จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย

กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

ประสิทธิผลของยีสต์บรรจุแคปซูลในการป้องกันโรคติดเชื้อสเตรปโตคอคโคซีสในปลานิล

โดย

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ชื่อโครงการ: ประสิทธิลของยีสต์บรรจุแคปซูลในการป้องกันโรคติดเชื้อสเตรปโตคอคโคซีสในปลานิล
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บทคัดย่อ

เชื้อยีสต์ **S. cerevisiae** JCM 7255จากอุตสาหกรรมการผลิตเบียร์ ถูกนำมาทดสอบความสามารถในการใช้เป็นโปรไบโอติกในสัตว์น้ำควบคู่กับเทคนิคเอนแคปซูลเลชันและการเก็บรักษาแบบแช่แข็งและทำแห้ง ทำการทดสอบอัตราการรอดชีวิตของเชื้อยีสต์และลักษณะรูปร่างการเปลี่ยนแปลงของเชื้อหลังจากบรรจุลงแคปซูลและอยู่ในสภาวะทางเดินอาหารจำลองของปลานิล วัดอัตราการเจริญเติบโต การเปลี่ยนแปลงของลักษณะเนื้อเยื่อทางเดินอาหาร และความสามารถในการป้องกันเชื้อสเตรปโตคอคโคซีส ผลการศึกษาการรอดชีวิตของเชื้อยีสต์ที่อุณหภูมิห้องพบว่ากลุ่มที่บรรจุลงแคปซูลมีอัตราการรอดชีวิตสูงหลังจากผ่านการเก็บรักษา 14 วันแต่ในกลุ่มควบคุมไม่พบการรอดชีวิตของเชื้อเลยหลังจากผ่านการเก็บรักษาได้เพียง 7 วัน อัตราการรอดชีวิตหลังจากผ่านสภาวะกรดและน้ำดีของปลานิลพบมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ลักษณะรูปร่างของเชื้อยีสต์เปลี่ยนแปลงผิดปกติ บิดเบี้ยว ขรุขระ เพิ่มจำนวนการแตกหน่อมากขึ้น เชื้อยีสต์สามารถยับยั้งการเจริญเติบโตของเชื้อเสตรปโตคอคัส อะกาเลคเทียในหลอดทดลอง 20 สายพันธุ์จากทั้งหมด 30 สายพันธุ์ การทดลอง ผลการทดลองภายหลังผสมอาหารให้ปลานิลพบว่า สามารถกระตุ้นการเจริญเติบโต เพิ่มความสูงของวิลไลในลำไส้ จำนวนของลิมโฟไซต์ที่อยู่ภายในเยื่อบุผิวทางเดินอาหารเพิ่มจำนวนมากขึ้นอย่างมีนัยสำคัญในลำไส้ส่วนต้นของปลานิลที่ได้รับเชื้อยีสต์ แต่เซลล์เมือกและเม็ดเลือดขาวอะซิฟิลลิกไม่มีความแตกต่างจากกลุ่มควบคุม ปลาที่ได้รับเชื้อเสตรปโตคอคโคซีสมีอัตราลดลงแต่ไม่แตกต่างจากกลุ่มควบคุม ผลการศึกษาทั้งหมดชี้ให้เห็นว่าแอลจิเนทแคปซูลห่อหุ้มเชื้อยีสต์สายพันธุ์ JCM 2755 สามารถเป็นโปรไบโอติกที่ใช้ในอุตสาหกรรมปลานิลได้

คำสำคัญ: : แซคคาโรไมเซส เซเรวิซิอี้ เอนแคปซูลเลชัน โปรไบโอติก อัลจิเนท ปลานิล

Project Title: Efficacy of encapsulated yeast (*Saccharomyces cerevisiae*) against streptococcosis in tilapia

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Year: June, 2015

Abstract

S. cerevisiae JCM 7255 from brewing industry was tested to be a possible probiotic candidate in aquaculture together with encapsulation and freeze-drying technique. I*n vitro* viability and morphology analysis of probiotic during storage and during transient with stimulated tilapia gut and bile conditions were evaluated as well as *In vivo* growth performance efficacy, gut mucosal immune parameters and anti-Streptococcal activity. The *In vitro* results showed that the viabilities of encapsulated yeasts remained in the high number after storage in room temperature for 14 days, while the viability of free *S. cerevisiae* could not be detected after 7 days pass. The viability of encapsulated yeast in simulated gastric condition and in tilapia bile was significantly higher than the free non-encapsulated group. The morphology of free *S. cerevisiae* revealed oval, rough bumpy surface and 2- 4 budding knots with rupture on the surface during incubation in gut and bile conditions. The *in vitro* anti-streptococcal activity of encapsulated yeast using agar spot test showed inhibitory reaction against 20 from 30 strains of *S. agalactiae.* The *in vivo* study showed that supplementation with encapsulated yeast improved the intestinal structure and growth performance in tilapias. A significantly increase number of intraepithelial lymphocytes in proximal intestine were observed while acidophilic granulocytes and mucous cells were not statistically different in any part of the intestine. Lowering the cumulative mortality after oral streptococcal challenge was also observed without statistical significance when compare with control group. The results suggested that encapsulated *S. cerevisiae* JCM 2755 could be a potential probiotic candidate in tilapia culture.

Keywords: *Saccharomyces cerevisiae*, encapsulation, probiotic, alginate, Nile Tilapia

บทนำ **(Introduction)**

ความเป็นมาและมูลเหตุจูงใจ **(Importance and Rationale)**

Tilapia production is rapidly growing in Thai aquaculture since tilapias are recognized as an important freshwater fish protein source for consumption among Thai population. In order to meet consumer demand, intensive tilapia culture is expanding all over the country. At the same time, outbreaks of infectious diseases, including streptococcosis, have been described in tilapia production. The disease, which is mainly caused by *Streptococcus agalactiae,* is considered to be one of the most important bacterial diseases in tropical tilapia culture marked by high morbidity and mortality (Suanyuk et al., 2008; Mian et al., 2009; Ye et al., 2011). The described symptoms included spiral or erratic swimming, periorbital and intraocular hemorrhage, opacity and exopthalmia, reddening or hemorrhage in the integument and musculoskeletal systems, ascites and ulceration. However, in some cases the fish may not show any clinical signs before death (Suanyuk et al., 2008). Antibiotics are commonly used to control bacterial diseases including streptococcosis, but many complications often follow such as residual drugs and drug resistance, which greatly concern the consumer (Smith et al., 1994; Agnew and Barnes, 2007). Although vaccines are also available for streptococcosis, vaccination of fish is limited by the number of fish and the cost involved, as well as the difficulty in administering the vaccine. An alternative aquaculture that is free from chemicals and drugs has been developed to replace the old ways of farming. Probiotics, live beneficial microorganisms that promote host health by improving intestinal microbial balance (Fuller, 1989), are suitable candidates in controlling infectious diseases and in promoting growth performance in fish (Verschuere et al., 2000; Kesarcodi-Watson et al., 2008). To sustainably and safely control the problem for both fish and humans, the use of probiotics derived from humans or from the food industry is of interest (Nikoskelainen et al., 2001; Li and Gatlin III, 2003; Li and Gatlin III, 2004). Among probiotic candidates, bacteria are recognized as the most common probiotic microorganisms. However the transfer of antibiotic resistance genes among probiotic bacteria is widely mentioned by scientific researchers (Nayak, 2011). Other microorganisms such as yeasts are increasingly being considered as alternative promising candidates for probiotics. *Saccharomyces* is yeast that has a long history of use in human and animal food industries. Several *S. cerevisiae* strains from baking and brewing industries have been examined as probiotic candidates in aquaculture, since they have high nutritional value; they contain enzymes, fatty acids, vitamins and amino acids, as well as the ability to promote food digestion (Fuller, 1989; Kesarcodi-Watson et al., 2008; Lazado and Caipang, 2014). Their

immunostimulant components such as beta glucans, nucleic acids and mannan oligosaccharides have been shown to activate the immune response (Nayak, 2011; De et al., 2014; Lazado and Caipang, 2014). *S. cerevisiae* isolated from food industry was capable of promoting growth performance as well as modulating the immune response in many fish species (Waché et al., 2006; Abdel-Tawwab et al., 2008; Chiu et al., 2010; Lazado and Caipang, 2014). In Nile tilapia fry, supplementation of probiotic *S. cerevisiae* was able to protect against *Aeromonas hydrophila* infection (Abdel-Tawwab et al., 2008). It also reduced the absorption of add supplied or environmental copper in tilapia (Abdel-Tawwab et al., 2010). Additionally, it reduced mortality from an *Streptococcus iniae* bath challenge in hybrid striped bass (Li and Gatlin III, 2004). While many *S. cerevisiae* strains have been promoted as probiotic candidates in humans (Moslehi-Jenabian et al., 2010; Rajkowska and Kunicka-Styczyńska, 2012) and animals including aquatic animals (Simon et al., 2001), the bio-protective properties and mechanisms of action have not been thoroughly characterized. *S. cerevisiae* JMC 7255 is a yeast strain derived from the brewing industry. It showed the ability to stimulate human intestinal epithelial cells, which is important for initiating and regulating mucosal immunity to foreign substances (Saegusa et al., 2004). This strain can enhance secretion of interleukin (IL)-Iβ, IL-6, IL-8, IL-12, Monocyte chemoattractant protein (MCP)-1/Chemokine (C-C motif) ligand (CCL2) from human neutrophil-like HL-60 cells (Saegusa et al., 2009). *S. cerevisiae* JMC 7255 is therefore one of the potential probiotic candidate. However, the probiotic potential of this specific strain in aquatic animals is not known.

There is one problem concerning the use of probiotics in aquaculture. Probiotics have to be viable in sufficient numbers at the possible sites of action (intestine) in the gastrointestinal tract. Due to the diverse conditions in the aquatic animal 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, is now being developed in aquaculture nutrition, in order to improve the viability of candidate probiotics in the gastrointestinal tract environment. Probiotic particles were coated or entrapped within capsule materials, varying from less than one micron to several hundred microns in size (Bansode et al., 2010). A previous *in vitro* study revealed that the type and concentration of capsular matrix, the concentration of calcium chloride and the hardening time are the main factors defining the characteristics of the capsule. Most of the *in vitro* studies using alginate, a non-toxic, water soluble, anionic polysaccharide derived from the cell wall of brown algae, showed satisfactory results as a microcapsule material and is widely used in food industry. It can

improve the survival of probiotic microbes up to 80-95% (Mandal et al., 2006). However, information regarding the use of alginate-encapsulated probiotics in aquaculture is limited.

In this study, we applied the encapsulation and freeze-drying technique to *S. cerevisiae* JCM 7255 and tested its potential probiotic properties in tilapias. The *in vitro* viability and morphology of the candidate probiotic during storage and during transit within simulated tilapia gut and bile conditions were evaluated. The growth performance efficacy, and gut mucosal immune parameters of tilapia were assessed and anti-Streptococcal activity of *S. cerevisiae* was analyzed as well.

วัตถุประสงค์ของโครงการ **(Objectives)**

1. To apply microencapsulation technique in probiotic yeast production for sustainable tilapia culture
2. To determine the effect of encapsulated yeast (*S. cerevisiae*) on gut morphology and gut immune response in nile tilapias against streptococcosis.

ผลงานวิจัยที่เกี่ยวข้อง **(Literature review)**

Tilapia

Tilapias are tropical freshwater cichlid fish, native to Africa and the Middle East (Fryer and Iles, 1972). They are classified into *Tilapia, Sarotherodon* and *Oreochromis* according to their reproductive behavior. Among the three genera, *Oreochromis* is commonly used in aquaculture. *Oreochromis niloticus* or Nile tilapia is one of the most popular species that has been recognized as a food source for rural communities and aquaculture industry worldwide (FAO Fisheries & Aquaculture Thailand, 2006). They were introduced to Thailand in 1965 from Japan (FAO *Oreochromis niloticus*, 2010) and had been developed to be the cultured. Tilapias have many interesting characteristics, making them suitable for farming. They are omnivorous - eating a variety of natural food organisms (Dong et al., 2010). They can tolerate poor water quality including high water temperature, high water salinity, low dissolved oxygen and high concentration of ammonia (Prunet and Bornancin 1989; Atwood et al., 2003; Sardella et al., 2004). They also can tolerate the stress resulting from high stock density (Siddiqui et al., 1989). They grow and attain market size rapidly and finally, they are palatable (FAO Fisheries & Aquaculture Thailand, 2006). Tilapia markets all over the world have been expanding in recent years. Tilapia culture has become the most important aquaculture crop in many countries including Thailand.

