



**Faculty of Resource Science and Technology**

**Selective Probiotics Mix Mechanism of Action in Gut of Empurau (*Tor  
tambroides*) Fingerlings**

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Selective Probiotics Mix Mechanism of Action in Gut of Empurau (*Tor  
tambroides*) Fingerlings

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## DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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## ABSTRACT

*Tor tambroides*, commonly known as Empurau or Malaysian mahseer, is Malaysia's most valuable freshwater fish and is native to Sarawak. Its substantial market value that pond reared Empurau is sold at RM500 per kilogram, led to Empurau (*T. tambroides*) increased popularity among fish farmers in Malaysia. However, this fish grows slowly, taking two to three years to reach a size that is marketable. Such an extended growth period underscores the importance of effective feeding management for maintain fish health. Probiotics have been shown to improve growth performance, feed utilization, immune response, and disease resistance in aquaculture. Lacto-sacc is the mixture of probiotics *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* commonly used as feed additives and a substitute for antibiotics in the diet. This research aims to evaluate the selective probiotics mix mechanism of action in gut of Empurau (*Tor tambroides*) fingerlings. The fingerlings, with an average body weight of  $6.53\text{g} \pm 0.17\text{g}$ , were stocked into twelve 650L tanks at a rate of 50 fingerlings/tank. Four experimental diets were formulated with 40% protein and supplemented with different concentration of Lacto-sacc forming 4 experimental diets; control diet (A), 0.5% Lacto-sacc (B), 1.0% Lacto-sacc (C) and 1.25% Lacto-sacc (D). The fingerlings were fed twice a day with 5% of the average body weight for 20 weeks in probiotics feeding trial (phase 1). The 8-week post-probiotics feeding trial (phase 2) was continued with commercial fish feed. Parameters including growth performance, feed utilization, relative protein digestibility, digestive enzyme activity and gut microbiota of *T. tambroides* were recorded. In both feeding trials, the study showed no significant difference ( $p>0.05$ ) in most of the parameters except lipase activity phase 1 and gut microbiota phase 2. However, Empurau fed with a diet supplemented with 0.5% Lacto-sacc (B) showed the highest numerical values and trends in most of parameters studied except lipase activity. Treatment B probiotics feeding trial showed the highest weight gain, specific growth rate (SGR), lipase and protease activity with lowest feed conversion rate (FCR) compared to

other treatments. On the other hand, 1.25% Lacto-sacc (D) gave the best numerical results and trends in post-probiotics feeding trial with best growth performance and better overall parameters. The dominant phyla found in gut of *T. tambroides* in both phases were the same which more than 95% were comprised by Fusobacteria, Proteobacteria, Bacteroidetes and Firmicutes. The dominant genera found in both phases were *Cetobacterium* (Fusobacteria), *ZOR0006* (Firmicutes), *Brevinema* (Spirochaetes), and *Aeromonas* (Proteobacteria). It is noticeable that the preferred concentration in each phase; 0.5% Lacto-sacc of Phase 1 (BP1) and 1.25% Lacto-sacc of Phase 2 (DP2) has lower Fusobacteria and an increase in other dominant bacteria compared to other treatment in their respective phases. The inclusion of Lacto-sacc to *T. tambroides* did benefit to the fish growth, feed utilization, digestive enzyme activities and provided a more even distribution and diverse gut microbiota.

**Keyword:** Digestive enzyme, growth performance, gut microbiota, Lacto-sacc, *Tor tambroides*

## ***Kesan Mekanisme Tindakan Campuran Probiotik Terpilih dalam Saluran Usus Benih***

### ***Empurau (Tor tambroides)***

#### **ABSTRAK**

Tor tambroides, yang biasanya dikenali sebagai Empurau atau ikan kelah Malaysia, merupakan ikan air tawar yang paling bernilai di Malaysia dan berasal dari Sarawak. Nilai pasarannya yang tinggi telah meningkatkan populariti Empurau (T. tambroides) di kalangan penternak ikan di Malaysia. Walau bagaimanapun, proses tumbesaran ikan agak perlahan sehingga mengambil masa dua hingga tiga tahun untuk mencapai saiz yang boleh dipasarkan. Tempoh pertumbuhan yang panjang ini menegaskan kepentingan pengurusan pemakanan yang berkesan untuk mengekalkan kesihatan ikan. Probiotik telah dibuktikan mampu meningkatkan prestasi pertumbuhan, penggunaan makanan, responsi imun, dan ketahanan terhadap penyakit dalam akuakultur. Lacto-sacc adalah campuran probiotik Lactobacillus acidophilus dan Saccharomyces cerevisiae yang biasanya digunakan sebagai tambahan makanan dan pengganti antibiotik dalam diet. Penyelidikan ini bertujuan untuk menilai mekanisme tindakan campuran probiotik pilihan dalam usus benih Empurau (Tor tambroides). Benih dengan berat badan purata  $6.53g \pm 0.17g$  diletakkan dalam dua belas tangki 650L pada kadar 50 ekor/tangki. Diet eksperimen diformulasikan dengan 40% protein dan disusuli dengan konsentrasi Lacto-sacc yang berbeza membentuk 4 diet eksperimen; diet kawalan (A), 0.5% Lacto-sacc (B), 1.0% Lacto-sacc (C) dan 1.25% Lacto-sacc (D). Benih T. tambroides diberi makan dua kali sehari mengikut 5% dari purata berat badan selama 20 minggu dalam ujian pemakanan probiotik. Ujian pemakanan pasca probiotik dilanjutkan dengan makanan ikan komersial selama 8 minggu. Dalam kedua-dua ujian pemakanan, tiada perbezaan yang signifikan ditunjukkan dalam hampir semua parameter. Walau bagaimanapun, Empurau yang diberi makan dengan diet yang disusuli dengan 0.5% Lacto-sacc (B) menunjukkan nilai-

nombor tertinggi dan trend yang terbaik untuk hampir semua parameter yang dikaji. Rawatan B menunjukkan peningkatan berat badan, kadar pertumbuhan spesifik (SGR), aktiviti lipase dan protease dengan kadar penukaran makanan (FCR) yang paling rendah berbanding rawatan lain. Sebaliknya, 1.25% Lacto-sacc (D) memberikan hasil numerik dan trend terbaik dalam ujian pemakanan pasca probiotik, dengan prestasi pertumbuhan terbaik dan parameter keseluruhan yang lebih baik. Phyla dominan yang ditemui dalam usus T. tambroides dalam kedua-dua fasa adalah sama, dengan lebih daripada 95% terdiri daripada Fusobacteria, Proteobacteria, Bacteroidetes, dan Firmicutes. Genera dominan yang ditemui dalam kedua-dua fasa adalah Cetobacterium (Fusobacteria), ZOR0006 (Firmicutes), Brevinema (Spirochaetes), dan Aeromonas (Proteobacteria). BP1 (0.5% Lacto-sacc) dan DP2 (1.25% Lacto-sacc) yang diutamakan dalam setiap fasa mempunyai Fusobacteria yang lebih rendah dan peningkatan bakteria dominan lain berbanding rawatan lain dalam fasa masing-masing. Penyertaan Lacto-sacc kepada T. tambroides memberi manfaat kepada pertumbuhan ikan, penggunaan makanan, aktiviti enzim pencernaan, dan menyediakan mikrobiota usus yang lebih seimbang dan sebagainya.

**Kata kunci:** Enzim pencernaan, prestasi pertumbuhan, mikrobiota usus, Lacto-sacc, Tor tambroides

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## LIST OF ABBREVIATIONS

|       |                                                     |
|-------|-----------------------------------------------------|
| ANOVA | Analysis of Variance                                |
| AOAC  | Official Methods of Analysis                        |
| ASV   | Amplicon Sequence Variants                          |
| BSA   | Bovine Serum Albumin                                |
| CFU   | Colony Forming Unit                                 |
| CMC   | Carboxymethyl Cellulose                             |
| DADA2 | Divisive Amplicon Denoising Algorithm 2             |
| DNA   | Deoxyribonucleic acid                               |
| DO    | Dissolved oxygen                                    |
| DOF   | Department of Fisheries                             |
| FAO   | Food and Agriculture Organization                   |
| FCR   | Feed Conversion Ratio                               |
| FITC  | Fluorescein Isothiocyanate                          |
| GI    | Gastrointestinal                                    |
| HCl   | Hydrochloric acid                                   |
| HSI   | Hepatosomatic Index                                 |
| HUFA  | Highly Unsaturated Fatty Acid                       |
| IFRPC | Indigenous Fisheries Research and Production Centre |
| IPF   | Intraperitoneal Fat                                 |
| IUCN  | International Union for Conservation of Nature      |
| KJ    | Kilojoule                                           |
| LAB   | Lactic acid bacteria                                |
| NACA  | Network of Aquaculture Centres in Asia-Pacific      |
| NaOH  | Sodium Hydroxide                                    |

|        |                                              |
|--------|----------------------------------------------|
| NGS    | Next Generation Sequencing                   |
| OTU    | Operational Taxonomic Unit                   |
| PacBio | Pacific Bioscience                           |
| PBS    | Phosphate Buffer Solution                    |
| PCoA   | Principal Coordinate Analysis                |
| PCR    | Polymerase Chain Reaction                    |
| PER    | Protein Efficiency Ratio                     |
| PUFA   | Polyunsaturated Fatty Acid                   |
| QIIME  | Quantitative Insights into Microbial Ecology |
| RG     | Relative Growth                              |
| RNA    | Ribonucleic acid                             |
| RPD    | Relative Protein Digestibility               |
| SBS    | Sequencing by Synthesis                      |
| SGR    | Specific Growth Rate                         |
| SMRT   | Single Molecule Real Time                    |
| SR     | Survival Rate                                |
| TAN    | Total Ammonia                                |
| TCA    | Trichloroacetic Acid                         |
| VSI    | Visceral Somatic Index                       |
| WHO    | World Health Organization                    |
| ZMW    | Zero-Mode Waveguide                          |

## CHAPTER 1

### INTRODUCTION

#### 1.1 Study Background

*Tor tambroides*, also known as Empurau, is the most valued freshwater fish in Malaysia (De Silva *et al.*, 2007) and is native to Sarawak. Empurau lives in most major river systems of the state of Sarawak. Empurau has great both commercial and conservation value whereas having high aquaculture production potential due to its high market demand and high flesh quality. However, Empurau is a slow-growing fish which reaches 500 to 600g in a year or more in wild situation (Ng, 2004). The challenges with *T. tambroides* is that the seed production for this fish is relatively low and does not meet the market demand. As feed becoming most of the expenses in culturing Empurau, there are many researches determined the feed formulations and feed additives and lots of studies done based on feed to improve the growth rate (Ng *et al.*, 2008; Misieng *et al.*, 2011; Ng & Andin, 2011; Kamarudin *et al.*, 2012; Ramezani-Fard *et al.*, 2012b; Ishak *et al.* 2016). Probiotics and prebiotics are able to provide antibiotics properties to animals and are suggested to replace the usage of antibiotics. Probiotics used in aquaculture is live microbial cultures added to feed or environment to increase viability or survival of the host, improve nutrient uptake and immunity, enhance growth performance and feed conversion rate. Accordingly, there has been a notable increase in interest in recent years to appraise the practicality and viability of incorporating non-nutrient dietary supplements, particularly prebiotics and probiotics. The objective is to enhance growth, stimulate immune system function, and improve fish resistance to infectious diseases.

#### 1.2 Problem statements

*Tor tambroides* is a slow growing fish, especially in the first year of its lifetime. *T. tambroides* may grow between 500 to 600g in a year in the wild and 800g in 2 years when

reared in captivity. The amount of wild Empurau in our river system decreases by time due to overfishing to meet the high market demand. As Empurau slow-growing characteristics, the cost of feeding becomes the most expensive maintenance to keep the Empurau with low feed conversion rate. Preventative measures, rather than the treatment of afflicted fish, are deemed a more effective strategy for controlling infectious diseases. Empurau commonly affected by infectious abdominal dropsy (IAD). According to Lau et al. (2021), the diseased *T. tambroides* found exhibits symptoms such as exophthalmia (pop-eye), coelomic distension (swollen abdomen) and petechial haemorrhage (small red spots on skin).

Also, there only several research determined the gut microbiota of *T. tambroides* (Tan et al. (2019), Iehata et al. (2021) and Lau et al. (2021). Tan et al. (2019) research on gut microbiota with wild and cultured *T. tambroides* while Iehate et al. (2021) compared the gut microbiota with cultured *T. tambroides* from different farm. Lau et al. (2021) determine the gut microbiota of healthy and diseased *T. tambroides*. However, there is no published report on the effect of Lacto-sacc or probiotics on the gut microbiota of the *T. tambroides*. It is essential determine gut microbiota of *T. tambroides* when fed with different concentration of Lacto-sacc and comparative with studies of gut microbiota of *T. tambroides* in different environment or condition.

### **1.3 Objectives**

This proposed research aims to investigate the effect of the selective probiotics mix mechanism of action in the gut of Empurau (*Tor tambroides*) fingerlings. The specific objectives are:

- i) To determine the effect of dietary probiotics mix on *Tor tambroides* growth performance, relative protein digestibility and digestive enzymes activity and
- ii) To determine the effect of different concentration of dietary probiotics mix on *Tor tambroides* gut microbiota

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Global Aquaculture and Challenges

Humans have first manipulated natural ecosystem processes through aquaculture in an attempt to satisfy the demand for food. Aquaculture has been found to cause environmental impacts such as eutrophication, oxygen depletion and pollution of the surrounding water and biota. An example can be seen in the mangrove ecosystem where destruction has occurred due to the culturing of brackish water shrimp and fishes. This has led to the development of aquaculture extension, which is set to produce more products without using more natural resources of land and water by utilizing a sustainable aquaculture system (Crab et al., 2012). One of the common challenges found in aquaculture is the susceptibility of fishes to the infectious pathogen in the form of bacteria, fungal, viral, and parasitic agents (Adams & Thompson, 2010). In Europe, empowered by the European Commission on 25 October 2018, non-therapeutic antibiotics are banned for use in aquaculture industries to enhance the growth and diseases prevention of animals. The intensive usage of antibiotics may lead to an increase in antibiotics-resistant bacteria (Abdel-Tawwab et al., 2008). The substitution for antibiotics usage can be alternate with probiotics, which help improve the immune system and act as biological control agents.

#### 2.2 Empurau, *Tor tambroides*

*Tor tambroides* (Bleeker, 1854), commonly known as Empurau, Malaysian mahseers or Kelah, is the most valued freshwater fish species in Malaysia that famous for its high flesh quality. Empurau is important for its prospect as potential as a protein source, as ornamental

fish and for recreation fishing (Nosi et al., 2018). It has high commercial and conservation value which resulted in many interests in its artificial propagation for aquaculture production.

Empurau has great both commercial and conservation value due to its potential for high aquaculture production, driven by its high market demand and high flesh quality. Ingram et al. (2005) agree that the *Tor* genus species has great aquaculture potential. According to Ingram et al. (2005), the open market in Kapit, Sarawak sells Empurau at a market price of RM400 per kilogram. Harith and Hassan (2011), highlight the economic importance of Empurau value at RM400 to RM600 per kilogram depending on its size, grade and whether it is wild or reared stock, and nowadays the price was far higher as RM500 per kilogram for pond reared Empurau that weighing between 2 to 5 kg (Chua, 2023). The Department of Fisheries (DOF) reported mahseer aquaculture in 2017 was 24.19 tonnes, 2018 with 12.31 tonnes and 2022 with 4.29 tonnes with respectively (DOF, 2017; 2018; 2022). Furthermore, *T. tambroides* has a potential wholesale value up to USD 53 per kilogram and achieved a production volume of 105904.01 tons in 2021 (DOF, 2021).

### **2.2.1 Taxonomy and Morphology of *Tor tambroides***

Empurau, *Tor tambroides* (Bleeker, 1854) is a species from family Cyprinidae. There are three main species of mahseer found in Malaysia: *T. tambroides*, *T. tambra* and *T. douronensis* (Kamarudin, 2015). *T. tambroides* is being classified as data deficient by IUCN (Kottelat et al., 2018).

**Kingdom:** Animalia

**Phylum:** Chordata

**Class:** Actinopterygii

**Order:** Cypriniformes

**Family:** Cyprinidae

**Genus:** *Tor*

**Scientific Name:** *Tor tambroides*



**Figure 2.1:** The image of Empurau (*Tor tambroides*).

*Tor tambroides* was initially morphologically characterized by Bleeker in 1854, featuring distinctive attribution such as four dorsal spines, eight dorsal soft rays, three anal spines, five anal soft rays, and a total of 39-41 vertebrae. The median lobe, a fleshy projection on the lower jaw, is notably elongated and more or less square, positioned on the lower lip and extending to an imaginary line between the curves of the mouth (Lau et al., 2021). An in-depth study focusing on the axial skeleton morphology of *T. tambroides* revealed it had four axial vertebrae bones within the *weberian apparatus*, 19 *ossa abdominal vertebrae*, 18 pairs *ossa costales*, 16 *ossa caudal vertebrae*, and one *os urostyles vertebrae* (Zulfami et al., 2018).

Empurau is morphologically similar to semah, *T. douronensis*, which causes little difficulty to distinguish by external appearance. However, Sim et al. (2008) have listed some diagnostic characters to distinguish them, such as Empurau has larger reddish and white scale

compared to semah's smaller yellowish, gold, and silvery scale. Empurau also has thicker and fleshier lips. Furthermore, Pinder et al. (2019) and Lau et al. (2021) mentioned that *T. tambroides* also often misidentified with *T. tambra*. *T. tambra* has thin lips, shorter median lobe and rounded snout compared to Empurau.

### **2.2.2 Biology of *Tor tambroides***

Empurau is sensitive to the surrounding environment and can serve as a bioindicator for water quality. According to a book published by Network of Aquaculture Centres in Asia-Pacific (NACA) in 2007, collaborated with Sim and colleagues (2008), Empurau lives in headwaters of most major river systems such as Batang Ai, Ulu Limbang and Ulu Baleh in Sarawak and could be found in Peninsular Malaysia including Perak, Kelantan, Pahang and Negeri Sembilan (Esa et al., 2008), further distributed throughout southeast Asia from Indonesia to Southern China. *T. tambroides* usually live in clear, swift-flowing water with stones, pebbles and rocky bottoms at mountainous streams and rivers (Harith & Hassan, 2011). They also mentioned that Empurau able to become bioindicator of a non-polluted river because they are sensitive to surrounding water quality. Rainboth (1996) mentions that adult fish always inhabits rivers that distribute in undisturbed forests that full of gravel and cobbles, while Kottelat (1998) states juvenile habitat in or near rapids and during dry season, they can be found in small rivers and streams then moving downstream on rainy season to avoid turbid water. Mature Empurau that are ready for spawning will migrate to clear, calm and slow-moving upstream with for two months then move to the mouth of small stream for breeding (Sim et al., 2008).

Empurau always lives in clean headwater with flowing water. For culturing Empurau, the water quality of the Empurau needs to be precisely cured. Water supply for aquaculture should be regular and reliable, free from pathogen and pollutants and pre-treatment of water is

required. Sim et al. in 2008, stated each fish species has favoured water quality parameter, reduced growth, diseases, and death may happen if water quality parameters get out of the range. They have listed a preferred water quality parameter for Empurau and semah aquaculture with historical data recorded for Empurau and semah rearing.

**Table 2.1:** Water quality preferred by Empurau and Semah. Adapted from Sim et al. (2008)

| Water Quality Parameter    | Range value preferred by Empurau and semah |
|----------------------------|--------------------------------------------|
| Water Temperature (°C)     | 22.5-35.1                                  |
| Dissolved Oxygen (mg/L)    | 2.4-15                                     |
| pH                         | 5.6-10.9                                   |
| Total ammonia (TAN) (mg/L) | 0.02-2.1                                   |
| Nitrite (mg/L)             | 0.001-0.067                                |
| Orthophosphate (mg/L)      | 0-0.55                                     |
| Turbidity (NTU)            | 0-522                                      |

According to the International Union for Conservation of Nature (IUCN) red list, the status of *T. tambroides* is currently data deficient but showing decreasing in population trend. The reasons for the decreasing population of Empurau are due to fishing and harvesting aquatic resources, dam and water management, agricultural and forestry effluents (Nosi et al., 2018). Due to the high demand for Empurau, there are reports that wild Empurau are experiencing ‘overfishing’ status now (Harith & Hassan, 2011). The Empurau is also facing a decrease in its natural food such as engkabang, *Shorea macrophylla* which is listed as least concern in the IUCN red list (Harith & Hassan, 2011). However, the Sarawak State Government has carried out a conservation program commenced in April 2001 to overcome the declination of the Empurau population, such as establishing Empurau artificial propagation research by the

Indigenous Fisheries Research and Production Centre (IFRPC) in Tarat, Sarawak to increase aquaculture production and produce fries for systematic re-stocking at the selected river.

### **2.2.3 The Diet and Nutrition of *Tor tambroides***

Empurau, being an omnivorous fish that feed on natural jungle fruits and nuts, insects and their larvae, small-size fishes, crustaceans, tadpoles, algae, zooplankton, prawn, and small frogs (Chan, 2009; Kamarudin et al., 2018). The natural diet of the Empurau includes some riverine fruits that contain a high lipid and saturated fatty acids percentages (Kamarudin et al., 2012). According to Wan (2015), they prefer fruit with high protein-energy content like the local forest fig (*Ficus auriculata*) and illipe fruit or known as buah engkabang (*Shorea macrophylla*). *F. auriculata* contains 46.64% of moisture, 5.32% of total protein and 27.09% carbohydrate, providing energy value of 135.51 kcal (Saklani & Chandra, 2012). *Shorea macrophylla*, which is least concern and decreasing in population trend, was listed in the IUCN red list in 2019. The seed with wings contains 8.42% protein, 14.6% oil and 62.53% carbohydrates, while the kernels alone contain 9.39% protein, 30.2% oil, and 42.11 carbohydrates (Kumar et al., 2016). The unique taste of the Empurau flesh is contributed by the illipe fruit. The study of Redhwan et al. (2023) mentions that, in addition to feeding on *Shorea macrophylla*, Sarawak locals observed that *T. tambroides* also consumes other fruit such as ensurai (*Dipterocarpus oblongifolius*), kelampok or jambu air (*Syzygium aquem*), buah era (*Ficus sp*) and fruit of the oil palm (*Elaeis guineensis*).

### **2.2.4 Growth Performance and Feed Utilization of *Tor tambroides***

Empurau is a slow-growing fish which reach 500 to 600g in a year or more in the wild situation (Ng, 2004) and 2 years to reach 800g when reared in captivity (Redhwan & Komilus, 2021). De Silva et al. in 2007 had raised Empurau to an average of 800g by 33 months of age

using prepared pond trails using captive-bred fish. Study of Ingram et al. (2005) grow *T. tambroides* to 142-179g in 60 weeks. Empurau also reaches its maturity at lengths ranging from 61cm to 74.9cm (Kottelat, 2000). Female mahseer sexually mature when over 2.5kg and males reach maturity in 20 months or 0.75kg (Ramenzani-Fard & Kamarudin, 2012).

However, the growth of the fish maybe affected by the diet. Research done by Ng et al. in 2008 shows that there is no interaction between the dietary protein and lipid level of feed for all growth parameter to Empurau. However, the result shows a trend that increasing protein levels with the same lipid level will increase the specific growth rate and decrease the feed conversion ratio (FCR) and suggested that the optimum dietary protein should fall between 42% to 50% and further increases in protein would not improve the FCR. They also suggested *T. tambroides* fingerling that weigh between 20 to 55g should require 48% of dietary protein. Misieng et al. (2011) experimented with different protein levels of feed on Empurau fingerlings and conclude that 40% of dietary protein ( $17.5\text{KJ g}^{-1}$ ) is the most optimum for fingerlings with the highest specific growth rate (SGR), lowest feed conversion rate (FCR) and highest protein efficiency ratio (PER).

