รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาอาหารเม็ดซินไบโอติกส์เสริม *Lactobacillus rhamnosus* GG พร้อมด้วยพรีไบโอติกแก่นตะวันและการประเมินผลของอาหารเม็ดซินไบโอติกส์ต่อการเจริญเติบโต สารเคมีในเลือด และ ลักษณะลำไส้ของปลานิล

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

Contents

Exclusive Summary

Tilapias are one of the economical protein sources proving nutrition and food security to human. Thailand is one of the top six countries globally producing tilapias. Thai tilapias are easily infected by bacteria leading to disease outbreaks because of high density culture. Since people now more concern about food safety, the use of antibiotics to treat the fish has been avoided and several fish farms attempt to produce antibiotic free Nile tilapia. Probiotics as *Lactobacillus rhamnosus* GG (LGG) and prebiotics as Jerusalem Artichoke (JA), called Kantawan in Thai, have been introduced to replace antibiotics. This study intended to develop Nile tilapia pelleted feed containing encapsulated LGG with JA (PFL) and to evaluate their effects on Nile tilapias. LGG and JA were encapsulated in alginate-skim milk matrix by two methods namely gel extrusion and spray drying process to form LGG with JA capsules and spray dried LGG with JA powders. Concentrations of JA were varied and evaluate for their protective effects on LGG viability after processing and under stimulated gastrointestinal (GI) tract of Nile tilapia. The spray dried LGG with JA was incorporated into feed ingredients prior to be extruded to be PFL. PFL was fed into the Nile tilapia to determine the synbiotic effects of LGG and JA on the fish growth performance, blood chemistry, and morphology changes of the intestines. The results indicated that JA could provide protective barriers to LGG capsules and spray dried LGG powders, helping increase LGG viability during freeze drying and spray drying and under stimulated fish GI tract. The highest survival rates of LGG were significantly observed when 20g/100mL of JA was added into the matrix to protect the cells from bile toxic at the concentration of 10 mL/100mL. Due to the dual effects of LGG and JA, the Nile tilapias fed with PFL showed significantly higher weight grain and specific growth rate and had higher villa length than controls. On the other hand, they had no effects on blood chemistry of the fish. This positive effects could be because LGG possibly increase the nutrient digestibility of the fish. Simultaneously, JA was fermented by indigenous microbiota, resulting in the production of short chain fatty acids that promote the healthy epithelium cells of the intestines. This results in the increase of Nile tilapia growth performance. This study indicates that Nile tilapia pelleted feed containing encapsulated LGG with JA had potentially to be used as practical diets for producing antibiotic free Nile tilapia.

บทคัดย่อ

จุลินทรีย์โพรไบโอติกส์สามารถช่วยเร่งการเจริญเติบโตและควบคุมการติดเชื้อของสัตว์น้ำได้ แต่อย่างไรก็ตามจุลินทรีย์โพรไบโอติกส์จำเป็นต้องสามารถรอดชีวิตได้จากกระบวนการผลิตและระหว่างการลำเลียงในระบบทางเดินอาหาร สารพรีไบโอติกส์แก่นตะวันสามารถป้องกันจุลินทรีย์โพรไบโอติกส์จากสภาวะที่ไม่เหมาะสม การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของแก่นตะวันต่อการป้องกันโพรไบโอติกส์ *Lactobacillus rhamnosus* GG (LGG) ในระหว่างการกระบวนการผลิต (การทำแห้งแบบแช่เยือกแข็งและการทำแห้งแบบพ่นฝอย) และในระบบทางเดินอาหาร พัฒนาอาหารปลานิลซินไบโอติกส์อัดเม็ดเสริม LGG และแก่นตะวัน และประเมินผลของปลานิลซินไบโอติกส์เสริม LGG และแก่นตะวันต่อสมรรถภาพการเจริญเติบโตของปลานิล ค่าเลือด และลักษณะทางสัณฐานวิทยาของลำไส้เล็กของปลานิล LGG ผสมด้วยแก่นตะวันที่ความเข้มข้นต่าง ๆ (10 g/100mL, 15g/100mL, และ 20g/100 mL) ถูกห่อหุ้มด้วยแอลจิเนตและนมพร่องมันเนยด้วยวิธีเจลเอ๊กทรูชั่น (gel extrusion) และ การทำแห้งแบบพ่นฝอย จนกลายเป็น เม็ดแคปซูล และ ผง LGG ผสมด้วยแก่นตะวันตามลำดับ LGG ในเม็ดแคปซูลและในรูปแบบผงถูกนำไปวิเคราะห์จำนวนจุลินทรีย์ที่รอดชีวิตหลังจากการทำแห้งแบบแช่เยือกแข็งและแบบพ่นฝอย รวมทั้งเมื่ออยู่ในสภาวะเลียนแบบระบบทางเดินอาหารของปลานิลที่มี pH และความเข้มข้นเกลือน้ำดีที่แตกต่างกัน หลังจากนั้นตัวอย่างที่มีการรอดชีวิตของ LGG สูงสุด ถูกนำมาผสมในอาหารปลา แล้วนำไปอัดเม็ดที่อุณหภูมิห้อง เพื่อผลิตอาหารปลานิลซินไบโอติกส์อัดเม็ดเสริม LGG และแก่นตะวัน หลังจากนั้น อาหารปลานิลซินไบโอติกส์อัดเม็ดถูกนำไปทดลองใช้เลี้ยงปลานิลเพื่อศึกษาผลของ LGG และแก่นตะวันในอาหารเม็ดต่อสมรรถภาพการเจริญเติบโตของปลานิล ค่าเลือด และลักษณะทางสัณฐานวิทยาของลำไส้ของปลานิล ผลการทดลองพบว่า แก่นตะวันสามารถป้องกัน LGG จากกระบวนการทำแห้งแบบแช่เยือกแข็งและแบบพ่นฝอยได้ รวมทั้งสามารถเพิ่มอัตราการรอดชีวิตของ LGG ในระบบทางเดินอาหารจำลองของปลานิลได้ ความเข้มข้นของแก่นตะวันที่ 20 g/100 mL จะสามารถช่วยเพิ่มอัตราการรอดชีวิต LGG ได้อย่างมีนัยสำคัญเมื่ออยู่ภายใต้สภาวะเกลือน้ำดีที่ความเข้มข้น 10mL/100mL เป็นเวลา 3 ชั่วโมง และ ผง LGG ผสมด้วยแก่นตะวันจะมีอัตราการรอดชีวิตในระบบทางเดินอาหารสูงกว่าแบบเม็ดแคปซูล เมื่อนำผง LGG ผสมด้วยแก่นตะวันไปผสมกับอาหารปลาเพื่อผลิตอาหารปลาอัดเม็ดซินไบโอติกส์ พบว่าการอัดเม็ดที่อุณหภูมิห้องและการทำแห้งเม็ดอาหารที่ 50C เป็นเวลา 8 ชั่วโมง ไม่ส่งผลต่อจำนวนอัตราการรอดชีวิตของ LGG ในอาหารปลาอัดเม็ด และมีจำนวนจุลินทรีย์รอดชีวิตมากกว่า 10^7 CFU/g ผลการทดลองการให้อาหารอัดเม็ดเสริมซินไบโอติกส์ในปลานิล (*Oreochromis niloticus*) แสดงให้เห็นว่า ปลาที่กินอาหารเม็ดเสริมซินไบโอติกส์มีการเจริญเติบโตที่ดีกว่าปลาที่กินอาหารควบคุม กล่าวคือ มีเปอร์เซ็นการเพิ่มขึ้นของน้ำหนัก และอัตราการเติบโตจำเพาะสูงกว่า นอกจากนี้ยังพบว่า การเสริม LGG และ แก่นตะวัน ช่วยเพิ่มความยาวให้กับวิลไลของลำไส้ทั้งส่วนต้นและส่วนปลายอย่างมีนัยสำคัญทางสถิติ อาหารเม็ดเสริมซินไบโฮติกส์ไม่มีผลต่อค่าเลือดของปลานิล ผลการวิจัยเหล่านี้แสดงให้เห็นว่าแก่นตะวันสามารถใช้ป้องกัน LGG จากสภาวะที่ไม่เหมาะสมได้ และการรวมกันของ LGG และแก่นตะวันช่วยส่งเสริมการเจริญเติบโตของปลานิลได้ ดังนั้นอาหารอัดเม็ดซินไบโอติกส์เสริม LGG และแก่นตะวันมีศักยภาพในการใช้เป็นอาหารปลา

Abstract

Probiotics can help promote growth and control infection of aquatic animals. However, they must survive during processing and under the digestive systems. Prebiotics Kantawan (Jerusalem Artichoke) can protect the probiotics from unfavorable conditions The objectives of this study were to study the effects of Kantawan on the prevention of probiotics, *Lactobacillus rhamnosus* GG (LGG), during processing, including freeze drying and spray drying, and under fish gastrointestinal tract, to develop tilapia pelleted feeds supplemented with LGG and Kantawan, and to evaluate the effects of the pelleted feeds on the growth performance of Nile tilapia, blood chemistry values and morphology of the fish intestine. LGG were mixed with Kantawan at various concentrations (10 g / 100mL, 15g / 100mL, and 20g / 100 mL) and encapsulated in alginate and skimmilk matrix using the gel extrusion and spraying drying methods to form the LGG with Kantawan capsules and powders, respectively. LGG in the capsules and in powders were analyzed for the number of viable cells after freezing and spray drying and under simulated gastrointestinal (GI) conditions of the tilapia at different pH and bile salt concentrations. After that, the samples that had the highest number of viable LGG were mixed with fish feed ingredients. The mixture was then pelleted at room temperature to produce Nile tilapia symbiotic pelleted feed supplemented with LGG and Kantawan. The synbiotic pellets were fed to Nile tilapia to evaluate the effects of LGG and Kantawan on the fish growth performance, blood chemistry values and morphology of the tilapia intestines. The results showed that Kantawan could protect LGG from freeze-drying and spray-drying processes and increase the survival of LGG under the simulated gastrointestinal tract of tilapia. The concentration of Kantawan at 20g/100mL could significantly enhance LGG survival under bile salt at a concentration of 10mL/100mL for 3 hours. LGG with Kantawan had higher survival rate in the GI tract than that of the capsules. When LGG with Kantawan powders were combined with fish feeds to produce synbiotic pelleted feed. It was found that pelletizing at room temperature and drying of pellets at 50 $^{\circ}$C for 8 hours did not affect the survival rate of LGG in the pelleted feed and the number of viable cells was more than 10^7 CFU/g. The results of feeding the Nile tilapia with the synbiotic pelleted feeds indicated that the fish fed synbiotics pelleted feeds had better growth than the control group, namely the weight gain and specific growth rates. In addition, LGG and Kantawan significantly increased the villi height. Synbiotics pellets did not affect the blood chemistry value of tilapia. These findings suggest that Kantawan could protect LGG from unfavorable conditions and the combination of LGG and Kantawan could help promote the growth of tilapia. Therefore, symbiotic pelleted feed supplemented with LGG and Kantawan have the potential to be used as fish diets.

1. Introduction

Tilapias are the second most farmed fish in the world. Their global production in 2011 was 3.96 million metric tons, and it has been expected to reach 7.3 million metric tons in 2030 (Juergen, 2015). There are more than 135 countries around the world have involved in the tilapia industry (FAO, 2014). Thailand is one of the top six countries globally producing tilapias (FAO 2012). Thai tilapias are commonly cultured at high density in ponds and floating cages placed on rivers or irrigation canals (Belton, Turongruang, Bhujel, & Little, 2009). Although the intensive fish culture production helps reduce production costs, it easily promotes susceptibility of tilapias to infectious bacteria (Shoemaker, Evans, & Klesius, 2000). The infection is one of the major threats affecting sustainability of the aquaculture industry. It causes disease outbreaks, resulting in huge economic losses. In worldwide, billions of dollars were lost annually from this problem.

To control bacterial infection, antibiotics are generally treated by mixing or/and coating with the fish diet without any diagnosis or prescriptions by veterinarians. The use of antibiotics is common, even though there are no apparent disease problems (Cabello, 2006). Misuse of antibiotics in aquaculture productions has become a large issue. It negatively affects food sustainability and security by developing and spreading antibiotic-resistant bacteria out to environments, which can harm to human health and ecosystems. Antibiotic-resistant bacteria possibly pass out to human through a food chain and ultimately result in human infections which are difficult or impossible to cure. The WHO reported that people in the European Union die from antibiotic-resistant bacteria infections more than 25,000 people per year (WHO, 2011). Large amounts of antibiotics from marine cage-based aquaculture farming remained in the surrounding ecosystems, resulting from leaching or sedimentation of medicated feeds and excretion from the cultured species (Capone, Weston, Miller, & Shoemaker, 1996). Intensive use of antibiotics in caged tilapia production in Thailand has a tendency to contaminate freshwater ecosystems (Rico et al., 2014). Thus, in order to culture tilapia sustainably unappropriated use of antibiotics in tilapia productions should be banned. Seeking for alternative ways to prevent and control bacterial infection is required.

In recent years, probiotics have gained attentions to be applied in aquaculture production as an alternative of antibiotics. Probiotics are beneficial microorganisms that can enhance fish growth, stimulate immune system functions, and improve the resistance of fish to infectious diseases (Welker & Lim, 2012). Potential probiotics are generally Lactobacillus sp, and Bacillus sp. (Apún-Molina, Santamaría- Miranda, Luna-González, Martínez-Díaz, & Rojas-Contreras, 2009), but also Aspergillus oryzae and Saccharomyces cerevisiae (Iwashita, Nakandakare, Terhune, Wood, & Ranzani-Paiva, 2015). However, studies of probiotics isolated from tilapias in term of human

toxicology have not been reviewed yet. Lactobacillus rhamnosus GG (LGG), a human-derived probiotic, has been introduced to tilapia culture.

LGG has been well known for its human health benefits such as preventing acute diarrhea in children and antibiotic-associated diarrhea, reducing cholesterol levels, simulating immune systems, and alleviating allergies (Anuradha & Rajeshwari, 2005). Besides human health promoting effects, LGG could inhibit some tilapia pathogenic bacteria such as Edwardsiella tarda and Streptococcus agalactiae (Pirarat, Kobayashi, Katagiri, Maita, & Endo, 2006; Pirarat et al., 2015), promote the intestinal structure, enhance the mucosal immunity of tilapia (Pirarat et al., 2011), and improve tilapia stress coping capacity (Gonçalves, Maita, Futami, Endo, & Katagiri, 2011). However, survival of LGG after administration is one of the major concerns. In order to confer health benefits the number of probiotics is required at least 106 to 107 CFU/g. As Probiotics are susceptible to acid, bile salts, oxygen, a huge loss of their viability during feed preparation and after exposure to stomach and upper intestine are possible. This leads to insufficient live probiotics reaching the target site, the lower intestinal tract. Therefore, protection of live probiotics during exposure to unfavorable conditions is necessary and seeking for an approach to improve their viability to be used as a feed supplement is challenging.

Kantawan or Jerusalem artichoke (Helianthus tuberosus) is a root vegetable, commonly grown in the northwest Thailand. It can be harvested after 100–140 days, and its crop yields are around 13–19 ton/ha. Kantawan is known as a good source of functional ingredients, particularly inulin (160–200 g/kg) and fructooligosaccharide (FOS) (120–150 g/kg) (Moshfegh et al., 1999). Inulin and FOS are fructan-type oligosaccharides, known as prebiotics, non-digestible foods that can be fermented by certain selective colonic bacteria, thus improving host health. They could enhance growth performance, modulate intestinal microbiota, and improve immune systems in fish, poultry, and swine. They could increase viability of Lactobacillus sp. to low pH and the presence of bile salt in simulated digestive systems (Krasaekoopt & Watcharapoka, 2014) and extend the retention period of live probiotic existing in the mice gut (Su, Henriksson, & Mitchell, 2007). However, applications of kantawan in aquaculture feeds as a functional ingredient have not been widespread yet. Therefore, this research proposal would like to combine kantawan with LGG as a synbiotic supplement for tilapias. Dual effects of kantawan and LGG could provide several benefits to tilapia, including inhibiting infectious bacteria, improving tilapia's growth performance, and enhancing the cell viability and their persistence in the gut system.

There are various forms of tilapia feeds in tilapia feed markets, but pellets are commonly used. Administration of a feed supplement for tilapia culture is generally done by separately mixing with feeds (Hai, 2015). Incorporation of synbiotic of kantawan and LGG in the feed pellets would be ideal as it is ready to use, can reduce time for feed preparation, and can easily control

a dose of the supplements. However, tilapia pellets are produced by an extrusion process with high pressure and temperature, which is definitely harmful for LGG probiotics. The bacteria are thus required protection prior to pelleting process. Encapsulation technology such as spray drying or gelation/extrusion process can serve a physical barrier to probiotics against adverse surrounding environments during processing and storage (i.e. heat, oxygen, and acid). It also can deliver them to a target site and control their release (Anal & Singh, 2007; de Vos, Faas, Spasojevic, & Sikkema, 2010) Alginate and skimmed milk has been reviewed as a remarkable matrix that can protect the cells from thermal conditions (Ding & Shah, 2007; Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, & Fisk, 2014). A previous study has proved a potency of alginate combined to skimmed milk to shield LGG against tilapia simulated gastrointestinal conditions and significantly improve tilapia growth performance as well as protection against Streptococcus agalactiae (Pirarat et al., 2015). However, incorporation of the encapsulated LGG with prebiotic kantawan into tilapia pellet feeds has not been conducted yet. Therefore, this research proposal is aimed to encapsulate LGG combined with prebiotic kantawan in alginate-skimmed milk matrixes as a synbiotic supplement and subsequently incorporate it into feed pellets as well as examining the efficacy of the encapsulated synbiotic feed pellets on growth performance, blood chemistry and gut morphology in tilapias. This proposed research would yield a novel functional feed, sustainably contributing to the tilapia production industry and lay a foundation on aquaculture feeds supplemented with encapsulated synbiotics.

