore, the effects of co-encapsulated cells on growth performance, survival and disease resistance in white shrimp, *Litopenaeus vannamei* also determined. The results showed that the highest survival after freeze-drying process of co-encapsulated cells was 3% alginate, 3% prebiotic and double coated with 0.8% chitosan condition which the survival percentage was 84.2% in inulin treatment and 85.3% treatment. In addition, the results showed that survival of co-encapsulated cells with inulin and Jerusalem artichoke were 62.8% and 68.16%, respectively that was significantly different higher than control after heat processing. From these results, co-encapsulated cells with Jerusalem artichoke could be useful to protect probiotic cells from feed processing. Consequently, the encapsulated cells were incorporated into shrimp feed supplement and cultured white shrimp for 60 days. The result showed that shrimp fed with co-encapsulated cells with Jerusalem artichoke had higher average shrimp weight gain and survival rate than control. Moreover, shrimp fed with feed supplement diet showed the cumulative mortality lower than control after challenge test with *Vibrio harveyi*. The study concluded that the *L. acidophilus* could be protected by microencapsulation technique, it can be further developed and applied in the aquaculture feed industry to prolong the life and viability of probiotic cells.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER 1
INTRODUCTION

Aquaculture is one of the fastest growing industries in the world, while shrimps from aquaculture continue to be the most important commodity traded in terms of value. As for Thailand, frozen and processed shrimp industry is the most considerably exporting fishery products in Thailand. Whereas there is an increasing intensification and commercialization in a shrimp production, disease is a major problem in the aquaculture industry. In recent years, a mass of antibiotics are used to control the disease outbreaks in shrimp culture industry, which led to a spread of antibiotic resistant pathogens in cultured species, environmental hazards and food safety problems. Therefore, it is necessary to search for natural and alternative feed additives to enhance immune capacity and to prevent the disease outbreaks in aquatic animal. Nowadays, probiotics and prebiotics have been widely accepted as a natural means to promote health for both humans and animals. They are used as health supplements in food and feeds to replace the use of antibiotics or chemical supplements which might have drug resistance problem and unacceptance from consumers. There is the need to look for viable alternatives to modulate gastrointestinal health and reduce the massive use of antibiotics; consequently, probiotics and prebiotics are natural strategies to defense mechanisms of human or animal health. Probiotic bacteria are defined as " live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). Probiotics beneficially affect host' s health by improving the gut microbiota balance and the defenses against pathogens, including stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenesis and anti-bacterial activities (Sarao & Arora, 2017). In the meantime, the use of probiotic bacteria in feed additives has become increasingly popular for improved nutrition, healthy digestion, and disease prevention. Whereas the latest prebiotics are defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). It has been suggested that adding prebiotics to certain foods may increase the viability of probiotic bacteria passing through gastrointestinal tract and thus exert a beneficial effect on host's health (Fooks,

Fuller, & Gibson, 1999; Iyer & Kailasapathy, 2005; Khalf, Dabour, Kheadr, & Fliss, 2010; Roberfroid, 2000). Commonly prebiotic oligosaccharides are used as consumer products such as inulin, fructooligosaccharides, mannooligosaccharides and galactooligosaccharides. The combination of probiotics and prebiotics are referred to as synbiotics (Gibson & Roberfroid, 1995) which is growing interest in the food industry, feed supplement and pharmaceuticals. However, the maintenance of the cell viability in probiotic-containing products is still considerably challenging because the probiotic must survive during the industrial processing, storage condition and gastrointestinal passage. The suitable technology for protection of probiotic cells has resulted in greatly enhanced viability of these microorganisms in food products as well as in the gastrointestinal tract, is microencapsulation technique. Microencapsulation is a process to entrap probiotic cells within a carrier material and it is a useful tool to keep living probiotic cells in foods, to protect them from the external environment and to extend their storage life (Capela, Hay, & Shah, 2007; Chavarri, Marañón, & Villarán, 2012). The most widely used matrix for microencapsulation is alginate which has been found to increase the survival of probiotics from 80 to 95% (Mandal, Puniya, & Singh, 2006). In addition, research is ongoing to develop microcapsule for a better resistance to stress environment by double or triple layers of alginate- chitosan matrix. *Lactobacillus acidophilus* 547 and *Lactobacillus casei* 01 encapsulated in alginate beads coated with chitosan has been reported to improve the survival of probiotics in both yogurt and severe conditions, such as in simulated gastric, intestinal juices and bile salt solution (Krasaekoopt, Bhandari, & Deeth, 2004). Another studies also demonstrated that microencapsulated *Lactobacillus plantarum* in double chitosan coat was improved the survivability of probiotic cells in pomegranate juice (Nualkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos, 2012). Moreover, several studies showed that in the combination of both prebiotics and alginate coating materials may better protect probiotic in food systems and the gastrointestinal tract due to synbiosis (Chen, Chen, Liu, Lin, & Chiu, 2005; Krasaekoopt & Watcharapoka, 2014; Nazzaroa, Fratiannia, Coppolaa, Sadaa, & Orlando, 2009; Okuro, Thomazini, Balieiro, Liberal, & Fávaro-Trindade, 2013). Some studies have shown that probiotics strain remain viable for prolonged periods in yogurts or refrigerated milk when co-encapsulated with prebiotic like inulin (Capela, Hay, & Shah, 2006; Desai, Powell, & Shah, 2004).

Galactooligosaccharides incorporated into microcapsules could increase the survival of *L. acidophilus* and *L. casei* in orange juice under refrigerated storage (Krasaekoopt & Watcharapoka, 2014). The most investigated prebiotic substances are fructan-based inulins and oligofructoses (Roberfroid, Van Loo, & Gibson, 1998). Jerusalem artichoke (*Helianthus tuberosus*) or Thai name "Kaentawan" is a tuberous annual crop of which is rich in fructooligosaccharide carbohydrates in the forms of inulin and fructans. The fructooligosaccharide are not digestible by the digestive enzymes but are readily digested by the beneficial microbes in gastrointestinal tract (Patkai, Barta, & Ivanics, 2002; Prosky, 1999). Conventionally, Jerusalem artichoke has been used for food or animal feed (Swanton & Hamill, 1994). Recently, alternative uses have been explored to produce functional food ingredients such as inulin, oligofructose and fructose (Panchev, Delchev, Kovacheva, & Slavov, 2011). However, there are few researches about Jerusalem artichoke on the synbiotic application.

Therefore, the aim of this work was to investigate the effect of Jerusalem artichoke and inulin on the survival of encapsulated *Lactobacillus acidophilus* TISTR 1338 within alginate matrix and double-coated with chitosan after freeze-drying process and heat processing. Moreover, the effects of co-encapsulated cells on growth performance, survival and disease resistance in white shrimp *Litopenaeus vannamei*, also determined.

CHAPTER 2
LITERATURE REVIEW

2.1 Pacific white shrimp, *Litopenaeus vannamei* (Boone, 1931)

Whiteleg shrimp (*Litopenaeus vannamei*), also known as Pacific white shrimp, King prawn, is native to the Eastern Pacific Coast from the Gulf of California, Mexico to Tumbes, North of Peru (Figure 2.1). Pacific white shrimp is the most important penaeid shrimp species farmed worldwide (Alcivar-Warren et al., 2007).

Figure 2.1 Pacific white shrimp, *Litopenaeus vannamei*
Source: http://www.sharkseafoods.com

According to the United Nations Food and Agriculture Organization, the growth of aquaculture sector is higher than any other types of animal food production systems which shrimp are the most valuable farmed aqua species worldwide. Farming of marine penaeid shrimp boomed from a mere 100 MT in the 80s to close to 4 million Mt in 2010 (Figure 2.2), with top five producers being China, Thailand, Vietnam, Indonesia and Ecuador. The shrimp industry, driven initially by high profitability which has immense economic importance for Thailand, but its rapid growth lead to disease outbreak in shrimp culture that ushered in some problems with the environment and food safety due to the use of antibiotics or chemotherapeutic agent. Hence, diseases are now considered as one of the critical limiting factor in the shrimp aquaculture. There became a fundamental need to establish standards to promote the sustainable development of

intensive shrimp farming in terms of quality and safety that would find acceptance at the domestic and international levels.

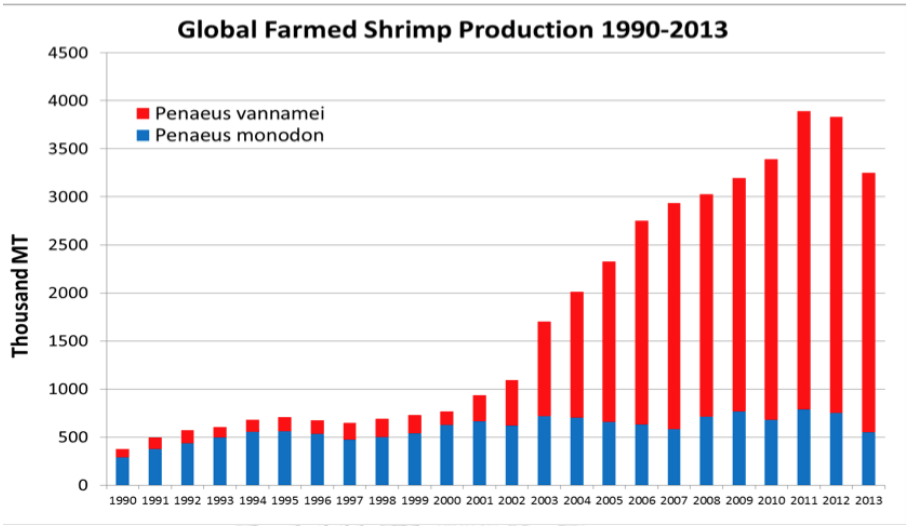

Figure 2.2 Global shrimp production for white shrimp (*P. vannamei*) and tiger shrimp (*P. monodon*) during 1990-2013.
Source: Fegan (2014) presented at the Aquaculture Roundtable (TARS) conference in Phuket, Thailand, held August 20-21.

2.2 Disease outbreaks in shrimp culture

Despite shrimp farming, an apparent success story in terms of production expansion, on the other hand, the fast development of these shrimp industry has produced various ecological, economic and social issues. Generally, intensive shrimp farming is the main aquaculture activity which has been frequently affected by bacterial pathogens especially in Asian countries. Shrimp production in many areas continues to suffer important economic losses due to the impact of a wide variety of diseases.

Recent events illustrate the impact of disease outbreaks on shrimp production in major producing countries. The white spot syndrome virus (WSSV), one of the main causes of the stagnating shrimp industry in the nineties, is significantly affecting shrimp production in recent years in Mexico and Brazil. Early Mortality Syndrome or Acute Hepatopancreatic Necrosis Disease (EMS/AHPND), is presently disrupting production in the three major shrimp producing countries China, Thailand and Vietnam. EMS was first reported in China in 2009, it has spread to Vietnam, Malaysia and Thailand, and

now causes annual losses of billions of USD. Among disease outbreaks in shrimp culture, Vibriosis is one of the major disease problems in shellfish and finfish aquaculture. Vibriosis is a bacterial disease responsible for mortality of cultured shrimp worldwide (Sivakumar, Sundararaman, & Selvakumar, 2012).

Vibriosis is caused by a number of Vibrio species of bacteria, including: *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida* (Brock & Lightner, 1990; Ishimaru, Akarawa-Matsushita, & Muroga, 1995). *Vibrio harveyi*, a gram-negative, luminous bacterium, is one of the important etiologic agents of mass mortalities of *Penaeus monodon* larval rearing systems. A large number of shrimp hatcheries along the coastline of our country involved in shrimp seed production often suffer setbacks due to luminescent bacterial disease and suffer enormous economic losses (Venkateswara, 2015). In some cases, antibiotics and other pharmaceuticals have been used to avoid disease outbreak of pathogen in shrimp aquaculture but there is no universally acceptable pharmaceutical agent approved by the FDA and does not imply an FAO recommendation for treating infections in shrimp aquaculture. Because, the development and spread of antimicrobial resistant pathogens were well documented (Cabello, 2006; Sorum, 2006). There is a risk associated with the transmission of resistant bacteria from aquaculture environments to humans, and risk associated with the introduction in the human environment of nonpathogenic bacteria, containing antimicrobial resistance genes, and the subsequent transfer of such genes to human pathogens (FAO, 2005). Considering these factors, there has been research in developing new dietary supplementation to prevent and control diseases in shrimp culture. A traditional approach to reduce the impact of shrimp diseases consists of increasing the level of key nutrients affecting the health and immunology of shrimp, including vitamin C and E, phospholipids, essential fatty acids, trace minerals and carotenoids. One of the methods gaining recognition for controlling pathogens is the use of a beneficial microorganism as an improving and growth promoting compounds in dietary supplements have been demonstrated. Moreover, synergistic blends between probiotic and prebiotic can be selected to promote shrimp health in form of feed additive by improving the immune potential of the aquatic animal and reducing pathogenic bacteria. Besides, feed additives are materials that are used to enhance the effectiveness of nutrients and exert their effects in the gut or on the gut wall cells to the

animal (McDonald et al., 2010). From the available alternative, there is presently an increased interest in using natural feed additives through their effect in increasing feed quality and palatability in feed industry to promoting aquatic animal health status.

2.3 Probiotic

Probiotics are organisms and substances that contribute to the intestinal microbial balance (Parker, 1974). Probiotics are a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). A definition provided by the Food and Agricultural Organization and World Health Organization was live microorganisms that when administered in adequate amounts confer a health benefit on the host. As for aquaculture, Probiotics are live microorganisms added to feed or rearing water that when administered to fish in adequate amounts confer increase in viability, enhance immune and digestive systems, promote growth and general welfare (Ringø et al., 2010). Probiotics are often defined as applications of entire or components of a microorganism which are beneficial to the health of the host (Irianto & Austin, 2002). Other probiotic definitions for aquaculture are more encompassing, for instance, the definition “microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health” (Gatesoupe, 1999). In the same way, a probiotic is defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by increase the host response towards disease, or by improving the quality of its environment (Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000). Probiotics are usually members of the healthy microbiota associated with the host; therefore, they may provide an alternative way to reduce the use of antibiotics in aquaculture (Perez et al., 2010).

The probiotic bacteria present in the aquatic environment influence the composition of the gut biota as the host and microorganisms share the ecosystem. Probiotics may act as a microbial dietary adjuvant that beneficially affects the host’s physiology by modulating mucosal and systemic immunity, as well as improving the nutritional and microbial balance in the intestinal tract. Probiotics can improve digestive activity by the synthesis of vitamins, cofactors or by improving the enzymatic

activity. These properties could facilitate the weight increase, improve digestion or nutrient absorption (Ninawe & Selvin, 2009). Though the probiotics might have potential nutritional improvement capacity, the main objective of probiotics would be to exploit their benefits by restricting the appearance of pathogenic bacteria in shrimp culture systems (Gullian, Thompson, & Rodriguez, 2004). The efficacy of probiotics to prevent disease and improve health management has been well established in white shrimp, *Litopenaeus vannamei*. Four bacterial strains isolated from the gastrointestinal tract of adult shrimp *L. vannamei* including *V. alginolyticus* UTM 102, *B. subtilis* UTM 126, *Roseobacter gallaeciensis* SLV03, and *Pseudomonas aestumarina* SLV22, were showed antagonism against the shrimp-pathogenic bacterium, *V. parahaemolyticus* PS-017 (Balcazar & Rojas-Luna, 2007). It has also been reported that lactic-acid bacteria increase the survival of marine shrimp, *L. vannamei*, after infection with *Vibrio harveyi* (Vieira, Pedrotti, & Buglione, 2007). This study evaluated the survival, post-larvae quality, and the population of bacteria in *L. vannamei* after the addition of two strains of lactic-acid bacteria (2 and B6) experimentally infected by *V. harveyi*. The survival of control shrimp was lower (21%) than that of animals fed with the strains B6 (50%) and 2 (44%). Although, the study showed the efficacy of lactic acid bacteria on the control of *V. harveyi*, the efficacy cannot be considered as significant, since the survival of treated shrimp was 29% and 23% respectively over the control. Balcazar and Rojas-Luna (2007) demonstrated inhibitory activity of probiotic *B. subtilis* UTM 126 against vibrio species confers protection against vibriosis in juvenile *L. vannamei*. Other studies, they reported that the beneficial effects of *Bacillus licheniformis* on the intestinal microbiota and immunity of the white shrimp, *Litopenaeus vannamei*. The administration of *B. licheniformis* improved the white shrimp's intestinal microbiota, and its immune ability (Li et al., 2007). Thus, the application of probiotics gets effective on the stabilization and improvement of gut microbiota in shrimp. Though shrimp immune system and physiology are different from higher animals, one of well-established mechanisms of action of probiotics in higher animals, stabilization of gut microbiota appear to be functional in shrimp body. Recent studies evidenced that shrimp immune system could be enhanced with the administration of probiotics. The administration of *Lactobacillus plantarum* in the diet induced immune modulation and enhanced the immune ability of *L. vannamei*, and increased its resistance to

V. alginolyticus infection (Chiu, Guu, Liu, Pan, & Cheng, 2007). Probiotics might be prevent bacterial diseases through a variety of mechanisms, such as the creation of a hostile environment for pathogens by the production of inhibitory compounds, by competing for essential nutrients and adhesion sites or by modulating the immune responses (Balcazar et al., 2006; Merrifield et al., 2010). There are many studies involving probiotics for use in shrimp culture (Table 2.1).

