ENRICHMENT OF LIVE FEED *Moina micrura* WITH PROBIOTICS AND ITS POTENTIAL IN PROTECTING RED HYBRID TILAPIA (*Oreochromis* spp.) AGAINST *Streptococcus agalactiae* INFECTION

By

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Although many newly-hatched larval fish species are still very much depending on live feeds as a starter diet, the mass production of high-quality live feeds proves to be challenging. The present study aimed to emphasize the utilization of probiotics to promote the production of *Moina micrura* and subsequently improve red hybrid tilapia survival when being challenged against pathogens. Four potential probiotics identified as *Lysinibacillus fusiformis* A1, *Lysinibacillus fusiformis* A2, *Bacillus pocheonensis* S2, and *Lysinibacillus fusiformis* Cl3 were used in enrichment assays. After 12 days of incubation, all isolates at 10^4 and 10^6 CFU mL^{-1} were able to enhance the population density, population growth rate, and mean body size of *M. micrura*. Furthermore, *M. micrura* enriched with *B. pocheonensis* S2 at 10^4 CFU mL^{-1} had the highest maximum population density (9 ± 0.07 ind./mL) and growth rate (0.4270 ± 0.001/day), whilst *M. micrura* enriched with *L. fusiformis* A1 at 10^4 CFU mL^{-1} had the longest mean body size (0.87 ± 0.007 mm). *In vitro* well-diffusion assay was carried out to evaluate the antagonism of all potential probiotics against two freshwater pathogens, *Aeromonas hydrophila* and *Streptococcus agalactiae* at three different concentrations of 10^5, 10^6, and 10^7 CFU mL^{-1}. The *in vitro* study revealed that *L. fusiformis* A1, *L. fusiformis* A2, and *B. pocheonensis* S2 were able to inhibit the growth of both pathogens, whereby *B. pocheonensis* S2 had the strongest antagonistic activities with inhibition zones ranging from 8 to 18 mm. The ecological consequences of increasing temperature, pH, and abnormal photoperiod on *M. micrura* enrichment with *L. fusiformis* A1 and *B. pocheonensis* S2 were examined for 12 days. It was revealed that *M. micrura* enriched with *B. pocheonensis* S2 at 30°C had the highest maximum population density (10 ± 0.2 ind./mL) and number of neonates produced (132 ± 6.43 ind.), meanwhile treatment at 20°C had the best growth rate (0.1863 ± 0.006/day). Similarly, *M. micrura* incubated with *B. pocheonensis* S2 at a normal photoperiod of 12L:12D had the highest maximum population density (10 ± 0.3 ind./mL) and number of

neonates produced (129 ± 4.58 ind.), meanwhile incubation at 8L:16D had the best growth rate (0.2879 ± 0.0007/day). Next, *M. micrura* enriched with *L. fusiformis* A1 at pH 8 had the highest maximum population density (11 ± 0.8 ind./mL), growth rate (0.5508 ± 0.04/day), and number of neonates produced (129 ± 4.36 ind.). *Moina micrura* and red hybrid tilapia larvae were used as biological models in *in vivo* bacterial challenge assays to evaluate the efficacy of *B. pocheonensis* S2 in protecting the host from diseases. Pre-enrichment of *M. micrura* with *B. pocheonensis* S2 at 10^4 CFU mL^{-1} for 24 h was able to significantly enhance the survival rate of *M. micrura* after being challenged with *S. agalactiae* (63 ± 3.33%) and *A. hydrophila* (63 ± 3.33%). Subsequently, red hybrid tilapia larvae fed with probiotic-enriched *M. micrura* had a significantly higher survival rate (77 ± 3.3%) than those in the control group (38 ± 4.4%) after being challenged with *S. agalactiae* following 10 days of experiment. The relative percentage survival rate of the larvae fed with probiotic-enriched *M. micrura* was recorded at 62.90. Furthermore, the bacterial counts study revealed that *B. pocheonensis* S2 was able to reduce *S. agalactiae* load in treated larvae (6.84 ± 0.39 CFU mL^{-1}) in comparison to untreated larvae (7.78 ± 0.09 CFU mL^{-1}), although the result was not statistically significant. In conclusion, due to the need for the increment of *M. micrura* production and disease resistance of red hybrid tilapia, the application of potential probiotic *B. pocheonensis* S2 in aquaculture practices worth to be further explored. This isolate has been proven to have a good potential in enriching and enhancing the population growth of *M. micrura* as well as to act as an excellent therapeutic in improving fish health.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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MEMPERKAYAKAN MAKANAN HIDUP *Moina micrura* DENGAN PROBIOTIK DAN POTENSINYA DALAM MELINDUNGI TILAPIA MERAH HIBRID (*Oreochromis* spp.) DARIPADA JANGKITAN *Streptococcus agalactiae*

