UNIVERSITI PUTRA MALAYSIA

ADDITION OF PROBIOTICS ON THE BIOFLOC SYSTEM FOR THE IMPROVEMENT OF GROWTH PERFORMANCE AND HEALTH OF RED HYBRID TILAPIA (Oreochromis spp.)

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Biofloc technology is the aggregation of microorganism bond in a matrix with the aim to improve water quality and as diseases management tools. However, biofloc system can be difficult to manage due to the high microbial load that may be pathogenic or cause no significant effect towards the cultured species. Probiotic addition in biofloc may provide promising results in cultured species and improve the culture system much efficiently from growth of cultured species and resistance to significant diseases. This study was carried out to evaluate the effectiveness of multi-strain probiotics addition in biofloc system and identify microbial composition in biofloc system with and without probiotic addition. Through *in vitro* analysis, four different probiotics *Lysinibacillus fusiformis* strain SPS11, *Bacillus amyloliquefaciens* strain L9, *Entrococcus hiriae* strain LAB3 and a commercial multi-train probiotic of *Lactobacillus* sp., *Aztobacter* sp., *Azospirillum* sp. (MG1©, Indonesia) at concentration of 10^8 CFU mL^{-1} were screened individually and as a mix using well-diffusion assay against *Streptococcus agalactiae* and *Streptococcus iniae* at two concentrations; 10^6 CFU mL^{-1} and 10^8 CFU mL^{-1}. The outcome showed that the selected probiotic multi-strain *Lysinibacillus fusiformis* strain SPS11, *Bacillus amyloliquefaciens* strain L9 and *Entrococcus hiriae* strain LAB3 showed antagonistic activity against *S. iniae* and *S. agalactiae* with diameter zone of 8.05±0.95 to 10.75±1 mm respectively. A 240 red hybrid tilapia fingerlings with an initial mean weight 50±0.64 mg and a total length of 3.56±0.21cm was used for *in vivo* assay with a duration of 4 weeks. The fish were cultivated in 6L aquarium with four treatments: biofloc only (B), biofloc enriched with probiotic strain *Lysinibacillus fusiformis* strain SPS11, *Bacillus amyloliquefaciens* strain L9 and *Entrococcus hiriae* strain LAB3 (B+PM), biofloc enriched with commercial probiotic (B+MG1) and freshwater as control. The survival of the fingerlings were higher in B+PM and control (90-91%) than other biofloc treatments (84-89%). The growth performance was significantly higher in biofloc treatments than control ($P<0.05$).

Treatment B+PM had the best specific growth rate (3.73±0.23 % day^{-1}), final body weight (15.07±1.30g) and feed conversion rate (0.76±0.04). Water quality parameters for all treatments were within suitable range for aquaculture. However, biofloc treatments had lower nitrogen concentration (NO_2-N, NO_3-N and NH_4-N). Biofloc+PM had lowest nitrite, NO_2-N, Nitrate, NO_3-N and ammonia concentration, NH_4-N (0.84±0.07 mg L^{-1}, 2.00±0.22 mg L^{-1}, 2.60±0.08 mg L^{-1}) as compared to control (2±0.14 mg L^{-1}, 0.8±0.31 mg L^{-1}, 3.8±0.3 mg L^{-1}). In a challenge test against *S. agalactiae*, the fingerlings from biofloc treatments B and B+PM showed significantly higher survival (73±1.2% and 83±1.43% respectively) than control (40±0.34%). For microbial composition analysis through metagenomics, biofloc water samples from taken at the end of the tilapia culture. Biofloc treatment showed composition of 18 phyla, from which the most abundant were Proteobacteria, Bacteroidetes, Chlamydiae and Actinobacteria. This study showed multi-strain probiotics of *Lysinibacillus fusiformis* strain SPS11*, Bacillus amyloliquefaciens* strain L9 *and Entrococcus hiriae* strain LAB3 showed great potential in improving water quality, growth peformance, diseases resistance of tilapia and biofloc microbial community. The applicability of this mix may further improve freshwater culture and the ability to control biofloc microbial community.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENAMBAHBAIKAN PROBIOTIK DALAM SISTEM BIOFLOK UNTUK MENINGKATKAN PERTUMBUHAN DAN KESIHATAN TILAPIA MERAH HIBRID (*Oreochromis* spp.)

