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METAGENETIC ANALYSIS OF GUT MICROBIAL COMMUNITY OF MALAYSIAN MAHSEER Tor tambroides (BLEEKER, 1854) (CYPRINIDAE) AND ITS PROBIOTICS POTENTIAL

TAN CHUN KEAT

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By

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December 2017

Chair: Natrah Fatin Mohd Ikhsan, PhD
Faculty: Agriculture

Gut microbiota in vertebrate is complex and contains abundant of diverse beneficial microorganisms important for a dynamic host-microbe interaction. Some of these bacteria may have probiotics properties. In this study, the gut microbiota in wild and captive *T. tambroide*s was identified through metagenetic sequencing of 16S rDNA V3-V4 hypervariable regions using Illumina MiSeq. The sequencing data were analyzed using Quantitative Insights into Microbial Ecology (QIIME). Bacteria were isolated from gut of *T. tambroides* to evaluate its safety and examined for its probiotics properties. These selected potential probiotics were fed to *T. tambroides* followed by challenge test to evaluate its effects on growth and disease resistance against *Aeromonas hydrophila*. The metagenetic analyses showed that the gut microbiota in *T. tambroides* was dominated by Firmicutes, Proteobacteria, Fusobacteria and Bacteroidetes. Wild *T. tambroides* gut contained *Cetobacterium* sp. (24.9%), unknown genus from Peprostreptococcaceae family (11.0%), *Bacteroides* sp. (10.1%), *PSB-M-3* from Erysipelotrichaceae family (7.89%) and *Vibrio* sp. (5.4%). Captive *T. tambroides* gut contained *Cetobacterium* sp. (27.9%), *Citrobacter* sp. (10.0%), unknown genus from Peptostreptococcaceae family (8.2%), unknown genus from Aeromonadaceae family (8.2%) and *Turicibacter* sp. (7.0%). The results showed that *Cetobacterium* sp. is the core microbiota in *T. tambroides* gut. Function of this bacterium in *T. tambroides* gut needed to be determined. Three *Aeromonas* sp., two *Bacillus* sp., two *Lysinibacillus* spp., and one *Pseudomonas* sp. were successfully isolated and identified from the wild *T. tambroides* gut sample. Both *Bacillus* sp. and *Pseudomonas* sp. showed quorum sensing inhibition activities while only *Pseudomonas* sp. showed mild antimicrobial activity against *A. hydrophila*. These two bacteria were selected for probiotics feeding experiment. Nevertheless, there was no significant difference in growth of *T. tambroides* fed with these probiotics. The *T. tambroides* juveniles were then challenged with *A. hydrophila* by intra-peritoneal injection after the probiotics feeding experiment. Both *Bacillus* sp. and *Pseudomonas* sp. appeared to be able to improve disease resistance of *T. tambroides* juveniles against *A. hydrophila* infection. Lower mortality was observed in fishes treated with *Bacillus* sp. and *Pseudomonas* sp. as compared to positive control. Lysozyme activities in *T. tambroides* juveniles fed with *Bacillus* sp. were significantly higher ($P<0.05$) than other treatments. In conclusion, *Cetobacterium* sp. is a core gut microbiota in *T. tambroides*. The other important gut microbiota in *T. tambroides* may include *Bacteroides* sp., *Citrobacter* sp., *Turicibacter* sp. and *Bacillus* sp. *Bacillus* sp. and *Pseudomonas* sp. isolated in this study showed QSI activity. *Bacillus* sp. could enhance the innate immune system of fishes by increase lysozyme activity in blood serum. Both *Bacillus* sp. and *Pseudomonas* sp. have the potential to be use as probiotics in *T. tambroides* aquaculture to improve disease resistance and immune system of fishes.

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

ANALISIS METAGENETIK UNTUK KOMUNITI MIKROBIOTA USUS IKAN KELAH *Tor tambroides* (BLEEKER, 1854) (CYPRINIDAE) DAN POTENSI PROBIOTIK

