Bioencapsulation and Colonization Characteristics of *Lactococcus lactis* subsp. *lactis* CF4MRS in *Artemia franciscana*: a Biological Approach for the Control of Edwardsiellosis in Larviculture

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Abstract

Predominance of beneficial bacteria helps to establish a healthy microbiota in fish gastrointestinal system and thus to reduce emerging pathogen. In this study, the colonization efficacy of *Lactococcus lactis* subsp. *lactis* CF4MRS in *Artemia franciscana* and its potential as a probiotic in suppressing *Edwardsiella* sp. infection were investigated in vivo. The colonization extent of the bioencapsulated *L. lactis* was established through visualization of *gfp* gene-transformed *L. lactis* in *A. franciscana*. Here, we demonstrate that when *A. franciscana* is administrated with *L. lactis* at 10^8 CFU mL^{-1} for 8 h, the highest relative percentage of survival (RPS = 50.0) is observed after inoculation with *Edwardsiella* sp. The total counts of *L. lactis* entrapped in *Artemia* were the highest (ranged from 3.2 to 5.1 × 10^8 CFU mL^{-1}), when 10^8–10^9 CFU mL^{-1} of *L. lactis* was used as starting inoculum, with the bioencapsulation performed within 8–24 h. Fluorescent microscopy showed *gfp*-transformed *L. lactis* colonized the external trunk surfaces, mid-gut and locomotion antennules of the *A. franciscana* nauplii. These illustrations elucidate the efficiency of colonization of *L. lactis* in the gastrointestinal tract and on the body surfaces of *Artemia*. In conclusion, *L. lactis* subsp. *lactis* CF4MRS shows a good efficacy of colonization in *Artemia* and has the potential for biocontrol/probiotic activity against *Edwardsiella* sp. infection.

Keywords *Artemia franciscana* · Biocontrol · *Edwardsiella* sp. · Green fluorescent protein · *Lactococcus lactis* subsp. *lactis*

Introduction

Aquaculture is the fastest growing food industry (Saravanan et al. 2013). However, the rapid expansion of the industry has resulted in massive occurrence of various fish diseases.

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Aquaculture of fish, in particularly, turbot *Scophthalmus maximus*, Japanese flounder *Paralichthys olivaceus*, Nile tilapia *Oreochromis niloticus* and catfish *Clarias batrachus*, often develop diseases such as edwardsiellosis (Sahoo et al. 1998; Kim et al. 2003; Zheng et al. 2004; Padros et al. 2006). The Gram-negative pathogen *Edwardsiella* sp. is the major cause of edwardsiellosis, which can cause hemorrhagic septicemia including lesions on the skin, muscles and internal organs in many aquatic species (Bullock and Herman 1985; Mohanty and Sahoo 2007). The spread of *Edwardsiella* sp. in fish culture systems can be transmitted via contaminated equipment and facilities used in cultivation, or during the harvesting process of the live feed (Mainous et al. 2010). Common live feed include *Artemia* and rotifers and are often used as natural food sources for post-larvae in finfish or shrimp hatchery. Studies showed that *Artemia* could be a potential bio-vehicle for the transmission of several fish diseases; evidence indicates these live feeds can be involved (directly or indirectly) in transmitting piscine cryptosporidiosis infection caused by *Cryptosporidium molnari* and *Cryptosporidium scophthalmi* (Méndez-Hermida et al.

2007). Sivakumar et al. (2009) claimed that *Artemia* might be a possible horizontal transmission pathway for Hepatopancreatic parvo-like virus (HPV) in post-larvae of *Penaeus monodon* (Sivakumar et al. 2009). More importantly, a recent study demonstrated *E. tarda* and *E. ictaluri* can be transmitted via the oral route, particularly when pathogen-infected *Artemia* was used as a food source for Nile tilapia *Oreochromis niloticus* larvae (Situmorang et al. 2014). To control edwardsiellosis, conventional chemotherapeutic approaches are carried out, including pre-disinfection of the facility and application of prophylactic antibiotics for preventative measures or treatment purposes. However, the excessive use of antibiotics raises many food safety concerns; therefore, probiotics would be an advantageous alternative to control *Edwardsiella* sp. infections.

Probiotics are defined as live microorganisms which, when administrated in adequate amounts, confer health benefits to the host (FAO and WHO 2001). In most cases, probiotics are supplied as live supplements in feed complex, which can then benefit the fish and shrimp through inhibition of pathogenic microbes, improving immune response, improving survival and growth rates, increasing feed utilization, enhancing digestion and promoting antimutagenic or anticarcinogenic activity, and furthermore, some species have been suggested for improvement of water quality in aquaculture systems (Harikrishnan et al. 2010; Andani et al. 2012). In hatchery production, probiotics are generally applied through the oral route through live feed (e.g. *Artemia* and rotifers) (Hai et al. 2010). Probiotics could help to prevent bacterial infections by preventing the colonization of pathogenic bacteria (Villamil et al. 2010). Effective probiotics used in the fish industry possess several characteristics, including antimicrobial properties, adhesion capacity and growth on the intestinal or external mucous of the fish, are also relatively important to determine the feasibility of the putative probiotic in the field. Chang and Liu (2002) reported only certain bacterial species that colonized the GI tract could exert the protection to the eel *Anguilla anguilla* L. from edwardsiellosis infection. Thus, probiotic colonization in the GI (gastrointestinal) tract should be of particular importance to determine the viability and functionality of the beneficial bacteria.

