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Bioencapsulation of probiotic *Lactococcus lactis* subsp. *lactis* on *Artemia franciscana* nauplii: Effects of encapsulation media on Nauplii survival and probiotic recovery

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ABSTRACT

Aims: This study aimed to investigate the suitability and efficacy of various encapsulation media in bioencapsulating the probiotic *Lactococcus lactis* subsp. *lactis* in *Artemia franciscana* nauplii. The impact of the encapsulation media on nauplii survival and probiotic recovery was also determined.
Methodology and results: Various encapsulation media (sodium alginate, palm oil, starch, gum Arabic and gelatin) were prepared by dissolving the respective media in artificial sea water. Each media was prepared in four different concentrations: 0.25, 0.5, 1.0 and 2.0 g/L. To determine the suitability of encapsulation media on the survivability of *A. franciscana*, survival rate (SR) of *Artemia* nauplii was determined after 8 hours post-encapsulation. Instar II stage *Artemia* nauplii at 1 nauplii per mL was used for each replicate. The result revealed that *A. franciscana* reached 100 % SR in the encapsulation media at ≤ 0.5 g/L. All media enabled > 23 % recovery of *L. lactis* subsp. *lactis* from encapsulated *A. franciscana*, which is similar (p > 0.05) to the recovery of free-cells (non-encapsulated) of *L. lactis* subsp. *lactis*. Noticeably in sodium alginate (E1) treatment, the total counts of *L. lactis* subsp. *lactis* in bioencapsulated *A. franciscana* were the highest among others, accounting for 2.44×10^7 CFU/mL per *A. franciscana* tissue homogenate.
Conclusion, significance and impact of study: *Artemia* nauplii bioencapsulated with *L. lactis* subsp. *lactis* using 0.5 g/L sodium alginate as encapsulation medium has the highest SR for nauplii and bioencapsulation efficiency, respectively. This result provides a basic guideline for *Artemia* bioencapsulation in fish/shrimp larval culture.

Keywords: *Artemia franciscana*, encapsulation media, *Lactococcus lactis* subsp. lactis, nauplii survival, probiotic recovery

INTRODUCTION

The use of *Artemia franciscana* nauplii as live food source for aquaculture species has long been established due to their nutritional and operational advantages (Lavens and Sorgeloos, 1986; Sorgeloos *et al.*, 1998). These live feed nauplii are being used as bio-vectors to deliver various nutritional (essential fatty acid, vitamins), and therapeutic (vaccines, probiotic) components to the larval stages of aquatic animals (Campbell *et al.*, 1993; Sorgeloos *et al.*, 1998; Hafezieh *et al.*, 2010; Gunasekara *et al.*, 2012). The process of incorporating those nutritional ingredients to the nauplii is termed as bioencapsulation (Sorgeloos *et al.*, 1991).

In recent years, *Artemia* nauplii have been used specifically in probiotic bioencapsulation to improve hatchery production and fish survival in aquaculture (Dagá *et al.*, 2013). At the initial stage of larval development, probiotics are often used as an alternative to antibiotics to control and prevent bacterial diseases (Gomez-Gil *et al.*, 2000; Burr and Gatlin 2005; Villamil *et al.*, 2010; Heo *et al.*, 2013). According to studies, bioencapsulated *Artemia* with probiotic strains such as *Lactobacillus* sp., *Bacillus* sp. and *Saccharomyces* sp., prior to larval feeding could effectively improve the survival, growth and the balance of gut microflora of fishes and shrimps (Deeseenthum *et al.*, 2007; Iranshahi *et al.*, 2011; Dagá *et al.*, 2013). The most commonly used method in bioencapsulation for aquaculture is through exposure of the *A. franciscana* nauplii (or other bio-vectors such as rotifers) to probiotic cultures in the liquid medium. This is usually done with bacterial free-cell suspensions (Martínez-Díaz *et al.*, 2003; Patra and Mohamed, 2003). Through this method, colonization of bacteria occurs via attachment to the body surfaces or ingestion by the live feed, which can further nourish their gut system, and maintain their viability and metabolic activity in the gut system (Grisez *et al.*, 1996; Gomez-Gil *et al.*, 1998; Picot and Lacroix, 2004). Nevertheless, encapsulation of free-cell forms to live feed is hampered by challenges, such as low survival rate of bacteria in high acidic conditions of the stomach and the small

