Enrichment of freshwater zooplankton *Moina micrura* with probiotics isolated from microalgae

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Abstract

Aim: The present study investigated the ability of probiotics to enrich freshwater zooplankton, *Moina micrura* based on population density, population growth rate and mean body size increment.

Methodology: Different concentrations of probiotics were introduced to one-day-old female *Moina* in filter-sterilized freshwater pond water. Addition to probiont, *Chlorella* sp. was added in culture media as a food source.

Results: Population density, population growth rates and increase in mean body size of *Moina* were observed and recorded to evaluate the ability of probiont to enrich *Moina*. All four probionts were able to enrich *Moina* in treatment PC 1 (10^4 CFU ml⁻¹) and PC 2 (10^6 CFU ml⁻¹) at a better rate compared to the control group (no probiont). Also, the specific population growth rate (μ) and mean body size of *Moina* in treatment PC 1 and PC 2 were significantly higher than in treatment PC 3 (10^8 CFU ml⁻¹) and control for all probionts.

Interpretation: All probionts were able to enrich *Moina* and increased their growth at lower concentrations (PC 1 and PC 2). Supplementation of probiont at high concentration was unnecessary.

Key words : *Moina micrura*, Pathogens, Probiont, Zooplankton

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Introduction

The availability of a suitable larval rearing diet is important for the propagation of many aquatic species because it plays a vital role in the growth, survival, and disease resistance at early stages (Sontakke *et al.*, 2019). Most newly-hatched fish and shrimp depend on live feeds as an important basic diet. Live feeds are regarded as 'living capsules of nutrition' for a wide range of aquatic animals. They are rich in proteins, lipids, carbohydrates, vitamins, minerals, amino acids, fatty acids, and carotenoids (Simhachalam *et al.*, 2015). Apart from nutritional profiles that meet larval fish requirements, some zooplankton also contains high levels of digestive enzymes and can stimulate larval appetite (Zheng *et al.*, 2018). Besides, live feeds are always available to fish larvae since they can swim in the water column, which is likely to stimulate the feeding response of larvae (Mondal *et al.*, 2018). While formulated feed tends to accumulate on the surface of water or sink quickly to the bottom are less accessible to the larvae. Zooplankton such as cladocerans, brine shrimps, copepods, and tubifex are among the most accepted live feeds worldwide that play a vital role in the cultivation of fishes and crustaceans. Jones *et al.* (1993) suggested that the cultivation and management of live feed for aquatic production is costly and unpredictable. Only a limited number of studies have demonstrated the success of replacement or reduction of live feed in aquaculture (Cahu and Zambonino-Infante, 2001; Curnow *et al.*, 2006). However, with recent knowledge and technologies, several research papers are available on the improvements in live feeds culture techniques (Alajmi *et al.*, 2014; Kline and Laidley, 2015). Also, many studies have reported a higher growth rate, survival, and disease resistance of larvae fed with live food than those fed with formulated diets (Sáez-Royuela *et al.*, 2007; Yanes-Roca *et al.*, 2018; Sontakke *et al.*, 2019).

Fuller (1989) defined probiotics as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. Probiotics were redefined as 'live microorganisms which when administered in adequate amount confer health benefits on the host' (FAO/WHO, 2002). Ideally, probiotics should have beneficial effect and should not cause any harm to the host. Some of the important criteria of probiotic candidates include, to be non-pathogenic (Gunasekara *et al.*, 2010), having no drug-resistant gene (Sapkota *et al.*, 2007), able to tolerate a wide range of pH (Sylvain *et al.*, 2016), able to adhere on gut epithelial mucosal surface (Panigrahi and Azad, 2007), possess antagonistic or inhibition ability (Hütt *et al.*, 2006), producing extracellular enzyme (Bairagi *et al.*, 2004), and indigenous in nature (Boutin *et al.*, 2013). Probiotic candidates are often evaluated for safety and pathogenicity (Cao *et al.*, 2012; Vijayan *et al.*, 2006; Irianto and Austin, 2003; Chythanya *et al.*, 2002). Also, Ryu *et al.* (2012) reported that commercial seafood can act as a reservoir for multi-drug resistant bacteria which then allow the spread of resistance genes. Therefore, the search for