In the present study, a potential probiotic strain, *Lactococcus lactis* subsp. *lactis* (CF4MRS), previously isolated from farmed fish (Loh et al. 2014; Loh and Ting 2016) was used to evaluate for inhibitory effect against *Edwardsiella* sp. using *A. franciscana* as a host in an in vivo pathogenic assays. The bacterial uptake, proliferation and colonization were determined through the employment of *gfp*-transformed *L. lactis* in the live feed. Efficacy of the bioencapsulation process and the probiotic dosage were also optimized in this study.

Materials and Methods

Bacterial Culture of *Lactococcus lactis* subsp. *lactis*

The bacterium *Lactococcus lactis* subsp. *lactis* CF4MRS (GenBank accession number: KM488626) was previously isolated from the GI tract of farmed freshwater catfish, *Clarias batrachus* (Monash University Animal Ethics Committee approval no.: MARP/2012/117) (Loh et al. 2014). The strain CF4MRS was sub-cultured regularly on de Man Rogosa and Sharp agar (MRS, Difco™, BD, USA). For the bioencapsulation experiment, the cell density of *L. lactis* in MRS broth (overnight culture) was adjusted to 10^6, 10^7, 10^8 and 10^9 CFU mL^{-1} (OD_{540}) using 10 g L^{-1} autoclaved artificial seawater (pH 7.5) (Instant Ocean® Sea Salt, USA). For the in vivo assay, the pathogenic *Edwardsiella* sp. BCRC 16703 (98% similarity to *E. anguillarum*, see supplementary BCRC 16703 gene sequences) (http://www.bcrc.firdi.org.tw) was adjusted to 10^5 CFU mL^{-1} using sterile artificial seawater.

Artemia Nauplii Hatching and Pre-disinfection

The cysts of *Artemia franciscana* (Great Lake *Artemia*, Salt Lake City, UT, USA) were exposed to UV radiation (254 nm) for pre-disinfection in a laminar flow hood for 30 min prior to hatching. The cysts (6 g L^{-1}) were placed in a 500-mL Artemio® set (JBL, Neuhofen, Germany) connected to an aerator and filled with sterile artificial seawater (20 g L^{-1}, pH 7.5). *Artemia* cysts were incubated under continuous aeration at 26 ± 2 °C for 20–24 h. (Touraki et al. 2013) and a photoperiod of 12:12 h (light:dark). The newly hatched nauplii (instar I) were collected, starved for 6 h and subsequently surface-disinfected with 10 ml L^{-1} Ovadine® (10% povidone-iodine) (Syndel Laboratories Ltd., Canada) for 10 min. The disinfected *Artemia* nauplii were then used for the in vivo pathogenic study.

In Vivo Challenge of *Artemia* Nauplii with *Edwardsiella* sp.

The surface-disinfected *Artemia* nauplii (Instar II, at approx. 28 h) were transferred into 250-mL conical flasks with a density of approx. 300 individuals per milliliter. This experiment consisted of seven treatments: positive control (nauplii with no *Lactococcus lactis* subsp. *lactis* were administered but challenged with *Edwardsiella* sp.), the negative control (nauplii were administrated with 10^6 CFU mL^{-1} *L. lactis* but without challenged with *Edwardsiella* sp.), blank treatment (nauplii with no *L. lactis* administration and without challenged with *Edwardsiella* sp.) and four different treatments of nauplii, each of which were administrated with 10^6, 10^7, 10^8 and 10^9 CFU mL^{-1} *L. lactis* and subsequently challenged with *Edwardsiella* sp.

Artemia nauplii were exposed to *L. lactis* for 1, 4, 8, 12 and 24 h. After bioencapsulation, a total of 20 *Artemia* nauplii per replicate were transferred aseptically from the flasks into 55-mm (in diameter) sterile Petri dishes, containing 10 mL of *Edwardsiella* sp. at 10^5 CFU mL^{-1} (except in the negative and blank treatments whereby *Edwardsiella* sp. was excluded). After 48 h of incubation, the *Edwardsiella* sp. suspensions were replaced with fresh suspensions at the same concentrations. A food source of autoclaved yeast *Saccharomyces cerevisiae* (Mauri-pan® instant yeast, Malaysia) (5.6×10^7 cells mL^{-1} in 10 g L^{-1} sterile artificial seawater, pH 7.5) was administered to the *Artemia* nauplii daily (100 μL $dish^{-1}$) throughout the experiment (Marques et al. 2004). Each treatment was performed in triplicate. The survival rates of the nauplii were monitored and recorded throughout the experiment until a complete mortality (100%) was achieved in the positive control. The relative percentage of survival (RPS) was calculated based on the following formula (Amend 1981):

RPS ð%Þ ¼ 1−ðM_t=M_cÞ × 100

where M_t is mortality from treatment and M_c mortality from control. RPS values (equivalent to ED_{50}) ≥ 50 indicated a positive effect (Cunningham et al. 2010) of *L. lactis* against the pathogen *Edwardsiella* sp.

