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Effects of *Lactobacillus* Probiotics on the Enhancement of Larval Survival of *Portunus pelagicus* (Linnaeus, 175) Fed Via Bioencapsulated in Live Feed

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Abstract: The aim of this study was to find out whether it is possible; the lactic acid bacteria (LAB) bioencapsulated with rotifers *Brachionus plicatilis* and *Artemia franciscana* can influence the survival of *Portunus pelagicus* larvae. Three indigenous LAB probiotics previously isolated from the gut of female *Portunus pelagicus* were used in the study. All LAB isolates were effectively grazed by *B. plicatilis* and *A. franciscana* when incubated at concentrations 10^7 cfu mL^{-1} for 2 hours. Mixture of three LAB effectively suppressed *Vibrio* in treated *Artemia*. LAB cfu per *Artemia* were decreased after inoculation in treated samples. No any LAB inhibited total growth of bacteria in *Artemia*. Survival of larvae was improved those fed with bioencapsulated live prey versus the untreated control. Enriched feed with *Lactobacillus plantarum* did produce highest survival 7.00 ± 0.87 %, followed by *L. rhamnosus* 6.83±1.26% survival. As an individual isolate, *L. plantarum* did show putative probiotic effect by producing better survival of larvae over other LAB probiotics. The survival counts of larvae were not statistically significant (P>0.05) in all cases. LAB was successfully recovered from the *Artemia*, rotifers, rearing water and larvae after post inoculation. The bioencapsulated live prey with LAB probiotics could be used as a tool for future studies on *P. pelagicus.*

Key words: Rotifer % *Artemia* % Probiotics % Putative % *Portunus Pelagicus*

INTRODUCTION

In hatcheries, low or even zero survival of *P. pelagicus* seed production achieved and mortality occurred owing to microbial infections particularly *V. harveyi* transmitted from adult female [1]. Antibiotics are used in general to control the infections in larviculture, which led to resistant bacteria and in case of *P. pelagicus* antibiotics are questionable [2]. In the past few years, efforts have been made to decrease the use of therapeutic chemicals and antibiotics [3] towards a more environmentally friendly and sustainable aquaculture. In intensive rearing of marine fish larvae suffers from heavy mortalities, due to bacteria introduced in the rearing system with live food [4, 5]. It has been widely recognized that probiotics constitute a potential tool in the reduction of mortalities in the rearing of aquatic organisms [6].

The search for alternative to antibiotic therapy has turned to the use of probiotics during the early larval stages. Several studies have reported encouraging results in the application of probiotics in aquaculture [7,9] and application of probiotics to fish larviculture in particular has yielded to positive effects, mainly in survival and growth rates [10,11].

The addition of bacteria to the rearing water at an early stage is one method that has proven effective [12] and other route of bacteria to fish larvae is by bioencapsulating bacteria in the live feed [13, 14].

Better strategy, that avoids the introduction of exotic bacteria to the system, is to select probiotic candidates among isolated strains from same healthy organism [15]. Replacement of the opportunistic bacteria with probiotics in water or live food can be a good strategy to provide protection to the larvae [16].

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Rotifers (*Brachionus plicatilis*) and *Artemia* are essential live prey in larval rearing of marine fish species and both are filter feeders [17]. Under culture conditions, it is possible to manipulate the associated microflora in the larval rearing environment with the aim of improving larval survival [18].

This is the first study to determine the influence of indigenous Lactic acid bacteria (LAB) on the survival of *P. pelagicus* larvae through bioencapsulation in live prey, rotifers and *Artemia*.

MATERIALS AND METHODS

Bacterial Isolates: Based on *in vitro* inhibition to indicator pathogens *Vibrio harveyi, V. parahaemolyticus* and *P. piscicida* and validated as probiotic via small scale *in vivo* model [19], three Lactic acid bacteria (LAB) including *L. plantarum, L salivarius* and *L. rhamnosus* which previously isolated from the gut microflora of female *P. pelagicus* were used in this study.