Table 2.1 Prospective probiotics evaluated for shrimp aquaculture applications

Strain	Source	Evaluated for
Bacillus S11	Black tiger shrimp	Growth and survival of black tiger shrimp
Bacillus subtilis BT23	Shrimp culture ponds	Against the growth of *Vibrio harveyi* isolated by agar antagonism assay from *Penaeus monodon*
Pseudomonas sp. PM11*Vibrio fluvialis* PM17	Gut of farm reared sub-adult shrimp	Immunity indicators of *Penaeus monodon*
Arthrobacter XE-7	Isolated from *Penaeus chinensis*	Protection of *Penaeus chinensis* post-larvae from pathogenic vibrios such as *Vibrio parahaemolyticus*, *Vibrio anguillarum* and *Vibrio nereis*
Bacillus licheniformis	Shrimp pond	Intestinal microbiota and immunity of the white shrimp *Litopenaeus vannamei*
Lactic-acid bacteria	Shrimp gut	Survival of marine shrimp,*Litopenaeus vannamei* challenged with *V. harveyi*
Lactobacillus plantarum	Shrimp isolate	Immune response and microbiota of shrimp digestive tract of *Litopenaeus vannamei* challenged with *V. alginolyticus* and *V. harveyi*

Reference: Adapted from Ninawe and Selvin (2009)

However, the probiotic potential of different bacterial strains, even within the same species, differs by means of adherence (site-specific), specific immunological effects, and actions on a healthy vs. an inflamed mucosal surfaces may be differ from

others. At present the researchers, aimed to characterize probiotic potentials of normal healthy gut microbiota of different aquatic species to understand microbe-microbe interactions in intestinal tract, host microbe interaction (include competitive exclusion of pathogens), their immunomodulation and nutritional importance (Soccol et al., 2010). Currently, it is widely accepted that the mechanism of probiotics include inhibitory interaction, production of inhibitory compounds, competition for chemicals and adhesion sites, improving the microbial balance, immune modulation and stimulation, and bioremediation of accumulated organic lead in the pond bottom (Lin, Browdy, & Hopkins, 1995; McCracken & Gaskin, 1999; Rengpipat, Phianpak, Piyatiratitivorakul, & Menasveta, 1998; Verschuere et al., 2000). The use of probiotic bacteria, based on the principle of competitive exclusion, and the use of immunostimulants are two of the most promising preventive methods developed to against diseases (Verschuere et al., 2000). One of the main challenges in developing probiotic bacteria is using appropriate selection and colonization methods. The selection criteria for probiotic bacteria should evaluate the colonization methods, competition ability against pathogens and the immunostimulatory growth effect on shrimp (Gatesoupe, 1999; Gomez-Gil, Roque, & Tumbull, 2000). By applying these bacteria in shrimp farms, a biological equilibrium between competing beneficial and deleterious microorganisms could be produced. The most commonly used probiotic in aquaculture are the strains such as *Lactobacillus* and *Bifidobacterium*.

2.3.1 *Lactobacillus* spp.

The *Lactobacillus* sp. is a group of lactic acid bacteria (LAB), Gram-positive bacteria, non-spore-forming and non-flagellated rods or coccobacilli that ferment carbohydrates to produce lactic acid as a major end-product (Hammes & Vogel, 1995). Reduction of pH and fermentation of large amount of carbohydrates are the primary actions by LAB to inhibit food borne pathogens. In technologically, they are regarded as non-pathogenic and safe microorganisms (Soccol et al., 2010). Lactic acid bacteria convert lactose into lactic acid, thereby reducing the pH in the gastro intestinal environment and naturally preventing the colonization by many bacteria (Klewicki & Klewicka, 2004). Lactic acid bacteria is great producers of bacteriocins and organic acids (lactic and acetic acids) which have inhibitory effects in vitro on the growth of

some aquatic pathogens (Planas et al., 2004). These antimicrobial substances have facilitating their environment with a competitive advantage over other microorganisms to be used as probiotics (Salminen, Deighton, Benno, & Gorbach, 1998). *Lactobacillus* is distributed in various ecological niches throughout the gastrointestinal tracts and constitutes an important part of the indigenous microflora of aquatic animals (Ringo & Gatesoupe, 1998). The *Lactobacillus* strains mainly used in animal feed are *L.casei*, *L.plantarum*, *L.acidophilus*, and *L.rhamnosus* (Anadon, Martínez-Larrañaga, & Martínez, 2006) and also has been used as probiotic in shrimp aquaculture. Several studies has been evaluated with different *Lactobacillus* sp. as probiotic in shrimp farming by their nutritional benefits and strong antimicrobial activity against pathogenic microorganisms (Gilliland, Nelson, & Maxwell, 1985; Ismail & Soliman, 2010; Qi, Zhang, Boon, & Bossier, 2009; Rossland, Andersen-Borge, Langsrud, & Sorhaug, 2003). Lactic acid bacteria has been used as beneficial microorganisms against shrimp pathogens (Corcoran, Ross, Fitzgerald, & Stanton, 2004; Senok, Ismaeel, & Botta, 2005). Probiotic preparations with Lactobacillus have recently received considerable interest in aquaculture as show in the Table 2.2.

Some important evidences for the use of Probiotic *Lactobacillus* sp. has been postulated several modes of action in the host which includes competition for nutrients, non-specific immune modulation, production of antimicrobial compounds and competition for site attachment. The bacteria that act as good probiotic must have the following properties;

1) adhere to intestinal epithelium
2) exclude pathogens
3) compete for essential nutrients
4) stimulate the immunity of the host
5) persist and multiply
6) produce antimicrobial substances and antagonistic to pathogen growth
7) be safe and nonpathogenic
8) improve the intestinal digestion
9) maintain the balanced microflora in the intestinal tract

These properties are mainly used to screen suitable probiotics for the aquaculture system (Sivakumar, Selvakumar, Varalakshmi, & Ashokkumar, 2014).

Table 2.2 Importance of lactic acid bacteria in aquaculture

Probiotic organism	Study
Lactobacillus acidophilus	Comparison of two strains of *Lactobacillus acidophilus* as dietary adjuncts for young calves
Lactobacillus plantarum	Psychrotrophic *Lactobacillus plantarum* from fish and its ability to produce antibiotic substances
Lactobacillus reuteri	The effect of yoghurt and milk fermented with a porcine intestinal strain of *Lactobacillus reuteri* on the performance and gastrointestinal flora of pigs weaned at two days of age
Lactobacillus spp	Probiotic use of *Lactobacillus* spp. for black tiger shrimp, *Penaeus monodon*
Lactobacillus rhamnosus	Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*).
Lactococcus lactis	An antibacterial effect of *Lactococcus lactis* isolated from the intestinal tract of the Amur catfish, *Silurus asotus*, Linnaeus
Lactobacillus plantarum	Time-related action of *Lactobacillus plantarum* in the bacterial microbiota of shrimp digestive tract and its action as immunostimulant
Lactobacillus plantarum	The effect of a *Lactobacillus plantarum*-supplemented diet on shrimp growth, digestive tract bacterial microbiota, survival, and some hemato-immunological parameters after an experimental challenge with *Vibrio harveyi was studied*.
Lactobacillus acidophilus	Efficacy of *Lactobacillus acidophilus* on the sperm quality and regeneration of spermatophores in *Penaeus monodon* (Fabricius, 1798)
Lactobacillus acidophilus	Probiotic effect of *Lactobacillus acidophilus* against vibriosis in juvenile shrimp (*Penaeus monodon*)

Reference: Adapted from Sivakumar et al. (2014)

Moreover, Dowarah, Verma, and Agarwal (2017) suggested that the criteria that can be used for the selection of microbial strains for feeding as probiotics should be the following properties;

1. Resistance to *in vitro/in vivo* conditions

They should be resistant to acidic pH and bile salt. After administration, the microbes should not be killed by the defense mechanisms of the host and should be resistant to the specific conditions occurring in the body.

2. Origin of the strain: probiotics are generally host-species specific

It is believed that probiotic organism is more effective if it is naturally occurring in the target species. The strains should be properly isolated and identified before use.

3. Biosafety

Lactobacillus, Bifidobacteria and *Enterococcus* are the microbes which fall in the category of generally recognized as safe and most widely used microorganisms as probiotics.

4. Viability/survivability and resistance during processing

Thermophilic/thermo-tolerant organisms have an advantage as they withstand higher temperature during processing and storage.

However, other criteria might also be considered for selection of mono or multi strains bacteria as probiotics like as probiotic-symbiotic interaction, stimulation of healthy microbiota and suppression of harmful bacteria. Adopting these predetermined criteria, it could be possible to select the best strains of probiotics which could be effective therapeutically and nutritionally (Dowarah et al., 2017).

Due to their health-promoting properties, some of these bacteria are classified as probiotic bacteria. *L. acidophilus* has been recognized as probiotic bacteria due to their ability to adhere to animal or human intestines and to release health advantages for the hosts. *Lactobacillus* also binds mutagens contributing to a protection mechanism against cancer. *L. acidophilus* has been extensively studied as a probiotic, and evidence has shown that it may provide a number of health benefits in human (Ljungh & Wadström, 2006). Previous studies suggested that certain probiotics can help reduce cholesterol levels and that *L. acidophilus* may be more effective than other types of probiotics (Cho & Kim, 2015; Shimizu, Hashiguchi, Shiga, Tamura, & Mochizuki, 2015). Gilliland et al. (1985) reported that *L. acidophilus* had the ability to resist bile

salt and decrease cholesterol levels. *L. acidophilus* is one of the most popular bacteria used as probiotics for human consumption (Mortazavian, Razavi, Ehsani, & Sohrabvandi, 2007). *Lactobacillus acidophilus* has been considered to be the predominant lactobacillus in the intestinal tract of healthy humans (Ray, 1996). Most strains of *L. acidophilus* can ferment amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose and trehalose (Gomes & Malcata, 1999). The *L. acidophilus* is capable of surviving in acidic environment with pH from 4 to 5 or below. It is able to pass harsh environments of the stomach and move through to the small intestine. The *L. acidophilus* have good survival rate as faced with pH 2.0 or mixed into the gastric juice/product at pH 1.4 and 45% survived in a medium that contain 0.3% bile for 3 hours (Deraz, Karlsson, Khalil, & Mattiasson, 2007). *L. acidophilus* strains have been widely utilized as a dairy starter culture for their therapeutic activities associated with an intestinal microbial balance. Such effects have been attributed to biochemical, physiological, and antimicrobial effects, as well as competitive exclusion in the intestinal tract (Goldin & Gorbach, 1992). The main therapeutic and health benefits of *L. acidophilus* are that they enhance the immune system, and prevent intestinal infections, diarrheal disease, colon cancer and upper gastrointestinal tract diseases (Kailasapathy & Chin, 2000). A number of studies have shown that probiotics like *L. acidophilus* may help prevent and reduce diarrhea that's associated with various diseases (Sazawal et al., 2006). Furthermore, *L. acidophilus* has been demonstrated in animal feeding to improve health such as Tortuer (1973) showed the influence of *L. acidophilus* in broiler chicks on growth, feed conversion and crude fat digestibility. The addition of *L. acidophilus* in broiler chick diet decreased the digestibility of crude fat. Karthikeyan and Santhosh (2009) reported that bacteriocin producing *L. acidophilus* strain was isolated from the gut of marine prawn (*P. monodon*). This bacteriocin has broad range of antibacterial activity against major food borne pathogens. The other studies demonstrate that the infected groups of fish maintained on probiotic diets yielded significantly better haematology parameters and histopathology than the infected groups fed with the non-probiotic diets. This supports the beneficial effects of *L. acidophilus* that were used as a bio-control agent in African catfish, *Clarias gariepinus*, juveniles (Al-Dohail, 2011). Also in the studies by Villamil, Reyes, and Martínez-Silva (2014) they reported that the *L. acidophilus* can increase

survival, weight gain of improvement in feed conversion, and stimulate humoral immune response, interfere with pathogenic bacteria survival in Nile tilapia. Sivakumar, et al. (2012) demonstrated that the incorporating *L. acidophilus* 04 in shrimp has potential probiotic to control pathogenic *V. alginolyticus* in *P.monodon* shrimp culture. However, there are still few studies has been reported for of probiotic *L. acidophilus* supplementation in white shrimp culture.

2.3.2 Applications of Probiotics in Aquaculture

The need for sustainable aquaculture has promoted research into the use of probiotics on aquatic organisms. The initial interest was focused on their use as growth promoters and to improve the health of animals; however, new areas have been found, such as their effect on reproduction or stress tolerance, although this requires a more scientific development.

1. Growth promoter: Probiotics have been used in aquaculture to increase the growth of cultivated species. According to Balcazar et al. (2006) probiotic microorganisms are able to colonize gastrointestinal tract when administered over a long period of time because they have a higher multiplication rate than the rate of expulsion, so as probiotics constantly added to fish cultures, they adhere to the intestinal mucosa of them, developing and exercising their multiple benefits. The use of probiotics as growth promoters of edible fishes has been reported.

2. Inhibition of pathogens: Antibiotics were used for a long time in aquaculture to prevent diseases in the crop. However, this caused various problems such as the presence of antibiotic residues in animal tissues, the generation of bacterial resistance mechanisms, as well as an imbalance in the gastrointestinal microbiota of aquatic species, which affected their health (Nakano, 2007). In fact, the European Union has regulated the use of antibiotics in organisms for human consumption (Ronson & Medina, 2002). Today, consumers demand natural products, free of additives such as antibiotics; moreover, there is a tendency for preventing diseases rather than treating them. Thus, the use of probiotics is a viable alternative for the inhibition of pathogens and disease control in aquaculture species (Cruz, Ana, Oscar, & Hugo, 2012). Probiotic microorganisms have the ability to release chemical substances with bactericidal or bacteriostatic effect on pathogenic bacteria that are in the intestine of the host, thus

constituting a barrier against the proliferation of opportunistic pathogens. In general, the antibacterial effect is due to one or more of the following factors: production of antibiotics, bacteriocins, siderophores, enzymes (lysozymes, proteases) and/or hydrogen peroxide, as well as alteration of the intestinal pH due to the generation of organic acids (Verschuere, et al., 2000). Gomez, Balcazar, and Shen (2007) reported the use of *Vibrio alginolyticus* strains as probiotics to increase survival and growth of white shrimp (*Litopenaeus vannamei*), also by using probiotics in Ecuadorian shrimp hatcheries, production increased by 35%, while with the use of antimicrobials it decreased by 94%.

3. Improvement in nutrient digestion: A study has suggested that probiotics have a beneficial effect on the digestive processes of aquatic animals because probiotic strains synthesize extracellular enzymes such as proteases, amylases, and lipases as well as provide growth factors such as vitamins, fatty acids, and amino acids (Balcazar et al., 2006). Therefore, nutrients are absorbed more efficiently when the feed is supplemented with probiotics (Haroun, Goda, & Kabir, 2006). In white shrimp *Litopenaeus vannamei* Boone and *Fenneropenaeus indicus*, various strains of *Bacillus* have been used as probiotics to increase apparent digestibility of dry matter, crude protein, and phosphorus. Results showed higher sizes when the diet is supplemented with 50 g of probiotic kg/1 of food (Heizhao, Zhixun, Yingying, Wenhui, & Zhuojia, 2004).

4. Improvement of water quality: In several studies, suggested that maintaining high levels of probiotics in production ponds, fish farmers can minimize the accumulation of dissolved and particulate organic carbon during the growing season. In addition, this can balance the production of phytoplankton (Balcazar et al., 2006). Wang, Lee, Najiah, Shariff, and Hassan (2000) showed that a commercial product made from *Bacillus* sp., *Saccharomyces cerevisiae*, *Nitrosomonas* sp., and *Nitrobacter* sp. had the ability to increase the beneficial bacterial microbiota of *Penaeus vannamei* shrimp, further reducing the concentrations of inorganic nitrogen from 3.74 to 1.79 mg/Land phosphate from 0.1105 to 0.0364 mg/L.

Probiotics are increasingly used in commercial animal production operations to advantageously alter gastrointestinal flora, thereby improving animal health and productivity. The major outcomes from using probiotics include improvement in

growth, reduction in mortality, and improvement in feed conversion efficiency. However, probiotics are sensitive to environmental conditions which might be affected during all steps involved in a delivery process through the exposure to different stress such as freezing, heating, drying and exposure to gastrointestinal environment. Therefore, the maintenance of the cell viability in probiotic-containing products under variety of harsh conditions is still considerably challenging.

2.4 Prebiotic

Besides, the use of Probiotics as biological control agents in shrimp aquaculture, the use of prebiotics that alter the conditions to favor certain bacterial species which may enhance fish growth efficiency and reduce disease susceptibility of the host organism (Burr, Hume, Ricke, Nisbet, & Gatlin, 2010; Grisdale-Helland, Helland, & Gatlin III, 2008).

Prebiotics are defined as non-digestible components that are metabolized by specific health-promoting bacteria such as Lactobacillus and Bifidobacterium. These bacteria are considered beneficial to the health and growth of the host by decreasing the presence of intestinal pathogens and/or changing the production of health related bacterial metabolites (Gibson & Roberfroid, 1995; Manning & Gibson, 2004). Whereas the latest prebiotics are defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). For a dietary substrate to be classed as a prebiotic, at least three criteria are required as follow (Manning & Gibson, 2004).

(1) The substrate must not be hydrolyzed or absorbed in the stomach or small intestine.

(2) It must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria.

(3) Fermentation of the substrate should induce beneficial luminal/systemic effects within the host.

Prebiotics are carbohydrates, which can be classified according to their molecular size or degree of polymerization (number of monosaccharide units), into monosaccharides, oligosaccharides or polysaccharides. The common prebiotics established in aquaculture to date include

- Inulin
- Fructooligosaccharides (FOS)
- Mannanoligosaccharides (MOS)
- Galactooligosaccharides (GOS)
- Isomaltooligosaccharide

The studies performed on prebiotics revealed promising results in case of improvement of disease resistance, growth performance, hormonal regulation as well as immune stimulation (Eshaghzadeh, Hoseinifar, Vahabzadeh, & Ringo, 2015). However, numerous studies have been conducted on administration of prebiotics in aquaculture, less information on the effects of inulin and FOS as prebiotic on shrimp culture.

2.4.1 Inulin

Inulin belongs to a class of dietary fibers known as fructans, composed of a polymer of β-D-fructose (F) attached by β -2-1 linkages. The first monomer of the chain is either a ß-D-glucopyranosyl or β-D-fructopyranosyl residue. D-fructose (F) link with D-glucose (G), with general structure of GFn. “n” refers to the degree of polymerization of inulin, and it’s usually 10 or so (Figure 2.3). A recent study showed that dietary supplementation of inulin decreased the prevalence of WSSV in *L. vannamei* and increased the phenoloxidase activity, but didn’t affect hemocyte number, growth, survival, and lactic acid bacteria in shrimp. It seems unclear whether inulin alone can act as an immunostimulant or can work through its fermented by-products, such as short chain FOS, so that certain immune defense activities occur in shrimp (Luna-González et al., 2012).