In addition, water quality was monitored throughout the experiment period for dissolved oxygen (DO), pH, temperature, total dissolved solid (TDS), salinity and total ammonia contents (Table 1). The DO, pH, temperature, TDS and salinity were determined using an Eutech instrument (PCD 650, Thermo Fisher Scientific, Singapore), while total ammonia nitrogen (NH_3-N), nitrite nitrogen (NO_2-N) and nitrate nitrogen (NO_3-N) were measured using a Hach colorimeter (DR890, Hach, USA) according to the Standard Methods for the Examination of Water and Wastewater (APHA 1985).

Optimization of Bioencapsulation

Firstly, new batches of *Artemia* nauplii were surface-disinfected using Ovadine® and then rinsed thoroughly with autoclaved artificial seawater. Nauplii (approx. 300 nauplii mL^{-1}) were transferred to 250-mL conical flasks containing 100 mL bacterial suspensions of various concentrations (10^6, 10^7, 10^8 and 10^9 CFU mL^{-1} in autoclaved artificial seawater). *Artemia* nauplii without treatment with *L. lactis* were used as the control. Mild aeration (filtered through 0.22-μm membrane filters) was provided at the bottom of the flasks to ensure sufficient oxygenation in the *Artemia* culture.

To quantify the total number of *L. lactis* cells bioencapsulated in the nauplii, 1 mL of *Artemia* nauplii of each treatment was collected after 1, 4, 8, 12 and 24 h of bioencapsulation. All nauplii were washed (5 mL sterile saline solution, NaCl, 0.85% *w*/*v*) and collected through sterile microclothes (Calbiochem, Merck, Germany). The nauplii were then transferred to 2 mL sterile saline solutions and macerated using a homogenizer (LabGEN®125, Cole-Parmar, USA). Serial dilutions of the homogenized suspensions were made down to 10^{-9}, plated (100 μL) on nutrient agar (Badhul Haq et al. 2012) and incubated at 26 ± 2 °C for 24–48 h. CFU were counted to enumerate the viable bacteria that were successfully encapsulated in the *Artemia* nauplii (all quantification experiments were done in triplicate).

Colonization of *gfp*-Transformed *L. lactis* in *A. franciscana*

Colonization and proliferation of *Lactococcus lactis* subsp. *lactis* CF4MRS in *Artemia franciscana* were detected via green fluorescent protein (GFP) (Fernández de Palencia et al. 2000). The *gfp*-transformation process was firstly initiated by culturing the lactococcal cells (CF4MRS) in GM17 medium (M17 broth (Oxoid Ltd., Hampshire, UK) supplemented with 1% glucose) at 37 °C overnight. Cultures were diluted 100-fold in SGM17 medium (GM17 supplemented with 0.5 M sucrose) containing 3% glycine (Vivantis Technologies Sdn Bhd., Malaysia) (Holo and Nes 1989). Exponential-phase cells were grown to 0.2–0.7 cell densities (OD_{600}) at 30 °C. The lactococcal cells were harvested by centrifugation at 6000×*g* for 5 min at 4 °C and washed following the protocol suggested by Dornan and Collins (1990). The lactococcal cells were re-suspended in 2 mL of 0.22 μm filtered Milli-Q water (Merck Millipore, USA), the cells were collected by centrifugation (6000×*g* for 5 min at 4 °C) and the supernatant was discarded. A second wash in 1 mL of

Table 1 Water quality in the *A. franciscana* experimental flasks

	Water quality physicochemical parameters							
	DO (mg L^{-1})	pH	Temperature (°C)	Salinity (ppt)	TDS (mg L^{-1})	NH_3-N (mgL^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mgL^{-1})
Control	3.15	7.4	22.5	20.0	15.97	0.80	0.025	0.02
Treatment	4.09–4.30	7.1–7.4	22.3–22.5	19.8–20.0	15.49–16.00	0.50–1.60	0.043–0.070	0.06–0.13

DO, dissolved oxygen; *TDS*, total dissolved solid; *NH_3-N*, total ammonia nitrogen; *NO_2-N*, total nitrite nitrogen; *NO_3-N*, total nitrate nitrogen

0.22 μm filtered Milli-Q water was used. The washing steps were repeated twice to collect the cell pellets. Immediately after washing, 1 mL of 50 mmol L^{-1} EDTA (Sigma-Aldrich, Co., USA) was used to re-suspend the cell pellets and placed on ice for 5 min. After incubation, cells were collected by centrifugation. The re-suspension step was repeated again with 1 mL of 0.22 μm filtered Milli-Q water. In the final step of re-suspension, the cells were re-suspended using the same protocol by replacing Milli-Q water with 1 mL of 0.3 mol L^{-1} sucrose (Systerm®, Malaysia). The lactococcal cells were then collected by centrifugation.