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intestine (due to enzymes and bile salts) (Li *et al.*, 2009). Hence, probiotic living cells requires a physical barrier in the live feed, which could resist the harsh gastrointestinal environment. As a solution, approaches of bioencapsulation using various encapsulation materials to increase the resistance of probiotic bacteria against adverse conditions have been proposed (Li *et al.*, 2009). De Vos *et al.* (2010) further revealed that the survival of probiotic bacteria was poor when they remained as free cells in liquid form. It is therefore crucial that the viability and attachment efficacy of probiotic in live feed at the point of consumption is kept optimum, to confer health benefit to the hosts.

To perform successful bioencapsulation on live feed, the following criteria are identified. Firstly, the encapsulation media should be harmless to the live feed, thus optimal survival of the live feed in the encapsulation media should be taken into consideration. Secondly, it is capable to entrap bacterial cells thus allowing the recovery of bacteria during larval digestion, and lastly the medium should have no chemical residues in tissues after consumed by the fish and crustacean larvae. As such, the common media used in the encapsulation of probiotics are usually materials used for food industry. These include polysaccharides (*k*-carrageenan, alginate), plants (starch and its derivatives, gum Arabic), bacteria derivatives (gellan, xanthan) and animal proteins (milk, gelatin); all of which have shown promising results in bacterial microencapsulation (Rokka and Rantamäki, 2010). This technique if apply to current bioencapsulation technology could potentially benefit the aquaculture industry from the points of probiotic recovery and media selection.

Due to the importance of bioencapsulation efficiency, the present study was aimed to investigate the suitability and efficacy of food-grade encapsulation media such as sodium alginate, palm oil, starch, gum Arabic and gelatin to improve the recovery of probiotic *Lactococcus lactis* subsp. *lactis* in the bioencapsulation process of *A. franciscana*.

MATERIALS AND METHODS

Preparation of gnotobiotic *Artemia*

Five grams of *Artemia franciscana* cysts (Great Lake Artemia, Salt Lake City, Utah, USA) were incubated in a 500 mL Artemio® set (JBL, Neuhofen, Germany) connected to an aerator, and filled with sterile artificial seawater (20 ppt, pH 7.5). The cysts were incubated under continuous aeration at 26 ± 2 °C for 20 – 24 h (Talpur *et al.*, 2012; Touraki *et al.*, 2013). Newly hatched nauplii were collected using a sieve net and surface-disinfected with 10 mL/L Ovadine® containing 10 % povidone-iodine (Syndel Laboratories Ltd., Canada) for 10 min.

Media for bioencapsulation

The encapsulation media used in this study are those commonly used for the encapsulation of probiotic bacteria in the food industry. These include sodium alginate, palm oil, starch, gum Arabic and gelatin (Rokka and Rantamäki, 2010; Burgain *et al.*, 2011). The stock media (2.0 g/L) were prepared by dissolving respective medium: Sodium alginate (R & M Chemicals, UK), starch (Fisher Scientific, UK), gum Arabic from acacia tree (Sigma, USA) and gelatin (R & M Chemicals, UK) in artificial sea water (sterile water contained 10 g/L Instant Ocean® Sea Salt, USA).

Crude palm oil was obtained from a palm oil mill (Tingkayu Sdn. Bhd.) located at Lahad Datu-Kunak, Sabah, Malaysia (4'49'39.49" N 118'3'47.0" E). The palm oil emulsion was prepared by mixing soybean-extracted emulsifier, L-α-phosphatidylcholine (Sigma-Aldrich™, USA) at a ratio of 4:1 (oil: emulsifier), based on emulsification methods described previously (Estévez *et al.*, 1998; Agh and Sorgeloos, 2005; Loh *et al.*, 2012). The mixtures were then blended vigorously with artificial sea water (10 ppt) using an electric blender (MX-799S, Panasonic, Malaysia) for 5 min. All encapsulation media were prepared in the stock solution at a concentration of 2.0 g/L. The media were then autoclaved at 121°C for 45 min and allowed to cool to room temperature prior to use.