Besides that, the *T. tambroides* is classified as semi-fatty fish as it contains 4.6- 5.2% of muscle crude lipids, and a fatty fish has 5-8% lipid in their edible tissues (Ozogul & Ozogul, 2007). The dietary lipid also provides an impact to the Empurau growth. An increase in lipid level from 10% to 15% does not contribute to the improvement of growth performance as 10% of lipids already provide enough metabolizable energy for Empurau fingerlings (Ng et al., 2008). Therefore, the most favourable dietary lipid requirement suggested by Ramezani-Fard et al. (2012b) is as low as 5% crude palm oil, with n-3 and n-6 Polyunsaturated Fatty Acid (PUFA) nutrient and a ratio of less than 0.3 (Ng & Andin, 2011) while saturated fatty acids to n-3 PUFA with the ratio of 15.3 is sufficient to provide best growth performance (Ramezani-

Fard et al., 2012a). Additionally, the study of Ng et al. (2008) suggests same as Ramenzani-Fard as above that the optimum dietary lipid for *T. tambroides* diet should be less than 5%. The inclusion of lipids level at 10% and 15% in diet results in increase of 1 KJ per gram of dietary gross energy. However, it does not show a significant enhancement in the growth or feed utilization efficiency of Empurau. The surplus dietary energy introduced to the feed is expected to be stored as fat in the visceral cavity, liver, and abdominal region (Ng et al., 2008).

There are studies that use dietary engkabang oil and crude palm oil, and other vegetable oils to substitute fish oil when making fish feed (Kamarudin et al., 2012; Bami et al., 2017; Redhwan et al., 2023). As study by Kamarudin et al. (2012) used vegetable oil (sunflower oil, linseed oil and palm oil) and fish oil as control and the result showed fish fed with 50% palm oil diet showed highest growth performance and daily feed intake. Whereas the study of Bami et al. (2017) recommended crude palm oil was the most cost-effective palm oil type as the lipid source in *T. tambroides* diet. On the other hand, Redhwan et al. (2023) incorporated the 2.5% *Shorea macrophylla* oil in diet gave positive impact to the growth performance of Empurau fingerlings.

Furthermore, the uses of brine shrimp, *Artemia* nauplii as a food source for the fish larvae is essential after they hatch because their hatching period is the most nutritious (Granvil, 2000). He stated that “the nutritious part which has high lipid level and fatty acid composition of the newly hatched *Artemia* nauplii is important in determining the best composition for the fish diet.” The high levels of unsaturated fatty acid (HUFA) in *Artemia* can influence the performance of larval fish hence the essential fatty acids needed by larval fish are the principal food. According to Granvil (2000), *Artemia* should be fed to the fish after they hatch because small sizes of *Artemia* can be caught easily by the fish. The study of Joshua et al. (2023) shows

that the *Chlorella vulgaris* enriched *Artemia* can improve the growth and survival of Empurau larvae, shorten the rearing and increase its yield in the hatcheries.

### 2.3 Probiotics

Probiotics arise from Greek words ‘pro’ and ‘bios’ which give the meaning ‘for live’. According to Food and Agriculture Organization (FAO) and World Health Organization (WHO), the probiotics are live organism which used orally to bring some tangible health benefits to the host. Probiotics are defined as live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). The host associated probiotics (autochthonous) are native to the fish, are more persistent in the GI tract of the fish and may exhibit longer-lasting effect to the host (Ige, 2013; Wuertz et al., 2021). On the other hand, probiotics that are not native but are introduced to the fish’s environment are known as allochthonous probiotics.

Probiotics used in aquaculture are live microbial cultures added to feed or environment to increase the viability or survival of the host (Ringo et al., 2016). Probiotics are able to stimulate digestive processes and immunity which reduce the probability of severity and amount of disease outbreaks in aquaculture. Probiotics can replace microbial chemotherapy as they are used as feed additives which able to secrete micro-toxins to inhibit the growth of virulent microorganisms such as *Escherichia coli* and *Salmonella*. The most widely used probiotics in research are Lactic acid bacteria (LAB) and spore-forming *Bacillus* sp. and yeast. LAB helps to reduce the pH in the gastrointestinal tract (GIT) that converts lactose to lactic acid that aims to prevent the colonization by many bacteria (Mombelli & Gismondo, 2000; Klewicki & Klewicka, 2004). The most widely used and researched LAB are lactobacilli and bifidobacteria. Probiotics are used to give its antibiotics characteristics to treat the amount and severity of disease outbreaks and thus enhance the growth performance and nutrient

digestibility and digestive enzymes activity. Probiotics have been shown to boost fish health and growth substantially without harming host, may be a promising eco-friendly alternative to conventional disease treatments (Bunner, 2019).

There are some studies that include probiotics into the diet of *T. tambroides*. Studies by Asaduzzaman et al. (2018a) and Asaduzzaman et al. (2018b) with *Bacillus* sp. AHG22, *Alcaligenes* sp. AFG22, and *Shewanella* sp. AFG21 were isolated from GI tract of *T. tambroides* and used to investigate gut morphology, microbiota composition, growth related gene expression, hypertrophic muscle progression and others. The result was the *Alcaligenes* sp. AFG22 gave the best result among the probiotics and was recommended as a potential autochthonous probiotic to enhance growth performance of Empurau. Furthermore, the study by Hossain et al. (2022) uses autochthonous probiotics of Empurau which were two different strain of *Enterococcus faecalis* on growth performance, gut morphology, and protection against *Aeromonas hydrophila*, resulting in *E. faecalis* FC11682 being most recommended as probiotics for *T. tambroides*. Additionally, research by Wan Alias et al. (2023) also uses dietary Lacto-sacc on the growth performance, food acceptability and body indices of Empurau reared in aquaponics. The result shows that the additional of Lacto-sacc did enhance the growth performance and hematological parameters of Empurau.

### **2.3.1 *Lactobacillus acidophilus***

*Lactobacillus acidophilus* is a well-known species in lactic acid bacteria (LAB), in family Lactobacillaceae that a non-spore forming gram-positive bacillus. *L. acidophilus* exists in the gastrointestinal (GI) tract of humans and animals where the environment can be quite acidic. *L. acidophilus* was initially isolated from GI tract of humans and its function was extensively researched since its identification in 1990 (Gao et al., 2022). They typical appear as slender rods with circular end, size measuring from 2-10 µm. Most of the *L. acidophilus* are

microaerobic, optimal growth temperature ranges between 35-38°C and optimum pH range of 5.5 to 6.0. They demonstrate a better resistance to both acid and bile salt (Wuertz et al., 2021; Gao et al., 2022).

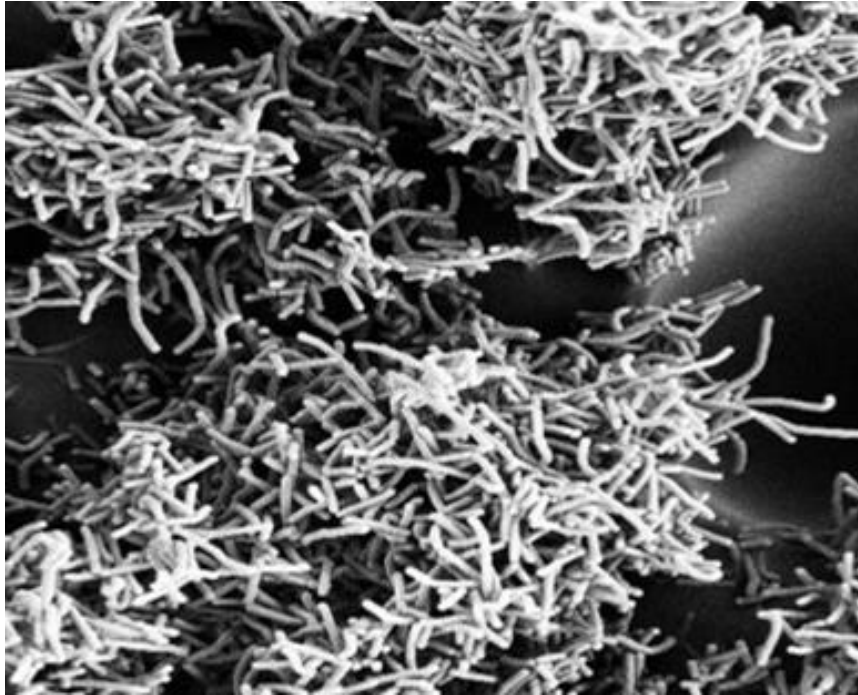


**Figure 2.2:** *Lactobacillus acidophilus* under scanning electron microscope with 10,000x magnification (Adapted from Pyar & Peh, 2014).

*Lactobacillus* sp. is an important probiotic in enhancing fish growth performance and immune responses (Panigrahi et al., 2011). Additional of *L. acidophilus* to animal diet is able to regulate host immune responses, reduction of host serum cholesterol, improvement of host lactose metabolism and prevention or treatment of infection (Gao et al., 2022). It also helps to enhance the growth performance and feed utilization of the host. The metabolic production of lactic acid by *L. acidophilus* effectively lower pH, inhibiting the growth of pathogenic bacteria. It eliminates the pathogenic bacteria that like neutral and alkaline environment and reduce the pathogenic microorganisms produce enzyme that can catalyze the conversion of carcinogenic precursors to carcinogens and inhibit enzyme activities. It also competes the site of adhesion with pathogenic bacteria (Singh et al., 2013).

*L. acidophilus* is found to increase body-weight gain which attributed to improved digestive activity by enhancing the synthesis of vitamins, cofactors, and enzymatic activity that results in an improvement in nutrient absorption and weight gain (Pérez-Cano et al., 2010). An experiment by Hoseinifar et al. in 2015 shows that the *L. acidophilus* is able to increase the total autochthonous bacteria and lactic acid bacteria levels in salinity stress resistance of black swordtail fish and significantly improve its weight, SGR and FCR. In research by Al-Dohail et al. (2011) shows *Lactobacillus acidophilus* is able to become a bio-control agent in African Catfish (*Clarias gariepinus*) to having better resistance to bacterial infections especially with *Staphylococcus xylosus*, *Aeromonas hydrophila* gr.2 and *Streptococcus agalactiae*. In study of Hosseini et al. (2016), the *L. acidophilus* fed treatment affects the appetite related genes expression by down-regulation of ghrelin, immune and skin mucus protein profile. There are also studies that show *Lactobacillus acidophilus* brings beneficial effect to the various fish species such as Nile tilapia, gold fish, Caspian white fish, marron, common carp and more (Hoseinifar et al., 2015; Hosseini et al., 2016; Adeshina et al., 2020; Foysal et al., 2020; Mousa et al., 2021).

### **2.3.2 *Saccharomyces cerevisiae***

*Saccharomyces cerevisiae*, known as yeast, bakers' yeast or brewers' yeast, is a unicellular fungus. It is famous for its fermentation characteristics, which are important in the production of beer, wine, and bread. It is a valuable protein source, compensating for amino acid and vitamin deficiencies. It is not pathogenic and able to resist bile and acidic pH. *S. cerevisiae* contains immunostimulating compounds such as  $\beta$ -1,3-glucans, nucleic acid, mannanoligosaccharides and chitin that able to enhance the immune response of fish and can be used as a become live food substitution or fish meal replacement (Abdel-Tawwab et al, 2008; Olivia-Teles & Concalves, 2001). It has demonstrated immunostimulatory activity and

the production of inhibitory substances (Dahan et al., 2003; Van der Aa Kühle et al., 2005). Moreover, it comprises cellular components other than  $\beta$ -glucan and mannaoligosaccharides, it also contains glucooligosaccharides and enzymes. All these constituents contribute to improve the growth, morphology, and physiology of the host, particularly in the context of digestive and immune responses. It is an acid-tolerant organism that is more effective in environment with lower pH value, the optimal pH range for yeast growth fluctuates between pH 4 to 6, with maximum production at pH 5.5, influenced by factor such as temperature, oxygen availability and strain of yeast (Narendranath & Power, 2005).

Yeast study of Abdel-Tawwab et al. (2008) found that yeast supplements were able to improve the protein content of body composition of Nile tilapia. It also increased glucose, lipid, protein, albumin, and globulin that reflect the diagnostic value on fish related to general nutrition status and the integrity of liver function in the vascular system with 1 g yeast per kg diet. Fish appetite increased resulting in higher feed intake and fish growth. The total mortality of fish injected with *Aeromonas hydrophila* in decreased with increase of yeast level. Its gives promising as antibiotics alternative for disease prevention. Moreover, in research by Gatesoupe (2007), they summerised the yeast as the risk of brutal invasion is low when fish rear in good condition. They also mention yeast provides a promising effect on the fish immune system, digestive system, and fish health. They also say yeast is able to secrete a killer toxin that is used to control pathogenic fungi. Yeast also can be used as partial protein alternative for fish meal, resulting improvement the growth, immunity, and disease resistance (Ozorio et al., 2012; Pongpet et al., 2016; Hassaan et al., 2018; Hao et al., 2022). There is a lot of study that *Saccharomyces cerevisiae* can bring beneficial effect to the fish host such as Tilapia, European Sea Bass, Sea Bream, and more (Abu-Elala et al., 2013; Iwashita et al., 2015; El-Bab et al., 2022; Perdichizzi et al., 2023)

### 2.3.3 Lacto-sacc

Lacto-sacc is the probiotics combination of *Lactobacillus acidophilus* and *Saccharomyces cerevisiae*. It has been used as a feed additive and antibiotics substitution in animal diet. The Lacto-sacc probiotics mixture was sold by Alltech company with content of crude protein 4%, crude fat 3.5% and crude fibre 20%. The probiotics concentration was *S. cerevisiae*  $3.80 \times 10^9$  CFU/lb, *L. acidophilus*  $2.10 \times 10^8$  CFU/lb, *Enterococcus faecium*  $1.50 \times 10^8$  CFU/lb. Lacto-sacc had been proven by some studies including fish, rabbit, and broiler chicken that able to improve the growth performance of the host, enhance the feed utilization and immune response, reduce pathogens, and improved digestive health and nutrient digestibility (Yamani et al., 1992; Gippert et al., 1992; Galal et al., 2000; Mahajan et al., 2000; Sotirov et al., 2001; Abou Zied et al., 2003; Wan Alias et al., 2023). The Lacto-sacc (mainly contributed by *L. acidophilus*) is able to stimulate the immune system, making them more resistant to diseases by the production of lactic acid and bacteriocin reducing pH and elimination pathogen (Gao et al., 2022; Ruiz-Ramirez et al., 2022).

## 2.4 Nutrient Digestibility and Digestive Enzyme Activities

### 2.4.1 Protein Digestibility

Relative protein digestibility (RPD) refers to the percentage of protein that is digested and absorbed by an animal relative to reference protein source. In aquaculture, RPD is used to evaluate the quality of different protein sources to determine their suitability for use in fish feed. Protein is the most costly component of fish feed, accounting for nearly 50% of the feed production costs.

The protein digestibility depends on several factors such as feed ingredient, intestine morphology and microbiota and enzyme supplementation. The digestibility of different feed

ingredient in fish feed give different protein digestibility. Experiment of Umalatha et al. (2018) shows that the *Catla catla* has higher protein digestibility of soybean diets at 40% incorporation compared to Azolla and silkworm diet. The protein digestibility may differ between fish species. The *Channa punctata* has lower RPD than *Anabas testudines* (Devi et al., 2022). The addition of enzyme in fish feed such as papain will improve the protein digestibility, growth, and survival rate.

There are several methods to measure protein digestibility in fish, including in vivo and in vitro methods. The most common method is in vitro method and was used in this study (Ali et al., 2009; Sultana et al., 2010). The in vitro method used crude enzymes that extracted from fish gut. The in vitro methods involve the pH drop method and it is comparatively rapid, reproducible and more cost and labour effective than other methods. The pH drop method stimulates the acidic condition of fish stomach to assess the susceptibility of dietary proteins to enzymatic digestion.

#### **2.4.2 Digestive Enzyme Activities**

Digestive enzymes are proteins that break down large molecules of food into smaller molecules facilitating their absorption into the cells of the body. The digestive enzyme can be produced by various organ in the digestive system including salivary glands, stomach, pancreases, and small intestine. There are several types of enzymes such as amylase, protease, lipases which break down carbohydrates, proteins, and fats, respectively. These enzymes increase the level of digestion in stomach, increasing the breakdown of food. The digestive enzyme activities can be influenced by diet, probiotics, and environmental pollution (Frank et al., 2023; Ameniyogbe et al., 2024). A study shows that the digestive enzyme activity correlates with the dietary habit of the fish. The indigenous fish species from India that omnivorous fish, *Puntius sophore* has highest total protease, trypsin, and amylase activity while carnivorous fish,

*Mystus tengara* has significant higher acid protease pepsin (Roy et al., 2022). Another study evaluated partial replacement of dietary fish meal by bioprocessed plant protein concentrates on the digestive enzyme activities in juvenile Pacific white shrimp and showed that bioprocessed plant protein was better (Moniruzzaman et al., 2020). Feeding frequencies could affect the digestive enzyme activities, as proven by study of Busti et al. (2020) that fish receiving more than one meal per day will decrease the pepsin and trypsin activity (Busti et al., 2020). The use of probiotics may increase the digestive enzyme activities in fish. Studies show that the inclusion of probiotics can improve digestive enzyme activities such as lipase, protease, amylase (Askarian et al., 2011; Castro et al., 2013; Hamza et al., 2016; Marzuqi et al., 2023). Enumeration of different digestive enzyme producing bacteria on *T. tambroides* with inclusion of probiotics to its diet was determined by Asaduzzaman et al. (2018a). *T. tambroides* juvenile fed with *Bacillus* sp. and *Alcaligenes* sp. had higher lipolytic and proteolytic bacterial number observed compared to control and *Shewanella* sp. group. Also, *Alcaligenes* sp. treated group had higher cellulolytic bacteria number compared to control and other probiotics group. The probiotics treatments had significant increase the total heterotrophic bacterial number compared to control group.

## **2.5 Gut Microbiota**

The gut microbiota refers to the community of microorganisms, including bacteria, fungi and viruses that reside in the gastrointestinal tract of the fish. The community usually comprises of symbiotic, commensal, and pathogenic microorganisms (Sandirini et al., 2015). The substantial influence of gut microbiota on host health has prompted a growing number of studies aimed at characterizing and determining the mechanisms of action of these microbes (Butt & Volkoff, 2019). Most of the microbes reside in the digestive tract, impacting a wide array of the host biological processes (Romero et al., 2014). In the other words, gut microbiota

is considered as an ‘extra organ’ and important in intestinal development and physiology and overall impact to the growth, health, and immunological protection of the host (Butt & Volkoff, 2019; Tan et al., 2019).

The microbial community can be categorized into allochthonous microbiota (only passes through lumen) and autochthonous microbiota (potentially resident and closely associated with host tissues). The autochthonous microbiota has the ability to tolerate the low pH in the gastric, resistant to the bile acid action and adhere to the epithelial surface of stomach, small and large intestine and colonize. The allochthonous microbiota is unable to colonize, temporarily available in the gastrointestinal (GI) tract. The autochthonous bacteria are used to bind to the intestinal tissue or mucus while digesta is utilized to characterize communities of allochthonous bacteria (Ringo et al., 2016).

### **2.5.1 The Gut of the Fish**

The gut is a tubular structure that the entire passage along the food passes through the body, from the mouth to the end of the anus including the oesophagus, stomach and intestines (Smith, 1980). The gut refers to the part of the digestive system that include stomach and intestines, where the food digestion and absorption take place. The fish gut is divided into three main sections, known as foregut, midgut and hindgut. The foregut initiates at the posterior edge of the gills and encompasses the oesophagus, stomach, and pylorus. The midgut usually is the largest portion of the gut and may form intricate loops. It includes the intestine situated posteriorly to the pylorus, usually lacking distinct demarcation between midgut and the hindgut. The midgut is the primary site of nutrient absorption as the highest nutrient absorption found in the first intestinal segment which is the midgut (Le et al., 2019). The hindgut continues from the midgut and ends at the anus. The beginning of the hind gut usually observed by an increase in diameter of the gut. The water reabsorption and the formation of feces happen in the hindgut.

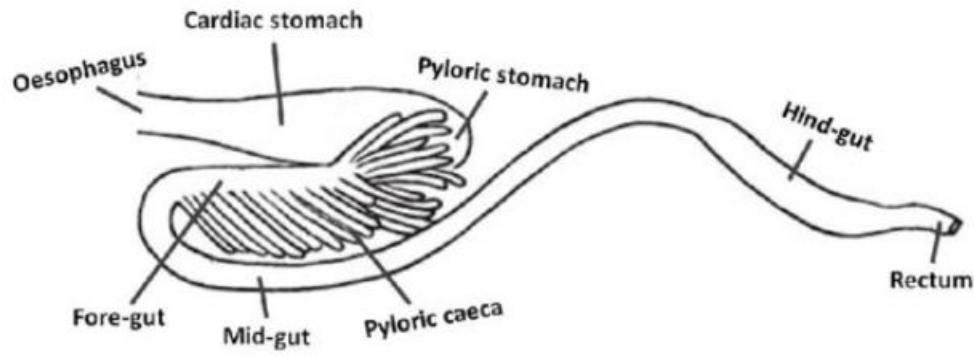


Figure 2.3: The digestive tract of fish (Adapted from Kamalam et al., 2020)

### 2.5.2 Role of Gut Microbiota in Fish

The gut microbiota community plays a crucial role in fish health and growth as it could affect fish physiology, immunity, development, and life span. The roles of the gut microbiota in fish were metabolism, immunity, development, and behaviour (Romero et al., 2014).

The gut microbiota is involved in the regulation of appetite, digestion, and metabolism. A germ-free individual that without a gut microbiota, exhibits leaner than normal individuals, even when consuming more calories as in the study of Duca et al. (2012) with mice. The germ-free mice were found reduced level of leptin and ghrelin that were appetite-regulating gene and showing the involvement of gut microbiota in regulation of appetite and metabolism. Also, the gut microbiota was found involving in modulating cholesterol metabolism, stimulate fatty acid uptake, and carbohydrates metabolism (Rawls et al., 2004; Semova et al., 2012; Rowland et al., 2018). The microbial secretion, consisting of specific metabolites such as short-chain fatty acid, indoles, propionate, butyrate, and acetate has an important impact on digestive process and metabolism. The microbiota also interacts with gastrointestinal (GI) neurotransmitters influencing their effects on GI motility, function, hormone release and feeding behaviour (Butt and Volkoff, 2019). It is believed that the dish gut microbiota contributes to host nutrition by secretion of enzyme and provide enzyme activity (Romero et al., 2014).

Furthermore, the gut microbiota takes a vital role in fish development and reproduction. The gut microbiota is important on the fish digestive tract development and function. The existence of microbiota in zebrafish serves as a stimulant for epithelial cell proliferation in developing GI tract, proving the role of gut microbiota in GI tract development (Cheesman et al., 2011). Bates et al. (2006) study show evidence that deficiency of brush border intestinal alkaline phosphatase activity, immature patterns of glycan expression, and reduction goblet cells responsible for mucus production, and decreased enteroendocrine cells were observed in microbiota absent zebrafish. The gut microbiota also contributed to the reproduction of the fish. The gut microbiota play role in the development of gonads and subsequent reproductive success of the host. The persistent administration of *Lactobacillus rhamnosus* from birth to sexual maturation, induce changes in gut microbiota and accelerate the larval development of zebrafish. This acceleration is attributed to enhancements in growth and sex differentiation (Avella et al., 2012; Carnevali et al., 2013). There is an increase in reproductive succession with increasing vitellogenic follicles and higher gonadosomatic index, higher ovulated egg and higher reproductive hormone expression in fish treated with *L. rhamnosus* zebrafish (Carnevali et al., 2013) and in ornamental livebearer fish species (Ghosh et al., 2007). Gut microbiota has the capacity to engage with sex hormone, influencing the regulation of the reproductive endocrine system (Xia et al., 2022)

The fish gut microbiota also contributes to fish health and immunity. The gut microbiota helps in development and maturation of immune system by decreasing immune effector gene expression and increasing the susceptibility to microbial pathogen in fish (Xia et al., 2022). Study of Bates et al. (2006) found the intestinal microbiota of zebrafish able to stimulate intestinal alkaline phosphatase, facilitating the dephosphorylation and detoxication of the endotoxin component within lipopolysaccharide that important in regulating inflammatory responses. The gut microbiota is essential for the host's health, the microbes can produce short-

chain fatty acids (conserve anti-inflammatory effect) through indigestible carbohydrate with anaerobic fermentation (Xia et al., 2022). Also, the microbiota able to protect host from colonization resistance that prevent colonization and proliferation of environmental pathogens (Butt & Volkoff, 2019).