Oleh

TAN CHUN KEAT

Pengerusi: Natrah Fatin Mohd Ikhsan, PhD
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Mikrobiota usus dalam vertebrata adalah kompleks dan mengandungi pelbagai mikroorganisma berfaedah yang penting untuk interaksi dinamik antara mikroorganisma dengan perumahnya. Sesetengah bakteria ini mungkin mempunyai ciri-ciri probiotik. Dalam kajian ini, mikrobiota usus dalam Ikan Kelah (*Tor tambroides*) dikenal pasti melalui penjujukan metagenetik untuk bahagian V3-V4 dalam 16S rDNA menggunakan Illumina MiSeq. Data penjujukan DNA dianalisis menggunakan Quantitative Insights into Microbial Ecology (QIIME). Bakteria diasingkan dari usus *T. tambroides* dikaji untuk keselamatan dan sifat-sifat probiotik. Probiotik berpotensi yang terpilih dibagi kepada *T. tambroides* melalui pemakanan diikuti dengan ujian cabaran untuk menilai kesan-kesan penambahbaikan pertumbuhan dan ketahanan penyakit. Analisis metagenomik menunjukkan bahawa mikrobiota perut di *T. tambroides* didominasi oleh Firmicutes, Proteobacteria, Fusobacteria dan Bacteroidetes. Usus *T. tambroides* liar mengandungi *Cetobacterium* sp. (24.9%), genus yang tidak diketahui dari keluarga Peprostreptococcaceae (11.0%), *Bacteroides* sp. (10.1%), PSB-M-3 dari keluarga Erysipelotrichaceae (7.89%) dan *Vibrio* sp. (5.4%). Usus *T. tambroides* peliharaan mengandungi *Cetobacterium* sp. (27.9%), *Citrobacter* sp. (10.0%), genus yang tidak diketahui dari keluarga Peptostreptococcaceae (8.2%), genus yang tidak diketahui dari keluarga Aeromonadaceae (8.2%) dan *Turicibacter* sp. (7.0%). Ini telah menunjukkan bahawa *Cetobacterium* spp. adalah mikrobiota utama dalam usus *T. tambroides*. Fungsi bakteria ini dalam usus *T. tambroides* perlu ditentukan. Beberapa bakteria berjaya diasingkan dan dikenal pasti dari sampel usus ikan kelah liar seperti tiga *Aeromonas* sp., dua *Bacillus* sp., dua *Lysinibacillus* sp., and satu *Pseudomonas* sp. Ujian QSI menunjukkan bahawa *Bacillus* sp. dan *Pseudomonas* sp. menunjukkan aktiviti perencatan penginderaan kuorum manakala hanya *Pseudomonas* sp. menunjukkan sifat antimikrobial yang sederhana terhadap *A. hydrophila*. Kedua-dua bakteria ini dipilih untuk eksperimen pemakanan berprobiotik. Walaubagaimanapun, tiada perbezaan yang ketara dalam pertumbuhan *T. tambroides* yang diberi makan dengan dua probiotik ini. Juvana *T. tambroides* telah dicabar dengan *A. hydrophila* melalui suntikan *intra-peritoneal* selepas eksperimen pemakanan berprobiotik. Kedua-dua *Bacillus* sp. dan *Pseudomonas* sp. dapat meningkatkan ketahanan penyakit juvana *T. tambroides* terhadap jangkitan *A. hydrophila*. Kadar kematian yang lebih rendah dikesan dalam ikan yang menerima rawatan *Bacillus* sp.

dan *Pseudomonas* sp. berbanding dengan rawatan kontrol positif. Aktiviti lisozim dalam juvana *T. tambroides* menerima rawatan *Bacillus* sp. adalah lebih tinggi (*P*<0.05) berbanding dengan rawatan yang lain. Secara kesimpulan, *Cetobacterium* sp. merupakan microbiota utama dalam usus ikan kelah. Mikrobiota lain yang penting termasuk *Bacteroides* sp., *Citrobacter* sp., *Turicibacter* sp. and *Bacillus* sp. *Bacillus* sp. and *Pseudomonas* sp. diasingkan dalam pengajian ini menunjukkan activity QSI. *Bacillus* sp. dapat meningkatkan sistem imun semula jadi dengan meningkatkan aktiviti lisozim dalam serum darah. Kedua-dua *Bacillus* sp. and *Pseudomonas* sp. mempunyai potensi untuk digunakan sebagai probiotik dalam akuakultur *T. tambroides* bagi meningkatkan ketahanan penyakit dan sistem imun ikan.

