Borneo Journal of Resource Science and Technology (2024), 14(2): 68-80
DOI: https:doi.org/10.33736/bjrst.7477.2024

Growth Performance, Digestive Enzymes Activities and Gut Microbiota of Malaysian Mahseer, *Tor tambroides* Fingerlings Affected by Various Probiotics Concentrations

SING YING CHUA[1*], MOHAMMAD BODRUL MUNIR[2], FATHURRAHMAN LANANAN[3], ROSLIANAH ASDARI[1*]

[1] Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia; [2] Faculty of Agriculture, Universiti Islam Sultan Sharif Ali, Senaut Campus, Jalan Tutong, Tutong TB1741, Negeri Brunei Darussalam; [3] East Coast Environmental Research Institute, Universiti Sultan Zainal Abidin, 21300 Kuala Nerus, Terengganu, Malaysia
*Corresponding author: singying0397@hotmail.com, aroslianah@unimas.my
Received: 25 July 2024 Accepted: 8 November 2024 Published: 31 December 2024

ABSTRACT

The most valued freshwater fish in Malaysia is the Malaysian mahseer, *Tor tambroides*, also known as Empurau. Due to the extended growing period, innovative feeding management is required to maintain fish health. This study looked at the effect of Lacto-sacc, a feed additive and antibiotic replacement made up of *Lactobacillus acidophilus*, *Saccharomyces cerevisiae* and *Enterococcus faecium*, on *T. tambroides* fingerlings' growth performance, digestive enzyme activities and gut microbiota. A total of 600 fingerlings, each weighing 6.53 g ± 0.17 g, were allocated into twelve 650 L tanks, with 50 fingerlings per tank. Over a period of 20 weeks, the fish were fed four different diets: 0% Lacto-sacc as control (A), 0.5% Lacto-sacc (B), 1.0% Lacto-sacc (C), and 1.25% Lacto-sacc (D), with each diet replicated in three tanks. Although statistic revealed no significant differences in growth performance among treatment group ($p > 0.05$), but it is noteworthy that fingerlings of *T. tambroides* were fed a diet containing 0.5% Lacto-sacc exhibited a trend toward improved growth performance with value higher SGR, along with elevated lipase and protease activities than other groups. Fusobacteria, Proteobacteria, Bacteroidetes, Firmicutes were the top four phyla in the gut microbiota of *T. tambroides*, accounting for more than 95%, with Fusobacteria dominating at around 70% of the gut microbiota. *Cetobacterium*, *ZOR0006*, *Brevinema*, and *Aeromonas* were the most common genera detected. *T. tambroides* fed a 0.5% Lacto-sacc (B) diet had lower Fusobacteria abundance while increasing other dominating bacteria compared to other treatments. Although there is no significant difference in gut microbiota, the gut microbiota of *T. tambroides* fed probiotics was more consistently disturbed and diversified, indicating less species dominance. The addition of Lacto-sacc, particularly at a concentration of 0.5%, appeared to enhance growth performance and increase the activity of digestive enzymes compared to the diet without Lacto-sacc, although the results were not statistically significant.

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

INTRODUCTION

Tor tambroides is known to be one of the most valuable riverine fish in Southeast Asia. The fish species which is found in Malaysia and Indonesia shares the same genus with *T. douronensis* and *T. tambra* (Lau *et al.,* 2021a). Due to its excellent commercial and conservation value, high market demand, and high flesh quality, it has significant potential in aquaculture, with market prices reaching up to USD 200 per kg in 2013, depending on grade and size (Redhwan *et al.,* 2022). However, despite its unique texture and taste, the slow-growing variant of *T. tambroides*, has low seed production which fails to meet the market demand, as they usually grow only to an average size of 800 g when reared in pond culture in around 33 months (Zomorni *et al.,* 2022). According to the Red List of Threatened Species provided by The International Union for Conservation of Nature (IUCN), study on *T. tambroides* population is still insufficient, though the population trend has decreased, primarily due to invasion events, massive destruction of habitats, and overfishing to fulfil market demand (Kottelat *et al.,* 2018).

In order to maintain the health of *T. tambroides*, which is a slow-growing fish, it is necessary to provide a feed that contains balanced nutrients and can support the fish's well-being over a prolonged period of time. Probiotics have been found to be effective in enhancing growth performance and boosting overall fish health through better nutrient uptake and enhanced immunity towards infectious diseases. The inclusion of probiotics in the diets of juvenile fish can provide nutritional and health benefits by detoxifying and denaturing indigestible compounds in feeds using hydrolytic enzymes, as well as stimulating the immunity of fishes (Bandyopadhyay *et al.,* 2015). Tachibana *et al.* (2021) found that feeding Nile Tilapia with probiotic strains of *Bacillus licheniformis* and *Bacillus subtilis* resulted in better overall net return and a 0.5 - 2% increase in fish production when compared to the control group. One commonly used probiotic in aquaculture is a lactic acid bacterium known as *Lactobacillus acidophilus*. The *Lactobacillus* genus has a long shelf life and good resistancy to environmental conditions, making it an ideal choice for aquaculture operations (Soltani *et al.,* 2017). *L. acidophilus* is found naturally as part of the microbiota at the larval, fry, and fingerling stages of fish development since they can be isolated from the skin, gills, and gut (Ige, 2013). *Saccharomyces cerevisiae*, on the other hand, is a type of Brewers' yeast with high protein content and can be used to compensate amino acid and vitamin deficiencies in fish diets. This microorganism is pathogenic and is capable of resisting bile and lower pH. *S. cerevisiae* also contains immunostimulating compounds such as β-1,3-glucans, mannan-oligosaccharieds (MOS) and chitin. These compounds can boost the immune response of fish and serve as a live food and fish meal replacement (Vallejos-Vidal *et al.,* 2016; Risjani *et al.,* 2021).

