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Enrichment of Brine Shrimp (*Artemia franciscana*) Nauplii with Potential Probiont Strains, Bacillus JAQ04 and Micrococcus JAQ07

Nora Azirah M. Z.[*1], Murni Karim[2,3], Ina-Salwany M. Y[2,3], Harmin S. A.[1], Marini I[1]

[1]Faculty of Science and Biotechnology
Universiti Selangor, 45600 Bestari Jaya, Selangor, Malaysia.
***E-mail: nora.azirah@unisel.edu.my**
[2]Department of Aquaculture, Faculty of Agriculture Universiti Putra Malaysia, 43400 Serdang Selangor, Malaysia
[3]Laboratory of Marine Biotechnology, Institute of Bioscience Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

The study of bacterial interaction with crustaceans used in aquaculture, such as brine shrimp *Artemia* is gaining importance. Thus, this study aims to evaluate the effect on survival, growth and development of *Artemia* nauplii enriched with potential probionts JAQ04 and JAQ07 in gnotobiotic condition. Brine shrimp lethality concentration assay (LC_{50}) was carried out under monoxenic condition to identify the cytotoxity of these probionts. After decapsulation of the cyst, the *Artemia* nauplii were pre-incubated with high concentration of probiont *Bacillus* JAQ04 and *Micrococcus* JAQ07 at concentration of 10^7 CFU ml^{-1}. These bacteria were previously isolated from juvenile of Tiger grouper (*Epinephelus fuscoguttatus*). Pathogenic and toxicity assay demonstrated that both probiont strains were not pathogenic to the host. Significant survival ($p<0.05$) was observed in *Artemia* nauplii enriched with *Bacillus* JAQ04 (73%) and *Micrococcus* JAQ07 (63%) as compared to the control (43%). This study also demonstrated that potential probionts significantly improve the growth of the *Artemia* nauplii as demonstrated by higher body length when compared to the control ($p<0.05$). Therefore, it is suggested that *Bacillus* JAQ04 and *Micrococcus* JAQ07 were not harmless, and had significant effect on the growth and development of *Artemia*.

Keywords: *Bacillus* JAQ04, gnotobiotic *Artemia*, *Micrococcus* JAQ07

INTRODUCTION

Brine shrimp *Artemia* is the planktonic form of crustaceans widely use in live feed for many cultured fish and shellfish larvae due to nutritional and operational advantage. In spite been used as a food source for marine animal, *Artemia* posed several characteristic and advantages that make them useful as a model organism for research in animal such as ecology, physiology, ecotoxicology, aquaculture and genetic (Ali and Sultana, 2011). It is also useful to study the biology of infections, effect of chemotherapeutic agent and probiont before testing to their target organism. Many studies have been performed to improve *Artemia* nauplii feature as a live feed or vector for transferring the probiont to the digestive tract of the target host through a process known as bio-encapsulation (Gomez-Gil et al., 1998). The probiotic beneficially affect the host animal by improving gut micro flora by outcompete the pathogenic microorganism or supply the larval gut with important micronutrient (Makridis & Vadstein, 1999).

Microbiological studies have demonstrated that *Artemia* cyst carried bacteria in their shell. The resting stage in the form of cyst make them easily available, convenient and cheaper, compared to the use of animals that require a long period of culturing. The nauplii (Instar 1) of 0.4 mm can be hatch out within 24 hours in a small container under axenic condition to eliminate microorganism (Das et al., 2012). Several studies have been performed on host-microbe interaction using gnotobiotic or germ-free *Artemia* as a test organism (Ali & Razia, 2011).

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Certain microorganism played a crucial role in aquaculture to control disease and water quality. Such microorganisms are called probiotic, range from gram positive or negative bacteria, yeast, and unicellular algae (Seenivasan et al., 2012). Generally, *Saccharomyces cerevisiae* yeast is an essential feed for *Artemia* (Marques, 2004). However, today the use of probiotic in *Artemia* is not restricted to the yeast. In recent year, much emphasis has been given to enhance the nutritional status of live food organism through various techniques of enrichment and bio encapsulation with some genus of bacteria such as lactic acid, bacillus, or other genera. These species contain the nutrient that boosts the live food when consumed by *Artemia* (Mahdhi et al, 2011; Seenivasan et al., 2012). Thus, the enrichment diet offers possibilities to cover the need of different species thereby, improving survival, higher growth rate and greater resistance to stress and disease (Sorgeloos et al., 2001). This study was aim to evaluate the effect on survival, growth and development of *Artemia* nauplii enriched with potential probionts *Bacillus* JAQ04 and *Micrococcus* JAQ07 in gnotobiotic condition.