Prior to electrotransformation, competent lactococcal cells were re-suspended in 0.2 mL of 0.3 mol L^{-1} sucrose. Immediately after re-suspension in sucrose, the cells were mixed with 2 μg of plasmid DNA. The plasmid DNA used in this study was pLS1GFP (KitMyGEN, Spain), a broad-host-range pMV158 replicon, which carries a *tetL* gene encoding a Tet^R determinant (Ruiz-Cruz et al. 2010), *ermAM* gene encoding an Erm^R determinant (Ruiz-Masó et al. 2012) and the *gfp* gene (Fernández de Palencia et al. 2000). Cell suspensions (100 μL) were transferred into a pre-chilled disposable electroporation cuvette (Bio-Rad Laboratories Inc., USA) (2-mm electrode gap) and subjected to a single pulse [2.5 kV (E = 12.4 kV cm^{-1}), 200 Ω and 25 μF corresponding to pulse length of 4.6 ms] using a Gene Pulser (Xcell™, Bio-Rad Laboratories Inc., USA). Immediately after discharge, the cell suspensions were transferred to 1 mL of ice-cooled MRS broth (supplemented with 5 μg mL^{-1} erythromycin) and placed on ice for 5 min. Cell suspensions were incubated at 37 °C for 2 h and 100 μL was plated on MRS agars (supplemented with 5 μg mL^{-1} erythromycin) for the selection of erythromycin-resistant transformants. The plates were incubated for 4 to 5 days at 30 °C and colonies were inoculated onto fresh MRS agars.

To detect *gfp*-transformed *L. lactis* in *A. franciscana*, *Artemia* cysts were surface-disinfected and followed by the hatching process as described previously (B*Artemia* Nauplii Hatching and Pre-disinfection^ section). *Artemia* nauplii were transferred to a conical flask containing 200 mL of *gfp*-transformed *L. lactis* bacterial suspension (10^8–10^9 CFU mL^{-1}) and mild aeration was provided during the experiment. After 6 and 12 h of bioencapsulation, 10–15 nauplii were collected using a Pasteur pipette and transferred into Petri dishes containing 10 mL sterile PBS (PBS containing 10 g L^{-1} Instant Ocean® Sea Salt). The nauplii were observed under a fluorescence microscope (Olympus BX43, Olympus Co., Japan) equipped with an excitation Standard FITC set, a DP26 digital camera system and UIS2 optical components, for colonization of the *gfp*-transformed *L. lactis*. GFP fluorescence was detected by exposing the *Artemia* to ultraviolet light at a wavelength of 450–490 nm. The images were captured using a DP26 digital camera system.

Statistical Analysis

The data were analyzed for normality and homogeneity of variance. For the data in percentage values, they were transformed to a square root value prior to the analysis using ANOVA (Tukey test). Significant values were accepted at P value < 0.05. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) Version 20.

Results

Generally, *Lactococcus lactis* subsp. *lactis* CF4MRS at 10^8 CFU mL^{-1} and bioencapsulated for 8 h to *Artemia franciscana* nauplii showed the highest protective effect against *Edwardsiella* sp. infection. The survival of both *A. franciscana* groups in the negative control and those administrated with 10^6 CFU mL^{-1} *L. lactis* showed a similar trend with no significant differences (P > 0.05) found between the blank treatment and positive control (Fig. 1a). Application of different *L. lactis* cell densities resulted in a slight increase in the relative percentage of survival (RPS) compared to the positive control (Table 2). No significant difference was found in the survival of *A. franciscana* in the negative and blank treatments (P > 0.05) (Fig. 2a). However, all *L. lactis* densities (10^6–10^9 CFU mL^{-1}) showed a significant reduction (P < 0.05) in the survival rate of *A. franciscana* (Fig. 2a) regardless of the bioencapsulation duration, when compared to the positive control. A lower survival was noticed when *A. franciscana* administrated with 10^6 CFU mL^{-1} *L. lactis* for 4 h (Fig. 1b). However, no significant difference was observed in the survival of *A. franciscana* administrated with 10^7–10^9 CFU mL^{-1} *L. lactis* (P > 0.05) (Fig. 2a). The RPS was recorded in the range of 31.7–48.3 within 4–12 h (Table 2). The survival of *A. franciscana* (10^8 CFU mL^{-1} *L. lactis*; 8 h) was higher than those fed with 10^9 CFU mL^{-1} *L. lactis* (Fig. 1c). In terms of RPS, *A. franciscana* administrated with 10^8 CFU mL^{-1} *L. lactis* showed the highest protective effect against *Edwardsiella* sp. (RPS = 50.0) (Table 2). Bioencapsulated *Artemia* with 10^8 CFU mL^{-1} *L. lactis* for 8-h duration showed 50% survival rate which was similar to those found in the negative control (nauplii not challenged with *Edwardsiella* sp.) (Fig. 2a). This trend, however, did not occur during the extended period of bioencapsulation (t = 12 and 24 h). The survival did not significantly differ (P > 0.05) in the *A. franciscana* treated with different *L. lactis* cell densities. In terms of bioencapsulation time, no significant difference (P > 0.05) was observed among the duration and cell density, except for the negative and blank treatments (Fig. 2b).