2. Objectives

Overall goal of this research proposal is to develop synbiotic feed pellets containing encapsulated *Lactobacillus rhamnosus* GG with prebiotic kantawan. In order to achieve the overall goal three internal studies will be conducted, which is aimed as follows:

1) To determine effects of prebiotic kantawan and alginate-skimmed milk matrixes on viability of *L. rhamnosus* GG under thermal conditions and simulated tilapia gastrointestinal tract

2) To develop an optimal formulation and condition for producing synbiotic feed pellets containing encapsulated *L. rhamnosus* GG with prebiotic kantawan

3) To determine the effect of the synbiotic feed pellets on growth performance, blood chemistry, and gut morphology in nile tilapias.

3. Literature reviews

3.1 Current situations of Nile Tilapia (*Oreochromis niloticus*) in Thailand

Nile tilapia (*Oreochromis niloticus*) is one of the most popular fishes used in aquacultures. They grow fast, reproduce easily, and resist to a variety of environment conditions (Martin *et al*., 2010). Thailand is one of the top six Nile tilapia producers worldwide. Approximately 200,000 metric tons of Nile tilapia are produced annually in Thailand, accounting for approximately 10% of the world's annual tilapia market (Macintosh and De Silva, 1984). They are commonly cultured in the central, northeastern and northern parts of the country. There are two types of Nile tilapia culture systems including pond systems and cage culture systems in natural rivers or reservoirs (Macintosh and De Silva, 1984). They prefer to stay in shallow waters for reproduction and feeding. As Nile tilapia is the highest produced fish species in Thailand and the demands has been increasing worldwide, intensive Nile tilapia culture has become a major problem causing the potential devastation of infectious diseases. The major diseases threatening intensively cultured tilapia farms have been identified as various bacterial infections such as *Streptococcus agalactiae* (Harikrishnan *et al*., 2010), *Flavobacterium columnare* (Figueiredo *et al*., 2005), *Francisella* sp. (Jeffery *et al*., 2010), *Edwardsiella* spp. (Soto *et al*., 2012), and *Aeromonas* spp *(Li and Cai, 2010.)* . Significantly, *S. agalactiae* called as streptococcosis has brought severe problems to Nile tilapia cultures, especially for grow-out fish (Esiobu *et al*., 2010). The outbreak is generally happened in the summer of Thailand (March to July) as the temperature is suitable for the bacteria to grow. After infection, the fish normally suffers a loss of appetite, and the mortality can increase by approximately 10-20% every day, depending upon the water quality (Jeffery *et al*., 2010). Beside, *F. columnare* infection is one of a major outbreak found in Nile tilapia culture. The bacteria can kill various stages of fish, ranging from fries in hatcheries to adults in culture systems (Siwicki *et al*., 1990). These two major pathogens have simultaneously hampered the Nile tilapia culture industry in Thailand and have caused severe economic losses to date. In order to manage these problems, the application of several chemicals and drugs by fish farmers is normally selected such as oxytetracycline, tetracycline, and chlortetracycline.

Obviously, antibiotics are generally used in the aquaculture industry not only for treatments but also for growth promotion and preventive purposes. This could bring about the emergence of drug resistant microorganisms and leave antibiotic residues in the fish and in the environment (Weston, 1996). In addition, 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 and WHO,

2001). Antibiotics could also inhibit or kill beneficial microbiota in the gastrointestinal (GI) ecosystem (Roy, 2005).

Alternative methods of preventing bacterial infections have been seeking for, which could be immune stimulation through alteration of the composition of fish diet and feeding practices as well as immersion or via injection. Many substances from different sources (bacterial components, chemical agents, animal or plant extracts, etc.) have also been studied as prospective immune stimulants for fish (Sakai, 1999). Several immune stimulants such as levamisole (Siwicki *et al*., 1990), chitin (Sakai, 1999), lactoferrin (Sakai *et al*., 1993), Nisin (Samanya and Yamauchi, 2002), recombinant transferrin (Stafford *et al*., 2004), modified carbohydrate (Misra *et al*., 2006), b-glucan (Das *et al*., 2009), chitosan (Geng *et al*., 2011), and various kinds of probiotics (Harikrishnan *et al*., 2011) have been reported. They could be used by injection or dietary administration. The substances could enhance the resistance of cultured fish against diseases. The probiotics administration is one of the strategies that has been launched. This approach provides several advantages over chemical and drug applications, especially for enhancing the growth and health of the host (Esteban *et al*., 2001). Probiotics could prevent several infected diseases and simultaneously enhance immune responses. Probiotics could improve fish growth performance by providing nutrients and enzymatic utilization and even help improve water quality (Sakai *et al*., 1993). However, the use of probiotics in aquaculture is limited as they have to be viable in sufficient numbers at in the gastrointestinal tract. Due to the diverse conditions in the fish gut, live probiotic bacteria often failed to reach the target site in the lower intestinal tract. Microencapsulation, a technology used to stabilize drugs for the controlled delivery and release of active ingredients, has been developed in aquaculture nutrition, in order to improve the probiotics viability in the gastrointestinal tract environment.

3.2 Probiotics

A term of probiotic has been defined for decades. It was first used for the substances produced by microorganisms that stimulate the growth of man and animals. This name was taken from the latin words "pro" and "bios" (Geng *et al*., 2011). The probiotic's definition has been revised for several times. Nowadays, it has been known as "live microorganisms that confer a health benefit to the host when administered in adequate amounts", which was defined by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO and WHO, 2001). Bacteria as probiotics are mostly a group of lactic acid bacteria, such as *Lactobacillus* sp., *Streptococcus* sp., *Enterococcus* sp. and *Lactococcus* sp. However, some *Bacillus* sp., fungi *Aspergillus* sp., and yeast, *Saccharomyces* sp., are also reported as probiotics.

Probiotics provide many health promoting effects, such as adjusting the balance of microorganisms, stimulating and enhancing immunity (Gilliland, 1989), inhibiting the growth of bacteria that cause the disease (Tripathi and Giri, 2014), reducing serum cholesterol levels and blood pressure, providing anti-carcinogenic activity (Lourens-Hattingh and Viljoen, 2001), and improving utilization of nutrients (Abbot *et al*., 2007). However, it was reported that probiotic could yield those health benefits when they were in sufficient amounts. International Dairy Federation (IDF) assigned to probiotic products that they should contain live bacteria at least 10^7 CFU/ml until they were consumed (OUWEHAND, 1998).

3.2.1 Mode of action of probiotics

1) Competitive exclusion

Competitive exclusion means the scenario in which one species of bacteria more vigorously competes for receptor sites in the intestinal tract than another species. The mechanisms are that one species of bacteria exclude or reduce the growth of another one by creation of a hostile micro ecology, production and secretion of antimicrobial substances and selective metabolites, elimination of available bacterial receptor sites, and competitive depletion of essential nutrients. Lactobacilli and Bifidobacteria have been shown to inhibit a broad range of pathogens, including *Escherichia coli*, *Salmonella* spp., *Helicobacter pylori*, *Listeria monocytogenes* and Rotavirus (Ng *et al*., 2009). They can modify the gut environment to make it less suitable for pathogenic bacteria by production of antimicrobial substances, such as lactic and acetic acid and hydrogen peroxide. Consequently, the pH of the gut is lowered below that essential for survival of pathogenic bacteria (Mack *et al*., 1999). Moreover, probiotics could produce the substance called bacteriocins, inhibiting growth of pathogenic microorganisms such as nisin, acidolina, acidofilina, lacatcyna, lacocydyna, reutryna, laktoline, and entrocine. They showed high antibacterial activities against *E. coli*, *Salmonella* sp., *Staphylococcus aureus*, *Clostridium perfringers*, and *Campylobacter* sp. (Caballero *et al*., 2007). Some lactobacilli and bifidobacteria could also share carbohydrate-binding specificities with some enter pathogens (Montalto *et al*., 2004), competing with specific pathogens for the receptor sites on host cells (Gómez-Llorente *et a*l., 2010). In addition, they can eject the colonization of pathogenic bacteria by attaching themselves to the surface of the gut through the interaction between surface proteins and mucins, resulting in prevention of the pathogenic bacteria adhesion to gastrointestinal epithelium (Yan *et al*., 2007). This effect has been found in *in vitro* human mucosal materials and *in vivo* pig mucosal material (Tuomola *et al*., 1999).

2) Production of Antimicrobial Substances

As mentioned above, probiotics can produce low molecular weight compounds, such as acetic acid, lactic acid, and bacteriocins. Acetic acid and lactic acid have been considered the main antimicrobial compounds responsible for the inhibitory activity of probiotics against pathogens especially gram-negative bacteria (Tuomola et al., 1999). The undissociated form of the organic acids enters the bacterial cells and dissociates inside its cytoplasm, lowering the intracellular pH or the intracellular accumulation of the ionized form of the organic acid which leads to the death of the pathogen (Hirn *et al*., 1992). Bacteriocins including lactacin B from *L. acidophilus*, plantaricin from *L. plantarum* and nisin from *Lactococcus lactis* could destroy target cells by pore formation and/or inhibition of cell wall synthesis.

3) Enhancement of the epithelial barrier

Probiotics can prevent the potential pathogen bacteria by enhancement of intestinal barrier function through modulation of cytoskeletal and epithelial tight junction in the intestinal mucosa (Schiffrin *et al.*, 1997). Under normal physiological conditions, intestinal barrier is maintained by several factors, such as mucus production, water and chloride secretion, and epithelial cells that form tight junction (Hirano *et al.*, 2003). Disruption of epithelial barrier has been reported in several clinical conditions such as enteric infections, celiac diseases and infection bowl disease (Hirano *et al*., 2003). Enhancement of epithelial barrier integrity may be an important mechanism through which the probiotic bacteria benefit the host in these disease conditions. The probiotic bacteria could increase the secretion of mucus by triggering inflammation in enterocytes of the intestines (González-Rodríguez *et al*., 2012).

4) Increased adhesion to intestinal mucosa

Adhesion to intestinal mucosa is regarded as a prerequisite for colonization and is important for the interaction between probiotic strains and the host (Ouwehand *et al*., 2002). Adhesion of probiotics to the intestinal mucosa is important for modulation of the immune system (Van Tassell and Miller, 2011), and antagonism against pathogens (Buck *et al*., 2005). Probiotics display various surface determinants that are involved in their interaction with intestinal epithelial cells (IECs) and mucus. IECs secrete mucin, which is a complex glycoprotein mixture that is the principal component of mucous, thereby preventing the adhesion of pathogenic bacteria (Hynönen *et al*., 2002). This specific interaction has indicated a possible association between the surface proteins of probiotic bacteria and the competitive exclusion of pathogens from the mucus (Hirn *et al*., 1992).

5) Stimulation of immune systems

It is well known that the immune system can be divided between the innate and adaptive systems. The adaptive immune response depends on B and T lymphocytes, which are specific for particular antigens. In contrast, the innate immune system responds to common structures called pathogen-associated molecular patterns (PAMPs) shared by the vast majority of pathogen (Hekmat and McMahon, 1992). Probiotic bacteria can exert an immunomodulatory effect. They have the ability to interact with epithelial and dendritic cells (DCs) and with monocytes/macrophages and lymphocytes, which have an important role in innate and adaptive immunity. Due to probiotic's ability of adhesion to the intestinal mucosa, it allows to create a natural barrier against potential pathogens, and thus enhances immunity by increasing production of immunoglobulins, activity of macrophages and lymphocytes, as well as stimulating the production of **γ**-interferon (Granato *et al*., 2010). It was reported that administration of *L. rhamnosus* resulted in enhancement of non-specific humoral responses by increasing production of IgG, IgA and IgM from circulating lymphocyte (Hirano *et al.*, 2003).

3.2.2 Probiotic in aquacultures

(F.a.m *et al*., 1993)The use of all sub-therapeutic antibiotics as growth-promoting agents in animal production has been banned since January 2006 by European Union. Alternative growth promoters to be used in aquatic feeds have received much attention (Klaver *et al*., 1993). Development of new dietary supplementation strategies, including health and growth-promoting compounds as probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been heightened (Dave and Shah, 1997). Particularly, the research of probiotics and prebiotics in fish nutrition has been increasing with the demand for consumers and environment-friendly aquaculture. Many published reports demonstrated positive effects of probiotics and prebiotics in feeds for various fish species, including rainbow trout, Common carp (Dave and Shah, 1997) Indian major carp, Mozambique, Nile tilapia, Atlantic cod, and European Sea bass juveniles (Marnett *et al*., 1985). Probiotics help increase feed conversion efficiency, live weight gain and confer protection against pathogens by competitive exclusion for adhesion sites (Charalampopoulos and Rastall, 2009), production of organic acids (formic acid, acetic acid, lactic acid), hydrogen peroxide and several other compounds such as antibiotics, bacteriocins, siderophores, lysozyme (Kailasapathy and Sultana, 2003) and also modulation of physiological and immunological responses in fish (Watson and Preedy, 2015). Apart from the nutritional and other health benefits (Galvão *et al*., 2015), certain probiotics as water additives can also play a significant role in decomposition of organic matter, reduction of nitrogen and phosphorus level as well as control of ammonia, nitrite, and hydrogen sulfide (PATHANIA *et al*., 2018). Several

probiotics either as monospecies or multispecies supplements are commercially available for aquaculture practices (Özkan and Bilek, 2014).

3.2.4 Factors affecting viability of probiotic

The ability of probiotics to survive and multiply in the host strongly influences their probiotic benefits. The bacteria should be metabolically stable and active in the products, survive passage through the upper digestive tract in large numbers and have beneficial effects when in the intestine of the host (Gilliland, 1989). The standard for any foods and feeds sold with health claims from the addition of probiotics is that it must contain per gram at least 10^6-10^7 CFU of viable probiotic bacteria (FAO and WHO, 2001). Therefore, probiotic survival is essential for organisms targeted to populate in the human gut. However, low viability of probiotic bacteria could happen due to several factors, namely pH, temperature, and oxygen (Ding and Shah, 2008).

Survival of probiotics is considerably affected by pH and titratable acidity of the products during storage as well as in gastrointestinal tracts (Pimentel *et al.*, 2015). A very low pH value increases the concentration of undissociated organic acids, enhancing the bactericidal effects of these acids. It was found that *L. acidophilus* were unable to recover cells of after exposure to a pH of 2.0 for 45 min, while no significant reduction in the number of cells was observed even after 2 h exposure at a pH of 4.0 (Tuorila and Cardello, 2002). Similar trends were found in survival of *L. rhamnosus* GG in stimulated gastric juices at pH values between 1.0 and 7.0. The optimum range of pH for growth of *Lactobacillus* spp. and Bifidobacteria is in the range of 5.5–6.0 and 6.0– 7.0, respectively (Zyrek *et al*., 2007). Lactobacilli are capable of growing and surviving in fermented products with pH values between 3.7 and 4.3 (Mortazavian *et al*., 2007). Bifidobacteria species are reported to be less acid tolerant, and a pH level below 4.6 is detrimental to their survival (Hood and Zottola, 1988).

Temperature is one of the crucial factors affecting probiotic viability during formulating, processing and storage (Park *et al*., 2016). Probiotic products should preferably be stored at low temperatures of 4–5 °C and depend on types of the strains (Vijaya Kumar *et al*., 2015). Highest viability of *L. acidophilus* LA-5 was observed for up to 20 days when stored at 2 °C, whereas for *Bifidobacterium lactis* BB-12, the optimum storage temperature was 8 °C (Vijaya Kumar *et al*., 2015). However, for long-term storage freeze drying of probiotics was recommended. a much lower storage temperature of −18 °C could maximize viability of certain probiotic strains such as Bifidobacteria. Storage temperatures of 15-25 °C could result in significant reductions in viable cell counts in the dried products. In addition, the low decrease in probiotic viability in sugar-containing products during storage was found when they were kept at high temperatures and/or relative humidity. This could be related to their glass transition temperatures (Vijaya Kumar

et al., 2015). Sugars could form high viscous glasses at room temperature when they are dehydrated, and the presence of a glassy state improves storage life of anhydrobiotes.

Besides, oxygen contents and redox potential are also the important factors affecting the viability of probiotics especially during the storage period (Hood and Zottola, 1988). Molecular oxygen is harmful to probiotic survival and growth, as most of the species are strictly anaerobic and saccharoclastic (Mortazavian *et al*., 2007). Oxygen affects probiotics in three ways as this following: it is directly toxic to some cells, certain cultures produce toxic peroxides in the presence of oxygen, and free radicals produced from the oxidation of components (e.g., fats) are toxic to probiotic cells (Tuorila and Cardello, 2002). Therefore, it has been recommended that the levels of oxygen within the package during storage of probiotic products should be as low as possible in order to avoid toxicity and death of the microorganism and the consequent loss of functionality of the product. The degree of oxygen sensitivity varies considerably among different species and strains of probiotics (Tamime, 2008). In general, Lactobacilli are more tolerant to oxygen than Bifidobacteria as Bifidobacteria are anaerobic naturally. To minimize effects of oxygen, vacuum packaging, using packaging materials with low oxygen permeability, adding antioxidants and oxygen scavengers to the product, and controlling the production process in such a way that minimum dissolved oxygen entered into product are suggested (ger and Santivarangkna, 2015).