Effect of encapsulation media on *Artemia* nauplii survival

Survival rate (SR) of *Artemia franciscana* in different encapsulation media and concentration was evaluated to determine the most suitable media and the respective concentration for subsequent bioencapsulation test. Encapsulation media were prepared at different concentrations of 0.25, 0.5, 1.0 and 2.0 g/L. Instar II stage *Artemia* were stocked at a density of 1 nauplii per mL in each of the encapsulation medium. A total of 20 nauplii were used for each replicate. Sterile artificial seawater (10 ppt) was used as control in this study. Three replicates were performed for the experiment. SR of *Artemia* nauplii in encapsulation media was calculated after 8 h of bioencapsulation, based on the formula (Singh *et al.*, 2011):

$$SR\ \% = (N_1 - N_2) \times 100$$

Where, N_1 = Total number of nauplii survived; and N_2 = Initial number of nauplii stocked in the experimental vessel.

Effect of encapsulation media on bacterial recovery from *Artemia* nauplii

Probiotic strain *Lactococcus lactis* subsp. *lactis* CF4MRS (GenBank accession number: KM488626) was cultured in 200 mL de Man Rogosa and Sharp broth (MRS broth, Difco™ BD, USA) at 26 ± 2 °C for overnight (Loh *et al.*, 2014). The cell density of *L. lactis* was adjusted to 10^8

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CFU/mL at an OD_{540} of 0.8 and the bacterial cells were collected by centrifugation (6000 × *g*, 24 °C, for 10 min). The liquid supernatant was then discarded. Bacterial cell pellets were collected and re-suspended in different encapsulation media.

The media concentration for subsequent bioencapsulation was selected based on 100 % SR of *Artemia* nauplii as described previously. Six encapsulation media at selected concentrations were prepared as follows: free-cell suspension of *Lactococcus lactis* subsp. *lactis* (control), sodium alginate + *L. lactis* (E1), palm oil emulsion+ *L. lactis* (E2), starch + *L. lactis* (E3), gum Arabic + *L. lactis* (E4) and gelatin + *L. lactis* (E5). All media were prepared at the concentration of 0.5 g/L. Approximately 300 – 350 nauplii per mL of surface-disinfected *A. franciscana* (instar II) were firstly collected using sterile Miracloth (Calbiochem, Merck, Germany), and then transferred into 30 mL of respective encapsulation media in 50 mL sterile tubes. The procedure was carried out in a laminar flow, and room temperature was maintained at 26 ± 2 °C. During the bioencapsulation process, tubes with *A. franciscana* were swirled gently at every 30 min in order to allow oxygen to dissolve into the liquid media. After 8 h of incubation, the *Artemia* nauplii were collected using sterile Miracloth, and washed with 1 mL sterile saline solution (0.85 %, NaCl), then macerated using a homogenizer (LabGEN®125, Cole-Parmar, USA). To estimate bacterial concentration in the tissues, the homogenate suspensions were serially diluted in sterile saline until dilution factor 10^{-9} (Badhul Haq *et al.*, 2012). A 100 µL aliquot was pipetted and spread-plated on de Man Rogosa and Sharp agar (MRS, Difco™ BD, USA), then followed by incubation at 26 ± 2 °C for 24-48 h. The experiment was performed in triplicates. Colony-forming units (CFU) on plates were counted to estimate the bacteria encapsulated in *Artemia nauplii*.

Statistical Analysis

The differences between groups in SR and bacterial counts of *A. franciscana* were analysed using one-way ANOVA and *post-hoc* analysis (Tukey test). All statistical significance was accepted at *P*-value < 0.05 (Zar, 1999). Statistical analysis was performed using SPSS® Statistics software Version 20.