The total estimated bacteria bioencapsulated in the *A. franciscana* increased relatively to the bacterial density and bioencapsulation time (Fig. 3). Only a cell density at 10^9 CFU mL^{-1} showed a significant difference (P > 0.05)

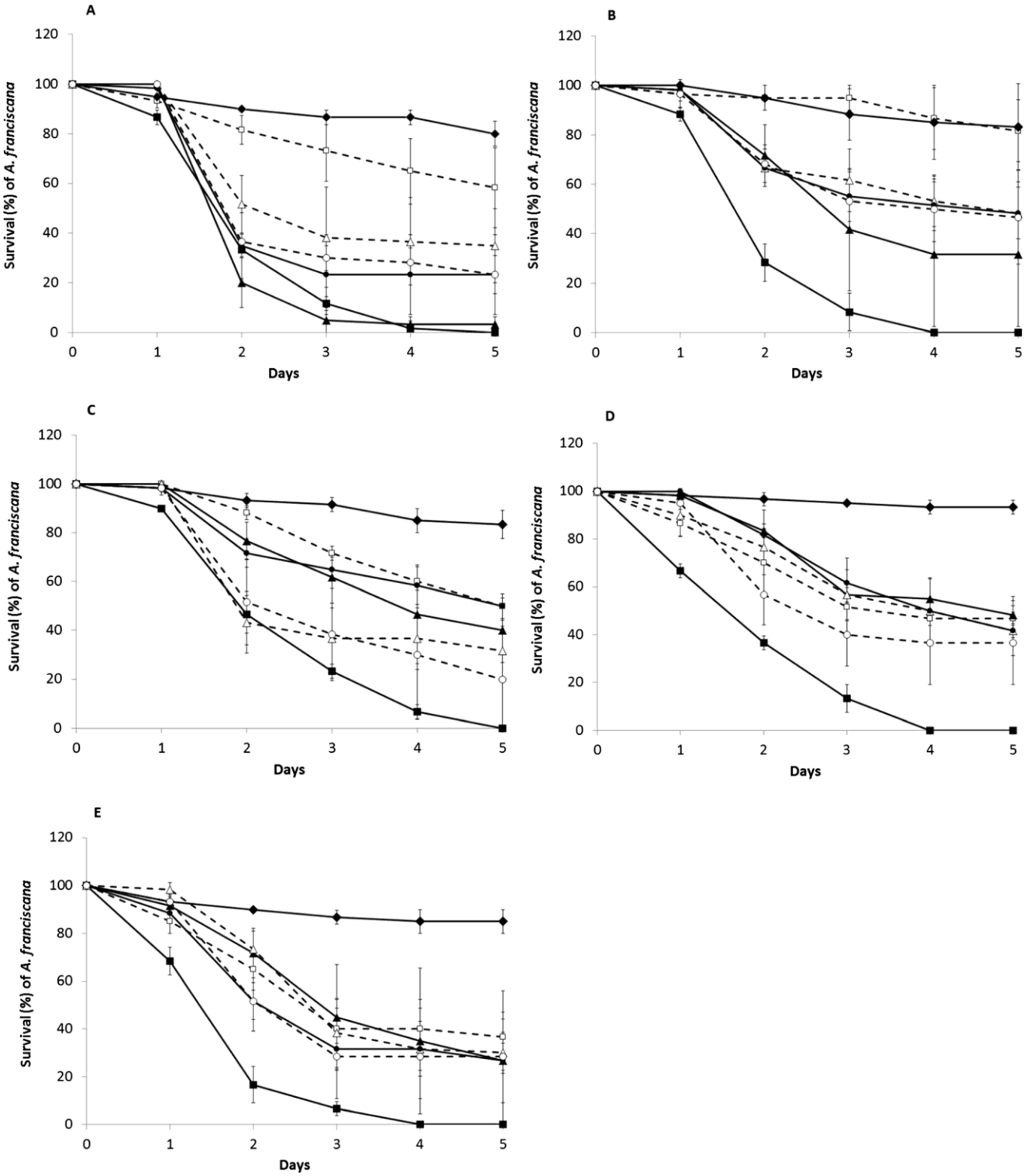

Fig. 1 Survival of *A. franciscana* administrated with or without *L. lactis* for a 1 h, b 4 h, c 8 h, d 12 h, and e 24 h in the presence of *Edwardsiella* sp. (in vivo challenging) or without *Edwardsiella* sp. ■ positive control, □ negative control, ◆ blank control, ▲ nauplii administered with 10^6 CFU mL^{-1}, △ 10^7 CFU mL^{-1}, ● 10^8 CFU mL^{-1}, and ○ 10^9 CFU mL^{-1} of *L. lactis*. Vertical error bars indicate standard deviation of means

compared to the lower bacterial densities and the control (without bioencapsulation). The total number of bacteria in the *A. franciscana* increased tenfold when compared to those in the control (1.7×10^6 CFU mL^{-1}), resulting in an average of 5.2×10^6 CFU mL^{-1} of bacteria when administrated with 10^9 CFU mL^{-1} of *L. lactis* in the first hour. The total estimated

Table 2 Mortality and relative percentage survival (RPS) of *L. lactis* subsp. *lactis*-bioencapsulated *A. franciscana* after challenged with *Edwardsiella* sp.