Moreover, the gut microbiota plays a significant role in the behaviour and stress response of fish. The gut microbiota interacts with the gut-brain axis, a bidirectional communication system between the gut and the brain mediated by hormonal, immune and neural signal. Through the interaction, the microbiota may affect behaviour including feeding behaviour, digestive or absorption processes, metabolism and immune response with effect on the energy homeostasis and health of the host (Butt & Volkoff, 2019). The guppy fish live in hydrocarbon and trace metal contaminated wastewater, the gut microbiota plays outstanding roles on adaptation mechanisms which degrade or tolerate gasoline and diesel fuel hydrocarbon, and exhibit tolerance to trace metals (Kayath et al., 2019). The increase in microbiome lowers corticotrophin-releasing hormone and cortisol level resulting in reduced anxiety-like behaviour and decreased stress response. The stress leads to changes in gut microbiota, potentially impacting immune response and increasing the vulnerability to colonization and invasion by pathogens, thereby increasing the risk of infection (Butt & Volkoff, 2019)

### **2.5.3 Factors Affecting Fish Gut Microbiota**

There are intrinsic and extrinsic factors that may affect the fish gut microbiota and influence its composition and diversity.

First, the environmental factors are largely be affected by the microbes present in the environment. In early stages, the fish embryos develop in relative constant and bacteria-free environments, either within the egg or the mother (Romero et al., 2014; Butt & Volkoff, 2019). Therefore, the fish are presumed to be without microbes at point of hatching, the introduction

of gut microbes post-hatch originates from their surrounding environments. The fish experience fluctuations in environmental factors including water composition, quality and temperature that significantly impact the gut microbiota over the course of their lifespan (Vatsos, 2017). Xiong et al. (2019) expected that the fish gut microbiota would be mainly impacted and high similarity between the environment (water) and was proven by studies (Wong et al., 2013; Giatsis et al., 2015). Xiong et al. (2019) also mentioned that fishes reared in similar environments exhibit a high degree of similarity in the structure of their gut microbial communities, regardless of their trophic level. The fishes in different water environments tend to have different gut microbial composition, this can be proven in study of wild and captive *T. tambroides* gut microbiota by Tan et al. (2019) that wild *T. tambroides* has higher species diversity. The gut microbiota varies over short times, longer times, and even seasonal changes. The intestinal microbiota of hybrid tilapia in winter was different with the microbiota community in the other seasons which *Pseudomonas*, *Micrococcus* and *Flavobacterium* only present in winter (Al-Harbi & Uddin, 2004). Other environmental factors such as the available of pollutant or toxin, salinity, and temperature were proven by studies (Schmidt et al., 2015; Huyben et al., 2018; Meng et al., 2018). The environmental factors significantly influence the gut microbiota composition of the fish (Yukgehnaish et al., 2020).

The fish gut microbiota is influenced by the host-specific intrinsic factors. There are several factors including phylogenies, genetics, life stages, diet and diseases cause variation in the microbiota composition. For genetics, the sex of the fish may influence the gut microbiota through sex-specific host-microbes interactions, diet preferences and immune responses (Romero et al., 2014; Butt & Volkoff, 2019). The difference in gut microbiota composition between sexes were found in Bolnick et al. (2014). However, studies on zebrafish and seals did not find specific differences in the gut microbiota composition between males and females. More research is needed about the gut microbiota between sexes to fully understand the impact

of sexes to microbiota composition. Moreover, the age or sexual maturity also affects the gut microbiota of the fish. Rosado et al. (2021) tested the effects of aging and sexual maturation on skin and gill microbiota of European seabass and gilthead seabream, concluding that the observed microbial differences attributed to age are likely driven by growth and sexual maturation. There are addition studies that investigate the microbial of fish with age (Stephens et al., 2016; Walburn et al., 2019) and some indicate that juveniles exhibit higher bacterial richness in their gut microbiota compared to elderly fish (Cantas et al., 2012; Yan et al., 2016).

Additionally, other host-specific intrinsic factors such as diet also contribute to the variation in fish gut microbiota. The diet, feeding habits, and nutrition can impact the structure and composition of the gut microbiota (Butt & Volkoff, 2019). The gut microbiota composition in animals exhibits a higher degree of similarity when host share a common diet or genetic ancestry, particularly noticeable in mammals. However, the correlation between microbiota composition and genetic ancestry is less pronounced in fish, reptiles, birds, and invertebrates (Lind & Pollard, 2021). The use of insect meals and probiotic feed as an alternative protein source to fish meal did affect the fish gut microbiota composition and abundance the bacteria (Hasan et al., 2023). The study of Xia et al. (2014) found that starvation on Asian seabass caused variation in the gut microbial communities which an increase in Bacteroidetes and decrease in Betaproteobacteria under starvation. According to Butt and Volkoff (2019), the feeding habit also influence the composition of gut microbiota as carnivores usually has lower bacterial diversity in the gut compared to omnivores and herbivores. The most abundant bacteria in carnivorous fish are *Clostridium*, *Cetobacterium* and *Holomonas*, while *Cetobacterium* and *Halomonas* in omnivorous fish and *Clostridium*, *Citrobacter* and *Leptotrichia* in herbivorous fish.

On the other hand, diseases will affect the fish gut microbiota pathogenic bacteria increased in the microbial communities. The studies of Liu et al. (2020), Parshukov et al. (2019)

and Ofek et al. (2022) show that *Cetobacterium* relative abundance were lower when infected or diseased compared to healthy fish. Ofek et al. (2022) also found that the abundance of Firmicutes ZOR0006 was higher in healthy tilapia compared to diseased tilapia. The study of Lau et al. (2021) on the gut microbiota of healthy and diseased *T. tambroides* shows reduced microbiota diversity and species richness in dropsy diseased *T. tambroides* gut. Some possible pathogens such as *Aeromonas*, *Arcobacter*, *Bacteroides*, *Vibrio*, *Pseudomonas*, and *Clostridium* was found higher abundance or uniquely in diseased Empurau. The use of antimicrobial treatment to fish might decrease the gut microbiota load or diversity of bacterial population after microbial exposure. The reduction of microbial level could leads decreasing of effective barrier by commensal microbiota (Romero et al., 2014). The use of antibiotics can eliminate susceptible microorganisms from the microbiota, facilitate the growth of resistant opportunistic pathogens. This occurs by reducing competition and promoting the colonization of opportunistic species, allowing them to occupy previously unavailable ecological niche.

#### **2.5.4 Dominant Gut Microbiota**

The dominant gut microbiota in fish can vary depending on the species and environmental factors. Studies have found that freshwater fish gut usually dominated by bacteria such as Proteobacteria, Firmicutes, Bacteroidetes, and Fusobacteria (Romero et al., 2014; Liu et al., 2016; Tan et al., 2019; Lau et al., 2021; Zhang et al., 2022). There are several studies on the gut microbiota of *Tor tambroides* (Tan et al., 2019; Iehata et al., 2021; Lau et al., 2021).

The experiment by Tan et al. (2019) compared the gut microbiota of wild and captive *T. tambroides*. Tan and other researchers found that Firmicutes, Proteobacteria, Fusobacteria and Bacteroidetes were the most abundant bacterial phyla in both wild and captive *T. tambroides*. The study showed that the wild Empurau had a more diverse gut microbiota than

captive Empurau. The wild *T. tambroides* have abundant in bacterial Operational Taxonomic Unit (OTU) such as *Cetobacterium* sp., Peptostreptococcaceae family, *Bacteroides* sp., Phosphate solubilizing bacteria PSB-M-3 and *Vibrio* sp. On the other hand, captive *T. tambroides* most abundant OTU are *Cetobacterium* sp., *Citrobacter* sp., Aeromonadaceae family, Peptostreptococcaceae family and *Turicibacter* sp. *Cetobacterium* sp. was the core microbiota in gut of *T. tambroides* as it is the most dominant genus found in both wild and captive Empurau.

Moreover, the study by Iehata et al. (2021) on gut microbiota with cultured *T. tambroides* from different farm. The core gut bacterial of Empurau is Proteobacteria, which was prominent phyla that more than 50% of gut microbiota in Kelantan and Terengganu samples and 37.4% of gut microbiota in Pahang sample. The overall dominant phylum was Proteobacteria, followed by Fusobacteria, Firmicutes, Bacteroidetes then Actinobacteria depending on their location. In the Terengganu sample, Actinobacteria exhibited both higher and lower levels of *Cetobacterium* (Fusobacteria), primarily due to a diet only composed of vegetable. In contrast, the Kelantan and Pahang samples demonstrated higher levels of *Cetobacterium*, attributed to a diet combination of artificial feed and fruit. The abundance of *Cetobacterium* higher in carnivores gut than herbivores. The difference in gut microbiota between locations may be due to environmental factors and diet.

On the other hand, Lau et al. (2021) study compared the gut microbiota of healthy and diseased *T. tambroides*. The healthy *T. tambroides* has higher gut microbiota species richness than dropsy diseased *T. tambroides* gut. Both healthy and disease *T. tambroides* gut microbiota were dominated by these 4 phyla which are Proteobacteria, Fusobacteria, Bacteroidetes and Firmicutes. These 4 phyla comprised almost 98% of the gut microbiota of healthy *T. tambroides* and 99.6% of diseased *T. tambroides* gut microbiota. However, diseased *T.*

*tambroides* comprises some opportunistic pathogens such as *Aeromonas*, *Enterobacteriaceae*, *Vibrio*, *Pseudomonas* and *Bacteroides*.

### **2.5.5 Next Generation Sequencing (NGS)**

Next generation sequencing (NGS) also known as massively parallel sequencing is a new sequencing method for DNA and RNA sequencing in basic, applied, and clinical research. Next generation sequencing (NGS) was introduced between 2004 to 2006, helps drastically increase in sequencing data output due to principles and innovations in nanotechnology that enable the massively parallel sequencing of single DNA molecules (Hu et al., 2021). The community was released from the more labor-intensive and inefficient previous Sanger sequencing methodologies, which allowed for an incredible increase in data output. This was made possible by the technology's evolving procedures, which were better blended with data collecting and processing.

NGS provide lower cost and higher throughput alternative to sequence data than Sanger sequencing (Granda & Weinbrecht, 2013; Ari & Arian, 2016; Hu et al., 2021). Also, NGS is widely used in sequencing of the human genome facilitating in finding genes and regulatory elements associated with diseases. Identification of disease-causing mutation for diagnose pathological condition with targeted sequencing. RNA-seq can offer information on a sample's whole transcriptome in a single study without requiring prior knowledge of an organism's genetic sequence. This method provides a viable alternative to microarrays for gene expression studies (Butt & Volkoff, 2019).

According to the studies of Slatko et al. (2018) and Hu et al. (2021), the DNA sequencing founding methods were Sanger dideoxy synthesis (Sanger & Coulson, 1975) and Maxam-Gilbert chemical cleavage (Maxam & Gilbert, 1980). The Original Maxam-Gilbert

method was modified to help eliminate toxic reagent, however Sanger sequencing by synthesis (SBS) dideoxy method becoming the sequencing standard. Sanger sequencing method was developed in 1977.

The second-generation sequencing (Short-read NGS) method were grouped into two categories; sequencing by hybridization and sequencing by synthesis (SBS). The Sanger sequencing further developed to SBS method without dideoxy terminators and combined with repeated cycles of synthesis, imaging, and methods that incorporate additional nucleotides in growing chain. However, the costs of the SBS were high and aiming to reduces the cost per base. On the other hand, sequencing by hybridization were developed in 1980s by arrayed DNA oligonucleotides of known sequence on filters that were hybridized to labelled fragment of the DNA to be sequenced. SBS differ with Sanger sequencing in relying on shorter reads about 300 to 500 bases. Ion Torrent platform and Illumina also approached in second generation sequencing.

Third generation sequencing (Long-read sequencing) aim to sequence long DNA and RNA molecules and achieve read length upwards of 10kb beyond Sanger or short-read sequencing technologies (Ari & Arikan, 2016; Slatko et al., 2018; Hu et al., 2021) Single Molecule Real time (SMRT) or Pacific Bioscience (PacBio) sequencing involve binding engineered DNA polymerase with bound DNA will be sequenced to the bottom of zero-mode waveguide (ZMW) well. In contrast to the second-generation methods, alterations in the process involve streamlined library preparation steps and the direct targeting of unfragmented DNA molecules in real-time. However, SMRT sequencing has a high error rate and overcome by the depth of the number of read passes obtained for each SMRT bell template in each ZMW.

The ongoing advancements in sequencing technologies, which offer potential for robust, reproducible, and accurate sequencing of extended fragments at increased throughput with reduced cost, are likely to be the next era of sequencing platform.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Research Design

The experiment aimed to investigate the effect of selective probiotics mix mechanism of action in the gut of Empurau (*Tor tambroides*) fingerlings. In this study, two phases of feeding trial were carried out, where Phase 1 was 20 weeks of probiotics mix diet feeding trial and Phase 2 was 8 weeks post-probiotics feeding trial with commercial fish feed (Figure 3.1). Diets were formulated with 40% protein and supplemented with different concentration of Lacto-sacc forming 4 treatment diets, control diet (T<sub>A</sub>), 0.5% Lacto-sacc (T<sub>B</sub>), 1.0% Lacto-sacc (T<sub>C</sub>) and 1.25% Lacto-sacc (T<sub>D</sub>). For each treatment, three replicates of tanks were prepared with each replicate containing 50 *T. tambroides* fingerlings with average weight of  $6.53 \pm 0.17$ g. The data for growth parameters, relative protein digestibility and digestive enzyme activity and gut microbiota were collected during the end of phase 1 and phase 2 of the study. Phase 2 will be continued with randomly selected 30 fishes and the remaining fishes will be sacrificed for relative protein digestibility, digestive enzyme activities and gut microbiota phase 1 experiment.

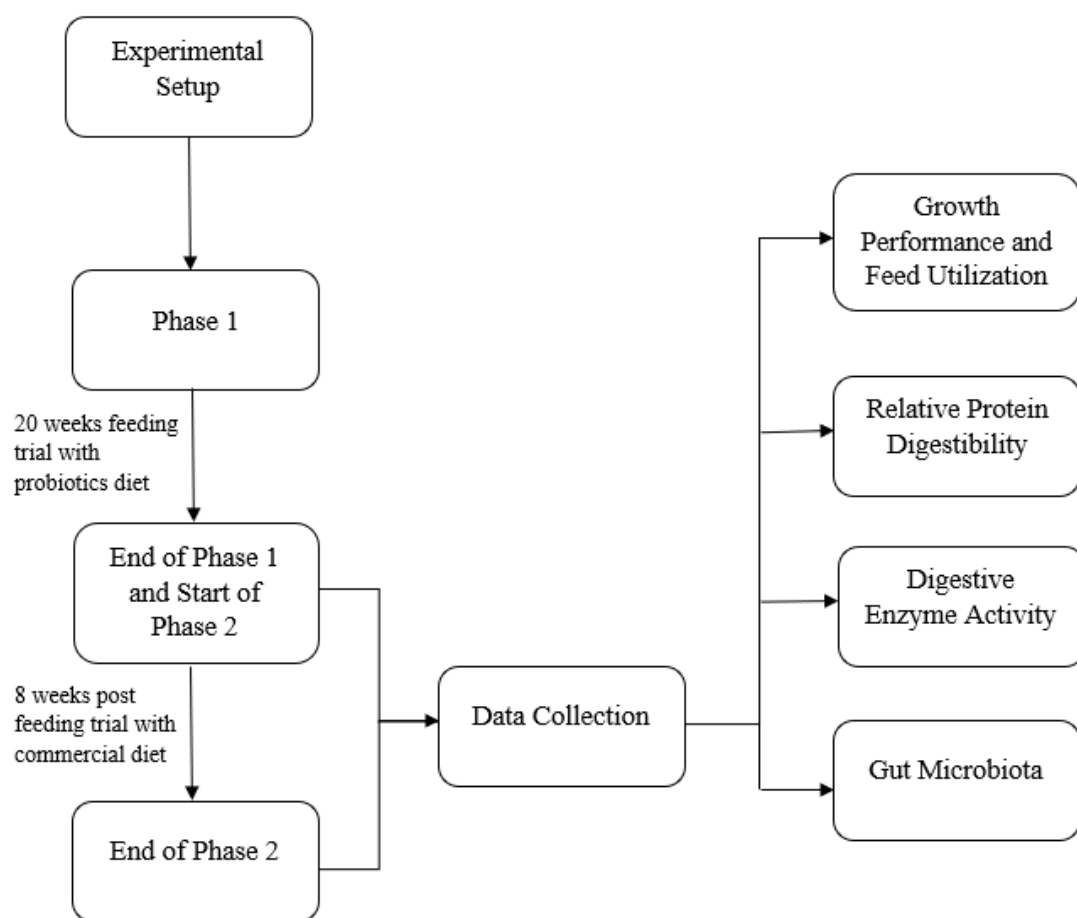


**Figure 3.1:** Study design for the effect of selective probiotics mix mechanism of action in the gut of Empurau (*Tor tambroides*) fingerlings

### 3.2 Experimental Fish and Husbandry Condition

Empurau, *Tor tambroides* fingerlings were obtained from Puri Johan Agro Sdn Bhd at Serian, Sarawak at size of 1-1.5inch per fingerlings. The fish were stocked into three 690 L HDPE tanks and acclimated for about 8 weeks with commercial catfish pellet (Uni-President, T502SV) containing minimum 40% crude protein and minimum 4% crude fat. Tank water maintenance was carried out daily with 50% of water changed. The fish were fed twice daily with 4% of the fish weight until satiation.

### 3.3 Experimental Setup

The experimental fish at average weight of  $6.53 \pm 0.17\text{g}$  were stocked randomly into twelve 680L HDPE tank with water volume of 580L at the density of 50 fingerlings per tank. The stocking density of each tank were around 0.086 fish/ litre. There were 12 tanks in total, divided into 4 treatments, with each treatment had 3 replicate tank. All treatment diets were prepared and fully defrosted prior feeding to the fish. Phase 1 feeding trial were conducted for 20 weeks with probiotics treatment feed and continued in Phase 2 with commercial catfish pellet for 8 weeks. The fish were fed twice daily at 5% of the average fish body weight at 8.30am and 4.30pm. The leftover fish feeds were taken out from tank and dried in oven for 48 hours at 35°C and weighted.

### 3.4 Probiotics Mix Diet Preparation

The treatment diets (probiotics mix diet) were made according to the formula of 40% crude protein and 12% lipid. The Lacto-sacc probiotics mix (Alltech, Lacto-sacc) was supplied by Alltech Sdn Bhd. The minimum amount of *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* per kilogram are 1980 billion cells and 110 billion colony forming unit (CFU), respectively. The concentration of probiotics in treatment diet were fixed as, control diet (A), 0.5% Lacto-sacc (B), 1.0% Lacto-sacc (C) and 1.25% Lacto-sacc (D). 1% of the mixture contains  $10^9$  CFU *Saccharomyces cerevisiae* and  $10^8$  CFU *Lactobacillus acidophilus*, selected from the previous study conducted by Munir *et al.* (2016a; 2016b). The experiment diet was made in a single batch with 20kg for each treatment. The fish feeds were prepared according to formulation listed in table 3.1. The fish feed were prepared by mixing well the dry ingredients (fish meal, corn starch, carboxymethyl cellulose (CMC) and cellulose), followed by fish oil- palm oil mixture. The probiotics mix was mixed with vitamin mix and mineral premix before adding to the dry ingredient mixture. The mixtures were then added with distilled

water up to desired moisture to form dough. The doughs were pelleted with meat mincer (Kimhill, Taiwan) with 0.2 mm size outlet. The pellets were air dried for 1-2 hour then dried in oven for 48 hours at 35°C. The pellets were break into smaller size (around 2mm) after 12 hours of oven drying to minimize the forming of powdery feed. The dried pellets were let cooled to room temperature, sieved, and packed into smaller container along with silica gel pack, seal well to maintain the freshness of the feed. The feeds were stored at -20°C freezer. The feeds were packed into smaller zip lock bag once a week and fully defrosted (defrost prior 30 minutes) before feeding. The fish feed proximate composition analysis including protein, lipid, moisture, ash, fibre, and gross energy were determined according to Official Methods of Analysis (AOAC), 1980.

**Table 3.1:** Feed formulation and proximate composition of ingredients used in *Tor tambroides* probiotics diet.

| <b>Ingredient (g)</b>               | <b>Control (A)</b> | <b>0.5% Lacto-sacc (B)</b> | <b>1.0% Lacto-sacc (C)</b> | <b>1.25% Lacto-sacc (D)</b> |
|-------------------------------------|--------------------|----------------------------|----------------------------|-----------------------------|
| Fish Meal                           | 594.00             | 594.00                     | 594.00                     | 594.00                      |
| Corn Starch                         | 287.60             | 287.60                     | 287.60                     | 287.60                      |
| Fish Oil                            | 17.4               | 17.4                       | 17.4                       | 17.4                        |
| Palm Oil                            | 40.00              | 40.00                      | 40.00                      | 40.00                       |
| Cellulose                           | 11.00              | 6.00                       | 1.00                       | 1.00                        |
| CMC                                 | 10.00              | 10.00                      | 10.00                      | 7.50                        |
| Vitamin mix                         | 20.00              | 20.00                      | 20.00                      | 20.00                       |
| Mineral mix                         | 20.00              | 20.00                      | 20.00                      | 20.00                       |
| Lacto-sacc mixture                  | 0.00               | 5.00                       | 10.00                      | 12.50                       |
| <b>Proximate Composition (g/kg)</b> |                    |                            |                            |                             |
| Protein                             | 41.82              | 40.41                      | 40.82                      | 41.74                       |
| Lipid                               | 12.99              | 13.34                      | 11.43                      | 11.78                       |
| Moisture                            | 7.23               | 7.7                        | 6.93                       | 6.27                        |
| Ash                                 | 4.13               | 4.13                       | 4.27                       | 4.33                        |
| Fibre                               | 0.27               | 0.27                       | 0.33                       | 0.33                        |
| NFE                                 | 33.65              | 34.15                      | 36.22                      | 35.55                       |
| GE(J/g)                             | 18418              | 19109.5                    | 18988.5                    | 18921                       |

### 3.4.1 Viability of *Lactobacillus acidophilus* in Diet

The viability of *Lactobacillus acidophilus* in probiotic diet was done after diet preparation after storage at -20 °C and at the end of the probiotics feeding trial. Rehydrate

solution was made with 1 gram of probiotics diet sample mixed with 9 ml MRS broth and incubated at 37 °C for 10 minutes. Serial dilution of rehydrate solution was prepared until 10<sup>10</sup> dilution. The warm autoclaved MRS agar was poured into the autoclaved petri dish and waited to solidify. After the agar was cooled and solidified, 1 ml of rehydrated solution and serially diluted rehydrated solution were pipetted on respective agar and spread well. The petri dish was then parafilm and incubated at 37 °C for 72 hours. After incubation, the visible colonies were counted manually. The validation of *Lactobacillus acidophilus* in probiotics diet was shown in the Appendix.