3.3 Prebiotics

Prebiotic was originally defined as “A non-digestible foods ingredients that beneficially affects the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon, and thus improves host health” (Charalampopoulos and Rastall, 2009). In the past decade a large number of studies have demonstrated that prebiotics have great potential as agents to improve or maintain a balanced intestinal microbiota to enhance health and wellbeing. The European market for health promoting prebiotics is growing rapidly (FAO and WHO, 2001). Many food oligosaccharides and polysaccharides (including dietary fiber) have been claimed to have prebiotic activity, but not all dietary carbohydrates are prebiotics (ger and Santivarangkna, 2015). In recent years, the use of prebiotics in fish aquacultures has been great interest (Fu and Chen, 2011). Various mechanisms have been proposed to explain their specific action, such as selective stimulation of beneficial microbiota, and improvement of immune functions, fish health, and productivity

1) Selective stimulation of beneficial microbiota

Among the group of beneficial bacteria present in the gastrointestinal (GI) tract are those that most utilize prebiotic oligosaccharides being considered as the only microorganisms able to beneficially affect the host's health. Numerous human and animal feeding studies have shown that they selectively stimulate one or a limited number of beneficial bacteria thus causing a selective modification of the host's intestinal microbiota. (Ma *et al*., 2008). Many prebiotics such as fructo oligosaccharides (FOS) and mannan-oligosaccarides (MOS) have been investigated for nutritional manipulation of the GI ecosystem of humans and animals, because they facilitate and support the symbiotic relationship between host and its microbiota (Urbanska *et al*., 2007). FOS and MOS are two classes of prebiotic oligosaccharides that are beneficial to enteric health, but they do so by different means. For example, dietary supplementation of prebiotic oligosaccharides has been shown to provide a nutrient source for beneficial bacteria and may promote the maintenance of bifidobacteria and certain lactic acid bacteria in the gut of humans and animals (Dave and Shah, 1997). FOS influence enteric microflora by 'feeding the good bacteria', which competitively excludes the colonization of pathogens and thus improving animal health and growth performance (Talwalkar and Kailasapathy, 2004).

2) Improvement of immune functions

Prebiotics have been demonstrated to possess the capability of modulating the fish immune system either by direct or indirect mechanisms. The direct immunostimulatory properties of prebiotics may be explained when the prebiotic ligands interact with their associated receptors such as b-glucan and dectin-1 receptors present in macrophages (Sultana et al., 2000). A down-stream process could occur when the ligand-receptor interaction activates signal transduction molecules such as NF-kB, which stimulate a number of immune cells (Cui *et al*., 2012). In another perspective, immune responses could be triggered when the saccharides interact with receptors in the form of microbe associated molecular patterns (MAMPs) such as teichoic acid, peptidoglycan glycosylated protein, or the capsular polysaccharide of bacteria (Soukoulis and Fisk, 2016). Prebiotics have been demonstrated to predominantly influence lysozyme, phagocytic, and respiratory burst activities in fish.

3) Effects on fish health and productivity

Evaluation of prebiotics as a feed additive and their effects on animal production has been developed. Inulin has been shown to have beneficial effects on growth and health status in mammals (Gani *et al*., 2019). However, little is known about its effects on fish (Wang *et al.*, 2015). It was reported dietary supplementation with inulin had a positive effect on

growth responses in various fish species, including Nile tilapia, Siberian sturgeon (*Acipenser baerii*), and rainbow trout (*Oncorhynchus mykiss*) (Shabbir *et al.*, 2011). However, dietary supplementation with inulin did not affect the grow the response in weaning turbot (*Psetta maxima*), Atlantic salmon (*Salmo salar*), hybrid striped bass (*Morone chrysops* x *Moronesaxatilis*) (Chotiko and Sathivel, 2016). Moreover, dietary FOS supplementation led to increase survival rate of common carp fry although it did not significantly improve growth performance (Urbanska *et al*., 2007). Comparative study on prebiotic effects *in vitro* between inulin and FOS revealed that they influenced different microbial community and proteolytic (Kaplan and Hutkins, 2000). Dietary supplementation with either inulin or FOS had similar effects on growth performance in rainbow trout [157] whereas supplementation with FOS had a more positive effect than inulin on the growth rate of turbot larvae (Heidebach *et al*., 2009).

3.4 Kantawan (Jerusalem artichoke)

Kantawan (Jerusalem artichoke; JA) is a plant native to North America. JA (Helianthus tuberosus) is a perennial plant which consists of a stem about 1–3m tall, small yellow flowers, hairy oval shaped leaves and an underground rhizome system which bears small tubers. It is an angiosperm plant species of the composite family, which is commonly referred to as the sunflower or daisy family (Alander *et al*., 2001). The stems are stout and ridged which can become woody overtime. Its leaves alternate near the top of the stem, the lower leaves are larger and broader, and can grow up to 30 cm long while the higher ones are smaller and narrower. In terms of flower heads, each is 5–7.5cm wide and formed by small, yellow, tubular disk flowers in the center and surrounded by florets, which occur separately or in groups at the end of alar branches and main stems. As for tubers, they are uneven and elongate varying from knobby to round clusters. The colors of tubers range from pale brown to white, red and purple [163]. JA has a number of advantageous characteristics over traditionally agricultural crops, including high growth rate, good tolerance to frost, drought and poor soil, strong resistance to pests and plant diseases, with minimal to zero fertilizer requirements (Alander *et al*., 2001). Conventionally, JA has been used for food or animal feed (Macfarlane, 2008), and for the past two decades, alternative uses have been explored especially for the production of functional food ingredients including inulin and fructose (Saavedra and Tschernia, 2002). JA contained high proportion of shorter fructan comparing to inulin from chicory. While high proportion (43–52%) of furctan in JA were short chain fructan (<9 degree of polymerization (dp)), 64–71% of fructan in inulin were medium chain fructan (10–40 dp) [154].

Inulin is a polysaccharide, similar to starch, and exists as a white powder with neutral taste. Chemically, it is a linear biopolymer of D-fructose units connected by glycosidic linkages, and terminated with one D-glucose molecule linked to the fructose chain (Sangwan *et al*., 2014). The degree of polymerization of inulin generally ranges from 2 to 60. To date, inulin has been increasingly used as functional ingredients in processed foods and feeds due to its unique characteristics (Playne and Crittenden, 2009). Inulin cannot be digested by human intestinal enzymes. When orally ingested, inulin passes through the mouth, stomach and small intestine without being metabolized, until it enters into the large intestine where it becomes fermented by the colonic microflora. Inulin as a prebiotic simulates the growth of existing strains of beneficial bacteria in the colon which enhances the absorption of important mineral components like calcium and magnesium as well as the synthesis of B vitamins (Sultana *et al*., 2000). In addition, it has been shown to exert a protective effect toward *L. acidophilus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *L. rhamnosus*, *Lactobacillus plantarum* and *Bifidobacterium* spp. (Böhm *et al*., 2005) by improving their survival and activity during storage. It was also found that the incorporation of inulin in a diet reduced the lipid content of blood and liver in saturated fat-fed rats. Recent studies observed that inulin played an important role in the prevention and inhibition of colorectal, colon and breast cancers (Zuidam and Nedović, 2013). However, it was reported that JA had positive effects on growth performance in Nile tilapia that were comparable to those of inulin. The growth performances (including final weight, specific growth rate, and Feed conversion ratio) of Nile tilapia fed the JA diets were superior to those of fish fed the inulin diets. Moreover, JA also contains various minerals and vitamins including iron, calcium, potassium, vitamin B complex, vitamin C, and vitamin A (Akalin and Erişir, 2008). These micronutrients in JA also may have had addition al positive effects on growth response and feed utilization in the Nile tilapia.

3.5 Encapsulation techniques

Encapsulation is a process to entrap an active substance within wall materials. The encapsulated substance can be called the core, fill, active, internal or payload phase, while the wall material that is encapsulating is often called the coating, membrane, shell, capsule, carrier material, external phase, or matrix (Rao *et al*., 1989). In recent years, several functional compounds such as antioxidants, vitamins, phytosterols, and probiotics are added into food and feed products. They are usually highly susceptible to environmental processing and/or gastrointestinal conditions. Therefore, encapsulation has imposed an approach for effective protection (Gbassi and Vandamme, 2012). This technology provides barriers between sensitive

bioactive agents and the environment. In addition, the techniques have been applied to modify physical characteristics of the original materials to allow easier handling, help separate the components of the mixture that would otherwise react with one another, and to provide an adequate concentration and uniform dispersion of an active agent (Rao *et al*., 1989). Many encapsulation technologies, such as emulsion technology, extrusion method and spray drying technology, have been reported (Voo *et al.*, 2011).

1) Emulsion techniques

The emulsion method is commonly used for encapsulation of microbial cells. This technique involves dispersion of the cells mixed in a polymer solution (dispersed phase) in an oil (continuous phase). The mixture forms a water-in-oil (W/O) emulsion with the aid of surfactant and stirring followed by the step in which the phases are separated and the dispersed phase encapsulates the probiotic microorganism as core material (Ma *et al*., 2008). Ingredients such as milk proteins or caseinate as well as alginate have mostly been used in the emulsion method. The main advantages of this technique are large particle size ranging from 0.2 to 5000 mm and upscale easy [184], achieved by controlling the stirring speed and water/oil ratio [185]. Encapsulation using the emulsion technique enhances protection of *L. plantarum* DPC206, *L. reuteri* DPC16, *Pediococcus acidilactici* DPC209 and *Bifidobacterium lactis* HN019 from gastrointestinal tract conditions and during storage (Charalampopoulos and Rastall, 2009).

2) Extrusion techniques

The extrusion technique is used to convert hydrocolloids into capsules. The technique consists of a core material completely mixed with the wall material which is projected into a nozzle in a hardening solution (Tamime, 2008). The droplet formation occurs in a controlled manner, which is done by pulsation of the jet or vibration of the nozzle (Zuidam and Nedovic, 2009). The main ingredients/materials used for this technique are alginate, carrageenan and pectin, which are able to form gels in presence of minerals such as, calcium and potassium and have been used successfully to entrap probiotic microorganisms inside the capsule. Gel is formed by the bonding of multiple free carboxylic radicals by gelling ions (ger and Santivarangkna, 2015). Also, the beads with probiotic microorganisms made by means of the extrusion technique can be mixed at the same time with different ingredients such as starch, milk protein and coated with chitosan to enhance the stability of the capsules throughout the process and better protect the probiotic in the gastrointestinal tract (ger and Santivarangkna, 2015). The extrusion technique is simple and gentle and does not harm probiotic cells. It does not require high temperatures and the use of organic solvents, which contribute to high probiotic viability. Moreover, it is cheap. All

conditions can be controlled, and it works well in either aerobic or anaerobic condition. Mass production of beads can either be achieved by multi-nozzle system or using a rotating disc. However, their use in large-scale production to date is limited due to slow formation of beads, technological advances are overcoming this disadvantage. These extrusion capsules have been added in different foods, mainly dairy products, especially cheese, yogurt drink and fermented milks (Doherty et al., 2012). This type of technique has also been tested in fermented meat products (Doherty *et al.*, 2012).

3) Spray drying

Spray drying is defined as the transformation of a fluid from a liquid state into a dried particulate form by spraying the fluid into a hot drying medium (Anandharamakrishnan and S, 2015). It is a suitable one-step process for the conversion of various liquid formulations into dry powders. The spray drying process consists of three fundamental steps as following; atomization of the liquid feed, drying of the sprayed droplets in the drying gas and formation of dry particles, and separation and collection of the dry product from the drying gas. Spray drying is a simple, fast, and scalable drying technology (Arpagaus *et al*., 2017). The equipment is commercially available and the production cost is lower compared to that of other drying technologies, such as freeze drying (Gharsallaoui *et al*., 2007). The powders produced are high in quality and have low moisture content, resulting in high shelf stability (Anandharamakrishnan and S, 2015). Spray drying is expected to be an alternative method for production of probiotic powders at an industrial scale. However, the harsh spray drying conditions, in particular high temperature exposure at the last stage of drying, limit the applicability of spray drying in probiotic production (Fu and Chen, 2011). Therefore, using a protective matrix as a drying medium represents has been used as an strategy to protect bacteria from spray drying (De Castro-Cislaghi *et al*., 2012).

Skim milk is regarded as a kind of material and proved to have remarkable effects on probiotic viability during dehydration (Manne and Guggenbuhl, 2000). There has been an increasing number of reports on the application of skim milk in the protection of probiotic cells in recent years, relating to roles in protection both while drying is occurring as well as during exposure to gastrointestinal or bile fluids (Judprasong *et al*., 2011). There have been a few reports specifically on the application of skim milk for the protection of probiotics during spray drying. These have demonstrated good survival; however, these studies utilized either whey skim milk in combination with carbohydrate or liquid whey which also contains lactose (Nyman, 2002). For example, skim milk coated inulin-sodium alginate (ISA) encapsulation beads improved the survival of *L. plantarum* during exposure to adverse environment. The viability of *L. plantarum* from ISA encapsulation beads did not change after 2 h incubation in simulant gastric fluid (SGF). In 1% bile

salt solution, the *L. plantarum* from ISA encapsulation beads only had 1.21 log CFU/mL reduction after 2 h treatment. After 7 weeks storage, the survival rate of *L. plantarum* was 20.89%, which showed reduction from 8.52 to 7.84 log CFU/mL (Playne and Crittenden, 2009).

3.6 Related research

The effects of dietary inulin and Jerusalem artichoke (JA) on intestinal microbiota and morphometry of Nile tilapia fingerlings were investigated. Five treatment diets were designed to supplement inulin at 0 (basal diet), 2.5 and 5.0 g/kg, and JA at 5.0 and 10.0 g/kg. The result showed that the fish fed with inulin and JA had different microbial community profiles compared to the basal diet group. The number of bacteria and *Bifidobacterium* spp were increased in the fish fed with dietary inulin at 5.0 g/kg and JA (at both levels) while the number of *Vibrio* spp was reduced. The inulin (5.0 g/kg) and JA at both levels improved intestinal villi height of the fish as well as A goblet cell number. The results indicated that dietary inulin (5.0 g/kg) and JA (5 and 10.0 g/kg) since the first feeding positively affected the intestinal microbiota and morphology of Nile tilapia fingerlings (Boonanuntanasarn *et al*., 2018).

The application of microencapsulation technique by alginate extrusion on a human-derived probiotic, Lactobacillus rhamnosus GG (LGG) and determination of the cell viability and morphology of the microcapsules while in transit through the tilapia gastrointestinal tract were studied. The *in vitro* results showed that the microencapsulated probiotics in alginate matrix and skim milk–alginate matrix under simulated had higher viability under gastric conditions and in tilapia bile than that of the free probiotics. The cell viability of both the alginate matrix and skim milk–alginate matrix microencapsulated probiotics was maintained after storage at room temperature for 14 days, while viable free LGG could not be detected after only 7 days. The results from the *in vivo* study revealed that the overall intestinal structure, growth performance and significant protection against *Streptococcus agalactiae* challenge of the fish were improved when they were fed with microencapsulated probiotic. These results suggested the potential application of alginate-microencapsulated LGG in tilapias (Pirarat *et al*., 2011).

Modulation of intestinal morphology and immunity in Nile tilapia by *L. rhamnosus* GG (LGG) were evaluated. The results suggested that LGG could promote the intestinal structure and the mucosal immunity of tilapia. The probiotic could increase the fish villous height in all parts of the intestines and enhanced the population of intraepithelial lymphocytes. The population of acidophilic granulocyte in the fish fed with LGG was significantly greater at the proximal and distal parts when compared with the control group. It was found that LGG could serve as an important regulator of gut associated immune systems indicated by the increase of serum complement activity as well as the phagocytosis and killing ability of the head kidney leukocytes in the probiotic supplemented fish (Nopadon *et al.*, 2015).

4. Materials and methods

4.1 Effects of Jerusalem artichoke on viability of *Lactobacillus rhamnosus* GG in alginate capsules after freezing, freeze drying, and in simulated Nile tilapia gastrointestinal conditions

4.1.1 Preparation of probiotics

Frozen cultures of *L. rhamnosus* GG (LGG) in 20% glycerol was reactivated into de Man Rogosa Sharpe (MRS) broth (BD Difco™, Maryland, USA) and incubated (BE 500, Schwa Bach, Germany) at 37 °C for 24 h. The bacteria (5%) was then subsequently inoculated into MRS broth and incubated at 37 °C until it reaches to stationary phase. The cells were harvested by centrifugation at 8000 rpm, for 10 min at 4 °C (Sigma 2-16PK, Sartorius, Germany). Afterwards, the cell pellets were dispersed into saline solution and used for encapsulation.