	Lactococcus lactis subsp. *lactis* CF4MRS concentration									
	Control[a]		10^6 CFU mL^{-1}		10^7 CFU mL^{-1}		10^8 CFU mL^{-1}		10^9 CFU mL^{-1}	
Time (h)	Mortality (%)	RPS	Mortality (%)	RPS	Mortality (%)	RPS	Mortality (%)	RPS	Mortality (%)	RPS
1	100	–	97	3.3	65	35.0	77	23.3	77	23.3
4	100	–	68	31.7	52	48.3	52	48.3	53	46.7
8	100	–	60	40.0	68	31.7	50	50.0	80	20.0
12	100	–	52	48.3	58	41.7	58	41.7	63	36.7
24	100	–	73	26.7	70	30.0	73	26.7	72	28.3

[a] *Artemia* did not receive administration of *Lactococcus lactis* subsp. *lactis* and are challenged with *Edwardsiella sp.*

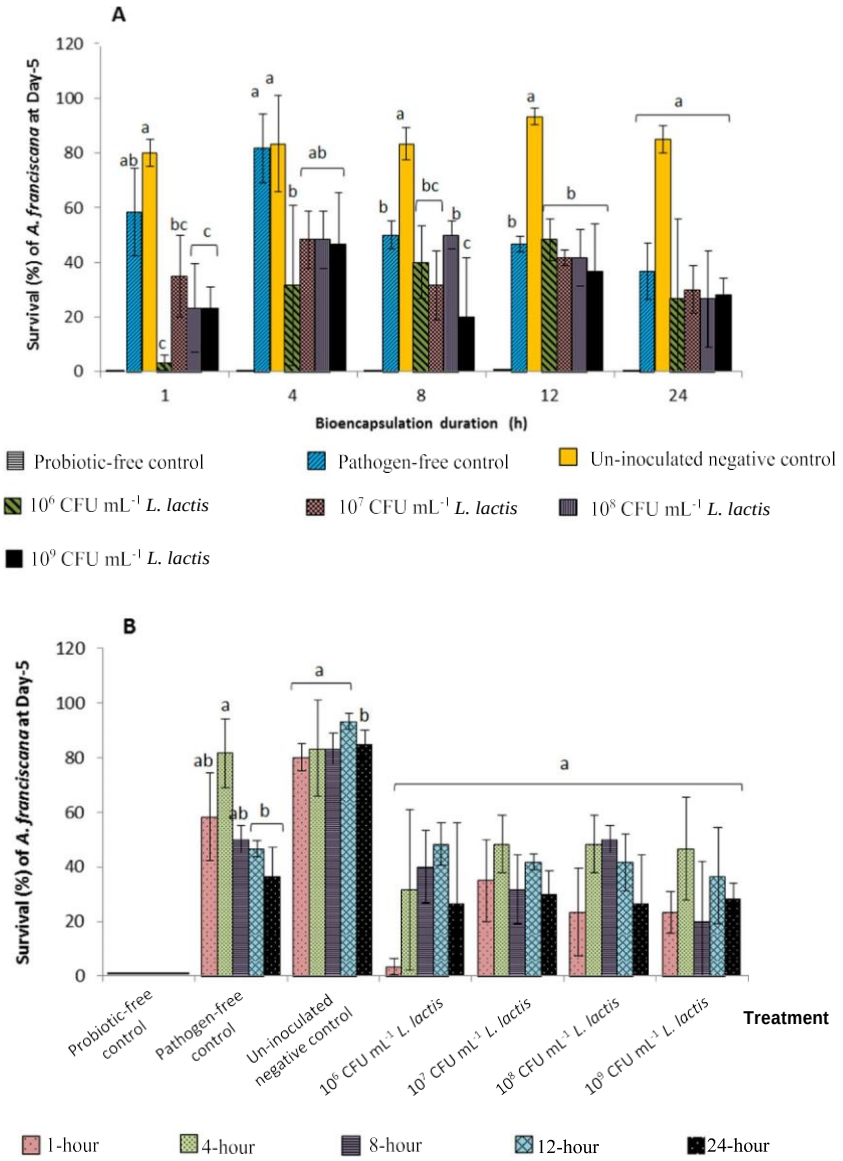

Fig. 2 Survival of *A. franciscana* at the end of experiment (5 days). a Survival rate at different bioencapsulation durations. b Survival rate at different treatments. Mean values with same letters within groups are not significantly different ($HSD_{0.05}$). Vertical bars indicate standard deviation of means