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Teknologi bioflok merupakan gabungan mikroorganisma dengan tujuan untuk meningkatkan kualiti air, hasil ikan, kecekapan makanan dan ketahanan penyakit tilapia merah hibrid (*Oreochromis* spp.) jika dibandingkan dengan sistem akuakultur lain. Namun begitu, sistem bioflok sukar dikehendalikan kerana mikrobia yang tinggi mungkin boleh menjadi pathogen terhadap ikan yang dibiakkan atau tidak memberikan sebarang kesan yang signifikan. Penambahan probiotik dalam bioflok mampu memberikan hasil yang baik pada spesies ternakan seperti meningkatkan pertumbuhan ikan dan teknik pengurusan penyakit. Kajian ini dilakukan untuk menilai kesan probiotik campuran melalui *in vitro* dan *in vivo*, terhadap ketahanan penyakit dan pertumbuhan tilapia dalam sistem bioflok, untuk menentukan kecekapan probiotik floc pada pengurusan penyakit setelah dicabar dengan *Streptococcus agalactiae* dan mengenalpasti komposisi mikrob dalam sistem bioflok dengan pertambahan campuran probiotik dan tanpa penambahan probiotik. Melalui analisis *in vitro*, empat sampel probiotik yang berbeza *Lysinibacillus fusiformis* strain SPS11, *Bacillus amyloliquefaciens* strain L9, *Entrococcus hiriae* strain LAB3 dan probiotik multi-strain *Lactobacillus* sp., *Aztobacter* sp., *Azospirillum* sp. (MG1©,Indonesia) pada kepekatan 10^7 CFU mL^{-1} disaring secara individu dan sebagai campuran menggunakan ujian difusi terhadap *Streptococcus agalactiae* dan *Streptococcus iniae* pada dua kepekatan; 10^6 CFU mL^{-1} dan 10^8 CFU mL^{-1}. Hasil kajian menunjukkan bahawa probiotik campuran *Lysinibacillus fusiformis* strain SPS11, *Bacillus amyloliquefaciens* strain L9 dan *Entrococcus hiriae* strain LAB3 menunjukkan aktiviti antagonis yang baik terhadap *S. iniae* dan *S. agalactiae* dengan zon diameter 8.05 ± 0.95 hingga 10.75 ± 1 mm. 240 tilapia dengan berat purata awal 50 ± 0.64 mg dan panjang keseluruhan 3.56 ± 0.21cm digunakan untuk ujian *in vivo* selama empat minggu. Ikan tilapia merah hibrid diternak di dalam akuarium 6L dengan empat rawatan: Bioflok sahaja (B), Bioflok dengan probiotik campuran *Lysinibacillus fusiformis* strain SPS11, *Bacillus amyloliquefaciens* strain L9 and *Entrococcus hiriae* strain LAB3 (B + PM), Bioflok dengan probiotik komersial (B + MG1) dan air tawar sebagai kawalan. Kelangsungan hidup ikan tilapia