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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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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABI	Agro-Biotechnology Institute
ACP	Alternative Complement Pathway
AHLs	N-acyl Homoserine Lactones
AI-2	Autoinducer-2
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CFU	Colony Forming Unit
CI	Chloroform:isoamyl alcohol
COI	Cytochrome C Oxidase subunit I
CytB	Cytochrome b
D-loop	Displacement Loop
DAH	Days After Hatching
DCs	Dendritic Cells
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DO	Dissolve Oxygen
DOF	Department Of Fisheries Malaysia
DS	Diffusion Sensing
dsDNA	Double Stranded Deoxyribonucleic Acid
EB	Elution Buffer
EFA	Essential Fatty Acids
ES	Efficiency Sensing
FAO	Food and Agricultural Organization of the United Nations
FCR	Feed Conversion Ratio
Gb	Giga Base Pair
GPS	Global Positioning System
HHL	N-Hexanoyl-L-Homoserine Lactone
IACUC	Institutional Animal Care and Use Committee
IFRPC	Indigenous Fish Research and Production Centre, Sarawak
Ig	Immunoglobulin
IgA	Immunoglobulin A
ITIS	Integrated Taxonomic Information System
IUCN	International Union for Conservation of Nature
LAB	Lactic Acid Bacteria
LB	Luria-Bertani Broth
LBA	Luria-Bertani Agar
MGI	Malaysia Genome Institute
MPO	Myeloperoxidase
MSR	MiSeq Reporter
n-3	Omega 3
n-6	Omega 6
n.d.	No Date
NA	Not Available
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing

NRC	National Research Council
OD	Optical Density
OTUs	Operational Taxonomic Units
PBS	Phosphate Buffered Saline
PC	Principal Coordinate
PCI	Phenol:chloroform:isoamyl alcohol
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PEAR	Paired-End reAd mergeR
PGM	Personal Genome Machine
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
ppm	Parts Per Million
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
QS	Quorum Sensing
QSI	Quorum Sensing Inhibition
QQ	Quorum Quenching
RAS	Recirculating Aquaculture System
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
SBS	Sequence By Synthesis
SD	Standard Deviation
SGR	Specific Growth Rate
SMRT	Single Molecule Real-Time Sequencing
SOD	Superoxide Dismutase
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
spp.	Species
SRA	Sequence Read Archive
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
tRNAs	Transfer Ribonucleic Acids
rRNAs	Ribosoma Ribonucleic Acids
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UPM	Universiti Putra Malaysia
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Aquaculture is considered as one of the fastest-growing sector in food producing industry (FAO, 2014). Besides its importance in food and nutrition security, it also provides employment to millions of people worldwide and supports their livelihood. Aquaculture involves the farming of aquatic organisms in inland and coastal areas that intervene in the rearing process to enhance production (FAO, 2015) as oppose to capture fishery which involves harvesting of naturally occurring living resources in both marine and freshwater environments. While capture fishery production remained relatively static since the late 1980s, aquaculture has been responsible for the impressive growth in the supply of fish for human consumption. Capture fisheries production achieved 93.4 million tonnes in 2014 as compared to 73.8 million tonnes in aquaculture which equals to 44.1% of total world production (FAO, 2016). Aquaculture production is expected to be increasing in the following years and may achieve over 50% of total world production. China has played a major role in this growth as it represents more than 60 percent of the world aquaculture production (FAO, 2016).

According to Department of Fisheries Malaysia statistics (DOF, 2016) in year 2015, total landing of capture fisheries in Malaysia was 1,486,051 tonnes as compared to 1,458,128 tonnes on year 2014 (DOF, 2015). Value of capture fisheries in year 2015 was RM 9.32 million which showed an increment of 6.1% from year 2014. Aquaculture production was 506,465.25 tonnes in year 2015 which equals to 25.4% of total production in Malaysia. Total fishes production in aquaculture of Malaysia in year 2015 were dominated by freshwater catfish (45.2%) followed by Red Tilapia (27.1%), River Catfish (12.4%), Black Tilapia (4.5%) and other species (10.8%).

Tor tambroides is one of the most expensive freshwater fish in Malaysia. The market price of this fish in Malaysia ranges from RM 370-700 per kg (Azuadi et al., 2013). However, *T. tambroides* production was only 24.7 tonnes in year 2015 which was 0.02% of total aquaculture production in year 2015 (DOF, 2016). This showed huge potential for development of *T. tambroides* aquaculture. However, there are some obstacles that needed to be overcome to enable success aquaculture for *T. tambroides*. IUCN Red List of Threatened Species did not classify *T. tambroides* as endangered species due to data deficiency but the global population of this species has been observed to be decreasing due to overfishing, logging, deforestation, agriculture activities and anthropogenic activities causing river morphology modification and water flow interruption (Kottetlat, 2012). The growth of mahseers reported to be slow (Lee et al., 2014; Chatta et al., 2015) which may discourage its aquaculture production.