alternatives to antibiotic therapy in aquaculture practices has turned attention towards the use of probiotics during early larval stages. Administration of probiotics via live feed as a vector is a promising approach. Probiotics remain viable and proliferate in the live feed, can be transported into the hosts effectively (Hai, 2015). Several reports are available on the positive effects of direct addition of probiotic in the culture water (Wang and Gu, 2010; Aguilar-Marcias *et al.*, 2010; Granados-Amores *et al.*, 2012; Boutin *et al.*, 2013; Chumpol *et al.*, 2017), however, studies on probiotic-enriched live feed, particularly freshwater Cladoceran *Moina* spp. are limited. Direct administration of probiotics to the culture water is risky as they are easily exposed to microbiological contamination (Sun *et al.*, 2013), and short survival period of probiotics in seawater is a major drawback (Gatesoupe, 2008).

Moina spp. in general can tolerate a wide range of water quality in terms of dissolved oxygen, ammonia, biochemical oxygen demand, salinity and pH level, respectively (Kibria *et al.*, 1995; Dawidowicz and Ozimek, 2013; He *et al.*, 2001; Sarma *et al.*, 2003). *Moina* spp. can grow and reproduce at temperature above its thermal optimum (Chen *et al.*, 2015; He *et al.*, 2001). These criteria are ideal for commercial fish farmers in both temperate and polar climate regions. Besides, Cladocerans feed on varied food size (Kamrunnahar *et al.*, 2019; Pagano, 2008). The nutritional value of *Moina* spp. varies considerably depending on their age and type of food they receive (Gogoi *et al.*, 2016; Kibria *et al.*, 1995; Watanabe *et al.*, 1983). The nutritional value of cladocerans can be manipulated by pre-feeding them in an enrichment method. In the past, fatty acids and vitamins have been incorporated in *Moina* spp. (Das *et al.*, 2007; Singh *et al.*, 2019). Even though commercial-scale quantities of *Moina* are not readily obtained from natural habitats, they can easily be farmed for use as live feed (Loh *et al.*, 2013).

In this study, an attempt was made to enrich freshwater cladoceran *M. micrura* with four probiotics previously isolated from microalgae. The ability of probiotics to enrich *Moina* was screened and analysed in terms of population density, specific growth rate, and mean body size increment of individual *Moina*.

Materials and Methods

Bacterial isolates : Four probionts, *Lysinibacillus fusiformis* strain A1 (Accession number: MK764897) and *Bacillus* sp. strain A2) (Accession number: MK764895) previously isolated from *Amphora* sp., *Lysinibacillus fusiformis* strain CI3 (Accession number: MK764896) from *Chlorella* sp., and *Bacillus pocheonensis* strain S2 (Accession number: MK764898) isolated from *Spirulina* sp. were used in this study. The *in-vitro* antagonism tests showed that the probiont candidates were able to inhibit the growth of common freshwater pathogens, *S. algalactiae* and *A. hydrophila*. All probionts were cultured in *Tryptic Soy Broth* (TSB, Difco, USA) prepared in distilled water with agitation 150 rpm for

24 hr. Probiotics were harvested by centrifugation at 5000 rpm for 10 min, the supernatant was discarded and the pellets were suspended in sterile distilled water with vortex for application. Bacterial concentration was measured at using a UV-1800 spectrophotometer.

Moina culture : *Moina micrura* was obtained from Marine Biotechnology Laboratory (MARSLAB), Institute of Bioscience, Universiti Putra Malaysia (UPM), Malaysia and then cultured in sterile-filtered pond water (0.45 µm fiberglass filters). *M. micrura* stock culture was sustained in a 2 l plastic aquarium and fed with *Chlorella* sp. (5×10^5 cells ml⁻¹) for two weeks before the experiment. *Chlorella* sp. that was fed to *M. micrura* was cultured in Bold's Basal Medium (Natrah et al., 2007), at laboratory conditions in a 2 l flask with mild continuous aeration for 7 days. The concentration of food *Chlorella* sp. provided was determined using a 0.1 mm improved Neubauer chamber using a light microscope according to the following formula:

$$\text{Density, } d \left(\frac{\text{cells}}{\text{ml}} \right) = \frac{\text{Average number of cells per square}}{4 \times 10^{-6}}$$

where, 4×10^{-6} is the volume of sample over the small square area which equivalent to 0.004 mm³ (0.2 x 0.2 x 0.1) expressed in cm³ (ml).

