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Supplementation of Indigenous *Lactobacillus* Bacteria in Live Prey and as Water Additive to Larviculture of *Portunus pelagicus* (Linnaeus, 1758).

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Abstract: Experimental trials were conducted to demarcate the effects of indigenous *Lactobacillus* probiotics as bioencapsulated in live prey (rotifers *Brachionus plicatilis* and *Artemia franciscana*) and water additives together on the survival of blue swimming crab, *Portunus pelagicus* larvae. Three LAB probiotics *L. plantarum*, *L. salivarius* and *L. rhamnosus* at final concentration $1x10^7$ cfu/mL were bioencapsulated in live prey added daily and same allowance was added to culture water on day 1, 3, 5, 7, 9, 11, 13 as a single isolates to treatments A, B, C and multi isolates to D with no probiotic added to control tanks. Bacteria were successfully accumulated in both rotifers and *Artemia* within two hours of incubation. Total viable count of bacteria in *Artemia* observed lower at sampling days in inoculated tanks compared to those at time of incubation, contrary it increased in controls and no *Vibrio* was determined in *Artemia* in LAB mixture isolate inoculated samples on the day 13. Highest LAB bacteria 4.10×10^3 was determined in *Artemia* on day 11 in those inoculated with mixture of LAB isolates. At the end of the trials, larvae treated with a mixture of LAB probiotics did produce significantly highest survival 13.83±0.76% over other LAB treatments. As a single isolate *L. plantarum* did produce survival 13.50±1.32% compared with those treated with *L. salivarius* and *L. rhamnosus* and those without probiotics (control). There was no statistical significance ($p>0.05$) in the survival of larvae in any treatment. Results indicate that LAB probiotics could be used to enhance survival of *P. pelagicus* larvae.

Key words: Accumulated, *Artemia franciscana*, *Brachionus plicatilis*, *Lactobacillus*, significance

INTRODUCTION

Blue swimming crab, *Portunus pelagicus* larvae are faint and susceptible to pathogenic bacteria particularly *Vibrio harveyi* transmitted through feces of adult female in hatching tanks which pave the way for mass mortalities (Talpur *et al*., 2011a). Another trail for the ingress of pathogenic bacteria into the larviculture of fish hatcheries is via live prey. With the development of commercial-scale aquaculture, it has become apparent that infections can be a significant limiting factor (Gomez-Gil *et al*., 2000). Due to the high mortality and transmittable nature of infections, large amounts of antibiotics and antiseptic chemicals are often used for therapy. However, indiscriminate use of antibiotics has led to development of drug-resistant bacteria that are becoming increasingly difficult to control and eradicate (Esiobu *et al*., 2002; Nomoto, 2005). In case of *P. pelagicus* a variety of pathogenic bacteria are drug resistant and the use of antibiotics is questionable (Talpur *et al*., 2011b). Therefore, the need for alternative techniques is increasing and the contribution of probiotics may be considerable. In the past years, efforts have been made to develop strategies for microbial control, to decrease the use of therapeutic chemicals and antibiotics towards a more environmentally friendly and sustainable aquaculture (Cabello, 2006). Among the possible alternatives for the improvement of microbial problem, avoiding the use of antibiotics and other therapeutic measures, the use of bacteria such as LAB (potential probiotics) has roused great interest during the last decade (Ringø and Gatesoupe, 1998).

According to original definition, probiotics are "organisms and substances which contribute to intestinal microbial balance". A large number of studies have dealt clearly with probiotics, and it is now possible to survey its state of the art, from practical use to the scientific approach (Gatesoupe, 1999; Wang and Xu, 2006; Kesarcodi-Watson *et al*., 2008).

Bacteria colonies in the intestines of the larvae enter from the mouth opening (Hansen and Olafsen, 1999). A better strategy, that avoids the introduction of exotic bacteria to the system, is to select probiotic candidates among isolated strains from same healthy organism (Westerdahl *et al*., 1991). As an inoculative mean for

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probiotic to digestive tract of larvae, live prey may be via bioencapsulating with probiotics (Planas *et al*., 2004). Substitution of the opportunistic bacteria by a preventive colonization with other nonaggressive bacteria with persistence in water or live food can be a good strategy to provide protection to the larvae (Gatesoupe, 1994; Ringø *et al*., 1996; Makridis *et al*., 2000b; Martínez-Diaz *et al*., 2003).