As aquaculture activities intensify and expand, there is higher chance of disease and problems caused by parasites, bacteria, viruses, fungi and other undiagnosed and emerging pathogens. This is because maintenance of large numbers of fish crowded together in a small area provides a very conducive environment for the development and spread of infectious diseases. Disease is a major constraint to the aquaculture industry, hampered both economic and social development in many countries (Bondad-Reantaso et al., 2005). The gills of Malaysian mahseer, *T. tambroides* obtained from Tasik Kenyir Reservoir Malaysia were frequently infected by a *Myxobolus* species (Székely et al., 2012). Golden mahseer, *Tor putitora* was subceptible to *Aeromonas hydrophila* infection (Kumar et al., 2016a). Eye lesions in golden mahseer, *T. putitora* was caused by *Pseudomonas koreensis* (Shahi and Mallik, 2014). *Tor tambra* from aquaculture fishponds were infested by Asian fish tapeworm (Muchlisin et al., 2015). Kesarcodi-Watson et al. (2008) reported the fastest treatment to diseases is the use of antimicrobial drugs but misuse of this drugs will eventually contributed to the emergence of antibiotic resistant bacteria. A feasible alternative to solve this problem is the use of probiotics or beneficial bacteria (Balcázar et al., 2006). Probiotics, prebiotics, immunostimulants, plant products and oral vaccines are some of the solutions in fish nutrition aiming to improve fish growth, feed efficiency, health, stress tolerance and resistance to diseases (Olivia-Teles, 2012; Newaj-Fyzul & Austin, 2014).

The term probiotic was defined as "a mono- or mixed culture of live microorganisms that when applied to animals or man, affect beneficially the host by improving the properties of the indigenous microflora" (Moriarty et al., 2005). Moriarty (1998) extended the definition for aquaculture to include the addition of natural bacteria to water environment in which the aquatic animals live. Probiotic strains have abilities to inhibit pathogenic bacteria both *in vitro* and *in vivo* through different mechanisms. These include enhancement of the epithelial barrier, increase adhesion to intestinal mucosa, competitive exclusion, production of anti-microorganism compounds, immune response enhancement, increase antiviral effects (Balcázar et al., 2006) and quorum sensing inhibition (Defoirdt et al., 2004; Kaufmann et al., 2008; Kalia, 2013). Addition of probiotics in feed may improve disease resistance and immune response of Malaysian mahseer. Besides, probiotics such as *Bacillus* spp. can produce digestive enzyme (Kesarcodi-Watson et al., 2008) to facilitate digestion of feed in the stomach and intestines of mahseer thus improve nutrient adsorption and growth rate. Lara-Flores et al. (2003) concluded that use of probiotic can reduce the amount of feed required for animal growth thus reduced the production cost.

There are more than 99% of prokaryotes in the environment that are unculturable in laboratory and this limits our understanding of microbial physiology, genetics and community ecology (Schloss & Handelsman, 2005). The development of Next Generation Sequencing (NGS) technology allows the recognition of discrete populations (culturable and unculturable) based on DNA sequences in the environmental samples (Konstantinidis & Rosselló-Móra, 2015). Gut microbiota of grass carp influenced by amount of food in the gut, diet and environment (Ni et al., 2014a). Fecal bacterial communities of *Epinephelus fuscoguttatus*, *Epinephelus sexfasciatus* and *Atule mate* collected from different geographical location showed same classes of bacteria as core microbiomes but the proportions of these bacteria were strongly varied (Hennersdorf et al., 2016). There were differences in gut microbial composition of wild and aquaculture origin *Seriola lalandi* (Ramírez & Romero, 2017). It is anticipated that the gut microbial community of wild and captive *T. tambroides* are different.

Since the wild stock of *T. tambroides* is decreasing and the growth of this species is remarkably slow, more research should be focus on the nutritional requirements, breeding and diseases control to develop a sustainable aquaculture production for this species. There has not been any report on the comparison of gut microbiome of wild and captive Malaysia mahseer. This study aims to identify and differentiate the gut microbiota in wild and captive Malaysian mahseer subsequently lead to the selection of bacteria with potential probiotics properties that can improve growth and disease resistance of Malaysian mahseer.

Objectives

1. To identify and compare gut microbiota of wild and captive *T. tambroides* by metagenetic sequencing
2. To examine the probiotics properties of bacteria isolated from gut of *T. tambroides*
3. To evaluate the effects of selected probiotics on growth and disease resistance of *T. tambroides* against *Aeromonas hydrophila*

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