The selection of safe probiotic candidates is of utmost importance. Lacto-sacc is probiotics mixture produced by AllTech Biotechnology for livestock use. Several studies involving Nile Tilapia, Koi Carp, rabbit, broiler chicken, turkey and quail hens have been conducted using *L. acidophilus* and *S. cerevisiae* combination (Gippert *et al.,* 1992; Mahajan *et al.,* 2000; Sotirov *et al.,* 2001; Abou Zied *et al.,* 2003; Dhanaraj *et al.,* 2010; Wan Alias *et al.,* 2023; Hannan *et al.,* 2024). Lacto-sacc had proven by these studies that able to improve the growth performance of the host, enhance the feed utilization and immune response, reduce pathogen and improve digestive health and nutrient digestibility. Hannan *et al.* (2024) study proven that inclusion of Lacto-sacc to diet also significantly enhance the immune responses and increase survival rate when challenged with pathogen such as *Vibrio parahaemolyticus* in gravid mud crab.

Additionally, it is crucial to establish the appropriate probiotic dosage before introducing it to the intensive aquaculture industry to avoid the risk of accidental overdose (Ige, 2013). Overdosing probiotics can lead to unwanted side effects and unnecessary costs, while underdosing probiotics may result in incurring costs without achieving the desired outcome of targeted parameters (Nikoskelainen *et al.,* 2001; Ghosh *et al.,* 2007).

Research conducted by Asaduzzaman *et al.* (2018a and 2018b), Hossain *et al.* (2022) and Wan Alias *et al.* (2023) are known to use probiotics on *T. tambroides*. Conversely, studies on the gut microbiota of *T. tambroides* have also been carried out to compare gut microbiota between wild and captive *T. tambroides*, batches of *T. tambroides* from different culture farms and also diseased and healthy *T. tambroides* (Tan *et al*., 2019; Iehata *et al*., 2021; Lau *et al*., 2021b). To date, there is still no published report on the effect of Lacto-sacc on the gut microbiota of the *T. tambroides*. The current study aims to determine the effects of different Lacto-sacc concentration on growth performance, digestive enzyme activities and gut microbiota of *T. tambroides.*

MATERIALS & METHODS

Experimental Fish and Husbandry Condition

The *T. tambroides* fingerlings were obtained from Puri Johan Agro Sdn Bhd at Serian, Sarawak at the size of 1–1.5 inch per fingerlings. The fish were fed with commercial catfish pellet (Uni-President, T502SV), which contain 40% crude protein and 5–8% crude fat, during the 8 weeks of acclimation prior to the experiment. A total of 12 High Density Polyethylene (HDPE) tanks, each with a capacity of 650 L, were utilized for running the current study. The tanks were stocked with experimental fish, which had an average weight and length of 6.53 ± 0.17 g

and 7.23 ± 0.14 cm, at a rate of 50 fingerlings per tank. Feeding was carried out twice per day at 08:30 and 16:30 at 5% of the average fish body weight for 20 weeks. The *in-situ* water quality measurements such as water temperature, pH and dissolved oxygen levels of the tanks were monitored twice daily.

Diet Preparation

Experimental diets containing 40% protein and 12% lipid were prepared with varying levels of Lacto-sacc (AllTech, Inc., USA) supplementation. The Lacto-sacc supplementation contains probiotics concentration was *S. cerevisiae* 3.80 X 10^9 CFU/lb, *L. acidophilus* 2.10 X 10^8 CFU/lb, *Enterococcus faecium* 1.50 X 10^8 CFU/lb. A total of four diets were formulated; 0% Lacto-sacc as control diet (A), 0.5% Lacto-sacc (B), 1.0% Lacto-sacc (C), and 1.25% Lacto-sacc (D). As shown in Table 1, all the raw ingredients were mixed with water and then pelleted with a pelletiser before drying the pellets in an oven at 35°C for 48 hours. Dried pellets were packed and stored in a -20 °C freezer and will only be defrosted before feeding. The proximate composition was analysed for experimental diets, as shown in Table 1.