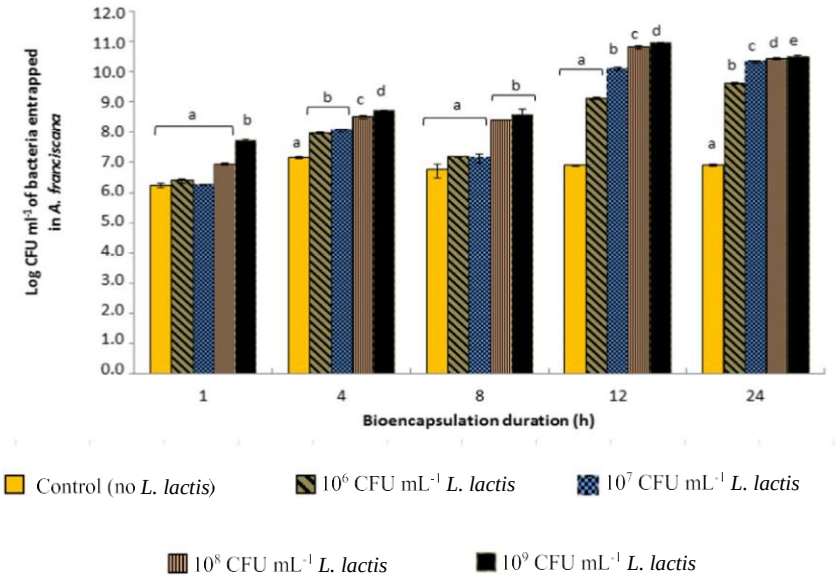

Fig. 3 Estimation of total bacteria bioencapsulated in *A. franciscana* at 1, 4, 8, 12, and 24 h, introduced by the initial cell densities of *L. lactococcus* subsp. *lactis* at 10^6, 10^7, 10^8, and 10^9 CFU mL^{-1}. Mean values with same letters within groups are not significantly different ($HSD_{0.05}$). Vertical bars indicate standard deviation of means

count of bacteria also increased tenfold when exposed to 10^8 and 10^9 CFU mL^{-1} of *L. lactis* (3.2 and 5.1 × 10^8 CFU mL^{-1}, respectively) for 4 h while the bacterial count in the control was only 1.4 × 10^7 CFU mL^{-1}. When the bioencapsulation time was extended to 8 h, a remarkable change was observed, whereby the total bacterial count increased to 20-fold at 10^8 (2.4 × 10^8) and 10^9 CFU mL^{-1} (3.9 × 10^8 CFU mL^{-1}) *L. lactis*, respectively. The total bacterial count continued to increase up to 40-fold compared to the control when the time was extended to 12 and 24 h.

In terms of bacterial uptake, proliferation and colonization, control *Artemia* did not have a fluorescent signal after ultraviolet light exposure (Fig. 4a, b). In contrast, green fluorescent spots were detected on the external trunk surfaces, including a pair of locomotion antennules of the instar after being administered with *gfp*-transformed *L. lactis* for 6 h. The fluorescence intensity was particularly intense in the areas of the esophagus up to the mid-gut (Fig. 4c), suggesting that the bacterial intake could have commenced at the stages of instar II to III.

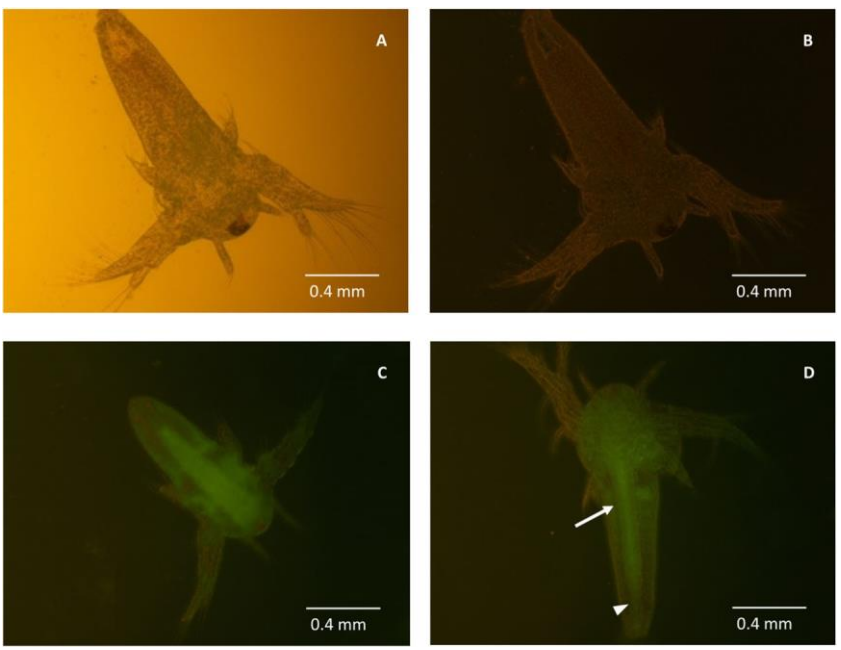

Fig. 4 Visualization of *gfp*-transformed *L. lactic* subsp. *lactis* in *A. franciscana*: a wild-type *A. franciscana* without administrated to *L. lactis* (control) under *bright-field-view* microscopy; b wild-type control *A. franciscana* under *dark-field-view* microcopy; c instar II of *A. franciscana* administrated to 10^8–10^9 CFU mL^{-1} *gfp*-transformed *L. lactic* for 6 h; d nauplii of *A. franciscana* administrated to the same concentration of *gfp*-transformed *L. lactic* for 12 h. Putative adhesion of bacteria (c, d) in the mid-gut (indicated with arrows) and hindgut areas (arrow heads) were viewed under *dark-field-view* microcopy with exposure of ultraviolet light in the range of 450–490 nm

Bacterial proliferation in gut epithelial cells was observed in the nauplii (12 h). Bacterial cells were mostly confined to the GI tract and colonized mainly to the anterior parts of the body surface (Fig. 4d). In comparison with instar, the bacteria in the nauplii colonized the entire digestive tract towards the hindgut.