4.1.2 Encapsulated alginate capsule preparation using gelation-extrusion method

The extrusion technique of encapsulation was modified from Krasaekoopt and Watcharapoka (2014). The cell pellets were resuspended in 10%w/v of skim milk (SM) mixed with 1.8%w/v food grade alginate and JA powders at different concentrations (10 g/100mL, 15 g/100 mL, and 20 g/100 mL). JA powders were obtained from Phetchabun Research Station, Agro-Ecological System Research and Development Institute, Kasetsart University, Thailand. JA was composed of 28 % of sugar, 39.09 % of inulin, 15.8 % of FOS (Sarote and Warangkana, 2011). The sample was mixed until it was homogenous. After that, the mixture was dropped into 0.1 M Calcium chloride ($CaCl_2$) (Univar®, Ajax Finechem, Australia) solution and kept hardening in the solution for additional 30 min. The $CaCl_2$ was decanted to collect the alginate capsules. After encapsulation, the capsules were determined for their encapsulation efficiency (%) = $(N/N_0) \times 100$, where N and N_0 were the number of viable cells encapsulated in alginate capsules after alginate encapsulation and the number of viable cells before encapsulation, respectively. The capsules were then placed in glass chamber and frozen in alcohol bath at -86 °C. All samples were subsequently dried in a freeze dryer (FDU-8624, Operon, Korea) for 48 h and stored in sealed aluminum bags at 4 °C. After freezing and freeze drying, the cell viability was measured. The un-encapsulated cells and only alginate encapsulated LGG were used as controls.

4.1.3 Determination of LGG viability in wet and dried AG-capsules under simulated gastrointestinal (GI) tract of Nile tilapia

1) Viability of freeze dried alginate capsules in simulated gastric conditions

Simulated gastric fluids (SGF) of Nile tilapia were prepared using normal saline solution, adjusted pH to 1.5, 2.0, and 3.0 (adjust pH by using 5 M hydrochloric solution (Lab scan, Analytical, Thailand)). Exactly 0.5 mL of free cells and 0.5 g of freeze dried alginate capsules were placed into separated test tubes containing 4.5 mL of the simulated gastric solutions. Triplicate samples were taken after incubation (JSGI-250J, Schwa Bach, Germany) at 25 °C in a water bath for 0, 1, and 2 h. The capsules were then disintegrated by soaking in 1 M phosphate buffer pH 7.0.and subsequently placing in a stomacher at 200 rpm for 3 min.

2) Viability of freeze dried alginate capsules in Nile tilapia bile salt solutions

Bile tolerance, 0.5 mL of un-encapsulated cells and 0.5 g of freeze dried capsules were added into 4.5 mL of SGF at pH 2.0 and incubated at 25 °C (BE 500, Schwa Bach, Germany) for 60 min. After the incubation, the SGF was removed and replaced with 4.5 mL of 3%w/v, 5%w/v, and 10%w/v tilapia bile salt. Triplicate samples were taken after further incubations of 1, 2, and 3 h at 25 °C. The capsules were then disintegrated as mentioned above. The cell counts of LGG were enumerated on MRS agars by using a pour-plate method. Un-encapsulated cells were used as a control.

4.2 Effects of Jerusalem artichoke on viability of *Lactobacillus rhamnosus* GG after spray drying

4.2.1 Spray drying of *L.rhamnosus* GG

Jerusalem artichoke (JA) was kindly provided by Phetchabun Research Station, Agro-Ecological System Research and Development Institute, Kasetsart University, Thailand. JA was composed of 28 g/100 mL of sugar, 39 g/100 mL of inulin and 16 g/ 100 mL of fructo-oligosaccharide (Sarote and Warangkana, 2011). Five feed solutions were prepared by resuspending LGG, prepared as described in 4.1.4, in these following solutions, alginate solution (18 g/L), alginate solution (18 g/L) with skim milk (200 g/L), and alginate solutions (18 g/L) with skim milk (200 g/L) and JA at different concentrations (100 g/L, 150 g/L, and 200 g/L). The solutions were homogenized prior to spray dry. Each feed solution was fed into in a laboratory scale spray dryer (Buchi B-290, Buchi, Germany) at inlet temperature of 125 °C and outlet temperature of 65 °C with the flow rate of 300L/h) to obtain LGG powder including LGG powder with only alginate (AL), used as a control, LGG powder with alginate and skim milk (AS), LGG powder with alginate,

skim milk, and JA at 100, 150, and 200 g/L (ASJ10, ASJ15, and ASJ20, respectively). AL and AS were used as a control. After spray drying, the number of viable LGG in the powders were enumerated as well as under simulated gastrointestinal tract and the cell viability (%) was calculated by the following equation. Cell viability (%) = $(N/N_0) \times 100$, where N and N_0 were the number of viable LGG in the spray dried powders (log CFU/g) and the number of viable cells before spray drying (log CFU/g), respectively.

4.2.2 Determination of LGG viability after drying processes and under simulated Nile tilapia gastrointestinal tract

1) After spray drying

One gram of LGG powder, namely AL, AS, ASJ10, ASJ15, and ASJ20, were resuspended in 0.85g/100 mL of normal saline solution (9 mL). The serial dilution was then conducted. A pour-plate method was used to enumerate the cell number of LGG powder on MRS agars. The samples were then incubated at 37 °C for 48 h.

2) *In vitro* simulated gastric conditions and bile salt solutions

Simulated gastric fluids (SGF) of Nile tilapia were prepared using normal saline solution, adjusted pH to 1.5, 2.0, and 3.0 by using 5 M hydrochloric solution (Lab scan, Analytical, Thailand). Exactly 0.5 mL of LGG fresh cells, cultured in MRS broth for 24 h at 37 °C were used as a control, and 0.5 g of LGG powders (AL, AS, ASJ10, ASJ15, and ASJ20) were placed into separated test tubes containing 4.5 mL of SGF. One mL of each sample was taken after incubation (JSGI-250J, Schwa Bach, Germany) at 25 °C in a water bath for 0, 1, and 2 h and enumerated for the cell counts on MRS agars by using a pour-plate method.

For bile tolerance determination, 0.5 mL of LGG fresh cells as control and 0.5 g of LGG powders (AL, AS, ASJ10, ASJ15, and ASJ20) were added into 4.5 mL of SGF at pH 2.0 and incubated at 25 °C for 60 min. After the incubation, the samples were then centrifuged. SGF was removed and separately replaced with 4.5 mL of 3 mL/10 mL, 5 mL/100 mL, and 10 mL/100 mL of tilapia bile salt. One mL of each sample was taken after further incubations of 1, 2, and 3 h at 25 °C and enumerated on MRS agars by using a pour-plate method to determine for the number of viable cells.

4.3 Development of Nile Tilapia Pelleted Feed Containing *Lactobacillus rhamnosus* GG and Jerusalem Articoke

4.3.1 Production of Nile tilapias pelleted feed containing LGG

To prepare the pellets, dry feed ingredients, including fish meal (300 g/kg of total weight), soybean meal (270 g/kg of total weight), rice bran (150 g/kg of total weight), corn meal (145 g/kg of total weight), cassava chips (120 g/kg of total weight), and premix (100 g/kg of total weight) were ground, sterilized at 121°C for 15 min, and dried in a hot-air oven (Contherm, Thermotec 2000, Germany) at 55°C until their moisture contents reached 12-13%. Dry feed ingredients were then mixed with starch binding agent (30 g/kg of total feed ingredients), coconut meal as a floating aid (200 g/kg of total feed ingredients) and the selected LGG powder (from 5.1.2) (100 g/kg of feed ingredients), having the highest cell viability under stimulated gastrointestinal tract. Tap water (300 g/kg of total feed ingredients) was then added to obtain a feed mash. The mash was conveyed to a single screw extruder and proceeded at room temperature to obtain pelleted feed containing LGG (PFL). They were then dried at 50 °C for 8 h. PFL was evaluated for proximate analysis and cell viability after drying and under simulated gastrointestinal tract.

4.3.2 Proximate analysis of Nile tilapia pelleted feed containing LGG

PFL samples were analyzed for their nutritional compositions. Moisture and protein contents were analyzed by using standard methods of ISO 6496:1999 and ISO 5983-2:2005, respectively. Ash and total fat were determined by following the methods of AOAC (2016) 942.05 and AOAC (2016) 954.02, respectively. The total carbohydrate was calculated by the following equation; Total carbohydrate content (%) = 100-(moisture content + protein content + total fat content + ash content).

4.3.3 Determination of LGG viability in pelleted feeds after drying processes and under simulated Nile tilapia gastrointestinal tract

1) After drying

Ten gram of PFL were ground in a stomarcher in 0.85g/100 mL of normal saline solution (90 mL) for 2 min. The serial dilution was then conducted. A pour-plate method was used to enumerate the cell number of LGG powder on MRS agars. The samples were then incubated at 37 ^{o}C for 48 h. LGG fresh cells and spray dried ASJ20 powders were used as controls.

2) In vitro simulated gastric conditions and bile salt solutions

Simulated gastric fluids (SGF) of Nile tilapia were prepared using normal saline solution, adjusted pH to 2.0 by using 5 M hydrochloric solution (Lab scan, Analytical, Thailand). PFL (1 g) were placed into separated bottoms containing 9 mL of SGF at 2.0. One mL of each sample was taken after incubation (JSGI-250J, Schwa Bach, Germany) at 25 °C in a water bath for 1. The viable cells were enumerated on MRS agars by using a pour-plate method. After that the sample was transferred to tilapia bile salt solution (10 mL/100 mL) and incubated for 3 h at 25 °C. The viable cells were enumerated on MRS agars by using a pour-plate method. LGG fresh cells and spray dried ASJ20 powders were used as controls.

4.4 Effects of Nile Tilapia pelleted feed containing *Lactobacillus rhamnosus* GG and Jerusalem Articoke on growth performance, blood chemistry and intestines of Nile tilapia

4.4.1 Fish, dietary supplementation and *in vivo* experimental designs

Three hundred tilapias (*Oreochromis niloticus*) with 20-30 g body weight were acclimatized for 7 days and randomly placed in 90-L tanks (30 fish per tank) for the control groups and the dietary supplementation groups. The tanks were filled with recycled water which was controlled for temperature (25-28 °C), dissolved oxygen (5.8–6.8 ppm), and pH (6.5–7.0) throughout the experiment. Five experimental groups was established as below. The fish was fed approximately 5% of body weight twice a day (Nopadon *et al*., 2015).

(1) Fish fed with tilapia feed pellets (control group) 60 fish
(2) Fish fed with cell free encapsulated capsules (AL+SM) 60 fish
(3) Fish fed with tilapia feed pellets incorporated with LGG (LGG) 60 fish
(4) Fish fed with tilapia feed pellets with JA (JA) 60 fish
(5) Fish fed with encapsulated synbiotic tilapia feed pellets (PFL) 60 fish

4.4.2 Growth performance determination

The fish was weighed at day 0 and 30 of feeding and calculated for weight gain, specific growth rate and feed conversion ratio. The equations are described below. By according to the following equations (Yanbo and Zirong, 2006).

$$\text{Weight gain (\%)} = \frac{(\text{final mean body weight} - \text{initial mean body weight})}{\text{initial mean body weight}} \times 100$$

$$\text{Specific growth rate (SGR)} = \frac{\ln(\text{final body weight}) - \ln(\text{initial body weight})}{\text{experimental days}} \times 100$$

$$\text{feed conversion ratio} = \frac{\text{feed intake (g)}}{\text{weight gain (g)}}$$

4.2.3 Blood chemistry analysis

At the end of the experimental period, fish was not fed for 18 h prior to blood collection. Seven fish from each group was removed from the tank and anesthetized with 0.2% of 2-phenoxyethanol. Blood samples were collected from the caudal vein using a hypodermic syringe and analyzed for glucose, triglyceride, cholesterol, total protein, glucose, triglyceride, cholesterol, total protein, albumin, blood urea nitrogen (BUN), total bilirubin, direct bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, calcium, chloride, magnesium, and iron.

4.4.4 Measurement of villous height

To carry out intestinal histopathology, intestinal samples were fixed in 10% buffered formalin. The fixed tissues were embedded into paraffin blocks according to standard histological techniques and the 4 μm tissue sections were stained with hematoxylin and eosin. The villous height was measured from the villous tip to the bottom of the 10 tallest villi per section. An average of these10 villi per section was expressed as the mean villous height for each section (Samanya and Yamauchi, 2002).

4.5 Statistical analysis

All values were means and standard deviations of determinations. Means values from statistical analysis was conducted with SPSS Statistics software (version 24). One-Way ANOVA and Duncan test were carried out to determine differences all treatments at the significance level of $P \leq 0.05$.

5 Results and Discussion

5.1 Effects of Jerusalem artichoke on viability of Lactobacillus rhamnosus GG in alginate capsules after freezing, freeze drying, and in simulated Nile tilapia gastrointestinal conditions

5.1.1 Encapsulation efficiency of alginate encapsulated capsules containing LGG

The result indicated that all treatments had less than one log reduction in cell viability after encapsulation, which were 0.53, 0.31 0.42, 0.45, and 0.42 log reduction, respectively. This suggested that the extrusion technique was suitable for encapsulation of LGG. According to Krasaekoopt *et al*. (2003) this technique is simple and gentle. It does not harm probiotic cells and does not require high temperatures and the use of organic solvents. Moreover, Gombotz *et al*. (1998) reported that alginate was categorized as a nanoporous polymer having pore size between 5 and 200 nm. As well known, bacteria size is about 0.2 μm in diameter and 2-8 μm in length were entrapped inside of the capsules. The %EE of all treatments was between 94.62±0.09% and 96.79±0.03% (Figure 1). The highest %EE was found in AS, while JA caused slight reduction of %EE. However, it was not significantly different. This was probably due to densely packed structure of added JA, causing partly block the cell entrapment (Idouraine *et al.,* 1996).

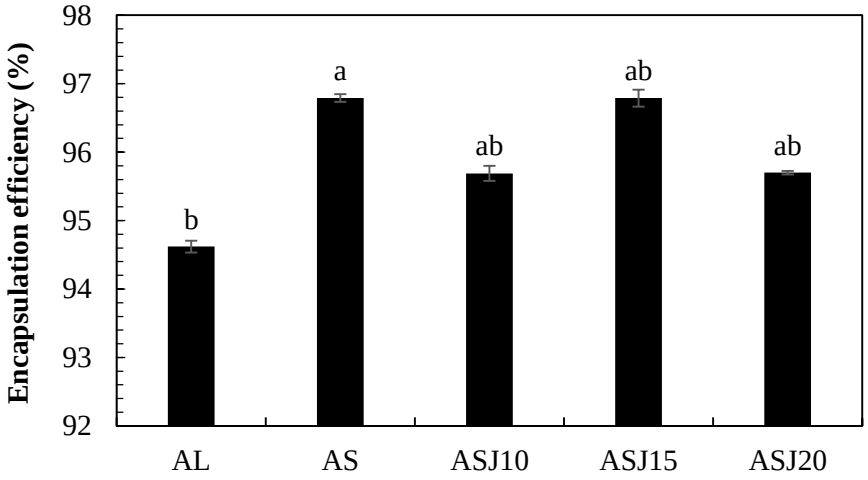

Figure 1. Encapsulation efficiency of alginate encapsulated capsules containing LGG; AL=Alginate, AS=Alginate+Skim milk, ASJ10, ASJ15, and ASJ20= Alginate+Skim milk+10%, 15%, and 20% Jerusalem artichoke, respectively. [a-b]Means±standard deviation with same letters were not significantly different (P > 0.05)

5.1.2 Effects of freezing and freeze drying on viability of LGG

For long-term storage, probiotics are usually preserved by freeze drying. In this study, the alginate capsules were freeze-dried. As shown in Figure 2, after freezing**,** the viability of LGG in un-encapsulated cells, AL and AS was reduced by 2.46, 2.05, and 1.46 log CFU/mL, respectively. Addition of JA could significantly increase the number of viable cells after freezing. However, the concentrations of JA did not significantly affect the cell viability. The number of viable cells in ASJ10, ASJ15, and ASJ20 was reduced by 1.07, 1.01, and 1.00 log CFU/mL, respectively. According to, Meng *et al*. (2008) the loss of cell viability after freezing was mainly caused by extracellular ice formation. Alginate encapsulation functioned as a physical barrier protecting the cells from the freezing process. Skim milk offered a surface for probiotic adherence, helping the cells avoid effects of ice crystallization and extracellular osmolality (Ding and Shah, 2008).

In addition, it was reported that JA contained large amount of inulin, which were 16-20% inulin with both low and high degree of polymerization (DP) (Gagga *et al*., 2011). Lingyun *et al*. (2007) mentioned that Low DP inulin in JA could penetrate into the cell membrane and acted as a buffer layer against ice formation when the beads encapsulated cells were frozen. Concurrently, high DP inulin adsorbed on the surface of cells and formed viscous layers, helping lower the rate of ice growth by increasing the solution viscosity (Judprasong *et al*., 2011). This would support our finding that the capsules with JA had better number of viable cells after freezing when compared to AS and controls.