Live feed rotifers (*Brachionus* spp.) have been used as a live food for feeding larval marine fishes for over 30 years (Yúfera, 2001) and are used as the first prey in larvae artificial food chain, are major carriers of bacteria (Munro *et al.*, 1993). Rotifers (*Brachionus plicatilis*) and *Artemia* both are filter feeders (Vadstein *et al*., 1993; Makridis and Vadstein, 1999) that are commonly used live feed in the intensive rearing of marine larvae. The ability of *Artemia* to feed on suspended particles allows ingestion of bioencapsulated nutrients including probionts (Gatesoupe, 1994).

Delivery of probiotic bacteria to live prey can not only serve as control agent of opportunistic or pathogenic bacteria but also be a vehicle for introducing probiotics to fish larvae (bioencapsulation) (Gatesoupe, 1994 , 1999; Ringø and Birkbeck, 1999; Skjermo and Vadstein, 1999; Makridis *et al*., 2000a). Effective transfer of probiotic bacteria to fish larvae is vital for the colonization potential of the bacteria.

This is the first report and the main aim of the current study was to evaluate the effects of Lactic Acid Bacteria (LAB) isolated from the gut of *P. pelagicus* including *L. plantarum*, *L. salivarius* and *L. rhamnosus* on survival of *P. pelagicus* larvae via supplementing live food and together as water additive to rearing system.

MATERIALS AND METHODS

Site of study: Present study was conducted during the year 2011 at the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu (UMT), Kuala Terengganu, Terengganu, W. Malaysia.

Experimental design: Live prey rotifers (*Brachionus plicatilis*) and *Artemia franciscana* nauplii was enriched with LAB probiotics *L. plantarum, L. salivarius* and *L. rhamnosus* at final concentration 1×10^7 cfu/mL and same allowance was added to culture water on day 1, 3, 5, 7, 9, 11, 13 as a single isolates to treatment A, B, C and multi isolates to D. Control A1, B1, C1 and D1with larvae without bacteria. In all experiments, treatments were replicated in triplicate. The aquaria tanks having capacity 10 L each were included in each of treatment at 20 larvae/L with sterilized seawater. Throughout the experiment temperature and salinity was maintained within the constant range and 12 h light and 12 h dark photoperiod period was maintained. A volume of 10-12% water was exchanged daily. However, in control, daily water changes accorded to 30-40%.

Bacterial cultures: Three LAB (*L. plantarum*, *L. salivarius* and *L. rhamnosus*), previously isolated from the gut microflora of female blue swimming crab, *P. pelagicus* were used in this study. These bacterial isolates have shown *in vitro* inhibition to indicator a pathogens *Vibrio harveyi, V. parahaemolyticus* and *P. piscicida* (Talpur *et al*., 2011c). LAB bacteria were cultured in MRS broth prepared in marine water with salinity 28 ppt at 37ºC at agitation 150 rpm for 48 h. Bacteria were harvested by centrifugation 15000 rpm for 15 min, the supernatant was discarded and the pellets were washed two times in sterilised seawater (28 ppt) and finally suspended in sterilised seawater. Bacterial density (1×10^7 cfu/mL) were determined through OD as standard set previously during the present study and bacterial suspensions were directly added for enrichment.

Seawater for larvae culture: Seawater was filtered through a 10 μm net and then sterilized with sodium hypochlorite (50 mg/L) for 24 h. This procedure, which eliminated almost all naturally occurring bacteria, was followed by neutralization with sodium thiosulphate at the beginning of the experiment.

Brood stock management, hatching and experimental larvae: Berried females were collected from Strait of Tebrau (1º22′ N and 103º38′ E), Johor, West Malaysia, and were transported to marine hatchery of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu (UMT), Malaysia. Females disinfected and placed in hatching tanks for breeding with sand substrate and adequate aeration according to Talpur *et al*. (2011a). Zoea (Z1) of *P. pelagicus* were used as an experimental larvae. Prior to exposing, to feed and disinfection, energetic larvae were acclimated and washed with sterilized seawater (SW) with similar parameters as in treated aquaria in order to minimise the bacterial load with larvae adhering from hatching tank water.