Streptococcosis in tilapia

Streptococosis is a disease cause by *Streptococcus sp.,* which is a Gram-positive, encapsulated, facultative anaerobic cocci bacterium in pairs or chains. The bacteria in this genus cause serious diseases in a number of different hosts such as mammals, fish and also humans. In aquatic animals, the diseases can occur in both wild and cultured fish. In 1957, the first identification of *Streptococcus* sp. in cultured fish (rainbow trout) in Japan was reported (Hoshina et al., 1958). The bacteria have been identified from various parts of the world particularly in intensive culture. Besides, an outbreak of infection is always followed by a serious economic loss due to high morbidity and mortality (Bromage and Owens, 2002). *S. agalactiae* and *S. iniae* are the two species that are most reported. Both of them show similar clinical signs - spiral or erratic swimming, ocular abnormalities such as peri-orbital and intraocular haemorrhage, opacity and exophthalmia, reddening or haemorrhage in integumental and musculoskeletal systems, ascites and ulceration (Bromage and Owens, 2002). However, in some cases the fish may not show any clinical signs before death. Traditional classification of Streptococci is based on serogrouping of carbohydrate antigens of the cell wall and their haemolytic activities. *S. agalactiae* is a group B Streptococcus that can be either haemolytic or non-haemolytic on a blood agar plate. It has been recognized as the cause of mastitis in bovine, neonatal meningitis, sepsis, and pneumonia in humans. Although *S. agalactiae* is found in both humans and animals, zoonotic transmission seems to be non-existent or insignificant (Robinson and Meyer, 1966). *S. iniae* is a non-group *Streptococcus* and always hemolytic on a blood agar plate. *S. iniae* was first identified from multiple subcutaneous abscesses in freshwater dolphins. Like *S. agalactiae*, they can cause diseases in fish, mammals, and also in humans, but in contrast, *S. iniae* is zoonotic. *S. iniae* infections in humans can occur mainly in people with skin injuries caused by handling live or fresh fish. In humans, it brings about the development of cellulitis, which is occasionally localized in organs or joints (Lau et al., 2003; Facklam et al., 2005). Streptococcosis can infect fish via contaminated water. It can also infect fish by oral route via contaminated food and cannibalism of infected fish (Roy et al., 2010). The conventional methods to prevent and control Streptococcosis are chemotherapy and vaccination. Regarding vaccination, there were reports of vaccination of *S. iniae* in rainbow trout farms from 1995 to 1997 with good results in decreasing the mortality rate from 50% to 5%, but shortly after vaccination, new massive outbreaks occurred (Bachrach et al., 2001). Until now, there has not been any commercial vaccine that can give satisfactory results. Although using antibiotics is an effective way for treating infected fish, drug resistance usually happens amongst dense populations (Agnew and Barnes, 2007).

Microencapsulation

Microencapsulation is a process of single or mixed particles being coated or entrapped with other material on a small scale (Risch, 2010; Bansode et al., 2010). Capsules less than one micron to several hundred microns in size are produce for different aims including immobilization, protection, release or structure the material in the capsule. The first application of this technique was produced in carbonless copy paper where the ink capsule on the copy paper will breaks down when compressed, thus transferring the image (Boh and Sumiga, 2008). It is now well recognized in many different kinds of manufacturing activities including printing, textile, chemical, agricultural, drugs, cosmetics and food industries (Boh and Sumiga, 2008). The microencapsule generally comprises of two main parts: core and shell. The core is the material to be coated and the shell is the wall that coats the core. There are two forms of microencapsules: core-shell-encapsulation where all the core material is coated by shell and matrix capsulation where the core material is diffused throughout the shell. The important part that will conduct characteristic properties of microencapsule is the shell. The general abilities of the shell are film-forming, stabilizing core material, inert toward active ingredients, non-hygroscopic, having no high viscosity, economical and facilitating controlled release under specific conditions. For probiotic encapsulation the most-often-selected material is alginate (Mandal et al., 2006). Alginate is anionic polysaccharides from the cell wall of brown algae and has been used in food for many purposes. It is water soluble and non-toxic to probiotic cell. Calcium alginate is one the form of alginate capsule that has been widely used to encapsulate probiotics. It can improve the survival of probiotic up to 80-95% (Mandal et al., 2006) Previously studies show that the concentration of alginate, concentration of calcium chloride and hardening time are the main factors that conduct characteristic of the capsule. The study of Chandramouli et al., 2004 reports the significantly increased survival of probiotic cells in gastric condition (*in vitro* study) by using 1.8% alginate, 30 min hardening in 0.1 M $CaCl_2$ and capsule size 450μm with 10^9 CFU/mL initial cell load.

Probiotic use in aquaculture

Probiotics are microbial cell preparations or components of microbial cells adjunct, which beneficially affect the host animals. (Fuller, 1989, Salminen et al., 1999). In aquaculture, Verschuere et al.(2000) gave the broader definition of probiotic as a live microbial adjunct, which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambience thanks

to the environment of aquatic animal which host and pathogen share the same ecosystem. The general mode of action of probiotic are modulating host immunity, enhancing digestion, providing minerals and vitamins to host, inhibiting pathogens by producing inhibitory compounds, competing for chemicals or energy and for adhesion sites. However the action of probiotics is controlled by many factors.

Saccharomyces cerevisiae (*S. cerevisiae*) is a probiotic in the group of budding yeast. It has a thick wall and a round to oval shape about 5-10 micrometer in diameter. It can be found on the skin of fruit such as grapes. *S. cerevisiae* is commonly known as "bakers yeast" or "brewers yeast. It has been longer used in the food and drink industry for bread, beer, and wine. The cell wall of *S. cerevisiae* contains main part as polysaccharides (85%) and the other part is protein (15%) (Lesage and Bussey, 2006). The polysaccharides comprise of 90% glucose, 1-2%N-acetylglucosamine and 10-20% mannose (Lesage and Bussey, 2006). Chitin is also found in the lateral cell wall of *S. cerevisiae*. In aquaculture, it was reported to be able to protect against bacterial infectious diseases such as *Aeromonas hydrophila* (Siwicki et al., 1994).

Gut immune system in fish

Apart from skin and gills, the gastrointestinal tract is a site that is fully exposed to pathogen via water and food. Hosts use complex mechanisms to protect themselves from pathogen. Gastrointestinal peristalsis, gut epithelial lining, commensal microorganism and mucus coat work as the first barrier against pathogens (Matsunaga, 1998). In fish, mucus that cover on epithelial layer does not contain only glycoprotein, but also humoral immune substance such as lysozyme, complement proteins, antibacterial peptides and Immunoglobulin M (MagnadÛttir 2006). Lysozyme is considered as one of the important innate immune response for protect against microorganism invasion that can be found in both invertebrates and vertebrates (Saurabh and Sahoo 2008). It is a mucolytic with a single polypeptide chain of about 120 amino acids. In fish, lysozyme is released from leukocytes and mainly distributed in the head kidney. It is found in gills, skin, gastrointestinal tract and eggs (Lie et al., 1989). Lysozyme has dissimilar pathways to prevent the invasion of gram-positive and gram-negative bacteria because of since the different cell wall's structure. Beta linkages between N-acetylmuramic acid and N-acetylglucosamine in cell wall (peptidoglycanlayers) of Gram-positive bacteria will be splitted by lysozyme while the outer layer of Gram-negative will be interrupted by complement and other enzymes before lysozyme become effective. Furthermore, lysozyme has ability to damage chitin, the main structure of fungi. It also enhances phagocytosis by activating

polymorphonuclear cells and macrophages directly or using opsonization (Saurabh and Sahoo 2008).

Although fish intestines are lack of Peyer's patches, M cells, IgA and J chain (Rombout et al., 2010). They contain population of leucocytes, including macrophages, lymphocytes, granulocytes and plasma cells that play important role in mucosal immune response. Intraepithelial lymphocytes (IELs) scattering among the epithelial layer mostly conserve the function and structure of T-lymphocytes with thymus dependent (Bernard et al., 2006; Matsunaga 1998). The function of IEL in fish is still unclear (Kiristioglu et al., 2002). The previous study in rainbow trout shows that IEL mainly comprises of T lymphocytes characteristic similar to human and mouse (Bernard et al., 2006). In trout, there are two types of IEL and both of them show cytotoxic activity (McMillan and Secombes, 1997). On the other hand, IELs in carp does not show any specific or non-specific cytotoxicity, but can express IL-1 beta, IL-10 and TNF-alpha (Huttenhuis et al., 2006; Rombout et al., 2010). Furthermore, they can up- regulate pro-inflammatory cytokines (Urán et al., 2008). This suggests other functions of IEL in regulating the immune responses.

Acidophilic granulocyte (AG) or eosinophilic granular cell is one of main population of immune cells that can be found in intestine. It is a mononuclear cell that contains eosinophilic granules. It has functions and structures similar to mast cells in mammals (Reite and Evensen, 2006), but some morphology, cytochemistry, and function of the AG are different. For example, granules in acidophilic granulocytes are lack of histamines (Reite 1998). In addition, AG also functions as neutrophils in higher vertebrates (Mulero et al., 2007). It is mainly localizes around the blood vessels in the organ that interfaces with the environment such as intestine and skin (Ellis 1997, Blackstock, N. and Pickering, A.D. 1980). In the intestinal tract of teleost fish, AG is a part of the defense mechanism against infection and involves in inflammatory response (Dezfuli and Giari, 2008) It acts as a second line of defense after mucous. AG is degranulated after being injected with antigen and is followed by accumulation of leukocytes (Matsuyama and Iida 1999). According to earlier studies of feeding probiotics on gilthead seabream and European sea bass, it is suggested that the increase of AG cells without damage of intestinal structure as a parameter of enhancement of innate immune response (Picchietti et al., 2007; Picchietti et al., 2009).

Specific humoral immune responses also play an important role in gut mucosal immunity in fish. In mammal and avian, immunoglobulin A (IgA) is diffused on mucosal surface, but in fish only IgM-like molecules can be detected (Rombout et al., 2010). It is

produced from local B-lymphocytes that are located in the intestine and has similar functions to IgA (Morrison 2002, Rombout et al., 2010).

The oral administration of probiotics activates the immune system in a non-specific way, providing resistance against a variety of pathogens. Many researchers were able to use the mRNA expression of cytokine genes, important mediators of the immune system and represent an essential part of the innate immune response in fish, as a tool for measuring immune responses against pathogenic bacteria (Low et al., 2003; Pierce et al., 2004; Panigrahi et al., 2007). It should be suggested that induction of IL1 and TNF alpha cytokines by *L. rhamnosus* served as an important regulator of gut associated immune systems eliciting better non-specific immune responses. Additional evidence exists in turbots where the feeding of nucleotide-supplemented diets increased the expression of pro-inflammatory cytokines including IL-1 and TNF (Low et al., 2003). The result is in agreement with another report on rainbow trout where the complement activity and IL-1 beta gene expression was enhanced upon feeding *Enterococcus faecium* and *L. rhamnosus* supplemented diet (Panigrahi et al., 2004; Panigrahi et al., 2007). In aquatic animals, there have been many studies reported about probiotics modulating immunity such as inducing proinflammatory cytokines, stimulating the activity of natural killer cells, increasing mucosal and systemic antibody production, activating phagocytic activity and increasing lysozyme and complement activity (Nikoskelainen et al., 2003; Pirarat et al., 2006).

วิธีการวิจัย **(Procedure)**

***In vitro* study**

The encapsulation method that gives the best results on the *in vitro* experiment will be selected to prepare microecncapsules for the *in vivo* experiment.

Probiotic preparation

S. cerevisiae (JCM 7255) from glycerol stock that was kept at -80°C and cultured in Sabouraud Dextrose agar (SDA) at 30°C for 24h, before transferred to 100 mL of Sabouraud Dextrose broth (SDB).