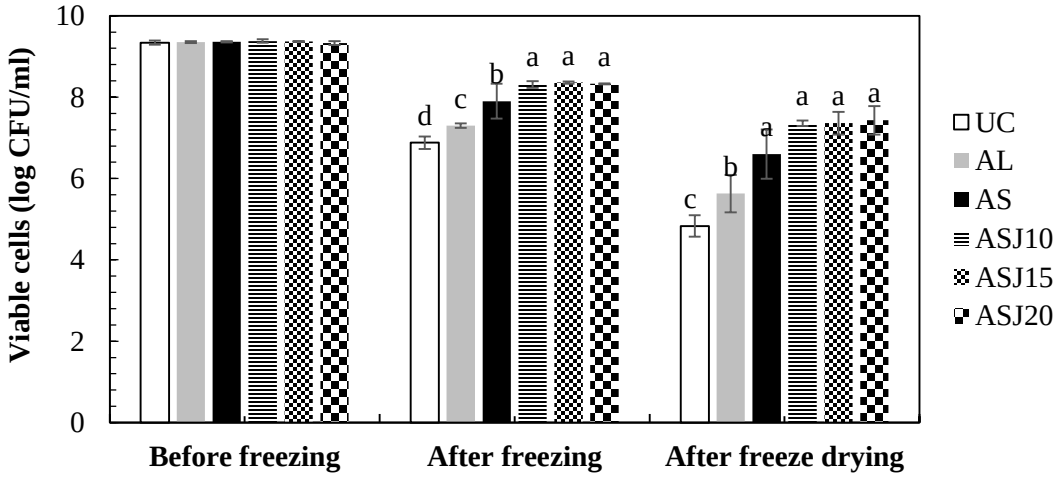

Figure 2. Viability of LGG in alginate encapsulated capsules before and after freezing and after freeze drying; AL = Alginate, AS = Alginate+ Skim milk, ASJ10, ASJ15, and ASJ20 = Alginate + Skim milk + 10%, 15%, and 20% Jerusalem artichoke, respectively. $^{a-d}$Means±standard deviation with same letters were not significant differences (P>0.05).

After freeze drying**,** the viability of LGG was continuously reduced. Our result suggested that AL had significant higher the number of viable cells than un-encapsulated cells. Removal of water from bacteria cells led to cell membrane transition and leakage (Doherty *et al*., 2011). The number of cell reduction in AL was 3.72 log cycles, while the fresh cell had 4.51 log reduction. Similarly, to the result of cell viability after freezing, skim milk combined with JA could increase the cell survival after freeze drying. The number of viable cells in AS was reduced by 2.76 log reduction. ASJ10, ASJ15, and ASJ20 had cell reduction at 2.02, 2.01, and 1.9 log cycles, respectively. However, they were not significantly different. The protein in skim milk can accumulate within the cells, resulting in reduction of the osmotic difference between the internal and external environments (Mille *et al*., 2004). Combination of JA and skim milk helped improve cell protection from freeze drying damage more effectively than only skim milk. Sugars and some polysaccharides can function as a water replacer (Doherty *et al*., 2011), helping stabilize the cell membrane during dehydration.

5.1.3 Viability of freeze dried alginate encapsulated LGG in simulated gastric conditions

The stomach is a crucial section for pH-sensitive components such as probiotic cells. In the fasted state, the stomach of healthy subjects has a range of pH from 1.3 to 2.5 (Nemeth *et al*., 2014). Figure 3, showed the number of viable cells in freeze dried capsules after exposure to SGF. The viability of encapsulated LGG at pH 3.0 and 2.0 significantly less decreased than that at pH 1.5.

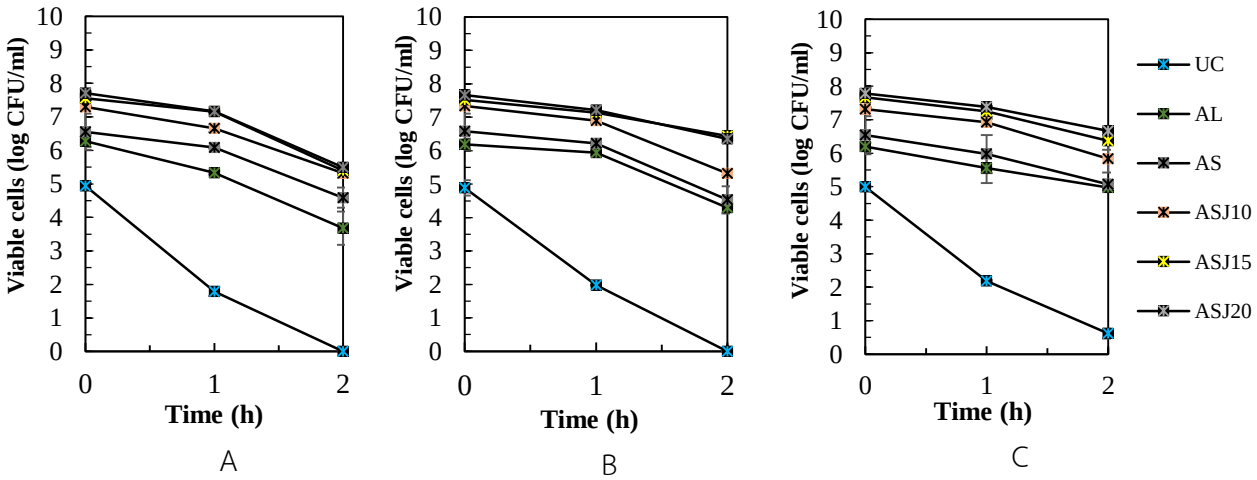

Figure 3. Viability of alginate encapsulated LGG (dried capsule) in low pH conditions (pH 1.5 (A), pH 2.0 (B), and pH 3.0 (C)); UC= Un-encapsulated cells, AL=Alginate, AS=Alginate+Skim milk, ASJ10, ASJ15, and ASJ20= Alginate+Skim milk+10%, 15%, and 20% Jerusalem artichoke, respectively.

This showed that pH in the medium played an important role in cell viability by inhibiting bacterial growth and their activity by passage of undissociated acid forms through the cell membrane (Luo *et al*., 2011). Additionally, the results indicated that after 2 h incubation in SGF, the cell viability was remarkably improved by alginate encapsulation, while no viable cells were detected in the un-encapsulated cells at pH 1.5 and 2.0. Regarding pH 2.0, it was found that JA helped increase LGG viability. ASJ15 had significantly greater survival rate than ASJ 20 and ASJ15, which were 85.65±0.03%, 82.82±0.12%, and 72.54±0.05%, respectively (Figure 4.). Survival rates of AL and AS had no significant difference.

5.1.4 Viability of alginate encapsulated LGG in bile salt conditions

After incubating in bile salt conditions (3%, 5%, and 10%) for 3 h, high bile salt solution tolerance was observed for encapsulated cells when compared with un-encapsulated cells. Regardless to JA concentrations, ASJ showed the highest number of viable cells, followed by AS and AL, respectively, while fresh cells had no viable cells detected after incubating for 3 h (Figure 5.). Ding and Shah, (2007) observed that *L. rhamnosus* GG were reduced by 4 log CFU/mL in 3% bile salt solution at 37 °C after 4 h incubation. This resulted from that bile salt functions as an emulsifier and fat solubilizer, hydrolyzing plasma membranes of bacteria cells (Begley *et al.,* 2005).

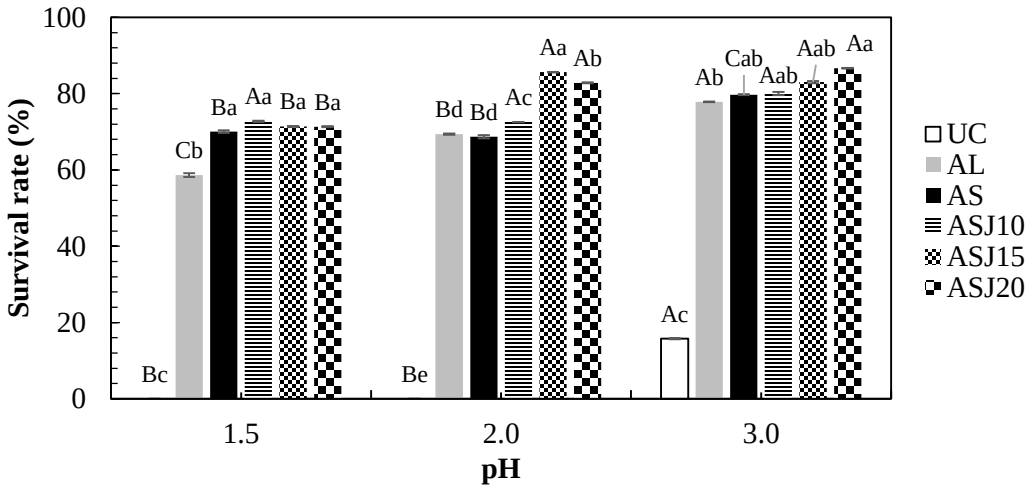

Figure 4. Survival rates of LGG after incubating in low pH conditions for 2 h; UC= Un-encapsulated cells, AL = Alginate, AS = Alginate+ Skim milk, ASJ10, ASJ15, and ASJ20 = Alginate + Skim milk + 10%, 15%, and 20% Jerusalem artichoke, respectively. $^{a-c}$Means ± standard deviation with same letter within the same treatments were not significant differences (P>0.05). ^{A,B}Means±standard deviation with same letters within the same color were not significant differences (P>0.05).

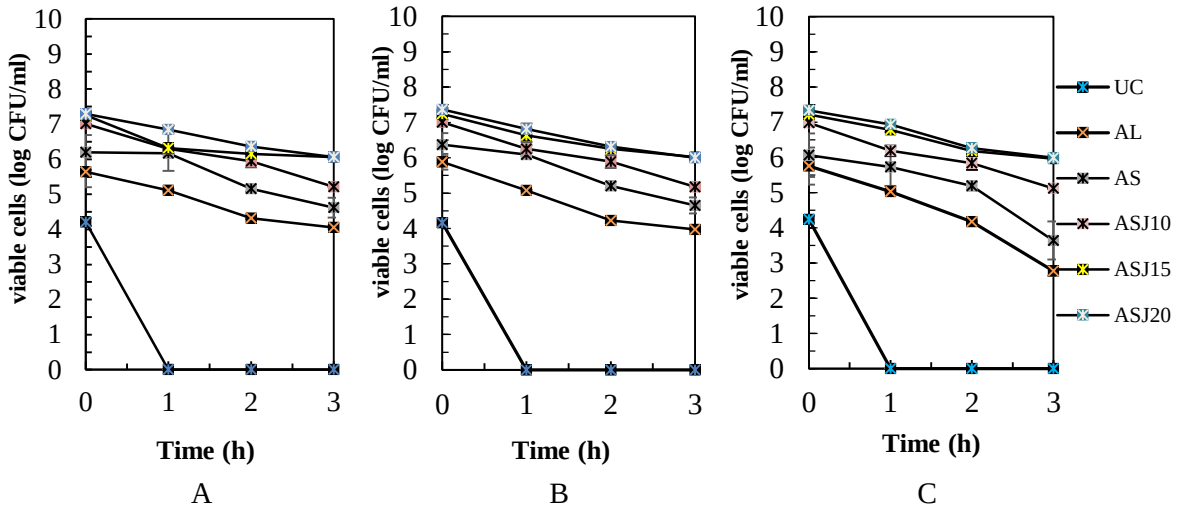

Figure 5. Viability of alginate encapsulated LGG (dried capsule) in bile salt conditions (bile salt 3% (A), bile salt 5% (B), and bile salt10% (C)); UC= Un-encapsulated cells, AL=Alginate, AS=Alginate+Skim milk, ASJ10, ASJ15, and ASJ20= Alginate+Skim milk+10%, 15%, and 20% Jerusalem artichoke, respectively.

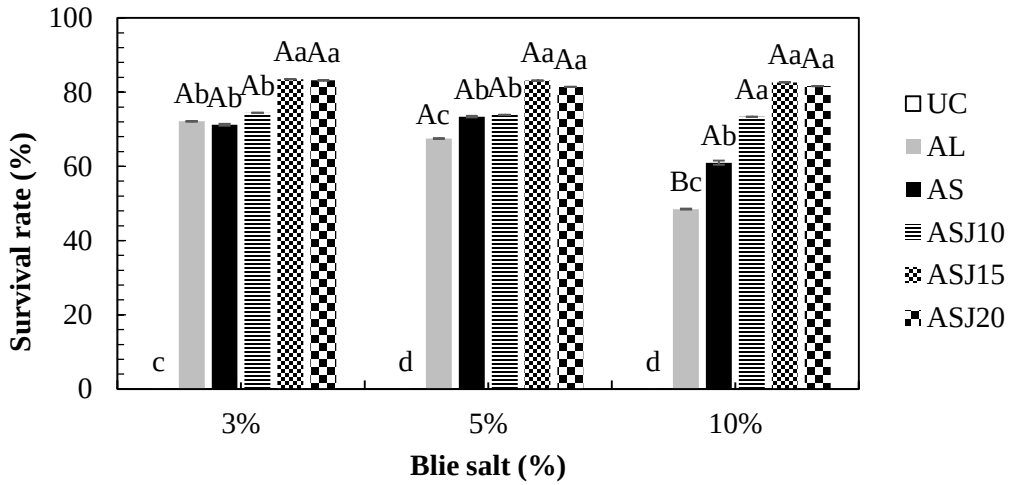

Figure 6. Survival rates of LGG after incubating in bile salt conditions for 3 h; UC= Un-encapsulated cells, AL = Alginate, AS = Alginate+ Skim milk, ASJ10, ASJ15, and ASJ20 = Alginate + Skim milk + 10%, 15%, and 20% Jerusalem artichoke, respectively. $^{a-d}$Means ± standard deviation with different letters within the same color indicate not significant differences (P≤0.05). ^{A,B}Means ± standard deviation with different letters within the same treatment indicate not significant differences (P≤0.05).

Krasaekoopt and Watcharapoka (2014) reported that the alginate beads could absorbed the bile salt, resulting in delay of bile salt permeability into the beads. Besides, addition of JA in to the alginate encapsulated capsules could enhance the survival rates of LGG. At 10% bile salt, survival rates of LGG in ASJ10, ASJ15, and ASJ20 were 73.36±0.02%, 82.62±0.05%, and 81.63±00.04%, respectively, which were significantly higher than AS (60.99±0.15%) and Al (48.47±0.13%) (Figure 6). Regarding to 3% and 5% bile salts, survival rates of ASJ15 and ASJ20 was significant higher than ASJ10. This was probably that JA possibly provided the capsules with greater networks and diffusion path lengths, reducing penetration of bile solution and consequently decreasing cell losses.

5.2 Effects of Jerusalem artichoke on viability of *Lactobacillus rhamnosus* GG after spray drying

5.2.1 Production of spray dried LGG

After spray drying, the viability of LGG was obviously reduced. The results (Figure 7) showed that the number of viable cells in AL (4±0.54 log CFU/g) was significantly lower than the AS (5.87±0.50 log CFU/g) and ASJ samples ($P<0.05$). The number of viable cells in ASJ samples were not significantly different, which were 7.01±0.11, 6.84±0.36, and 6.31±0.16 log CFU/g for ASJ20, ASJ15, and ASJ10, respectively. However, ASJ20 and ASJ15 had significantly higher number of viable cells than AS ($P<0.05$). The results thus indicated that skim milk and JA played important roles in the cell viability during spray drying. Skim milk has been well-known for their ability to protect the cells from dehydration processes. The proteins in skim milk were accumulated within the cells, helping stabilize cell membrane constituents and reduce the osmotic difference between the internal and external environments (Mille et al., 2004). Besides skim milk, sugars and some polysaccharides such as fructooligosaccharides (FOS) and inulin were reported to be able protect the cells from dehydration. The JA were mainly composed of 39 g/100 mL of inulin and 16 g/ 100 mL of fructo-oligosaccharide (Sarote and Warangkana, 2011). It was reported that FOS and inulin could function as a water replacer, helping stabilize the cell membrane during dehydration (Doherty et al., 2011).

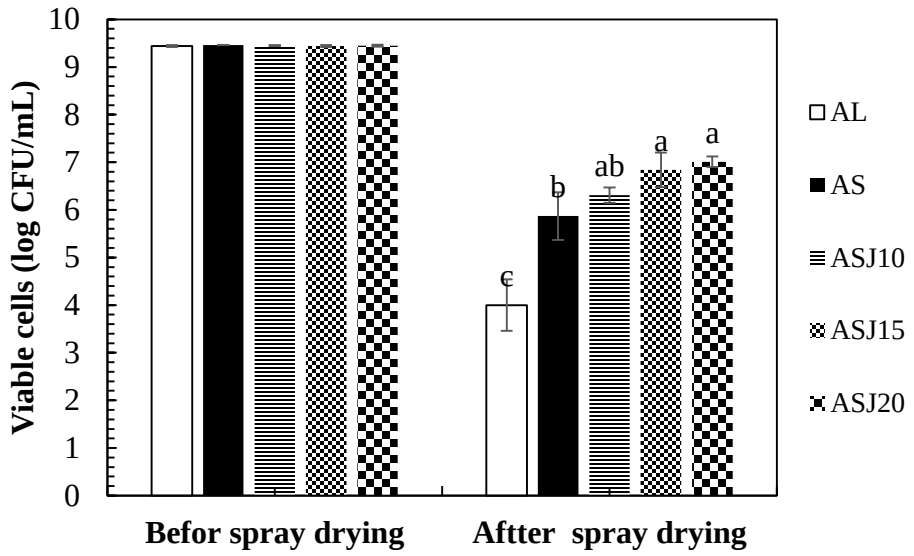

Figure 7. Viability of LGG powders before and after spray drying; AL= LGG powder with only alginate, AS = LGG powder with alginate and skim milk, ASJ10, ASJ15, and ASJ20 = LGG powder with alginate, skim milk, and JA at 100, 150, and 200 g/L respectively. [a–b]Means±standard deviation with same letters were not significant differences (P>0.05).