merah hibrid lebih tinggi pada B + PM dan kawalan (90-91%) daripada rawatan bioflok lain (84-89%). Prestasi pertumbuhan jauh lebih tinggi dalam rawatan bioflok daripada kawalan (P <0,05). Rawatan B + PM mempunyai kadar pertumbuhan spesifik terbaik (3.73±0.23% hari^{-1}), berat badan akhir (15.07 ± 1.30g) dan kadar penukaran makanan (0.76±0.04). Parameter kualiti air untuk semua rawatan berbeza dan masin dalam lingkungan yang sesuai untuk akuakultur. Walau bagaimanapun, rawatan Biofloc mempunyai kepekatan nitrogen yang lebih rendah (NO_2-N, NO_3-N dan NH_4-N). Biofloc + PM mempunyai kepekatan Nitrit, NO_2-N, Nitrat, NO_3-N dan Ammonia, NH_4-N terendah (0.84±0.07 mg L^{-1}, 2.00± 0.22 mg L^{-1}, 2.60±0.08 mg L^{-1}) berbanding untuk Mengawal (2±0.14 mg L^{-1}, 0.8± 0.31 mg L^{-1}, 3.8±0.3 mg L^{-1}). Setalah melalui experiment pertumbuhan selama empat minggu, ikan tilapia yang sama direndam dalam suspensi *S. agalactiae* untuk tujuan ujian cabaran. Dalam ujian cabaran terhadap *S. agalactiae*, ikan tilapia dari rawatan Bioflok B dan B + PM menunjukkan kelangsungan hidup yang lebih tinggi (73±1.2%-83±1.43%) daripada kawalan (40±0.34%). Untuk analisis komposisi mikroba melalui analisa metagenomik, sampel air diambil dari setiap rawatan pada penghujung experiment terutama rawatan Bioflok sahaja (Bioflok) dan bioflok dengan pertambahan probiotik (Bioflok+MP). Rawatan bioflok menunjukkan komposisi 18 phyla, dari mana yang paling banyak adalah Proteobacteria, Bacteroidetes, Chlamydiae dan Actinobacteria. Kajian ini menunjukkan probiotik campuran *Lysinibacillus fusiformis* strain SPS11, *Bacillus amyloliquefaciens* strain L9 *and Entrococcus hiriae* strain LAB3 menunjukkan potensi besar dalam meningkatkan kualiti air, peningkatan pertumbuhan, ketahanan penyakit ikan tilapia merah hibrid dan komposisi mikrobia bioflok. Kebolehlaksanaan campuran ini dapat meningkatkan keberkesanan penternakan ikan air tawar dan kemampuan untuk mengawal komposisi mikrobia bioflok.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

CFU	Colony Forming Units
BFT	Biofloc Technology
°C	Degree centrigrade
%	Percent
mm	Milimeter
µl	Microlitre
mL	Mililitre
L	Litre
g	Grams
ppm	Parts per million
mg L^{-1}	Milligram per Litre
m^3	Cubic metre
hr	hour
C:N	Carbon and Nitrogen Ratio
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
LC50	Lethal Concentration 50

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Tilapia is a freshwater finfish with a high economical yield and being cultured intensively, with China having the highest production roughly 1.7 million tonnes per year throughout Asia (FAO, 2018). Tilapia is also the highest cultured freshwater species in Malaysia due to its fast growth in tropical climates. By 2018, the world production of farmed tilapia reached 6.03 million tonnes, which further place tilapia as the second most farmed aquatic animals exceeding 5 million tonnes. Between 1999 and 2013, farmed tilapia output grew at the fastest pace, with an average annual growth rate of 11.8 percent Between 1999 and 2018, the average growth rate of farmed tilapia output was 10.1%.. Between 2014 and 2018, the increase of farmed tilapia output decreased to an average of 5% each year, varying between 1.6 and 8.9%, with the lowest growth of 1.6 % in 2016 (Miao, 2020; FAO, 2020). In 2019, Malaysia produced up to 31, 884 metric tonnes of red hybrid tilapia contributing to 30% from total Malaysian Aquaculture production (Annual Fisheries Statistics, 2019). Intensive aquaculture systems are well known for high stocking density culture to maximize production. However, such system produces large amount of agricultural waste that requires complex filtration system. Production of tilapia throughout the world is greatly affected by stressful conditions and diseases, which have caused a serious economic loss in this species alone (Dhar et al., 2014).

The most popular disease caused by bacteria in tilapia production is streptococcal infection due to *Streptococcus iniae* and *Streptococcus agalactiae* which have been known to cause mass mortalities in warm freshwater fish aquaculture (Agnew and Barnes, 2007). *Streptococcus agalactiae* and *S. iniae* are known as opportunistic bacteria which are lethal to tilapia when being exposed within three to five days and it mainly infects tilapia stocked in high density. Due to the fast spreading of such diseases, most farmer rely on antibiotic usage to counter the problem, which in return give rise to antibiotic resistant strains. In aquaculture, antibiotics have been used for generations to treat and prevent disease though it should not be used in commercial aquaculture due to various effects.