Oleh

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Walaupun terdapat pelbagai spesies larva ikan yang masih bergantung kepada makanan hidup sebagai makanan permulaan, pengeluaran makanan hidup yang berkualiti tinggi dalam kuantiti yang besar terbukti mencabar. Kajian ini dijalankan bertujuan untuk memberi penekanan kepada penggunaan probiotik untuk menggalakkan pengeluaran *Moina micrura* dan seterusnya meningkatkan kelangsungan hidup tilapia merah hibrid apabila ditentang dengan patogen. Empat probiotik berpotensi yang telah dikenal pasti sebagai *Lysinibacillus fusiformis* A1, *Lysinibacillus fusiformis* A2, *Bacillus pocheonensis* S2, dan *Lysinibacillus fusiformis* Cl3 digunakan dalam ujian pengayaan. Selepas 12 hari tempoh inkubasi, kesemua isolat pada kepekatan 10^4 dan 10^6 CFU mL^{-1} dapat meningkatkan kepadatan populasi, kadar pertumbuhan, dan ukuran badan *M. micrura*. Disamping itu, *M. micrura* yang diperkaya dengan *B. pocheonensis* S2 pada kepekatan 10^4 CFU mL^{-1} memiliki kepadatan populasi maksimum (9 ± 0.07 ind./mL) dan kadar pertumbuhan (0.4270 ± 0.001/hari) paling tinggi, manakala *M. micrura* yang diperkaya dengan *L. fusiformis* A1 pada kepekatan 10^4 CFU mL^{-1} memiliki purata ukuran badan paling panjang (0.87 ± 0.007 mm). Ujian *in vitro* susupan telaga dijalankan untuk menilai antagonisme kesemua probiotik berpotensi keatas dua patogen air tawar, *Aeromonas hydrophila* dan *Streptococcus agalactiae* pada kepekatan 10^5, 10^6, dan 10^7 CFU mL^{-1}. Ujian *in vitro* menunjukkan hanya *L. fusiformis* A1, *L. fusiformis* A2, dan *B. pocheonensis* S2 dapat merencatkan pertumbuhan kedua-dua patogen tersebut dimana *B. pocheonensis* S2 menunjukkan aktiviti antagonis paling kuat dengan zon perencatan dicatatkan dari 8 hingga 18 mm. Kesan ekologi peningkatan suhu, pH, dan fotoperiod yang tidak normal keatas pengayaan *M. micrura* dengan *L. fusiformis* A1 dan *B. pocheonensis* S2 dinilai selama 12 hari. Didapati bahawa *M. micrura* yang diperkaya dengan dengan *B. pocheonensis* S2 pada suhu 30°C memiliki kepadatan populasi maksimum (10 ± 0.2 ind./mL) dan bilangan neonat (132 ± 6.43 ind.) paling tinggi, manakala rawatan pada suhu 20°C (0.1863 ±