Experimental design : Ten one-day-old *M. micrura* were transferred to a 50 ml Falcon tube containing 30 ml of filter-sterilized freshwater pond water (0.45 µm fiberglass filters). They were fed once every two days with *Chlorella* sp. at a concentration of 5×10^5 cells ml⁻¹. *M. micrura* were supplemented with different concentrations of probiotic *L. fusiformis* strain A1 5×10^4 CFU ml⁻¹ (probiotic concentration 1-PC 1), 5×10^6 CFU ml⁻¹ (probiotic concentration 2-PC 2), 5×10^8 CFU ml⁻¹ (probiotic concentration 3-PC 3) and no probiotic supply (control) on the first day of treatment. Each treatment was in triplicate. Cultures were maintained for 12 days with exposure to 12 hr light and 12 hr dark. The culture water was changed every two days. The population density of *M. micrura* was determined by examining the sample in a Petri dish. The number of *M. micrura* were recorded daily throughout the experiment. The population growth rate was calculated according to the following (Khatoon et al., 2013; Azuraiddi et al., 2013):

$$\text{Population growth rate, } \mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

where, X_1 = number of *M. micrura* at the start of selected time interval; X_2 = number of *M. micrura* at the final (highest) of selected time interval; $t_1 - t_2$ = selected time (in days). The experimental design was applied using other probiotics; *Bacillus* sp. strain A2, *L. fusiformis* strain CI3, and *B. pocheonensis* strain S2.

Statistical analyses : The data collected were analysed using one-way ANOVA. A significant difference in growth rates and body

sizes among treatments was determined using Tukey's multiple comparisons test at a 0.05 level of probability. Statistical analyses were carried out using GraphPad Prism 8.0.2.

Results and Discussion

Moina spp. are highly nutritious and suitable for many fish and shrimp larvae (He et al., 2001; Martin et al., 2006; Peña-Aguado et al., 2009). Ovie and Ovie (2006) reported the approximate value of moisture and protein contents of freshwater zooplankton *M. micrura* were 89% and 52.4%, respectively. They also reported that the main essential amino acids in *M. micrura* were lysine, arginine, leucine and histidine, which were found to be much higher compared to the minimum essential amino acid requirements for finfish for both omnivorous and carnivorous fry. The same study also revealed that the concentrations of amino acid of *Moina* were higher than those obtained for *Artemia* by Watanabe et al. (1983) except for alanine, glycine, proline, serine and tyrosine. Accordingly, the enrichment with probiotic may further boost the nutritional value of *Moina* by providing essential compounds such as vitamins or inorganic nutrients lacking in the diets (Douillet, 2000). This study demonstrated that all four probiotics tested were able to enrich *Moina* and contribute to its growth (Fig. 1, 2).

Population density is an appropriate method to evaluate the effect of a diet on zooplankton growth (Peña-Aguado et al., 2005). A review by Sarma et al. (2005) reported that as small-sized cladoceran genera including *Moina* are often exposed to invertebrate predators, they produce large offspring to ensure survivability. While cladoceran genera of larger size tend to maximize early growth to a larger size. Furthermore, *Moina* population density and growth can be further amplified through increasing fecundity and decreasing the reproductive period by manipulating the quality and quantity of the feed (Hakima et al., 2013; Mubarak et al., 2017; Damayanti et al., 2020). By taking advantage of primitive feeding characteristics, it is possible to manipulate the nutritional value of *Moina* through enrichment process (Das et al., 2007; Singh et al., 2019). Additionally, the abundance of microbial species and diversity of microflora in live feed can be enhanced with probiotics (Jiang et al., 2018).