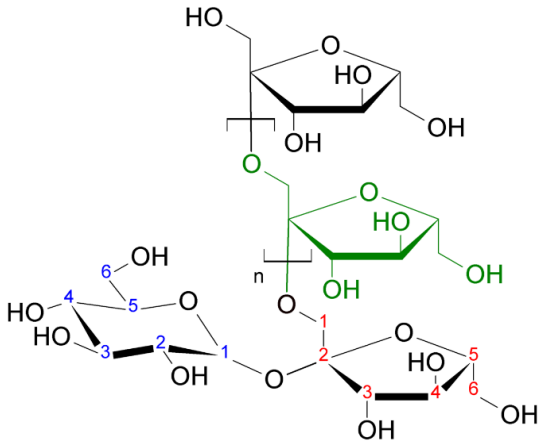

Figure 2.3 Inulin structure
Source: https://en.wikipedia.org/wiki/Inulin

2.4.2 Fructooligosaccharides

One of the most common prebiotics studied in humans and terrestrial animals is FOS. FOS refers to short and medium chains of β-D-fructans in which fructosyl units are bound by β-(2-1) glycosidic linkages and attached to a terminal glucose unit (Figure 2.4). FOS is an inulin-like ingredient, having the same general formula of GFn, with n ranging from 1 to 5. FOS can be fermented by certain bacteria expressing this enzyme, such as lactobacilli and bifidobacteria (Manning & Gibson, 2004). Dietary inclusions of FOS will thus selectively support the growth and survival of such bacteria in the GI tract of animals. Dietary supplementation of FOS has been shown to enhance growth rate of some aquatic animals such as Atlantic salmon, hybrid tilapia, turbot larvae and soft-shell turtle (Grisdale-Helland et al., 2008; He, Xu, Wu, Weng, & Xie, 2003; Mahious, Gatesoupe, Hervi, Metailler, & Ollevier, 2006).

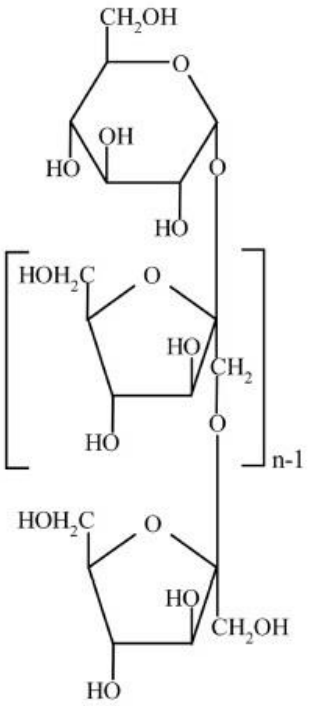

Figure 2.4 Fructooligosaccharides structure
Source: Wayne, et al. (2012)

Some studies have investigated the effect of dietary supplementation of short-chain fructooligosaccharide (scFOS) on intestinal microbiota of white shrimp. Li et al. (2007) showed that different inclusion levels of scFOS (0.25, 0.5, 0.75, 1, 2, 4 and 8 g/kg) did not improve weight gain, feed conversion or survival of shrimp. However, DGGE analysis suggested that the gut microbiota was affected by scFOS compared to shrimp fed a basal diet and that the gut microbial community from shrimp fed the scFOS supplemented diets (1-8 g/kg) were very similar. In the report of Zhou, Ding, and Huiyan (2007) shown that dietary scFOS supplementation at concentrations from 0.04% to 0.16% improved specific growth rate and feed conversion of white shrimp cultured in a recirculation system, although survival was relatively low (42-61%) for all treatments. Significant differences were observed in the counts of *V. parahemolyticus*, *A. hydrophila, Lactobacillus* sp. and *S. faecalis*. The counts of *V. parahemolyticus* were the highest in the gut of shrimp fed 0.04% and 0.08% scFOS, while the population level of *S. faecalis* was the highest when the shrimps were fed 0.12% and 0.16% scFOS. Whether the microbial shift had any positive effect on the

fish health, contribution to inhibit colonization of pathogenic bacteria in the gut or to improve innate immunity remains to be elucidated.

2.4.3 Jerusalem artichoke

Jerusalem artichoke (JA) (*Helianthus tuberosus*) or Thai name “Kaentawan” is a tuberous annual crop of which is rich in fructooligosaccharide carbohydrates in the forms of inulin and fructans (Figure 2.5). Jerusalem artichoke is widely used as forage for animal, biofuel production for automotive transport, food and medicinal uses for human and chemical raw materials (Ahmed, El-Sakhawy, Soliman, & Abou, 2005; Pan et al., 2009; Yuan et al., 2008; Yuan, Gao, Xiao, Tan, & Du, 2012). These Jerusalem artichoke compounds were found to possess antioxidant, antimicrobial, antifungal and anti-cancer activities for medicinal uses (Ma et al., 2011).

Figure 2.5 Jerusalem artichoke
Source: https://www.growthis.com/how-to-grow-jerusalem-artichokes/

The previous studied showed that feeding FOS from Jerusalem artichoke to animals helped improve gut health by balancing microbial population, lowering intestinal pH and stimulating development of gut wall resulting in improved absorption (Farnworth et al., 1992).

2.4.4 Mode of action of prebiotic in aquaculture

Prebiotics can be considered as a beneficial dietary supplement for improving growth performance, boosted immune responses, and increased stress resistance, improving digestive enzyme activities. Several studies analyzed the survival of the

specimens after fed the prebiotic. The results demonstrated that usually prebiotic improve the growth factors (daily weight gain, final weight, weight gain, specific growth rate, condition factor, food conversion ratio, feed efficiency ratio, and protein efficiency ratio) (Gultepe et al., 2012; Hoseinifar, Khalili, Rostami, & Esteban, 2013; Xu, Wang, Li, & Lin, 2008) or not produce any effect on them (Burr et al., 2010; Hoseinifar, Ringo, Shenavar Masouleh, & Esteban, 2014). This could be explained taken into account that the effects of prebiotics may vary depending upon the fish species to be analyzed.

2.5 Synbiotic

Additionally, a large number of studies have combined probiotics with prebiotics, a selectively fermented ingredient that allows specific changes both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health (Gibson, Probert, Van-Loo, Rastall, & Roberfroid, 2004). Thus the synbiotics, as a combination of probiotics and prebiotics, have been studied to expect the synergistic effects.

The effect of dietary application of a commercial probiotic (*Bacillus* spp.) and MOS, used singularly and combined, on the survival, growth performance and feed cost-benefit of European lobster (*Homarus gammarus*) larval was assessed and the results in this study strongly suggest that the dietary combination of *Bacillus* spp. and MOS is cost effective when used to promote survival and provides the added benefits of improved growth performance, compared to their individual supplementation (Daniels et al., 2010). Similar results have been reported on shrimp, *L. vannamei*, and the disease resistance was also improve by enhancing immunity, as well as presumably modulating microflora in the shrimp's gut (Li, Chen, Cha, Park, & Liu, 2009). It suggested that the combined application of probiotics and prebiotics is an interesting prospect for replacement of growth-promoting chemotherapeutics in the aquaculture industry and could be a useful tool in the rearing of certain aquatic animals. In addition, Hai and Fotedar (2009) reported that the addition of Bio-Mos® and β-1,3-D-glucan into feed has increased growth, survival rates and immune responses in king prawns (*Penaeus latisulcatus*).

2.6 Microencapsulation

The maintenance of the cell viability in probiotic-containing products is still considerably challenging because the probiotic must survive during the industrial processing, storage condition and gastrointestinal passage. The suitable technology for protection of probiotic cells has resulted in greatly enhanced viability of these microorganisms in food products as well as in the gastrointestinal tract, is microencapsulation technique.

Encapsulation is a physicochemical or mechanical process to entrap a substance in a material in order to produce particles with diameters of a few nanometers to a few millimeters (Chen & Chen, 2007). Encapsulation of bioactive components can be used in many applications in the food industry: controlling oxidative reaction, masking flavours, colours and odours, providing sustained and controlled release, extending shelf life, etc. (Burgain, Gaiani, Linder, & Scher, 2011).

Microencapsulation is a process to entrap probiotic cells within a carrier material and it is a useful tool to keep living probiotic cells in foods, to protect them from the external environment and to extend their storage life (Capela et al., 2007; Chavarri, Maranon, & Carmen, 2012).

In aquaculture, probiotics can also be encapsulated infeed (Ramos, Relucio, & Torres-Villanueva, 2005) or in live food, such as rotifers and Artemia (Mahdhi, Amoun, & Bakhrouf, 2011). Another efficient application of probiotics to aquatic animals is through bio-encapsulation or infusions in diets. According to the FAO and WHO guidelines, probiotic organisms used in food must be capable of surviving passage through the gut. They must have the ability to resist gastric juices and exposure to bile. In addition, probiotics must be able to proliferate and colonize in the digestive tract, to be safe, effective, and maintain their effectiveness and potency for the duration of the shelf life of the product (Senok et al., 2005). The benefits of inclusion of bacterial strains into feed ingredients include improvements in feed values, contributions to enzymatic digestion, inhibition of pathogenic microorganisms, anti-mutagenic and anti-carcinogenic activity, growth-promoting factors, and enhanced immune response (Ambas, Suriawan, & Fotedar, 2013; Kuhlwein, Merrifield, Rawling, Foey, & Davies, 2013; Wang, 2007; Wang & Xu, 2006). Microencapsulation technologies were developed and applied successfully to protect probiotic bacteria from damage caused

by external factors such as drying, packaging and storage conditions (e.g., time, temperature, moisture and oxygen), and the degradation in the gastrointestinal tract, especially due to extreme pH (2.5 to 3.5) of gastric juices and bile salts (Kailasapathy, 2006). The selection of the encapsulation method depends on the required particle average size, the physical and chemical properties of the carrier material, the applications of the encapsulated material, the required release mechanism and cost.

2.6.1 Extrusion Techniques for encapsulated microbial cells

Extrusion is commonly employed for the microencapsulation of microbial cells (Koyama & Seki, 2004; Özer, Uzun, & Kirmaci, 2008). In employing extrusion, a polymeric solution is first mixed with the microbial cells and forming droplets by extruding the suspension through a syringe needle (laboratory scale) or a extruder (pilot scale) to free-fall into a hardening solution (e.g., calcium chloride) (Figure 2.6).

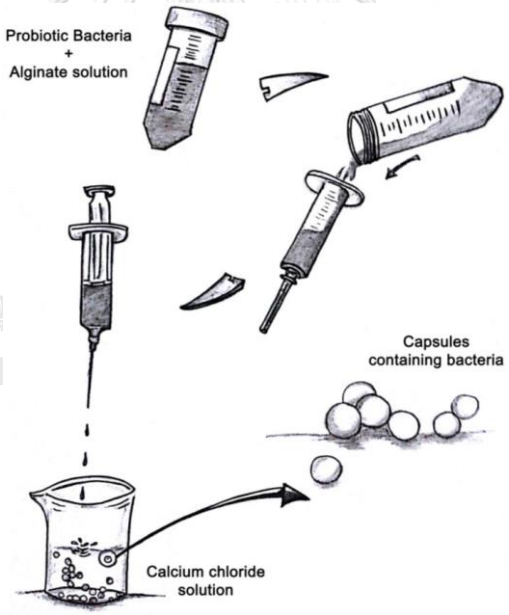

Figure 2.6 Schematic diagram of the extrusion encapsulation method.
Source: Serna-Cock and Vallejo-Castillo (2013)

The size and shape of the microcapsule formed depend on the diameter of the nozzle and the distance between the nozzle and the $CaCl_2$ solution. The major

advantages of the extrusion method are simplicity of its operation, lower cost, and mild operational conditions ensuring high cell viability (de Vos, Faas, Spasojevic, & Sikkema, 2010). It does not cause cell damage and results in high cell viability (Krasaekoopt, Bhandari, & Deeth, 2003). The technology does not use harmful solvents and can be done under both, aerobic and anaerobic conditions. However, there are also a few drawbacks such as its inefficiency in producing microspheres smaller than 500 μm, requirement of low to moderate viscosity polymer solutions and relatively large diameter nozzles (Reis, Neufeld, Vilela, Ribeiro, & Veiga, 2006). In addition, rapid cross-linking and hardening at the surfaces of the microspheres delay the movement of cross-linking ions into the inner core, resulting in less stable microspheres (Liu et al., 2002). Although microspheres are conveniently produced at laboratory-scale, the scaling up of the process is generally difficult due to the slow production of microspheres (Burgain et al., 2011). The survival of the probiotic microorganisms *L. acidophilus* 547, *B. bifidum* ATCC 1994, and *L. casei* 01 microencapsulated in chitosan-coated alginate pearls was evaluated in yogurt made with UHT milk and conventional pasteurization during storage at 4°C for 4 weeks. Sodium alginate 2% and chitosan 4% were used to prepare the microcapsule. The results showed that the survival of the encapsulated probiotic bacteria was greater than free cells in approximately 1 log cycle. During storage, the number of probiotic bacteria, with the exception of *B. bifidum*, remained above 10^7 cfu/g, minimum recommended to ensure a therapeutic effect. The *B. bifidum* count fell below 10^7 cfu/g after 2 weeks of storage. The UHT treatment in yogurt did not alter the probiotic bacteria viability when compared with conventional thermal treatment (Krasaekoopt, Bhandari, & Deeth, 2006; Soto et al., 2011).

2.6.2 Materials used for encapsulated microbial cells

The encapsulated substance called the core material is dispersed in a matrix also named coating or shell. This carrier material must be food grade if used in food or feed industry, and able to form a barrier to protect the encapsulated substance. Producing stable microspheres for microbial cell immobilization starts with the selection of an appropriate encapsulation material. Studies have shown that polymer types play a dominant role in determining the properties of the microspheres (Jen, Wake, & Mikos,

1996). Owing to, the viability of encapsulated probiotic cells depend on the physico-chemical properties of the capsules. In fact, the type and the concentration of the coating material, particle size, initial cell numbers and bacterial strains are some parameters to evaluate the cell viability (chen and chen, 2007). In the other case of probiotic encapsulation, the objective is not only to protect the cells against adverse environment, but also to allow their release in a viable and metabolically active state in the intestine (Picot & Lacroix, 2004).

The most widely used matrix for microencapsulation is alginate which has been found to increase the survival of probiotics from 80 to 95% (Mandal, Puniya, & Singh, 2006). Alginate is a naturally derived polysaccharide extracted from various species of algae and composed of β-D-mannuronic and α-L-guluronic acids (Figure 2.7).

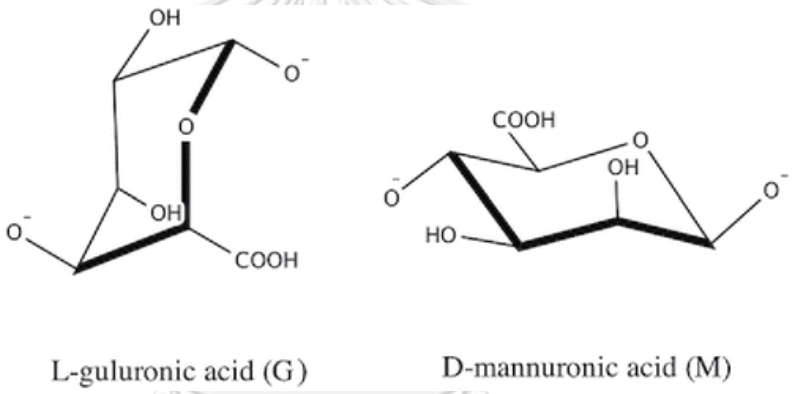

Figure 2.7 Chemical structure of alginate monomers.
Source: Ching, Bansal, and Bhandari (2017)

The composition of the polymer chain varies in amount and in sequential distribution according to the source of the alginate and this influences functional properties of alginate as supporting material. The alginate polymer may contain regions consisting of exclusively one type of monomer (M-blocks or G-blocks) or an alternating sequence of M and G residues (MG-blocks) (Figure 2.8). Commercially, alginates are available in the form of sodium, potassium, or ammonium salts. Molecular weights of alginate typically range from 60,000 to 700,000 Daltons depending on the application (Draget, Simensen, Onsoyen, & Smidsrod, 1993). Compared to other polysaccharides such as gelatin or agar, alginate is able to form gel independent of temperature. One of the most highly valued properties of alginate in the food industry is the ability to form

ionic gel in the presence of multivalent cations to formation of alginate gels by ionic crosslinking with cations. The gel formed by this interaction is widely utilized in the encapsulation of bioactive in the food industry, drugs in pharmaceutical industry and cell immobilization in the biotechnology industry. Moreover, Ca is a nontoxic and hence is widely used to form ionic alginate gels (Ching et al., 2017). The addition of Ca ions into the alginate polymer causes the binding of two G chains on opposite sides. This tightly bound polymer configuration results in the formation of a junction zone shaped like an "egg-box" (Figure 2.9). Each cation binds with four G residues in the egg-box formation to form a 3-D network of these interconnected regions (Clare, 1993).

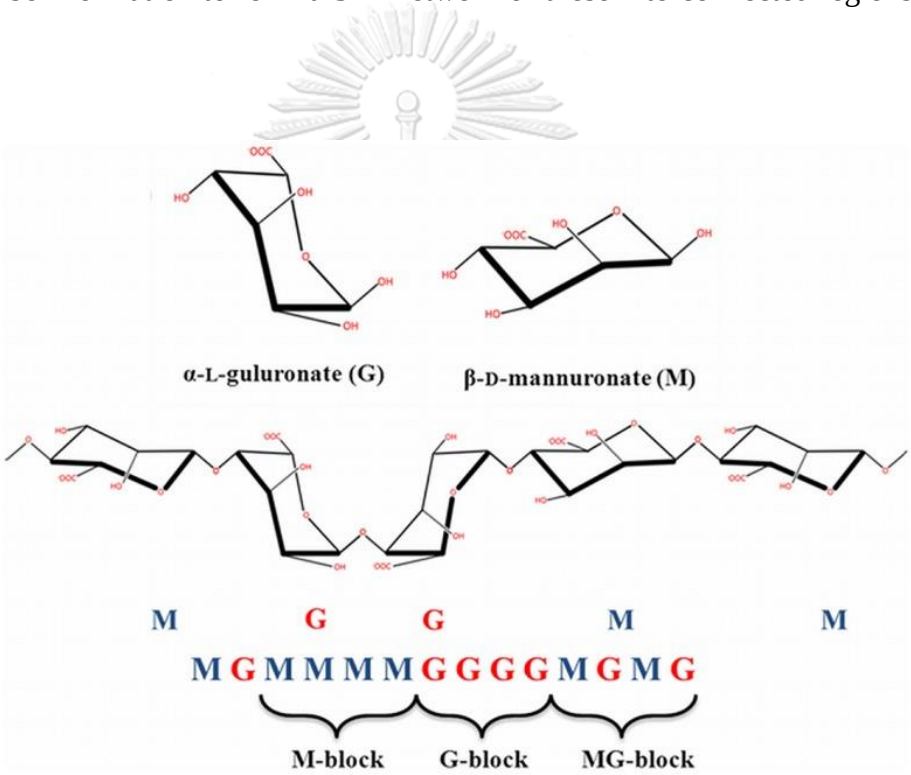

Figure 2.8 Chemical structure of alginate: Linear block polymers of L-guluronate (G) and D-mannuronate (M) with a variation in composition and sequential arrangements.
Source: Paredes-Juárez, Spasojevic, Marijke, and deVos (2014)

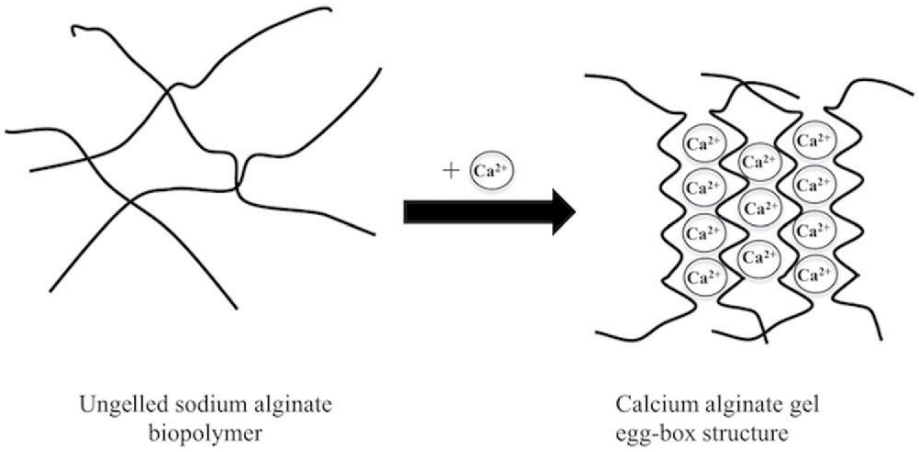

Figure 2.9 Alginate and $CaCl_2$ formation during the ionic gelation.
Source: Ching, et al. (2017)

Alginate hydrogels are extensively used in cell encapsulation and calcium alginate is preferred for encapsulating probiotics because of its simplicity, non-toxicity, biocompatibility and low cost (Krasaekoopt et al., 2003). The encapsulation method, with sodium alginate in calcium chloride has been used to encapsulate *L. acidophilus* to protect this organism from the harsh acidic conditions in gastric fluid. Studies have shown that calcium-alginate immobilized cell cultures are better protected, shown by an increase in the survival of bacteria under different conditions, than the non-encapsulated state. The results from these studies indicate that the viability of encapsulated bacteria in simulated gastric fluid (SGF) increases with an increase in capsule size (Anal & Singh, 2007).