### **3.5 Water Quality Management**

The water exchange was carried out daily to avoid poor water quality. Sixty percent of the water were drawn out and exchanged with dechlorinated tap water after water collection for water quality parameter analysis. The uneaten feeds, sediments and faeces were drawn out once daily after an hour after feeding.

#### **3.5.1 In Situ Water Quality**

The *in-situ* water quality parameters (temperature, dissolved oxygen and pH) were taken twice daily. The dissolved oxygen (DO) and temperature were measured using a DO meter (Extech instruments, SDL 150) whereas pH was measured with pH meter (Extech instruments, SDL 100).

#### **3.5.2 Ex Situ Water Quality**

The *ex-situ* water quality parameters such as nitrate (Appendix A) and ammonia (Appendix B) were measured weekly with spectrometer (Hach, DR2800) according to each parameter analysis guidelines. The water sample bottles were prepared by acid wash in 10% hydrochloric acid (HCl) for at least 6 hours and dried. The water samples were collected using sample bottles and stored in cooler box before conducting water analysis.

### 3.6 Data Collection

The data of growth performance, relative protein digestibility, digestive enzyme activities and gut microbiota were collected at the end of each phase.

#### 3.6.1 Growth Performance

The initial fish body weight and length were measured and recorded prior to the commencement of study at each phase. The measurements were collected by taking every fish for weight. The fish growth parameters were calculated as relative growth (RG); the specific growth rate (SGR) and survival rate (SR) according to the formula below:

$$\text{RG (\%)}: \{(\text{Final weight} - \text{Initial Weight} / \text{initial weight}) \times 100\} \quad \text{Equation 3.1}$$

$$\text{SGR (\%)}: \{(\ln \text{ final weight} - \ln \text{ initial weight} / \text{nos. of days}) \times 100\} \quad \text{Equation 3.2}$$

$$\text{SR (\%)}: \{(\text{Final Number of Fish} / \text{Initial Number of Fish}) \times 100\} \quad \text{Equation 3.3}$$

The measurement for hepatosomatic index (HSI), visceral somatic index (VSI) and intraperitoneal fat (IPF) were done at the end of feeding trials of Phase 1 and Phase 2. The measurements were done sacrificed 3 fish per replicate tank following the formula of below:

$$\text{HSI (\%)}: \{(\text{Liver Weight} / \text{Fish Weight}) \times 100\} \quad \text{Equation 3.4}$$

$$\text{VSI (\%)}: \{(\text{Viscera Weight} / \text{Fish Weight}) \times 100\} \quad \text{Equation 3.5}$$

$$\text{IPF (\%)}: \{(\text{IPF Weight} / \text{Fish Weight}) \times 100\} \quad \text{Equation 3.6}$$

The total feed consumed by the fish per replicate tank were recorded daily. The parameter for feed utilization in term of the feed conversion ratio (FCR) and protein efficiency rate (PER) were calculated using these formulas:

$$\text{FCR}: (\text{Total Feed Consumption} / \text{Weight Gain of Fish}) \quad \text{Equation 3.7}$$

$$\text{PER}: \{(\text{Final Weight} - \text{Initial Weight}) / \text{Protein Intake}\} \quad \text{Equation 3.8}$$

The proximate composition analysis including crude protein, fat, crude fibre, moisture, ash and gross energy of fish was carried out according to the Official Methods of Analysis (AOAC), 1980.

### **3.6.2 Relative Protein Digestibility**

Relative Protein Digestibility (RPD) was found out by *in vitro* using pH drop method to determine the intestinal enzymes using method of Munir et al. (2016b).

#### **3.6.2.1 Crude Intestinal Enzyme Extraction**

The method of extraction of crude intestinal enzymes were carried out following method of Munir et al. (2016b). Six fishes were randomly collected from each tank forming 18 fish per treatment group. The fish were sacrificed for crude intestinal enzyme extraction from the intestines after 4 hours of feeding. The following procedure were conducted in 16 °C room temperature and samples were always kept on distilled water ice block and dissection were conducted on metal tray above ice block. Gut of the fish was collected by carefully cutting the end region of stomach till the end of intestine, held with forceps. Intestine was collected and rinsed with cold autoclaved distilled water and weighted. The gut cut into smaller pieces, mixed with 10 parts of cold Phosphate Buffer Solution (PBS) pH 7.4 in 50 mL Falcon tube and homogenized using homogenizer (Witeg, HG-15A). The mixture was centrifuged at 12000 rpm for 15 minutes at 4 °C. The supernatant transferred into 2 mL Eppendorf tube, labelled and seal with paraffin. The Eppendorf tubes were kept frozen at -80 °C deep freezer.

#### **3.6.2.2 pH Drop Method**

The protein suspension solution (6.25 mg crude protein/ml) were prepared using diet from each treatment mixed with distilled water. The treatment diet was crushed and sieved through 250 µm meshed sieve. The treatment diet that weighted equivalent to 3125 mg crude protein were mixed with 500 mL of autoclaved distilled water in a Schott bottle. The mixtures

were kept in room temperature for 1.5 hour and stir occasionally. The mixture was shaken well and transferred into 50 mL Falcon tube. The tubes were then vortexed and centrifuged at 10000 rpm. The supernatant was collected and transferred to a new autoclaved Schott bottle and stored in fridge at 4 °C before usage. The casein (control solution) was prepared by mixing with distilled water to form casein solution with 6.25 mg crude protein/mL.

The pH drop method was performed first by mixing 30 mL of protein suspension solution with 3 mL intestinal enzyme extracted into 50 mL autoclaved glass test tube in 37 °C water bath. The pH was quickly adjusted to 8 with 0.01M Hydrochloric acid (HCl) and 0.01M Sodium hydroxide (NaOH). The changes of pH were measured and recorded for 10 minutes with every minute interval. The pH drop method procedure were repeated by replacing the protein suspension solution with casein solution as control. The relative protein digestibility (RPD) was calculated by using the following formula:

$$\text{RPD} = \{(-\Delta\text{pH feedstuff}) / (-\Delta\text{pH casein})\} \times 100 \quad \text{Equation 3.9}$$

### **3.6.2.3 Protein Content of Intestinal Enzyme**

The protein content of intestinal enzyme was measured using Bradford method (Bradford, 1976; Kruger, 2009) and Bovine Serum Albumin (BSA) as standard. The Bradford method was carried out using Bio-Rad Protein Assay Kit II #5000002. Dye was mixed with autoclaved distilled water with a ratio of 1:4 and filtered with filter paper. The diluted dye can be stored in room temperature for 2 weeks. 7 dilution of protein standard (0.125, 0.5, 0.75, 1.0, 1.5, 2) were prepared by dilute BSA with autoclaved distilled water. 10 µL of each protein standard, enzyme sample were pipetted into assigned wells in 96 wells flat bottom cell culture plate. Dye reagent of 200 µL were pipetted into each well and mixed well by repetitive pipetting. The multiwell plate was left to incubate at room temperature for at least 5 minutes, absorbance

was increased over time but sample incubation in room temperature should not be more than 1 hour. The absorbance of the samples was measured at 595 nm.

### **3.6.3 Digestive Enzyme Activities**

The digestive enzyme assays (amylase, lipase and protease) were done to examine the fish intestinal digestive enzyme. The sample used was intestinal enzyme extracted from fish using the same procedure in section 3.6.2.1. All enzyme samples were stored in small amount (200  $\mu$ L) in -80 °C freezer. Samples were fully thawed before being used and disposed after every use to avoid freeze-thaw action that may destroy enzyme activity.

#### **3.6.3.1 Amylase Activity Assay**

The amylase activity assay was carried out using Sigma Aldrich Amylase activity assay kit (Sigma-Aldrich, MAK009). Standards were prepared with pipette 0, 2, 4, 6, 8, 10  $\mu$ L of 2 mM of nitrophenol standard with autoclaved distilled water to 50  $\mu$ L to form 0, 4, 8, 12, 16, 20 nmole/well in assigned well of 96 well flat bottom cell culture plate. Samples of 1-50  $\mu$ L were added to the assigned wells and 50  $\mu$ L amylase assay buffer was added to each sample well. For positive control, 5  $\mu$ L of amylase positive control and 45  $\mu$ L amylase assay buffer was added to assigned control well. After every standard, sample and control were added to assigned well, 50  $\mu$ L of amylase assay buffer and 50  $\mu$ L of amylase substrate mix were added to each well and repetitive pipetting to mix well. The multiwell plate was shaken for 5 second in spectrophotometric multiwell plate reader then incubated for 2-3 minutes. The initial absorbance was measured at 405 nm. The sample were then incubated in 25 °C inside spectrophotometric multiwell plate reader (away from light), the absorbance was measured every 5 minutes at 405 nm. The measurement continues and stops until the most active sample was higher than the highest standard (20 nmole/well) as final absorbance recorded. The procedures were repeated twice for replication.

### **3.6.3.2 Lipase Activity Assay**

Lipase activity assay was done by using Sigma Aldrich Lipase Activity Assay kit (Sigma-Aldrich, MAK046). Glycerol standard was prepared by mixing 10  $\mu\text{L}$  of glycerol standard with 990  $\mu\text{L}$  of lipase assay buffer to form 1 mM standard. The standard then prepared by adding 0, 2, 4, 6, 8, 10  $\mu\text{L}$  of diluted glycerol standard with lipase assay buffer to form 50  $\mu\text{L}$  in each well. For sample, 1-50  $\mu\text{L}$  sample were pipetted into assigned well and filled up to 50  $\mu\text{L}$  with lipase assay buffer. Positive control was prepared by mixing 5  $\mu\text{L}$  of lipase positive control with 45  $\mu\text{L}$  of lipase assay buffer. Master mixes were made by mixing a ratio of 93  $\mu\text{L}$  lipase assay buffer, 2  $\mu\text{L}$  of peroxide substrate, 2  $\mu\text{L}$  of enzyme mix and 3  $\mu\text{L}$  of lipase substrate to standard and sample well. Master mix of positive control was 96  $\mu\text{L}$  of lipase assay buffer, 2  $\mu\text{L}$  peroxide substrate and 2  $\mu\text{L}$  of enzyme mix. The master mix were mixed well by repetitive pipetting with standard, samples and positive control in respective well. The multiwell plate was shaken for 5 second in spectrophotometric multiwell plate reader then incubated at 37 °C for 2-3 minutes. The initial absorbance measured at 570 nm. The multiwell was incubated in multiwell plate reader at 37 °C (away from light) and measurement were taken every 5 minutes until most active sample was higher than highest standard (10 nmole/well).

### **3.6.3.3 Protease Activity Assay**

The protease activity assay was conducted using Sigma Aldrich protease fluorescent detection kit (Sigma-Aldrich, PF0100). The kit was required to be carried out in dark room and experiment items with samples were always kept in the dark and wrapped with aluminium foil. Black 96 well flat bottom cell culture plate was used in this experiment. For blank sample, 20  $\mu\text{L}$  of incubation buffer, 20  $\mu\text{L}$  of Fluorescein Isothiocyanate (FITC)-casein substrate and 10  $\mu\text{L}$  of ultrapure water were added into microcentrifuge tube. For control, 20  $\mu\text{L}$  of incubation buffer, 20  $\mu\text{L}$  FITC-casein substrate and 10  $\mu\text{L}$  of trypsin control solution were added to

another microcentrifuge tube. For sample, 20  $\mu$ L of incubation buffer, 20  $\mu$ L of FITC-casein substrate and 10  $\mu$ L of test sample were added into a microcentrifuge tube. The chemicals were mixed gently, and tubes were incubated at 37 °C in dark for 60 minutes. After an hour of incubation, 150  $\mu$ L of diluted 0.6N Trichloroacetic Acid (TCA) solution was gently mixed to each microcentrifuge tube. The microcentrifuge tubes were incubated at 37 °C for 30 minutes in incubator. After incubation, the tubes were centrifuged at 10000 xg for 10 minutes. The supernatant was collected into new microcentrifuge tube and able to store in dark at 2-8 °C for less than 24 hours before absorbance measurement. Supernatant of 2  $\mu$ L was added with 200  $\mu$ L of assay buffer to black 96 well flat bottom cell culture plate. The fluorescence intensity with excitation of 485 nm and emission wavelength were monitored at 535 nm.

#### **3.6.4 Gut Microbiota**

The gut microbiota was analysed with Next Generation Sequencing (NGS) for qualitative and quantitative data (Aloisio et al., 2016; Hardwick et al., 2017; UCD Centre for Food safety et al., 2018; Gaebler et al., 2019). Three fish sample from every treatment group were sacrificed and dissected at the end of both phase 1 and phase 2 feeding trial.

##### **3.6.4.1 DNA Extraction**

The fish was rinsed with cold autoclaved distilled water and wiped dry before dissection. The gut was cut from the end of stomach to anus. The gut was cut and put into a microcentrifuge tube. The DNA extraction was done with Qiagen DNeasy Powersoil Pro Kit. First, the PowerBead Pro Tube was spun to settle down the bead at the bottom of the tube. A total of 250 mg of fish gut sample and 800  $\mu$ L of Solution CD1 was added to the PowerBead Pro Tube. The PowerBead Pro tube was horizontally vortexed at maximum speed for 20 minutes. The PowerBead Pro tube was centrifuged at 15000 xg for 1 minute. The supernatant was transferred to a new 2 mL microcentrifuge tube and 200  $\mu$ L Solution CD2 was added. The tube was

vortexed for 5 second. The microcentrifuge tube was centrifuged at 15000 xg for 1 minute again. 700 µL of supernatant was transferred to a new microcentrifuge tube, added 600 µL of Solution CD3 and vortexed 5 second. 650 µL of lysate was loaded to MB Spin Column and centrifuged at 15000 xg for a minute. The flow-through was discarded and load 650 µL lysate and centrifuge again. The MB Spin Column was transferred into a new 2 mL Collection tube. 500 µL of Solution EA was added to MB spin column and centrifuged at 15000 xg for 1 minute. Flow-through was discarded and MB spin column was placed back to same collection tube. A total of 500 µL of Solution C5 added to MB Spin Column and centrifuged at 15000 xg for 1 minute. The MB Spin Column is transferred into new 2 mL Collection tube and centrifuged at 16000 xg for 2 minutes. The MB spin column was placed into new 1.5 mL Elution tube. 50 µL of Solution C6 was added to the centre of white filter membrane then centrifuged at 15000 xg for 1 minute. MB Spin Column was discarded, and the DNA was ready inside the 1.5 mL Elution tube. 1 µL of DNA was taken to check its concentration using Nanodrop (DeNovix; USA) and ultrapure water as blank sample. DNA sample purity that around 1.8 at 260 nm/280 nm with concentration more than 25 ng/µL were used for further experiment.

#### **3.6.4.2 Library Preparation and Sequencing**

The V3-V4 hypervariable regions of 16S rRNA genes of gut microbiota were selected for Polymerase Chain Reaction (PCR) amplification with the following primers: 5' CCTACGGGNGGCWGCAG and 5' GACTACHVGGGTATCTAATCC 3' were the forward and reverse primers, respectively (Klindworth et al., 2013). PCR reaction mix was prepared as listed in Table 3.2 prior conduct PCR reaction. The PCR mixes were thermally amplified using the settings specified in Table 3.3.

**Table 3.2:** PCR reaction components for amplification of V3-V4 hypervariable regions of 16s rRNA gene

| Component                      | Final Concentration | Volume (1X), $\mu$ L |
|--------------------------------|---------------------|----------------------|
| 2X KAPA HiFi HotStart ReadyMix | 1X                  | 12.5                 |
| 10 $\mu$ M Forward Primer      | 10 $\mu$ M          | 5.0                  |
| 10 $\mu$ M Reverse Primer      | 10 $\mu$ M          | 5.0                  |
| DNA Template                   | 5ng                 | 2.5                  |
| Final Volume                   | 25.0                |                      |

**Table 3.3:** Thermal cycling parameters used for PCR amplification

| Stage                | Temperature, $^{\circ}$ C | Duration | No of cycle |
|----------------------|---------------------------|----------|-------------|
| Initial Denaturation | 95.0                      | 3 min    | 1           |
| Denaturation         | 95.0                      | 30s      | 25          |
| Annealing            | 55.0                      | 30s      | 25          |
| Extension            | 72.0                      | 30s      | 25          |
| Final Extension      | 72.0                      | 5 min    | 1           |

Illumina MiSeq was selected as the NGS platform for this study. It is selected because to its paired-end sequencing with output of 13.2 to 15.6 Gb per run in about 56 hours. It is equipped with paired end NGS technology, which sequences both ends (forward and reverse reads) of the template to increase the accuracy of the aligned sequences. DNA samples were subsequently treated to library preparation prior to sequencing. The bacterial V3-V4 region of the 16S rRNA gene was amplified during first part of library construction with using locus-specific sequencing primers with overhanging adapters, forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. Nextera XT Index kit (Illumina) was used to clean the amplicons in preparation for the attachment of a unique dual index adapter to the amplicons (Illumina). Dual-indexed sequencing involve index 1 (i7) and index 2 (i5) sequences to generate libraries with unique tags. The DNA libraries were purified with Agencourt AMPure XP (Beckman Coulter, USA). The concentrations of libraries were quantified with a Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer (Thermo Fisher

Scientific, USA) and their sizes were validated with an Agilent 2100 Bioanalyzer (Agilent, USA). The libraries were then normalised and pooled prior to the protocol using MiSeq sequencing (2 x 300 bp paired-end).

### **3.7 Data Analysis**

The significance of growth performance, relative protein digestibility and digestive enzyme activities were analysed with One-way Analysis of Variance (ANOVA) using IBM SPSS Statistics Version 25 at 95% significance difference.

The MiSeq sequencing data were analysed using Quantitative Insights into Microbial Ecology (QIIME) 2 v2020.8 (Bolyen et al., 2019) and was installed on Linux Ubuntu System v18.04. The sequences were quality checked with FastQC v0.11.5 before further analysis to ensure sequences were in good quality (Andrew, 2010). Illumina sequencing assigns a Q-score to each base in order to measure the accuracy of that base's assignment to the sequence length. Higher Q score indicate better quality of sequencing data with high level of confidence and less likely to contain errors. Conversely, lower Q score indicate lower quality sequencing data and base call was less reliable which may lead to incorrect conclusion as a result of false-positive variant calls. Sequence reads having a Q score greater than 30 were included and proceed for further analysis. Before trimming chimeric sequences, adapter sequences were cleaved from both paired-end forward and reverse reads using the 'cutadapt' command. The sequences were quality checked with FastQC to evaluated on the length of sequences to be retained and base with poor Q score were cut off prior to denoising. The trimmed sequences were imported into QIIME 2 and Divisive Amplicon Denoising Algorithm 2 (DADA2) was utilised to denoise, and filter chimeric sequences based on a parametric model that infer actual biological sequences from reads (Prodan et al., 2020). Checking the percentage of filtered sequences in the denoised statistics to retain the most of sequence information. Forward and reverse reads were individually denoised and merged prior to the removal of chimeric

sequences using 'removeChimeraDenovo,' resulting in the formation of Amplicon sequence variant (ASV). The feature table and feature data were generated for each sample group, providing information on the number of samples and features, the frequency of samples, the frequency per feature, and the ideal sampling depth for retaining the most number of feature counts and feature details. Subsequently, a phylogenetic tree was constructed using sequences of features that accounted for each feature. The features were categorized taxonomically using Greengenes classifiers, yielding in taxonomy mapped to each ASVs.

The statistical analysis was examined into two section which were alpha rarefaction curve and beta diversity. The alpha rarefaction curve was plotted in order to determine the sequencing depth. Several alpha diversity index parameters (Chao1, Simpson, Shannon) were used to clarify the species richness and diversity of each sample within the gut microbiota of each *T. tambroides* treatment. The value of the Chao1 index is directly proportional to the species richness. The Simpson index ranges from 0 to 1, higher value mean lower diversity. Shannon index ranges from 0 to 5 and is directly proportional to species richness and evenness. The objective of beta diversity analysis is to assess the diversity of gut microbiota in *T. tambroides*. Consequently, principal coordinate analysis (PCoA) was plotted to visualize differences or similarities in the diversity of gut microbiota in each treatment of *T. tambroides*. Weighted UniFrac was selected for PCoA in this study because it can account for differences in the relative abundances of each taxon within communities (Lozupone et al., 2007).

## CHAPTER 4

### RESULTS

#### 4.1 Growth Performance of *Tor tambroides*

##### 4.1.1 Phase 1

In the result, there was no significant difference in growth performance, feed utilisation and body indices between treatments (Table 4.1). All the probiotics supplemented groups perform better than control group, especially treatment B, followed by C, D then A. Treatment B had the highest weight gain, relative growth, specific growth rate with best feed conversion ratio were found in treatment B, although it was not significant to other treatments. The SGR values was relatively consistent across all group with treatment B had the slightly higher value of 0.73%. The survival rates were high across all treatment groups, with treatment C achieved a 100% survival rate. Treatment with lower FCR indicated better feed efficiency. Treatment B had the lowest FCR and highest PER. All the preferable trend for weight gain, RG, SGR, FCR and PER were treatment B, C, followed by D and A. Moreover, treatment B had lowest IPF with highest HSI while Treatment A had lowest VSI and HSI. The RPD of all the lacto-sacc supplemented group were higher than control group, especially 0.5% group had the highest value of 42.06. As for digestive enzyme activities, there were 3 types of digestive enzyme tested. The amylase concentration was higher with lacto-sacc concentration of 1.25% lacto-sacc group with value of 3886.90. Treatment B had significantly higher lipase activity than the other treatment groups and highest in protease activity. Amylase activity increased with increasing Lacto-sacc concentration.

**Table 4.1:** Phase 1 growth performance, feed utilization and body indices of *Tor tambroides*

| Parameter         | Control (A) | 0.5% Lacto-sacc (B) | 1.0% Lacto-sacc (C) | 1.25% Lacto-sacc (D) |
|-------------------|-------------|---------------------|---------------------|----------------------|
| Initial Weight, g | 6.52±0.09   | 6.48±0.16           | 6.55±0.11           | 6.57±0.22            |
| Final Weight, g   | 17.36±0.45  | 18.14±0.89          | 17.94±1.33          | 17.79±1.38           |
| Weight Gain, g    | 10.84±0.57  | 11.66±0.97          | 11.39±1.43          | 11.23±1.36           |
| RG, %             | 166.28±9.44 | 180.64±18.12        | 174.76±24.84        | 170.85±20.23         |
| SGR, %            | 0.70±0.03   | 0.73±0.04           | 0.72±0.06           | 0.71±0.05            |
| SR, %             | 98.67±0.67  | 99.33±0.67          | 100±0.00            | 98.67±1.33           |
| FCR               | 7.34±0.31   | 6.39±0.28           | 6.49±0.41           | 7.00±0.71            |
| PER               | 0.33±0.01   | 0.38±0.02           | 0.37±0.02           | 0.35±0.03            |
| VSI               | 8.28±0.52   | 8.81±0.77           | 8.81±0.49           | 8.98±0.35            |
| HSI               | 0.44±0.08   | 0.60±0.05           | 0.53±0.11           | 0.59±0.08            |
| IPF               | 0.83±0.28   | 0.66±0.15           | 1.57±0.33           | 1.70±0.26            |

\* Means with standard error

**Table 4.2:** Phase 1 relative protein digestibility and digestive enzyme activities of *Tor tambroides*

| Parameter             | Control (A)             | 0.5% Lacto-sacc (B)     | 1.0% Lacto-sacc (C)     | 1.25% Lacto-sacc (D)    |
|-----------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| RPD                   | 42.06±5.97              | 56.12±11.37             | 52.44±11.78             | 52.65±12.31             |
| Amylase, nmole/min/mL | 2059.62±102.03          | 2821.63±1126.14         | 3591.27±1664.69         | 3886.90±371.16          |
| Lipase, nmole/min/mL  | 14.51±1.38 <sup>a</sup> | 62.90±8.80 <sup>b</sup> | 24.50±1.69 <sup>a</sup> | 21.17±2.47 <sup>a</sup> |
| Protease, ug/mL       | 2700.25±170.18          | 3173.87±826.72          | 2969.48±481.47          | 2929.54±309.62          |

\*Means with standard error within the same column followed by the same letter are non-significantly different (p<0.05) based on Tukey HSD.