Disinfection of *P. pelagicus* larvae: Disinfection was carried out according to Planas *et al*. (2006). Newly hatched larvae of *P. pelagicus* were collected and transferred to a 30 L transparent aquaria tank provided with sterilised sea water (28 ppt) previously supplemented with Dismozon Pur (1%), larvae were placed in tank for 4 h. Tank was supplied adequate aeration and temperature was maintained at 28ºC. Before to start the process of culture, larvae were washed with sterilised seawater (28 ppt) to remove the residue of Dismozon Pur and were kept in aerated seawater for 1 h.

Bioencapsulation of live feed:

Rotifer (*Brachionus plicatilis*) culture: Rotifers *Brachionus plicatilis* were cultured in sterile seawater with 22±1 ppt under the usual rearing hatchery conditions. Rotifers were fed with Selco Plus (INVE Aquaculture, Belgium) daily. Before enrichment with bacteria the rotifers were sieved, washed with sterilised seawater (28 ppt) and resuspended in filtered sterilised seawater (22 ppt) adjusted to 20×10^6 cells/mL of the microalgae *Nannochloropsis* sp. for 24 h. Aeration was continuous and lighting was supplied from 40-W two fluorescent lamps. Rotifers were maintained at ambient temperature.

Bioencapsulation of probiotic bacteria in the rotifer *Brachionus plicatilis*: Suspensions of bacteria were prepared in seawater for the two treatments single isolate and multi isolate at a final concentration of approximately 10^7 bacteria per mL. Equal numbers of the three candidates of LAB probiotics were used in one suspension of multi isolate. Rotifers were collected from the culture tank, washed carefully with filtered sterilised seawater (22 ppt) in a 40-µm pore size sterile net and transferred to a sterile 1500-ml conical flask containing 1000 mL of sterilised seawater (22 ppt). The conical flask was capped with sterilised cotton and placed under a laminar flow hood. The rotifers at density 800 ind/mL grazed in the bacterial suspensions (10^7 cfu/mL) for 2 h with aeration, were rinsed with autoclaved seawater (22 ppt) for 5 min, and then added to the rearing tanks. An allowance of 30-40 rotifers per mL was added daily to culture tanks. Rotifers fed to control tanks were treated in the same way, but bacteria were not added. All manipulations were carried out aseptically under a laminar flow hood and all tools were previously autoclaved at 120ºC for 20 min.

***Artemia franciscana* culture:** *Artemia franciscana* cysts (Great Lake *Artemia*, Salt Lake City, Utah, USA) were incubated for 24 h at 28±1.0ºC in sterile seawater with adequate aeration. The newly hatched nauplii were rinsed in sterilised seawater after hatching and enriched overnight with Selco Plus (INVE Aquaculture, Belgium) in 10 L cylindroconical tanks with adequate aeration.

Bioencapsulation of *Artemia*: The *Artemia* were bioencapsulated with probiotics according the procedure of Makridis *et al*. (2000b). *Artemia* were collected in 2500 mL conical flask. During the experiment, *A. franciscana* (100 ind/mL) were starved for two hours and rinsed in sterilised seawater (28 ppt) before incubated for two hour in cultures of probiotic bacteria diluted in autoclaved seawater (28 ppt) at a final concentration 1×10^7 cells/mL. Enriched *Artemia* were collected in 100 µ pore size nylon net and were rinsed with sterilised seawater (28 ppt) for while before they were fed to the larvae. Treatment A, B, C and D received *A. franciscana* incubated in diluted cultures of the probiotic bacteria *L. plantarum*, *L. salivarius* and *L. rhamnosus* as single isolate and a mixture of equal doses of bacterial isolates, respectively. Treatment control A1, B1, C1 and D1 received rotifers and *A. franciscana* that were not incubated in bacteria. Enriched *A. franciscana* were added to the larval tanks at ratio 4-5 *Artemia nauplii* per mL once a day from day 9 to day 13. The feeding intensity was remained constant during the experiment from four to five *A. franciscana* per mL.