However, some disadvantages are attributed to the use of alginate. For instance, alginate beads are sensitive to the acidic environment (Mortazavian, Razavi, et al., 2007) which is not compatible for the resistance of the microparticles in the stomach conditions. In addition, the microparticles obtained are very porous which a drawback when the aim is to protect the cells from its environment (Gouin, 2004). Besides, it was reported that very large calcium alginate beads cause a coarseness of texture in live microbial feed supplements and that small beads of size less than 100mm do not significantly protect the bacteria in SGF, compared with free cells. These studies indicate that these bacteria should be encapsulated within a particular size range. They

tested nine different strains of Bifidobacterium spp. for their tolerance to simulated gastrointestinal conditions, and observed some variations among the strains for resistance to gastric fluid (pH 2–3) and bile salts (5 and 10 g/L). Among these strains, only a strain *B. lactis* Bb-12 was found to be resistant to low pH and bile salts. They also encapsulated some of the strains in alginate microspheres to evaluate their resistance properties in gastric fluid and to bile salts. They obtained alginate microspheres (20-70 μm) by emulsifying the mixture of cells and sodium alginate in vegetable oil and subsequently cross-linking with $CaCl_2$. Cryo-scanning electron microscopy revealed that these microparticles were densely loaded with probiotic bacteria and were porous. The loaded alginate microparticles remained stable during storage at 4ºC in 0.05 M $CaCl_2$ and in milk (2% fat), sour cream and yogurt for up to 16 days and in SGF (pH 2.0) for 1 hrs at 37ºC. However, the microparticles exposed to low pH did not improve the survival of acid sensitive bifidobacteria. They also showed that *B. bifidum* survived in higher numbers in frozen milk in beads made from alginate than in beads made from k-carrageenan (Truelstrup-Hansen, Jin, Allan-Wojtas, & Paulson, 2002). Nevertheless, the defects can be compensated by mixing alginates with other polymer compounds, coating the capsules by another compound or applying structural modification of the alginate by using different additives (Krasaekoopt et al., 2003). In addition, research is ongoing to develop microcapsule for a better resistance to stress environment by double or triple layers of alginate- chitosan matrix. *Lactobacillus acidophilus* 547 and *Lactobacillus casei* 01 encapsulated in alginate beads coated with chitosan has been reported to improve the survival of probiotics in both yogurt and severe conditions, such as in simulated gastric, intestinal juices and bile salt solution (Krasaekoopt et al., 2004). The studies from Nualkaekul, et al. (2012) also demonstrated that microencapsulated *Lactobacillus plantarum* in double chitosan coat was improved the survivability of probiotic cells in pomegranate juice. Polycations such as chitosan promotes strong complexes with alginate, leading to the stability in the presence of calcium ions chelators and reduction of the porosity of microcapsules (Wee & Gombotz, 1998).

Chitosan is a linear polysaccharide composed of glucosamine units, which consists of two types of repeating units, N-acetyl-d-glucosamine and d-glucosamine, linked by (1-4)-β-glycosidic linkage. It is a bio-polyaminosaccharide cationic polymer

that is obtained from chitin by alkaline deacetylation and characterized by the presence of a large numbers of amino groups on its chain (Figure 2.10). Although chitosan is obtained from chitin, the applications of the latter compared to chitosan are limited because it is chemically inert. A common method for chitosan synthesis is the deacetylation of chitin, usually derived from the shells of shrimp and other sea crustaceans, using excess aqueous sodium hydroxide solution as a reagent. Chitosan is insoluble in water but soluble in dilute acidic solutions of acetic, citric, and tartaric but not phosphoric or sulfuric at pH less than 6.5 (Ahmed & Aljaeid, 2016; LeHoux & Grondin, 1993).

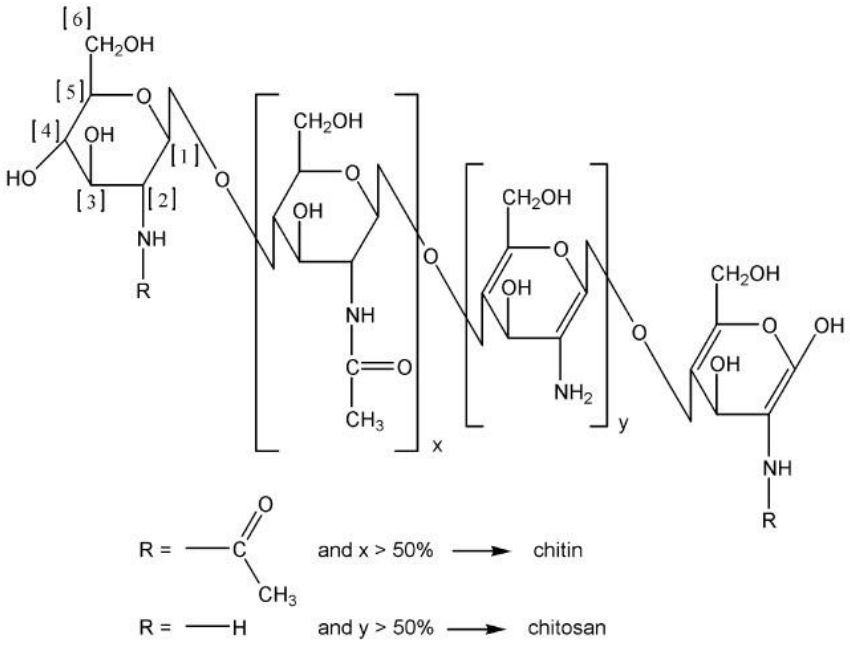

Figure 2.10 Structure of chitin and chitosan.
Source: Pillai, Paul, and Chandra (2009)

Chitosan has attracted attention because of its biological properties and effective uses in the medical field, food industries, and agricultural sector (Li, Dunn, & Grandmaison, 1997). Moreover, chitosan is a promising material for coating due to its non-toxic character, biocompatibility, an ease of handling, inexpensive, owing to its abundance in nature and biodegradability (Pillai et al., 2009). Chitosan has been used as a coating material for alginate beads, and has been shown to increase the survival of probiotics in simulated gastric and intestinal juices compared to uncoated alginate beads

(Chavarri et al., 2010; Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011). Therefore, it is a good way of delivery of viable bacterial cells to the colon. As in figure 2.10, the negatively charged alginate forms a semi-permeable membrane with the positively charged chitosan and as a result the capsule has a smoother surface and is less permeable to water soluble molecules (Koo, Cho, Huh, Baek, & Park, 2001; Krasaekoopt & Watcharapoka, 2014). A polycation of chitosan can complex with above hydrogel to form alginate-chitosan microcapsules for probiotic encapsulation (Ortakci & Sert, 2012; Pimentel-González, Campos-Montiel, Lobato-Calleros, & Vernon-Carter, 2009). As Krasaekoopt et al. (2004) revealed that *L. acidophilus* 547 and *L. casei* 01 encapsulated in alginate beads coated with chitosan which improve the survival of probiotics in both yogurt and severe conditions, such as in simulated gastric, intestinal juices and bile salt solution. Koo et al. (2001) showing alginate-chitosan encapsulated cells showed higher survival rate than alginate encapsulated cells. They suggested that encapsulation technique with double coating material such as chitosan may provide better protection for probiotic from cold damage during freeze-drying process. Moreover, several studies showed that the combination of prebiotics and alginate coating materials may better protect probiotic in food systems and the gastrointestinal tract due to synbiosis (Chen, Chen, Liu, Lin, & Chiu, 2005; Krasaekoopt & Watcharapoka, 2014; Nazzaroa, Fratiannia, Coppolaa, Sadaa, & Orlando, 2009; Okuro, Thomazini, Balieiro, Liberal, & Fávaro-Trindade, 2013).

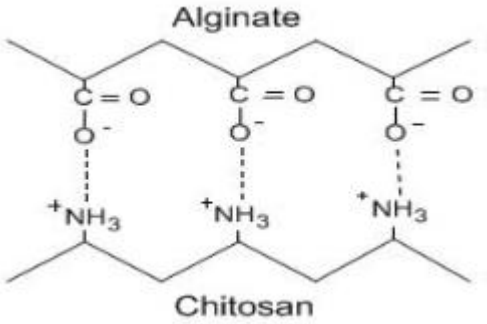

Figure 2.11 Schematic representation of the ionic interactions between alginate and chitosan.
Source: Mohy-Eldin et al. (2015)

CHAPTER 3

MATERIALS AND METHODS

3.1 Probiotics and prebiotics preparations

3.1.1 Probiotics preparations

All the glassware and solutions used for the experiments were autoclaved at 121°C for 15 min. The probiotic strain *Lactobacillus acidophilus* TISTR 1338 was obtained from Thailand Institute of Scientific and Technological Research. The culture was transferred twice in MRS broth and incubated at 37°C for 24 hrs under facultative anaerobic condition in vacuum jar. Cells were harvested by centrifugation at 3000×g for 15 min at 4°C. The cell pellet was washed twice with sterile saline solution. The cell pellet was then re-suspended in 10 mL of 5.5% (w/v) MRS broth to obtain a final cell counts about 8-9 log CFU/mL. The cells were then either directly used in the assay or subjected to microencapsulation.

Growth curve of *L. acidophilus* TISTR 1338 was measured by cell culture in MRS broth incubated at 37°C. The samples were collected every 2 hrs for 48 hrs to measure the optical density (turbidity) at 600 nm by UV-7504C spectrophotometer. The growth curve was obtained.

3.1.2 Prebiotics preparations

Fibruline® which is commercial inulin was used as prebiotic. Fibruline® was obtained from Nutrition SC Company, Thailand (Appendix B). Another prebiotic that is Jerusalem artichoke powder. Jerusalem artichoke JA102 tubers were purchased from Piriya farm (Nakhon Ratchasima, Thailand). The tubers were washed, peeled, sliced, dried in hot air oven 60°C for 24 hrs, grinded to powder with high speed electric grain grinder powder machine and stored in a tightly boxes to protect Jerusalem artichoke powder from humidity at 4°C for further use in microencapsulation. (Figure 3.1).

Figure 3.1 Preparation of Jerusalem artichoke powder

3.2 Selection of complementary prebiotics by *in vitro* fermentation

A minimal medium free of carbon source was used to investigate the ability of *L. acidophilus* TISTR 1338 to grow on different prebiotic, inulin and Jerusalem artichoke, and also in minimal medium, which acted as control. The minimal growth medium (pH 6.8) contained 1.0% beef extract, 0.3% yeast extract, 1.0% pancreatic digest of casein, and 0.5% NaCl. The prebiotics were added to the medium at 2.0% (w/v). The minimal medium and treatment medium were sterilized by autoclaving at 121°C for 15 min and cooled in room temperature. The fermentation which initial cell load about log 5 CFU/ml was carried out in a 250 mL and incubated at 37 °C for 48 hrs. Criteria for the evaluation of the use of prebiotics were the growth of *Lactobacillus* and acidification in medium which compared with the results obtained in the control medium. Growth was measured by serial dilutions method and viable numbers enumerated using spread plating on MRS agar, and colonies were counted after 48 hrs at 37 °C incubation. The acidification by these strains was determined as changes in pH using a pH meter.

3.3 Microencapsulation and coating procedure

3.3.1 Preparation of encapsulation matrix

Microencapsulating materials were sodium alginate (Sigma Aldrich), inulin (Fibruline® instant: Crosuca CO.Ltd), Jerusalem artichoke powder (local farm), chitosan 95% deacetylation (Bonafides Marketing CO. Ltd), MRS media, $CaCl_2$, peptone solution (Himedia). The encapsulated solution were sterilized by autoclaving at 121°C for 15 min and cooled at room temperature.

The cells suspension were prepared as previous section in 3.1.1. Different concentration of alginate, 1%, 2%, and 3% (w/v), was used as encapsulation matrix. Optimal alginate concentration was mixed with prebiotic in ranged of 1%, 2%, and 3% (w/v) concentration. The microcapsule of alginate and prebiotic were sterilized by autoclaving at 121°C for 15 min, Jerusalem artichoke encapsulation solution was aseptically filtered by colander to remove solid particle. The holes in this colander are small enough to filter the insoluble particle out.

3.3.2 Microencapsulation of probiotic bacteria

The beads were prepared by the extrusion method according to the method described by Trabelsi et al. (2013) with some modification as shown in Figure 3.2. The cells suspension was mixed well in encapsulating material i.e. sodium alginate solution and sodium alginate solution mixed with prebiotic. After that the solution was injected through a 30G needle into sterile 0.1 M $CaCl_2$ solution in a drop wise manner. The syringe was hold 5 cm above $CaCl_2$ solution. The droplets immediately formed in gel spheres. The microcapsules were allowed to harden for 30 min in $CaCl_2$ and then washed twice with 0.1% peptone solution to remove excess calcium ions and un-trapped cells. The beads were separated by filtration using filter paper and subsequently frozen beads at -35°C for 12 hrs and freeze dried beads in vacuum freeze-dryer at -46°C for 36 hrs. The freeze-dried beads were tightly packed and stored at 4°C for further analysis.

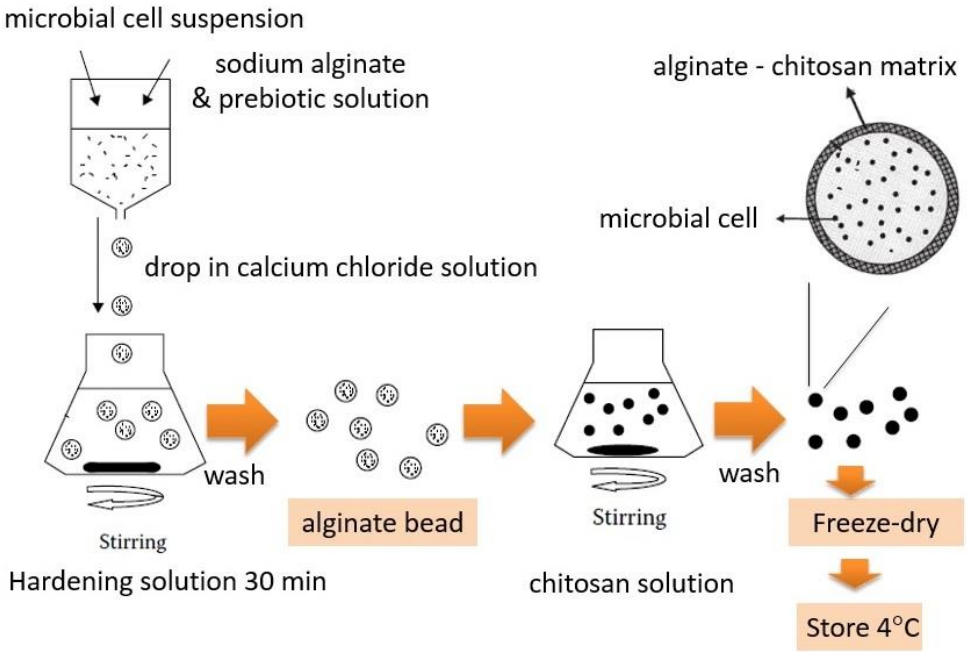

Figure 3.2 Microencapsulation process

3.3.3 Double-coating microcapsule with chitosan

The alginate beads were then further coated with chitosan in the range of 0%, 0.4%, and 0.8% (w/v) chitosan concentration for double-coated microcapsule after stress test condition. The chitosan solution was prepared according to the method described by Zhou, Martins, Groboilloot, Champagne, and Neufeld (1998) with some modification. Chitosan solution was prepared by dissolving 4 g or 8 g of chitosan in 950 mL of 0.1 M acetic acid to achieve a final chitosan concentration of 0.4% and 0.8% (w/v). After dissolution, the pH of chitosan solution was adjusted to 5.7 - 6.0 with 1 M NaOH. The chitosan solution was filtered through Whatman No.1 filter paper, adjusted to 1 L by distilled water and then autoclaved at 121°C for 15 min and cooled at room temperature. The alginate beads prepared in section 3.3.2 were transferred to chitosan solution and stirred gently with a magnetic bar at 150 rpm for 60 min to ensure the evenly coated of the surface of the microcapsules. Such microcapsules were then separated by using a filter paper Whatman No. 1 and rinsed twice with 0.1% peptone solution to remove the excess chitosan and then frozen beads at -35°C for 12 hrs. The final microcapsules were obtained using a vacuum freeze-dryer at -46°C for 36 hrs. The freeze-dried beads were packed in tightly boxes and stored at 4°C for further analysis.