#### 4.1.2 Phase 2

As for post-probiotics feeding trial with commercial feed, the *Tor tambroides* growth was positively influenced by Lacto-sacc supplementation, especially in treatment D gave the best result in growth performance as it has the highest weight gain, RG and SGR as shown in Table 4.3. However, for feed utilization, treatment D has highest PER same as treatment B but treatment D with worst FCR. The viscera somatic index (VSI) of fish were generally decreased with lacto-sacc supplement, especially in treatment B and C. Lowest VSI was observed in treatment C with value of 7.60 and highest VSI found in control group. Treatment D have best

result for post-probiotics feeding growth performance followed by C, A then B. Protein efficiency ratio is positive correlation with relative protein digestibility result with following order, B, D, C, A. The higher value of relative protein digestibility in 0.5% and 1.25% Lactosacc treatment was found. In term of digestive enzyme activity, a consistent pattern emerged both amylase and lipase. Treatment D exhibited the highest enzyme activity, followed by B, A, and then C. this trend was observed across both amylase and lipase activity. Conversely, for protease activity, a different trend was identified. The order of protease activity was C, followed by B, then D and finally A.

**Table 4.3:** Phase 2 growth performance, feed utilization and body indices of *Tor tambroides*

| Parameter         | Control (A) | 0.5% Lactosacc (B) | 1.0% Lactosacc (C) | 1.25% Lactosacc (D) |
|-------------------|-------------|--------------------|--------------------|---------------------|
| Initial Weight, g | 18.82±0.98  | 19.94±1.00         | 19.03±2.57         | 18.51±1.69          |
| Final Weight, g   | 29.51±1.19  | 30.69±2.37         | 29.92±2.73         | 29.68±2.84          |
| Weight Gain, g    | 10.69±0.79  | 10.76±1.70         | 10.89±0.18         | 11.17±2.39          |
| RG, %             | 57.17±5.59  | 53.82±7.15         | 59.04±6.79         | 61.74±14.46         |
| SGR, %            | 0.81±0.06   | 0.77±0.08          | 0.83±0.77          | 0.84±0.17           |
| SR, %             | 100.00±0    | 100.00±0           | 100.00±0           | 100.00±0            |
| FCR               | 3.08±0.08   | 3.03±0.27          | 3.08±0.12          | 3.23±0.71           |
| PER               | 0.78±0.02   | 0.81±0.07          | 0.78±0.03          | 0.81±0.15           |
| VSI               | 8.48±0.17   | 7.93±0.47          | 7.60±0.38          | 8.23±0.37           |
| HSI               | 0.71±0.03   | 0.65±0.02          | 0.63±0.02          | 0.74±0.11           |
| IPF               | 1.46±0.41   | 1.31±0.22          | 1.57±0.11          | 1.62±0.12           |

\* Means with standard error

**Table 4.4:** Phase 2 relative protein digestibility and digestive enzyme activities of *Tor tambroides*

| Parameter             | Control (A)    | 0.5% Lactosacc (B) | 1.0% Lactosacc (C) | 1.25% Lactosacc (D) |
|-----------------------|----------------|--------------------|--------------------|---------------------|
| RPD                   | 37.70±8.25     | 49.47±10.34        | 38.94±3.11         | 40.87±8.43          |
| Amylase, nmole/min/mL | 9085.94±335.03 | 9633.49±420.53     | 8527.66±336.74     | 9705.37±264.24      |
| Lipase, nmole/min/mL  | 8.90±0.19      | 9.31±0.56          | 8.73±0.11          | 9.83±1.17           |
| Protease, ug/mL       | 3757.32±144.96 | 3930.75±520.97     | 4420.31±1173.20    | 3906.93±156.97      |

\* Means with standard error

## 4.2 *Tor tambroides* Gut Microbiota

### 4.2.1 The Quality of DNA Extract

The DNA quality that extracted from *T. tambroides* were checked using Nanodrop (DeNovix, DS-11). All samples were qualified for good quality that concentration was more than 20ng/μl and the purity of the sample OD260/280 were around 1.8 to 2.0 as shown in table 4.5. The PCR product was visualized on a 1.7% TAE agarose gel (Figure 4.1). The qualified samples were sent for 16S rDNA metagenomic sequencing at Apical Scientific Sdn Bhd, Selangor.

**Table 4.5:** DNA quantification using Nanodrop

| Sample | Sample Name | Sample Concentration (ng/μl) | Volume (μl) | Total Amount (μg) | OD260/280 | OD260/230 |
|--------|-------------|------------------------------|-------------|-------------------|-----------|-----------|
| 1      | A1P1        | 558.10                       | 20          | 11.16             | 1.898     | 2.003     |
| 2      | A2P1        | 588.90                       | 20          | 11.78             | 1.893     | 2.402     |
| 3      | A3P1        | 331.20                       | 20          | 6.62              | 1.887     | 1.055     |
| 4      | B1P1        | 130.60                       | 18          | 2.35              | 1.656     | 1.062     |
| 5      | B2P1        | 285.95                       | 18          | 5.15              | 1.876     | 1.862     |
| 6      | B3P1        | 511.55                       | 17          | 8.70              | 1.896     | 1.360     |
| 7      | C1P1        | 351.25                       | 18          | 6.32              | 1.902     | 1.605     |
| 8      | C2P1        | 508.45                       | 19          | 9.66              | 1.896     | 2.121     |
| 9      | C3P1        | 383.00                       | 17          | 6.51              | 1.992     | 2.225     |
| 10     | D1P1        | 242.20                       | 16          | 3.88              | 1.908     | 2.049     |
| 11     | D2P1        | 394.90                       | 18          | 7.11              | 1.922     | 2.249     |
| 12     | D3P1        | 236.50                       | 18          | 4.26              | 1.894     | 2.195     |
| 13     | A1P2        | 135.90                       | 16          | 2.17              | 1.894     | 2.021     |
| 14     | A2P2        | 198.95                       | 17          | 3.38              | 1.824     | 1.961     |
| 15     | A3P2        | 90.45                        | 16          | 1.45              | 1.798     | 0.182     |
| 16     | B1P2        | 116.10                       | 18          | 2.09              | 1.840     | 0.670     |
| 17     | B2P2        | 97.30                        | 19          | 1.85              | 1.859     | 1.886     |
| 18     | B3P2        | 316.35                       | 20          | 6.33              | 1.880     | 2.195     |
| 19     | C1P2        | 356.30                       | 17          | 6.06              | 1.877     | 1.383     |
| 20     | C2P2        | 128.00                       | 20          | 2.56              | 1.810     | 0.299     |
| 21     | C3P2        | 580.80                       | 20          | 11.62             | 1.884     | 2.302     |
| 22     | D1P2        | 38.40                        | 16          | 0.61              | 1.842     | 1.536     |
| 23     | D2P2        | 395.20                       | 20          | 7.90              | 1.881     | 1.404     |
| 24     | D3P2        | 109.65                       | 17          | 1.86              | 1.851     | 1.542     |

\* DNA sample extracted from *Tor tambroides* gut from their respectively treatment which A1P1-A3P1: Control Phase 1; B1P1-B3P1: 0.5% Lacto-sacc Phase 1; C1P1-C3P1: 1.0% Lacto-sacc Phase 1; D1P1-D3P1: 1.25% Lacto-sacc Phase 1; A1P2-A3P2: Control Phase 2;

B1P2-B3P2: 0.5% Lacto-sacc Phase 2; C1P2-C3P2: 1.0% Lacto-sacc Phase 2; D1P2-D3P2:  
1.25% Lacto-sacc Phase 2

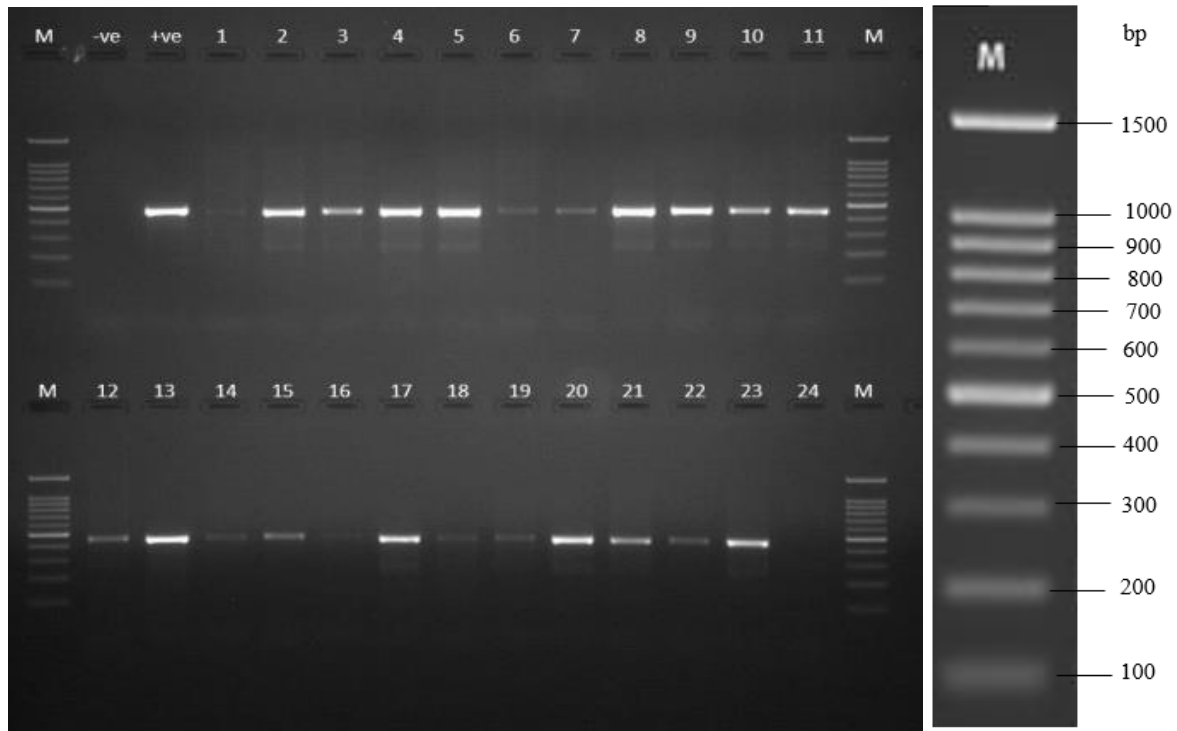


**Figure 4.1:** All sample PCR amplicons were visualised on agarose gel using primers

#### 4.2.2 *Tor tambroides* Gut Microbiota Sequencing with QIIME Analysis

In the study of *T. tambroides* gut microbiome, we found a total of 825 unique ASVs across 24 samples. The sequence information of gut microbial communities was summarised in Table 4.6. Figure 4.2 showed the Venn chart that the number of shared and unique ASVs among treatments of each phases. The study was divided into two phases, Phase 1 had 515 ASVs, Phase 2 had 443 ASVs, and both phases shared 133 ASVs (16.1%) in common. In Phase 1, treatment A to D revealed ASV counts of 247, 187, 184 and 235 respectively. Meanwhile, Phase 2 displayed ASV counts of 148, 176, 170 and 179 for treatment A to D respectively. All treatment between phase 1 and phase 2 exhibited ASVs overlap ranging from approximately 16% to 21%. Specifically, treatment A displayed a commonality with 55 ASVs (16.2%) shared between AP1 and AP2 for their microbial compositions. Similarly, treatment B shared 63 ASVs

(21%) in both phases, while treatment C exhibited 54 ASVs (18%) in common and treatment D had 68 ASVs (19.7%) shared between the two phases. It is noticeable that treatment A demonstrated the lowest similarity of ASVs (16.2%) among the two phases. Upon closer examination of each sample, it found both samples B1P1 and D2P1 had the lowest ASVs observed, amounting 63 ASVs, while D3P1 had the highest 165 ASVs observed for Phase 1. Moving on to Phase 2, samples that has the lowest and highest ASVs observed were A1P2 and C2P2 with 34 and 125 ASVs respectively.

**Table 4.6:** Summary of gut microbiome of *T. tambroides*

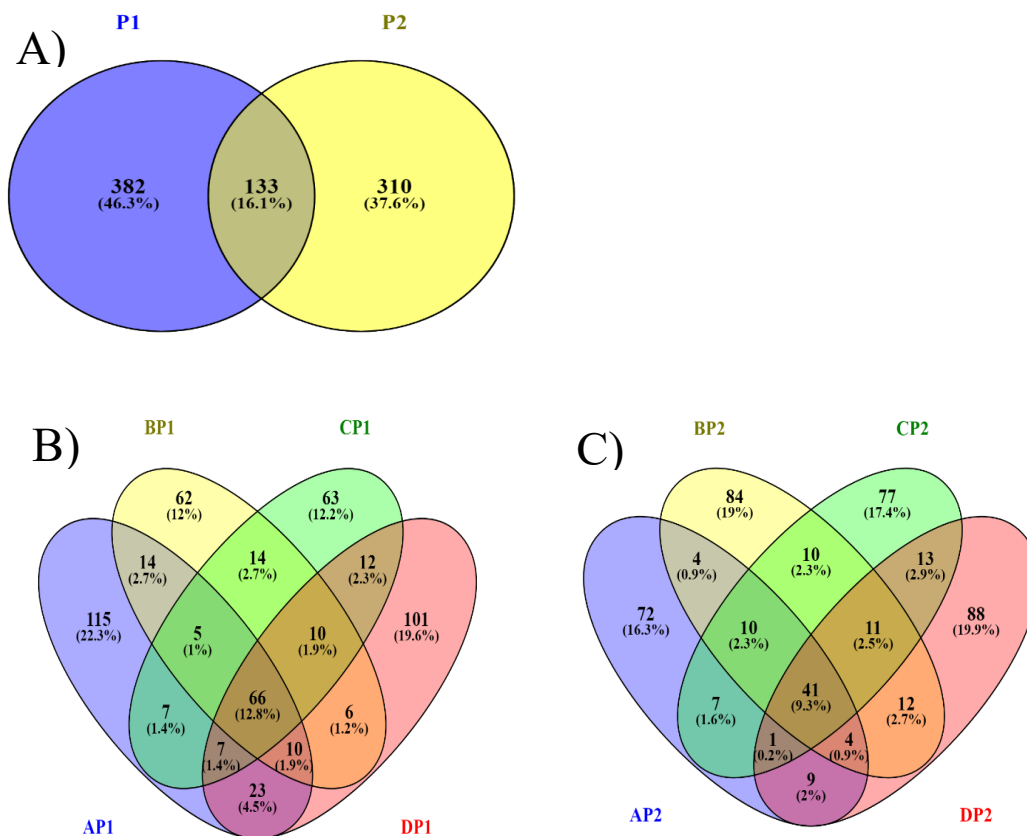
| Sample | Sequence after filter | Sequence after denoised | Sequence after merged | ASV |
|--------|-----------------------|-------------------------|-----------------------|-----|
| A1P1   | 135779                | 135141                  | 133848                | 120 |
| A2P1   | 126189                | 124676                  | 119540                | 125 |
| A3P1   | 141125                | 140278                  | 137538                | 84  |
| B1P1   | 134022                | 133084                  | 128574                | 63  |
| B2P1   | 124706                | 123608                  | 117692                | 93  |
| B3P1   | 91926                 | 90992                   | 88560                 | 129 |
| C1P1   | 121449                | 120808                  | 118700                | 81  |
| C2P1   | 86502                 | 85384                   | 82138                 | 86  |
| C3P1   | 123274                | 121181                  | 114784                | 95  |
| D1P1   | 140030                | 139241                  | 135874                | 106 |
| D2P1   | 127061                | 126304                  | 123832                | 63  |
| D3P1   | 123376                | 121255                  | 114866                | 165 |
| A1P2   | 131727                | 131416                  | 130652                | 34  |
| A2P2   | 126443                | 125540                  | 124112                | 130 |
| A3P2   | 125712                | 124498                  | 120949                | 82  |
| B1P2   | 81568                 | 80702                   | 79360                 | 102 |
| B2P2   | 116935                | 115486                  | 110491                | 101 |
| B3P2   | 114559                | 113615                  | 111172                | 103 |
| C1P2   | 106016                | 102472                  | 94099                 | 106 |
| C2P2   | 114008                | 112135                  | 107182                | 125 |
| C3P2   | 119906                | 119033                  | 115811                | 87  |
| D1P2   | 116673                | 115480                  | 112123                | 112 |
| D2P2   | 123531                | 122251                  | 117266                | 107 |
| D3P2   | 120255                | 118315                  | 111693                | 109 |

\* DNA sample extracted from *Tor tambroides* gut from their respectively treatment which A1P1-A3P1: Control Phase 1; B1P1-B3P1: 0.5% Lacto-sacc Phase 1; C1P1-C3P1: 1.0% Lacto-sacc Phase 1; D1P1-D3P1: 1.25% Lacto-sacc Phase 1; A1P2-A3P2: Control Phase 2; B1P2-B3P2: 0.5% Lacto-sacc Phase 2; C1P2-C3P2; 1.0% Lacto-sacc Phase 2; D1P2-D3P2: 1.25% Lacto-sacc Phase 2

**Table 4.7:** Summary of gut microbiome for each treatment of *T. tambroides*

| Sample | Sequence After Filter | Sequence After Denoised | Sequence After Merged | ASV   |
|--------|-----------------------|-------------------------|-----------------------|-------|
| AP1    | 134364.3              | 133365.0                | 130308.7              | 109.7 |
| BP1    | 116884.7              | 115894.7                | 111608.7              | 95.0  |
| CP1    | 110408.3              | 109124.3                | 105207.3              | 87.3  |
| DP1    | 130155.7              | 128933.3                | 124857.3              | 111.3 |
| AP2    | 127960.7              | 127151.3                | 125237.7              | 82.0  |
| BP2    | 104354.0              | 103267.7                | 100341.0              | 102.0 |
| CP2    | 113310.0              | 111213.3                | 105697.3              | 106.0 |
| DP2    | 120153.0              | 118682.0                | 113694.0              | 109.3 |

\* AP1: Control Phase 1; BP1: 0.5% Lacto-sacc Phase 1; CP1: 1.0% Lacto-sacc Phase1; DP1: 1.25% Lacto-sacc Phase 1; AP2: Control Phase 2; BP2: 0.5% Lacto-sacc Phase 2; CP2: 1.0% Lacto-sacc Phase 2; DP2: 1.25% Lacto-sacc Phase 2



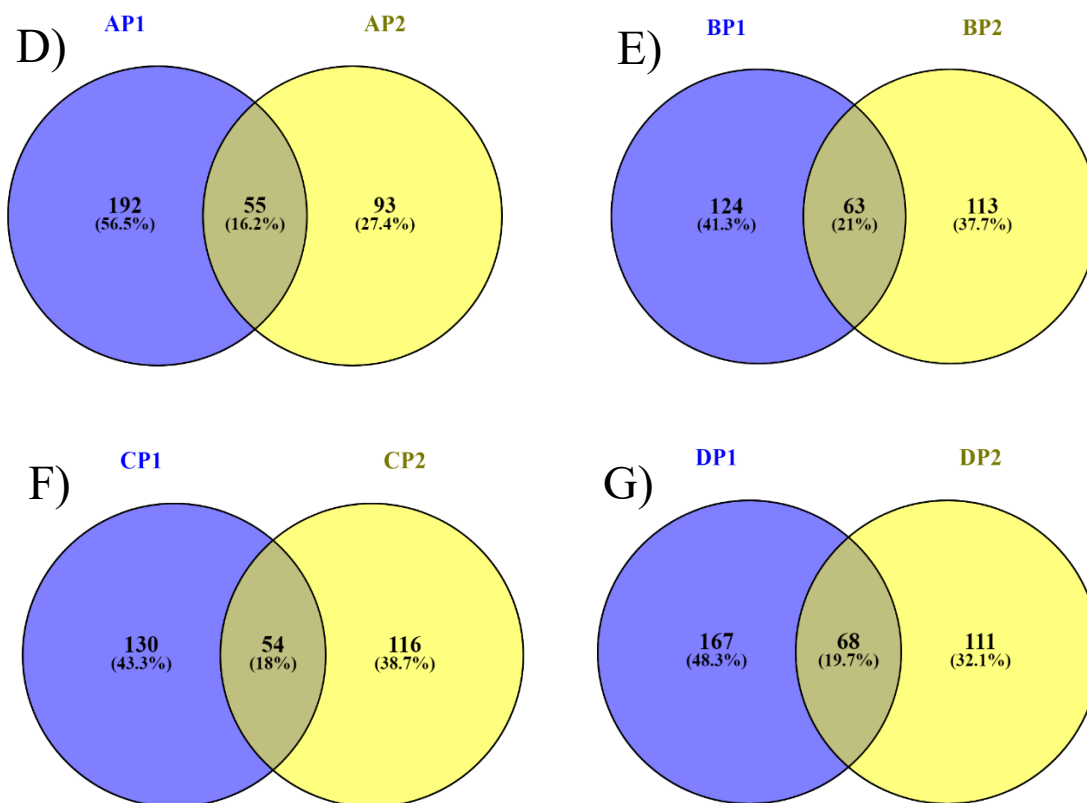


**Figure 4.2:** Venn chart showing the number of shared and unique ASVs (A) in Phase 1 and Phase 2 *Tor tambroides* gut (B) each treatment in Phase 1 *Tor tambroides* gut (C) each treatment in Phase 2 *Tor tambroides* gut (D) in Treatment A of both phase *Tor tambroides* gut (E) in Treatment B of both phase *Tor tambroides* gut (F) in Treatment C of both phase *Tor tambroides* gut (G) in Treatment D of both phase *Tor tambroides* gut

### 4.2.3 Alpha Diversity Analysis of *Tor tambroides*

The sequencing depth was normalised for all *T. tambroides* samples as plateau was observed when curve flattened gradually as the sequence depth increase as shown in Figure 4.3. This plateau indicates that the current sequencing depth is adequate for capturing the diversity present in each sample. Furthermore, the probability of discovering more ASVs beyond this depth is considered negligible. Therefore, the data obtained from the current sequencing depth can reliably reflect the diversity within each sample.