Table 1. Feed formulation used in *Tor tambroides* feeding trial

Ingredient (g/kg)	Control (A)	0.5% Lacto-sacc (B)	1.0% Lacto-sacc (C)	1.25% Lacto-sacc (D)
Fish Meal[1]	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[2]	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
Proximate Composition (%)				
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
Gross Energy (J/g)	18418	19109.5	18988.5	18921

[1] Sri Putra Trading, Alor Star, Kedah. Crude protein 67.38%; Crude fat 10.03%
[2] CMC: carboxymethyl cellulose

Relative Protein Digestibility

The relative protein digestibility (RPD) was determined *in vitro* using the pH drop method and carried out according to the Munir *et al.* (2016) method. Six fish were randomly collected from each tank, forming 18 fish per treatment, and dissected after 4 hours of feeding. The casein was chosen as standard. The RPD was calculated using the formula Eq. (1):

$$RPD = \{(-\Delta pH \text{ feedstuff}) / (-\Delta pH \text{ casein})\} \times 100 \quad \text{Eq.(1)}$$

Digestive Enzymes Activity Assay

The crude intestinal enzyme extraction method was carried out according to Munir *et al.* (2016) with modification. The fish gut was rinsed with 4 °C cold distilled water and cut into pieces, then weighed. The gut was then mixed with 4 °C cold phosphate buffer saline (PBS) at a ratio of 1:10 in a 50 ml centrifuge tube and homogenized using a homogenizer. Subsequently, the mixture was centrifuged at 12000 RPM for 15 minutes at 4 °C. The supernatants were then transferred into 2 ml centrifuge tubes and stored in a -80 °C deep freezer. The activity of digestive enzymes (amylase, lipase, and protease) was assessed using the Sigma-Aldrich MAK009, MAK046 and PF0100 kits, respectively according to the manufacturer's instructions.

Data Collection

Growth and survival parameter was determined. Measurements were taken by weighing each fish fingerling at four weeks intervals. Ten fish were selected from each tank, and their total length, standard length and body weight were recorded. The final weight of each fish was measured at the end of the experiment. The relative growth (RG)[Eq.(2)], specific growth rate (SGR)[Eq.(3)] and survival rate (SR)[Eq.(4)] were calculated using the following equations:

$$\text{RG (\%): } 100\ \left(\frac{Final\ Weight - Initial\ Weight}{Initial\ Weight}\right) \quad \text{Eq.(2)}$$

$$\text{SGR (\%/day): } 100\ \left(\frac{In\ Final\ Weight - In\ Initial\ Weight}{Nos.of\ Days}\right) \quad \text{Eq.(3)}$$

$$\text{SR (\%): } 100\ \left(\frac{Final\ Number\ of\ Fish}{Initial\ Number\ of\ Fish}\right) \quad \text{Eq.(4)}$$

DNA Extraction

Fish were rinsed with 4 ℃ cold autoclaved distilled water and wiped dry before dissection. The gut was cut from the end of the stomach to the anus and cut into smaller pieces in a microcentrifuge tube. The DNA extraction was performed with the Qiagen DNeasy Powersoil Pro Kit. The concentration of eluted DNA was checked using Nanodrop (DeNovix, USA). DNA sample purity around 1.8 at 260 nm/280 nm with a concentration of more than 20 ng/µl was used for further experiments.

Library Preparation and Sequencing

The V3-V4 hypervariable regions of 16S rRNA genes of gut microbiota were selected for Polymerase Chain Reaction (PCR). PCR amplification was carried out using the following primers: the forward primer 5' CCTACGGGNGGCWGCAG 3' and the reverse primer 5' GACTACHVGGGTATCTAATCC 3'. The PCR reaction mixture (25 µl) was prepared with 12.5 µl of 2X KAPA HiFi HotStart ReadyMix, 5 µl of 10 µM Forward Primer, 5 µl of 10 µM Reverse Primer and 2.5 µl of DNA template. The PCR amplification conditions included an initial denaturation at 95 ℃ for 3 minutes, followed by 25 cycles of denaturation at 95 ℃ for 30 seconds, annealing at 55 ℃ for 30 seconds, elongation at 72 ℃ for 30 seconds, and final elongation at 72 ℃ for 5 minutes.

The DNA samples were sequenced using an Illumina MiSeq platform. The DNA samples were subjected to library preparation before sequencing. The amplicons underwent purification and were linked with distinctive index adapter pairs using the Nextera XT Index kit. Following this, the indexed DNA libraries underwent purification using Agencourt AMPure XP (Beckman Coulter, USA). The concentration of these libraries was determined using a Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA). At the same time, their size was confirmed using the Agilent 2100 Bioanalyzer (Agilent, USA). Subsequently, the libraries were standardized and combined for subsequent MiSeq sequencing (2 X 300 bp paired-end).

Data Analysis

The significance of growth performance, RPD, and digestive enzyme activities was analysed using One-way Analysis of Variance (ANOVA) at a 95% significance difference. Analysis was carried out using IBM SPSS Statistics Version 25.