Experimental Design: Live prey was enriched with LAB probiotics at a final concentration $1x10^7$ cfu mL^{-1} as a single isolate to T-1, T-2, T-3 and multi isolate to T-4 added to culture tanks. Control was employed with larvae but no bioencapsulated feed provided to T-1C T-2C, T-3C and T-4C. Each experiment was replicated and carried for 14 days. All aquarium were confined in 3' x 5' x 1.5' flat bottom tanks and filled with fresh tap water and tank supplied with submerged heater in order to maintain the temperature of aquaria to 28°C. A 12 h dark/12h light photoperiod was maintained during the entire trials. Dead larvae, debris were siphoned out daily and water amount discharged during cleaning of aquaria, same quantity was compensated. Any live larva found was transferred to respective tank with big bore pipette tip. Water exchange was started at the day second of experiment and maintained at 10-12 % of the total aquarium volume daily. In controls, water was changed 30-40 % daily from day two of the experiment to reduce the pathogenic bacterial risk which multiplying in aquaria or may be ingress via untreated live feed. During the study period, temperature, salinity, dissolved oxygen (DO) and pH were monitored daily using YSI 556 MPS multi meter (USA). A quantity of 20 larvae per liter was introduced in each 10 liter inoculated and non-inoculated aquaria with sterilized seawater (28ppt) and aeration system. Each aquarium was inoculated until day 13 and the trials were terminated on day 14.

Bacterial Cultures: LAB were cultured in MRS (deMan Rogosa and Sharpe) broth prepared in sea water with salinity 28ppt at 37°C with agitation 150 rpm for 48 hrs. Bacteria were harvested by centrifugation 15000 rpm for 15 minutes, the supernatant was discarded and the pellets were washed two times in sterilized seawater (28ppt) and finally suspended in sterilized seawater for application. Bacterial density (1×10^7 cfu mL^{-1}) was measured in OD 630_{nm} using a UV-1800 spectrophotometer (Shimadzu, Japan) according to the standard set previously.

Brood Stock Management and Hatching and Experimental Larvae: Berried females were collected from Strait of Tebrau (1°22' N and 103° 38' E), Johor, West Malaysia and were transported to marine hatchery of Institute of Tropical Aquaculture, Universiti Malaysia Terengganu for breeding. Females disinfected and placed in hatching tanks with sand substrate and adequate aeration according to Talpur *et al*., [1].

Zoeas 1 of *P. pelagicus* were used as experimental larvae. Prior to exposing, to feed and disinfection, energetic larvae were washed with sterilized seawater with similar parameters used for lariviculture in order to minimize the bacterial load with larvae adhering from hatching tank water.

Seawater for Larvae Culture: UV treated seawater was filtered through a 10 µm net and then sterilized with sodium hypochlorite (50 mg L^{-1}) for 24 h. This procedure, almost eliminated all naturally occurring bacteria in water, was followed by neutralization with sodium thiosulphate at the beginning of the experiment.

Disinfection of *P. pelagicus* Larvae: Disinfection was carried out according to Planas *et al*., [20]. Larvae were transferred to a 30 liter transparent aquaria tank provided with sterilised sea water (28ppt) previously supplemented with Dismozon Pur (1%), larvae were placed in tank for 4 hours with adequate aeration and temperature was maintained at 28 °C. Larvae were kept in aerated seawater (28ppt) for 1 hour to remove the residue of Dismozon Pur.

Rotifer Culture and Bioencapsulation of Probiotic Bacteria: Rotifers, *Brachionus plicatilis* were cultured according to Talpur *et al*., [21]. Suspensions of bacteria were prepared in seawater for the two treatments single isolate and multi isolate (1:1:1) at 10^7 cells mL^{-1}. Rotifers fed with microalgae *Nannochloropsis* sp. were washed carefully with autoclaved seawater (22ppt) in a 40-µm pore

size sterile net and then transferred to a sterile 1500-ml conical flask containing 1000 mL of sterilized seawater (22ppt). The rotifers at density 800 ind mL^{-1} grazed in the bacterial suspensions for 2 hours with aeration, were rinsed with autoclaved seawater (22ppt) for 5 min and then added to the rearing tanks at allowance of 30-40 rotifers mL^{-1} daily. Rotifers fed to control tanks without bacteria. All manipulations were carried out aseptically under a laminar flow with sterilized material to maintain the hygiene.