MATERIALS AND METHODS

Preparation of probiotic candidates. A pure colony of putative probiotics was inoculated in Marine Broth for 24 hours at 30°C of 120rpm (Shazwani et al., 2015). Both strains were harvested by centrifugation (1500 rpm x 10 min x 3) after 24 hours of incubation. The bacterial pellet was re-suspended in saline solution and the densities of the new bacterial suspensions were determined by measuring their optical density at 550 nm with a spectrophotometer, assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml according to the McFarland standard (Novriadi & Haw 2014). The bacteria suspension adjusted were added to *Artemia* at final concentration of 10^7 CFU/mL.

Axenic hatching of *Artemia*. A 200mg commercial *Artemia* cyst (Ocean Nutrition) was placed in 18 ml tapwater for 1 hour. Sterile cyst and nauplii were obtained by employing the methodology by Defoirdt et al., (2008), a process known as decapsulation. Hydrated cyst were decapsulated by transferring them into new screw cap tubes containing 10 ml sodium hypochlorite (50%) and 660ul sodium hydroxide (32%) solution. The decapsulation was stopped after 2 minutes by adding 14 ml of sodium thiosulphate (10g/l). Decapsulated cyst then washed to remove the trace of chemical and allow hatching under hatching condition according the protocol as described by the manufacturer with temperature; 27-30°C, salinity: 25-35g/L (30-35 ppt), 18-30 hours, vigorous aeration and light and light intensity. During reaction, 0.22 um filter aeration was provided.

Survival and development of *Artemia*. The experiment was carried out using standard procedure as describe by Orozco-Medina et al. (2002); Baruah et al. (2010). The probionts were applied 4- 6 hours after hatching (mouth open and nauplii became capable of ingestion). All tests included the following treatment: (1) The treatment with *Artemia* alone used as a control (2) Treatment *Artemia* enriched with *Bacillus* JAQ04 (3) Treatment *Artemia* enriched with *Micrococcus* JAQ07. The tubes were placed on rotator 4 cycles per min to provide aeration and constant incandescent light. All treatments were run in triplicate with 10-bacteria free *Artemia* nauplii in 20 ml sterile artificial seawater. The mortality rate will be analyzed daily by counting number of survive and dead *Artemia* in each treatment sample. Each treatment was carried out in triplicate under sterile condition. Larval survival was expressed as the percentage that successfully incorporated by Ali & Sultana (2011) by the following formula:

Larval survival (%) = (Total number of live Artemia larvae/ Initial number of Artemia larvae stocked) x 100

The development of larvae was determined according to the procedure as previously described by (Marques et al. (2005). The living *Artemia* were fixed in lugol iodine and their length was measured from the top of the head to the end of abdomen under dissecting microscope with the aid of digital measure, drawing mirror, and software *Artemia* 1.0. As criterion that combines both the effects of survival and individual length (IL), the total biomass production or total length (TBP) was determined according the following equation:

TBP = number of survivors x mean IL

Inoculation and monitoring of bacteria. The presence and absence of bacteria in each treatment were monitored at the end of the culture period. The bacteria from each sample were inoculated by transferring nauplii and 100ul of culture water on marine agar (MA). After incubation, the purity of isolates was confirmed through gram staining, oxidase, catalase, indole and spore forming test. The presence of probionts *Bacillus* JAQ04 and *Micrococcus* JAQ07 in gut also was confirmed by the observation under microscope.

RESULTS

Table 1. Percent survival of *Artemia* nauplii after 72 hours enriched with probionts *Bacillus* JAQ04 and *Micrococcus* JAQ07

Strains	Survival rate	Percent Survival (%)
Bacillus JAQ04	7.33 ± 0.58^{b}	73 ± 5.77^{b}
Micrococcus JAQ07	6.33 ± 0.58^{b}	63 ± 5.77^{b}
Control	4.33 ± 0.58^{a}	43 ± 5.77^{a}

All analysis is the mean of triplicate percent survival ± standard deviation. Means not sharing the same letter in the same column were significantly different at $p<0.05$.

In control group, *Artemia* were starved and no probiont were introduced in the culture medium. In the absence of feed (*Artemia* alone), more than 50% mortalities were observed after 4 days of hatching. The results demonstrated that the *Artemia* nauplii able to survive in high concentration of probionts when compared to the control treatment. At the end of treatment, there was no significant different were found between the nauplii survive in treatment with *Bacillus* and *Micrococcus* ($p>0.05$). However, the probionts with concentration 10^7 CFU/ml had significant effect ($P<0.05$) on the survival of *Artemia* compared to the control treatment.