RESULTS

Effect of encapsulation media on *Artemia* survival

Starch and gelatin as bioencapsulation media allowed better SR (> 95%) of *A. franciscana*, regardless of their concentrations. This is followed by sodium alginate, palm oil emulsion and gum Arabic (Figure 1). When referring to media concentration, the SR of *A. franciscana* in control (sterile seawater) was recorded at 100%. No mortality of *A. franciscana* was found in all encapsulation media at 0.5 g/L. When the concentration of media was increased to 1.0 g/L, only *A. franciscana* that enriched in palm oil emulsion, starch and gelatin media achieved 100% survival (Figure 1). The SR of *A. franciscana* in sodium alginate medium at same concentration (1.0 g/L) showed 88 % of SR. Among the encapsulation media, 98% of SR in *A. franciscana* was recorded in starch and gelatin, followed by 85 % *A. franciscana* survived in gum Arabic at the highest concentration of 2.0 g/L. At this concentration, SR of *A. franciscana* was only 55% in palm oil emulsion (Figure 1).

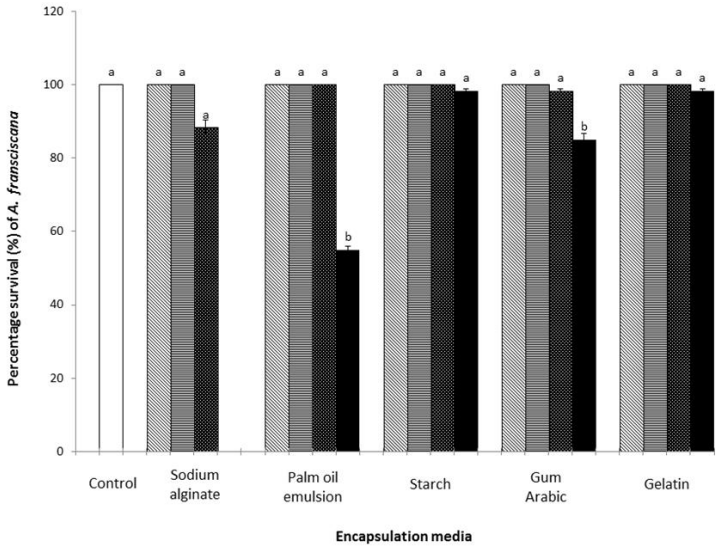

Figure 1: The survival rate (in %) of *A. franciscana* in different concentrations of encapsulation media: 0.25; 0.5; 1.0 and 2.0 g/L. Mean values with different alphabets within media are significantly different ($HSD_{0.05}$). Vertical bars indicate standard deviation of means.

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Effect of encapsulation media on bacterial recovery from *Artemia* nauplii

Total bacterial count in treatments (E1, E2, E3, E4 and E5) showed an increasing trend compared to the control (*A. franciscana* administrated *L. Lactis* free-cell). In the control treatment, total bacterial recovery was 1.42×10^7 CFU/mL from an initial inoculum of 1×10^8 CFU/mL (Figure 2). There is no significant difference ($p > 0.05$) in the total bacterial recovery between treatments and control. However, the total bacterial counts in *A. franciscana* using sodium alginate (E1) were the highest accounting for 2.44×10^7 CFU/mL, and the lowest count was from encapsulation with palm oil emulsion (E2) with 1.75×10^7 CFU/mL.

Bacterial recovery from encapsulation using starch (E3) was also relatively higher (2.24×10^7 CFU/mL), followed by gum Arabic (E4) (2.03×10^7 CFU/mL), and gelatin (E5) (2.06×10^7 CFU/mL) (Figure 2). The results demonstrated that higher bacterial count from encapsulated *A. franciscana* is recovered compared to the control, suggesting the benefits of bioencapsulating *L. Lactis* to *A. franciscana*. Generally, the optimal SR of *A. franciscana* could be achieved when using sodium alginate as a bioencapsulation medium at the concentration of 0.5 g/L.