3.3.4 Bacterial enumeration and determination of encapsulation efficiency

To determine the viable cells of the microencapsulated *L. acidophilus* TISTR 1338, One gram of microcapsule was re-suspended in 10 ml phosphate buffer (0.1 M, pH 7.4), gently shaken at 100 rpm on magnetic stirrer for 30 min at room temperature. Samples were taken to determine the release of encapsulated bacteria by spread plating on MRS agar. The plates were incubated at 37°C for 48 hrs. Sterile sodium chloride solution 0.85% (w/v) was used to prepare the serial dilutions in plate count method. Free bacteria were also enumerated on MRS agar using the same technique.

The encapsulation efficiency (EE), the efficiency of entrapment and survival of viable cells during the microencapsulation process, was calculated by the following expression;

$$\text{Encapsulat ion efficiency } (\%) = \frac{Xt}{Xi} \times 100 \qquad (1)$$

Where Xt is the total amount of probiotic loaded in microcapsules and Xi is the initial amount of probiotic added in the preparation process.

3.3.5 Viability of microencapsulated cells after freeze-drying process and heat processing

Survival of the viable cells of the encapsulated *L. acidophilus* TISTR 1338 after stress test in harsh condition by freeze-dried process and heat processing was calculated by the following expression;

$$\text{Survival } (\%) = \frac{Ha}{Hb} \times 100 \qquad (2)$$

Where Ha is the amounts of viable cells in microcapsules after test in harsh condition and Hb is the amounts of viable cells in microcapsules before test in harsh condition.

3.3.6 Survival of encapsulated cells after heat treatment

To investigate the survival of microencapsulated cells after heat treatment, freeze-dried microcapsules 0.1 g were transferred into dry test tube and stress test in water bath. The free and encapsulated *L. acidophilus* TISTR 1338 were heat stress test at 70°C for 60 min which is the temperature used in shrimp feed processing. After heat stress, test tubes were cooled in 25°C and then re-suspended freeze-dried microcapsules in 10 ml phosphate buffer (0.1 M, pH 7.4), gently shaken at 100 rpm on magnetic stirrer for 30 min at room temperature. The survival encapsulated cells were enumerated by plating on MRS agar as described in section 3.3.4. The encapsulated cell viability after heat treatment was calculated by the expression (2) in section 3.3.5.

3.3.7 Viability of microencapsulated cells during refrigerated storage

The viability of free cell, microencapsulated cells, and freeze-dried microencapsulated cells were evaluated during storage at 4°C for 60 days. Microcapsules (1 g) and freeze-dried microcapsules (0.1 g) were re-suspended in 10 ml phosphate buffer (0.1 M, pH 7.4), gently shaken at 100 rpm on magnetic stirrer for 30 min at room temperature and enumerated on MRS agar, as described in section 3.3.4. The encapsulated cell viability during storage was calculated by the following expression;

$$\text{Survival} \quad (\%) = \frac{Vt}{Vi} \times 100 \qquad (3)$$

Where Vt is the number of viable cell at a particular storage period, Vi represent the number of viable cells at the beginning of storage.

3.4 White shrimp culture

3.4.1 The effect of microencapsulated cell in shrimp feeding on growth performance and survival rate in white shrimp

White shrimps, *Litopenaeus vannamei* post-larvae 30, were obtained from commercial farm in Pathumtani province, Thailand. Shrimps were cultured in the hatchery at Center of Excellence for Marine Biotechnology, Chulalongkorn University. The experimental design was completely randomized with four treatment diets, each of which was done in triplicated. The four treatment diets were as follows:

Treatment 1: Commercial basal diet (Control)

Treatment 2: 1% of free cell *L. acidophilus* spray on basal diet every week

Treatment 3: 1% of microencapsulated cells without prebiotic

Treatment 4: 1% of co-encapsulated cells with prebiotic

Microencapsulated cells and co-encapsulated cells were mixed in commercial basal diet and then each of treatment was pellet feed by extruder machine. The pellets feed were dried in hot air oven at 70°C for 1 hrs. The experimental diets were packed were packed in tightly boxes and stored at 4°C for further shrimp culture.

Twenty shrimps were randomly distributed into each rectangular plastic tank with a volume of 150L under continuous aeration. Each tank was provided with an internal filter system consisting of water flow through sponge, oyster shell and bio-balls. To acclimatize the shrimp to the experimental conditions, the shrimp was fed with commercial basal diet for 1 week and then, the experimental diets were provided in three times (8:00, 13:00, and 17:00) daily at the ration of 5% body weight per day to all tanks for 60 days. During the experiments, salinity was maintained at 5ppm. Uneaten feed and feces were removed every day, and sufficient seawater was added to maintain 150 L in each tank and the seawater in experimental tank were changed every three days. The temperature ranged from 28 to 30 °C. The pH value, nitrites, nitrates and ammonia were analyzed every week.

After the 60 days experimental period, shrimp in each tank were counted and weighed to determine survival rate, final body weight, weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) as follow;

Weight gain (g/shrimp) = Final weight (g) - Initial weight (g)

Feed conversion ratio (FCR) = Total Feed Given (g) / Weight Gain (g)

Specific growth rate (SGR) = ([Final weight – Initial weight] / Days) x 100

Survival rate (%) = Final numbers / Initial numbers x 100

3.4.2 Challenge test

Ten shrimps from each treatment were collected for challenge test. The challenge test was conducted by immersion method that was observed in 7 days with zero water change and did not fed shrimp. Shrimp in all treatments were exposed to challenge test with *V. harveyi* (10^5 CFU/ml) per tank by immersion for 7 days. During 7 days of challenge test, the cumulative mortality of the shrimps was observed. The cumulative mortality was calculated by the following expression;

$$\text{Cumulative mortality } (\%) = \frac{St}{Si} \times 100 \qquad (4)$$

Where St is the number of deaths shrimp at a particular challenge period, Si represent the number of shrimp at the beginning of challenge test.

Before and after challenging test, the total bacteria, total lactic acid bacteria, and total *Vibrio* in the gastrointestinal tract shrimp were obtained by plating on plate count agar (PCA), MRS agar, and thiosulfate-citrate-bile salts-sucrose agar plates (TCBS), respectively. Bacterial population numbers were recorded as a logarithm of colony-forming units per gram of intestine.

3.5 Statistical analysis

All experiments and analyses are presented as mean ± standard deviation in triplicate trial. The data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P<0.05$) using SPSS version 21 for windows.

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1 Growth curve of *L. acidophilus* TISTR 1338

The growth curve of *L. acidophilus* TISTR 1338 cultured in MRS medium incubated at 37°C for 48 hrs and collected the samples every 2 hrs to measure the optical density (turbidity) at 600 nm by UV-7504C spectrophotometer was obtained (Figure 4.1). The growth curve is useful for selecting the time to harvest cells. The cells at early stationary stage (24 hrs of cultivation) which are the fully mature cells were harvested to use in encapsulation process. The number of cells at 24 hrs were about 8-9 log CFU/mL.

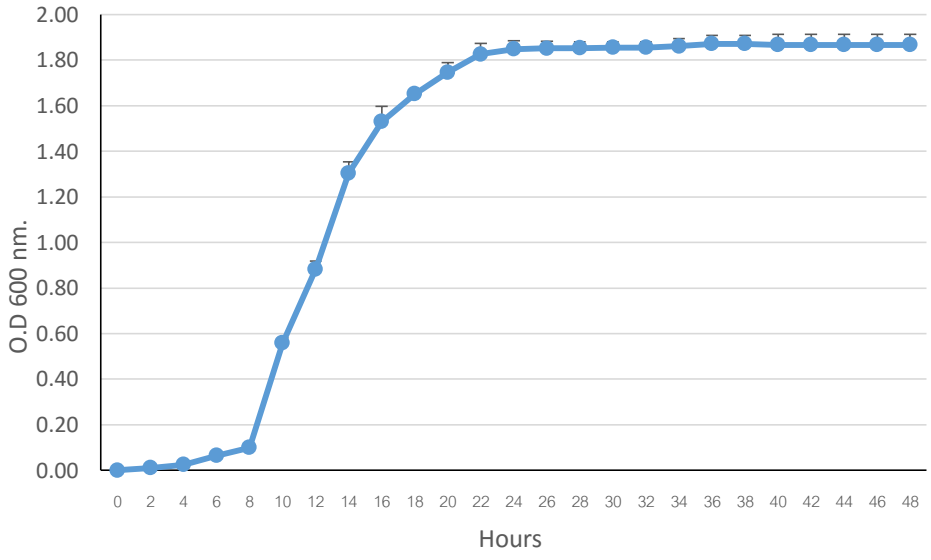

Figure 4.1 Growth curve of *L. acidophilus* TISTR 1338 in MRS medium. The error bars represent standard deviation of means (n=3)

4.2 Selection of complementary prebiotics

In order to investigate the effect of inulin or fructooligosaccharides on the growth of *L. acidophilus* TISTR 1338. Commercial inulin and Jerusalem artichoke which is rich in fructooligosaccharides carbohydrates in the forms of inulin and fructans was used to investigate the ability of *L. acidophilus* TISTR 1338 to grow on different prebiotic, inulin and Jerusalem artichoke, and also in a minimal medium, which acted as control. The selection of complementary prebiotic showed that there was no significant difference in the prebiotics tested between inulin and Jerusalem artichoke tuber powder on bacterial growth. The total number of bacteria reached log 8 CFU/ml in 48 hrs of cultivation which initial cell load about log 5 CFU/ml and was significantly higher than control medium (Figure 4.2). In the case of cultivation of the *L. acidophilus* TISTR 1338 in minimal medium without the addition of prebiotic, the total number of bacteria did not increase.

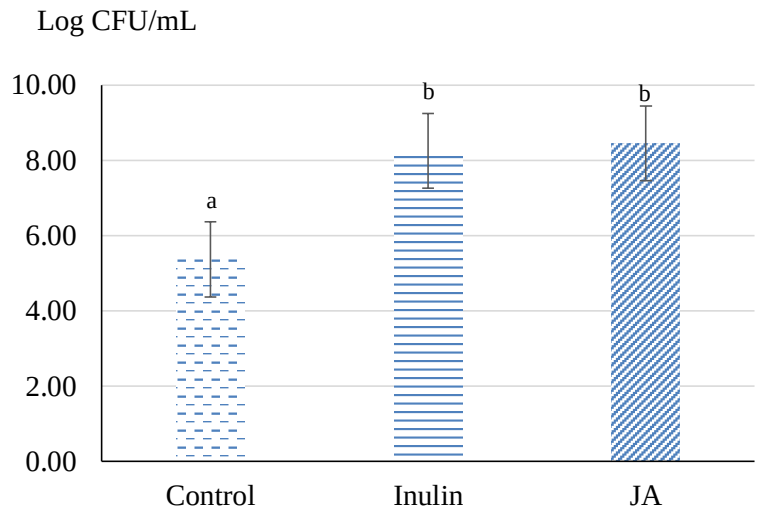

Figure 4.2 Selection of complementary prebiotics by *in vitro* fermentation of *L. acidophilus* TISTR 1338 in minimal media and media containing different prebiotic incubated at 37°C for 48 hrs.

Growth was measured as log CFU/mL. The error bars represent standard deviation of means (n=3). Different superscripts in each column indicated significant difference (P<0.05).

*JA = Jerusalem artichoke powder

Goderska, Nowak, and Czarnecki-Acta (2008) revealed that the *Lactobacillus* strains also utilized Raftilose® (oligofructose) and Raftiline® (inulin) in the medium as carbon sources. Although total number of bacteria developed during 48 hours of cultivation were not as high as in the case of easily-available carbon sources, however their numbers differed significantly in comparison with the control medium. Iyer and Kailasapathy (2005) suggested that *L. acidophilus* strains was able to grow well on oligosaccharides composed predominantly of fructose moieties than the monosaccharide fructose. Biedrzycka and Bielecka (2004) reported that the *in vitro* consumption of inulin by bifidobacteria depended on purity and DP of FOS chains. Sathyabama, Ranjith-Kumar, Bruntha-Devi, Vijayabharathi, and Brindha-Priyadharisini (2014) revealed that the variety of oligosaccharide composition could be the reason for difference in bacterial growth rate. Accordingly, *L. acidophilus* TISTR 1338 was grown in inulin and Jerusalem artichoke powder indicated that it could use the inulin and Jerusalem artichoke as a carbon source to sustain their growth.

There was no significant difference among the prebiotics tested on the amount of cell growth after incubated at 37°C for 48 hours while it grew very poorly on the control treatment. As the same result in the acidification by *L. acidophilus* TISTR 1338, the acidification of the strain in these two prebiotics were not significantly different whereas prebiotic treatment was significantly better compared with control treatment (Table 4.1). The pH values of the medium supplemented with inulin and Jerusalem artichoke were 4.90 and 5.10 respectively. These values were lower in comparison with control medium. Similarly, Sathyabama et al. (2014) showed that the acidification rate of the *S. succinus* and *E. fecium* strain with different prebiotics medium (oats, sugarbeet, chicory) was lower than minimal medium. They indicated that the variation in the prebiotic composition might be the reason for difference in growth rate. The rate at which oligosaccharides are fermented depends on the degree of polymerization, sugar and glycosidic linkage and degree of branching, synergy between bacteria during fermentation, relationship between substrate bacteria and fermentation products, nature of the fermentations and saccharolytic capacity (Voragen, 1998). Elaheh, Sani-Ali, Elnaz, and Ladan (2016) indicated that the degree of polymerization of fructans was an important factor that provided the accessibility of fructans to the bacteria.

Table 4.1 Acidification of *L. acidophilus* TISTR 1338 in minimal media and media containing different prebiotic incubated at 37°C for 48 hrs.

Prebiotic	pH
Control	6.70±0.03[a]
Inulin	4.90±0.14[b]
Jerusalem artichoke	5.10±0.16[b]

[a,b] Means±standard deviation with different superscript within the same column are significantly different (P<0.05)

Analyzing the correlation of pH changes of the medium with the total number of bacteria during the cultivation in media containing prebiotic and without prebiotic, it can be noticed that the pH reduction was accompanied by the increase in the total number of bacteria (Goderska et al., 2008).

In this part, the results showed that inulin and Jerusalem artichoke tuber can be used as a complementary prebiotic for encapsulated *L. acidophilus* TISTR 1338.

4.3 Optimum condition for encapsulated cell *L. acidophilus* TISTR 1338

4.3.1 The effect of different alginate concentration on microcapsule

Alginate is the biopolymer most used and investigated for encapsulation. Microencapsulated cells of *L. acidophilus* in alginate beads survived better than free cells after sequential incubation in simulated gastric and intestinal juices (Krasaekoopt et al., 2004). Higher survival was also reported when lactobacilli immobilized in alginate beads were incubated in simulated gastric fluid (Lee, Cha, & Park, 2004). Alginates are natural occurring marine polysaccharides extracted from seaweed. Entrapment of probiotic bacteria in alginate is possible due to alginate gel is recognized as a rapid, non-toxic and versatile method for macromolecules and cells (Melvik & Dornish, 2004). The kinetics of alginate gelation can be affected by the temperature at which gelation occurs, as well as alginate concentration and ion concentration (Draget, 2000).

In order to evaluate the effect of alginate concentration on microencapsulation process to maintain *L. acidophilus* TISTR 1338 cells viability after freeze-drying, in this study, the tests were done at 1%, 2%, and 3% alginate concentration. The result

showed that the viability of encapsulated *L. acidophilus* TISTR 1338 cells improved with increasing alginate concentration (Table 4.2). Viable cells of 3% alginate concentration showed the highest survival rate after freeze-drying. Similar results were observed by Mandal et al. (2006) showing that the viability increased with increasing concentration of alginate and the highest viability was obtained from the usage of 4% alginate. Chandramouli, Kailasapathy, Peiris, and Jones (2004) found that raising the concentration of alginate solution from 0.75% to 1.5% had noticeable effects on *L. acidophilus* viability under simulated gastric conditions. This concurs with the studies of Lee and Heo (2000) revealed that the mortality rate of *Bifidobacteria longum* encapsulated in calcium alginate beads containing 2%, 3%, and 4% sodium alginate. The death rate of the cells in the beads decreased proportionally with increasing concentration of alginate. Moreover, Anekella and Orsat (2013) also reported that the increase in the concentration of microencapsulating material induced an increase in the survival rate of probiotic bacteria.

Table 4.2 Number of encapsulated *L. acidophilus* TISTR 1338 cells after encapsulation with different concentration of alginate and survival of encapsulated *L. acidophilus* TISTR 1338 after freeze-dried.

Alginate Conc.	Number of cells (log cfu/ml)			EE (%)	%Survival after freeze-drying process
	Initial cells	Encapsulated cells	Freeze-dry encapsulated cells		
1%	9.60±0.40	7.21±0.19[a]	4.04±0.06[a]	75.10±1.65[a]	56.15±2.07[a]
2%	9.73±0.15	7.45±0.08[a]	4.42±0.19[b]	76.58±0.52[a]	59.36±2.16[a]
3%	9.65±0.49	8.64±0.28[b]	5.74±0.19[c]	89.70±5.33[b]	66.42±4.37[b]

[a,b] Means±standard deviation with different superscript within the same column are significantly different and without superscript in the same column are not significantly different (P<0.05).

*EE (%) = Encapsulation efficiency (%)

However, the alginate bead obtained are very porous, which is hindrance when trying to protect viable cells from environmental stress (Gouin, 2004). Alginate gel is formed in the presence of calcium ions, its integrity is deteriorated when subjected to monovalent ions or chelating agents which absorb calcium ions such as phosphates,

lactates and citrates (Mortazavian, Ehsani, et al., 2007). The increase in pore size and porosity can be attributed to the formation of ice structures during the freezing of the gels. Lower polymer concentration and higher water content in gels resulted in maximal pore size increase in 1% alginate matrices. The increased pore size or porosity can decrease the integrity of the hydrogel matrices. Mohanty, Wu, Chakraborty, Mohanty, and Ghosh (2016) revealed that the increase in alginate concentration resulted in a significant decrease in pore size of alginate matrix. The mentioned defects can be efficiently compensated by blending of alginate with other polymer compounds, coating other compounds on its capsules and structural modification of the alginate by using various additives (Krasaekoopt et al., 2003).