Sampling procedures: Samples from rotifers before (control) and after incubations were taken for detection of LAB probiotics were harvested in 20µ nylon net, washed thrice with sterilised seawater (22 ppt) and homogenised in 1mL sterilised seawater. Samples of rotifers from treated tanks were taken after 24 h. Serial dilutions of the samples in autoclaved seawater (28 ppt) were plated on MRS agar. Petri dishes were incubated at 37ºC for 3 days. Plates grown with LAB were recorded for the study.

Three *A. franciscana* were taken before (control untreated) and after the bioencapsulation process for LAB detection. At least three *A. franciscana* from each replicate tank of treatment and control were collected aseptically with a pipette on day 10, 11 and 13 of the experiment before addition of new bioencapsulated *Artemia*. *A. franciscana* nauplii were aseptically homogenized in five ml of autoclaved seawater (28 ppt). On day 14 of the experiment, samples of larvae for detection of LAB were collected before termination of experiment. Three larvae from each tank were surface-disinfected for 60 sec with benzalkonium chloride (0.1% w/v), rinsed in sterilised non ionic water and homogenized by use of glass homogenizers in 5 mL autoclaved seawater (28 ppt) (Muroga *et al*., 1987; Munro *et al*., 1994). Serial dilutions of the samples in autoclaved seawater (28ppt) were plated on Marine agar (Difco), TCBS agar and MRS agar. All agars prepared in seawater with 28 ppt . Petri dishes were incubated at 37ºC for 24 h (Marine agar and TCBS) and 3 days (MRS). Plates with 30 cfu or above were studied for the study. Samples of tank water were taken on day 2, 4, 6, 8, 10, 12, and 14 for detection LAB.

Bacterial count and identification: Total bacterial count was estimated on marine agar while presumptive *Vibrio* was enumerated on TCBS and LAB were isolated from the MRS agar. Bacteria from TCBS were identified through BD BBL Crystal™ kit identification system (USA). LAB was identified based on morphology and Gram reaction.

Survival of larvae: At the end of the experiment, the percent survival of larvae was determined by direct counting of larvae or using following formula:

$$\text{Survival rate} = \frac{\text{Total number of larvae survive} \times 100}{\text{Initial number of larvae stocked}}$$

Water parameters: During the study period, water parameters such as temperature, salinity, dissolved oxygen (DO) and pH were monitored using YSI 556 MPS multi meter (USA). Readings were taken once a day from all treatments.

Statistical analysis: One-way ANOVA was used to test null-hypothesis, while Tukey's test was used for multiple comparisons between the different treatments.

RESULTS

Both *B. plicatilis* and *A. franciscana* were looking efficient grazers to accumulate the LAB bacteria present in solution during 2 h incubation period and rotifers successfully retained LAB after 24 h. No LAB was detected from the control or untreated rotifers or *Artemia*. Results obtained for LAB detection from rotifers are given in Table 1.

It was well observed that all three probiotic bacterial as single isolates and multi isolates added were efficiently accumulated in the *A. franciscana* nauplii after incubation in the bacterial suspensions during the incubation experiment (2 h).

The total bacteria per *A. franciscana* for *L. plantarum* was determined to 6.5×10^3, 2.2×10^2, 0 and 2.5×10^2, 0.72×10^2 and 4.7×10^3 on Marine agar, TCBS agar and MRS agar before and after inoculation respectively. The total bacteria per *A. franciscana* for *L. salivarius*, 5.3×10^3, 2.12×10^2, 0 and 2.62×10^2, 0.90×10^2, 4.22×10^3 bacteria before and after inoculation on Marine agar, TCBS agar and MRS agar respectively. The total bacteria per *A. franciscana* for *L. rhamnosus* 5.55×10^3, 2.32×10^2, 0 and 2.54×10^2, 0.82×10^2 and 4.26×10^3 bacteria before and after inoculation on marine agar, TCBS agar and MRS agar respectively. However, all three together in mixture $6.33\times10^{3,}$ 2.30×10^2, 0 and 2.14×10^2, 0.52×10^2, 5.22×10^3 bacteria per *A. franciscana* in the treatments respectively in first part of the study (bacteria added via bioencapsulation only) Table 2.