The MiSeq sequencing data were analysed using Quantitative Insights into Microbial Ecology (QIIME 2 v2020.8) and was installed on Linux Ubuntu System v18.04. The sequence was quality checked with FastQC v0.11.5 before further analysis to ensure sequences were of good quality. Adapter sequences were removed from both paired-end forward and reverse reads using the cutadapt command before trimming chimeric sequences. The Divisive Amplicon Denoising Algorithm 2 (DADA2) was utilized to denoise, and filter chimeric sequences based on the parametric model that infers true biological sequences from reads. Forward and reverse reads were individually denoised and merged before removing chimeric sequences using 'removeChimeraDenovo', resulting in the formation of Amplicon Sequence Variants (ASVs). Clustered ASVs were then summarised into various taxonomic levels based on GreenGenes database at a 99% identity threshold.

The statistical analysis was examined into two sections, which are alpha diversity and beta diversity. The alpha diversity index parameter chosen was Shannon, Chao1 and Simpson to evaluate the species richness and diversity of each treatment among the gut microbiota of *T. tambroides*. The beta diversity analysis aims to quantify the difference in species composition among the gut microbiota of *T. tambroides*.

Weighted UniFrac was selected for Principal Coordinate Analysis (PCoA) to visualize the variation in gut microbiota composition across different treatments. One-way ANOVA at 95% significance difference was chosen for the check significance of gut microbiota relative abundance of phyla and genera of *T. tambroides*.

RESULTS

Growth Performance, Relative Protein Digestibility and Digestive Enzyme Activities of *T. tambroides*

The presence of Lacto-sacc has been shown to influence the growth performance of fish, with the probiotic concentration listed in Table 2. The statistical analysis revealed no significant difference in all growth performance and survival rate among the treatment groups ($p>0.05$). However, it is noteworthy that treatment B (0.5% Lacto-sacc) exhibited a trend toward improved performance and survival rate with value consistently higher in weight gain, RG, SGR and SR than other treatment groups. These observations indicate that, although not statistically significant, treatment B may offer potential benefits that should be investigated further. As shown in Table 2, the results indicated a trend observed that 0.5% Lacto-sacc was the ideal concentration for raising *T. tambroides* fingerlings, followed by 1.0% Lacto-sacc, 1.25% Lacto-sacc, and control (0% Lacto-sacc), based on their high weight gain, RG, SGR and SR. The addition of probiotics positively influenced growth performance.

Although there was no significant effect ($p>0.05$) found between treatments for amylase and protease activity using One Way ANOVA in Table 3, numerically higher digestive enzyme activities were observed in all probiotic treatments compared to the control group. Treatment D recorded the highest amylase activity, followed by C, B, and A. For protease activity, treatment B showed highest value, followed by C, D, and A. Notably, treatment B had a statistically significant effect ($p<0.05$) on lipase activity, exhibiting higher levels compared to the other treatments.

Gut Microbiota of *T. tambroides*

The sample extracted from *T. tambroides* was quantified for good quality that concentration was more than 20ng/µl, and the purity of the sample OD260/280 was around 1.8 to 2.0. The sequencing depth was normalised for all *T. tambroides* samples as a plateau was observed when the curve flattened gradually, as the sequencing depth is adequate for capturing the diversity present in each sample. Alpha diversity assesses the richness and evenness of species within a specific area without considering differences between areas or samples. As shown in Table 4, the Shannon, Chao1 and Simpson index were shown for each treatment. Treatment B had the highest Shannon (2.8394) and Simpson index (0.8981), while treatment D had the highest Chao1 index (112.3333). Conversely, treatment C had the lowest Shannon, Chao1 and Simpson index.

The 12 samples of *T. tambroides* were observed at Principle Coordinate 1 vs Principle Coordinate 2 in Figure 1. The figure shows that the overall distribution distance between gut microbiota diversity of *T. tambroides* was closely related. Some samples were more dispersed, and A1 at the lower part, and C3 at the upper part of the plot.

Gut Taxonomy of *T. tambroides*

The gut taxonomy data for 12 samples of *T. tambroides* were categorized into their respective groups, the group means were calculated and summarised for each top 10 phyla (Table 5) and genera level (Table 6). No significant differences were found between treatments in both the top 10 phyla and genera of gut microbiota of *T. tambroides*.

The phylum Fusobacteria dominated the gut microbiota of *T. tambroides*. The relative abundance of Fusobacteria varied between 71.79% to 78.31%, with treatment B exhibiting the lowest abundance and treatment A having the highest abundance. On the other hand, the highest Proteobacteria abundance was found in treatment B and the highest Bacteroidetes found in treatment D. Spirochaetes was absent in treatment D.