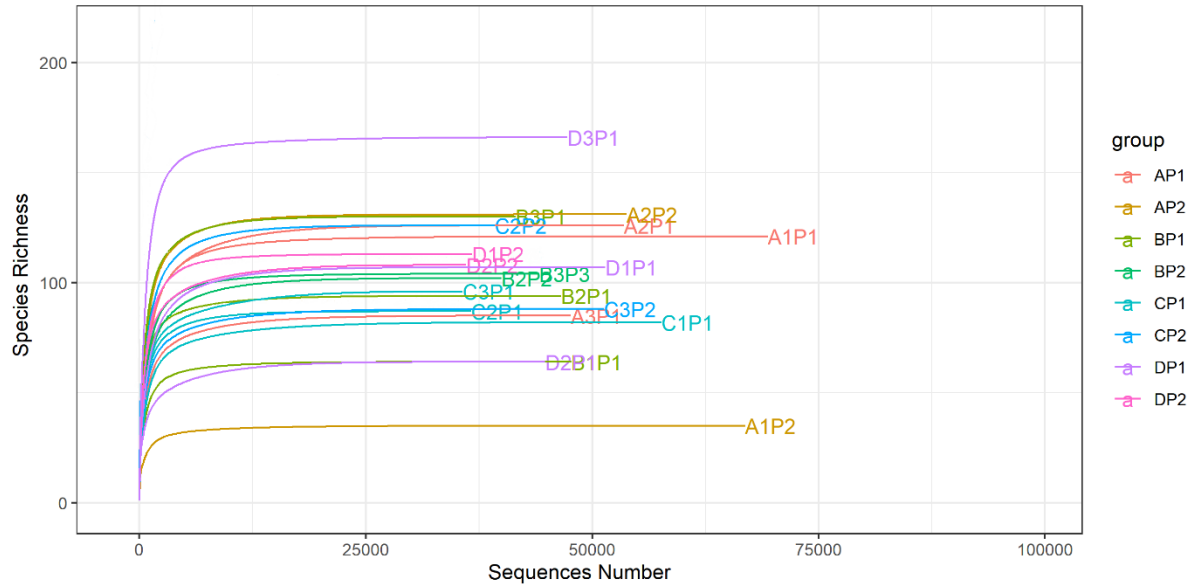


**Figure 4.3:** The alpha rarefaction curve of *Tor tambroides* gut microbiota  
 \* AP1: Control Phase 1; BP1: 0.5% Lacto-sacc Phase 1; CP1: 1.0% Lacto-sacc Phase1; DP1: 1.25% Lacto-sacc Phase 1; AP2: Control Phase 2; BP2: 0.5% Lacto-sacc Phase 2; CP2: 1.0% Lacto-sacc Phase 2; DP2: 1.25% Lacto-sacc Phase 2

Alpha Diversity is a measure of biodiversity that quantifies the diversity of species within a sample or habitat. It is assessing the richness and evenness of species within a specific area without considering differences between areas or samples. The summary of the alpha diversity was summarised in Table 4.8 while box plot for each parameters were visualized in Figure 4.4. For Shannon index that measure diversity and evenness, DP1 and DP2 had the highest Shannon index value of 2.8360 and 3.1068 respectively, indicating higher diversity and more even distribution of species abundances compared to other treatment within their respective phase. In the meanwhile, Chao1 index estimate the species richness, treatment D showed highest Chao1 indexes value for both phases, with DP1 value of 112.3333 and DP2 value of 110.5000. Simpson indices measure the species dominance within a community which the probability that two randomly chosen individuals in the sample belong to the same species. CP1 and AP2 had the lowest Simpson index value of 0.8840 and 0.8729 in their phase respectively. They are indicating higher species diversity and less dominance by a few species

compared to other treatment in their phase. Overall, the of alpha diversity indices for phase 1 and phase 2 suggest that DP1 and DP2 had relatively higher diversity and richness of species, also show more even distribution of species abundance with highest dominance. Conversely, CP1 from Phase 1 and AP2 from Phase 2 had the lowest species diversity and richness with relative lower evenness distribution and dominance from their respective phase.

**Table 4.8:** Summary of alpha diversity of *Tor tambroides* gut microbiota

| <b>Treatment</b> | <b>Shannon index</b> | <b>Chao1 index</b> | <b>Simpson index</b> |
|------------------|----------------------|--------------------|----------------------|
| AP1              | 2.7017               | 110.667            | 0.8859               |
| BP1              | 2.8394               | 96.0000            | 0.8981               |
| CP1              | 2.6704               | 88.3333            | 0.8840               |
| DP1              | 2.8360               | 112.3333           | 0.8958               |
| AP2              | 2.5347               | 83.0000            | 0.8729               |
| BP2              | 2.7846               | 103.0000           | 0.8958               |
| CP2              | 2.7810               | 107.0000           | 0.8932               |
| DP2              | 3.1068               | 110.5000           | 0.9235               |

\* AP1: Control Phase 1; BP1: 0.5% Lacto-sacc Phase 1; CP1: 1.0% Lacto-sacc Phase1; DP1: 1.25% Lacto-sacc Phase 1; AP2: Control Phase 2; BP2: 0.5% Lacto-sacc Phase 2; CP2: 1.0% Lacto-sacc Phase 2; DP2: 1.25% Lacto-sacc Phase 2

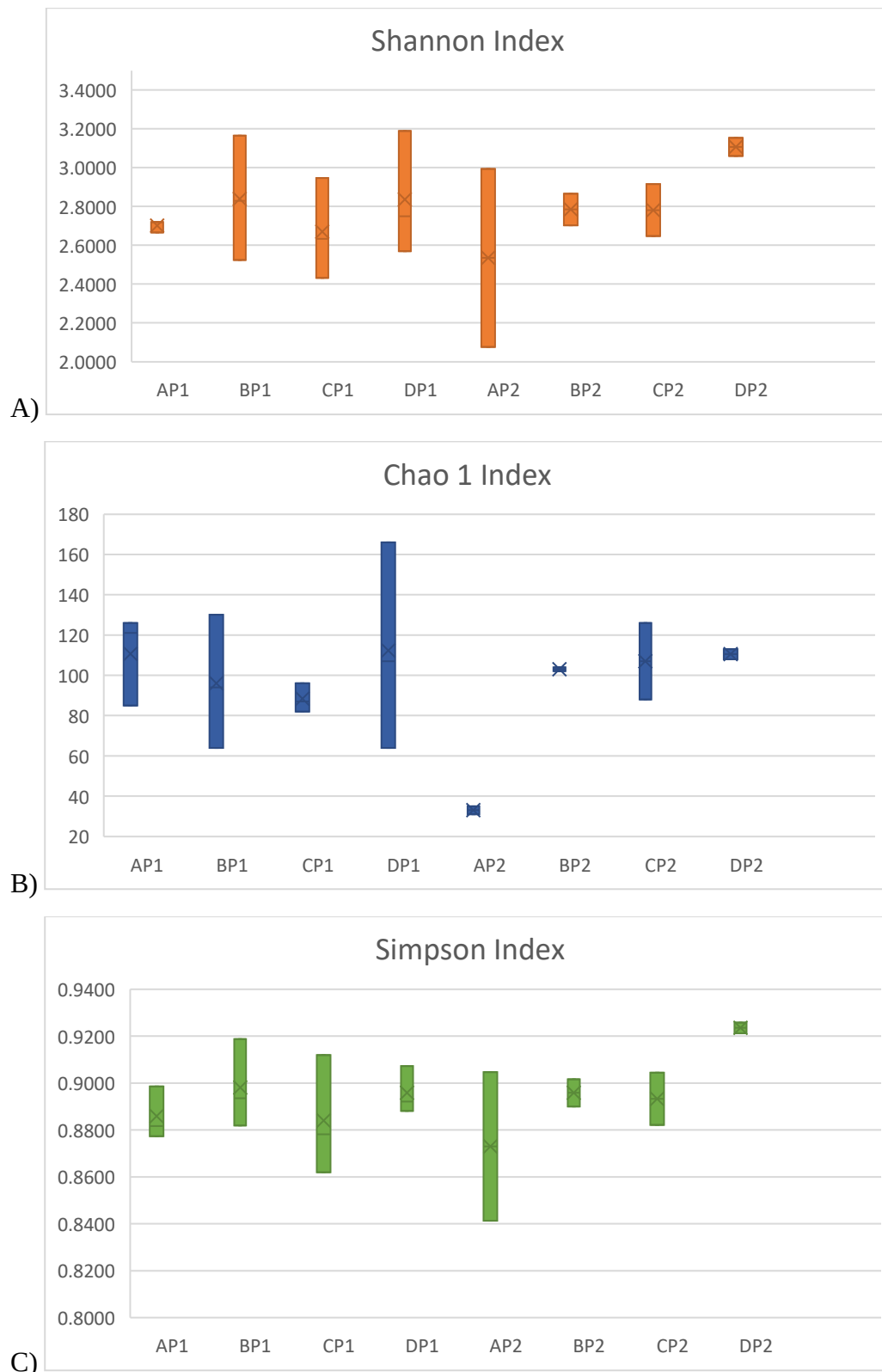


**Figure 4.4:** Alpha diversity indices boxplot of *Tor tambroides*. (A) Shannon index (B) Chao 1 index (C) Simpson index

\* AP1: Control Phase 1; BP1: 0.5% Lacto-sacc Phase 1; CP1: 1.0% Lacto-sacc Phase1; DP1: 1.25% Lacto-sacc Phase 1; AP2: Control Phase 2; BP2: 0.5% Lacto-sacc Phase 2; CP2: 1.0% Lacto-sacc Phase 2; DP2: 1.25% Lacto-sacc Phase 2

#### 4.2.4 Beta Diversity Analysis of *Tor tambroides*

The 24 samples of *T. tambroides* (triplicate for each treatment in both phases) were observed at Principal Coordinate1 (Axis 1) vs Principal Coordinate 2 (Axis 2) in Figure 4.5. From the figure, most of the sample were closely proximity. A1P1, A1P2 were at lower part of the axis while D1P2 and D2P2 were at the higher part of the axis.

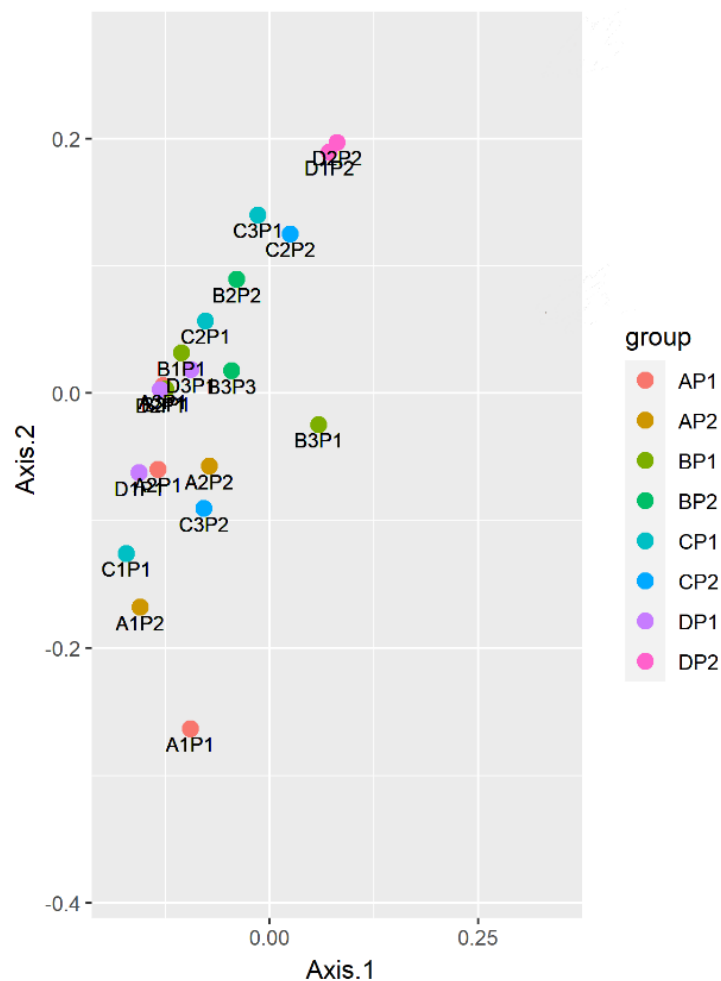


**Figure 4.5:** PCoA plot of beta diversity analysis based on weighted UniFrac distance metric.  
 \* AP1: Control Phase 1; BP1: 0.5% Lacto-sacc Phase 1; CP1: 1.0% Lacto-sacc Phase1; DP1: 1.25% Lacto-sacc Phase 1; AP2: Control Phase 2; BP2: 0.5% Lacto-sacc Phase 2; CP2: 1.0% Lacto-sacc Phase 2; DP2: 1.25% Lacto-sacc Phase 2

#### 4.3.5 Gut Taxonomy of *Tor tambroides*

The gut taxonomy data for 24 samples of *T. tambroides* were categorized into their respective groups, and the group means were calculated and summarised for each top 10 phyla (Table 4.9 and Table 4.10) and genera level (Table 4.11 and Table 4.12) of each phase. These summaries provided an overview of the average taxonomic composition at both phylum and genus level for each group of *T. tambroides* sample. As NGS of the 16S rRNA V3-V4 region has limitation in achieving accurate species level classification of bacteria, it often only reaching the genus level (Martinez-Porchas et al., 2016; Rizal et al., 2020). It is noticeable that gut microbiota phyla in Phase 1 had no significant difference, whereas certain phyla in Phase 2 exhibited significant difference between treatments.

The gut microbiota of *T. tambroides* exhibited a prevailing dominance of the phylum Fusobacteria, which constituted the most abundant taxonomic group. Among the top ten identified phyla in the gut of *T. tambroides*, Fusobacteria took precedence, followed by Proteobacteria, Bacteroidetes, Firmicutes, Spirochaetes, Actinobacteria, Planctomycetes, Verrucomicrobia, Chlamydiae, and Cyanobacteria. In Phase 1, the relative abundance of Fusobacteria ranged from 71.79% to 78.31%, while in Phase 2, it varied from 61.39% to 84.71%. Particularly in Phase 2, Treatment AP2 exhibited a significantly higher relative abundance of Fusobacteria compared to other treatments within Phase 2. On the other hand, Treatment DP2 displayed a significantly lower relative abundance of Fusobacteria compared to AP2, BP2, and CP2. The Proteobacteria emerged as the second most abundance phylum found in gut. Phase 1 relative abundance of Proteobacteria fall between 6.79% to 8.06%, while phase 2 ranging from 7.48% to 16.77%. The treatment group DP2 displayed the significantly highest abundance of Proteobacteria in Phase, surpassing the other treatment. The Bacteroidetes was highest abundance found in treatment D and lowest in treatment A in both phases. In phase 1, the abundance of Bacteroidetes in DP1 was (13.06%) and lowest in BP1

(7.17%), whereas in phase 2, DP2 (16.77%) significantly higher than other treatment. Firmicutes was found highest in BP1 and DP1, conversely, DP1 and AP1 had the lowest. It is noticeable that Spirochaetes was absent in treatment D of both phases. For phase 1, abundance of Actinobacteria were found highest in DP1 (1.94%) and lowest in CP1 (0.24%) whereas in phase 2 Actinobacteria abundance were highest in CP2 (2.01%) and lowest in Bp2 (0.95%). Other abundance of phyla was detected less than 1.0%. Cyanobacteria were absent in CP1, AP2 and DP2.

The top 10 genera found in *T. tambroides* gut microbiota were shown in table 4.11 and table 4.12. Phase 1 showed no significant difference while some of the phase 2 genus (*Cetobacterium*, *ZOR0006* and *Aeromonas*) showed significant difference between group. *Cetobacterium* dominated in all treatment group with relatively high abundance. The highest abundance of *Cetobacterium* in phase 1 was observed in CP1 (78.27%) and the abundance remains consistent across all treatment group with small variation. Moreover, highest abundance of *Cetobacterium* in phase 2 was AP2 (84.71%) and decrease slightly in the other groups, with the lowest in DP2 (61.39%). The second most abundance genus found in *T. tambroides* gut was *ZOR0006*, a type of Firmicutes bacterium. The abundance of *ZOR0006* varies across treatment group, highest abundance was found in both phase treatment B where BP1 (6.78%) and BP2 (5.45%), AP2 abundance was significant lower (2.09%). As for *Brevinema*, it was absent in both treatment D in both phases, Bp1 show visibly higher result (4.48%) and CP1 with relatively lower result with value 0.03%. Furthermore, abundance of *Brevinema* in CP2 (1.80%) was highest among the treatment while AP2 observed to be lowest with value of 0.08%. There was quite a lot of uncultured genera found within the gut of *T. tambroides*, the abundance of uncultured genus ranging from 5.96 to 11.67% and phase 2 range from 1.29 to 4.71%. Highest abundance of *Aeromonas* found in Bp1 (3.4%) in phase 1 while *Aeromonas* abundance in phase 2 increase from AP2 (1.81%) to DP2 (11.16%).

*Cloacibacterium* was only found in phase 2, highest abundance 2.12% in AP2 and lowest abundance in BP2 with value of 0.03%. The *Bacteroides* found in phase 1 were less than 1% and highest in phase AP1 (0.90%). Moreover, *Bacteroides* abundance highest in 6.20% and relatively lower in AP2 (0.52%). *Macellibacteroides* abundance was highest in CP1 (1.5033%) and BP2 (0.85%). Other genera such as *Mycobacterium*, *Dechloromonas*, *Bosea* were lower abundance (< 1.0%).

**Table 4.9:** The relative abundance for top 10 phyla detected in Phase 1 *Tor tambroides* gut

| Phylum          | AP1              | BP1              | CP1              | DP1              |
|-----------------|------------------|------------------|------------------|------------------|
| Fusobacteria    | 78.3133 ± 3.5158 | 71.7900 ± 7.0800 | 78.2700 ± 8.8803 | 73.9833 ± 3.1083 |
| Proteobacteria  | 7.0667 ± 3.1086  | 8.0633 ± 4.3664  | 6.7867 ± 0.8756  | 7.6000 ± 4.7219  |
| Bacteroidetes   | 7.9333 ± 10.4787 | 7.1733 ± 2.8856  | 9.4200 ± 4.6252  | 13.0633 ± 6.0600 |
| Firmicutes      | 3.5833 ± 3.2124  | 7.6167 ± 3.9730  | 4.7200 ± 4.6627  | 2.3967 ± 2.0467  |
| Spirochaetes    | 1.9767 ± 3.2012  | 4.4767 ± 7.7538  | 0.0267 ± 0.0462  | 0 ± 0            |
| Actinobacteria  | 1.3233 ± 0.9592  | 0.3733 ± 0.3347  | 0.2400 ± 0.1054  | 1.9400 ± 2.3601  |
| Planctomycetes  | 0.3867 ± 0.2367  | 0.2500 ± 0.4330  | 0.1467 ± 0.1185  | 0.4667 ± 0.5807  |
| Verrucomicrobia | 0.1033 ± 0.1274  | 0.1167 ± 0.0586  | 0.3267 ± 0.4535  | 0.4167 ± 0.4565  |
| Chlamydiae      | 0.0267 ± 0.0379  | 0.0200 ± 0.0346  | 0.0067 ± 0.0115  | 0.0467 ± 0.0808  |
| Cyanobacteria   | 0.0367 ± 0.0551  | 0.0067 ± 0.115   | 0 ± 0            | 0.0233 ± 0.0404  |

\*Means with standard error

**Table 4.10:** The relative abundance for top 10 phyla detected in Phase 2 *Tor tambroides* gut

| Phylum         | AP2                           | BP2                            | CP2                            | DP2                           |
|----------------|-------------------------------|--------------------------------|--------------------------------|-------------------------------|
| Fusobacteria   | 84.7067 ± 8.1674 <sup>b</sup> | 75.1767 ± 2.2405 <sup>ab</sup> | 75.2067 ± 7.3488 <sup>ab</sup> | 60.7233 ± 0.3550 <sup>a</sup> |
| Proteobacteria | 7.4800 ± 5.2100 <sup>a</sup>  | 10.0133 ± 1.5250 <sup>ab</sup> | 10.5333 ± 2.5550 <sup>ab</sup> | 16.7700 ± 1.9700 <sup>b</sup> |
| Bacteroidetes  | 3.0400 ± 1.8055 <sup>a</sup>  | 6.3033 ± 4.9524 <sup>ab</sup>  | 4.2567 ± 0.2517 <sup>a</sup>   | 13.1500 ± 1.5156 <sup>b</sup> |

**Table 4.10** continued

|                 |                                 |                                  |                                  |                                 |
|-----------------|---------------------------------|----------------------------------|----------------------------------|---------------------------------|
| Firmicutes      | 2.2400 ±<br>0.0300 <sup>a</sup> | 5.7433 ±<br>0.8450 <sup>ab</sup> | 5.9100 ±<br>3.6300 <sup>ab</sup> | 7.2467 ±<br>0.0451 <sup>b</sup> |
| Spirochaetes    | 0.0800 ±<br>0.0800              | 0.5300 ±<br>0.5300               | 1.7933 ±<br>1.7950               | 0 ± 0                           |
| Actinobacteria  | 1.4233 ±<br>1.4250              | 0.9500 ±<br>0.5400               | 2.0100 ±<br>0.3000               | 1.3667 ±<br>0.5752              |
| Planctomycetes  | 0.3867 ±<br>0.3850              | 0.1367 ±<br>0.0252               | 0.4167 ±<br>0.2950               | 0.2500 ±<br>0.2000              |
| Verrucomicrobia | 0.0667 ±<br>0.0651 <sup>a</sup> | 0.3500 ±<br>0.1200 <sup>b</sup>  | 0.0133 ±<br>0.0153 <sup>a</sup>  | 0.4467 ±<br>0.0058 <sup>b</sup> |
| Chlamydiae      | 0.2700 ±<br>0.2700              | 0.0867 ±<br>0.0651               | 0.1100 ±<br>0.1100               | 0.0100 ±<br>0.0100              |
| Cyanobacteria   | 0 ± 0                           | 0.1500 ±<br>0.1500               | 0.0400 ±<br>0.0400               | 0 ± 0                           |

\*Means with standard error within the same column followed by the same letter are non-significantly different ( $p < 0.05$ ) based on Tukey HSD.

**Table 4.11:** The relative abundance for top 10 genera detected in Phase 1 *Tor tambroides* gut

| Genus                     | AP1                 | BP1                 | CP1                | DP1                 |
|---------------------------|---------------------|---------------------|--------------------|---------------------|
| <i>Cetobacterium</i>      | 77.3133 ±<br>5.1683 | 71.7900 ±<br>7.0800 | 78.2700 ±<br>8.880 | 73.9833 ±<br>3.1083 |
| <i>ZOR0006</i>            | 3.2333 ±<br>3.4469  | 6.7800 ±<br>4.0661  | 3.6700 ±<br>3.7326 | 2.2033 ±<br>2.0410  |
| <i>Brevinema</i>          | 1.9767 ±<br>3.201   | 4.4767 ±<br>7.7538  | 0.0267 ±<br>0.0462 | 0 ± 0               |
| <i>Uncultured</i>         | 7.1600 ±<br>9.0179  | 5.9567 ±<br>1.8700  | 8.4700 ±<br>5.5209 | 11.6733 ±<br>5.5256 |
| <i>Aeromonas</i>          | 1.8967 ±<br>0.9577  | 3.4000 ±<br>1.0714  | 2.0033 ±<br>0.8394 | 1.7367 ±<br>0.9182  |
| <i>Bacteroides</i>        | 0.9033 ±<br>1.2989  | 0.7733 ±<br>0.4692  | 0.5667 ±<br>0.0551 | 0.8500 ±<br>0.4349  |
| <i>Macellibacteroides</i> | 0.4667 ±<br>0.2103  | 0.7733 ±<br>0.3844  | 1.5033 ±<br>1.0111 | 1.5000 ±<br>1.5862  |
| <i>Mycobacterium</i>      | 0.7433 ±<br>0.8846  | 0.0933 ±<br>0.1365  | 0.0467 ±<br>0.0808 | 0.8300 ±<br>1.0306  |
| <i>Dechloromonas</i>      | 0.4000 ±<br>0.3345  | 0.4233 ±<br>0.5689  | 0.2100 ±<br>0.2456 | 0.6900 ±<br>0.6986  |
| <i>Plesiomonas</i>        | 0.6867 ±<br>0.8171  | 0.2900 ±<br>0.0400  | 0.2367 ±<br>0.1234 | 0.1433 ±<br>0.1401  |

\*Means with standard error

**Table 4.12:** The relative abundance for top 10 genera detected in Phase 2 *Tor tambroides* gut

| Genus                     | AP2                           | BP2                            | CP2                            | DP2                           |
|---------------------------|-------------------------------|--------------------------------|--------------------------------|-------------------------------|
| <i>Cetobacterium</i>      | 84.7067 ± 9.3950 <sup>b</sup> | 75.7100 ± 2.5858 <sup>ab</sup> | 74.8733 ± 4.8106 <sup>ab</sup> | 61.3900 ± 1.2053 <sup>a</sup> |
| <i>ZOR0006</i>            | 2.0933 ± 0.0351 <sup>a</sup>  | 5.4467 ± 0.9243 <sup>ab</sup>  | 5.6533 ± 3.0418 <sup>ab</sup>  | 6.6400 ± 0.1600 <sup>b</sup>  |
| <i>Brevinema</i>          | 0.0800 ± 0.8000               | 0.3700 ± 0.5981                | 1.7967 ± 1.7950                | 0 ± 0                         |
| <i>Uncultured</i>         | 1.2933 ± 1.0500               | 3.2800 ± 2.6100                | 3.0933 ± 0.0058                | 4.7100 ± 0.6000               |
| <i>Aeromonas</i>          | 1.8100 ± 0.4813 <sup>a</sup>  | 5.1167 ± 1.7390 <sup>ab</sup>  | 7.0233 ± 1.0821 <sup>b</sup>   | 11.1633 ± 1.7051 <sup>c</sup> |
| <i>Cloacibacterium</i>    | 2.1200 ± 1.8377               | 0.3000 ± 0.3000                | 0.4733 ± 0.7853                | 1.8367 ± 0.7853               |
| <i>Bacteroides</i>        | 0.5233 ± 0.3750               | 2.4400 ± 2.0525                | 0.7767 ± 0.1710                | 6.1967 ± 3.8150               |
| <i>Macellibacteroides</i> | 0.0600 ± 0.0600               | 0.8467 ± 0.7976                | 0.5300 ± 0.2207                | 0.6733 ± 0.3530               |
| <i>Mycobacterium</i>      | 0.5167 ± 0.4881 <sup>a</sup>  | 0.5533 ± 0.3650 <sup>a</sup>   | 1.6800 ± 0.1800 <sup>b</sup>   | 0.2400 ± 0.1000 <sup>a</sup>  |
| <i>Dechloromonas</i>      | 1.6833 ± 1.6850               | 0.3233 ± 0.1450                | 0.2667 ± 0.3553                | 0.8567 ± 0.6550               |

\*Means with standard error within the same column followed by the same letter are non-significantly different (p<0.05) based on Tukey HSD.