5.2.2 Viability of spray dried LGG powders in simulated gastrointestinal conditions

Figure 8 showed the number of viable cells in LGG powders after 2-h exposure to simulated gastric fluids (SGF) at different pH. LGG could obviously survive in the SGF at pH 3.0 and 2.0 and the number of viable cells of all treatments at pH 3.0 and 2.0 were not significantly difference. However, the remarkable decreases in the number of viable cells were significantly observed in SGF at pH 1.5. Regarding pH 1.5, the viable cell counts were enhanced by encapsulating LGG in skim milk and JA. The cell log reductions of ASJ20, ASJ15, and ASJ10 were 1.21, 1.67, and 2.31, respectively, while AS, having only skim milk, had 3.02 log reduction and no viable cells were detected in the control or AL. When consider the survival rate (Figure 9), ASJ20 and ASJ15 had survival rate at 84.10% and 75.82%, respectively which were significantly greater than that of ASJ10 (65.25%) and AS (49.69%). The results indicated that JA could prevent LGG from high acid conditions. It was mentioned that the presence of inulin in alginate matrix could decrease the porosity of the alginate when it was in gastric environment with low pH (Atia et al., 2016).

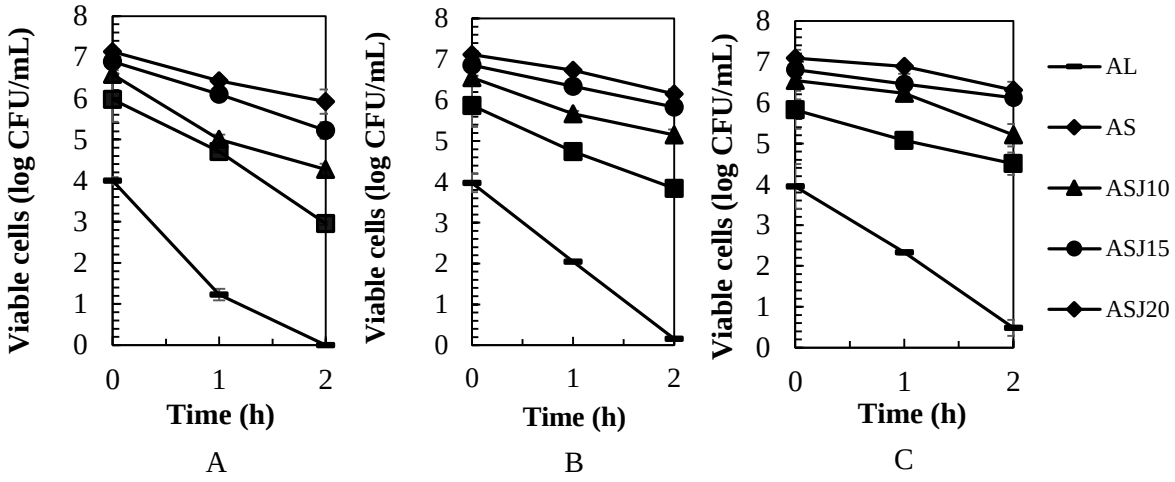

Figure 8 Viability of LGG powders in low pH conditions (pH 1.5 (A), pH 2.0 (B), and pH 3.0 (C)); AL= LGG powder with only alginate, AS = LGG powder with alginate and skim milk, ASJ10, ASJ15, and ASJ20 = LGG powder with alginate, skim milk, and JA at 100, 150, and 200 g/L respectively.

The viability of LGG powders after incubating in bile salt conditions (3 mL/10 mL, 5 mL/100 mL, and 10 mL/100 mL of fluids) for 3 h was shown in Figure 10. Bile salt is as an emulsifier and fat solubilizer that could hydrolyze plasma membranes of bacteria cells. This causes the cells to lose their cell wall integrity (Bustos et al., 2018). As the results, no viable cells were detected in the control (AL) of all bile salt conditions after exposure for 1 h. The cell losses were increased with the increasing of bile salt concentrations in all treatments. Regardless to bile salt concentrations, after 3-h incubating in bile salts, ASJ20 had the lowest cell reductions, which were 0.86, 1.10, and 1.27 log reductions for 3 mL/100 mL, 5 mL/100 mL, and 10 mL/100 mL of tilapia bile salt, respectively, followed by ASJ15, ASJ10, and AS. Regarding to survival rates of LGG, at 3 mL/100 mL of fluids and 5 mL/100 mL of fluids, ASJ20 had significantly greater survival rates than ASJ15 and ASJ10, while AS had the lowest survival rate. Similarly, the LGG survival rate of ASJ 20 in bile salt at the concentration of 10 mL/100 mL of fluids was 80.52±0.05%, which was significantly higher than ASJ15 (62.78±0.47%) and ASJ10 (61.26±0.07%) and followed by AS (44.93±0.10%) (Figure 11). Therefore, this indicated that skim milk could protect LGG in the powders from bile salt effects, simultaneously JA had ability to enhance the protection of the cells during bile salt conditions. The results were confirmed by Pirarat et al. (2015) mentioning that skimmilk-alginate matrix could improve cell viability of *L. rhamnosus* GG after being exposed in 10% tilapia bile when compared with only alginate matrix and fresh cells. This could be due to protein in skim milk possibly acting as an insoluble matrix protecting probiotics during gastrointestinal tract (Ying, Schwander, Weerakkody, Sanguansri, Gantenbein-Demarchi, & Augustin,

2013). As mentioned earlier, JA was a rich source of inulin and FOS. *L. plantarum* that were encapsulated in skim milk coated inulin-alginate beads by extrusion method had 1.21 log CFU/mL reduction after 2 h incubation in 1% bile salt solution treatment (Wang, Yu, Xu, Aguilar, & Wei, 2016). Addition of inulin could enhance the acid and bile tolerance of encapsulated *L. acidophilus* and *L. casei* powders by freeze drying, resulting in higher number of cells than cultured cells after they were put into simulated gastric juice (pH 1.55) for 2 h, followed by intestinal juice containing 0.6% bile salt for 2.5 h (Krasaekoopt & Watcharapoka, 2014).

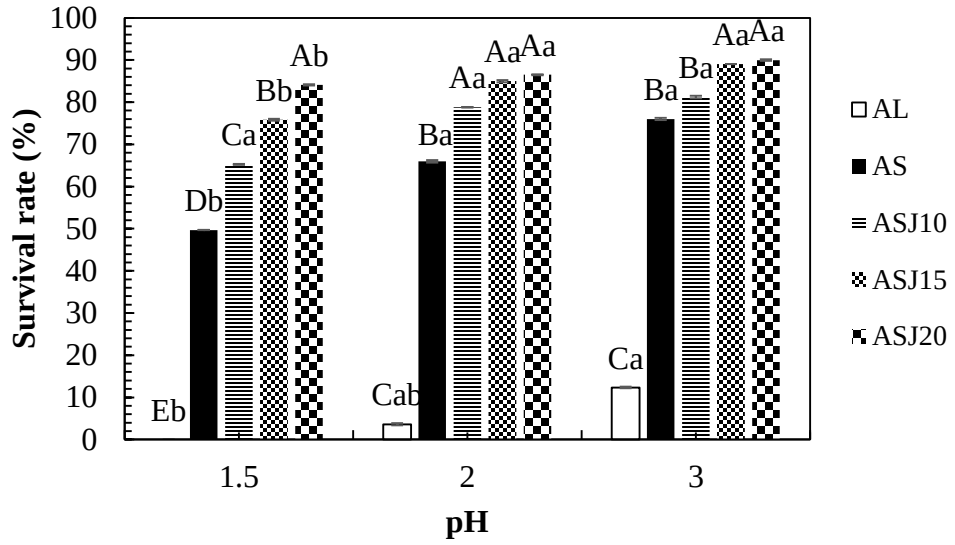

Figure 9. Survival rates of LGG powders after incubating in low pH conditions for 2 h; AL= LGG powders with only alginate, AS = LGG powder with alginate and skim milk, ASJ10, ASJ15, and ASJ20 = LGG powder with alginate, skim milk, and JA at 100, 150, and 200 g/L respectively. [a–c]Means ± standard deviation with different letter within the same color were significant differences (P>0.05). [A,B]Means±standard deviation with different letters within the same pH were significant differences (P>0.05).

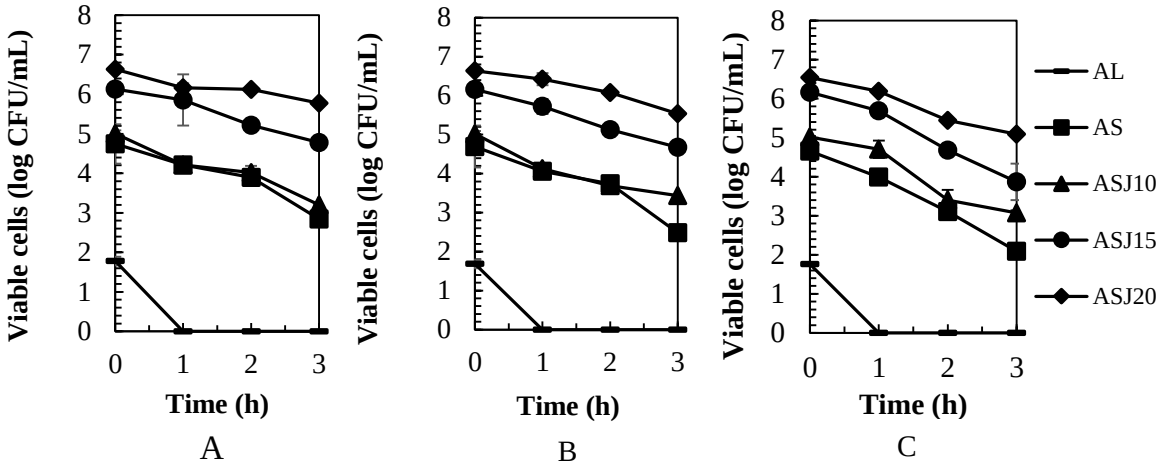

Figure 10. Viability of LGG powders in bile salt conditions (bile salt 3 mL/10 mL (A), bile salt 5 mL/100 mL (B), and bile salt 10 mL/100 mL (C)); AL= LGG powder with only alginate, AS = LGG powder with alginate and skim milk, ASJ10, ASJ15, and ASJ20 = LGG powder with alginate, skim milk, and JA at 100, 150, and 200 g/L respectively.

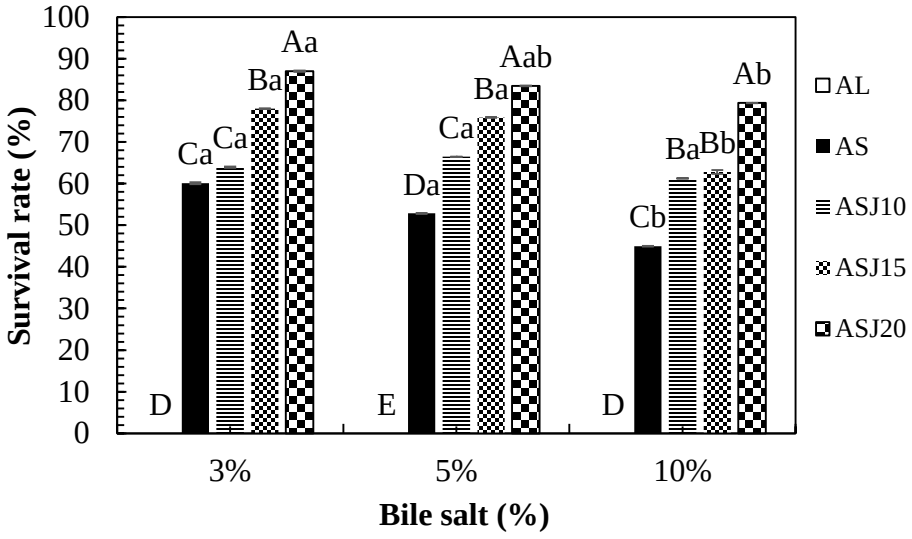

Figure 11. Survival rates of LGG powders after incubating in bile salt conditions for 3 h; AL= LGG powder with only alginate, AS = LGG powder with alginate and skim milk, ASJ10, ASJ15, and ASJ20 = LGG powder with alginate, skim milk, and JA at 100, 150, and 200 g/L respectively. $^{a-c}$Means ± standard deviation with different letter within the same color were significant differences (P>0.05). ^{A,B}Means±standard deviation with different letters within the same pH were significant differences (P>0.05).

5.3 Development of Nile Tilapia Pelleted Feed Containing *Lactobacillus rhamnosus* GG and Jerusalem Articoke

Nile tilapia pelleted feeds containing LGG (PFL) were composed of 55.35 g/100 g dry feed matter of carbohydrate, 24.54 g/100 g dry feed matter of protein, 8.66 g/100g dry feed matter of fat, and 11.13 g/100 g dry feed matter of ash. The moisture content of PFL was 2.71 g/100 g of PFL after drying at 50°C for 8 h. It was recommended that crude protein levels of tilapia feeds should not under 20 % due to poor palatability. The feed containing approximately 25% of crude protein were commonly used in Thailand as it was cost effective (Bhujel, 2013). After pelleting and drying, the final cell number of LGG in PFL was 7.08±0.04 log CFU/g, which was not significantly different with the spray dried LGG powder (7.23±0.06 log CFU/g). This indicated that the pelleting and drying processes did not affect the cell viability of LGG powder. The efficient number of viable cells in probiotic products to provide benefits to the host was recommended to be at least 10^6 CFU/g. In the simulated gastrointestinal tract, after incubating the PFL in a low pH condition (pH 2.0) for 1 h prior to expose to bile salt solution at 10mL/100mL for 3 h, as shown in Figure 12, the cell viability was reduced for 0.57 log CFU/g, which was not significantly different with the spray dried LGG (0.41 log reduction), while rapid loss of LGG fresh cells was detected (3.94 log reduction). The number of viable cells after 1 h incubation in SGF at pH 2.0 were 6.51±0.02, 6.82±0.02, and 5.83±0.04 log CFU/g for PFL, spray dried LGG, and fresh cells, respectively. Regarding bile salt tolerance, the results showed that the number of viable cells in PFL was 5.04±0.03 log CFU/g, followed by the spray dried LGG (5.22±0.04 log CFU/g), which was undergone for 2.04 and 2.01 log reduction, respectively. The results were not significant difference. On the contrary, massive decrease in viable cells of the fresh cell treatment was undergone for 7.84 log cycles. Only 1.93±0.02 log CFU/g could survive. The survival rates after 3 h in the bile salt solution were 71.17%, 72.25%, and 19.79% for for PFL, spray dried LGG, and fresh cells, respectively.

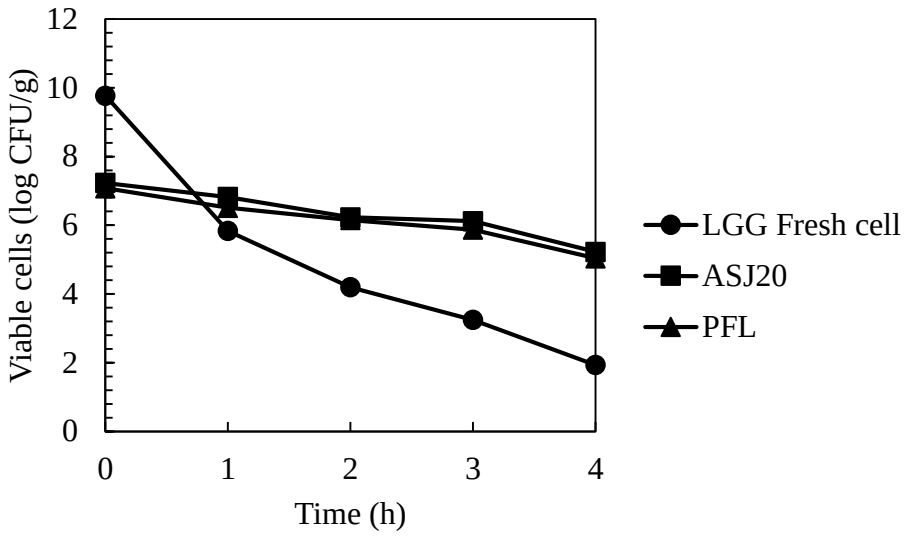

Figure 12 Viability of LGG Nile tilapia pelleted feeds after incubating in SGF at pH 2.0 for 1 h and in bile salt conditions (10 mL/100 mL) for 3 h

5.4 Effects of Nile Tilapia pelleted feed containing Lactobacillus rhamnosus GG and Jerusalem Articoke on growth performance, blood chemistry and intestines of Nile tilapia

5.4.1 Growth performance

Probiotics are considered as a good alternative to promote fish growth performance by increasing digestibility and nutrient utilization, inhibiting some pathogenic bacteria, and resisting some diseases. Table 1 showed the growth performance of tilapias fed with pelleted feeds containing LGG and JA (PFL), including weight gain (%), specific growth rate, feed conversion ratio (FCR), and survival rate (%). Probiotic (LGG), prebiotic (JA), and synbiotics (LGG+JA) in PFL could increase weight gain and specific growth rate of Nile tilapia, while reduce feed conversion ratio when compared to the control group. PFL had significantly greater weight gain and (79.86±25.08%) specific growth rate (1.940±0.466) than other groups. Control had significantly higher feed conversion ratio (2.393±0.376) than other treatments, while that of PFL (1.976±0.621) was significantly lower than JA (1.963±0.313) but higher than LLG (1.963±0.313). The result indicated that combination of LGG and JA could enhance the growth of Nile tilapia remarkably. Similarly, Sewaka et al. (2019) separately mixed LGG and/or JA with red Nile basal tilapia diets for 30 days and determined for their growth performance. It was reported that JA and LGG could improve the weight gain and specific growth rate of red Nile tilapia when they were compared with basal diets but they were not significantly different with symbiotic JA+LGG. This mean that corporation JA and LGG into the pelleted feed could improve the fish growth performance more effectively than separately mixing them with diets. Moreover, skim milk may play an important

role in improving fish growth performance due to lactoferrin, a milk glycoprotein that showed inhibitory activity against bacterial pathogens (Ward and Conneely, 2004).