The most abundant genus found in the gut microbiota of *T. tambroides* was *Cetobacterium*, followed by *ZOR0006*, *Brevinema*, Uncultured and *Aeromonas*. The highest Cetobacterium abundance (77.31%) was found in treatment A, and the lowest abundance (71.79%) was

observed in treatment B. The second most abundance genus found was *ZOR0006*, which varied from 2.20% to 6.78%, with the lowest being treatment D and the highest abundance being treatment B. The *Brevinema* was absent in treatment D, and the highest abundance was found in treatment B

Table 2. Growth performance parameter of *Tor tambroides* fingerlings

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[1], %	166.28±9.44	180.64±18.12	174.76±24.84	170.85±20.23
SGR[2], %	0.70±0.03	0.73±0.04	0.72±0.06	0.71±0.05
SR[3], %	98.67±0.67	99.33±0.67	100±0.00	98.67±1.33
VSI[4]	8.28±0.52	8.81±0.77	8.81±0.49	8.98±0.35
HSI[5]	0.44±0.08	0.60±0.05	0.53±0.11	0.59±0.08
IPF[6]	0.83±0.28	0.66±0.15	1.57±0.33	1.70±0.26

Values are the means of triplicate groups ± S.E.
[1]RG: Relative Growth
[2]SGR: Specific Growth Rate
[3]SR: Survival Rate
[4]VSI: Viscerosomatic Index
[5]HSI: Hepatosomatic Index
[6]IPF: Intraperitoneal Fat

Table 3. Digestive enzyme activities of *Tor tambroides* fingerlings

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[a]	62.90±8.80[b]	24.50±1.69[a]	21.17±2.47[a]
Protease, ug/ml	2700.25±170.18	3173.87±826.72	2969.48±481.47	2929.54±309.62

Values are the means of triplicate groups ± S.E. Data with a different superscript in same row indicate significant differences ($p<0.05$).
[1] One unit of amylase is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 µmole of *p*-nitrophenol per minute at 25 °C.
[2] One unit is the amount of lipase that generates 1.0 µmol glycerol from triglycerides per mg of protein per minute at 37 °C.

Table 4. Summary of alpha diversity of *Tor tambroides* gut microbiota

Treatment	Shannon index	Chao1 index	Simpson index
Control (A)	2.7017	110.667	0.8859
0.5% Lacto-sacc (B)	2.8394	96.0000	0.8981
1.0% Lacto-sacc (C)	2.6704	88.3333	0.8840
1.25% Lacto-sacc (D)	2.8360	112.3333	0.8958

.

Table 5. The relative abundance for top 10 phyla detected in Phase 1 *Tor tambroides* gut

Phylum	Control (A)	0.5% Lacto-sacc (B)	1.0% Lacto-sacc (C)	1.25% Lacto-sacc (D)
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

Table 6. The relative abundance for top 10 genera detected in Phase 1 *Tor tambroides* gut

Genus	Control (A)	0.5% Lacto-sacc (B)	1.0% Lacto-sacc (C)	1.25% Lacto-sacc (D)
Cetobacterium	77.3133 ± 5.1683	71.7900 ± 7.0800	78.2700 ± 8.880	73.9833 ± 3.1083
ZOR0006	3.2333 ± 3.4469	6.7800 ± 4.0661	3.6700 ± 3.7326	2.2033 ± 2.0410
Brevinema	1.9767 ± 3.201	4.4767 ± 7.7538	0.0267 ± 0.0462	0 ± 0
Uncultured	7.1600 ± 9.0179	5.9567 ± 1.8700	8.4700 ± 5.5209	11.6733 ± 5.5256
Aeromonas	1.8967 ± 0.9577	3.4000 ± 1.0714	2.0033 ± 0.8394	1.7367 ± 0.9182
Bacteroides	0.9033 ± 1.2989	0.7733 ± 0.4692	0.5667 ± 0.0551	0.8500 ± 0.4349
Macellibacteroides	0.4667 ± 0.2103	0.7733 ± 0.3844	1.5033 ± 1.0111	1.5000 ± 1.5862
Mycobacterium	0.7433 ± 0.8846	0.0933 ± 0.1365	0.0467 ± 0.0808	0.8300 ± 1.0306
Dechloromonas	0.4000 ± 0.3345	0.4233 ± 0.5689	0.2100 ± 0.2456	0.6900 ± 0.6986
Plesiomonas	0.6867 ± 0.8171	0.2900 ± 0.0400	0.2367 ± 0.1234	0.1433 ± 0.1401

DISCUSSION

Growth Performance, Relative Protein Digestibility and Digestive Enzyme Activities of *T. tambroides*

Although no significant difference in growth performance was observed between treatments in this study, various other studies have reported a positive effect on growth when using higher concentrations of probiotics. For instance, Soltani *et al.* (2017) found that common carp exhibited significant growth improvement when administered 1.2 x 10^6 CFU of *L. plantarum*, while Abou Zied *et al.* (2003) observed significant growth enhancement in Nile Tilapia with 0.1% Lacto-sacc, and Abass *et al.* (2018) reported improved growth performance in Nile Tilapia with 7% yeast. However, these outcomes can be affected by various factors, including species, life stage, dosage, and experimental conditions, as noted by Hosseini *et al.* (2016), who suggested assessing different probiotics for desired species.