The bacteria added via bioencapsulation and to rearing water of larviculture, total viable count of bacteria in *Artemia* on marine agar observed lower at each day in inoculated tanks compared those after time of incubation. Contrary it increased in controls during the same days. With regard to presumptive *Vibrio* on TCBS agar similar results were observed to those of inoculated with single isolate, however, no *Vibrio* was determined in *Artemia* in multi isolate inoculated samples on the day 13. Highest LAB bacteria 4.10×10^3 was determined in *Artemia* on day 11 in those incubated with mixture of LAB isolates. In all samples of *Artemia*, LAB count was decreased as compared to those at the time of incubation. No LAB was detected from *Artemia* sampled from the control. The total cfu of bacteria in *Artemia* collected from the water of control increased from day 10 to day 13 in all experiments compared to *Artemia* from treated groups (Table 2).

Table 1: Detection of LAB probiotics (on MRS agar) in rotifer (*B. plicatilis*) before and after incubation and after 24 h inoculation in bioencapsulation treatment dose

LAB isolates	Before	After	After 24 h	Control
L. Plantarum	-	+	+	-
L. Salivarius	-	+	+	-
L. Rhamnosus	-	+	+	-
L. Plantarum, *L. Salivarius* and *L. Rhamnosus*	-	+	+	-

Furthermore, *L. plantarum*, *L. salivarius* and *L. rhamnosus* were evident on days 2 to day 14 of the experiment. Mixture of LAB showed their evidence through out the experiment but was not identified to species level in the application trials. No LAB was detected in any of the control. It was noted that LAB were detected in all larvae collected from the treated tanks but it was not detected in any control larvae (Table 3).

Survival of larvae: By the end of the larval period, larval survival (±95% confidence intervals) those inoculated with *L. plantarum* was 13.50±1.32% over the non inoculated control 2.83±0.76%. The larval survival those inoculated with *L. salivarius* achieved to 12.33±1.76% over the untreated control 1.83±0.29% and larvae exposed to *L. rhamnosus* did produce12.50±1.00% compared to untreated control 2.50±1.00% For mixture of LAB probiotics did produce survival 13.83±0.76% over the non treated control 1.67±1.53% (Fig. 1).

There was no statistical significance ($p>0.05$) in the survival of larvae in any treatment fed with bioencapsulated feed rotifers and *Artemia* and inoculated to water.

The obtained survival in experimental treatments of larvae did show significant higher survival in comparison to non inoculated controls.

The results showed that mixture of three LAB did produced highest survival followed by *L. plantarum* isolate as single probiotic inoculation during the present study. All LAB probiotics did produce considerably better survival over the control. In addition, the total bacterial count of flora in *Artemia* from rearing water was affected by the addition of probiotic candidates; and decrease in *Vibrio* count was noticed accordingly.

Water parameters: The water parameter in the treatments inoculated with bioencapsulation live feed and probiotics added to water on the day first and subsequently every after one day were recorded. Temperature ranged, 28.16-28.19ºC, salinity 28.16-28.18 ppt, DO 6.07-6.09 mg/L and pH 7.97-7.98 within the

Table 2: Mean bacteria per *Artemia* of LAB probiotics in bioencapsulation treatment before and after incubation and post inoculations dose with water additive

		Bacteria per *Artemia* (Control) and after incubation (at Day-9)			Bacteria per *Artemia* after inoculation to rearing system Day-10		
Treatment		MA	TCBS	MRS	MA	TCBS	MRS
Control		6.5×10^3	2.2×10^2	-	9.78×10^3	6.34×10^3	-
L. plantarum	After	2.5×10^2	0.72×10^2	4.7×10^3	1.48×10^2	0.18×10^2	2.68×10^3
Contro		5.3×10^3	2.12×10^2	-	6.88×10^4	6.78×10^3	-
L. salivarius	After	2.62×10^2	0.90×10^2	4.22×10^3	2.12×10^2	0.22×10^2	2.16×10^3
Control		5.55×10^3	2.32×10^2	-	7.62×10^4	6.12×10^3	-
L. rhamnosus	After	2.54×10^2	0.82×10^2	4.26×10^3	1.78×10^2	0.18×10^2	2.46×10^3
Control		6.33×10^3	2.30×10^2	-	7.68×10^4	6.81×10^3	-
Mixture of three LAB	After	2.14×10^2	0.52×10^2	5.22×10^3	1.84×10^2	0.24×10^2	2.16×10^3