## CHAPTER 5

### DISCUSSION

#### 5.1 Growth Performance of *Tor tambroides*

##### 5.1.1 Phase 1

Based on the results, there was no significant difference in growth performance and feed utilisation of *T. tambroides* across the various treatments. However, numerous studies demonstrated notable enhancements in growth by increasing the concentration of probiotics. For instance, Soltani et al. (2017) observed improved growth in common carp with a concentration of  $1.2 \times 10^6$  *L. plantarum*. Similarly, Abou Zied et al. (2003) found positive effects on Nile tilapia with 0.1% Lacto-sacc, while Abass et al. (2018) reported growth improvement in Nile tilapia with 7% of yeast. The inconsistent outcomes may arise from variation in species, life stage, dosage and experimental condition and suggest the necessity to evaluate several probiotics on target species (Hosseini et al., 2016).

In contrast, several studies have shown that higher probiotic concentrations may not consistently result in a beneficial impact when compared to a lower dose. For instance, study of Hosseini et al. (2016) shows that the concentration of *L. acidophilus* had no significant impact on the growth performance of goldfish (*Carassius auratus gibelio*). Nikoskelainen et al. (2001) illustrated that the administration of  $10^{12}$  CFU of *L. rhamnosus* did not provide greater protection against furunculosis in rainbow trout compared to the protection achieved with a lower dose of  $10^9$  CFU. Similarly, Pooramini et al. (2009) discovered that feeding rainbow trout with 10% yeast did not lead to improved development and feed utilization when compared to the benefits reported with 5% yeast.

Moreover, *S. cerevisiae* has beneficial alters intestinal absorption, leading to increased nutrient digestibility, better integrity of intestinal mucosa, and higher density of intestinal villi

(Abass et al. 2018). Additionally, the stimulatory effect of mannan-oligosaccharide (MOS) aids *Lactobacilli* in enhancing the food digestion and assimilation. The studies by Hoseinifar et al. (2015) prove that *L. acidophilus* improves growth performance, boosts immunological response, stress resistance and modulates intestinal microbiota toward beneficial bacteria. Conversely, Hosseini et al. (2016) discovered that the concentration of *L. acidophilus* has no significant effect on the growth performance of gold fish. Incorporating *L. acidophilus* into diet down regulate the ghrelin gene, which is a appetite related gene in intestinal of gold fish. This leads to a decrease in appetite and a reduction in body glucose levels.

All tested treatment group exhibited a higher survival rate. From observation, the mortality in Treatment B and Treatment D was attributed by overfeeding of bigger size fish. There are some smaller size fish that could not get enough feed but bigger size fish were fight for feed in the same tank, resulting bigger size fish consuming too much feed and died. In contrast, mortality of Treatment A was suspected due to disease infection, including pop eye and red spot on the fish's skin. Study conducted by Abass (2018) and Hoseinifar et al. (2015) have proved that inclusion of probiotics in the diet of fish improved their survival rate.

All groups treated with probiotics showed better digestive enzyme activity compared to the control group, suggesting probiotics contributed to enhance fish digestion. *Lactobacillus* sp. able to secrete a diverse variety of exogenous enzymes has improve the digestion process by increasing intestine enzymatic activity in striped catfish juvenile (Akter et al. 2019). They correlated between consumption of *L. acidophilus* and an increase in LAB, resulting alterations in activity of amylase and lipase. Additionally, some studies showed that *L. acidophilus* did enhance the digestive enzyme activities of fish (Wang, 2011; Akter et al., 2019; Mohammadian et al., 2019). Most probiotics have capability to secrete lipase enzymes that digest lipid resulting in triggering essential fatty acid production (Mohapatra et al., 2012). Askarian et al. (2012) reported that LAB able to synthesize digestive enzymes, including amylase, lipase, and

protease. In a study by Darafsh et al. (2020), Persian sturgeon exhibited higher amylase, protease and lipase activities when fed with *S. cerevisiae* compared to the control group.

Probiotics have the ability to impact the digestive process by increasing the population of beneficial microbes and microbial enzymes which promotes feed digestibility, absorption, and utilisation leading to an improvement in the growth performance and feed utilization of the fish (Allameh et al., 2017). The Treatment B exhibited the highest growth, protease, and lipase activity. The high protease activity found in Treatment B help digest protein and increase the protein efficiency rate, as shown in Table 4.1. The fish enzyme activity does not show a significant effect with probiotics dose possibly because the bacteria retained in the gut did not differ significantly among the treated fish (Dawood et al., 2019).

In the study by Abou Zied et al. (2003), a higher concentration (0.1%) of Lacto-sacc did give a better result than 0.05% Lacto-sacc for Nile tilapia. Dhanaraj et al. (2010) found the combination of *L. acidophilus* and *S. cerevisiae*, each at 0.25% did improve the growth performance and microbial load in the gut of Koi Carp, but a diet with 0.5% of brewer's yeast was the best. As a higher initial concentration was being used in our experiment, the ideal concentration may fall between 0.1%-0.5% of Lacto-sacc. Soltani et al. (2017) suggested higher concentration of probiotics does not correlate with the best result in terms of growth and other physiological status and overdosing it could lead to undesired side effects and unnecessary costs. A higher concentration of probiotics may cause bias in gut microflora, disrupt the natural diversity and balance of the gut microbial community (Bauer-Estrada et al., 2023; Alvanou et al., 2023). The *L. acidophilus* can produce bacteriocins and ferment lactose to lactic acid, reducing pH that may eliminate harmful bacteria and/or beneficial bacteria. Feeding Empurau with more than 0.5% Lacto-sacc diet does not bring additional effects in growth, feed utilisation and digestive enzyme activities.

### 5.1.2 Phase 2

From the growth performance results, *T. tambroides* fed with higher probiotics feed during post-probiotics feeding trial has greater growth performance than the lower one. The abundance of probiotics and its effectiveness declined over time. As our probiotics supplemented feeding trial takes 20 weeks, the Lacto-sacc has already adapted to the gut and formed population community inside as probiotics supplemented treatment group grow better than unsupplemented after withdrawal of probiotics in phase 2 feeding trial. The abundance of probiotics gradually declined over time after stop probiotics supplementation also shown in Nikoskelainen et al. (2003) and Tapia-Paniagua et al. (2014). This result in higher probiotics concentration treatment performed better in growth, feed utilization and digestive activity.

On the other hand, the impact of the probiotics when stopped being supplemented the fish with probiotics (Topic Popovic et al., 2016). Balcazar et al. (2007) studied that LAB is capable of antagonizing resident microbiota and will gradually reduce this effect once the probiotics supplemented feed is withdrawn. The abundance of probiotics will drop, especially if they do not colonize inside the gut of the host when probiotics supplement cessation. The probiotics may only briefly adhere to the gut lining or interact with the residents of the gut microbiota (Monteagudo-Mera et al., 2019). The effectiveness of the non-colonizing probiotics (*Saccharomyces cerevisiae*) may vary from person to person and depend on the strain and dosage used. Probiotics can easily be lost from the intestine within a few days following their application, even in indoor systems that are considered relatively stable (Xia et al., 2019; Li et al., 2022). Research by Nikoskelainen et al. (2003) found that the *Lactobacillus* sp. was not found after the supplement was stopped for the fish in 2 weeks and probiotics dropped drastically after a week in tank water, fish's intestines and skin and the adhesion properties of probiotics may influence the speed of the washout. Higher supplemented treatment groups showed no traces of supplemented *Lactobacillus*, but lower supplemented treatment found low

amount of *Lactobacillus*. Since the experiment of Nikoskelainen et al. (2003) both feeding trial and post-probiotics feeding trial only takes 2 weeks as short-term feeding trial, the probiotics might not persist in the gut after feeding trial ends and provided acute benefits. Aubin et al. (2005) also mentioned that a 20 days short probiotics treatment was insufficient to be effective. However, as our probiotics feeding trial and post-probiotics feeding trial only took 20 weeks and 8 weeks respectively, the bacteria might colonize in the gut, resulting better growth performance to higher probiotics treatment groups. However, longer term of post-probiotics feeding effect could not be foreseen.

For VSI, HSI and IPF, the result does not show significant difference as the fish were normally behave without any symptom of sickness and no mortality found in phase 2 experiment. As the fish nutrient baseline diet were the same, and the fish were provided with uniform and equally amount of feed, resulting body composition of the fish almost the same. Furthermore, Empurau had relatively lean and streamlined body which naturally result in lower VSI and IPF compared to other fish. The Lacto-sacc level might need a threshold level to trigger changes in body composition of Empurau.

Gut bacteria or probiotics may generate enzymes such as cellulase, amylase, lipase, protease, and chitinase (Bairagi et al., 2002), which can also modulate the synthesis of important nutrients for fish, such as amino acids, fatty acids, and vitamins (Nayak, 2010; Semova et al., 2012; Mohammadi et al., 2021). Microbes may improve fatty acid bioavailability by altering the synthesis of bile salt (Swann et al., 2011), by increasing absorption by lipolytic activity (Ringø et al., 1995; Osman et al., 2021), and by influence on physiological responses to absorption in the intestinal epithelium (Van Doan et al., 2020).

### 5.1.3 Overall Growth Performance

The growth performance between treatments were insignificant for both phases might be due to water change. Water changing affected the growth performance of the fish. The frequent water change in a closed system was necessary because the water quality was unfavourable for the fish and Empurau was sensitive to water quality. However, the water change had affected the biological environment of fish, fish may take time to adapt the water quality changed and nutrient flow were also disturbed. The fish may also be infected with pathogen and diseases as new water introduced. Pathogens can be present in untreated or contaminated water, when added to the tank, pathogen can pose a risk to the health of the fish (Li et al., 2023). The introduction of new water to tank will cause stress to the fish due to alteration in water chemistry, include changes in water temperature, pH, dissolved oxygen and other water parameter (Conte, 2004; Wu et al., 2015).

The growth performance of phase 2 was better than phase 1 might be due to the quality of the feed. In Phase 1, water exchange was carried out daily, due to the handmade probiotics diet was more loose than commercial, more easily disperse in the water and required to break into small pieces before feeding. The easily disperse fish feed causes the water quality to become unfavourable to Empurau. On the other hands, phase 2 water was changed twice every week. Also, Phase 2 fish densities was lowered to 30 fishes/ tank compared to phase 1 was 50 fishes/ tank.

The accuracy of phase 1 FCR might not as accurate as Phase 2 due to Phase 1 fish feed easily disperse to water and hard to collect back, may loss part of the feed when collect back causing higher FCR (Irza et al., 2023). There also difference between the handmade probiotics fish feed and commercial feed including the nutritional composition, size, and gross energy. Furthermore, smaller fish also tend to have higher FCR, similar to the study of Sweilum et al.

(2005). The higher FCR in smaller size fish may be due to higher metabolism rates and lower energy efficiency.

As conclude for growth performance, even among the treatment did not show significant, there still trend of preference result can be observed. For continuously supplement of the probiotics, 0.5% Lacto-sacc was recommended to the *T. tambroides* diet. On the other hand, for more cost-effective way, 1.25% Lacto-sacc diet was recommended to supply to *T. tambroides* occasionally or during fingerling period.

## **5.2 Gut Microbiota of *Tor tambroides***

### **5.2.1 Alpha and Beta Diversity of Gut Microbiota of *Tor tambroides***

In Alpha Diversity that used to measure of biodiversity that quantifies the diversity of species within a sample or habitat. BP1 and DP2 has the highest Shannon Index that indicating higher diversity and more even distribution of species abundance compared to other treatment in their respective phases. Treatment D of both phases showed highest Chao 1 indexes value (112.3333 and 110.5000 respectively) representing they had highest species richness within their phases. For Simpson index, CP1 and AP2 had the lowest Simpson Index value indicating they had higher diversity, and all species were more equally abundant.

Treatment BP1 has the highest Shannon and Simpson index but with lower Chao1 index. This result can be interpreted as treatment has mix of both abundant and rare species with fewer estimated total species and dominance of a few species that cause uneven distribution (Kim et al., 2017). Treatment DP2 can be accounts highest in Shannon Index, Chao1 Index and Simpson Index. The result means that treatment DP2 might have a highly diverse community with a rich variety of species and be influenced by one or a few dominant species at the same time. The species community might have a mix of both rare and abundant species, contributing

to the high diversity indices. Some studies show improvement in Alpha diversity with supplement of probiotics (Ramos et al., 2013; Stevick et al., 2019; Li et al., 2022)

Beta Diversity analysis was showed by the Principal Coordinate Analysis (PCoA) and visualized patterns in dissimilarity. Most of the sample were closed proximity and clustered together. A1P1, A1P2, C1P1 were at the lower part of the axis while B3P1 was more at the right of plot and D1P2, D2P2, C3P1, C2P2 were at higher part of the plot. It can be seemed that treatment that belongs to same treatment were more likely to clump together.

### **5.2.2 Phylum and Genus Distribution of Gut Microbiota in *Tor tambroides***

The most abundant phyla that had been found in gut microbiota of Empurau of both phases were Fusobacteria, Proteobacteria, Bacteroidetes and Firmicutes, similar with studies of Tan et al. (2019) and Lau et al. (2021) on Empurau. However, in study of Lau et al. (2021) with microbiome analysis of gut bacterial communities of healthy and diseased *T. tambroides* studies, Proteobacteria was the most abundant among them all whereas studies of Tan et al. (2019) on wild and captive *T. tambroides*, Fusobacteria was highest abundant (26.8%) but almost similar with abundance of Firmicutes (25.8%) and Proteobacteria (25.2%). The total relative abundance of Fusobacteria, Proteobacteria, Bacteroidetes and Firmicutes were more than 95% of the total gut microbiota of the *T. tambroides*.

The Fusobacteria were dominant in the gut microbiota of *T. tambroides* of every treatment of both phases with a range of 60% to 84% of the total gut microbiota. Fusobacteria can be found as most abundance in gut and this finding agreed with some studies with different fish species such as *Atractosteus tropicus* (tropical Gar), *Petromyzone marinus* (Sea Lamprey), *Symphysodon haraldi* (discus fish), *Cyprinus carpio* (carp), *Panaque* sp. (catfish) (van Kessel et al., 2011; Di Maiuta et al., 2013; Mendez-Perez et al., 2020; Mathai et al., 2021; Zhang et al., 2021). Fusobacteria, particularly *Cetobacterium* were found prevalent in gut of microbiota

of freshwater fish. The presence of this microbial contributed to host nutrition, immunity, and protection. They produced B12 vitamins and obligate gut associate and promote overall health of the fish. They characterized as gram-negative obligate with fermentative capabilities. Fusobacteria capable to fermentation amino acids such as aspartate, cysteine, glutamate, histidine, lysine, methionine, serine and threonine, produce butyrate and acetate as fermentation product (Loesche & Gibbons, 1968; Duncan et al., 2002) They able to produce butyric acid, that final product of carbohydrates fermentation, having inhibitory effect on potential pathogens in freshwater fish and butyrate sodium is marked as food additive further promoting the health and growth of the fish (Yoshida et al., 2022). There was significant difference between the phase 2, treatment D were significantly different with treatment A. This may be due to sudden stop supplement of Lacto-sacc causing a different in gut microbiota community. This can be seemed that higher concentration of Lacto-sacc supplied, the lower Fusobacteria were. The sudden withdrawal of Lacto-sac caused biased and may change the gut microbiota to revert to the composition before probiotics supplementation. Topic Popovic et al. (2016) study on the effect of *L. rhamnosus* on rainbow trout found the fish microflora reverted to control composition after three weeks of probiotics cessation.

*Cetobacterium*, as a genus of the phylum Fusobacteria, it is important commensal bacterium and account more than 70% of the gut microbial fish community in many fish species (van Kessel et al., 2011; Ray et al., 2017; Xie et al., 2022). *Cetobacterium* also dominate the gut of *T. tambroides* in treatment groups at both probiotics feeding and post-probiotics feeding phase. In studies of Qi et al. (2023), it proved that *Cetobacterium* able to act as a sensor of health especially in pathogenic bacteria infected fish. It induces alterations in the structure and function of gut microbiome, improvements in microbials interactions and enhance the stability of the gut microbiota network by synthesize vitamin B12. The vitamin B12 does not direct influence on pathogen resistance but upregulated the tight junction protein of intestine as

protective barrier against pathogen infection. Also, studies of Liu et al. (2020), Parshukov et al. (2019) and Ofek et al. (2022) shows that *Cetobacterium* relative abundance were lower when infected or diseased compared to healthy fish. The decrease in the abundance of Fusobacteria including *Cetobacterium* might because of *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and Fusobacteria that were play crucial role in carbohydrates fermentation. They both competed for carbohydrates and the introduction of Lacto-sacc in diet might impact the growth of *Cetobacterium*, resulting its abundance drop. Also, high-fat diet will accelerate the establishment of characteristic bacteria in fish such as Fusobacteria (Xia et al., 2022).

Ofek et al. (2022) observed Proteobacteria was most dominant (53.5%) in sick fish compared to second dominant phylum (around 19%) in healthy fish and agreed with study of Li et al. (2016) with largemouth bronze gudgeon. Study of Siddik et al. (2022) found out that the abundance of Proteobacteria and Firmicutes increased when fed with *Saccharomyces cerevisiae* and *Lactobacillus casei* on juvenile barramundi. On the other hand, Ofek et al. (2022) study found that Proteobacteria was increased at the expense of Fusobacteria in the intestine of diseased fish. Among top 10 genera, there are 3 genera belongs to Proteobacteria which were *Aeromonas*, *Dechloromonas* and *Plesiomonas*. *Aeromonas* is bacteria that could bring both beneficial and harmful effect. Some *Aeromonas* species can help break down organic matter and carry out denitrification (Fialkowska et al., 2019). On the other hand, *Aeromonas* was an opportunistic pathogen in diseased fish (Ofek et al., 2022), specifically *Aeromonas hydrophila* and *Aeromonas salmonicida* was found cause infectious disease. However, the *Aeromonas* abundance were higher approximately according to the trend of growth performance of each treatment in both phases and significantly found between Phase 2 treatment which control, treatment C and treatment D, it is suggested that *Aeromonas* in this study were beneficial *Aeromonas* rather than pathogenic. Nonetheless, this study only gathered phylogenetic data up to genus level, as the V3-V4 region exhibited limitation in confidently identifying bacteria at

the species level (Siddik et al., 2022). *Dechloromonas* might be beneficial to host especially *Dechloromonas aromatica* able to perform diverse function in environmental microbiology such as bioremediation, benzene reduction, denitrification, chlorite and bromite decomposition and detoxification (Deng et al., 2020; Geeraerts et al., 2022; Parida et al., 2022).

Bacteroidetes were found third dominant phylum in gut of Empurau of both phases. Study of Duan et al. (2021), Bacteroidetes can support the metabolism of carbohydrate. Also, Duan et al. (2021) found out that there was a decrease in the abundance of Bacteroidetes (especially the Bacteroidia class) observed in the stomach, foregut and hindgut but increased in the midgut and faeces as the fish underwent growth. This variation may potentially influence the utilization of carbohydrates by intestinal bacteria in different sections of the gastrointestinal tract. There were 3 genera among top 10 genera was found belongs to phylum Bacteroidetes which were *Bacteroides*, *Macellibacteroides* and *Cloacibacterium* that only present in probiotics feeding phases. DP2 Bacteroidetes abundance was found significantly higher than AP2 and look into the genus level, *Bacteroides* was found higher in treatment in D. *Bacteroides* able to degrade organic aromatic compound and play a vital role in the carbon cycle (Wang et al., 2023). The *Bacteroides* was also believed as cellulose degraders that played important role in digestion, physiology, metabolism, and health of Rohu fish (Tyagi & Singh, 2017).

Firmicutes, characterized as advantageous intestinal bacteria, it recognized for positive impact on the growth performance, immunity, digestion, and disease resistance in aquatic animals (Xu et al., 2021). Firmicutes also able facilitate energy harvesting. The *ZOR0006* was a genus of phylum Firmicutes and were observed as second dominant genus in the study. The *Cetobacterium* and *ZOR0006* were the two most dominant gut associated bacteria on the medaka fish in studies of Duperron et al. (2019), Foucault et al. (2022) and Gallet et al. (2023). In the study conducted by Ofek et al. (2022), the abundance of *ZOR0006* was greater (9.0%) in intestine of healthy tilapia compared diseased tilapia, constituting only one-third of the

abundance observed in healthy fish intestine. Furthermore, majority of the functions found in *ZOR0006* were similarly encoded by other bacteria, except for the distinctive lactate-pyruvate interconversion module that otherwise only found in *Cetobacterium*. The lactate homeostasis was crucial in the gut and able to inhibit the growth of the pathogenic bacteria. The abundance of *ZOR0006* also followed approximately to the trend of the growth performance of the fish resulting the beneficial effects of the *ZOR0006* was bring to the host (Xu et al., 2021).

The phylum Spirochaetes was the fifth dominant phylum within the gut microbiota of the *T. tambroides*. Spirochaetes were absent in treatment D of both phases. Spirochaetes could be found one of the dominants in *C. semilaevis* when infected with *Shewanella algae* (Han et al., 2018). *Brevinema* was present at top 10 genera, member of the phylum Spirochaetes. *Brevinema* was suggested as opportunistic pathogen as it increase its abundance in gut microbiota in susceptible rainbow trout (Brown et al., 2019), in studies of Castaneda-Monslave et al. (2019) that dominated on white Cachama, Shiina et al. (2006) on Grass Puffer and Wang et al. (2020) both healthy and diseased seahorses. *Brevinema* was said was a pathogenic bacterium in aquaculture and was originally an infectious pathogen isolated from short-tailed shrews and white-footed mice. However, this bacterium was conditional pathogen that occurrence of intestinal diseases when found high abundance in intestine (Kong et al., 2023).

It is noticeable that the more preferred concentration in each phase, BP1 and DP2 has lower *Cetobacterium* and higher other dominant bacteria. As shown in alpha diversity, they are more even distributed and more diverse than other treatment. In accordance with the diversity resistance hypothesis, a microbial community that exhibits greater diversity is more likely to include a species possessing antagonistic traits toward an invader or pathogen (Xiong et al., 2019). Also, study of Yang et al. (2023) found out that diseased yellow catfish has significant lower gut microbial richness and diversity than healthy individuals.