In contrast, higher probiotic concentrations do not always result in a positive effect on growth compared to lower concentrations. For

instance, Hosseini *et al.* (2016) found that the growth performance and survival of goldfish, *Carassius auratus gibelio* were not affected by the concentration of *L. acidophilus*. A report by Nikoskelainen *et al.* (2001) also shows that administering a high dosage of *L. rhamnosus* at 10^{12} CFU does not enhance the resistance of rainbow trout to furunculosis compared to a lower dose of 10^{9} CFU. Additionally, Pooramini *et al.* (2009) also discovered that feed with 5% and 10% yeast yield similar results in terms of growth for rainbow trout.

Additionally, *S. cerevisiae* can enhance nutrient digestibility and improve the overall health of the intestinal mucosa and the density of intestinal villi. At the same time, MOS stimulates the growth of beneficial bacteria, such as *Lactobacilli*, which improve food digestion and assimilation (Abass *et al.,* 2018). *L. acidophilus* has been reported to enhance growth performance, mucosal immune response, and stress resistance and modulate intestinal microbiota towards beneficial bacteria (Hoseinifar *et al*., 2015). Furthermore, Hosseini *et al.* (2016) have found that dietary *L. acidophilus* could help in reducing the ghrelin gene, an appetite-related gene in the intestinal tract of goldfish, resulting in reduced body glucose levels and overall appetite.

The highest survival rate, 100% was demonstrated by 1.0% Lacto-sacc (C), followed by 99.33% in 0.5% Lacto-sacc (B) and 98.67% in both 1.25% Lacto-sacc (D) and 0% Lacto-sacc (A). The death fish in treatment 0.5% Lacto-sacc (B) and 1.25% Lacto-sacc (D) resulted from overfeeding larger fish. The death in the control group, 0% Lacto-sacc (A) was found to be pop eye infection and red spot symptoms that happened in week 13 in tank A2 (second replicate of control group) and week 17 in tank A3 (third replicate). Two additional fish in tank A2 exhibited similar symptoms. The introduction of probiotics in fish diets improved survival rates as reported by Abass *et al.* (2018) and Hoseinifar *et al.* (2015).

Better digestive enzyme activity was observed in fish treated with probiotics compared to the control group, indicating that probiotics could enhance fish digestion. Gram-positive bacteria such as *Lactobacillus* sp. could increase intestine enzymatic activity by secreting exogenous enzymes that are beneficial to the digestion process (Akter *et al.,* 2019). The intake of *L. acidophilus* has been associated with the increase of lactic acid bacteria, resulting in changes in both amylase and lipase activity. Moreover, *L. acidophilus* has also been proven to effectively improve dish digestive enzyme activities (Akter *et al.,* 2019; Mohammadian *et al.,* 2019). According to Mohapatra *et al.,* (2012), most probiotics are able to trigger essential fatty acid production through the secretion of lipid digesting enzymes. Digestive enzymes such as amylase, lipase, and protease can also be produced by lactic acid bacteria (Askarian *et al.,* 2012). In a study by Darafsh *et al.* (2020), Persian sturgeon fed with *S. cerevisiae* showed higher amylase, protease, and lipase activities compared to the control group.

The introduction of probiotics also increases the population of beneficial microbes and microbial enzymes that promote feed digestibility and absorption. As a result, the growth performance of fish will be improved (Allameh *et al*., 2017). The best results were observed in 0.5% Lacto-sacc in terms of growth, protease and lipase activity. Treatment B shows high protease activity which helps in protein digestion (Table 3). Probiotic dosage does not have noticeable effects on fish enzymes because there were no significant differences in the bacteria retained in the gut among the fish in treatments (Dawood *et al.,* 2019).

However, the treatment showed not significantly on growth performance and amylase and protease activity might be due to short duration of the probiotics feeding trial as *Tor tambroides* is slow growing fish. Also, *T. tambroides* prefer flowing and clean water environment. To maintain a good water quality for *T. tambroides*, 50% of the water in the closed tank was changed daily. However, this water change may disrupt the nutrient flow and lead to the loss of nutrient and probiotics, potentially affecting the fish's nutrient uptake and leading to no significant differences between treatments.

As seen in a study on Nile Tilapia, a higher concentration (0.1%) of Lacto-sacc yields better results than lower concentration (0.05%) Lacto-sacc (Zied *et al*., 2003). The combination of both *L. acidophilus* and *S. cerevisiae* at a concentration of 0.25% has been shown to effectively improve the growth performance and microbial load in the gut of Koi Carp, with

brewer's yeast at a concentration of 0.5% showing the best results (Dhanaraj *et al*., 2010). Given that a higher initial concentration was used in this experiment, the optimal concentration of Lacto-sacc may fall between 0.1% and 0.5%. Higher concentration of probiotics will not always result in better growth and improvement in physiological status, while overdosing might lead to higher costs and undesired effects (Soltani *et al*., 2017). As Lacto-sacc becomes dominant in the gut, the gut microbiota may become imbalanced. This will induce *L. acidophilus* to produce bacteriocins while also fermenting lactose into lactic acid. These processes will reduce the pH within the gut which will then eliminate both harmful and beneficial bacteria. Therefore, a Lacto-sacc diet of more than 0.5% concentration is unnecessary for *T. tambroides* feeding since there will not be any improvements to the growth and digestive enzyme activities.