		Bacteria per *Artemia* after inoculation to rearing system					
		Day-11			Day-13		
		MA	TCBS	MRS	MA	TCBS	MRS
Control		6.92×10^4	7.16×10^3	-	7.12×10^4	7.84×10^3	-
L. plantarum	After	1.28×10^2	0.24×10^2	3.18×10^3	1.16×10^2	0.18×10^2	3.12×10^3
Control		7.22×10^4	6.96×10^3	-	8.24×10^4	8.32×10^3	-
L. salivarius	After	1.62×10^2	0.28×10^2	2.92×10^3	2.12×10^2	0.20×10^2	2.98×10^3
Control		8.11×10^4	6.52×10^2	-	8.62×10^4	7.92×10^3	-
L. rhamnosus	After	1.61×10^2	0.24×10^2	3.12×10^3	1.56×10^2	0.20×10^2	3.10×10^3
Control		8.12×10^4	7.22×10^2	-	7.24×10^4	7.32×10^3	-
Mixture of three LAB	After	1.56×10^2	0.14×10^2	4.10×10^3	1.22×10^2	-	3.10×10^3

MA: Marine agar; TCBS: Thiosulphate Citrate Bile Salts Sucrose Agar; MRS: de Man, Rogosa and Sharpe

Table 3: Detection of probiotics (LAB) in water and larvae fed with bioencapsulated feed (rotifers and *Artemia*) and added to water

Days		2	4	6	8	10	12	14	14
Probiotics		w	w	w	w	w	w	w	L
L. plantarum	Control	-	-	-	-	-	-	-	-
	Treated	+	+	+	+	+	+	+	+
L. salivarius	Control	-	-	-	-	-	-	-	-
	Treated	+	+	+	+	+	+	+	+
L. rhamnosus	Control	-	-	-	-	-	-	-	-
	Treated	+	+	+	+	+	+	+	+
L. plantarum L. salivarius	Control	-	-	-	-	-	-	-	-
L. rhamnosus	Treated	+	+	+	+	+	+	+	+

W: water; L: larvae; - No; + Yes

Table 4: Mean, standard deviation and range for temperature, salinity, DO and pH for control and bioencapsulated and water additive probiotic treatments. There were three replicates per treatment

	Single isolates treatment			
Parameters	Temp (ºC)	Salinity (ppt)	DO (mg/L)	pH
Control	28.16±0.04	28.18±0.04	6.05±0.08	8.16±0.02
L. plantarum	28.19±0.05	28.18±0.05	6.09±0.06	7.97±0.07
L. salivarius	28.18±0.06	28.18±0.05	6.07±0.12	7.97±0.08
L. rhamnosus	28.16±0.06	28.16±0.04	6.07±0.07	7.98±0.11
	Multi isolates treatment			
Control	28.21±0.06	28.19±0.04	6.03±0.11	8.14±0.03
Mixture of three LAB	28.22±0.06	28.17±0.04	6.02±0.11	7.98±0.07

treated groups while in control 28.16ºC, 28.18 ppt, 6.05 mg/L and 8.16, respectively in single isolates treatments Table 4. However in multi isolate mixture treatment as bioencapsulation and water additive, the water parameters such as temperature, salinity, DO and pH were 28.22ºC, 28.17 ppt, 6.02 mg/L and 7.98 in treated groups while in control parameters were 28.21ºC, 28.19 ppt, 6.03 mg/L and 8.14, respectively (Table 4).