4.3.2 The effect of different prebiotic concentration on microcapsule

As mentioned previously about the drawback of alginate matrix, which is undesirable as the objective of encapsulation is protecting the cells from the harsh environmental conditions. These problems could be overcome by blending alginate with prebiotic which is one of the practices and it has been shown that encapsulation effectiveness of different bacterial cells were improved by applying this method (Chen et al., 2005; Iyer and Kailasapathy, 2005; Nazzaro et al., 2009; Chavarri et al., 2010; Krasaekoopt & Watcharapoka, 2014; Sathyabama et al., 2014). Their advantages over other covering materials rely on the fact that, besides being non-digestible carbohydrates, they also have beneficial effects for the host by selectively stimulating the growth and/or activity of probiotic bacteria within the colon (Fritzen-Freire et al., 2012).

In order to increase the viability of the encapsulated probiotic bacteria in the microcapsule, an enrichment step was necessary. As the *L. acidophilus* TISTR 1338 can grow in both prebiotics tested, individual prebiotic was further examined to co-encapsulate the probiotic bacteria in encapsulated solution mixed with 3% alginate concentration. In this part, *L. acidophilus* TISTR 1338 was separately encapsulated with inulin or Jerusalem artichoke powder at 1%, 2%, and 3% concentration, respectively.

The viability of encapsulated cells with different prebiotic in alginate matrix coated were analysed after freeze-dried process. The results showed that the survival rate of encapsulated *L. acidophilus* TISTR 1338 with inulin and Jerusalem artichoke at 3% concentration were significantly ($P<0.05$) higher than 1% and 2% concentration as show in Table 4.3. Fritzen-Freire, et al. (2012) noticed that the properties of oligosaccharides, primarily through their impact on the glass transition process enhanced cell survival is expected when their level in the drying media is increased.

The previous study showed that the addition of starch to an alginate mixture increased recovery of encapsulated cells (Sultana, Godward, Reynolds, Arumugaswamy, & Peiris, 2000). Chen, et al. (2005) revealed that adding prebiotics fructooligosaccharide or isomaltooligosaccharide into alginate beads improved the stability and survival of bifidobacteria during storage in unfermented milk as well as in simulated gastric solutions. Iyer and Kailasapathy (2005) revealed that co-encapsulated probiotic bacteria with resistant starch Hi-maize survived better than the encapsulated bacteria without the prebiotic. Dianawati and Shah (2011) indicated that encapsulation using alginate-mannitol provided higher survival compared to alginate alone after freeze drying. They suggested that mannitol might interact with the polar sites of the phospholipid bilayer of *B. animalis* through H-bond interaction. Moreover, the addition of FOS provided the best protection during freeze-drying of *L. reuteri* TMW1 (Schwab, Vogel, & Ganzle, 2007). The application of sucrose and maltodextrins was previously shown to improve the viability of bacteria during drying and revealed that the direct interaction of sucrose with membranes was suggested to contribute to the protective effects of sucrose on dried cells of *Lactobacillus bulgaricus* (Oldenhof, Wolkers, Fonseca, Passot, & Marin, 2005). During freeze-drying intracellular ice formation reduces the cell viability due to membrane injury. Therefore, interaction between the cells membrane and fructans of the FOS maintains the membrane fluidity (Schwab, et al., 2007). Krasaekoopt and Watcharapoka (2014) suggested that the addition of prebiotics during microencapsulation was not only providing better protection to probiotics, but also enhancing the growth of these microorganisms in simulated digestive system.

Table 4.3 Number of encapsulated *L. acidophilus* TISTR 1338 cells after encapsulation with different prebiotic concentration and survival of encapsulated *L. acidophilus* TISTR 1338 after freeze-dried.

	Prebiotic conc.	Number of cells log cfu/ml			EE %	%Survival after freeze-drying process
		Initial cells	Encapsulated cells	Freeze-dry encapsulated cells		
Inulin	1%	9.92±0.29	8.81±0.40	6.08±0.09[a]	88.80±3.98	69.14±3.11[a]
	2%	9.85±0.16	8.87±0.07	6.23±0.17[a]	90.11±0.87	70.25±1.88[a]
	3%	9.92±0.34	8.95±0.22	6.70±0.20[b]	90.31±0.85	74.89±2.46[b]
Jerusalem artichoke	1%	9.90±0.22	8.67±0.19	6.03±0.08[a]	87.56±0.76	69.54±0.85[a]
	2%	9.73±0.46	8.68±0.12	6.16±0.13[a]	89.28±4.96	71.03±2.07[a]
	3%	10.03±0.25	8.80±0.49	6.62±0.19[b]	87.72±4.10	75.27±1.58[b]

[a,b] Means±standard deviation with different superscript within the same column are significantly different and without superscript in the same column are not significantly different $P<0.05$.

*EE % = Encapsulation efficiency %

On the other hand, some authors reported that the increase concentration of oligofructose-enriched inulin from 1.5 to 5% w/w did not provide the increase in cell survival or insignificantly improved the survival rate during the spray-and freeze-drying processes (Ivanovska, Zhivikj, Mladenovska, & Petrushevska-Tozi, 2015). Similar study was observed when concentrations of FOS were increased from 1.5 to 5% w/w, they noticed that higher oligosaccharide level in the drying media probably contribute to increased osmotic stress which negatively affects the cell survival (Ivanovska et al., 2012).

In this part, it can be shown that separate blending with inulin or Jerusalem artichoke tuber powder at 3% concentration in 3% alginate solution to microencapsulation provide better protection and increase cell viability after freeze-drying process.

4.3.3 The effect of different chitosan concentration on microcapsule

Apart from blending other polymer in alginate beads to solve some disadvantages of alginate matrices such as low stability and high porosity of the structure. These limitations of alginate can be efficiently improved by coating the alginate beads with other polymers such as resistant starch and chitosan (Sultana et al., 2000; Truelstrup-Hansen et al., 2002; Krasaekoopt et al., 2003).

Chitosan is biodegradable and biocompatible. Due to the possibility of a negative impact in the viability of bacteria, and that chitosan has a very good film-forming ability, chitosan is more used as external shell in capsules made with anionic polymers as alginate. In addition, research is ongoing to develop microcapsule for a better resistance to stress environment by double or triple layers of alginate-chitosan matrix. *L. acidophilus* 547 and *L. casei* 01 encapsulated in alginate beads coated with chitosan has been reported to improve the survival of probiotics in both yogurt and severe conditions, such as in simulated gastric, intestinal juices and bile salt solution (Krasaekoopt *et al*., 2004).

To investigate the effect of chitosan concentrations on the viability of the encapsulated probiotic bacteria in the microcapsule, 0%, 0.4%, and 0.8% chitosan solutions were used to double coat on alginate beads. The viability of encapsulated cells

in alginate beads with no chitosan coat (0% chitosan) and coated with 0.4% and 0.8% chitosan were analyzed after freeze-dried process. The results showed that the survival of encapsulated *L. acidophilus* TISTR 1338 in double coated beads with 0.4% and 0.8% chitosan were significantly ($p<0.05$) higher than alginate bead (with no chitosan coated) in both prebiotics (Table 4.4). There was no significant difference between the 0.4% and 0.8% chitosan on the survival rate after freeze-drying.

Similar results were observed by Koo et al. (2001) showing alginate-chitosan encapsulated *Lactobacillus casei* YIT 9018 had higher survival rate than alginate encapsulated cells. They suggested that encapsulation technique with double coating material such as chitosan may provide better protection for probiotic from frozen damage during freeze-drying process. Ivanovska et al. (2015) showed that the viability of co-encapsulate *L. casei* 01 in chitosan-Ca-alginate microparticles in the presence of oligofructose-enriched inulin after freeze-drying was significantly increased when compared to the formulation without prebiotic. Abbaszadeh, Gandomi, Misaghi, Bokaei, and Noori (2014) found that the combination with 2% and 3% alginate and increasing the chitosan concentration from 0.2% to 1% to coat alginate gel induced a significant increase in gastric survival rate of *L. rhamnosus* GG. Besides, the intestinal survival rate of the encapsulated *L. rhamnosus* GG increased in the range 24.4-37.2% with increasing alginate and chitosan concentrations, compared with a survival rate of 8% for free cells. Survival of probiotic bacteria in alginate beads containing chitosan was higher than in alginate beads, and it is an approach that can be applied for increasing the survival of strains (Nualkaekul et al., 2012). Several studies have indicated differences among strains of probiotic bacteria with respect to their survival in acid environment (Kailasapathy, 2006; Truelstrup Hansen *et al*., 2002). Krasaekoopt et al. (2004) found that microencapsulation of *Lactobacillus* in alginate coated with chitosan beads was the most efficient treatment to protect strains for all conditions tested. Anekella and Orsat (2013) also reported that the increase in the concentration of microencapsulating material induced an increase in the survival rate of the probiotic bacteria, these results showed that chitosan enhanced the viability of the probiotic strain *L. plantarum* TN9 at refrigeration conditions. The *L. plantarum* TN9 cells encapsulated in alginate beads gave slightly better results than the free cells.

Table 4.4 Number of encapsulated *L. acidophilus* TISTR 1338 cells after encapsulation with different chitosan concentration and survival of encapsulated *L. acidophilus* TISTR 1338 after freeze-dried.

	Chitosan conc.	Number of cells log cfu/ml			EE %	Survival rate after freeze-drying process %
		Initial cells	Encapsulated cells	Freeze-dry encapsulated cells		
Inulin	0%	9.77±0.32	8.86±0.14	6.46±0.15[a]	90.69±1.62	72.95±1.17[a]
	0.4%	9.87±0.08	8.77±0.20	7.11±0.15[b]	88.87±1.70	81.03±3.17[b]
	0.8%	9.83±0.20	8.76±0.25	7.38±0.35[b]	89.11±1.88	84.23±2.01[b]
Jerusalem artichoke	0%	9.89±0.08	8.89±0.06	6.55±0.27[a]	89.86±0.94	73.71±2.65[a]
	0.4%	9.64±0.14	8.70±0.19	7.24±0.15[b]	90.27±1.05	81.56±0.14[b]
	0.8%	9.54±0.30	8.75±0.37	7.47±0.56[b]	91.62±2.15	85.37±2.91[b]

[a,b] Means±standard deviation with different superscript within the same column are significantly different and without superscript in the same column are not significantly different $P<0.05$.

*EE % = Encapsulation efficiency %

Formation of a coat/shell around the alginate capsule has been verified to considerably improve its physicochemical characteristics. It has been reported that by coating semipermeable layers of chitosan polymer (as a polycationic compound) around the alginate capsules (which have negative charges), beads with improved physical and chemical stability were produced. Also structurally, the beads were denser and much stronger, thus avoiding breaking and cells release (Krasaekoopt, Bhandari, & Deeth, 2003; Smidsrod & Skjak-Braek, 1990; Zhou, Martins, Groboilloot, Champagne, & Neufeld, 1998). The alginate-chitosan is a polymer blend of alginate and chitosan whose structure is configurationally different. Alginate is an anionic polysaccharide composed of alternating blocks of 1-4 linked L-guluronic and D-mannuronic acid residues, while chitosan is a cationic polysaccharide composed of randomly distributed ß-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine. The network occurred due to the electrostatic attraction between the negatively charged carboxyl group of alginate and the positively charged amino group of chitosan. After the complexation between these two polymers, the carboxyl group of alginate was additionally cross-linked with calcium ions. The double coated microcapsule has a better protect compared with either of these alone owing to its double network structure and has less porous than the alginate microcapsule; therefore, it is more resistant to a harsh condition in feed processing.

From the results, it seems that increasing chitosan concentrations resulted in higher survival rates. On the contrary, after ingestion the feed will stay in a digestive system of the shrimp only 2-3 hrs. If the concentration of chitosan is too high, microcapsule might release cells slowly. Shrimp might not get the probiotic cells that are entrapped inside. Thus, in this study, the 3% alginate and 3% prebiotic and further coat with 0.4% and 0.8% chitosan was selected as the microencapsulating material.

4.3.4 Viability of encapsulated cells after heat treatment

In the present, the use of probiotic bacteria has become increasingly popular in feed additive for improved nutrition, healthy digestion, and disease prevention. However, probiotic bacteria are sensitive to harsh conditions during feed processing, storage and in gastrointestinal tract. Therefore, the investigation of the efficacy of microcapsules for protecting probiotic cells during feed processing which often

employs high temperature. So, dry heat treatment to the free cell and encapsulated cells for heat resistance was evaluated.

The survival of free cells and encapsulated *L. acidophilus* TISTR 1338 exposed to 70 °C for 60 min which is the condition in shrimp feeding process was determined (Table 4.5). The results showed that the free cells and encapsulated cells in alginate bead (0% chitosan) were very sensitive to heat treatment and were completely destroyed at the end of treatment, while both encapsulated cells double-coated with chitosan still survived after heat treatment. The survival rate of the 0.8% chitosan treatment showed significant higher than 0.4% chitosan treatment. While, both of the prebiotics, inulin and Jerusalem artichoke treatment did not show the significant difference on survivability after exposed to high temperature.

Table 4.5 Survival of encapsulated *L. acidophilus* TISTR 1338 after dry heat treatment in water bath at 70 °C for 60 min.

	Chitosan concentration	Survival after heat processing (%)
		70 °C for 60 min
Free cells		0.00±0.00[a]
Inulin	0%	0.00±0.00[a]
	0.4%	41.78±4.05[b]
	0.8%	62.89±0.72[c]
Jerusalem artichoke	0%	0.00±0.00[a]
	0.4%	43.29±4.66[b]
	0.8%	68.16±1.58[c]

[a,b,c] Means±standard deviation with different superscript in the same column are significantly different (P<0.05).

These results are in accordance with previous studied which reported that encapsulation by chitosan double coating could enhance the thermal resistance of probiotic bacteria. Peck, Mirhosseini, Mustafa, Manap, and Yazid (2011) reported that free cells experienced about 5 log cycles reduction after heat exposure at 60°C for 30 min, whereas encapsulated *L. acidophilus* LA-5 was reduced by 1.99 log cycles. Moreover, Tárrega, Rocafull, and Costell (2010) revealed that long-chain inulin, with a high degree of polymerization, was more thermally stable thus rendering higher

protection for *Bifidobacterium* BB-12. The results of present study suggested that encapsulation by double-coating alginate bead with 0.8% (w/v) chitosan proved to be good combination to provide thermal resistance to probiotic bacteria. Microencapsulated *Bifidobacterium* BB-12 showed higher bacterial count (after spray-drying) with encapsulating agent reconstituted skim milk (RSM) and prebiotics inulin or oligofructose-enriched inulin in a ratio 1:1, when compared to microcapsules produced with RSM only and with oligofructose (Fritzen-Freire et al., 2012). The inulin was reported to act as a thermoprotector for the cells undergoing the drying process. Inulin applied at concentration of 0.5, 1, and 1.5% was also reported to provide improved survival during microencapsulation of *L. acidophilus* 5 and *L. casei* 01 with alginate and chitosan (Krasaekoopt and Watcharapoka, 2014). Several researches revealed that incorporating both prebiotics and alginate-chitosan coating materials for encapsulate probiotic may better protect probiotic in food systems due to synbiosis (Chen et al., 2005; Nazzaroa et al., 2009).

The survival rate of Jerusalem artichoke treatment was 68.16% at 70 °C for 60 min which is higher than inulin treatment. The results indicated that the double coated with 0.8% chitosan on alginate matrices blending with Jerusalem artichoke was the most effective combination in protecting probiotic bacteria from high temperature and freeze-drying process. It was plausible that the slower water diffusion in the alginate matrix, with a consequence of slower heat penetration into the core, offers protection to the cells. Moreover, chitosan coating was believed to exert an extra protective effect for the probiotics that were situated in the inner core. The addition of calcium ions into chitosan solution was thought to increase the binding capacity of alginate to chitosan, thereby giving the capsules additional mechanical strength.

The variation in the composition of Jerusalem artichoke such as the degree of polymerization, sugar and glycosidic linkage, a degree of branching might be the reason for differing survivability of encapsulated cell. Moreover, Jerusalem artichoke is a natural compound with some new and interesting revelations about its potential health and healing use. Thereby, the Jerusalem artichoke is the intensive material which has the potential to use in the production of white shrimp feed in the next step.

4.3.5 Viability of encapsulated cells during refrigerated storage

One of the most considerable prerequisites for the use of probiotics in the feed is that they must survive throughout the production process and shelf life of the probiotics in the feed should survive for a certain time during storage. Therefore, free cell, microcapsule and dried microcapsule which was coated by alginate treatment and double-coated by 0.8% chitosan treatment were stored at 4°C (refrigerator temperature) for 60 days to investigate the microencapsulated cell viability.

As shown in figure 4.3, the viability of the microencapsulated cells showed better stability than the free cells under the same storage conditions. After 60 days, the survival of *L. acidophilus* TISTR 1338 in alginate-chitosan dried bead decreased from around log 7 to log 5 CFU/g which was still significantly higher than in alginate dried bead (decreased from around log 6 to log 2 CFU/g).

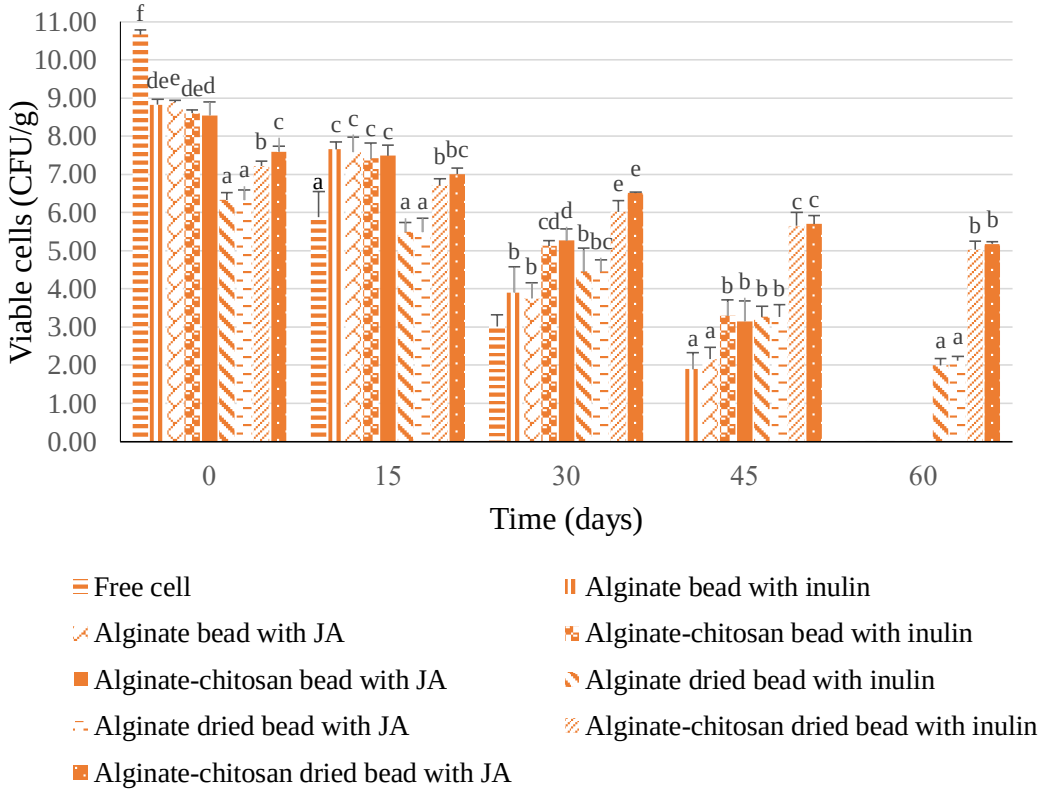

Figure 4.3 The viability of the free cell, microencapsulated cells, and freeze dried microencapsulated cells at 4 °C refrigerator temperature after 60 days.
Different letters over each treatment column bar (mean ± SE) indicate significant difference (P<0.05).
*JA = Jerusalem artichoke

Several studies showed that the survival of microencapsulated bacteria during the storage was improved in alginate microcapsule as compared to free bacteria. For instance, Koo et al. (2001) reported that *L. bulgaricus* loaded in alginate microcapsule exhibited higher storage stability than free cell cultures. Moreover, they revealed that lactic acid bacteria loaded in alginate coated-chitosan beads showed higher storage stability than free cell culture. Anekella and Orsat (2013) also reported that the increase in the concentration of microencapsulating material induced an increase in the survival rate of the probiotic bacteria.