Microencapsulation

The probiotic, *S. cerevisiae* was cultured in SDB at 30°C for 48h, refrigerated centrifuged at 4500x g at 4°C for 15 min, and washed with sterile Peptone Dilution Saline (PDS) three times. The *S. cerevisiae* was encapsulated in two groups. The first group (group A) was prepared by resuspending the *S. cerevisiae* in PDS at a final concentration of 10^9cfu/mL. The second group

(group B) was prepared by resuspending the *S. cerevisiae* in 10% skim milk at a final concentration of 10^9cfu/mL. Under sterile conditions, *S. cerevisiae* was encapsulated according to the method described by Chandramouli et al. (2004) with certain modifications. Alginate matrix was prepared by mixing 1.8% sterile sodium alginate solution with yeast suspension. The capsules were prepared aseptically by dropping the alginate mixture 30 cm from a 10-mL syringe through a 25G needle to sterile 0.1M calcium chloride solutions. The capsules were hardened in 0.1M $CaCl_2$ solutions for 30 minutes and washed with sterile distilled water. The yeast cells in calcium alginate capsules were determined the density.

Viability after being freeze-dried

To determine the loss of encapsulated yeast during freeze-dried process. The calcium alginate capsules were freeze-dried by freezing at -80°C for 2h followed by drying at -50°C for 48h. The numbers of yeast were determined before and after the freeze-dried process. Dried Calcium alginate yeast capsules were measured in weight and the yeast cell densities were determined.

Viability during storage

To assess the viability of encapsulated yeast during storage at room temperature (25°C). The encapsulated *S. cerevisiae* were kept at room temperature. 0, 7, and 14days after drying, 0.5 g of capsules was resuspended in sodium citrate and then shook at room temperature for 30 minutes. The complete release of yeast cells was determined by serializing the dilution in PDS before plating on SDA (ISO 6887-1).

Viability of free and encapsulated *S. cerevisiae* in simulated gastric conditions and bile salts
Acid tolerance

Simulated gastric solution was prepared with normal saline pH 1.5 (adjust pH by using 5M HCl). 0.5 g of each type of microcapsules was placed separately in test tubes containing 4.5 mL of simulated gastric solution then incubated at 25°C. Triplicate samples were taken after incubation for 0, 1, 2, 3 and 4h. The cell counts of encapsulated yeast were enumerated on SDA to determine the survival of free and encapsulated yeast in acid environment.

Bile tolerance

For bile salt tolerance, 0.5 g of each type of microcapsules were placed separately in each test tubes, containing 4.5 mL simulated gastric solution at 25°C. After incubation for 60min. the simulated gastric solution was removed and the microcapsules were placed in 10% tilapia bile

salts (Nikoskelainen et al., 2001). Triplicate samples were drawn after incubation at 30°C for 1, 2, and 3h. The cell counts of encapsulated yeast were enumerated on SDA agar.

Antimicrobial activity

The antimicrobial activity of fresh *S. cerevisiae*, encapsulated *S. cerevisiae* in PDS solution and encapsulated *S. cerevisiae* in 10% skim milk solution was determined by using agar spot test according to Pirarat et al., (2009) with modifications.

Agar spot test

S. cerevisiae from an overnight culture (24h) in SDB and each type of encapsulated *S. cerevisiae* were spotted on the surface of SDA after incubating at 30°C for 24 hours to allow the development of the colonies. After 24h, 50 µl of each strain of *S. agalactiae* (5×10^6cfu/ml) (25 strains) was inoculated in semi-solid TSA (TSB with yeast extract of 0.6%+ Agar 0.75%) and poured over the SDA (spotted with grown *S. cerevisiae*). The plates were incubated at 30°C for 24h and checked for inhibition zone. The inhibition zones were classified as (-) no visible inhibition, (+) 0.5 to 6 mm inhibition, (++) 7 to 12 mm, and (+++) more than 12 mm inhibition. The strains of *S. agalactiae* that were used in this study were isolated from the outbreak in tilapia farm in the central area of Thailand during 2008-2011 that confirmed by biochemical method and PCR.

***In vivo* study**

Fish, probiotic supplementation and *in vivo* experimental designs

420 tilapias, *Oreochromis niloticus*, with 20-30 g body weight, will be acclimatized for 7 days and randomly placed in ten 90-L tanks (30 fish per tank) for the control groups and the dietary supplementation groups. The tanks will be filled with recycled water that will be kept at 25°-28° C, 5.8–6.8 ppm dissolved oxygen (DO) and 6.5–7.0 in pH throughout the experiment.

Under sterile conditions, each dietary supplement will be mixed into commercial dry pellets and feed the fish in each treatment groups. The fish in the control group will be fed with the commercial dry pellets. They will be fed approximately 3% of body weight twice a day. At day 0, 14, 30 of feeding, the fish will be weighed for the growth performance.

The group of the experiment will be separated as follow:

Control	60	fish
Control free encapsulated capsule	60	fish
Encapsulated LGG 10^{10} cfu/g	60	fish

Growth parameters

After feeding the fish with the dietary supplement for a month, growth performance parameters, weight gain (%), specific growth rate and feed conversion ratio (FCR) were calculated by using the following equations (Yanbo and Zirong, 2005):

Weight gain (%) = 100 X (final mean body weight-initial mean body weight)/initial mean bodyweight

Specific growth rate = [(ln (final body weight) – ln (initial body weight)/days] x 100

Feed conversion ratio= feed intake (g) / Weight gain

Measurement of villous height

After 4 weeks of feeding three parts of the intestine, the proximal part (from after the pyloric part of the stomach to before the spiral part of the intestines), the middle part (the spiral part of the intestines) and distal part (from after spiral part of the intestines to 2 cm. before anus), from fish in the dietary supplement (n=6) and control groups (n=6) will be collected and fixed in 10% buffered formalin. The fixed tissues will be processed according to standard histological techniques and the tissue sections will be stained with haematoxylin and eosin (H&E). For the villous height measurement 10 highest villi will be selected per section. The villous length will be measured from the villous tip to the base. An average of these 10 villi per section will be expressed as the mean villous height for each section (Samanya and Yamauchi, 2002).

Intraepithelial lymphocytes (IEL) and Acidophilic granulocytes (AG)

After 4 weeks, the tissue of intestine of 6 fish in each group will be collected and separated into 3 parts following the above criteria and fixed in 10% buffered formalin. The fixed tissues will be processed according to standard histological techniques and the tissue sections will be stained with haematoxylin and eosin (H&E)

For IEL, the 10 highest villi which are selected to measure the villous height will be given an arbitrary score from 0 to 3 based on the frequency and population number: 0 - none, 1 - mild, 2 - moderate and 3 - marked IEL.

For measurement of acidophilic granulocytes, the presence of cells in lamina propia of each intestinal part will be totally counted (Gargiulo et al., 1998; Picchietti et al., 2009).

Immune related gene expression (Real Time RT-PCR)

Total RNA will be extracted from the intestine using a Trizol reagent. After the final wash with ethanol, the pellet will be air-dried, dissolved in diethylpyrocarbonate (DEPC)-treated

water and stored at -80 °C. The total RNA concentration and purity of the samples will be measured. For the TNF alpha, 5'-GCTGGAGGCCAATAAAATCA-3' and 5'-CCTTCGTCAGTCTCCAGCTC-3 will be used for sense and anti-sense primers, which span the region from 489 to 827 on the TNF alpha coding sequence to amplify a band of 339 bp. For the IL-1, the sense 5'-TGCTGAGCACAGAATTCCAG-3' and the antisense 5'-GCTGTGGAGAAGAACCAAGC-3' primers will be used, which span the region from 459 to 829 on the IL-1 coding sequence to amplify a band of 371-bp. L32 amplification will be used as a control to normalize the amounts of input RNA. The RT-PCR reaction mixture (20 µl) contained 1x iQ^{TM} SYBR Green Supermix (Bio-Rad, CA), 4 µl cDNA sample and 2 µl of the appropriate forward and reverse PCR primers. PCR conditions included an initial denaturation at 95 °C for 3 min, followed by a 40-cycle amplification consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 sec and extension at 72°C for 15 sec. As a control for each primer pair and each RNA sample, the cDNA synthesis reaction will be carried out in the absence of reverse transcriptase in order to identify whether the RNA samples will be contaminated by residual genomic DNA. Standard curves will be used for transformation of the C_t values to the relative number of cDNA molecules.

Statistical Analysis

The data were expressed as a mean ± S.D. and evaluated using one-way ANOVA followed by the Bonferroni-type multiple *t*-test. All tests used a significant difference level of $p< 0.05$.

ผลการวิจัย **(Result)**

Number of cell entrapped

The microcapsules were prepared by using 1.8% alginate mixed with suspension of *S. cerevisiae* in different solutions. The cell loading ranged from 1-2 x 10^8 cfu/g beads in both microcapsules.

Viability after being freeze-dried

The survival of skim milk group was significantly higher than Peptone distilled saline group (PDS); 7.9 log cfu/g beads, and 6.7 log cfu/g beads respectively (Fig.1)

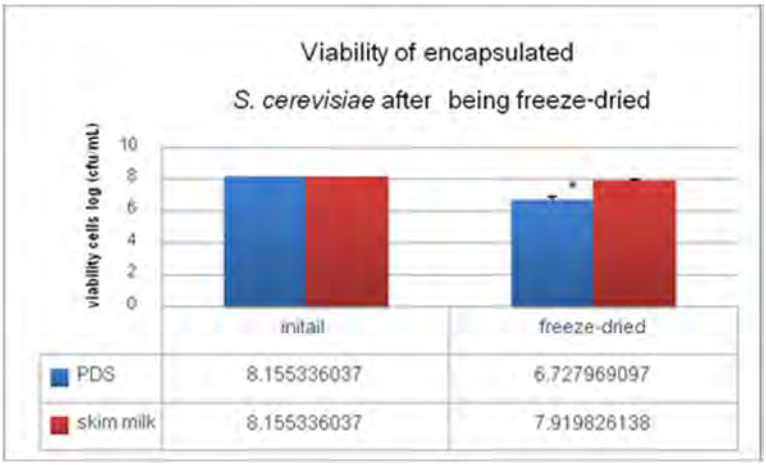


Figure 1. Viability of encapsulated *S. cerevisiae* after being freeze-dried was significantly different between the groups at p <0.05 (*)

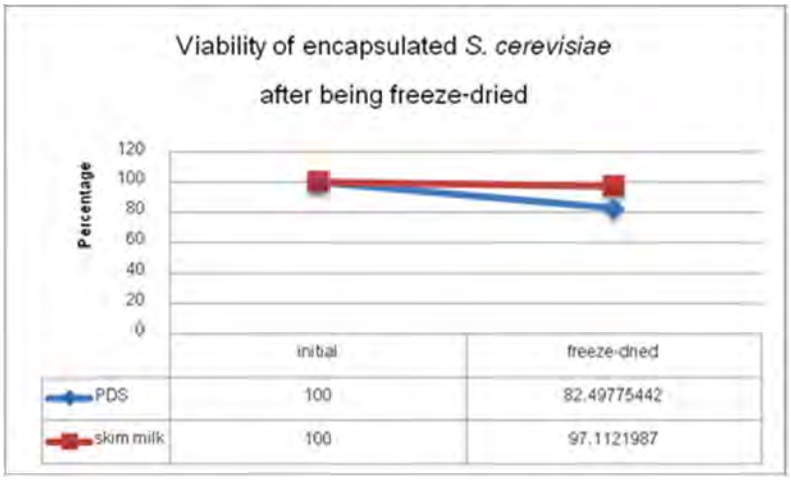


Figure 2. Percent viability of encapsulated *S. cerevisiae* after being freeze-dried

Viability of encapsulated *S. cerevisiae* during storage

The viability of free and encapsulated *S. cerivisiae* during storage at room temperature was shown in Fig 3. No viability of yeast cells was detected after 7 days in free cells. At 7 and 14 days of storage the viability of group B was significantly higher than encapsulated *S. cerivisiae* used PDS; 91% survival was recorded in group B and 81.8% and 79.4% survival was in group A at 7 and 14 days respectively.