0.006/hari) mencatatkan kadar pertumbuhan tertinggi. Begitu juga, inkubasi *M. micrura* dengan *B. pocheonensis* S2 pada fotoperiod normal 12L:12D menghasilkan kepadatan populasi maksimum (10 ± 0.3 ind./mL) dan bilangan neonat (129 ± 4.58 ind.) tertinggi, manakala inkubasi pada fotoperiod 8L:16D mencatatkan kadar pertumbuhan (0.2879 ± 0.0007/hari) paling tinggi Seterusnya, pengayaan *M. micrura* dengan *L. fusiformis* A1 pada pH 8 menghasilkan kepadatan populasi maksimum (11 ± 0.8 ind./mL), kadar pertumbuhan (0.5508 ± 0.04/hari), dan bilangan neonat (129 ± 4.36 ind.) paling tinggi. *Moina micrura* dan tilapia merah hibrid digunakan sebagai model biologi dalam ujian *in vivo* tentangan bakteria untuk menilai keberkesanan *B. pocheonensis* S2 dalam melindungi hos daripada penyakit. *Moina micrura* yang diperkaya dengan *B. pocheonensis* S2 pada kepekatan 10^4 CFU mL^{-1} selama 24 jam mencatatkan kadar kelangsungan hidup yang lebih tinggi berbanding kumpulan kawalan dengan signifikan setelah ditentang dengan *S. agalactiae* (63 ± 3.33%) dan *A. hydrophila* (63 ± 3.33%). Seterusnya, larva tilapia merah hibrid yang diberi makan *M. micrura* yang telah diperkaya dengan probiotik mencatatkan kadar kelangsungan hidup yang lebih tinggi (77 ± 3.3%) berbanding kumpulan kawalan (38 ± 4.4%) dengan signifikan setelah ditentang dengan *S. agalactiae* selepas 10 hari tempoh eksperimen. Peratusan relatif kadar kelangsungan hidup larva tilapia merah hibrid yang diberi makan *M. micrura* yang telah diperkaya dengan probiotik dicatatkan pada 62.90. Di samping itu, ujian pengiraan bakteria menunjukkan bahawa *B. pocheonensis* S2 dapat mengurangkan kiraan *S. agalactiae* dalam larva tilapia merah hibrid (6.84 ± 0.39 CFU mL^{-1}) berbanding larva yang tidak dirawat (7.78 ± 0.09 CFU mL^{-1}), walau bagaimanapun pengurangan tersebut tidak signifikan. Kesimpulannya, disebabkan oleh keperluan untuk meningkatan pengeluaran *M. micrura* dan ketahanan penyakit tilapia merah hibrid, penggunaan *B. pocheonensis* S2 dalam aktiviti akuakultur wajar diterokai. Isolat ini terbukti berpotensi dalam memperkaya dan meningkatkan pertumbuhan populasi *M. micrura* disamping menjadi terapi yang baik untuk meningkatkan kesihatan ikan.

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%	Percentage
v/v	Volume per volume
h	Hour
min	Minute
µm	Micrometer
°C	Degree Celsius
g	Gram
N	Normality
rRNA	Ribosomal ribonucleic acid
mL	Mililiter
TSA	Tryptic soy agar
TSB	Tryptic soy broth
rpm	Revolutions per minute
x *g*	Times gravity
nm	Nanometer
OD	Optical density
BBM	Bold's basal medium
mm	Milimeter
mm^3	Cubic milimeter
cm^3	Cubic centimeter
CFU mL^{-1}	Colony-forming unit per mililiter
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction

sec	Second
TBE	Trisborate-EDTA (Ethylene-diamine-tetraacetic acid)
kb	Kilobase
UV	Ultraviolet
ind	Individual
SEM	Standard error mean
BHI	Brain heart infusion
LC_{50}	Lethal concentration 50
L	Litre
µL	Microliter
kg	Kilogram
mg	Miligram
mg/L	Miligram per litre
mg/kg	Miligram per kilogram
DW	Dry weight
Se	Selenium
I	Iodine
NaI	Sodium iodide
GI	Gastrointestinal tract
FAO	Food and Agriculture Organization
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Live feeds as the fundamental needs for larval rearing and fry production of most aquatic species have to be prioritized to ensure sustainable farming operations. Farmers' reliance on imported live feeds will only elevate the cost of production. Therefore, continuous research efforts involving screening, stocking, maintaining, and preserving local zooplankton species to establish stable cultures for mass production are economically necessary. Live feeds which are commonly regarded as 'living capsules of nutrition' naturally contain high dietary components of macro- and micronutrients essential for larval growth and survival (Radhakrishnan *et al*., 2020). Moreover, in terms of acceptance, live feeds are superior compared to artificial larval feed (Das *et al.*, 2012).