Furthermore, the results showed that alginate-chitosan bead enhanced the viability of the probiotic strain *L. acidophilus* TISTR 1338 at refrigeration conditions. This was probably due to the protection effect of thicker membranes with chitosan.

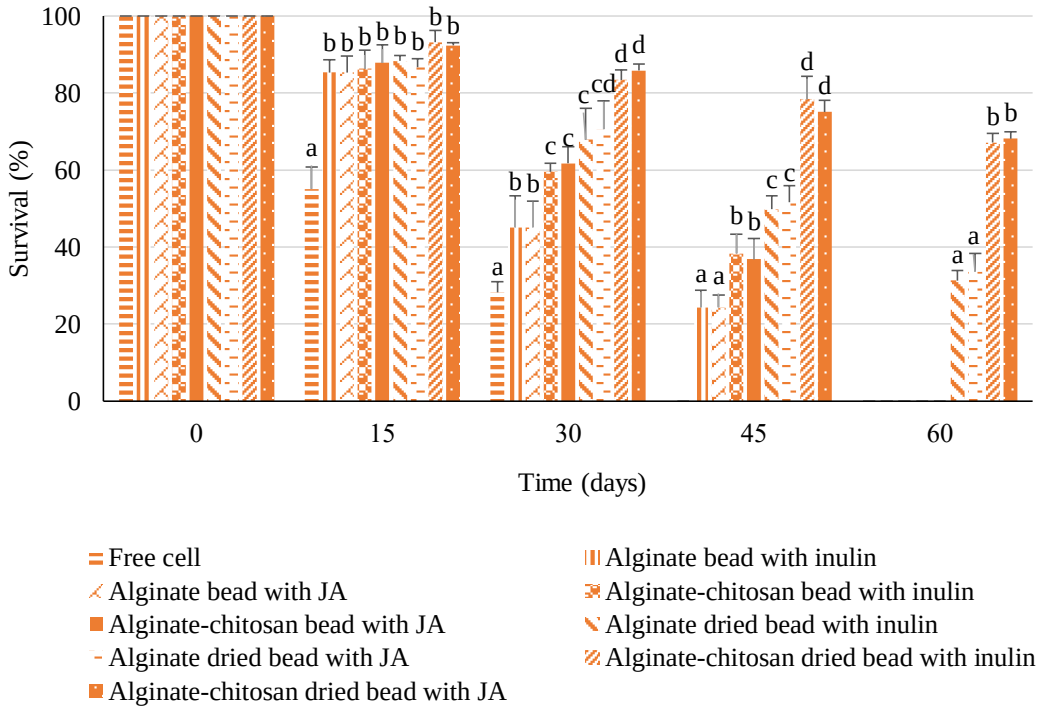

Figure 4.4 The survival (%) of the free cell, microencapsulated cells, and freeze dried microencapsulated cells at 4 °C refrigerator temperature for 60 days.
Different letters over each treatment column bar (mean ± SE) indicate significant difference (P<0.05).
*JA = Jerusalem artichoke

4.4 White shrimp culture

4.4.1 Growth performance and survival of white shrimp after fed with experimental diet

Application of biotherapeutics such as prebiotics, probiotics, and synbiotics as alternatives to replace chemotherapy in shrimp production holds to prevent disease outbreak challenge. Therefore, dietary co-supplementation with probiotics and prebiotics is a promising alternative biotherapeutic approach for improving the health of aquatic animals. Especially, the potential for positive synergistic effects to use as feed additives in shrimp culture.

One percentage (w/w) of free cell, microencapsulated cell and co-encapsulated cell with Jerusalem artichoke were added to commercial shrimp feed which acts as control. The *L. acidophilus* TISTR 1338 was incorporated in the range of 10^6 CFU/g in shrimp feed. The growth performance of white shrimp are presented in Table 4.6. There was no significant difference in term of initial weight among the treatment and control, however, after 60 days treated groups fed with free cell, microencapsulated without prebiotic and co-encapsulated cells with prebiotic showed about 4.26%, 3.83% and 4.34% increase in weight gain, respectively, in comparison with the control group. The highest average of final weight and weight gain was observed in Treatment4. Even though, there was no significant differences between Treatment2 and Treatment4. The addition of microencapsulated cell to the feed also produced particularly better survival rate in white shrimp with values significantly higher than control, more specially in groups treated co-encapsulated cell with Jerusalem artichoke as shown in Table 4.6.

Similarly, the previous research showed that the dietary supplementation of *L. acidophilus* and *L. sporogenes* for *Macrobrachium rosenbergii* increased shrimp growth rate (Venkat, Sahu, & Jain, 2004). Karthik, Jaffar Hussain, and Muthezhilan (2014) indicated that the significant difference was observed in final weight of shrimp fed with the potential strain *Lactobacillus* sp AMET1506. Kumar, Jyothsna, Reddy, and Sreevani (2013) also reported that, they observed the increase growth pattern of *P. vannamei* when fed with *B. subtilis* incorporated diet and *L. rhamnosus* incorporated diet compared to control groups.

Table 4.6 Effects of feed supplement with free cell, microencapsulated cell and co-encapsulated cell on growth performance and survival rate in white shrimp after 60 days feeding trial.

Parameters	Feed treatment			
	Control	Free cell	Microencapsulated cells	Co-encapsulated cells
Initial weight (g)	5.41±0.51	5.61±0.65	5.58±0.82	5.64±0.45
Final weight (g)	7.75±0.77^{a}	9.88±0.80^{c}	9.42±0.50^{bc}	9.98±0.72^{c}
WG (g)	2.33±0.65^{a}	4.26±0.59^{b}	3.83±0.93^{b}	4.34±0.42^{b}
SGR %	3.88±1.08^{a}	7.10±0.98^{b}	6.38±1.55^{b}	7.23±0.71^{b}
FCR	8.24±3.15	4.51±1.03	5.13±1.79	4.42±0.81
Survival %	40±5.00^{a}	66.67±2.89^{b}	61.67±7.64^{b}	70±5.00^{b}

^{a,b,c} Means±standard deviation with different superscript in the same row are significantly different and without superscript in the same row are not significantly different $P<0.05$.

Weight gain g/shrimp = Final weight g - Initial weight g

Feed conversion ratio FCR = Total Feed Given g / Weight Gain g

Specific growth rate SGR = [Final weight – Initial weight] / Days x 100

Survival % = Final numbers / Initial numbers x 100

Moreover, the previous study showed that the combination between the probiotic bacteria *Vibrio alginolyticus* SKT-b and prebiotic extracted from the sweet potato *Ipomoea batatas* was used on the juvenile stage of Pacific white shrimp and the results showed that probiotic and prebiotic could be improved the shrimp growth rate, immune response, and resistance (Oktaviana, Widanarni, & Yuhana, 2014).

The higher survival and growth rate of shrimp fed with probiotic supplemented feed might be related to an immune reactive effect of probiotics on the host immune system, and the lactic acid bacteria are the main microbes which produce extracellular compounds to stimulate the non-specific immune response in vertebrates (Gill, 2003; Marteau, Seksik, & Jian, 2002). Moreover, shrimp fed with prebiotics administered to the host would be actively fermented by intestinal bacteria and would modulate bacterial activity (Ai et al., 2011). Prebiotics also increase the length of intestinal microvilli (Zhang et al., 2010)which help increase nutrient absorption and thus improve growth performance (Cerezuela, Meseguer, & Esteban, 2011). Therefore, the application between probiotic and prebiotic in this study might be resulted in better shrimp growth and survival. This is assumed to be due to the synergistic effect of the joint administration of the prebiotic and probiotic, increasing the activity of the intestinal microflora. The increase of microflora activity was the result of the prebiotic administered through feed which could become a source of nutrients for the intestinal bacteria. This could increase feed digestibility which in turn would affect growth (Merrifield et al., 2010).

4.4.2 Immersion challenge test

Vibriosis is one of the major pathogenic bacterial diseases in shrimp aquaculture. Therefore, shrimp in all treatments were exposed to *V. harveyi* (10^5 CFU/ml) which caused diseases in aquaculture. The challenge test was conducted by immersion method after 60 days feeding trial that was observed in 7 days without water change. After 7 days, the final mortality of the shrimps was observed. Shrimp fed with free cell, microencapsulated cell and co-encapsulated cell showed greater survival than control group with 60%, 80%, 40% mortality, respectively, whereas 100% mortality in control feed group (Figure 4.5).

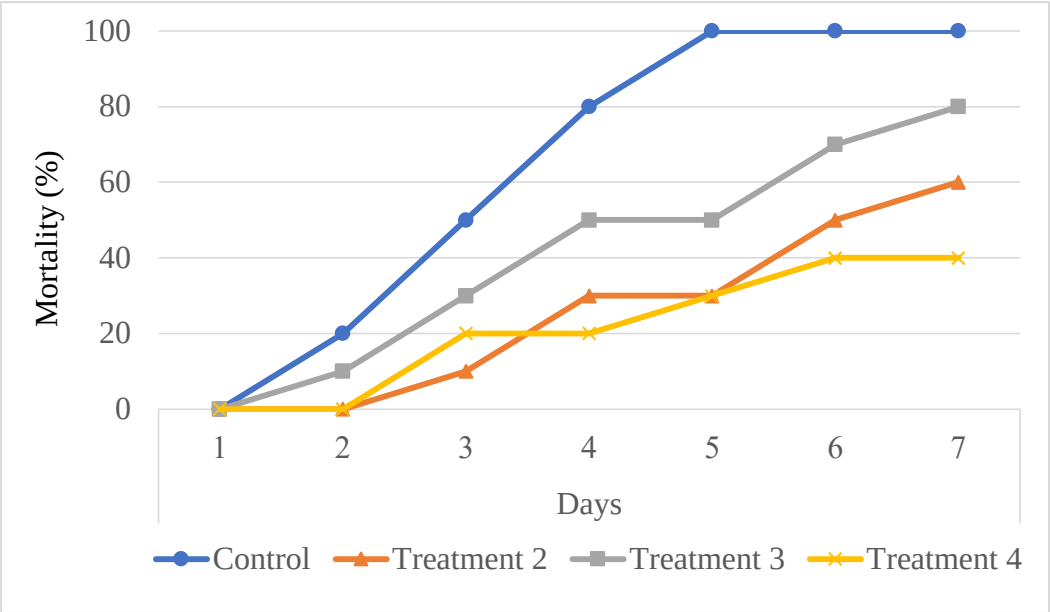

Figure 4.5 Cumulative mortality of *L. vannamei* following challenges by immersion with *V. harveyi* for 7 days.
Treatment 1: Commercial basal diet (Control)
Treatment 2: 1% of free cell L. acidophilus
Treatment 3: 1% of microencapsulated cells without Jerusalem artichoke
Treatment 4: 1% of co-encapsulated cells with Jerusalem artichoke

The population of microbiota; total bacteria, *Lactobacillus*, *Vibrio* count in the GI tract shrimp were evaluated before and after challenging test (Table 4.7). Throughout the experimental period, total bacteria count were similar among the experimental group. At the end of feed trial experiment, there were no significant differences in shrimp fed with Treatment2, Treatment 3 and Treatment 4 on the amount of lactic acid bacteria. However, higher total bacterial count was recorded in shrimp fed with feed Treatment 2, total *Lactobacillus* count of the GI tract was also higher in Treatment 2. Moreover, shrimp fed with Treatment 2, Treatment 3 and Treatment 4 contained significantly fewer intestinal *Vibrio* bacteria compared to shrimp fed with control group. Average total *Lactobacillus* counts in the GI tract after *Vibrio* challenging study decreased in both probiotic and control groups. Karthik et al. (2014) also showed 12% final mortality of *L. vannamei* treated with *Lactobacillus* sp AMET1506 after challenging test with *V. harveyi*. *Lactobacillus* sp count also increased in experimental shrimp intestine while the vibrio load mostly decreased in the groups fed with probiotic *Lactobacillus* sp AMET1506 supplemented feed.

Table 4.7 Microbiota population in the intestine of white shrimp that were fed with the experimental diet before and after challenging test.

	Microbiological evaluation of the digestive tract (CFU/g)		
	Total bacteria	Total LAB	Total *Vibrio* spp.
Before challenge			
Control	7.76±0.13	4.44±0.32[a]	5.82±0.13[b]
Treatment 2	7.56±0.43	6.74±0.21[c]	5.18±0.26[a]
Treatment 3	7.42±0.39	5.67±0.23[b]	5.27±0.16[b]
Treatment 4	7.71±0.22	6.65±0.08[c]	5.25±0.24[b]
After challenge			
Control	7.05±0.10	3.77±0.13[a]	6.31±0.35
Treatment 2	6.98±0.09	5.81±0.12[c]	5.97±0.07
Treatment 3	6.97±0.13	4.93±0.06[b]	6.13±0.28
Treatment 4	7.00±0.21	5.82±0.15[c]	6.06±0.19

[a,b,c] Means±standard deviation with different superscript in the same column of before and after challenge are significantly different and without superscript in the same column are not significantly different ($P<0.05$).

Treatment 1: Commercial basal diet (Control)

Treatment 2: 1% of free cell *L. acidophilus*

Treatment 3: 1% of microencapsulated cells without Jerusalem artichoke

Treatment 4: 1% of co-encapsulated cells with Jerusalem artichoke

The previous research also revealed that the antibacterial activity of *Lactobacillus* sp. against the pathogenic microbes may be due to the production of its metabolites such as organic acids (lactic acid and acetic acid), hydrogen peroxide, diacetyl and bacteriocins (Valenzuela, Ben-Omar, Abriouel, Martinez-Canamero, & Galvez, 2010). Therefore, the reduction of pathogenic microbial load in the shrimp intestine may be due to the production of acid end products and antimicrobial peptides produced by the lactic acid bacteria (Karthik et al., 2014). Similarly the previous research, Widanarni, Nababan, and Yuhana (2015) observed synbiotic between the probiotic bacteria *Vibrio alginolyticus* SKT-b and a prebiotic extracted from the sweet potato *Ipomoea batatas* on *L. vannamei*. They showed that the highest total bacterial count and highest total probiotic *Vibrio alginolyticus* SKT-b were found in larvae

treated with the synbiotic. The administration of prebiotic is postulated to stimulate the growth of microflora besides the administered probiotic in the digestive tract of the Pacific white shrimp larvae, resulting in a higher total bacterial count in the synbiotic treatment compared to the other treatments. The administration of synbiotic could improve the function and increase the number of beneficial bacteria in the intestines (Delgado, Tamashiro, Junior, Moreno, & Pastore, 2011). This experiment showed that shrimp fed with experimental diets Treatment 2 had no significant difference with Treatment 4. Treatment 2, the experimental diets had to mix with free cells every week to maintain the same number of probiotic bacteria in the feed. Shrimp fed with Treatment 2 and Treatment 4 obtained the cells in nearly the same amount throughout the experiment, whereas the number of cells in experimental diets Treatment 3 is likely to decline after the feed processing and during storage. The results indicated that co-encapsulation probiotic could be protected and prolonged during storage. Furthermore, cell encapsulation can also improve shrimp growth and resistance to disease, as well as free cell.

Nowadays, the use of probiotics or prebiotic in aquaculture might be a valuable mechanism to increase shrimp growth, survival rate and disease resistance. Therefore, the incorporation of co-encapsulated probiotic bacteria *L. acidophilus* TISTR 1338 and Jerusalem artichoke in feed could help in the prevention of *Vibrio* disease and modify its gastrointestinal tract and improve the health status of culture organism, *L. vannamei*.

CHAPTER 5
CONCLUSIONS AND SUGGESTIONS

In shrimp farming, the application of biological therapy as probiotics and prebiotics act as an alternative treatment to antibiotics and chemicals, and play the role of alarm molecules to activate the immune system in shrimp culture which would aid in sustainable shrimp production. Moreover, recent researches indicated that the microencapsulation techniques presented as one of the most promising and efficient techniques to enhance viability and stability of probiotics bacteria. This technique has also often used to protect the cells from heat treatment in feed processing to reduce cells injury and death.

Alginate-chitosan microcapsule was used to encapsulate *L. acidophilus* TISTR 1338 with prebiotic (inulin and Jerusalem artichoke) by extrusion method. The results of the present study showed that the co-encapsulation of probiotic *L. acidophilus* TISTR 1338 with 3% prebiotic in 3% alginate bead and further coated with 0.8% chitosan increased probiotic bacterial survival after freeze-drying process and heat condition. Moreover, microcapsules which double coated with chitosan exhibited higher survival after exposure to high temperature compared with the free cells and single coated microcapsules with alginate.

In addition, the administration of probiotics, *L. acidophilus* TISTR 1338 and prebiotic, Jerusalem artichoke, in the form of microcapsule and double coated with chitosan could increase shrimp growth, survival rates, and disease resistance. Probiotic and prebiotic might constitute a valuable mechanism to increase shrimp growth and survival rates. In the present study, one percentage of free cells, microencapsulated cells and co-encapsulated cells with Jerusalem artichoke were incorporated in supplement feed shrimp and cultivated shrimp for 60 days. The result showed that average shrimp weight gain and survival rate of the treatment group were significantly greater than those of the control after 60 days. Furthermore, significant differences for the survival after challenge test with *V.harveyi* in the experimental groups were observed compare with control group.