DISCUSSION

The larvae of *P. pelagicus* are faint, low immune and easily susceptible to pathogenic bacteria. Rotifers (*Brachionus plicatilis)* culture is a crucial feature of marine larvae production and are used as live feed as first feeding during early larval stages including *P. pelagicus*. However, during the mass culture of rotifers, a complex

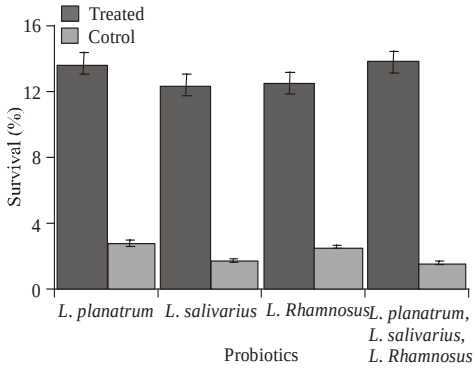

Fig. 1: Survival (%) of larvae fed to bioencapsulated rotifer and *Artemia* and probiotics as water additive

bacterial ecosystem develops and as a consequence the rotifers can be considered as an actual and important source of bacteria for fish larvae (Verdonck *et al*., 1994). As part of the first food, the rotifer-associated bacteria modify the gut flora present during early larval stages (Muroga *et al*., 1987; Nicolas *et al*., 1989). In nutritional terms, the bacteria present or added in the rotifer cultures can improve the dietary value of rotifers for fish larvae (Gatesoupe, 1989b; Gatesoupe, 1991); however, the rotifer-associated pathogenic bacteria could be detrimental to larval performance and survival (Gatesoupe, 1982; Perez-Benavente and Gatesoupe, 1988; Gatesoupe, 1989b). Other live prey, *Artemia* nauplii are widely recognized as the best natural storable live feed available, and are extensively used in marine finfish and crustacean hatcheries throughout the world because of their nutritional and operational advantages (Lavens and Sorgeloos, 1996). Both rotifers and *Artemia* have been used as a vector for the delivery of different materials, such as nutrients (Watanabe *et al*., 1983), antimicrobial agents (Dixon *et al*., 1995), vaccines (Campbell *et al*., 1993), and probiotics (Gatesoupe, 1994).

In the present study rotifers *B. plicatilis* and nauplii of *A. franciscana* were bioencapsulated with *Lactobacillus* probiotics and it was observed that both live prey organisms could have ability to accumulate LAB probiotics. However, when *A. franciscana* enriched with the LAB probiotics it was observed it inhibited the bacterial growth compared to control. An incubation of live food organisms in a bacterial suspension, consisting of one or several probiotic strains is a possible approach to replace opportunists with other less aggressive bacteria. In the present study, LAB was successfully recovered from *B. plicatilis*, *A. franciscana*, culture water and larvae after inoculations. It has been observed that during the present study, when *Artemia* enriched with LAB probiotics, the total bacteria per *Artemia* was significantly lower compared to *Artemia* not added with bacteria. It is therefore possible that probiotic isolates produced substances that inhibited the growth of other bacteria (Gatesoupe, 1997; Jöborn *et al*., 1997; Sugita *et al*., 1998). The result of present study was in agreement of previous findings. Live food organisms may reside in the rearing tanks for several hours before ingested by the larvae (Reitan *et al*., 1993). Once the bacteria have been bioencapsulated in the live food, it is important to determine the rate of loss of the bioencapsulated bacteria, and whether the changed bacterial composition persists, as live food organisms may be depleted of the specific bacteria before they are ingested by fish larvae. It was successfully observed during the present study that rotifers were able to accumulate the LAB probiotics when incubated either in single or in combinations. Bioencapsulation of LAB bacteria in live feed was achieved after short incubations (2 h) in bacterial suspensions. A decrease in the bioencapsulated bacteria was determined after transfer to first feeding; on the contrary, the bacteria were still present in the live feed 24 hours after transfer. *Artemia* did retain LAB after incubation but the frequency of the bacteria was not same compared to those at the time of incubation. In all experiments LAB isolates, in particular, when *Artemia* nauplii were maintained in the bacterial suspension, the quantity of bacteria bioencapsulated rapidly increased after 2 h with a sustained level, followed by a decline after 24 h post inoculations. When *L. plantarum* as sole probiotic was inoculated to *Artemia* after 2 h of incubation, it was detected at 4.7×10^3 bacteria per *Artemia* but decreased to 2.68×10^3 bacteria per *Artemia* after 24 h. Again a slight increase was seen on day 11 and day 12 with 3.18×10^3, 3.12×10^3 bacteria per *Artemia* respectively. Similar results were observed when *Artemia* was inoculated with *L. salivarius* and *L. rhamnosus* and mixture of three LAB. Therefore, it could be depicted from the results that both live prey have the ability to retain or accumulate the LAB probiotics after incubation and inoculation to culture water.