Table 2. Length of *Artemia* nauplii after 72 hours enriched with probionts *Bacillus* JAQ04 and *Micrococcus* JAQ07

Strains	Concentration		Mean length (mm)
Bacillus JAQ04	10^7	CFU/ml	1.49 ± 0.05^{a}
Micrococcus JAQ07	10^7	CFU/ml	1.05 ± 0.05^{b}
Control	10^0	CFU/ml	0.77 ± 0.04^{c}

All analysis is the mean of triplicate length (mm)± standard deviation. Means not sharing the same letter in the same column were significantly different at $p<0.05$. Introduction of bacteria into *Artemia* was induced the growth with *Bacillus* and *Micrococcus* (1.49±0.05 mm and 1.05±0.05 mm) and significant difference was noted. Under axenic condition, the mean length of survived *Artemia* at

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day 4 was 0.77±0.04 mm. The mean lengths were significantly different among different groups ($p<0.05$).

Table 3. Percent Survival, Length and Total Biomass production of enriched *Artemia* with *Bacillus* JAQ04 and *Micrococcus* JAQ07

Bacteria strains	Survival (%)	Length(mm)	Total Biomass Production (TBP)
Bacillus JAQ04	70±1[a]	1.49±0.05[a]	10.92±0.51[a]
Micrococcus JAQ07	60±1[b]	1.05±0.05[b]	6.65±0.31[b]
Control	40±1[c]	0.77±0.04[c]	3.33±0.25[c]

All analysis is the mean of triplicate reading± standard deviation. Means not sharing the same letter in the same column were significantly different at $p<0.05$.

In term of total biomass production or total length, *Bacillus* JAQ04 strains significantly better in *Artemia* TBP compare to the *Micrococcus* JAQ07. It is mainly due to the characteristic of individual strains. The nauplii inoculated with *Bacillus* JAQ04 and *Micrococcus* JAQ07 significantly improve compared to the nauplii without bacteria (Figure 1).

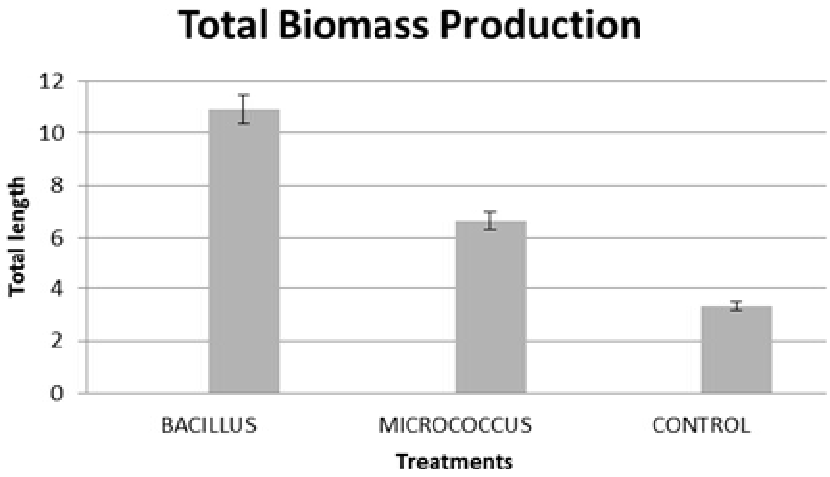

Figure 1. Total Biomass Production of enriched and non-enriched *Artemia* nauplil.

Table 4. Biochemical properties of *Bacillus* JAQ04 and *Micrococcus* JAQ07 isolated from enriched *Artemia* nauplii.

	Bacillus JAQ04	*Micrococcus*JAQ07
Gram staining	+	+
Oxidase test	-	-
Indole test	-	-
Catalase test	+	+
Spore forming	+	-
Swarming on TSA + 1.5% NaCl	+	-
Grow on TCBS media	nd	Nd

Note: TCBS, thiosulphate citrate bile salt agar; +,positive; -, negative; nd, not determined.

The higher survival rate was observed in *Bacillus* JAQ04 and *Micrococcus* JAQ07 enriched *Artemia* may be due to adherence, attachment and colonization in the population of gut flora. In the present study, the bacteria colony from the survive enriched-*Artemia* were successfully isolated on MA. The two isolates of the probiont were confirmed belong to the *Bacillus* and *Micrococcus* bacteria based on the physical and biochemical characters in Table 4. The bacteria detected could be one colonizing the interior or firmly attached to the exterior surface. In axenic group no bacteria were

observed at the beginning and the end of the procedure.