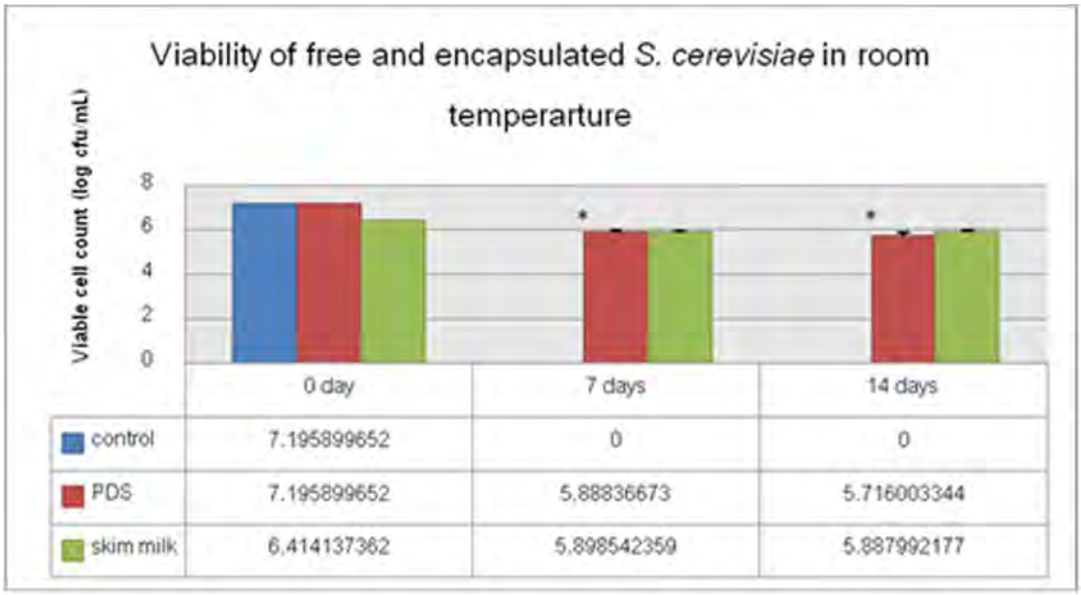


Figure 3. Viability of free and encapsulated *S.cerevisiae* during storage. Significantly different between the groups at $p < 0.05$ (*)

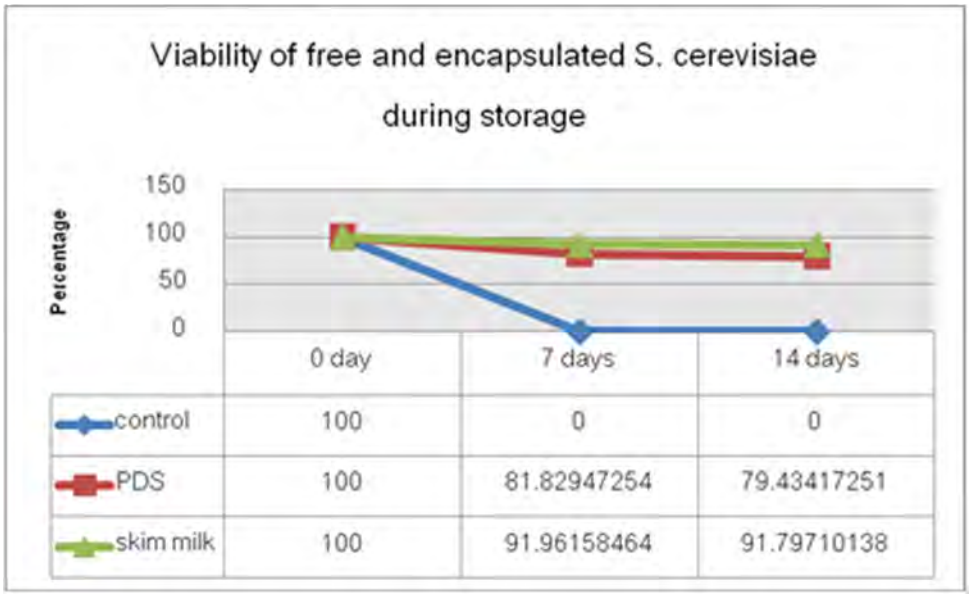
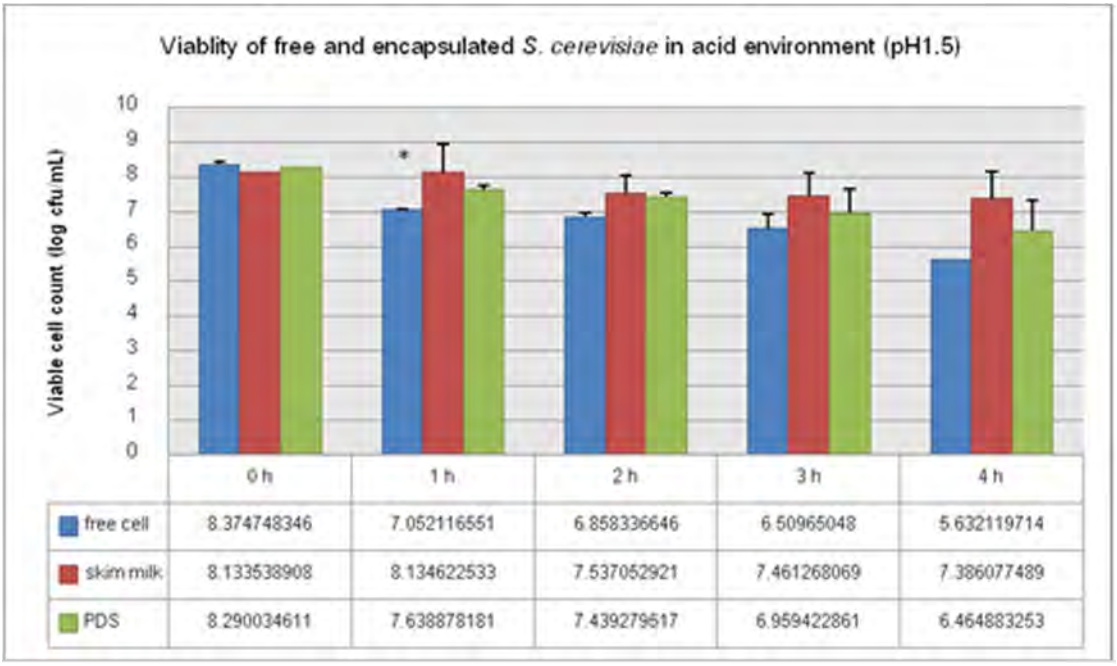


Figure 4. Percent viability of free and encapsulated *S.cerevisiae* during storage

Survival of free and encapsulated yeast in simulated gastric conditions and bile salts

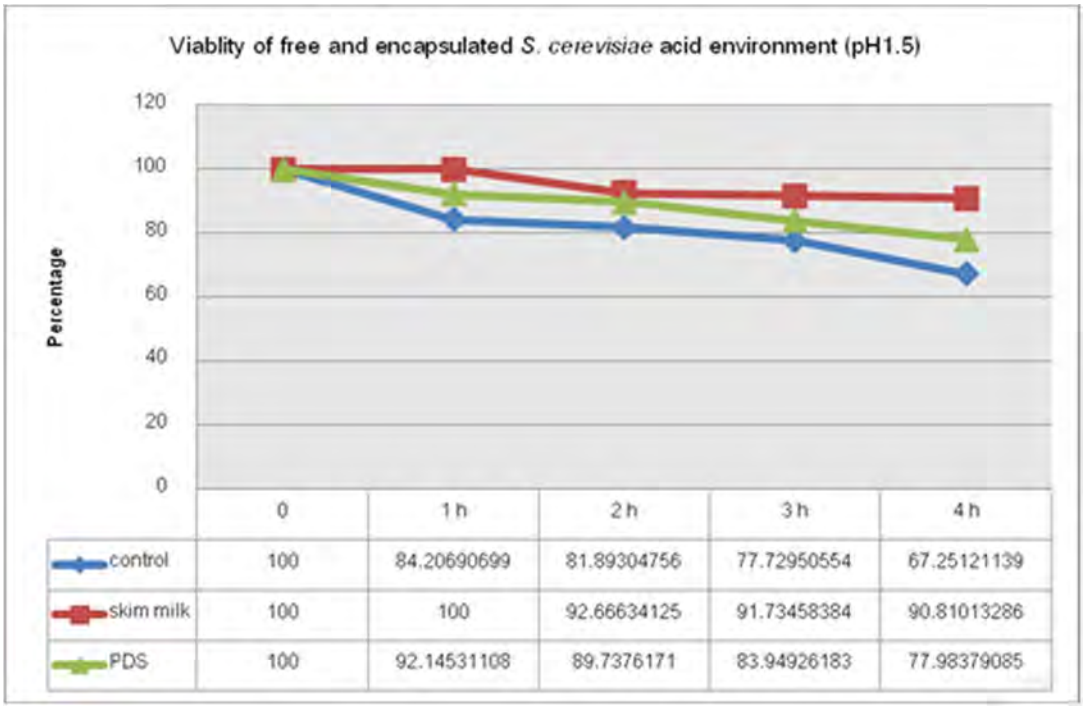
The survivals of free and encapsulted *S. cerevisiae* (PDS and skim milk) in gastric condition (NSS pH 1.5) in 0, 1, 2, 3, 4 h were represented in Fig. 5. The survival of *S. cerevisiae* continued to decreased in every passing hour but not at the same rate; at 0 h – 8.3 log cfu/mL, after 1 h – 7 log cfu/mL, 2 h – 6.8 log cfu/mL, 3 h – 6.5 log cfu/mL, 4 h – 5.6 log cfu/mL. The

viability of group A solution also decreased which was similar to free cells but slower by 0h – 8.2 log cfu/mL, 1 h – 7.6 log cfu/mL, 2 h – 7.4 log cfu/mL, 3 h – 6.9 log cfu/mL, 4 h – 6.4 log cfu/mL. However, there was no decrease in the viability of group B after being exposed to NSS pH 1.5 for 1 h which was significantly different from that of both free cells and group A. After 2 h, 3 h, and 4 h the survival rate of group B were 7.5 log cfu/mL, 7.4 log cfu/mL and 7.3 log cfu/mL, from 8.1 log cfu/mL (0 h).



	0 h	1 h	2 h	3 h	4 h
free cell	8.374748346	7.052116551	6.858336646	6.50965048	5.632119714
skim milk	8.133538908	8.134622533	7.537052921	7.461268069	7.386077489
PDS	8.290034611	7.638878181	7.439279517	6.959422861	6.464883253

Figure 5. Viability of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in acid environment was significantly different among the groups at $p < 0.05$ (*)



	0	1 h	2 h	3 h	4 h
control	100	84.20690699	81.89304756	77.72950554	67.25121139
skim milk	100	100	92.66634125	91.73458384	90.81013286
PDS	100	92.14531108	89.7376171	83.94926183	77.98379085

Figure 6. Percent viability of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in acid environment

Survival of free and encapsulated *S. cerevisiae* in tilapia bile salts

To determine the tolerance of free and encapsulated *S. cerevisiae* in *in vitro* proximal intestine, the samples were exposed to NSS pH 1.5 for 60 min, followed by a further incubation in 10% tilapia bile salts for 1 h, 2 h and 3 h. The results were represented in Fig.6. The survivals of free *S. cerevisiae* and encapsulated *S. cerevisiae* (both groups) continued to decrease from hour to hour with significantly difference in every hour that passed. At 1 h and 2 h after being exposed to 10% tilapia bile, the highest survival was group B followed by that of group A and that of free *S. cerevisiae.* At 3 h there was similar trend in the decrease among these three groups with the survivals of encapsulated *S. cerevisiae* using PDS and used 10% skim milk being significantly higher than free *S. cerevisiae* but there was no significant difference among encapsulated groups.