During the present study, it was observed that probiotics were able to enrich *Moina* at a better rate than the control group (without probiont). The highest peak population density of *Moina* enriched with all probionts (A1, A2, S2, and CI3) at PC 1 and PC 2 were observed on 10th day as compared to the control group, on 9th day (Fig. 1). On the following day, the population density started to decline probably due to highly crowded condition (Fig. 1). Morales-Ventura et al. (2012) observed a peak population density of *Moina* between 18 to 21 days, which was much longer as compared to the present study. These were probably due to the difference in the setup and duration of the experiment. As compared to 50 ml culture medium in 100 ml jars used by Morales-

Ventura *et al.* (2012), only 30 ml culture medium in 50 ml tubes were used in this study, therefore, results showed earlier overcrowding and decline in population density. In addition, the present study was conducted for 12 days only. This study also showed that the increase in the population density of *Moina* does not depend on the concentrations of probiont supplied. The highest population growth was recorded in cultures with the lowest probiont concentration (PC 1), except probiont CI3 which logged the highest population growth in cultures with PC 2 (Fig. 1). Additionally, in the cultures of treatment PC 1, PC 2, and control group, *Moina* reproduced as early on 4th day of culture, whilst in treatment PC 3, the first neonates were produced between 5th and 9th day (Fig. 1). It is interesting to note that treatment PC 3 with the highest concentration of probionts supplied recorded a much lower neonates' production than the control group (no probiont

supplied) (Fig. 1). It was noted that very high probiotic supplementation is unnecessary for *Moina* enrichment. Makridis *et al.* (2000) suggested that the concentrations of bacteria bioencapsulated in live food should be minimal. Also, individuals in treatment PC 1 and PC 2 were observed to be very active as compared to those supplemented with the highest probiont concentration (PC 3) and control. However, it was observed that the absence of probiont could still allow the population growth of *Moina* when the source of food (*Chlorella* sp.) was present but at a lower rate compared to treatment PC 1 and PC 2 (Fig. 1). Feeding *Moina* with high concentration of microalgae can enhance the growth and reduce maturity age of *Moina* (Azuraiddi *et al.*, 2013; Hakima *et al.*, 2013; Morales-Ventura *et al.*, 2012). Additionally, dissolved organic carbon (DOC) compounds released by microalgae could influence zooplankton biomass (Bowszys *et al.*,

Fig. 1 : Changes in mean population densities of *M. micrura* in cultures with different probiont concentrations (probiotic concentration 1 (PC 1) = 5×10^4 CFU ml⁻¹; probiont concentration 2 (PC 2) = 5×10^6 CFU ml⁻¹; probiont concentration 3 (PC 3) = 5×10^8 CFU ml⁻¹; and control = no probiont supply). (A) Probiotic A1, (B) Probiotic A2, (C) Probiotic S2 and (D) Probiotic CI3. Verticals bars indicate mean of three replicates ± SE.

2020). Size and age at first reproduction may have a great ecological impact on a population (Sarma et al., 2005). Cladoceran population may delay their maturity to conserve limited resources in unfavourable conditions (Sarma et al., 2005). Generally, tropical cladoceran *M. micrura* may reach maturity as early as day three (Azuraidei et al., 2013), which contributes to the higher reproductive output. Theoretically, early production of offspring at larger sizes is accompanied by the production of larger broods, which will eventually generate a larger number of neonates per reproductive female. Additionally, the fact that *M. micrura* was able to reproduce parthenogenetically at lower densities in the control group is probably due to the presence of other bacteria following filter-sterilization of freshwater pond water using 0.45 μm fiberglass filters. Wang et al. (2007) were able to enrich freshwater bacterial communities that pass through 0.45 μm pore size filters at significantly increased percentages.

Generally, *Moina* can feed efficiently on a wide range of phytoplankton particles sizes, from unicellular picoplankton *Chlorella* sp. (2 to 4 μm spherical diameter) to large *Coelastrum reticulatum* coenobia (20 to 40 μm spherical diameter) (Pagano, 2008). However, Gliwicz and Siedlar (1980) suggested that cladocerans are relatively selective due to their ability to adjust their gape as an adaptation mechanism to avoid larger and elongated particles which are difficult to handle.

In the present study, three probionts A1, A2 and S2 produced the best mean specific population growth rates in treatment PC 1, while probiont C13 produced the best population growth in treatment PC 2 (Fig. 2). *Moina* enriched with probiont S2 in treatment PC 1 recorded the highest mean specific growth at 0.288. There were significant increase in specific population growth rates of probiont-enriched *Moina* in treatment PC 1 and

Fig. 2 : Mean specific growth rate (μ) of *M. micrura* supplemented with different concentrations of probiont bacteria (probiont concentration 1 (PC 1) = 5×10^4 CFU ml^{-1} ; probiont concentration 2 (PC 2) = 5×10^5 CFU ml^{-1} ; probiont concentration 3 (PC 3) = 5×10^6 CFU ml^{-1} ; and control = no probiont supply). (A) Probiont A1, (B) Probiont A2, (C) Probiont S2, and (D) Probiont C13. Values are mean $\pm$ SD. Bars with different letters are significantly different at $p < 0.05$. Vertical bars indicate mean of three replicates $\pm$ S.E.