Table. 1 Growth performance of Nile tilapia after feeding pelleted feed containing LGG and JA for 30 days

Treatments	Initial mean body weight (g)	Mean body weight at 30 days (g)	Weight gain at 30 days (%)	Specific growth rate at 30 days	Feed conversion ratio at 30 days
Control	30.37±2.40	49.48±1.78	63.47±9.95^{a}	1.635±0.202^{a}	2.393±0.376^{e}
AL+SM	28.19±4.75	49.64±4.69	78.32±18.97^{d}	1.919±0.356^{d}	1.973±0.478^{b}
LGG	35.27±2.47	62.35±1.31	77.41±12.35^{c}	1.907±0.232^{c}	1.963±0.313^{a}
JA	32.28±4.44	54.60±3.22	71.00±19.15^{b}	1.778±0.374^{b}	2.192±0.591^{d}
PFL	35.64±7.64	62.74±7.41	79.86±25.08^{e}	1.940±0.466^{e}	1.976±0.621^{c}

Mean values in the same row with different superscripts indicate significant differences ($P < 0.05$)

5.4.2 Blood chemistry

Blood chemical parameters are able to indicate the nutritional and health status of animals. Table 2 demonstrated blood chemical parameters of Nile tilapia fed with different diets for 30 days. The result showed that the fish fed with LGG had the highest total glucose but was not significantly different with JA and PFL as well as AL+SM. The control group showed the lowest total glucose contents. This indicated that LGG could contribute to digestibility improvement which was related to energy contribution and growth performance of the Nile tilapia. Sewaka et al. (2019) reported that symbiotic JA+LGG could increase increased the glucose, total cholesterol, indicating improvement of digestive enzyme activities such as amylase, protease and lipase. Regarding to T-bilirubin and D-bilirubin values, the results showed that there were no significant differences in all groups as well as AST and BUN, demonstrating that LGG, JA and the diet had no effect on the majority of blood serum biochemical parameters in the fish.

Table. 2 Blood chemistry of Nile tilapia fed experimental diets for 30 days

Parameters	Experimental groups				
	Control	AL+SM	LGG	JA	PFL
ALT (SGPT) (U/L)	5.85±2.41^{ab}	6.00±2.16^{ab}	5.28±1.38^{ab}	4.57±2.07^{a}	17.00±22.55^{b}
AST (SGOT) (U/L)	25.57±12.30^{a}	30.00±25.65^{a}	26.42±7.23^{a}	27.00±11.59^{a}	75.14±94.59^{a}
BUN (mg/dL)	2.00±0.82^{a}	2.00±1.15^{a}	2.14±0.38^{a}	1.42±0.53^{a}	2.00±1.10^{a}
Glucose (mg/dL)	51.85±25.80^{a}	72.28±14.94^{ab}	81.00±12.01^{b}	72.71±19.23^{ab}	66.85±21.57 ^{ab}
Total protein (g/dL)	3.04±0.24^{b}	2.75±0.18^{a}	2.74±0.13^{a}	2.78±0.26^{a}	2.57±0.08^{a}
Triglyceride (mg/dL)	392.85±164.12^{a}	410.57±172.82^{a}	373.42±179.35^{a}	287.14±75.14^{a}	271.71±237.67^{a}
Cholesterol (mg/dL)	224.42±48.16^{b}	171.71±17.89^{a}	185.00±33.95^{a}	179.71±24.11^{a}	149.42±35.20^{a}
Total Bilirubin (mg/dL)	0.03±0.01^{a}	0.03±0.01^{a}	0.03±0.01^{a}	0.02±0.01^{a}	0.04±0.01^{b}

Means with different superscripts in each row differ significantly from each other (*P<0.05*).
SGPT = serum glutamic pyruvic transaminase; SGOT = serum glutamic oxaloacetic transaminase; BUN = blood urea nitrogen.

5.4.3 Villous height

After 30 days on the treatment diets, all treatments had higher villi height in the proximal intestine than the distal part. The fish fed with PFL showed the highest villi height in proximal part which was 933.36±199.371 μm, followed by JA and LGG groups. This indicated that combination of LGG with JA could enhance the intestinal morphology. The results were confirmed with the study of Pirarat et al. (2011). The villi functions are associated with nutrient absorption, contributing to the fish growth. The higher the villi were, the greater chance more nutrients were absorbed (Caspary, 1992; Pirarat et al., 2011). Moreover, it was possible that JA as prebiotics could be fermented by the fish gut microbiota, resulting in the release of short chain fatty acids for

intestinal epithelium cells to be used as an energy sources (Blottiere et al., 2003; Pelicano et al., 2005). It was reported that direct supplementation with JA exhibited better effects than the purified inulin at the equivalent inulin levels (Boonanuntanasarn et al., 2015).

Table. 3 Average villus height of the intestine of fish after feeding pellets feed containing JA and LGG for 30 days

Treatments	Villous height (μm)	
	Proximal	Distal
Control	611.1±68.4[a]	415.8±45.7[b]
AL+SK	673.0±102.5[ab]	394.5±59.5 [a]
LGG	681.5±86.1[b]	653.6±72.4 [d]
JA	876.5±165.4[c]	687.2±352.9[e]
PFL	933.3±199.3[c]	528.6±56.9[c]

Means with different superscripts in each row differ significantly from each other (*P<0.05*).

6. Conclusion

The data from the experiment indicated that applications of microencapsulation by either gel extrusion or spray drying methods by using alginate-skim milk matrix could protect and improve viability of *L. rhamnosus* GG from freezing and drying and under stimulated gastrointestinal tracts of Nile tilapia. Kantawan or Jerusalem Artichoke (JA) could enhance the cell viability in both capsule and spray dried powder forms. After processing, survival rates of LGG in both capsules and powders were not significantly different. However, addition of JA at 20g/100 mL significantly yielded the highest cell viability when the LGG exposed to tilapia bile salt at 10mL/100mL. Spray dried LGG with JA powders had cell viability higher than LGG capsules with JA. Production of Nile tilapia pelleted feed containing LGG and JA (PFL) by pelleting at ambient temperature and drying at 50°C had no negative effects on cell viability. After pelleting and drying the number of viable cell was over 10^7 CFU/g as it was recommended for probiotic products. Corporation of spray dried LGG with JA powders into the pelleted feeds did not enhance cell viability during exposure to simulated gastrointestinal tract. However, PFL could yield higher weight gain and specific grow rate of the fish and improve the villi height after feeding for 30 days. LGG increased glucose levels in the fish blood as well as the LGG with JA (PFL) group and JA alone. However, PFL and JA groups had no significantly different in glucose levels when compared with that of the fish fed with basal diet. In summary JA could be used as a potential protective agent in for LGG. Corporation of JA and LGG into pelleted feed could be practical nutritional supplement in red tilapia.

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Marisa Kongboonkird, Kiattisak Duangmal, Nawanon Chantaprasan and Sarn Settachaimongkon

CSB-P-04 Detection of cordycepin using indicator displacement assay of cucurbit[7]uril and 2-p-toluidinylnaphthalene-6-sulphonate
Arinta Agnie Dewantari, Tanapat Nakprach, Akio Ojida and Jirarut Wongkongkatep

CSB-P-05 Preparation and Characterization of Iron(III)-Quercetin Complex as Contrast Enhancer for Magnetic Resonance Imaging and Their Biological Effects in Human Breast Cancer
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FBS-O-04 Chain length distribution of pectic-oligosaccharides derived from alkaline extracted pomelo peel pectin by endo-polygalacturonase
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Thanawat Thumrongtaradol, Onrapak Reamtong, Nisa Patikarnmonthon and Watanalai Panbangred

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FBS-O-08 Development of multiplex PCR assay for detection of *Salmonella* serotypes Enteritidis and Typhimurium
Phutthaphorn Phaophu, Bhunika Ngamwongsatit, Ratchaneewan Aunpad and Soraya Chaturongakul

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FBS-P-07 Application of intelligent gelatin films for monitoring the degree of fermentation of Pla-Som, a Thai fermented fish product
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FBS-P-09 Physical and antimicrobial properties of banana flour films incorporated with holy basil essential oil and cassia essential oil
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FBS-P-10 Effects of Jerusalem artichoke on viability of *Lactobacillus rhamnosus* GG in alginate capsules after freezing, freeze drying, and in simulated Nile tilapia gastrointestinal conditions
Unchaleeporn Sribounoy, Nopadon Pirarat, Subramaniam Sathivel and Arranee Chotiko

FBS-P-10

Effects of Jerusalem artichoke on viability of *Lactobacillus rhamnosus* GG in alginate capsules after freezing, freeze drying, and in simulated Nile tilapia gastrointestinal conditions

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ABSTRACT

Probiotics have been well documented for their health benefits. Unfortunately, they are sensitive to acids, bile salts, and heat, causing their low viable cells during formulation, process, and storage, as well as after consumption. An alginate gelation-extrusion with protective agents is an encapsulation method having been used to maintain probiotic viability. Jerusalem artichoke (JA) is a source of prebiotics that could help enhance the probiotic viability in those unfavorable environments. The objective of this study is to investigate effects of JA on protection of *Lactobacillus rhamnosus* GG (LGG) in alginate capsules under undesired conditions. LGG were encapsulated by using an alginate gelation extrusion technique. Skim milk (SM) (10%w/v) and JA (10%w/v, 15%w/v, and 20%w/v) were added into alginate capsules containing LGG as protective agents. Un-encapsulated cells (UC) and only LGG in alginate capsules were used as controls. The encapsulated probiotics were determined for their viability after freezing and freeze drying as well as in simulated gastrointestinal tract. The result indicated that addition of JA had no effects on encapsulation efficiency of alginate capsules. Combination of SM with JA could provide better protection to LGG during freezing and when they were in bile salt conditions than having only SM or alginate control. In conclusion, the study indicates that SM and JA could be used as potential protective agents for enhancing encapsulated probiotic viability after freezing and in simulated gastrointestinal tract.

KEYWORDS: Alginate encapsulation; Probiotics; Jerusalem artichoke (JA)

INTRODUCTION

Fish farms has been growing tremendously and becoming an economically important industry (Passot *et al.,* 2012). With the increasing of intensification and commercialization of fish production, disease outbreaks are a crucial problem in the fish farming industry. To control any possible outbreaks and improve the fish performance, antibiotics have been extensively used, leading to public concerns and criticisms about their health and environmental safety. In 2006, European Union announced a ban for the use of all sub-therapeutic antibiotics as growth-promoting agents in animal production. This led fish farms and the animal feed industry to search for and develop natural alternative growth promoters that could be used in aquatic feeds.

According to FAO/WHO (2001), probiotics are "live microorganisms in which an adequate consumption of probiotics confers many beneficial effects on the host". Many published reports demonstrated positive effects of probiotics and prebiotics in feeds for various fish species, including rainbow trout (Klaver *et al.,* 1993), Common carp (Roy, 2005) and Nile tilapia (Dave and Shah, 1997). However, it has been recommended that the number of probiotics in foods or feeds, at the time of consumption, should contain at least 10^6 to 10^7 CFU/mL (Kim *et al*., 1988). Probiotics has been well-known for their susceptibility to heat, ice crystals, acids, hydrogen peroxide, and oxygen (Anal *et al*., 2006). This could limit the applications of probiotics in animal feeds, as low survival of probiotics during formulating, processing, and storage as well as after consumption could occur. To prevent probiotics from the mentioned factors, it is recommended that probiotics should be encapsulated. A gelation-extrusion technique has been commonly suggested. This technique is relatively simple, gentle and no toxicity (Krasaekoopt and Watcharapoka, 2014). However, a drawback of this method is porosity of the microspheres, allowing fast and easy diffusion of water and other fluids in and out of the matrix.

Jerusalem artichoke (JA) is a rich source for prebiotics. JA tuber contains fructooligosaccharide (FOS) and inulin. Prebiotics are defined as non-digestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of healthful bacteria and by combating undesired bacteria in the intestinal tract (Gibson and Roberfroid, 1995). Inulin, which belongs to a class of carbohydrates known as fructans, is one of the most common prebiotics used in feed for livestock and aquatic animals. It could enhance animal growth performance, modulate intestinal microbiota, and improve hematological and immune parameters in fish, poultry, and swine (Mourino *et al.,* 2012). Therefore, this study aims to investigate effects of JA on protection of *Lactobacillus rhamnosus* GG in alginate capsules during processing (freeze drying) and in simulated gastrointestinal tract.

MATERIALS AND METHODS

Preparation of probiotics

Frozen cultures of *L. rhamnosus* GG (LGG) in 20% glycerol was reactivated into de Man Rogosa Sharpe (MRS) broth (BD Difco™, Maryland, USA) and incubated (BE 500, Schwa Bach, Germany) at 37 °C for 24 h. The bacteria (5%) was then subsequently inoculated into MRS broth and incubated at 37 °C until it reaches to stationary phase. The cells were harvested by centrifugation at 8000 rpm, for 10 min at 4 °C (Sigma 2-16PK, Sartorius, Germany). Afterwards, the cell pellets were dispersed into saline solution and used for encapsulation.

Encapsulated alginate capsule preparation using gelation-extrusion method

The extrusion technique of encapsulation was modified from Krasaekoopt and Watcharapoka (2014). The cell pellets were resuspended in 10%w/v of skim milk (SM) mixed with 1.8%w/v food grade alginate. JA powders were obtained from Phetchabun Research Station, Agro-Ecological System Research and Development Institute, Kasetsart University, Thailand. JA was composed of 28 % of sugar, 39.09 % of inulin, 15.8 % of FOS (Sarote and Warangkana, 2011). The sample was mixed until it was homogenous. After that, the mixture was dropped into 0.1 M Calcium chloride ($CaCl_2$) (Univar®, Ajax Finechem, Australia) solution and kept hardening in the solution for additional 30 min. The $CaCl_2$ was decanted to collect the alginate capsules. After encapsulation, the capsules were determined for their encapsulation efficiency (%) = $(N/N_0) \times 100$, where N and N_0 were the number of viable cells encapsulated in alginate capsules after alginate encapsulation and the number of viable cells before encapsulation, respectively.

Freeze drying of LGG encapsulated alginate capsules

The sample was placed in glass chamber and frozen in alcohol bath at -86 °C. All samples were subsequently dried in a freeze dryer (FDU-8624, Operon, Korea) for 48 h and stored in sealed aluminum bags at 4 °C. After freezing and freeze drying, the cell viability was measured. The un-encapsulated cells and only alginate encapsulated LGG were used as controls.

Determination of LGG viability in wet and dried AG-capsules under simulated gastrointestinal (GI) tract of Nile tilapia

Viability of freeze dried alginate capsules in simulated gastric conditions

Simulated gastric fluids (SGF) of Nile tilapia were prepared using normal saline solution, adjusted pH to 1.5, 2.0, and 3.0 (adjust pH by using 5 M hydrochloric solution (Lab scan, Analytical, Thailand)). Exactly 0.5 mL of free cells and 0.5 g of freeze dried alginate capsules were placed into separated test tubes containing 4.5 mL of the simulated gastric solutions. Triplicate samples were taken after incubation (JSGI-250J, Schwa Bach, Germany) at 25 °C in a water bath for 0, 1, and 2 h. The capsules were then disintegrated by soaking in 1 M phosphate buffer pH 7.0.and subsequently placing in a stomacher at 200 rpm for 3 min.

Viability of freeze dried alginate capsules in Nile tilapia bile salt solutions

Bile tolerance, 0.5 mL of un-encapsulated cells and 0.5 g of freeze dried capsules were added into 4.5 mL of SGF at pH 2.0 and incubated at 25 °C (BE 500, Schwa Bach, Germany) for 60 min. After the incubation, the SGF was removed and replaced with 4.5 mL of 3%w/v, 5%w/v, and 10%w/v tilapia bile salt. Triplicate samples were taken after further incubations of 1, 2, and 3 h at 25 °C. The capsules were then disintegrated as mentioned above. The cell counts of LGG were enumerated on MRS agars by using a pour-plate method. Un-encapsulated cells were used as a control.

Statistical analysis

All values were means and standard deviations of determinations. Means values from statistical analysis was conducted with SPSS Statistics software (version 24). One-Way ANOVA and Duncan test were carried out to determine differences all treatments at the significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Encapsulation efficiency of alginate encapsulated capsules containing LGG

The result indicated that all treatments had less than one log reduction in cell viability after encapsulation, which were 0.53, 0.31 0.42, 0.45, and 0.42 log reduction, respectively. This suggested that the extrusion technique was suitable for encapsulation of LGG. According to Krasaekoopt *et al*. (2003) this technique is simple and gentle. It does not harm probiotic cells and does not require high temperatures and the use of organic solvents. Moreover, Gombotz *et al*. (1998) reported that alginate was categorized as a nanoporous polymer having pore size between 5 and 200 nm. As well known, bacteria size is about 0.2 µm in diameter and 2-8 µm in length were entrapped inside of the capsules. The %EE of all treatments was between 94.62±0.09% and 96.79±0.03% (Figure 1). The highest %EE was found in AS, while JA caused slight reduction of %EE. However, it was not significantly different. This was probably due to densely packed structure of added JA, causing partly block the cell entrapment (Idouraine *et al.,* 1996).