Artemia Culture and Bioencapsulation: *Artemia franciscana* cysts (Great Lake *Artemia*, Salt Lake City, Utah, USA) were incubated for 24 h in sterile seawater (28ppt) and newly hatched nauplii were enriched overnight with Selco Plus (INVE Aquaculture, Belgium) in 10L cylindroconical tank with adequate aeration.

The *A. franciscana* were bioencapsulated with probiotics according the procedure of Makridis *et al*., [22]. *A. franciscana* were collected in 2500 mL conical flask at 100 ind mL^{-1} and were incubated for two hour in cultures of probiotic bacteria at 1 x 10^7 cells mL^{-1}. *Artemia* were rinsed in sterilised seawater (28ppt) in 100 µm pore size nylon net then fed at ratio 4-5 *Artemia* per larvae once a day from day 9 until day 13.

Sampling Procedures: Rotifers samples (5mL) before (control), after incubation (treated) and after 24h from treated tanks were taken in 20 µm nylon net, washed thrice with sterilised seawater (22ppt) and were homogenised in 1mL sterilized seawater. Samples were serially diluted, plated on MRS (de Man Rogosa and Sharpe) agar to determine the evidence of LAB and Petri dishes incubated at 37 °C for 3 days. Plates grown with LAB were recorded for the study.

At least three *A. franciscana* were taken aseptically with a pipette before and after incubation from each treatment and control on day 10, 11 and 13 before addition of new bioencapsulated *Artemia*. Each sample was washed three times with sterilized seawater to remove the adhering bacteria and was homogenized in 5mL autoclaved seawater (28ppt). On day 14 three larvae from each tank were surface-disinfected for 60 seconds with benzalkonium chloride (0.1% w/v), rinsed in sterilized distilled water and homogenized in 5mL autoclaved seawater (28ppt) according to Muroga *et al*., [23]. Samples of tank water were taken on day 2, 4, 6, 8, 10, 12 and 14. Serial dilutions of the samples were plated on (28ppt) marine agar (Difco), Thiosulphate Citrate Bile Salts Sucrose Agar (TCBS) and MRS agar prepared with seawater. Petri dishes were incubated at 37 °C for 24 hours (TCBS) and 3 days (MRS).

Total Bacterial Count and Bacterial Identification: Total bacterial count of *Artemia* samples was estimated on marine agar. TCBS agar was used to count the *Vibrio* and MRS agar was used for LAB determination. *Vibrio* was identified through BD BBL crystal kit identification system (USA). However, LAB were identified based on morphology and Gram reaction.

Survival of Larvae: At the end of the experiment, larvae were directly counted and the percent survival was determined by using following formula:

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

Statistical Analysis: Survival was compared by ANOVA analysis of variance using statistical software (SPSS 16.0 for windows). Post hoc test and Tukey's tests were used, if they were significant (confidence level±95).

RESULTS

Detection of LAB and Bacterial Count: *B. plicatilis* accumulated the LAB bacteria within 2 hours incubation period, were also detected after 24-post enrichment in samples. No LAB was detected from the control or untreated rotifers Table1.

The total LAB bacteria (cfu) per *A. franciscana* on MRS agar were determined decreased after inoculation to rearing water. No probiotic bacteria found with *A. franciscana* from control. Determination of cfu per *A. franciscana* after incubation with LAB (on MRS) was different with each isolate. Highest LAB cfu per *A. franciscana* after incubation was determined with mixture of three LAB followed by *L. plantarum* and lowest cfu with *L. salivarius* Table 2. In all control total viable bacteria and *Vibrio* was escalated with time elapse.

The total bacteria (cfu) per *A. franciscana* enriched with *L. plantarum* taken from culture water were found lowest $2.22x10^2$ on marine agar on day 13 and highest $2.98x10^2$ on day 11. Lowest *Vibrio* count $0.74x10^2$ on TCBS agar was observed on day 11. Highest LAB $3.44x10^2$ was seen on day 13 of the experiment T-1 and Table 2.