To conclude, although the study results were not statistically different in most of the bacteria between treatment, but comparing to the control group, generally probiotics treated group were decreased in abundance of Fusobacteria and increased in Proteobacteria, Bacteroidetes, Firmicutes. The elevation of the other bacteria levels may be attributed to the decrease of the Fusobacteria abundance which might be due to nutrient competition such as carbohydrate as they have overlapping nutrient utilization ability with probiotics (Tramontano et al., 2018; Horrocks et al., 2023). The introduction of Lacto-sacc bring beneficial effect to the gut, resulting increase in these beneficial bacteria and bring more even distributed gut microbiota (Nayak, 2010).

## CHAPTER 6

### CONCLUSION AND RECOMMENDATIONS

#### 6.1 Conclusion

To conclude this study, the probiotics did give positive effect to the growth performance and feed utilization and digestive enzyme activities of *T. tambroides*. Treatment B (0.5% Lacto-sacc) performed the best growth performance in specific growth rate and feed utilization in feed conversion ratio and protein efficiency ratio in probiotic feeding trial. It also showed the highest relative protein digestibility, lipase, and protease activity. For post-probiotics feeding trial, treatment D (1.25% Lacto-sacc) had the best growth performance and feed utilization with highest amylase and lipase activity and protein digestibility.

The gut microbiota of *T. tambroides* was dominated by phyla Fusobacteria, Proteobacteria, Bacteroides and Firmicutes and genera of *Cetobacterium*, *ZOR0006*, *Brevinema* and *Aeromonas*. Gut microbiota of Empurau with probiotics fed treatment also showed a more even disturbed and diverse that less dominance by a species.

#### 6.2 Recommendations

One of the recommendations of this study was a lower starter Lacto-sacc concentration can be investigated for more cost effectively. Post-probiotic feeding duration can be lengthened, the effect of Lacto-sacc after longer duration can be investigated. Furthermore, the microbiota identification method can be improved by involving more variable region of 16S rRNA gene to explore more gut microbial community. The relative abundance of *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* should be investigated to observe if they colonize in gut. The studies can also be replaced with other fast grower fish such as Tilapia, Pangasius and Grouper for more obvious result.

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## APPENDIX

### Appendix A: Average water quality throughout the study.

| Parameters              | A                | B                | C                | D                |
|-------------------------|------------------|------------------|------------------|------------------|
| pH                      | 6.3999 ± 0.0155  | 6.4346 ± 0.0555  | 6.4411 ± 0.0272  | 6.4407 ± 0.0402  |
| Dissolved Oxygen (mg/L) | 3.8221 ± 0.0432  | 3.8278 ± 0.0356  | 3.8326 ± 0.1044  | 3.9211 ± 0.1367  |
| Temperature (°C)        | 26.3471 ± 0.1213 | 26.5987 ± 0.0637 | 26.5125 ± 0.0265 | 26.5541 ± 0.0323 |
| Nitrate (mg/L)          | 0.5865 ± 0.0031  | 0.6884 ± 0.0643  | 0.6642 ± 0.0918  | 0.6980 ± 0.0805  |
| Ammonia (mg/L)          | 0.3820 ± 0.0441  | 0.4607 ± 0.0683  | 0.3842 ± 0.0185  | 0.3635 ± 0.0806  |

\*Average water quality with standard error throughout the study.

### Appendix B: The validation of *Lactobacillus acidophilus* in the probiotics diet

| Validation peroid               | 0.5% Lacto-sacc                   | 1.0% Lacto-sacc                   | 1.25% Lacto-sacc                  |
|---------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Initial                         | 4.28 ± 0.33 X 10 <sup>7</sup> CFU | 4.31 ± 0.09 X 10 <sup>8</sup> CFU | 5.94 ± 0.20 X 10 <sup>8</sup> CFU |
| End of probiotics feeding trial | 3.48 ± 0.13 X 10 <sup>7</sup> CFU | 3.62 ± 0.09 X 10 <sup>8</sup> CFU | 4.81 ± 0.10 X 10 <sup>8</sup> CFU |

\*The average validation of *Lactobacillus acidophilus* with standard error.

### Appendix C: Nitrate Nutrient Analysis

According to Hach manual book, program 351, the method of nitrate nutrient analysis was showed below, 15 ml of water sample was poured into measuring cylinder, NitriVer-3 reagent powder pillow to measuring cylinder and shaken for 3 minutes. Water sample was allowed to rest for 2 minutes and 10 ml of water sample was poured into a spectrophotometer cuvette. NitriVer-6 reagent powder pillow was added and inverted to dissolve for 30 second. The water sample was rested for 15 minutes before reading. Blank cell was prepared by 10 ml water sample poured into spectrophotometer cuvette. The blank cell was cleaned with kimwipe (Kimtech) and inserted into cell holder then the 'Zero' button was pressed. The sample cell was cleaned and replace the blank cell then "Read" button was pressed. The displayed value was showed in mg/L and the reading was recorded.

#### **Appendix D: Ammonia Nutrient Analysis**

Based on Hach manual book, the ammonia analysis program 380 was chosen on the spectrophotometer. The ammonia water analysis was carried out by Nessler method. 25 ml of water sample and deionized water were added to each measuring cylinder for sample cell and blank cell respectively. Three drops of Mineral Stabilizer were added to both measuring cylinder and inverted several times until dissolved. Both measuring cylinders were then added 3 drop of Polyvinyl Alcohol dispersing agent and inverted until dissolved. 1 ml of Nessler Reagent was added to each measuring cylinder and inverted to mix into solution. The mixture was rested to react for 1 minutes. 10 ml of water from each measuring cylinder were poured into spectrophotometer cuvette respectively. The blank cell was cleaned with kimwipe and inserted into cell holder then the 'Zero' button was pressed. The sample cell was cleaned and replace the blank cell then "Read" button was pressed. The displayed value was showed in mg/L and the reading was recorded.



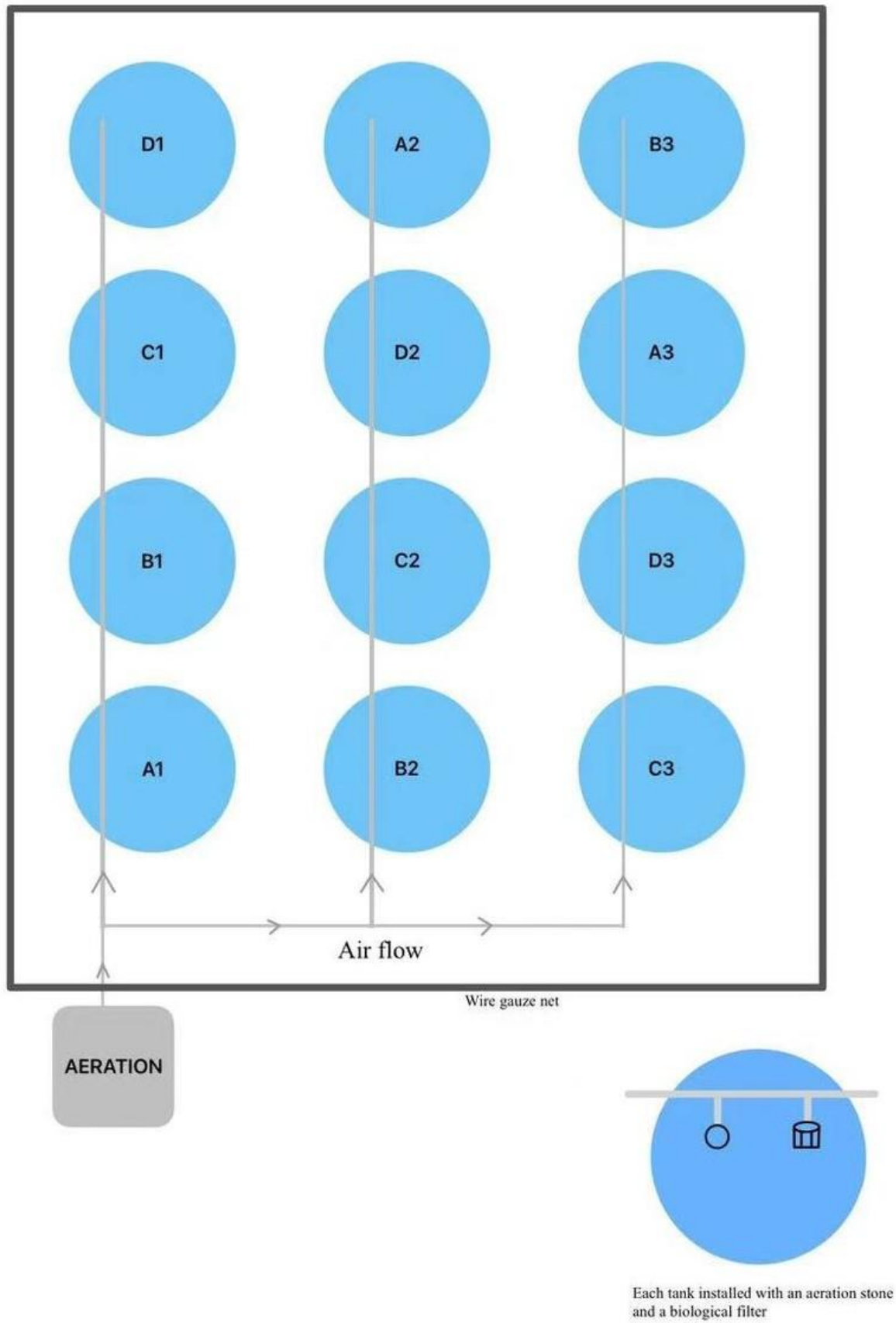
Making of Phase 1 (probiotic feeding trial) feed



The acclimation of Empurau into 3 rectangular tanks.



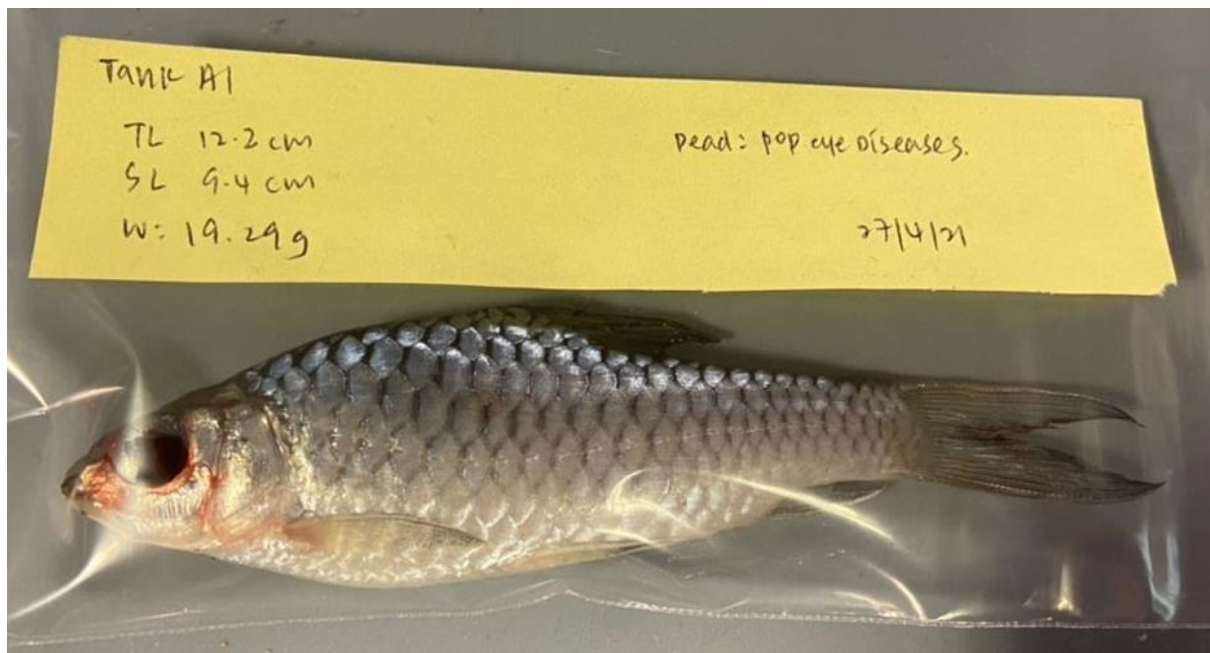
Experimental Setup



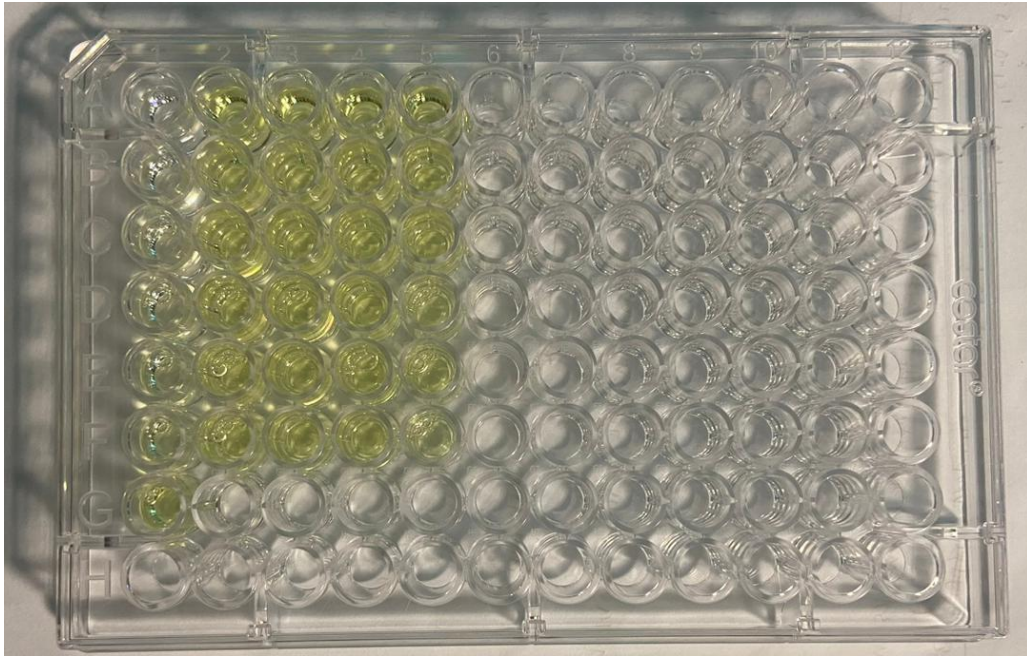
Experimental setup plan



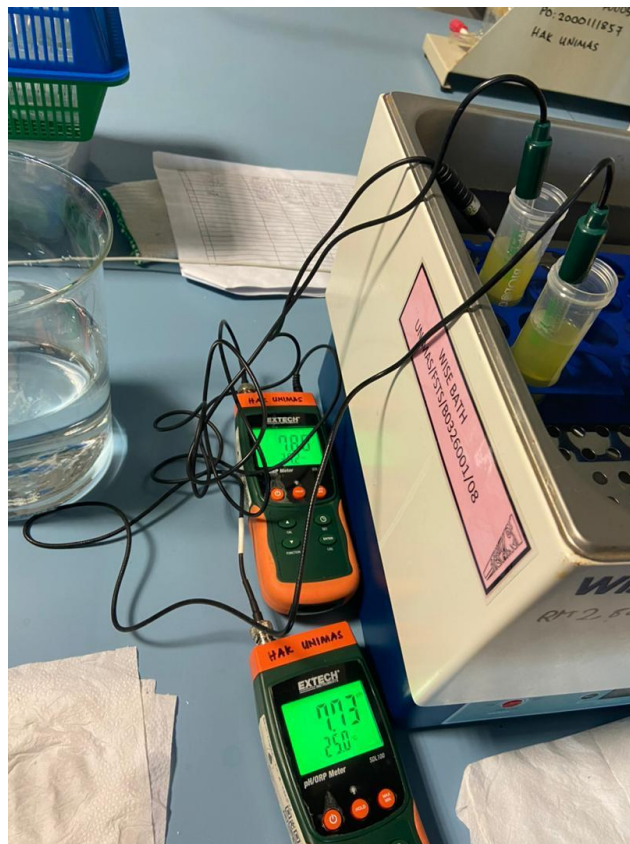
Fish measurement



Fish died with symptoms of pop-eye from Control Tank 1, Phase 1



96 well plate of amylase activity assay



Relative protein digestibility- pH drop

Phase 1

| Tank | Initial Weight, g | Final Weight, g | Weight Gain, g | FCR    | PER      | RG     | SGR, %/day | SR, % | VSI    | HSI    | IPF    |
|------|-------------------|-----------------|----------------|--------|----------|--------|------------|-------|--------|--------|--------|
| A1   | 6.5226            | 18.4806         | 11.9580        | 6.7250 | 183.3318 | 0.3556 | 0.7439     | 100   | 8.9075 | 0.4808 | 1.3527 |
| A2   | 6.3632            | 16.8498         | 10.4866        | 7.6762 | 164.8007 | 0.3115 | 0.6956     | 98    | 7.2563 | 0.2843 | 0.3848 |
| A3   | 6.6856            | 16.7629         | 10.0773        | 7.6381 | 150.7314 | 0.3131 | 0.6566     | 98    | 8.6894 | 0.5485 | 0.7649 |
| B1   | 6.2810            | 19.8833         | 13.6023        | 5.8628 | 216.5626 | 0.4079 | 0.8231     | 100   | 9.5845 | 0.6484 | 0.8096 |
| B2   | 6.3621            | 16.9787         | 10.6166        | 6.5250 | 166.8726 | 0.3665 | 0.7011     | 100   | 9.5845 | 0.6484 | 0.8096 |
| B3   | 6.7892            | 17.5491         | 10.7599        | 6.8090 | 158.4855 | 0.3512 | 0.6783     | 98    | 7.2690 | 0.5115 | 0.3469 |
| C1   | 6.5534            | 17.1375         | 10.5841        | 6.7365 | 161.5055 | 0.3550 | 0.6866     | 100   | 9.7842 | 0.7534 | 0.9879 |
| C2   | 6.3620            | 20.5390         | 14.1770        | 5.6889 | 222.8387 | 0.4203 | 0.8371     | 100   | 8.3056 | 0.4305 | 2.1326 |
| C3   | 6.7264            | 16.1378         | 9.4114         | 7.0410 | 139.9173 | 0.3396 | 0.6251     | 100   | 8.3491 | 0.3967 | 1.6014 |
| D1   | 6.5942            | 20.0276         | 13.4334        | 5.9694 | 203.7154 | 0.4006 | 0.7935     | 100   | 9.6771 | 0.7440 | 2.0130 |
| D2   | 6.5232            | 15.2637         | 8.7405         | 8.3728 | 133.9910 | 0.2856 | 0.6072     | 96    | 8.6674 | 0.4832 | 1.8855 |
| D3   | 6.5830            | 18.0920         | 11.5090        | 6.6872 | 174.8291 | 0.3576 | 0.7221     | 100   | 8.6021 | 0.5358 | 1.1908 |

| Tank | RPD, %  | Amylase, nmole/min/mL | Lipase, nmole/min/mL | Protease, ug/mL |
|------|---------|-----------------------|----------------------|-----------------|
| A1   | 46.3229 | 1909.3276             | 14.4395              | 2586.3995       |
| A2   | 30.2629 | 2254.3018             | 16.9305              | 2479.3962       |
| A3   | 49.5959 | 2015.2401             | 12.1648              | 3034.9553       |
| B1   | 73.7137 | 5068.3823             | 47.7355              | 1633.7305       |
| B2   | 34.8416 | 1834.8243             | 62.7456              | 4464.8937       |
| B3   | 59.8160 | 1561.6955             | 78.2338              | 3422.9852       |
| C1   | 45.8865 | 954.1451              | 27.8694              | 2979.1122       |
| C2   | 36.1177 | 3149.7927             | 22.7972              | 2130.7732       |
| C3   | 75.3219 | 6669.8684             | 22.8277              | 3798.5396       |
| D1   | 62.8889 | 4453.9773             | 20.0449              | 3353.9735       |
| D2   | 56.0845 | 4018.2185             | 25.8910              | 3107.8285       |
| D3   | 38.9888 | 3188.5189             | 17.5762              | 2326.8179       |

Phase 2

| Tank | Initial Weight, g | Final Weight, g | Weight Gain, g | FCR    | PER    | RG      | SGR, %/day | SR, % | VSI    | HSI    | IPF    |
|------|-------------------|-----------------|----------------|--------|--------|---------|------------|-------|--------|--------|--------|
| A1   | 20.7627           | 31.5220         | 10.7593        | 3.1007 | 0.7736 | 51.8203 | 0.7456     | 100   | 8.5562 | 0.7708 | 1.1947 |
| A2   | 17.5963           | 29.6220         | 12.0257        | 3.2125 | 0.7467 | 68.3422 | 0.9301     | 100   | 8.1546 | 0.6964 | 0.9265 |
| A3   | 18.1040           | 27.4023         | 9.2983         | 2.9363 | 0.8169 | 51.3605 | 0.7402     | 100   | 8.7192 | 0.6773 | 2.2601 |
| B1   | 21.3867           | 35.4187         | 14.0320        | 2.5546 | 0.9389 | 65.6109 | 0.9008     | 100   | 7.0070 | 0.6243 | 1.0856 |
| B2   | 18.0183           | 27.9167         | 9.8984         | 3.0320 | 0.7911 | 54.9353 | 0.7819     | 100   | 8.2886 | 0.6430 | 1.0978 |
| B3   | 20.4010           | 28.7480         | 8.3470         | 3.4979 | 0.6857 | 40.9147 | 0.6125     | 100   | 8.4990 | 0.6859 | 1.7467 |
| C1   | 17.7937           | 28.4240         | 10.6303        | 3.2184 | 0.7453 | 59.7419 | 0.8364     | 100   | 8.0680 | 0.6690 | 1.6778 |
| C2   | 23.9610           | 35.2100         | 11.2490        | 3.1811 | 0.7540 | 46.9471 | 0.6873     | 100   | 7.8889 | 0.6362 | 1.3415 |
| C3   | 15.3290           | 26.1247         | 10.7957        | 2.8541 | 0.8404 | 70.4266 | 0.9520     | 100   | 6.8511 | 0.5845 | 1.6776 |
| D1   | 20.6797           | 35.3020         | 14.6223        | 2.3170 | 1.0352 | 70.7085 | 0.9550     | 100   | 8.9296 | 0.8841 | 1.7502 |
| D2   | 15.1807           | 27.4877         | 12.3070        | 2.7450 | 0.8738 | 81.0698 | 1.0602     | 100   | 8.1207 | 0.8067 | 1.3895 |
| D3   | 19.6643           | 26.2417         | 6.5774         | 4.6301 | 0.5181 | 33.4484 | 0.5153     | 100   | 7.6584 | 0.5333 | 1.7190 |

| Tank | RPD, %  | Amylase, nmole/min/mL | Lipase, nmole/min/mL | Protease, ug/mL |
|------|---------|-----------------------|----------------------|-----------------|
| A1   | 49.2313 | 8434.4513             | 8.6760               | 3937.9492       |
| A2   | 21.7085 | 9547.3727             | 9.2887               | 3470.6102       |
| A3   | 42.1724 | 9276.0049             | 8.7498               | 3863.3928       |
| B1   | 69.0559 | 8927.7484             | 10.2040              | 4867.0824       |
| B2   | 33.9110 | 10382.5663            | 8.2701               | 3858.4422       |
| B3   | 45.4579 | 9590.1541             | 9.4585               | 3066.7383       |
| C1   | 43.1696 | 7929.5519             | 8.8679               | 5337.3918       |
| C2   | 40.7667 | 9094.8290             | 8.5063               | 2091.3662       |
| C3   | 32.8819 | 8558.5879             | 8.8237               | 5832.1572       |
| D1   | 55.5865 | 10085.5839            | 12.0641              | 4196.0748       |
| D2   | 26.3900 | 9833.1447             | 9.3404               | 3656.4567       |
| D3   | 40.6339 | 9197.3716             | 8.1003               | 3868.2444       |