Despite all the merit on live feeds, zooplankton rearing is quite laborious (Rasdi & Qin, 2016). Ensuring a constant and sufficient supply of high-quality live feeds at the lowest cost is tricky and occasional culture crashes remain a reality. Through the advancement of live feed enrichment techniques, the quality of zooplankton can be manipulated to enhance feed utilization and efficiency (Hamre *et al*., 2008b; Singh *et al*., 2019). The nutritional profile of *Artemia*, rotifers, copepod, and *Moina* have been further improved in various enrichment methods (Rasdi & Qin, 2016; Cavrois-Rogacki *et al*., 2020). Live feeds have been previously enriched with minerals, vitamins, fatty acids, and probiotics to enhance their nutritional value, population density, growth, reproduction, and survival (Rasdi & Qin, 2016).

In mass cultured conditions, zooplankton can feed on a wide range of microalgal species and bacteria (Rasdi & Qin, 2016). Therefore, enrichment of zooplankton with probiotics to enhance their nutritional level and establish stable production is a promising approach. In recent years, the enrichment of live feeds with probiotics in the form of single or multiple strains has become a popular approach as a method of probiotic delivery to protect fish from bacterial diseases (Rasmussen *et al*., 2018; Rasmussen *et al*., 2019). Cladocerans *Moina* spp. are equally important zooplankton species in aquaculture and are frequently used as food for larval and post-larval rearing of crustaceans and teleost fish (Poynton *et al*., 2013). Although *Moina* is considered as a superior live feed compared to *Artemia* in terms of nutrient content (Loh *et al*., 2012), they are rarely used in nutrient enrichment studies. Moreover, it is more fitting to use freshwater zooplankton for feeding freshwater species than *Artemia* and marine rotifer (Alam *et al*., 1993; Loh *et al*., 2012).

Aquaculture is responsible for the significant increase in the total fish production for direct human consumption from 67% in the 1960s to 88% in 2016 (FAO, 2018). As capture fisheries becoming increasingly unsustainable, aquaculture is expected to fill the supply-demand gap (FAO, 2018). However, factors such as income, population growth, the potential decline in capture fisheries production, the slowdown in aquaculture production growth, and cost pressure are likely to result in increased economic adversity in countries that depend heavily upon fisheries for their livelihood (FAO, 2018). Meanwhile, widespread disease outbreak remains the biggest constraint that hurt the livelihood of farmers (Assefa & Abunna, 2018). In Malaysia, *Streptococcus agalactiae* is commonly reported as the causative agent of streptococcosis in red tilapia (*Oreochromis* spp.) (Zamri-Saad *et al*., 2010).

Control of *S. agalactiae* infection mainly relies on the use of antibiotics and vaccinations which are commonly studied in Nile tilapia (Amal & Zamri-Saad, 2011; Laith *et al*., 2019). The abuse of antibiotics in aquaculture practices eventually lead to the emergence of aquatic antimicrobial-resistant bacteria, which taint edible products marketed for human consumption (Ryu *et al*., 2012). It has been reported that resistance genes can be transferred from aquatic bacteria to terrestrial bacteria including bacteria of animals and human pathogens (Schmidt *et al.*, 2001). Additionally, the accumulation of antibiotic residues has led to undetected consumption of antibiotics by fish consumers which could potentially modify their normal flora and eventually increases their susceptibility to bacterial infection (Angulo *et al*., 2004). Therefore, the application of probiotics in aquaculture practices as a disease preventive tool is regarded as a safer alternative to antibiotics.

1.2 Problem statement

Probiotics have been intensively studied as a safer alternative to antibiotics to prevent and control disease outbreaks in the aquaculture system. The method of administering probiotics via live feeds as vectors is logical due to the short survival period of probiotics in seawater and the risk of microbiological contamination (Gatesoupe, 2008; Sun *et al*., 2013). In addition, the swimming motion of live feeds in the water column is likely to stimulate larval feeding response, making them readily available to fish larvae (Mondal *et al*., 2018). However, despite the superiority of live feeds to artificial larval feeds in terms of acceptance, zooplankton rearing is laborious and unstable (Rasdi & Qin, 2016). The mass production of high-quality live feeds is the bottleneck for sustainable aquaculture production. Through the advancement of live feeds enrichment technique, the quality of zooplankton can be manipulated by means of probiotics to stabilize the production of high-quality zooplankton and enhance their growth and population density at a lower cost (Hamre *et al*., 2008b; Singh *et al*., 2019).