Effects of freezing and freeze drying on viability of LGG

For long-term storage, probiotics are usually preserved by freeze drying. In this study, the alginate capsules were freeze-dried. As shown in Figure 2, after freezing**,** the viability of LGG in un-encapsulated cells, AL and AS was reduced by 2.46, 2.05, and 1.46 log CFU/mL, respectively. Addition of JA could significantly increase the number of viable cells after freezing. However, the concentrations of JA did not significantly affect the cell viability. The number of viable cells in ASJ10, ASJ15, and ASJ20 was reduced by 1.07, 1.01, and 1.00 log CFU/mL, respectively. According to, Meng *et al*. (2008) the loss of cell viability after freezing was mainly caused by extracellular ice formation. Alginate encapsulation functioned as a physical barrier protecting the cells from the

freezing process. Skim milk offered a surface for probiotic adherence, helping the cells avoid effects of ice crystallization and extracellular osmolality (Ding and Shah, 2008).

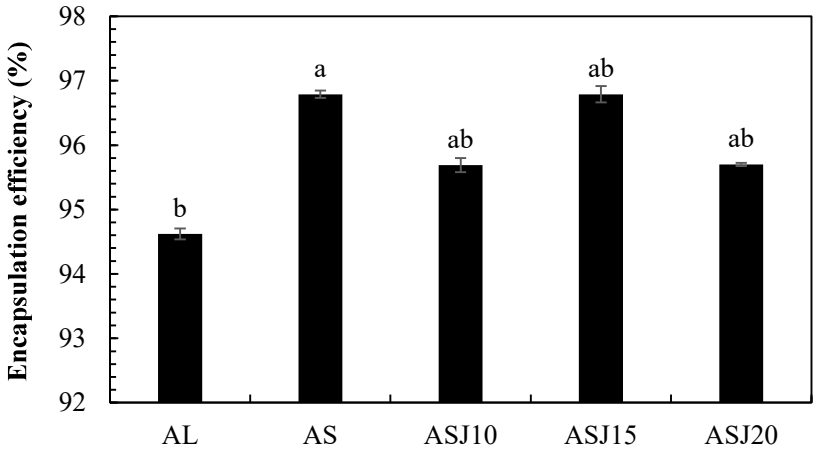

Figure 1. Encapsulation efficiency of alginate encapsulated capsules containing LGG; AL=Alginate, AS=Alginate+Skim milk, ASJ10, ASJ15, and ASJ20= Alginate+Skim milk+10%, 15%, and 20% Jerusalem artichoke, respectively. [a-b]Means±standard deviation with same letters were not significantly different (P > 0.05)

In addition, it was reported that JA contained large amount of inulin, which were 16-20% inulin with both low and high degree of polymerization (DP) (Gagga *et al*., 2011). Lingyun *et al*. (2007) mentioned that Low DP inulin in JA could penetrate into the cell membrane and acted as a buffer layer against ice formation when the beads encapsulated cells were frozen. Concurrently, high DP inulin adsorbed on the surface of cells and formed viscous layers, helping lower the rate of ice growth by increasing the solution viscosity (Judprasong *et al*., 2011). This would support our finding that the capsules with JA had better number of viable cells after freezing when compared to AS and controls.

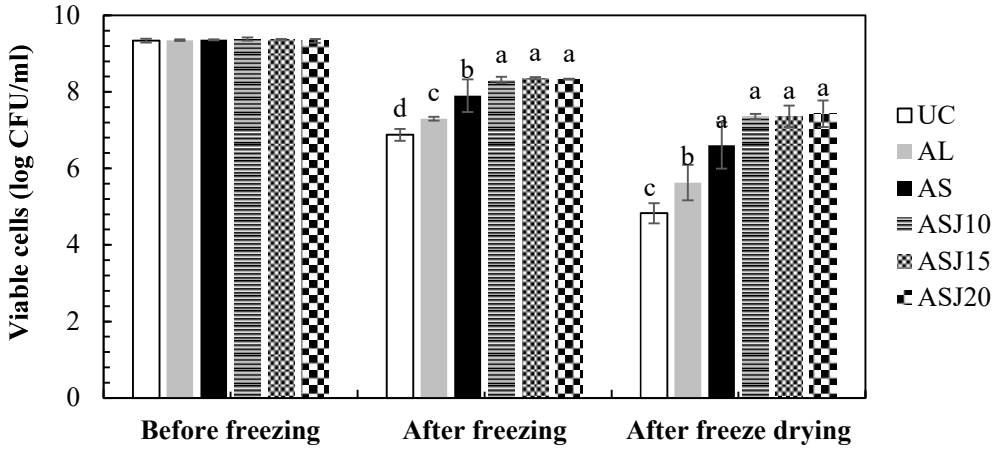

Figure 2. Viability of LGG in alginate encapsulated capsules before and after freezing and after freeze drying; AL = Alginate, AS = Alginate+ Skim milk, ASJ10, ASJ15, and ASJ20 = Alginate +

Skim milk + 10%, 15%, and 20% Jerusalem artichoke, respectively. [a–d]Means±standard deviation with same letters were not significant differences (P>0.05).

After freeze drying**,** the viability of LGG was continuously reduced. Our result suggested that AL had significant higher the number of viable cells than un-encapsulated cells. Removal of water from bacteria cells led to cell membrane transition and leakage (Doherty *et al*., 2011). The number of cell reduction in AL was 3.72 log cycles, while the fresh cell had 4.51 log reduction. Similarly to the result of cell viability after freezing, skim milk combined with JA could increase the cell survival after freeze drying. The number of viable cells in AS was reduced by 2.76 log reduction. ASJ10, ASJ15, and ASJ20 had cell reduction at 2.02, 2.01, and 1.9 log cycles, respectively. However, they were not significantly different. The protein in skim milk can accumulate within the cells, resulting in reduction of the osmotic difference between the internal and external environments (Mille *et al*., 2004). Combination of JA and skim milk helped improve cell protection from freeze drying damage more effectively than only skim milk. Sugars and some polysaccharides can function as a water replacer (Doherty *et al*., 2011), helping stabilize the cell membrane during dehydration.

Viability of freeze dried alginate encapsulated LGG in simulated gastric conditions

The stomach is a crucial section for pH-sensitive components such as probiotic cells. In the fasted state, the stomach of healthy subjects has a range of pH from 1.3 to 2.5 (Nemeth *et al*., 2014). Figure 3, showed the number of viable cells in freeze dried capsules after exposure to SGF. The viability of encapsulated LGG at pH 3.0 and 2.0 significantly less decreased than that at pH 1.5.

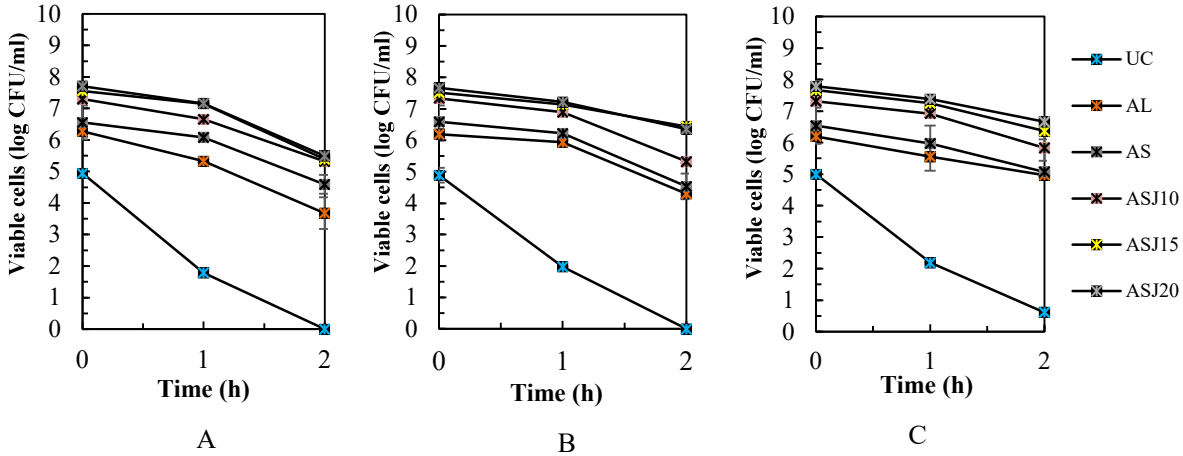

Figure 3. Viability of alginate encapsulated LGG (dried capsule) in low pH conditions (pH 1.5 (A), pH 2.0 (B), and pH 3.0 (C)); UC= Un-encapsulated cells, AL=Alginate, AS=Alginate+Skim milk, ASJ10, ASJ15, and ASJ20= Alginate+Skim milk+10%, 15%, and 20% Jerusalem artichoke, respectively.

This showed that pH in the medium played an important role in cell viability by inhibiting bacterial growth and their activity by passage of undissociated acid forms through the cell membrane (Luo *et*

al., 2011). Additionally, the results indicated that after 2 h incubation in SGF, the cell viability was remarkably improved by alginate encapsulation, while no viable cells were detected in the un-encapsulated cells at pH 1.5 and 2.0. Regarding pH 2.0, it was found that JA helped increase LGG viability. ASJ15 had significantly greater survival rate than ASJ 20 and ASJ15, which were 85.65±0.03%, 82.82±0.12%, and 72.54±0.05%, respectively (Figure 4.). Survival rates of AL and AS had no significant difference.

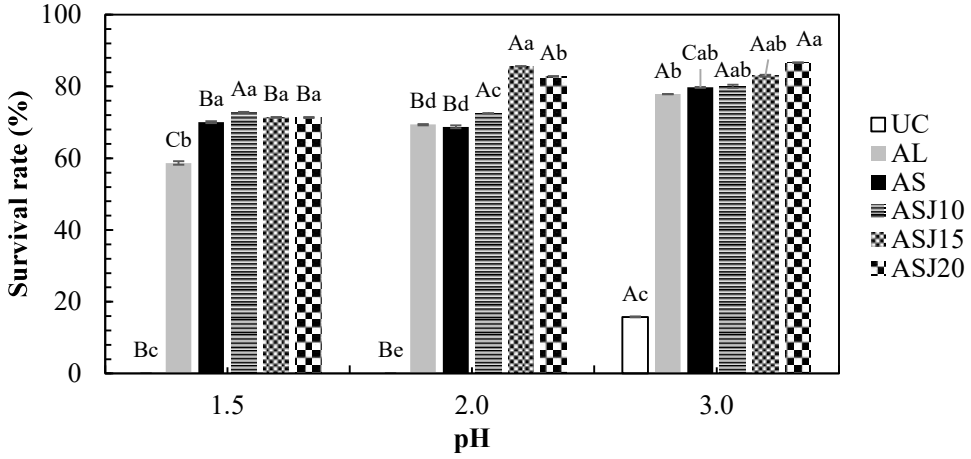

Figure 4. Survival rates of LGG after incubating in low pH conditions for 2 h; UC= Un-encapsulated cells, AL = Alginate, AS = Alginate+ Skim milk, ASJ10, ASJ15, and ASJ20 = Alginate + Skim milk + 10%, 15%, and 20% Jerusalem artichoke, respectively. $^{a-c}$Means ± standard deviation with same letter within the same treatments were not significant differences (P>0.05). ^{A,B}Means±standard deviation with same letters within the same color were not significant differences (P>0.05).

Viability of alginate encapsulated LGG in bile salt conditions

After incubating in bile salt conditions (3%, 5%, and 10%) for 3 h, high bile salt solution tolerance was observed for encapsulated cells when compared with un-encapsulated cells. Regardless to JA concentrations, ASJ showed the highest number of viable cells, followed by AS and AL, respectively, while fresh cells had no viable cells detected after incubating for 3 h (Figure 5.). Ding and Shah, (2007) observed that *L. rhamnosus* GG were reduced by 4 log CFU/mL in 3% bile salt solution at 37 °C after 4 h incubation. This resulted from that bile salt functions as an emulsifier and fat solubilizer, hydrolyzing plasma membranes of bacteria cells (Begley *et al.,* 2005). Krasaekoopt and Watcharapoka (2014) reported that the alginate beads could absorbed the bile salt, resulting in delay of bile salt permeability into the beads. Besides, addition of JA in to the alginate encapsulated capsules could enhance the survival rates of LGG. At 10% bile salt, survival rates of LGG in ASJ10, ASJ15, and ASJ20 were 73.36±0.02%, 82.62±0.05%, and 81.63±00.04%, respectively, which were significantly higher than AS (60.99±0.15%) and Al (48.47±0.13%) (Figure 6). Regarding to 3% and 5% bile salts, survival rates of ASJ15 and ASJ20 was significant higher than ASJ10. This was

probably that JA possibly provided the capsules with greater networks and diffusion path lengths, reducing penetration of bile solution and consequently decreasing cell losses.

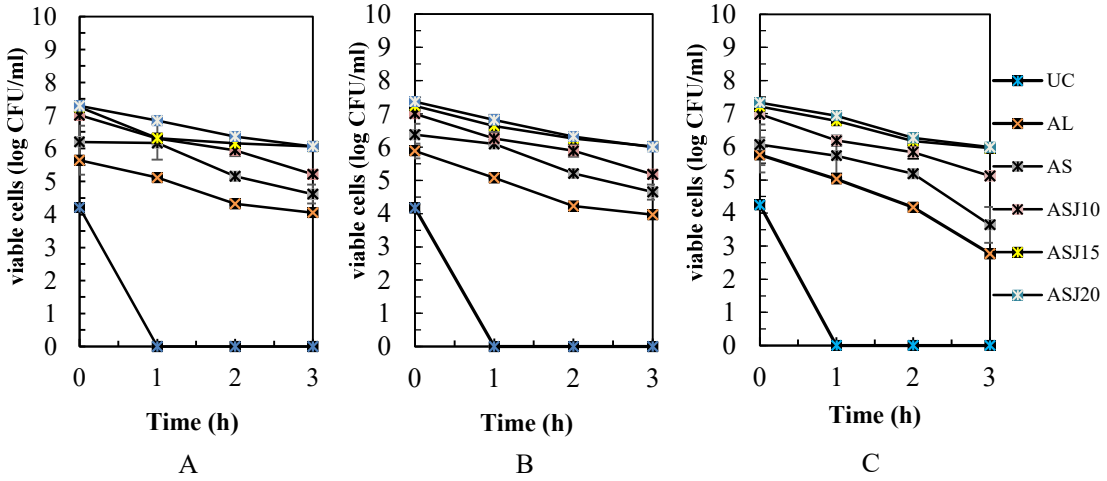

Figure 5. Viability of alginate encapsulated LGG (dried capsule) in bile salt conditions (bile salt 3% (A), bile salt 5% (B), and bile salt10% (C)); UC= Un-encapsulated cells, AL=Alginate, AS=Alginate+Skim milk, ASJ10, ASJ15, and ASJ20= Alginate+Skim milk+10%, 15%, and 20% Jerusalem artichoke, respectively.

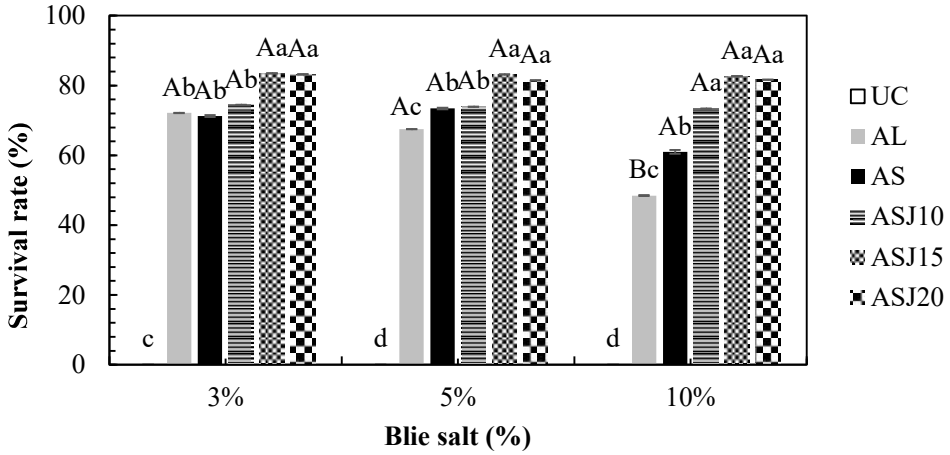

Figure 6. Survival rates of LGG after incubating in bile salt conditions for 3 h; UC= Un-encapsulated cells, AL = Alginate, AS = Alginate+ Skim milk, ASJ10, ASJ15, and ASJ20 = Alginate + Skim milk + 10%, 15%, and 20% Jerusalem artichoke, respectively. $^{a-d}$Means ± standard deviation with different letters within the same color indicate not significant differences (P≤0.05). ^{A,B}Means ± standard deviation with different letters within the same treatment indicate not significant differences (P≤0.05).

CONCLUSION

In conclusion, the study indicated that alginate-extrusion encapsulation technique offered high encapsulation efficiency. Addition of skim milk could enhance %EE, while, Jerusalem artichoke (JA) had no effect on the encapsulation efficiency. Alginate capsules with skim milk and JA significantly improved cell viability of the encapsulated cells under freezing condition. Supplementation of protective agents as JA significantly enhanced the number of viable cells under bile salt conditions. The result suggests that JA could be used as a potential protective agent in probiotic capsules.

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