The highest average of final weight and weight gain were observed in shrimp fed with co-encapsulated with prebiotic. Even though, there was no significant differences in free cell treatment. Due to the fact that free cell is sensitive to environment damage, so shrimp feed in Treatment 2 have to prepare every week to maintain the high number of probiotic bacteria in a shrimp feed. On the other hand, microencapsulated cell can retain for long periods after shrimp feed processing and kept in refrigerated storage. It seems that microencapsulation technique is the best alternative to overcome the problem of poor survivability and could improve the viability of probiotic bacteria in feed processing and storage.

This new finding on the effect of Jerusalem artichoke being used as prebiotics to improve viability of encapsulated probiotic bacteria during heat process and the effect of combination between Jerusalem artichoke and probiotic bacteria on the growth and survival in white shrimp will be beneficial to the shrimp industry. Therefore, the study concluded that the co-encapsulation probiotic *L. acidophilus* and Jerusalem artichoke might be useful to develop thermal resistance property of probiotic cells during heat process in shrimp feed processing and incorporated co-encapsulated beads in feed shrimp to increase shrimp growth, survival rate and disease resistance. So, the microencapsulation of the probiotic and prebiotic used in this study can be applied in shrimp feed for aquaculture in the future.

Suggestions:

- During shrimp culture experimental, *Lactobacillus* in GI tract shrimp should be enumerated to evaluate the probiotic during feed trial compare with at the end of experiment.
- The viability of freeze-dried microcapsule at room temperature (25°C) should be investigated and compare with refrigerated storage (4°C). Because normally, shrimp feed is kept at room temperature.

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APPENDIX

APPENDIX A

บัญชีรายชื่อเชื้อจุลินทรีย์ที่เป็นจุลินทรีย์โพรไบโอติกสำหรับใช้ในอาหาร

แนบท้ายประกาศกระทรวงสาธารณสุข เรื่อง การใช้จุลินทรีย์โพรไบโอติกในอาหาร

เล่ม ๑๒๘ ตอนพิเศษ ๘๖ ง ราชกิจจานุเบกษา ๓ สิงหาคม ๒๕๕๔

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๑. บาซิลลัส โคแอกกูแลน	*Bacillus coagulans*
๒. บิฟิโดแบคทีเรียม อะโดเลสเซนทิส	*Bifidibacterium adolescentis*
๓. บิฟิโดแบคทีเรียม อะนิมอลิส	*Bifidobacterium animalis*
๔. บิฟิโดแบคทีเรียม บิฟิดัม	*Bifidobacterium bifidum*
๕. บิฟิโดแบคทีเรียม เบรเว	*Bifidobacterium breve*
๖. บิฟิโดแบคทีเรียม อินฟานทิส	*Bifidobacterium infantis*
๗. บิฟิโดแบคทีเรียม แล็กทิส	*Bifidobacterium lactis*
๘. บิฟิโดแบคทีเรียม ลองกัม	*Bifidobacterium longum*
๙. บิฟิโดแบคทีเรียม ซูโดลองกัม	*Bifidobacterium pseudolongum*
๑๐. เอ็นเทอโรค็อกคัส ดูแรน	*Enterococcus durans*
๑๑. เอ็นเทอโรค็อกคัส เฟเซียม	*Enterococcus faecium*
๑๒. แล็กโทบาซิลลัส แอซิโดฟิลัส	*Lactobacillus acidophilus*
๑๓. แล็กโทบาซิลลัส คริสปาทัส	*Lactobacillus crispatus*
๑๔. แล็กโทบาซิลลัส แก็สเซอรี	*Lactobacillus gasseri*
๑๕. แล็กโทบาซิลลัส จอห์นโซนอิ	*Lactobacillus johnsonii*
๑๖. แล็กโทบาซิลลัส พาราคาเซอิ	*Lactobacillus paracasei*
๑๗. แล็กโทบาซิลลัส รียูเทอรี	*Lactobacillus reuteri*
๑๘. แล็กโทบาซิลลัส รามโนซัส	*Lactobacillus rhamnosus*
๑๙. แล็กโทบาซิลลัส ซาลิวาเรียส	*Lactobacillus salivarius*
๒๐. แล็กโทบาซิลลัส ซีอี	*Lactobacillus zeae*
๒๑. โพรพิโอนิแบคทีเรียม อะราไบโนซัม	*Propionibacterium arabinosum*
๒๒. สแตปฟิโลคอคคัส ไซน์ยูรี	*Staphylococcus sciuri*
๒๓. แซ็กคาโรไมซีส เซรีวิซิอี สับสปีชีย์ บัวลาดิอิ	*Saccharomyces cerevisiae subsp. Boulardii*

อ้างอิงจาก Bulletin of the International Dairy Federation No.377/2002

ประกาศกระทรวงเกษตรและสหกรณ์
เรื่อง กำหนดวัตถุที่เติมในอาหารสัตว์
ปริมาณการใช้และเงื่อนไขในการห้ามผลิต นำเข้าหรือขายอาหารสัตว์
พ.ศ. ๒๕๕๙

ข้อ ๙ กำหนดให้ชีวผลิตภัณฑ์ซึ่งมีชื่อทางวิชาการอาหารสัตว์ว่า “สารเสริมชีวนะ” ต่อไปนี้เป็นวัตถุที่เติมในอาหารสัตว์ โดยมีปริมาณที่ใช้ผสมในอาหารสัตว์ผสมสำเร็จรูปแล้วจะต้องมีอัตราส่วนหรือปริมาณของสารเสริมชีวนะชนิดเดียวหรือหลายชนิดรวมกันไม่น้อยกว่า ๑×๑๐๕ ซี.เอฟ.ยู (CFU) ต่ออาหารสัตว์ ๑ กิโลกรัม และสารเสริมชีวนะที่ให้ใช้เป็นวัตถุที่เติมในอาหารสัตว์ ดังนี้

(ก) จำพวกเชื้อแบคทีเรีย

(๑) แล็กโทบาซิลลัส แพลนทารัม (*Lactobacillus plantarum*)

(๒) แล็กโทบาซิลลัส เคซีไอ (*Lactobacillus casei*)

(๓) แล็กโทบาซิลลัส เฟอร์เมนตัม (*Lactobacillus fermentum*)

(๔) แล็กโทบาซิลลัส เบรวิส (*Lactobacillus brevis*)

(๕) แล็กโทบาซิลลัส บัลการิคัส (*Lactobacillus bulgaricus*)

(๖) แล็กโทบาซิลลัส แอซิโดฟิลัส (*Lactobacillus acidophilus*)

(๗) แล็กโทบาซิลลัส เซลโลไบโอซัส (*Lactobacillus cellobiosus*)

(๘) แล็กโทบาซิลลัส เคอร์วาตัส (*Lactobacillus curvatus*)

(๙) แล็กโทบาซิลลัส เดลบรูคิไอ (*Lactobacillus delbruekii*)

(๑๐) แล็กโทบาซิลลัส แล็กติส (*Lactobacillus lactis*)

(๑๑) แล็กโทบาซิลลัส ริวเทอริไอ (*Lactobacillus reuterii*)

(๑๒) แล็กโทบาซิลลิส เฮลวีทิคัส (*Lactobacillus helveticus*)

(๑๓) ลิวโคนอสตอก มีเซนเทอรอยเดส (*Leuconostoc mesenteroides*)

(๑๔) สเตรปโทค็อกคัส ฟิเซียม เซอร์เนลส์ ๖๘ (*Streptococcus faecium cernelle* ๖๘)

(๑๕) สเตรปโทค็อกคัส เทอร์โมฟิลัส (*Streptococcus thermophilus*)

(๑๖) สเตรปโทค็อกคัส ฟีเซียม (*Streptococcus faecium*)

(๑๗) สเตรปโทค็อกคัส ครีโมริส (*Streptococcus cremoris*)

(๑๘) สเตรปโทค็อกคัส ไดอะซิทีแล็กติส (*Streptococcus diacetylactis*)

(๑๙) สเตรปโทค็อกคัส แลกติส (*Streptococcus lactis*)

(๒๐) สเตรปโทค็อกคัส อินเตอร์มีเดียส (*Streptococcus intermedius*)

(๒๑) บาซิลลัส ซับทิลิส สเตรน บีเอ็น (*Bacillus subtilis* strain BN)

(๒๒) บาซิลลัส โคแอกูแลน (*Bacillus coagulan*)

(๒๓) บาซิลลัส เลนตัส (*Bacillus lentus*)

(๒๔) บาซิลลัส ไลเคนิเฟอร์มิส (*Bacillus lichenifermis*)

(๒๕) บาซิลลัส พูมิลัส (*Bacillus pumilus*)

(๒๖) บาซิลลัส ซับทิลิส (*Bacillus subtilis*) (สเตรนที่ไม่สร้างยาปฏิชีวนะ) (non-antibiotic producing strains only)

(๒๗) บาซิลลัส โทโยอิ (*Bacillus toyoi*)

(๒๘) แบคทีรอยเดส แอมิโลฟิลัส (*Bacteroides amylophilus*)

(๒๙) แบคทีรอยเดส คาพิลโลซัส (*Bacteroides capillosus*)

(๓๐) แบคทีรอยเดส รูมิโนโคลา (*Bacteroides ruminocola*)

(๓๑) แบคทีรอยเดส ซูอิส (*Bacteroides suis*)

(๓๒) ไบฟิโดแบคทีเรียม แอโดเลสเซนติส (*Bifidobacterium adolescentis*)

(๓๓) ไบฟิโดแบคทีเรียม แอนิมาลิส (*Bifidobacterium animalis*)

(๓๔) ไบฟิโดแบคทีเรียม ไบฟิดัม (*Bifidobacterium bifidum*)

(๓๕) ไบฟิโดแบคทีเรียม อินแฟนติส (*Bifidobacterium infantis*)

(๓๖) ไบฟิโดแบคทีเรียม ลองกัม (*Bifidobacterium longum*)

(๓๗) ไบฟิโดแบคทีเรียม เทอร์โมฟีลัม (*Bifidobacterium thermophilum*)

(๓๘) พีดิโอค็อกคัส แอซิดิแล็กทิซิไอ (*Pediococcus acidilacticii*)

(๓๙) พีดิโอค็อกคัส เซอรีวิซิอี (*Pediococcus cerevisiae*) ดอมโมซัส (domosus)

(๔๐) พีดิโอค็อกคัส เพนโทซาเซียส (*Pediococcus pentosaceus*)

(๔๑) โพรพิโอนิแบคทีเรียม ฟริวเดนไรชิไอ (*Propionibacterium freudenreichii*)

(๔๒) โพรพิโอนิแบคทีเรียม เชอร์มานิไอ (***Propionibacterium shermanii***)

APPENDIX B

อินนูลินและฟรุกโตโอลิโกแซคคาไรด์ในแก่นตะวันสายพันธุ์ต่างๆ

Inulin and fructooligosacharides in different varieties of Jerusalem artichoke (*Helianthus tuberosus* L.)

เปรียบเทียบปริมาณอินนูลินและฟรุกโตโอลิโกแซคคาไรด์ในแก่นตะวันที่ปอกเปลือก
(กรัมต่อน้ำหนักแห้ง 100 กรัม)

สายพันธุ์	อินนูลิน	ฟรุกโต-โอลิโกแซคคาร์ไรด์ (FOS)			
		GF2	GF3	GF4	Sum
CN 52867 แบบปอกเปลือก	77.56	7.04	6.22	6.40	19.66
HEL 231 แบบปอกเปลือก	65.59	6.24	5.99	5.81	18.04
HEL 335 แบบปอกเปลือก	64.69	6.62	3.43	6.27	16.32
HEL 53 แบบปอกเปลือก	66.05	9.28	8.64	8.53	26.44
HEL 61 แบบปอกเปลือก	72.33	5.36	4.78	4.17	14.31
HEL 62 แบบปอกเปลือก	66.58	7.26	6.32	5.44	19.01
HEL 65 แบบปอกเปลือก	60.89	8.17	7.92	7.66	23.75
HEL 66 แบบปอกเปลือก	67.86	7.96	6.36	5.07	19.40
HEL 68 แบบปอกเปลือก	64.15	5.03	4.64	4.08	13.76
HEL 69 แบบปอกเปลือก	63.06	6.87	6.89	7.07	20.83
JA 102 แบบปอกเปลือก	67.86	8.09	7.07	6.37	21.53
JA 37 แบบปอกเปลือก	68.39	6.56	6.67	6.27	19.50
JA 38 แบบปอกเปลือก	79.17	7.07	6.97	6.90	20.94
JA 67 แบบปอกเปลือก	71.87	8.54	8.90	8.55	25.99
JA 89 แบบปอกเปลือก	74.61	4.80	5.03	5.13	14.95
KKU Ac 001 แบบปอกเปลือก	72.85	7.56	6.56	5.20	19.32
ค่าเฉลี่ย (Mean)	**68.97**	**7.03**[a]	**6.40**	**6.18**	**19.61**
ส่วนเบี่ยงเบนมาตรฐาน (SD)	**5.26**	**1.26**	**1.44**	**1.35**	**3.74**

Source: Tanjor, Judprasong, Chaito, and Jogloy (2012)

Composition of commercial inulin: Fibruline® instant (NutritionSc Co., Ltd.)

Product Sheet **Fibruline® Instant**

Tel +32(0) 69 44 66 00
Fax +32(0) 69 44 66 22

Email
sales@cosucra.com

visit our website :
http://www.cosucra.com

Fibruline®, chicory inulin, is a soluble dietary fibre extracted from chicory roots by a natural process. It's a naturally-sourced food ingredient. Fibruline® Instant is an easy to disperse fine granulated white powder, giving mouthfeel to a wide range of food applications.
Belonging to the fructan group, inulin is a non-digestible oligosaccharide built up of fructose units with β2-1 bonds, mostly ending by a glucose unit.

Guaranteed specifications (Analytical methods available on request)

Dry matter (D.M.)	96 +/- 1%
Composition based on D.M	
Ash	max 0.3%
Total carbohydrates	min 99.7%
Inulin	min 90%
Free fructose, glucose & sucrose	max 10%
Microbiology	
Bacillus cereus	max 100/g
Enterobacteriaceae	absent/1g
E. Coli	absent/1g
Moulds	max 20/g
Salmonella	absent/100g
S. Aureus	absent/1g
Aerobic plate count	max 1000/g
Aero thermo count	max 2000/g
Yeasts	max 20/g
Characteristics	
Heavy metals (Pb, Cd, Hg, As)	max 0.5 ppm

Typical data (indicative values)

General Characteristics

Average DP (degree of polymerisation)	~10	pH (30% in water)	~ 6.0
Colour	white	Solubility	≥ 100g/l
Density - Tapped	~ 0.6 kg/l	Stability	Heat stable, at pH<3.5, hydrolysis risk increases significantly
Dispersibility	~ 1% lumps		
Granulometry	< 500 µm	Taste	neutral / slightly sweet

Labelling

(Chicory) inulin, Chicory (dietary) fibre, Chicory (Vegetable) fibre, (Chicory) Fructo-Oligosaccharide, (Chicory) Oligofructose.

Nutrition Labelling (values expressed per 100g commercial product)

Caloric Value (energy)	208 kcal (1) , 840 kJ	Fat	0 g (2)
Carbohydrates	8 g (96g) (3)	of which saturated	0 g (2)
of which sugars	8 g	Protein	0 g (2)
Dietary Fibre	88g	Sodium	0.1g

(1) Based on a caloric value of 2kcal/g for pure inulin/oligofructose & subject to local regulations, (2) Non detectable, (3) Including dietary fibre

Method of analysis

Inulin and oligofructose (fructan) level in food products can be analysed by the following method: AOAC 997.08.

Certification

KOSHER & HALAL (on demand) certified ingredient.
Does not contain GMO's or GMO-derived components. Not produced using GMO-based technology (not concerned by EC 1829/2003 and EC 1830/2003).

Allergens

No labelling required according to EC legal requirements for Allergen Labelling 1169/2011/EC, repealing Directive 2000/13/EC.

Safety

Food grade, suitable for human consumption. Free from any harmful or toxic substances. Food Safety System Certification 22000 : 2010 in compliance with ISO 22000: 2005 and PAS 220: 2008 certified by SGS Systems and Services Certification. Certificates recognized by Global Food Safety Initiative (GFSI)

Shelf-life

4 years in dry conditions (Max 30°C, Max 60% R.H.), in original sealed bag.

Packaging

Multi-layer paper bags (with blue PE liner inside) 20 kg net on pallet.
Industrial Pallet 960 kg net (120x120xmax190cm)*, Export-pallet 960 kg net (115x115xmax190 cm)*, Euro-pallet 720kg net (120X80Xmax180 cm)*. On demand: Plastic pallet and Big Bag.
*(LengthxWidthxHeight cm)

Produced by

Cosucra Groupe Warcoing S.A., Belgium (Manufacturing site : Warcoing Industrie, Belgium).

SOLUTIONS

fibruline®

- Fibre enrichment
- Calorie reduction
- Gut health
- Calcium absorption

APPLICATIONS

- Dairy
- Bakery
- Confectionery
- Savoury
- Powder blends
- Beverages

PRODUCTS

- Fibruline® Instant, native inulin
- Fibruline® S20, soluble inulin
- Fibruline® DS2, desugared inulin
- Fibrulose® F97, oligofructose
- Fibruline® XL, long chain inulin

VITA

Miss Suppharada Jantarathin was born on August 27th, 1983 in Phatthalung, Thailand. In 2005, she received a Bachelor degree in Bio-Industrial Management from Prince of Songkla University. After that, she received a Master degree in Program of Biotechnology, Faculty of Science, Chulalongkorn University in 2009. After graduation, she pursued her graduate study for Doctoral Degree in Program of Biotechnology, Faculty of Science, Chulalongkorn University.

Her address is 139/19 Nararom village, Bangkruai-sainoi Road, Bangkruai, Nonthaburi, Thailand 11130.

Journal Contributions:

Jantarathin, S., Borompichaichartkul, C., & Sanguandeekul, R. 2017. Co-encapsulation of Lactobacillus acidophilus with Jerusalem artichoke in alginate-chitosan matrix. KHON KAEN AGR. J. 45 SUPPL. 1:1126-1135.

Jantarathin, S., Borompichaichartkul, C., & Sanguandeekul, R. 2017. Microencapsulation of probiotic and prebiotic in alginate-chitosan capsules and its effect on viability under heat process in shrimp feeding. Materials Today: Proceedings 4:6166-6172.