Gut Microbiota of *T. tambroides*

In Alpha diversity, treatment B has the highest Shannon Index that indicating higher diversity and more even distribution of species abundance compared to other treatment. Treatment D showed highest Chao 1 indexes value representing they had highest species richness. For Simpson index, CP1 had the lowest Simpson Index value indicating they had higher diversity, and all species were more equally abundant. Treatment B that has the highest Shannon and Simpson index but with lower Chao1 index 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.

Beta Diversity was shown by the Principal Coordinate Analysis (PCoA) that visualized patterns in dissimilarity. Most of the samples were close clustering, which means most of the samples were more similar to each other microbial community composition. For the distal treatment that more far apart from other samples are more dissimilar microbial communities with others.

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

Fusobacteria can be found in most abundance in the gut, and this finding agrees with some studies with different fish species (Mathai *et al.*, 2021; Zhang *et al.*, 2021). Fusobacteria, particularly *Cetobacterium* were found to be prevalent in gut of microbiota of freshwater fish and account for 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). The decrease in the abundance of Fusobacteria including *Cetobacterium* might because of *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and Fusobacteria which played a crucial role in carbohydrates fermentation. In the meantime, Ofek *et al.* (2022) study found that Proteobacteria was increased at the expense of Fusobacteria in the intestine of diseased fish. Also, 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. Among top 10 genera, there are 3 genera belongs to Proteobacteria which were *Aeromonas*, *Dechloromonas* and *Plesiomonas*.

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). In the study, the *ZOR0006* was a genus of phylum Firmicutes and were observed as second most abundance genus. While in Duperron *et al.* (2019), Foucault *et al.* (2022) and Gallet *et al.* (2023) studies, the two most prevalent gut associated bacteria on the medaka fish were *Cetobacterium* and *ZOR0006*. 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. Spirochaetes were absent in treatment D. *Brevinema* was present at top 10 genera, member of the phylum Spirochaetes and it was suggested as opportunistic pathogen. However, this bacterium was a conditional pathogen that occurrence of intestinal diseases only when found high abundance in intestine (Kong *et al.*, 2023).

It is noticeable that the more preferred concentration in growth performance which is treatment B has lower *Cetobacterium* and higher other more abundance 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.

Although the study results were not statistically different in most of the bacteria between treatment, and 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. The introduction of Lacto-sacc bring beneficial effect to the gut, resulting increase in these beneficial bacteria and bring more even distributed gut microbiota.

CONCLUSION

This study demonstrated that adding 0.5% Lacto-sacc to the diet of *T. tambroides* fingerlings improved growth performance and digestive enzyme activity when compared to the control (0% Lacto-sacc) and other treatment groups. However, no additional benefits were observed at concentrations greater than 0.5%. Fusobacteria, Proteobacteria, Bacteroidetes, Firmicutes were the top four phyla in the gut microbiota of *T. tambroides*, accounting for more than 95%, with Fusobacteria dominating at around 70% of the gut microbiota. *Cetobacterium*, *ZOR0006*, *Brevinema*, and *Aeromonas* were the most common genera detected. *T. tambroides* fed a 0.5% Lacto-sacc diet had lower Fusobacteria abundance while increasing other bacteria compared to other treatments. Although there is no significant in gut microbiota, the gut microbiota of *T. tambroides* fed probiotics was likewise more consistently disturbed and diversified, indicating less species dominance. Future research should focus on the appropriate Lacto-sacc concentration to enhance cost efficiency, as well as the long-term effects of probiotics on growth.

ACKNOWLEDGEMENTS

This project was funded by the Ministry of Higher Education (MOHE), Malaysia through Fundamental Research Grant Scheme (FRGS) FRGS/1/2019/WAB01/UNIMAS/03/2.

DECLARATION OF INTERESTS

The authors have declared that there are no potential conflicts of interest. The data produced and/or analysed in this study can be obtained from the corresponding author upon request.

REFERENCES

Abass, D.A., Obirikorang, K.A., Campion, B.B., Edziyie, R.E. & Skov, P.V. (2018). Dietary supplementation of yeast (*Saccharomyces cerevisiae*) improves growth, stress tolerance, and disease resistance in juvenile Nile tilapia (*Oreochromis niloticus*). *Aquaculture International*, 26: 843-855. DOI: 10.1007/s10499-018-0255-1

Abou Zied, R., Abd El-Maksoud, A. & Ali, A. (2003). Effect of virginiamycin or lacto-sacc on the growth performance of nile tilapia fish during the nursing period. *Egyptian Journal of Nutrition and Feeds*, 6: 481-489.