Antibiotics and chemicals are relatively high cost, the application is restricted due to the uncertainty of its regulation, toxicity, poor absorption, application routes, pathogen resistance, cause gut microbe imbalance and various effects to the environment including the possibility of being transferred to humans through handling, consumption causing a high rish towards humans (Weston, 2000; Holmström et al., 2003; Gatlin, 2007). Alternative methods through biocontrol can be done by using probiotics. Probiotic bacteria can be fed, infused, or immersed in water for aquaculture management (Irianto and Austin, 2002). According to FAO and WHO standards, probiotic species utilised in food must be able to endure passages through the gut, such as avoiding gastric acids and bile exposure (Senok AC et al., 2005). They must also be able to colonise and multiply in the digestive system, and they must be healthy, efficient, and keep their efficiency and potency for the duration of the product's shelf life (Senok AC et al., 2005). Injections of probiotics are another option. Austin et al. introduced the concept of freeze-drying the probiont as a vaccination in 1995 and applying it through bathing or injection. Yassir et al., 2002, found that injecting probiotic *Micrococcus luteus* into *Oreochromis niloticus* via the intraperitoneal route resulted in only 25% mortality compared to 90% with *Pseudomonas* via the same route. According to Yassir et al., 2002, probiotics boost Rainbow trout immunity by boosting phagocyte activity, supplementing mediated bacterial death, and increasing immunoglobulin synthesis.

Biofloc Technology (BFT) is a closed system that process nitrogenous waste in a culture system with the help of heterotrophic bacteria that converted nitrogen into natural food for the culture system (De Schryver et al., 2008). Heterotrophs are essential bacteria in a biofloc, which may occur naturally in the system or through the addition of probiotic products. Heterotrophs helps to assimilate organic and inorganic nitrogen with the continuous supply of carbon source that is readily available from decomposition and addition of complex carbon sources such as molasses.

Sufficient carbon sources and nitrogen with a ratio of 20:1 reduces the concentration of ammonia in the culture system (Schneider et al., 2006). Hence, various studies have been made on beneficial bacteria that may improve the culture system much efficiently in terms of growth of the cultured species and resistance to significant diseases (Katia, 2018). Biofloc integrated with probiotics is recently a new strategy of integrating both biofloc and probiotics were introduced. However, selecting probiotics is crucial, and it is best that the microorganisms are endemic to its purpose, which will avoid the introduction of other bacteria into the system (Hansen, 1993; Abraham, 2016). Probiotics can be utilised as a source of microbial biomass for larger species, but they can also be exploited to produce virulence factors in reaction to environmental changes (De Schryver et al., 2008). Furthermore, the primary bacteria claimed to be present in commercial probiotics (Noor-Uddin et al., 2015) are highly abundant in biofloc (*Bacillus* spp.) and supply an infinite number of additional heterotrophic and autotrophic bacteria that also contribute to system equilibrium maintenance (Zhao et al., 2012; Ferreira et al., 2015). As a result of the contradictory facts, determining the effectiveness of probiotics in BFT systems is difficult.

Microbial intervention may play a critical role in aquaculture production, and effective probiotic treatment may provide broad spectrum and higher nonspecific disease protection (Rengpipat et al., 2000; Panigrahi and Azad, 2007). Probiotic microorganisms investigated for use in aquaculture include Gram-negative and Gram-positive bacteria, bacteriophages, yeasts, and unicellular algae. Irianto and Austin (2002; Irianto and Austin, 2002). *In vitro* antagonistic activity analysis allows for the selection of probiotic candidates, which includes colonisation, adhesion, and development in intestinal mucus (Verschuere et al. 2000).