Freshwater fish making up 39% of aquaculture production in Southeast Asia, whereby tilapia is in the top five most cultured species (FAO, 2004). Over the years, *S. agalactiae* has been widely reported as one of the major pathogens in

tilapia causing serious global economic losses of approximately 250 million USD in 2008 (Iregui *et al*., 2014; Barkham *et al*., 2019). In 2015, *S. agalactiae* was identified as the causative agent to the major outbreak associated with the consumption of a Chinese-style raw fish dish in Singapore (Tan *et al*., 2016). Furthermore, isolates of similar sequence types were later found in all tilapia samples from 14 outbreak sites in Malaysia and Vietnam (Barkham *et al*., 2019). The use of antibiotics in aquaculture has become a controversial issue due to the development and dissemination of bacterial resistance (Cabello, 2006).

1.3 Significance of the study

To consistently produce high quality live feeds at a low cost proves to be challenging and the production of live feeds population with higher survival rate, growth, and stability remains a major problem. Through probiotics enrichment of live feeds, stable production of high-quality zooplankton can be achieved. Consequently, the growth, population density, reproduction, and survival of zooplankton can be enhanced. Moreover, the nutritional profile of zooplankton can be further improved and manipulated in various enrichment methods.

Probiotics are among the most heavily researched alternatives to antibiotics for disease treatment and prevention. However, the effects of direct administration of probiotics and the fate of added strain in culture water are unpredictable. Therefore, the method of administering probiotics via live feeds as vectors is proposed. The enrichment of live feeds with probiotics is potentially a cost-effective practice for fish larval rearing while simultaneously enhancing the nutritional value of live feeds.

In this study, previously isolated probiotic candidates with the ability to enhance the population growth and reproduction of zooplankton when being exposed to different environmental parameters would be valuable to the aquaculture industry. Furthermore, the ability of the selected probiotic candidates to protect zooplankton and fish against pathogens would be beneficial in reducing the prevalence of bacterial infections in freshwater fish.

1.4 Objective of the study

The utilization of probiotics in *M. micrura* cultures to enhance the production of high-quality live feeds and in tilapia cultures for improvement of disease resistance in fish are worth considering. Hence, the objectives of this study were:

1. To screen and select potential probiotics based on their ability to enrich *M. micrura* and inhibit the growth of freshwater pathogens *in vitro*.

2. To determine the efficacy of the selected potential probiotics in improving the population density, growth rate, and neonates production of *M. micrura* when being exposed to different environmental parameters.

3. To evaluate the efficacy of the selected potential probiotic in protecting *M. micrura* and enhancing the survival of red hybrid tilapia larvae after being challenge with *S. agalactiae*.

1.5 Hypothesis of the study

The hypothesis of the study:

Objective 1:

Null hypothesis: The selected probiotic strains are unable to enrich *M. micrura* and inhibit the growth of *A. hydrophila* and *S. agalactiae* in *in vitro* assay.

Alternative hypothesis: The selected probiotic strains are able to enrich *M. micrura* and inhibit the growth of *A. hydrophila* and *S. agalactiae* in *in vitro* assay.

Objective 2:

Null hypothesis: The selected probiotic strains are unable to enhance the population growth and reproduction of *M. micrura* at different temperatures, pH, and photoperiod levels.

Alternative hypothesis: The selected probiotic strains are able to enhance the population growth and reproduction of *M. micrura* at different temperatures, pH, and photoperiod levels.

Objective 3:

Null hypothesis: The selected probiotic strain is unable to enhance the survival of *M. micrura* after challenge with *S. agalactiae* and *A. hydrophila* and is unable to protect red hybrid tilapia larvae against *S. agalactiae* infection.

Alternative hypothesis: The selected probiotic strain is able to enhance the survival of *M. micrura* after challenge with *S. agalactiae* and *A. hydrophila* and is able to protect red hybrid tilapia larvae against *S. agalactiae* infection.

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