Table 1: Detection of LAB in rotifers

Isolates	Control	Treated	After 24 from culture water
L. plantarum	-	+	+
L. salivarius	-	+	+
L. rhamnosus	-	+	+
Mixture of three LAB	-	+	+

Note: +, Yes,-, No

Table 2: Mean bacteria (cfu) per *Artemia* of LAB probiotics in bioencapsulation treatment post inoculation dose (after 24 h)

		Accumulation of LAB at time of incubation	Day-10			Day-11			Day-13		
Days	Treatment	MRS	MA	TCBS	MRS	MA	TCBS	MRS	MA	TCBS	MRS
L. plantarum	T-1	$4.7x10^3$	$2.55x10^2$	$0.78x10^2$	$2.92x10^2$	$2.98x10^2$	$0.74x10^2$	$3.12x10^2$	$2.22x10^2$	$0.78x10^2$	$3.44x10^2$
Control		-	$7.55x10^4$	$6.12x10^3$	-	$7.94x10^4$	$7.68x10^3$	-	$8.26x10^4$	$8.66x10^3$	-
L. salivarius	T-2	$4.22x10^3$	$2.86x10^2$	$0.86x10^2$	$2.36x10^2$	$3.18x10^2$	$1.04x10^2$	$2.82x10^2$	$3.22x10^3$	$0.92x10^2$	$2.66x10^2$
Control		-	$8.06x10^4$	$7.10x10^3$	-	$8.84x10^4$	$7.98x10^3$	-	$8.78x10^4$	$9.06x10^3$	-
L. rhamnosus	T-3	$4.26x10^3$	$2.72x10^2$	$0.82x10^2$	$2.38x10^2$	$2.98x10^2$	$0.92x10^2$	$2.94x10^2$	$4.22x10^2$	$0.82x10^2$	$2.88x10^2$
Control		-	$7.12x10^4$	$7.08x10^3$	-	$8.14x10^4$	$6.98x10^2$	-	$8.68x10^4$	$8.78x10^3$	-
L. plantarum *L. salivarius* and *L. rhamnosus*	T-4	$5.22x10^3$	$2.11x10^2$	$0.62x10^2$	$2.17x10^2$	$2.88x10^2$	$0.48x10^2$	$2.15x10^2$	$2.20x10^2$	$0.62x10^2$	$2.26x10^2$
Control		-	$6.94x10^4$	$5.16x10^3$	-	$7.48x10^4$	$8.58x10^2$	-	$7.82x10^4$	$7.84x10^3$	-

Note:-Nil, 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*)

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	-	-	-	-	+	+	+	+
Mixture of three LAB									
	Control	-	-	-	-	-	-	-	-
	Treated	-	-	-	+	+	+	+	+

Note: W-water, L-larvae.-. No, +. Yes

The total bacteria (cfu) per *A. franciscana* enriched with *L. salivarius* taken from culture water were found lowest $2.86x10^2$ on marine agar on day 10 and highest $3.22x10^3$ on day 13. Lowest *Vibrio* count $0.86x10^2$ on TCBS agar was observed on day 10 and highest $1.04x10^2$ on day 11. Highest LAB $2.98x10_2$ was observed on day 11 and lowest $2.72x10_2$ on day 10 of the experiment T-3 but on day 13 *Vibrio* found suppressed. An increase in total viable count of bacteria was witnessed with time elapse Table 2.

The total bacteria (cfu) per *A. franciscana* enriched with *L. rhamnosus* taken from culture water were found lowest $2.72x10^2$ on marine agar on day 10 and highest $4.22x10^2$ on day 13 of the experiment. Lowest *Vibrio* count $0.82x10^2$ on TCBS agar was observed on day 10 and day 13 respectively and highest $0.92x10^2$ on day 11. Highest LAB $2.98x10^{2\ was}$ observed on day 11 and lowest $2.72x10^2$ on day 10 of the experiment T-3 but on day 13 *Vibrio* found suppressed. An increase in total viable count of bacteria was witnessed with time elapse Table 2.

The total bacteria (cfu) per *A. franciscana* enriched with mixture of three LAB (*L. plantarum*, *L. salivarius* and *L. rhamnosus*) taken from culture water were found lowest $2.11x10^2$ on marine agar on day 10 and highest $2.88x10^2$ on day 11 of the experiment. Lowest *Vibrio* count $0.48x10^2$ on TCBS agar was observed on day 11 and highest $0.62x10^2$ on day 10 and day 13 respectively. Highest LAB $2.26x10^2$ was observed on day 13 and lowest $2.17x10^2$ on day 10 of the experiment T-3. An increase in total viable count of bacteria was witnessed with time elapse Table 2.