It has been shown in previous studies that bacteria added in the live food inhibited the total growth of bacteria in the rearing system of turbot larvae (*Scophthalmus maximus* L.) (Gatesoupe, 1989a; Kennedy *et al*., 1998). During the present study, total viable count of bacteria and presumptive *Vibrio* in *Artemia* observed lower at sampling days in inoculated tanks and only mixture of LAB inhibited *Vibrio* growth on day 13 in *Artemia* sample. It could be assumed that LAB might have produced antibacteriocin substance that inhibited or lowered the growth of bacteria in treated *Artemia*. Moreover, only mixture of LAB did inhibit total *Vibrio* on day 13. It was observed that none of any LAB probiotics did inhibit the total growth of viable bacteria in *Artemia* at the time of incubation or *Artemia* collected from the culture water. Result of present study could indicate the new findings and somewhat differ from the findings of previously mentioned.

In the present study, nauplii of *A. franciscana* and *B. plicatilis* were used as a vector to carry the probiotic *Lactobacillus* to digestive tract of *P. pelagicus* larvae. The probiotics in this study promoted the survival of *P. pelagicus* larvae and influence the water parameter particularly pH in the experimental treatments in comparison to non inoculated control. It was apparent from present study that the application of LAB probiotic via bioencapsulation and the water had beneficial effects on the survival rate of *P. pelagicus* larvae and did show a significantly higher survival when compared with those the non inoculated control. Lactic Acid Bacteria (LAB) strains applied to different fish species led to increased survival compared with control groups, as in trout (Nikoskelainen *et al*., 2001; Irianto and Austin, 2002; Vendrell *et al*., 2008) and tilapia (Aly *et al*., 2008). The previous study showed that supplementation of the commercial LAB, *Bacillus* probiotic significantly increased the survival rate of *P. vannamei* (Zhou *et al*., 2009), Indian white shrimp (*Fenneropenaeus indicus*) in the treatments over the controls (Ziaei-Nejad *et al*., 2006). A similar finding was obtained by Nogami and Maeda (1992), Maeda (1992) and Nogami *et al*., (1997), who inoculated a non LAB bacterial strain PM-4 *Thalassobacter utilis*, into blue crab (*P. trituberculatus*) larval rearing tanks at the concentrations of 10^6 cells/mL achieved better survival over non inoculated control. However, when LAB added to rotifers, *Artemia* and inoculated in rearing water in combination of three it achieved highest ever survival 13.83% during the present study compared to those inoculated with single isolates. In addition, *L. plantarum* as a single isolate, when inoculated to live feed and added to rearing water together did significantly produce better survival 13.50% compared to those of treated with *L. salivarius* 12.33% survival and *L. rhamnosus* 12.50% survival respectively. There was no major difference among the survivals of larvae inoculated either with single or mixture of LAB probiotics. The use of LAB significantly influences the survival and affects the pH of treated water during the trials of experiment. LAB has capacity to influence the pH and water quality. When Zhou *et al*. (2009) added lactic acid bacteria *B. coagulans* SC8168 as water additive to larviculture of shrimp *Penaeus vannamei*, it significantly influenced the pH and water quality. The results of present study are in match of previously mentioned findings. This is the first report where indigenous LAB were used as a single and in mixture of three via bioencapsulation and water additive together in the larviculture of *P. pelagicus*.

It can be concluded that *Lactobacillus* probiotics promoted the survival in all experimental treatments in comparison to control treatments and significantly influenced the pH. LAB reflected positive effects on larvae and can be used as tool for the larviculture of *P. pelagicus*.

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