Fig. 3 : Mean body size of individual *M. micrura* according to concentration of probiont supplied (probiont concentration 1 (PC 1) = 5×10^4 CFU ml⁻¹; probiont concentration 2 (PC 2) = 5×10^6 CFU ml⁻¹; probiont concentration 3 (PC 3) = 5×10^8 CFU ml⁻¹; and control = no probiont supply). (A) Probiont A1, (B) Probiont A2, (C) Probiont S2, and (D) Probiont Cl3. Verticals bars indicate mean of six replicates $\pm$ SE. Bars with different letters are significantly different at $p < 0.05$.

PC 2 than in the control group for probiont A1, S2 and Cl3 (Fig. 2). In the case of probiont A2, treatment PC 1 was significantly higher than in the control group, while treatment PC 2 was lower (Fig. 2). Treatment PC 3 of all four probionts produced the lowest population growth rates among all treatments, in which probiont A1, A2 and S2 recorded significantly lower growth rates than in the control group (Fig. 2). Siddque *et al.* (2004) suggested that lower number of neonate production may be due to the growth of thread algae. In this study, the culture water was changed every two days however, it is recommended to change the culture water every day to maximize the capacity of neonates production. Interestingly, the reproduction and maturation of *Moina* were induced in the absence of aeration system. It was suggested that small bubbles produced by aerator can get trapped under the carapace, causing *Moina* to float to the surface and eventually kill them. A significant increase was observed in the mean size of individual *Moina* supplemented with all probiont A1, A2, S2 and Cl3 in the treatment PC 1 and PC 2 than in the control group (Fig.

3). However, the mean size of all four probiont-enriched *Moina* in treatment PC 3 were not significantly different than control group (Fig. 3). It is clear that high levels of probiont supplementation can hinder the size increment and reproduction of *Moina*. In most cladocerans, offspring production or fecundity increases as body size increases when food is readily available (Sarma *et al.*, 2005). Generally, the size of tropical cladoceran genera are smaller and factors such as quality and quantity of food are beneficial to these smaller-sized cladocerans (Sarma *et al.*, 2005).

However, a study on the effects of probiont addition to the composition and diversity of microflora in *Moina* is limited. It is important to ensure that probionts can serve its utmost purpose. Addition of probionts, either directly to the water in rearing tanks or via live food influence the species-composition of microflora (Makridis *et al.*, 2000). Jiang *et al.* (2018) observed the increase in the abundance of microbial species and diversity of microflora in rotifers and *Artemia sinica* when probiont was added. Despite the

changes in the structure of microbiota, the abundance of probiont was relatively low in live feed (Jiang et al., 2019). This effect may indicate that probiont can inhibit the growth and reproduction of some bacteria through competition for nutrients and adhesion site while improving the growth and reproduction of other microflora via mutual benefits. Talpur et al. (2012) observed effective grazing of three lactic acid bacteria (LAB) by rotifers and *A. franciscana* when incubated at for 2 hrs. Additionally, a mixture of three lactic acid bacteria effectively suppressed *Vibrio* in treated *Artemia*. However, few studies have been carried out on the addition of probionts to freshwater *Moina*. Therefore, future studies can be conducted to observe the composition and diversity of microflora in *M. micrura* when probiont A1, A2, S2 and C13 are added.

The present study suggested that all four probionts were able to enrich *Moina* at low concentrations (PC 1 and PC 2). *M. micrura* was smaller in size and less active when no probiont was added. In addition, there was no significant difference in size between *M. micrura* supplemented with probiont at high concentration (PC 3) and cultures without probiont (control). In spite of significant difference in the size of *M. micrura*, between low (PC 1 and PC 2) and high (PC 3) probiont supplementation, *M. micrura* still reproduced but at a significantly lower ($p < 0.05$) rate. This study suggests that the concentration of probiont for *M. micrura* enrichment should be kept minimal.

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