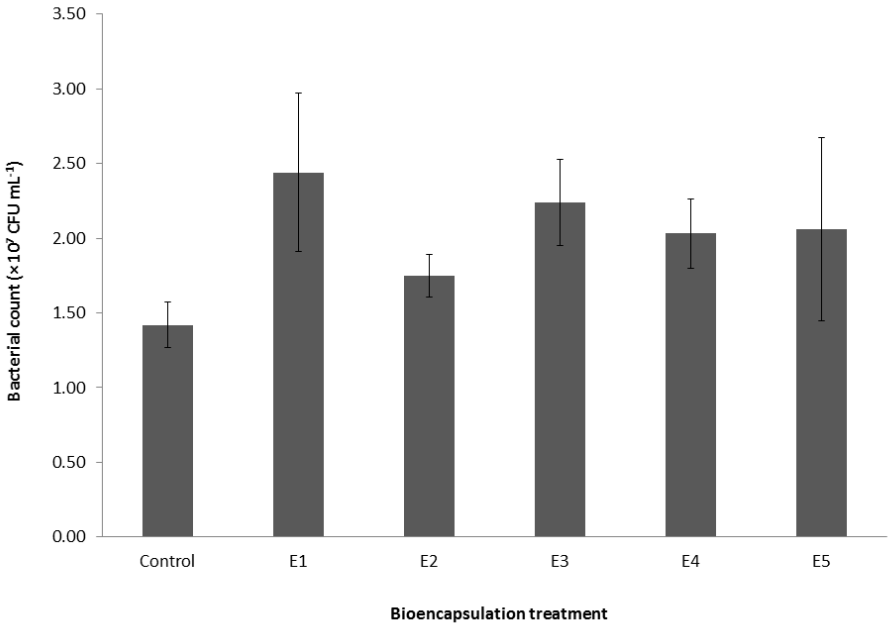

Figure 2: Recovery of *L. lactis* from *A. franciscana* after 8 h of bioencapsulation in *L. lactis* free-cell suspension (control), and after exposure to 0.5 g/L of: sodium alginate + *L. lactis* (E1); palm oil emulsion + *L. lactis* (E2); starch + *L. lactis* (E3); gum Arabic + *L. lactis* (E4) and gelatin + *L. lactis* (E5). Vertical bars indicate standard deviation of means. No significant difference among treatments ($p > 0.05$).

DISCUSSION

The present study reveals the SR of *A. franciscana* was not significantly affected by the type of media used. However, the media concentrations have a more important role. In the experiment, *A. franciscana* achieved 100 % SR in different encapsulation media at lower concentrations such as 0.25 and 0.5 g/L before introduction of probiotic bacteria *L. lactis* subsp. *lactis*. Nevertheless, increase in the media concentration to 2.0 g/L in sodium alginate (E1), palm oil emulsion (E2) and gum Arabic (E4) seemed to be a limiting factor to the SR of *Artemia* (Figure 1). This could be probably due to high viscosity of the media, and this was particularly observed in sodium alginate medium. High viscosity in the media demands the generation of large amounts of energy by the crustaceans for them to clean up the thoracic limbs clogged by high particulate concentrations. The large energy consumption due to the cleanup may consequently lead to the starvation of the crustaceans (Porter *et al.*, 1982; Burak, 1997; Nandini and Sarma, 2000). Similar observation was reported in laboratory-cultured *Moina macrocopa*, whereby overall biological performance such as fecundity, longevity, life expectancy and cumulative birth rate deteriorated when the cladocerans were cultivated using food particulate at concentrations higher than 0.0625 g/L (Loh *et al.*, 2013). This perhaps explained the mortality of *A. franciscana* caused by the increasing concentration of media in the present study.

Rapid probiotic recovery in larval gastrointestinal system is vital to establish a balance microflora

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ecosystem prior to colonization of potential pathogenic bacteria. Therefore, it is important to understand the recovery rate of encapsulated probiotics. In the present study, the probiotic bacterial concentration was pre-determined at 10^8 CFU/mL to standardize the initial inoculation dosage. The test concentration (0.5 g/L) was then selected based on the SR of *A. franciscana* from earlier observation. Results showed that total bacterial count in *A. franciscana* using sodium alginate as an encapsulation medium for *L. lactis* was the highest (2.44 × 10^7 CFU/mL) compared to other media. This result was in agreement with Li *et al.* (2009), the authors also demonstrated that microencapsulation of probiotic bacteria with alginate alone or together with gelatin could recover up to 10^7 CFU/g of *Lactobacillus casei* ATCC 393. Nevertheless, the recovery efficiency and viability rate are usually pH dependent (gastric and intestinal fluids). The high bacterial recovery on *A. franciscana* using sodium alginate could be due to its physical properties. In term of fluid property of viscosity, sodium alginate was relatively higher than others. In this case, the bioencapsulation of bacteria in the *Artemia* nauplii could have occurred either externally via attachment to the body surfaces, or internally by ingestion, or both (Grisez *et al.*, 1996; Gomez-Gil *et al.*, 1998).