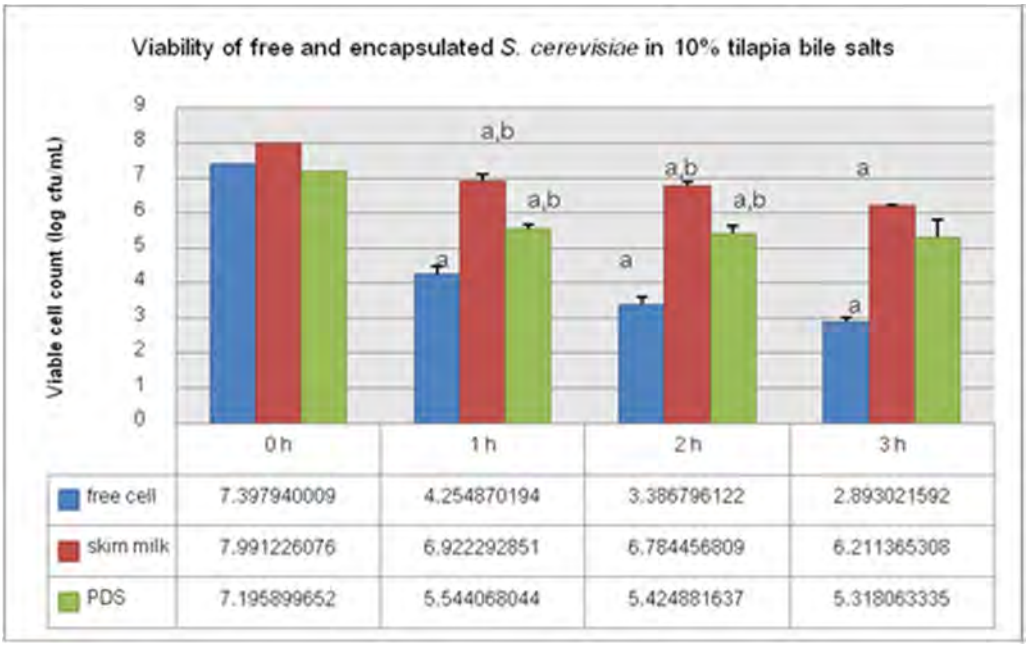


Figure 7 Viability of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in 10% tilapia bile salts. Means having the same letter are significantly different at $P < 0.05$

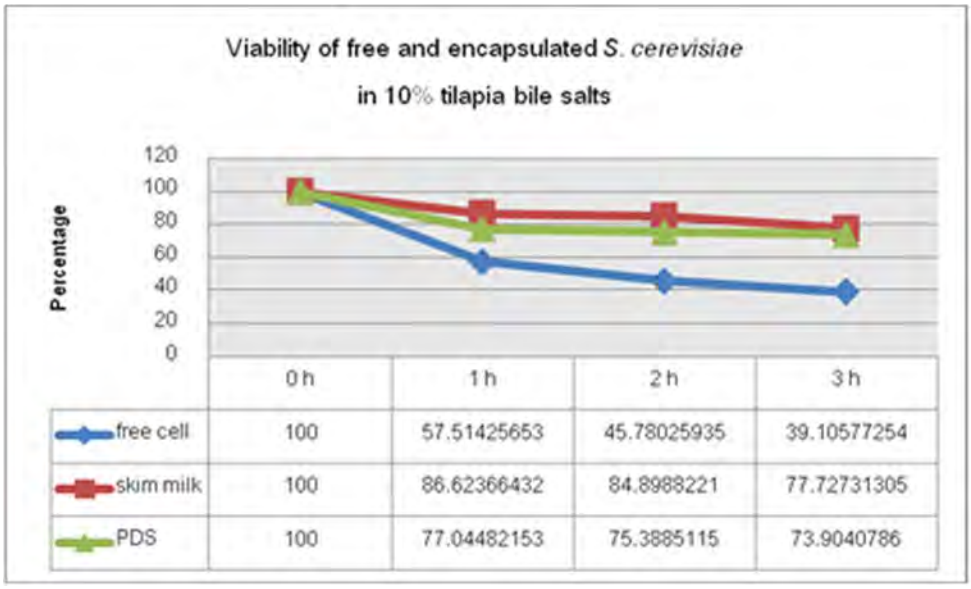


Figure 8 Percentage of survivals of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in 10% tilapia bile salts

Antimicrobial activity

The agar spot test of using free *S. cerivisiae* on SDA showed inhibition zones of 17 from 25 stains of *S. agalactiae* (Table 1). There was no inhibition zone in any strains of *agalactiae* undergoing agar spot test with killed yeast.

Isolation no.	Free S. cerevisiae	Group A (PDS)	Group B (skim milk)	Killed S. cerevisiae
SA065301	+++	++	++	-
SA075302	++	++	++	-
SA075303	-	-	-	-
SA075304	+	+	+	-
SA075305	-	-	-	-
SA075306	+	+	+	-
SA075314	+	+	+	-
SA075315	-	-	-	-
SA075316	+	++	++	-
SA075317	-	-	-	-
SA075318	-	-	-	-
SA075319	++	++	++	-
SA075320	+	+	+	-
SA015401	++	++	++	-
SA015403	-	-	-	-
SA015404	+	+	+	-
S.aga 1	++	+	++	-
S.aga 3	+	+	+	-
S.aga 4	-	-	-	-
SAT 1	++	++	++	-
SAT 2	-	-	-	-
SAT 3	+	++	+	-
SAT 4	+	+	+	-
CRCU1	+	+	+	-
SA CP	+	+	+	-

Table 1. Inhibition zone

Growth performance

Data on the growth performance of tilapias, including weight gain (%), specific growth rate, and feed conversion ratio (FCR) are shown in Table 2. The fish fed with encapsulated *S. cerevisiae* showed significantly higher weight gain (%) and specific growth rate than the fish fed with the control diet. The largest increases were observed in the fish feeding on encapsulated *S. cerevisiae* after 14 and 30 days, respectively. FCR in the encapsulated *S. cerevisiae* -treated fish was also significantly lower than in the control in the first (14 days) and second (30 days) phases of feeding.

Growth performance	Control	*S. cerevisiae* Capsule
Initial mean body weight	26.12 ± 1.5	24.95 ± 0.1	26.26 ± 0.19
Mean body weight 14 days	32.54 ± 0.1	32.51 ± 0.3	33.12 ± 0.3
Mean body weight 30 days	34.15 ± 0.16	35.53 ± 0.15	36.63 ± 0.09
Weight gain % 14 days	24.58^{a} ± 0.54	30.26^{b} ± 1.14	26.16^{a} ± 1.42
Weight gain % 30 days	30.76^{a} ± 0.32	$42.39^{b,d}$ ± 1.12	39.53^{d} ± 0.94
Specific growth rate 14 days	1.57^{a} ± 0.03	1.89^{b} ± 0.06	1.66^{a} ± 0.08
Specific growth rate 30 days	0.89^{a} ± 0.01	1.18^{b} ± 0.02	1.11^{d} ± 0.02
Feed conversion ratios 14 days	1.70^{a} ± 0.03	1.38^{b} ± 0.05	1.6^{a} ± 0.09
Feed conversion ratios 30 days	2.92^{a} ± 0.03	2.12^{b} ± 0.06	2.27^{b} ± 0.05

Table 2 Growth performance of Nile tilapia after feeding probiotics supplemented diet for 30 days. Mean values in same row with different superscripts differ ($p < 0.05$) among groups

Intestinal histopathology and measurement of intestinal fold height

After 30 days, the fish fed on encapsulated *S. cerevisiae* had higher intestinal folds than the control group. Significantly higher intestinal folds were observed in the proximal and distal parts of the intestines of the encapsulated *S. cerevisiae* group compared to control group (Fig 9). There was no significant difference in the middle part of the intestine among three groups. For the distal part, the villous heights in the capsule and encapsulated *S. cerevisiae* groups were significantly higher than the control group.

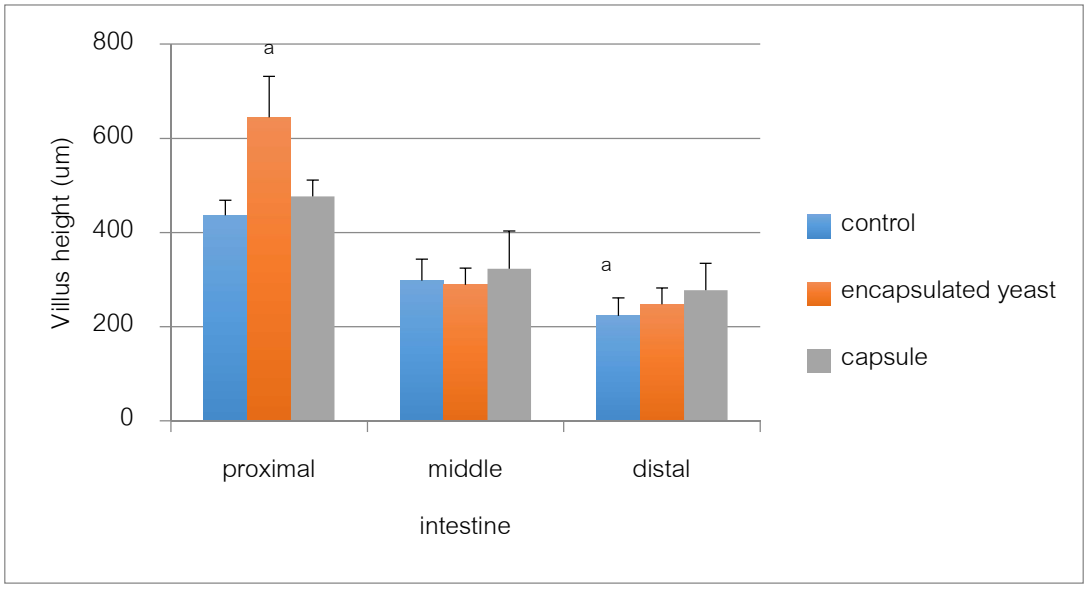


Fig. 9 Average villus height of the intestine of encapsulated yeast, capsule and control group fish after feeding for 30days. Data for the different sampling regions with different letters significantly differ ($p < 0.05$) among groups

Acidophilic granulocytes (AG), intraepithelial lymphocytes (IEL) and mucous cells

The acidophilic granulocytes in the capsule group were higher than in the control and encapsulated *S. cerevisiae* groups in the proximal and middle parts of the intestines (Fig. 10). The acidophilic granulocytes in the distal part of the intestine in the encapsulated *S. cerevisiae* group were higher than in the control and capsule groups. Intraepithelial lymphocytes in the encapsulated *S. cerevisiae* group were significantly greater than in the control and capsule groups in the proximal part of the intestine (Fig 11). Among the three groups, there was no significant difference in the IEL in the middle and distal parts of the intestine.

The mucous cells were stained with a combination of Alcian blue (AB) and periodic

acid-Schiff reagent (PAS). The mucous cells were blue, red or purple. There was no significant difference in the proximal and distal parts of the intestine among the three groups. The mucous cells in the middle part of the intestine for the capsule group were significantly higher than the encapsulated *S. cerevisiae* group, but were not significantly different when compared to the control group (Fig 12).