Discussion

In this present study, *Artemia franciscana* administrated with *Lactococcus lactic* subsp. *lactis* CF4MRS showed a significant survivorship after challenged with *Edwardsiella* sp. RPS showed *A. franciscana* administrated with 10^8 CFU mL^{-1} for 8-h bioencapsulation offered the best protection against *Edwardsiella* sp. (Table 2). However, the cell density of *L. lactis* in the range of 10^6–10^9 CFU mL^{-1} shared the same protection as the survival of *A. franciscana* in the pathogenic challenging de facto. This result is in agreement with Touraki et al. (2013) where the authors found no significant effect on the survival of *A. franciscana* regardless of the dosage concentration of *L. lactis* subsp. *lactis* used in *Vibrio anguillarum* challenge study. And yet, some studies suggest that a probiotic concentration of at least 10^8 CFU mL^{-1} is required to protect *Artemia* from *Vibrio* spp. infection (Lamari et al. 2013). When *L. lactis* is used as a prophylactic treatment for sea bass to prevent against vibriosis infection, the application dosage of the probiotic is suggested to be within the range of 10^6–10^7 CFU mL^{-1} (Touraki et al. 2013). There are nu- merous studies on the application of lactic acid bacteria in fish and shrimp cultures; all of these showed promising results in disease protection. However, it is worth men- tioning that when *L. lactis* was supplied solely in live feed cultivation (*Artemia* in our case), without a pathogen, a noticeable mortality rate of *Artemia* was observed in the culture (Figs. 1 and 2); this is presumably attributed to starvation as no food was provided during the bioencapsulation process.

The administration of the probiotic before the first exogenous feeding can facilitate the establishment of beneficial bacteria through their colonization in the digestive tract (Motlagh et al. 2012). Bacterial colonization in the digestive tract relies mainly on the number of bacteria being introduced and its proliferation capability in the intestinal system (Ziaei-Nejad et al. 2006). Therefore, it is crucial to understand the effects of bacterial density and incubation time, and the interaction between these two factors with/without exposure to the pathogen. The total bacterial count entrapped in *A. franciscana* exposed to *L. lactis* at 10^8–10^9 CFU mL^{-1} showed a significant increment at prolonged bioencapsulation duration i.e. > 4 h. Although *Artemia* cysts were surface-sterilized with povidone-iodine (Ovadine®), some bacteria were still observable on the surface of *A. franciscana* in the control group. This presumably could be due to the presence of natural microflora in the hatched nauplii. According to Sahul Hameed (1993) and Phatarpekar et al. (2002), the crustacean *Penaeus indicus* and *Macrobrachium rosenbergii* larvae were found to be dominated by some bacteria species upon hatching, even though their egg homogenates were sterile (Colorni 1985). This might be primarily due to the natural microflora present in the digestive tract of the animals (Colorni 1985). Our results indicate that bioencapsulation using *L. lactis* at 10^8–10^9 CFU mL^{-1} could improve the total bacterial count in *A. franciscana* from 10- to 40-fold compared to those in the control. The recovery of the probiotic was relatively higher than that of other strains such as *Bacillus subtilis* and *B. licheniformis*, where only 10^4–10^6 CFU mL^{-1} of the total bacterial count were recovered from bioencapsulated *A. urmiana* (Motlagh et al. 2012). The higher CFU mL^{-1} presented in this study could be due to different probiotic strains, *Artemia* species and also the enumeration technique used as well.

In terms of bacterial uptake, proliferation and colonization, results showed that *L. lactis* mainly localized at the external trunk surfaces and in the digestive tracts of the zooplankton (*Artemia* nauplii). Probiotic adhesion capacity on the intestinal tract is a fundamental prerequisite in probiotic treatments to transient colonization, while others include antagonistic activity against invasive pathogens and the stimulation of innate immunity (Lauzon et al. 2008; Fjellheim et al. 2010; Motlagh et al. 2012). The adhesion capacity of a putative probiotic can be assessed through different approaches such as a cell line attachment assay. Lauzon et al. (2008) showed that the adhesion capacity of an actinobacteria group of probiotic which was isolated from salmon was only prominent in certain cell lines. Lactic acid-producing bacteria, especially *Carnobacterium divergens* V41, do not adhere to Epithelioma papulosum cyprini (EPC) cell lines. The authors suggested that the evaluation of adhesion capacity solely based on cell lines could be a limitation. Furthermore, no cell line has yet been established for freshwater fish (Lauzon et al. 2008). Our study showed that the *gfp*-transformed *L. lactis* subsp. *lactis* has shown a significant capability of proliferation and colonization of the probiotic. More importantly, it provides a real-time observation for the presence of bacteria on the surfaces and in the digestive tract of *A. franciscana*. To our knowledge, this is the first report on gut colonization and proliferation in the GI of *Artemia* through the employment of *gfp*.

In conclusion, our study suggests that bioencapsulated *Artemia* with *L. lactis* subsp. *lactis* CF4MRS at 10^8 CFU mL^{-1} for 8 h could effectively increase the survival rate of live feed up to 50% against *Edwardsiella* sp. infection. Extensive colonization of this bacterium on *Artemia* also confirms the potential use of this probiotic in larviculture.

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