As indicated in our results, the capability of sodium alginate to encapsulate probiotic bacteria on *A. franciscana* is comparatively higher than others, although the bacterial counts are insignificantly different among the encapsulation media used. Each medium offered good adhesion ability to *L. lactis* subsp. *lactis* on this crustacean. The use of sodium alginate seems to be the most preferable bioencapsulation medium due to its stronger adhesive capability and low solubility in water. The polysaccharides derived from seaweed found in alginate have a gel-like structure (mainly comprised of 1, 4 linked copolymer of β-D-mannuronic acid and α-L-guluronic acid) (Li *et al.*, 2009), which can form a viscous gum when binding with water and naturally have a lower solubility at normal room temperature. Due to the alginate's biocompatibility, low toxicity, relatively low cost, and mild gelation by addition of divalent cations such as Ca^{2+}, it is therefore concluded that alginate can be widely used for cell immobilization and encapsulation (Lee and Mooney, 2012). These advantages, in turn, render better efficacy in bacterial entrapment compared to high solubility materials such as starch, gelatin and gum Arabic. With reference to biofilm adhesion on *A. franciscana*, the gel-like structure of alginate could also adhere firmly and fill the gaps between the body segments of the organisms, thus allowing the formation of biofilm. On the other hand, bacterial adhesion on *A. franciscana* bioencapsulated with free-cell suspension of *L. lactis* (control) would be attributed to their own secretion such as exopolysaccharides (EPS), a polymer of bacterial origin produced by lactic acid bacteria (Mozzi *et al.*, 2009).

Other factors such as probiotic strain, the time of exposure of the bacteria, and status (live or dead) of the bacteria used could also influence the rate of bacterial recovery (Gomez-gil *et al.*, 1998). In the present study, live *L. lactis* subsp. *lactis* was used instead of dead bacteria. Our results showed that at least 10^7 CFU/mL of *L. lactis* was successfully recovered from *A. franciscana* tissue homogenate after 8 h of bioencapsulation with different encapsulation media. Commonly, probiotic inoculation concentrations for terrestrial animals were usually in the range of 10^7 – 10^9 CFU/mL, depending on the probiotic strain used (Ouwehand and Salminen, 1998). However, no specific range of concentration was suggested for aquatic animals due to their huge species diversity and the complexity of aquatic environment. Ziaei-Nejad *et al.* (2006) reported that total bacterial flora of shrimp larvae, *F. indicus*, increased to 61.5 – 93.0 % (10^4 – 10^5 CFU per larvae), when the larvae fed with *Artemia* enriched by 10^6 CFU/mL commercial probiotic product containing *Bacillus*. Seenivasan *et al.* (2012) also suggested bioencapsulation of *Artemia* nauplii with 10^7 CFU/mL *L. sporegenes* could significantly improve the growth and survival of *M. rosenbergii* post larvae. These findings corroborated *L. lactis* subsp. *lactis* at the initial concentration of 10^8 CFU/mL could be effectively used to enrich *A. franciscana*, as the recommended concentration (10^7 CFU/mL) of probiotic could be recovered in 0.5 g/L of various types of encapsulation media.

In conclusion, the optimal *L. lactis* recovery and SR of *Artemia nauplii* could be achieved at the medium concentration of 0.5 g/L. The total bacterial counts in sodium alginate-encapsulated *A. franciscana* were the highest among others. Our study suggests that sodium alginate at 0.5 g/L could be potentially used as a safe and effective delivery bioencapsulation medium, for *Artemia* to transmit viable probiotic bacteria into fish and shrimp gastrointestinal system.

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