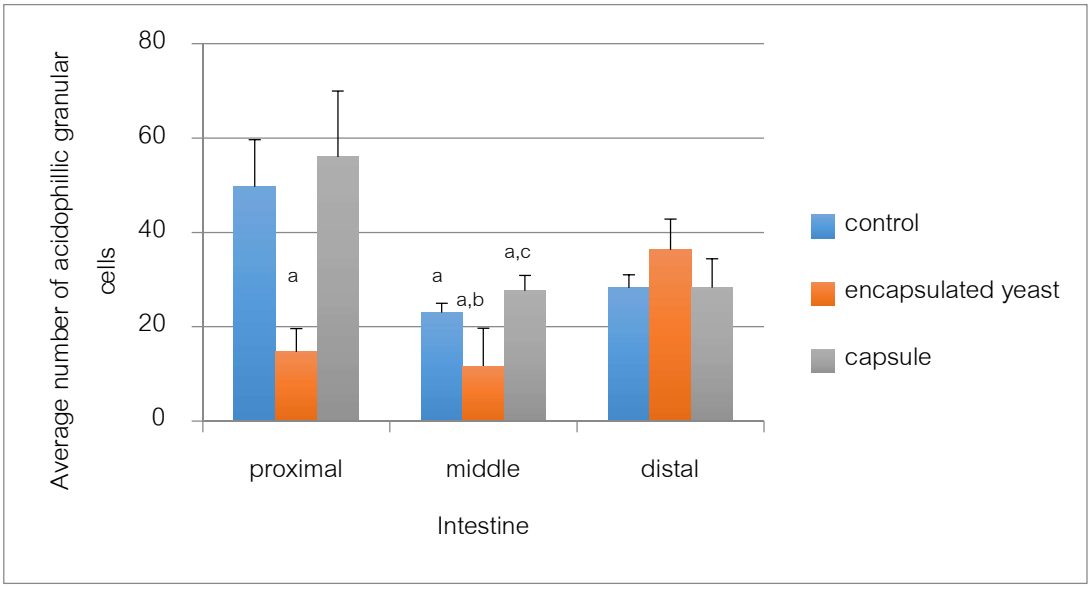


Fig. 10 Average number of acidophilic granulocytes in intestines of encapsulated yeast, capsule and control group fish after feeding for 30 days. Data for the same sampling region with different letters significantly differ ($p < 0.05$) among groups

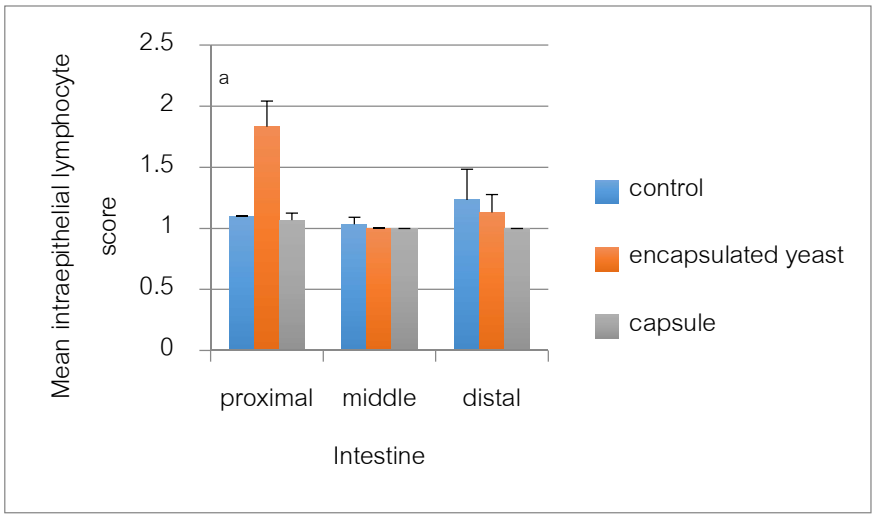


Fig. 11 Mean score of intraepithelial lymphocytes in intestines of encapsulated yeast, capsule and control group fish after feeding for 30 days. Data at the same sampling time with different letters significantly differ ($p < 0.05$) among groups

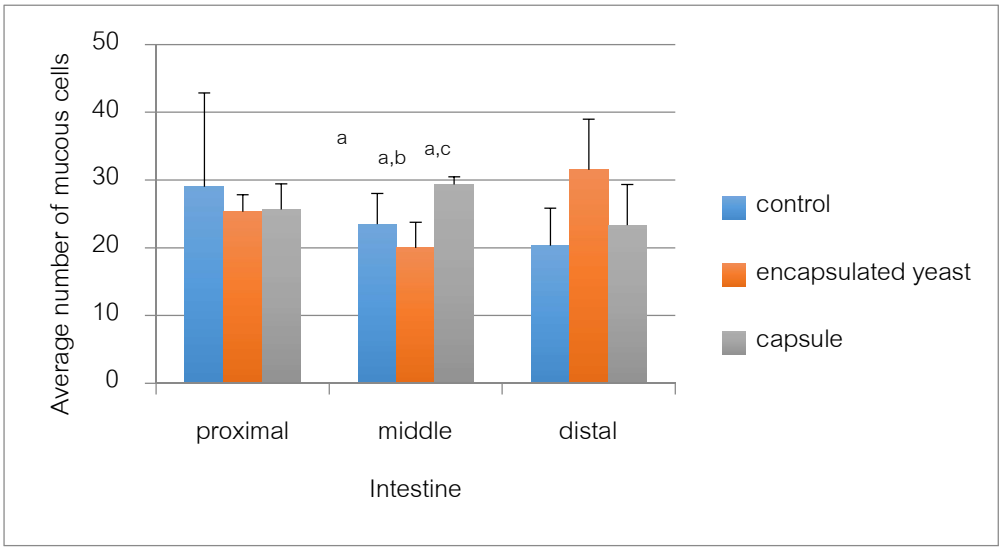


Fig. 12 Average number of mucous cells in intestines of encapsulated yeast, capsule and control group fish after feeding for 30 days. Data at the same sampling time with different letters significantly differ ($p < 0.05$) among groups

Challenge with *S. agalactiae*

The mortality of fish in the *S. agalactiae* challenge was initially observed on the first day post infection (DPI) in all test groups. The mortality ceased on 3 DPI in the encapsulated *S. cerevisiae* group, while in the control and capsule groups, mortality occurred continuously until 11 and 14 DPI, respectively. Cumulative mortality in the probiotic group (70%) was statistically insignificant from the control (90%) and capsule groups (84%) (Fig 13). *S. agalactiae* was recovered from spleens and livers of death fish in all groups (100%).

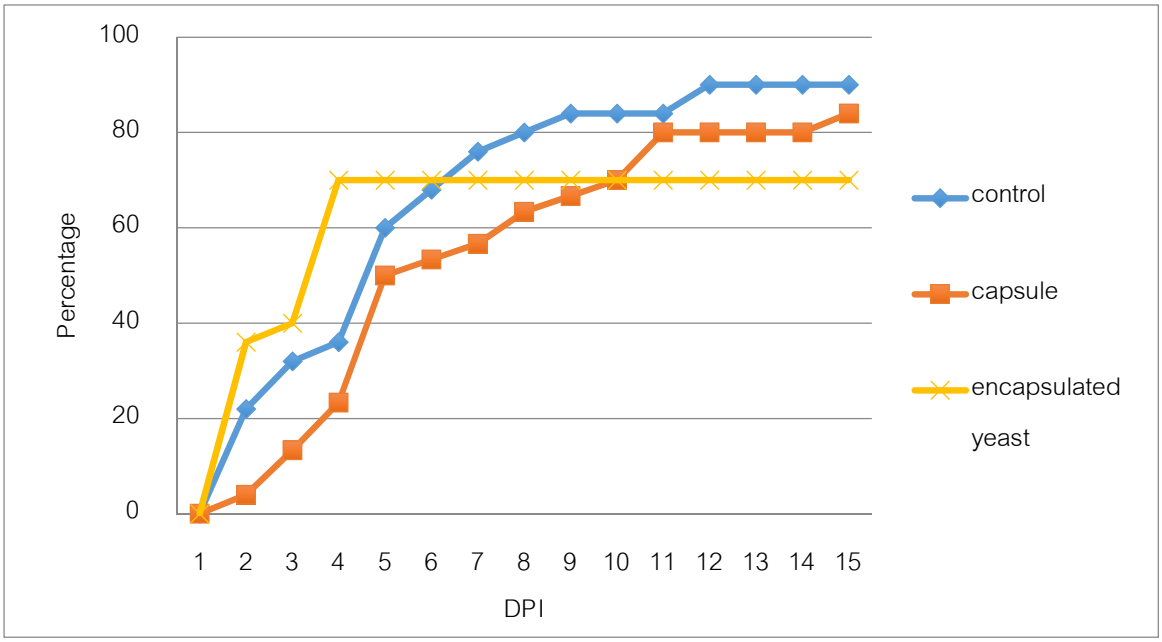


Fig. 13 Average cumulative mortality of encapsulated yeast, capsule and control group fish during *S. agalactiae* challenge trial

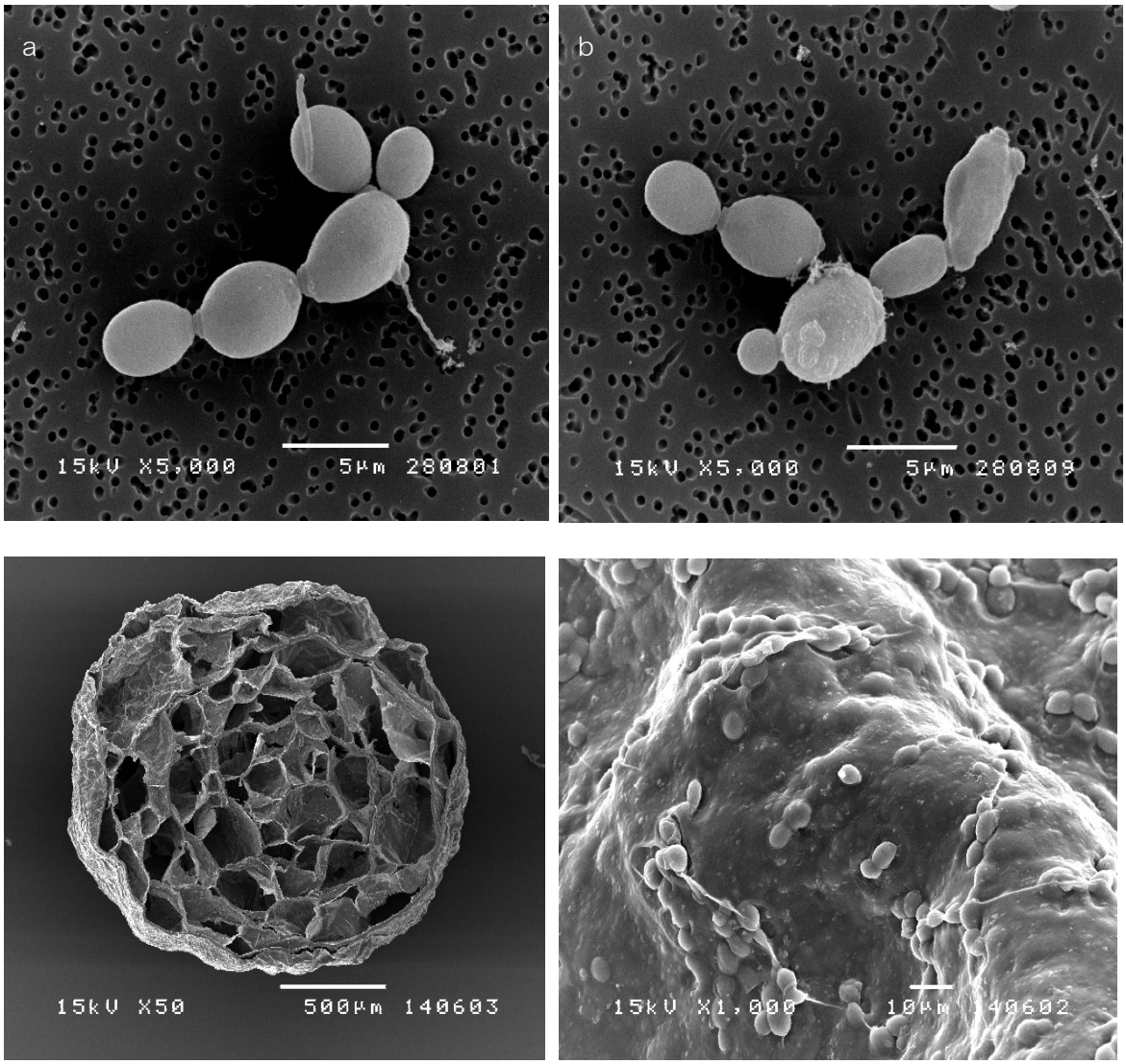


Fig 14. *S. cerevisiae* 0h (a): had round to oval shape with a smooth slightly bumpy surface. *S. cerevisiae* 1h treated with NSS pH 1.5 (b): had slightly flatten oval shape, with a smooth bumpy surface and 1-2 bud scars, erosion on the surface (c): spherical shape with wrinkled surface, cross section of microencapsule of *S. cerevisiae* in alginate-skim milk capsule(c) surface of microencapsule of *S. cerevisiae* in alginate-skimmed milk capsule (d)