1.2 Problem Statement

Diseases outbreaks has been a major challenge in the aquaculture industry while obtaining great loss globally. One of those is streptococcal infection caused by *Streptococcus* sp., which has becoming one of the great concerns over the years especially in intensive aquaculture systems (Buchanan et al., 2006). *Streptococcus* known to be the most significant bacterial diseases causing major mortality in Nile Tilapia, *Oreochromis niloticus* (Shoemaker et al., 2001). Various disease prevention techniques have been developed to control diseases includes vaccines and probiotic addition into feed (Welker and Lim, 2011) which greatly improves their overall growth and health.

However, continuous probiotic addition in an intensive culture system may be harder to manage. Despite the fact that farmers are using biofloc and/or probiotics for the practical reasons mentioned, some pathogens are still prevalent in fish, as seen by reduced farm survival rates (Su et al., 2008). Since animal diseases are frequently connected to certain bacteria, the activity of specific antagonistic/beneficial bacteria might help to alleviate these issues (Wang, 2007). As a result, it was anticipated that adding certain, well-known probiotic bacteria to the biofloc would increase the bacterial population in the water or in the animals' stomach, suppressing potentially dangerous pathogenic strains. Thus, BFT is well recommended to maintain an efficient bacterial load which includes beneficial bacteria that will both prevent bacterial diseases and improve the growth performance of cultured fish in an intensive culture system especially in tilapia culture (Ekasari et al., 2015). Enhanced immunity was observed in BFT that include associated microbes, which aids in protection against diseases while providing nonspecific defense against various pathogens (Kim et al., 2014). Including beneficial probiotics in a biofloc system and producing quality floc may improve cultured organism and eradicate bacterial disease as a whole. Selecting and testing which probiotic combination suits certain diseases is the key to bacterial disease prevention. In terms of monostrain or multi-strain probiotic usage, the impact varied considerably depending on the culture setting, and multiple strain probiotics were more effective at promoting immunity than single strain probiotics as observed by Mohammadi et al., 2021 on Nile Tilapia (*Oreochromis niloticus*) in bioflloc system. Multi-strain probiotics have a synergistic impact, providing additional protection for animal health (Timmerman et al., 2004).

1.3 Significant of Study

Over the years, application of probiotics in aquaculture is one of the major interesting research subjects due to their capability to control diseases in aquatic farms. It could be expressed by probiotic in ability to provide nutritional substances as well as enzymes, which aids in digestion process, improve, water quality and immune response and also confer resistance towards diseases (Qi et al., 2009; Tuan et al., 2013).
This study can aid in identifying the most suitable probiotic to be used for culture species through careful selection and elimination following *in vitro* assays against major pathogens. In addition, to improve the current biofloc system by reducing the need to constantly adding probiotics into the culture and introducing a more pathogen resistant

probiotic that is compatible with the biofloc microbial community. Furthermore, this study may further improve the effectiveness of mixed probiotic addition in biofloc system and serve as a reference for other biofloc studies focusing on improving biofloc system.

1.4 Objective of Study

The overall goal of this study was to evaluate the effect ot probiotic enrinchment to a tilapia biofloc system on water quality, growth performance, disease resistance, and the microbial community of red hybrid tilapia, *Oreochromis* sp.. Specific objectives include:
.

1. To evaluate the effect of probiotics addition thru *in vitro* and *in vivo* trials on the survival, water quality and growth performance of red hybrid tilapia fingerlings in a biofloc system.

2. To determine the probiotic efficiency of floc on disease resistance in immersion challenge assay with *Streptococcus agalactiae.*

3. To analyze the microbial composition of water sample in biofloc system with and without probiotic addition.

1.5 Hypothesis of Study

The hypothesis of the study:

Null hypothesis: Probiotic addition in biofloc system unable to improve the growth performance and disease resistance towards *Streptococcus agalactiae* and does not affect the biofloc microbial community.

Alternative hypothesis: Probiotic addition in biofloc system effectively improve the growth performance and disease resistance towards *Streptococcus agalactiae* effecting the biofloc microbial community.

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