No LAB was detected in *A. franciscana* from non inoculated controls on day 10, 11 and day 13 of the trials. cfu per *Artemia* collected from the water of control increased from day 2 to day 13 in all experiments compared to *Artemia* from treated groups. In multi isolate treatment, it was hard to distinguish morphologically microflora on same plate, therefore it was observed only as an evident of LAB presence.

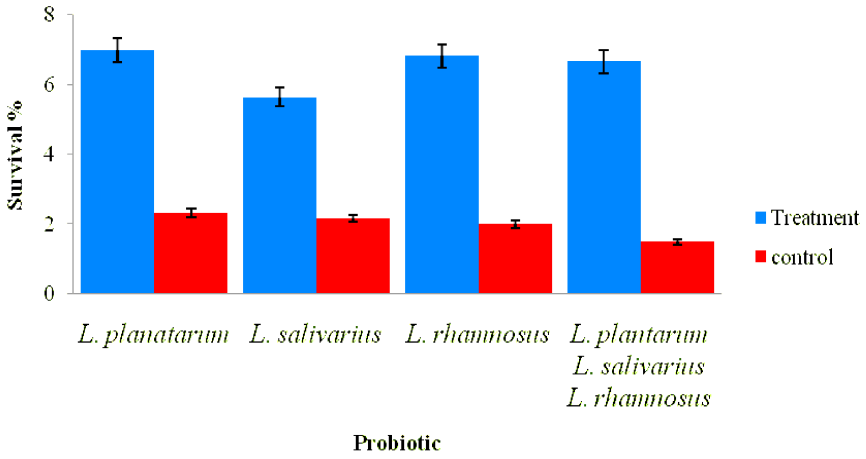

Fig. 1: Survival (%) of larvae fed bioencapsulated rotifer and *Artemia*

L. plantarum was not detected on day 2, 4 and 10 in the rearing water. *L. salivarius* did not detect on day 2, 4 and day 6. *L. rhamnosus* was not evident on days 2, 4, 6 and 8th day. However, mixture of three LAB detected on day 8, 10, 12 and day 14 and the LAB were successfully detected in all treated larvae and not detected in control larvae Table 3.

Survival of Larvae: There was no statistical significance ($p > 0.05$) in the survival of larvae in any treatment fed with treated rotifers and *Artemia*. By the end of the larval period, larval survival (± 95% confidence intervals), inoculated with *L. plantarum* was 7.00 ± 0.87 % over the non inoculated control 2.50 ± 0.50 %. Survival of larvae with *L. salivarius* was achieved 5.67 ± 0.76 87% compared to that of control 2.33 ± 0.29 %. However, larvae feed with *L. rhamnosus* did produce survival 6.83 ± 1.26% over the control 2.17 ± 1.15%. Furthermore, a mixture of three LAB did enhance the survival to 6.67 ± 0.58% over the control 1.50±0.87 (control) Fig.1.

Water Parameters: Temperature ranged 28.23-28.27°C between treated tanks over the controls 28.21°C. Salinity remained 28.16-28.20 ppt in treated groups over controls 28.17-28.23 ppt. Dissolved oxygen ranged 6.01-6.05 mg/L in treated groups over controls 6.04 mg/L. pH ranged 8.13-8.15 in treated over the controls 8.14-8.15.

DISCUSSIONS

Rotifers (*Brachionus plicatilis)* are used as live feed as first feeding during early larval stages including *P. pelagicus*. In nutritional terms, the bacteria present or added in the rotifer cultures can improve the dietary value of rotifers for fish larvae [24]. During the present study, it was observed that rotifers did accumulate the LAB after short incubations (2h) in bacterial suspensions and LAB bacteria were still present in rotifers 24 hours after transfer. In study by Talpur *et al*., [21] who reported similar results when incubated rotifers with LAB probiotics. Therefore the present result was in match of previously mentioned study.