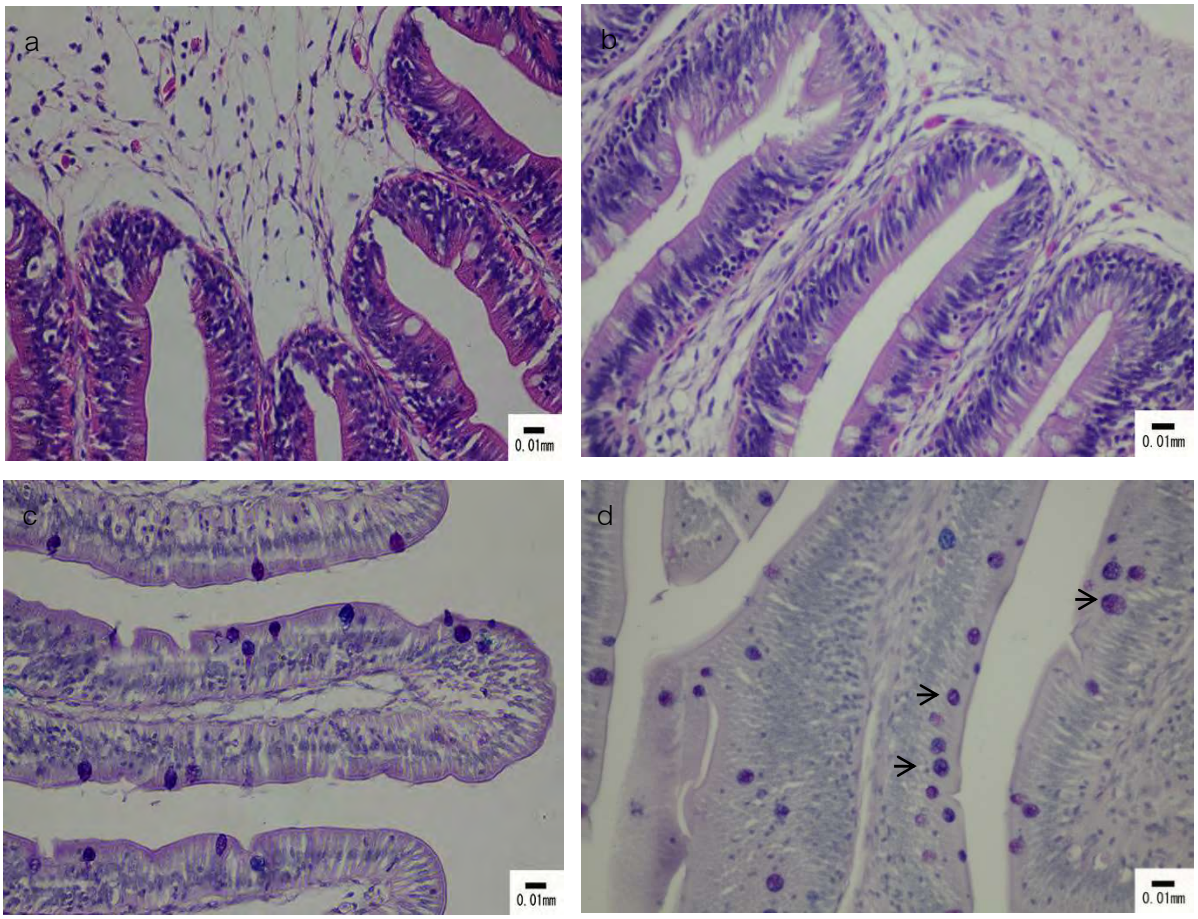


Fig. 15 Histology of proximal part of intestine of control (a) and probiotic (b) fish after feeding for 30 days (a); acidophilic granulocytes (arrow) (H&E) (b) Histology of proximal part of intestine of control (c) and probiotic (d) fish after feeding for 30 days; mucous cells (arrow) PAS and AB

Immune related genes expression

After oral challenge with *S. agalactiae*, the relative expression of the IL-1 and TNF alpha gene was high in variation among the intestine and spleen of probiotic fish and control when compensated with that of the L-32 gene (Fig. 16-21). Interpretation of immune related gene expression could not be specifically determined.

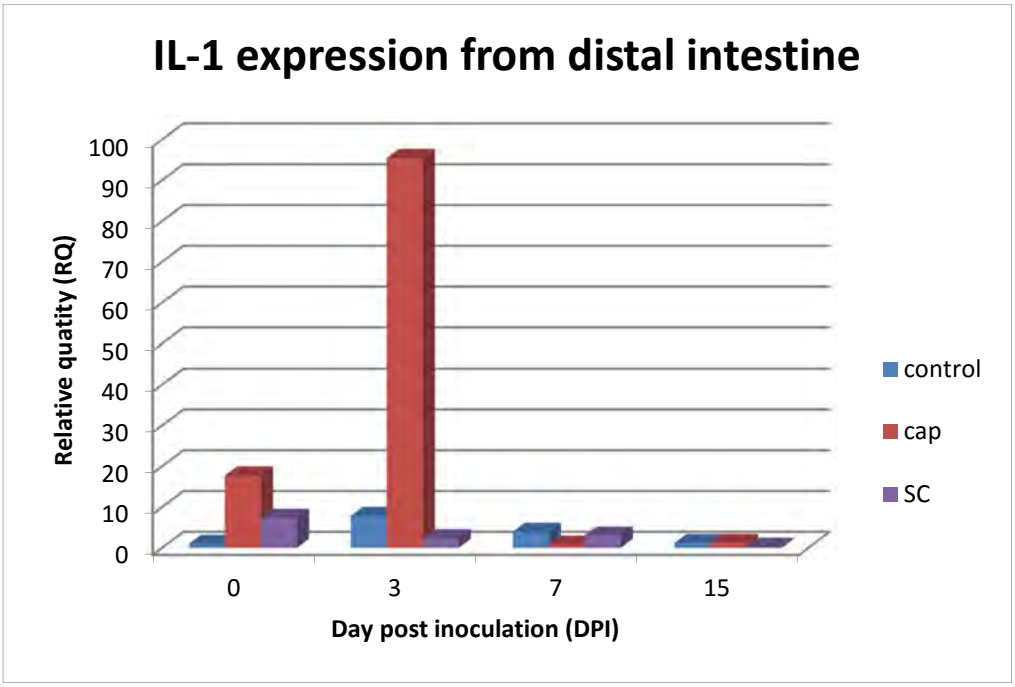


Fig 16. Immune response (IL-1 expression) of probiotic and control tilapia (distal intestine) after oral challenge with *S. agalactiae* (* significant difference between the groups; ANOVA with Bonferroni-type multiple t-test, $P < 0.05$)

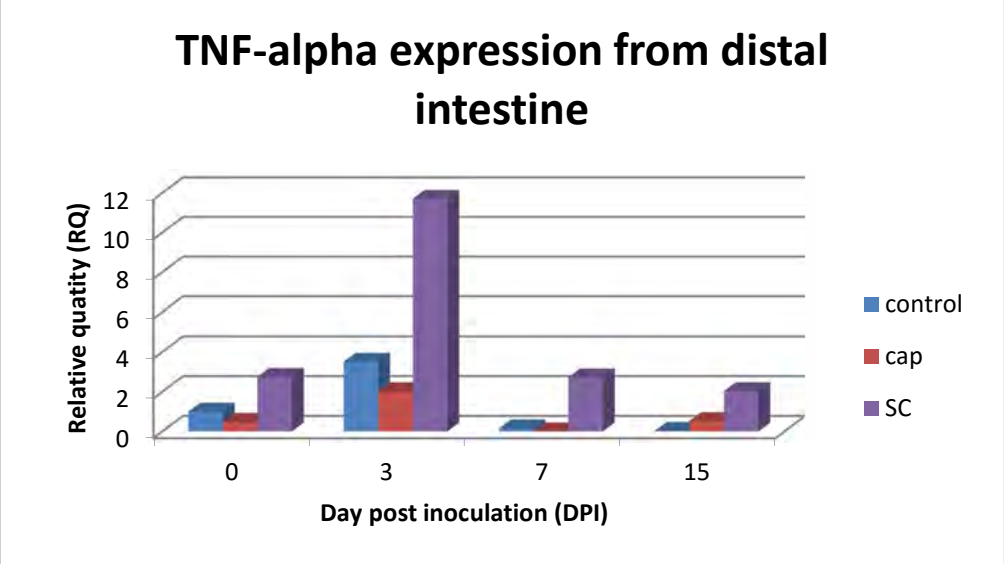


Fig 17. Immune response (TNF-alpha expression) of probiotic and control tilapia (distal intestine) after oral challenge with *S. agalactiae* (* significant difference between the groups; ANOVA with Bonferroni-type multiple t-test, $P < 0.05$)

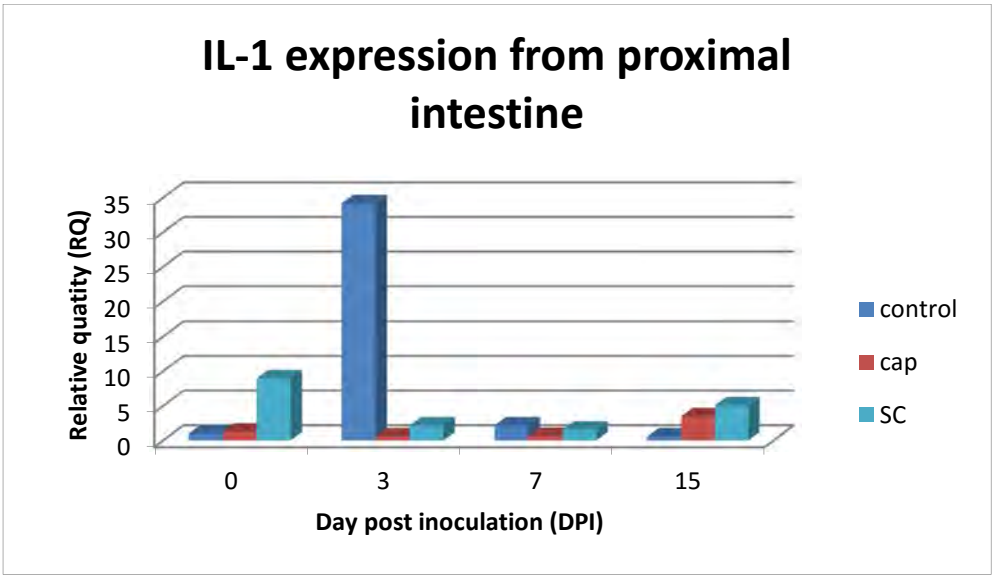


Fig 18. Immune response (IL-1 expression) of probiotic and control tilapia (proximal intestine) after oral challenge with *S. agalactiae* (* significant difference between the groups; ANOVA with Bonferroni-type multiple t-test, $P < 0.05$)

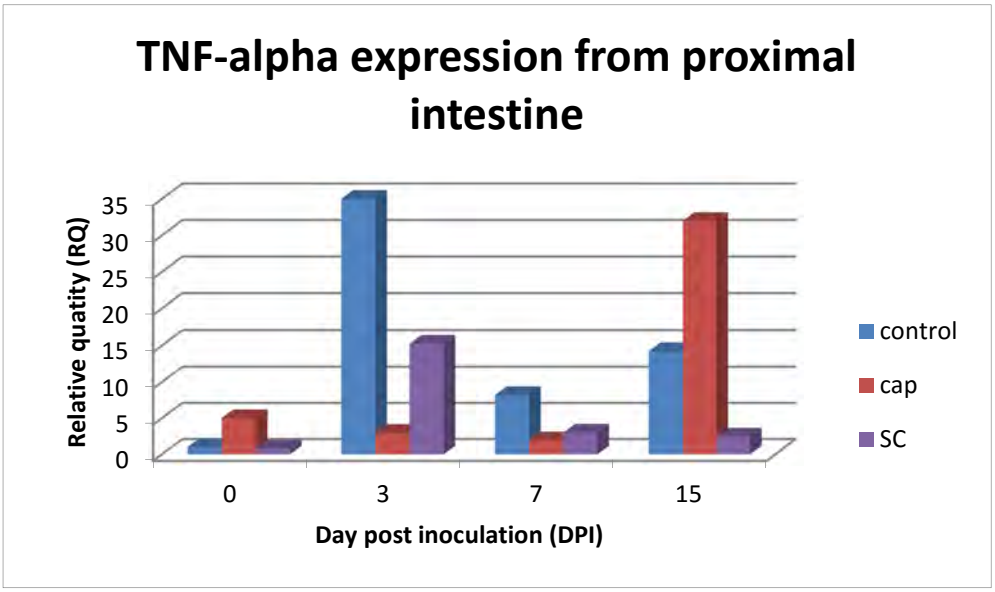


Fig 19. Immune response (TNF-alpha expression) of probiotic and control tilapia (proximal intestine) after oral challenge with *S. agalactiae* (* significant difference between the groups; ANOVA with Bonferroni-type multiple t-test, $P < 0.05$)