As the nauplii were transparent, presence of the probionts in gut could readily assessed by the yellowish gut. Results showed the gut of enriched *Artemia* full with *Bacillus* JAQ04 and *Micrococcus* JAQ07 under light microscope (Figure 2).

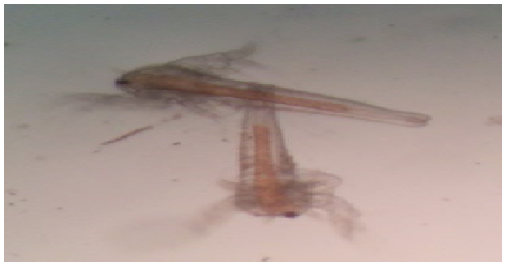

Figure 2. Photomicrograph showing gut region *Artemia* nauplii enriched with bacteria strains

DICUSSION

Artemia can ingest the small particle ranging from 1 to 50 µm in size after the mouth is open. Thus, it is possible bacteria can be used as a direct feed source for *Artemia* instead of yeast, microalgae, and waste product from food industry (Soltanian, 2007). However, it is essential to know the safety (non-pathogenic) and ability of the strain to survive in the gastrointestinal tract of the host (e.g. resistant to bile salt, low pH and protease) (Balcazar et al., 2006).

In the present study, the *Artemia* fed with probionts were able to resist longer compared to the unfed *Artemia*. The result obtained in control treatment was similar with previous treatment by Orozco-Medina et al. (2002) resulted not more than 4 days survival of axenic *Artemia* nauplii without beaker yeast (treatment "*Artemia* alone").These results clearly demonstrated that both bacteria strains were not harmless to the *Artemia* with over than 50% survival at the end of treatment. Similar finding was observed by Patimar (2014) in using *Bacillus* species. The *Artemia* were used as a vector to carry the probiotic *Bacillus* to the digestive tract of larvae promoted better growth and feeding parameter on Rainbow trout. The diet containing *Micrococcus leteus* considered as a growth promoter in aquaculture due to increase the final weight, weight gain, specific growth rate, survival rate, and feed intake and protein efficiency in *Oreochromis niloticus* (Sayed, 2014).

Artemia were extensively used as a live feed source for rearing of fish larvae. However, nutritive deficiencies in *Artemia* have developed bio-encapsulation technique to improve their nutritional level. It was found that bacteria enriched with *Artemia* provide nutritional element such as vitamin, essential amino acid, fatty acids and enzyme. The enrichment process in *Artemia* generally known as bio-encapsulation process whereby the *Artemia* ingest enrichment particle until gut is full. As the nauplii were transparent, the enriched *Artemia* were assessed by yellowish gut under microscope where the bacteria could readily be ingested by nauplii (Akbar et al., 2014). In this experiment, the bacteria from *Artemia* enriched with *Bacillus* were swarmed in solid media and produced white colonies on MA. On the other hand, the sample from the *Artemia* enriched with *Micrococcus* produced yellow colonies on MA. With this characteristic, it was identified as *Bacillus* and *Micrococcous* strains (Nurhidayu et al., 2012). Isolates proved that it was successfully incorporated into the *Artemia* nauplii. Bacteria colonization of *Artemia* could occur externally via attachment to the body surface or internally by ingestion (Gomez-Gil et al., 1998). Although, the *Artemia* enriched with *Bacillus* and *Micrococcus* cultured under identical conditions, the *Artemia* fed with *Bacillus* showed an overall increase in total biomass production. This could be due to some bacteria may produce essential proteins, amino acid,

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vitamins or active enzyme that enhance the growth of the *Artemia* (Balcazar et al., 2006). *Bacillus* also widely uses as putative probiotic due to ability of bacteria to secrete many exoenzymes (Moriarty, 1998). Thus, *Artemia* enriched is a better feed source for marine larvae compared with the non-enriched *Artemia*.

CONCLUSION

The present study showed that *Bacillus* JAQ04 and *Micrococcus* JAQ07 significantly improve survival and growth of *Artemia*. The bacteria strains ingested by *Artemia* possibly can be used as a vector to carry probionts matters for shellfish or fish larvae. However, it is recommended to conduct further study in order to understand the exact mode of action, the dose- response relationship and duration of effect after the intake of probiont.

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