In the use of 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 [25] and have been used as a vector for the carrying of different materials, including probiotics [13]. It was seen during the present study the *Artemia* was actively grazing the LAB when incubated in probiotic suspension. In the study by Gomez-Gil *et al.*, [26] reported that when two *Vibrio* sp. including bacterium HL57 at 1.487 x 10^8 cfu mLG^1 was added to *Artemia* for 2 h, the *Artemia* accumulated 4.55 x 10^3 cfu/nauplii after incubation and bacterium C7b at 1.512 x 10^8 cfu mLG^1 the *Artemia* accumulated 4.77 x 10^3 cfu/nauplii respectively. *L. plantarum*, *L. salivarius*, *L. rhamnosus* and mixture of three LAB after 2h incubation, *Artemia* accumulated 4.7x10^3, 4.22x10^3, 4.26x10^3 and 5.22x10^3 cfu /*Artemia* respectively, similar results have already been reported by Talpur *et al*., [21] and it can be said that *Artemia* has ability to accumulate the bacteria when treated for appropriate time.

Moreover, it has been observed during the present study, when *Artemia* enriched with LAB probiotics, the total cfu per *Artemia* decreased in comparison to *Artemia* not added with bacteria. Similarly, Swain *et al*., [8]

found LAB being able to inhibit other bacteria and because the LAB has exhibiting probiotic profiles [27]. It could be assumed that probiotic profile LAB has inhibited bacteria in *Artemia*. After inoculation of enriched *Artemia* to rearing water, total LAB cfu per *Artemia* found decreased to a level as compared to those at the time of incubation with LAB and *Vibrio* count were evident lower compared to control. One common thing in all inoculated treatments was observed that LAB was found highest in *Artemia* on day 13 of the experiment. In general, total viable count of bacteria per *Artemia* was determined escalated with time elapse in all treated and untreated groups. Only mixture of LAB effectively suppressed to some degree the *Vibrio* in *Artemia* in treated groups. It has been shown in previous studies that bacteria added in the live food inhibited the growth of bacteria in the rearing system of turbot larvae (*Scophthalmus maximus* L.) [28]. The present study showed that LAB added to live prey particularly *Artemia* did effectively inhibited bacteria to a certain extent but not all bacteria were inhibited, therefore present results are in agreement of previous studies. Fluctuation in bacteria count per *Artemia* from treated groups might be due to random collection of *Artemia* samples. It was possible *Artemia* which treated on day nine may not be ingested by larvae were collected on day 11 may have low bacteria count or vice versa. Nevertheless, LAB count was found higher in all day 13 *Artemia* samples, this might be due to multiplication of LAB in *Artemia*.

The previous study showed that supplementation of the commercial LAB, *Bacillus* probiotic significantly increased the survival rate of *Penaeus vannamei* [29] and LAB are well known to provide nutritional elements which play essential roles in fish growth and development [30]. Other authors reported that LAB probiotic bacteria enhance the general welfare [31] and contribute to the health of host [32, 33]. However, during the present study, when LAB added to rotifers and *Artemia* and fed to larvae it achieved better survival of *P. pelagicus* larvae over the control, therefore, the results are in agreement with previous studies. Talpur *et al*., [21] reported higher survival of *P. pelagicus* larvae when larvae were fed bioencapsulated LAB together with addition of LAB to a rearing system, in all treated groups survival was above the 12% and *L. plantarum* as a single isolate and mixture of three LAB (*L. plantarum L. salivarius* and *L. rhamnosus*) did produce highest survival. In the present study as a single isolate *L. plantarum* showed more effect on survival when fed via bioencapsulation produced highest survival compared to other treated groups. Therefore the results are similar to some degree to previously mentioned study. In the purview of previous research it could be said that only bioencapsulation of LAB probiotics in live feed have not influenced the survival of larvae at maximum level compared to those reported by Talpur *et al*., [21] when added LAB to rearing system of *P. pelagicus* larvae along with bioencapsulation of live prey.

The probiotics used in the present study promoted the survival of larvae in all experimental treatments compared to those of untreated control. Moreover, LAB was successfully recovered from the *Artemia*, rotifers, rearing water and larvae after post inoculation.

CONCLUSION

LAB probiotics have ability to accumulate in live prey and inhibit the other bacteria. Different isolates of LAB probiotic had shown different effects on the survival of *P. pelagicus* larvae. *L. plantarum* as single isolate showed better probiotics effects on larval survival compared to other isolates of LAB. In general, LAB enhanced the survival of larvae when fed through bioencapsulation in live prey.

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