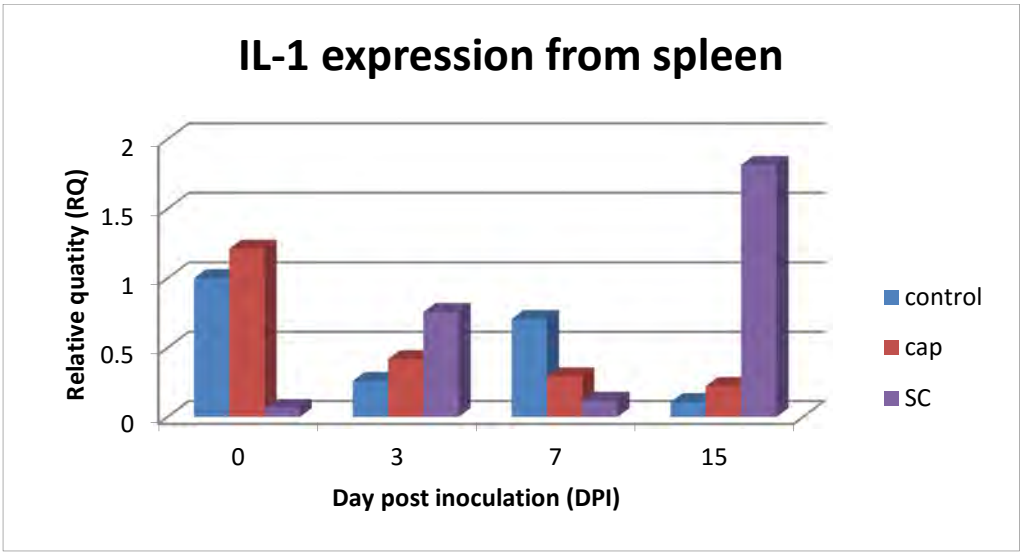


Fig 20. Immune response (IL-1 expression) of probiotic and control tilapia (spleen) after oral challenge with *S. agalactiae* (* significant difference between the groups; ANOVA with Bonferroni-type multiple t-test, $P < 0.05$)

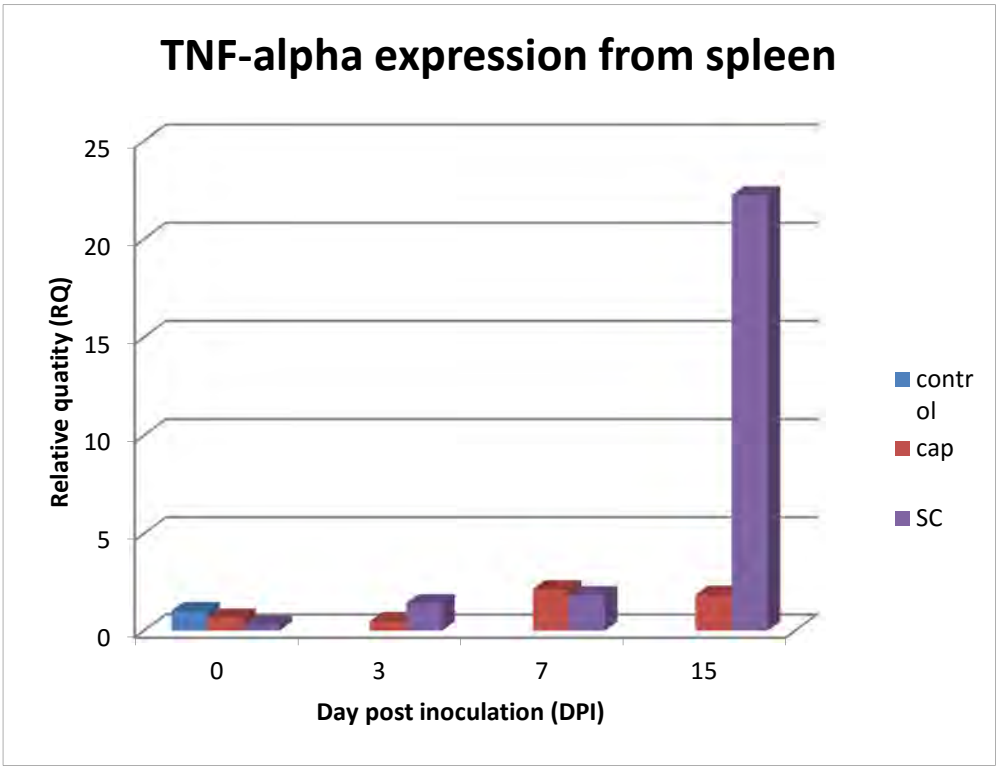


Fig 21. Immune response (TNF-alpha expression) of probiotic and control tilapia (spleen) after oral challenge with *S. agalactiae* (* significant difference between the groups; ANOVA with Bonferroni-type multiple t-test, $P < 0.05$)

วิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต **(Discussion and further research)**

Encapsulation is a method that has been used to improve the viability of probiotics in unflavorable condition. There are numerous materials and techniques that can be used to give different advantages. In this study, the extrusion and alginate were used.

The loss of viability during encapsulation in this present study was extremely low in both microencapsules using PDS and 10 % skim milk. There was no significant difference of viability between two groups of microencapsule, which suggested that using PDS and 10% skim milk as solution had no effect on cell viability during encapsulation process. The loss of viability during encapsulation process was due to two main factors, the material and the method. Alginate, anionic polysaccharide from algae is the material that was used in this study, which was reported to be non-toxic to the probiotic cells. The alginate matrix has less than 17 nm pore size (Klein et al., 1983) which can retain *S. cerevisiae*, with has diameter around 5-10 μm. This result was similar to the studies using the extrusion method for encapsulation (Krasaekoopt et al., 2003; 2006). The extrusion technique was reported to be a gentle method to encapsulate living cells with less viability loss during the process, but then may not be practical for large-scale production because of slow formation of the microcapsule (Mortazavian et al., 2007; Burgain et al., 2011). The size of both types of microcapsules was ranged from 1-1.5 mm. which was rather smaller when compared with other encapsulated probiotic from the extrusion method through the syringe. The size of microcapsule depended on many factors including the viscosity of alginate, the distance between the syringe and batch, and the diameter of extruder hole (needle) (Mortazavian et al., 2007).

Freeze-drying is a technique used to immobilize living cells, which is essential to its long-term preservation. The technique, however, may damage the cells in many ways. During the process, cells are exposed to the risks of chemical imbalance and temperature that can cause membrane damage, macromolecule denaturation and cell rupture from internal ice formation. The removal of water may also affect the properties of many hydrophilic macromolecules (Khoramnia et al., 2011). Different microorganisms have different responses to the freeze-drying procedure due to the various physiological states of the cells (Blanquet et al., 2005). The reported survival rates of *S. cerevisiae* ranged from 0.1% to 98% due to the strains and the conducted freeze-drying conditions (Lodato et al., 1999; Blanquet et al., 2005; Cerrutti et al., 2000). In this experiment, the survival rate of group A was significantly higher than group B. This variance in the survival rates may be the result of different solutions used to re-suspend the yeast cells, as skim milk has been known and used in the food industry as an effective cryo-protectant. The skim milk protects the cells by stabilizing their membranes and creating a sponge-like structure

that assists the rehydration process (Selmer-Olsen et al., 1999). Above all, the skim milk provides the cells protective coating during freeze-drying process. 5-20% of skim milk is suggested to be the most appropriate concentration, which was used in this study. After being freeze-dried, the PDS and 10% skim milk encapsulated *S. cerevisiae* was stored in room temperature. On the 7^{th} and 14^{th} day after being freeze-dried the yeast cells were detected; 1-log decrease was found in the encapsulated *S. cerevisiae* using 10% skim milk and 2-log decrease was found in the encapsulated *S. cerevisiae* using PDS. The higher viability of the 10% skim milk encapsulated *S. cerevisiae* might be related to the microcapsule dissolving process due to skim milk is a good media that can stimulate the growth of microorganisms.

From light microscope, the yeast cells could be seen inside the microcapsule using PDS, but the yeast cells were invisible in the microcapsule using 10% skim milk. Smooth, yellowish white substance was seen instead. This may happen because of the turbidity of skim milk that was a suspension. After freeze-dried the microcapsules were examined by SEM, which revealed the different surface of two types of microcapsules. The microcapsule group B presented rough surface while the microcapsule group A presented rough with concave polygonal ridges. The difference of surface appearance could be a result from different chemical compositions (Chen et al., 2005).

Among the groups of microorganisms that have been used to be probiotics, yeast is generally believed to be the most tolerant specie that can survive in unflavorable environment such as low pH in stomach or bile salt from proximal intestine. However, this present study showed that the survival of *S. cerevisiae* continued to decrease with time in low pH condition. Furthermore, the survival rate of *S. cerevisiae* significantly decreased after being exposed to NSS pH 1.5 and transferred to 10% tilapia bile salts which remained around 57%. On the other hand, the survival of encapsulated *S. cerevisiae* in gastric condition and 10% bile salts was higher in both types of alginate-microencapsules when compared with free cells. The results of this study was in contrast with the previous study by Sultana et al. (2000) which reported that alginate used as the matrix for encapsulation could not protect the organisms from high acid environment. On the other hand, the results were compatible to several studies that showed the improvement of the survival of microorganisms that used alginate as the matrix (Graff et al., 2007). The difference in the results from various reports was possible due to the difference in velocity, concentration of alginate, and the sizes of microcapsule that were used in each study. Several studies revealed improvement of the survival of probiotic when used in combination with alginate and other materials since alginate could be affected by acidic environment and chelating agent that can absorb calcium ion such as phosphorus. In this study skim milk was used to mix

with alginate. The microcapsules group B gave the better protection in low pH and 10% tilapia bile salts than the microcapsule group A. Since calcium ion in the skim milk could inhibit the leaching of calcium ions from microcapsule (Truelstrup-Hansen et al., 2002), therefore, reduced the deterioration of the capsules.

The present study on the agar spot test showed the efficacy of the antimicrobial activity of *S. cerevisiae* on 17 from 25 strains of *S. agalactiae*. The agar spot test using killed yeast, which showed no inhibition zones, which demonstrated that only the metabolite products, not the yeast cells, are involved in the growth inhibition of *S. agalactiae*. In this study, fresh *S. cerevisiae* and encapsulated *S. cerevisiae* gave similar results in agar spot test, which indicated that encapsulation did not affect the antimicrobial characteristic of *S. cerevisiae*.

The data from this experiment showed that microencapsulation of *S. cerevisiae* in alginate matrix combine with 10% skim milk can limit the degradation of *S. cerevisiae in vitro*. Microencapsulation method could be used as a major procedure in probiotic application in aquaculture.

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ภาคผนวก

Output จากโครงการวิจัยที่ได้รับทุนรัชดาภิเษกสมโภช

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ **(Proceeding)**

1.1 *in vitro* antimicrobial activity of saccharomyces cerevisiae against streptococcus agalactiae isolated from nile tilapia (The 5th Conference and Congress of Asian Society of Veterinary Pathology (ASVP), Bogor, Indonesia 2011)

1.2 *In vitro* viability study of calcium alginate-encapsulated probiotic *Saccharomyces cerevisiae* in gastric condition and bile salt of tilapia. The 38th International conference on Veterinary Science, Bangkok, Thailand 2013.

2. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ **(Journal)**

2.1 The study on probiotic properties of encapsulated yeast, *Saccharomyces cerevisiae* JCM 2755, in nile tilapias (*Oreochromis niloticus*)
(Research in Veterinary Science Journal)