Akter, M.N., Hashim, R., Sutriana, A., Siti Azizah, M.N. & Asaduzzaman, M. (2019). Effect of *Lactobacillus acidophilus* supplementation on growth performances, digestive enzyme activities and gut histomorphology of striped catfish (*Pangasianodon hypophthalmus* Sauvage, 1878) juveniles. *Aquaculture Research*, 50(3): 786-797. DOI: https://doi.org/10.1111/are.13938

Allameh, S., Noaman, V. & Nahavandi, R. (2017). Effects of probiotic bacteria on fish performance.

Advanced Techniques in Clinical Microbiology, 1(2): 11.

Asaduzzaman, M.D., Iehata, S., Akter, S., Kader, M.A., Ghosh, S.K., Khan, M.N.A. & Abol-Munafi, A.B. (2018a). Effects of host gut-derived probiotic bacteria on gut morphology, microbiota composition and volatile short chain fatty acids production of Malaysian Mahseer *Tor tambroides*. *Aquaculture Reports*, 9: 53-61. DOI: 10.1016/j.aqrep.2017.12.003

Asaduzzaman, M., Sofia, E., Shakil, A., Haque, N.F., Khan, M.N.A., Ikeda, D., Kinoshita, S. & Abol-Munafi, A.B. (2018b). Host gut-derived probiotic bacteria promote hypertrophic muscle progression and upregulate growth-related gene expression of slow-growing Malaysian Mahseer *Tor tambroides*. *Aquaculture Reports*, 9: 37-45. DOI:10.1016/j.aqrep.2017.12.001

Askarian, F., Zhou, Z., Olsen, R.E., Sperstad, S. & Ringø, E. (2012). Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar L*.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. *Aquaculture*, 326: 1-8. DOI: 10.1016/j.aquaculture.2011.10.016

Bandyopadhyay, P., Mishra, S., Sarkar, B., Swain, S.K., Pal, A., Tripathy, P.P. & Ojha, S.K. (2015). Dietary *Saccharomyces cerevisiae* boosts growth and immunity of IMC *Labeo rohita* (Ham.) juveniles. *Indian Journal of Microbiology*, 55: 81-87. DOI: 10.1007/s12088-014-0500-x

Darafsh, F., Soltani, M., Abdolhay, H.A. & Shamsaei Mehrejan, M. (2020). Improvement of growth performance, digestive enzymes and body composition of Persian sturgeon (*Acipenser persicus*) following feeding on probiotics: *Bacillus licheniformis*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. *Aquaculture Research*, 51(3): 957-964. DOI: 10.1111/are.14440

Dawood, M.A., Magouz, F.I., Salem, M.F. & Abdel-Daim, H.A. (2019). Modulation of digestive enzyme activity, blood health, oxidative responses and growth-related gene expression in GIFT by heat-killed *Lactobacillus plantarum* (L-137). *Aquaculture*, 505: 127-136. DOI: 10.1016/j.aquaculture.2019.02.053

Dhanaraj, M., Haniffa, M., Singh, S.A., Arockiaraj, A.J., Ramakrishanan, C.M., Seetharaman, S. & Arthimanju, R. (2010). Effect of probiotics on growth performance of koi carp (*Cyprinus carpio*). *Journal of Applied Aquaculture*, 22(3): 202-209. DOI: 10.1080/10454438.2010.

Duperron, S., Halary, S., Habiballah, M., Gallet, A., Huet, H., Duval, C., Bernard, C. & Marie, B. (2019). Response of fish gut microbiota to toxin-containing cyanobacterial extracts: a microcosm study on the medaka (*Oryzias latipes*). *Environmental Science & Technology Letters*, 6(6): 341-347. DOI: 10.1021/acs.estlett.9b00297.s004

Foucault, P., Gallet, A., Duval, C., Marie, B. & Duperron, S. (2022). Gut microbiota and holobiont metabolome composition of the medaka fish (*Oryzias latipes*) are affected by a short exposure to the cyanobacterium *Microcystis aeruginosa*. *Aquatic Toxicology*, 253: 106329. DOI: 10.1016/j.aquatox.2022.106329

Gallet, A., Halary, S., Duval, C., Huet, H., Duperron, S. & Marie, B. (2023). Disruption of fish gut microbiota composition and holobiont's metabolome during a simulated *Microcystis aeruginosa* (Cyanobacteria) bloom. *Microbiome*, 11(1): 108. DOI: 10.1186/s40168-023-01558-2

Ghosh, S., Sinha, A. & Sahu, C. (2007). Effect of probiotic on reproductive performance in female livebearing ornamental fish. *Aquaculture Research*, 38(5): 518-526. DOI: 10.1111/j.1365-2109.2007.01696.x

Gippert, T., Virag, G. & Nagy, I. (1992). Lacto-Sacc in rabbit nutrition. *Journal of Applied Rabbit Research*, 15: 1101-1101.

Hannan, M.A., Munir, M.B., Asdari, R., Islam, M.S., Lima, R.A., Islam, H.R., Rashid, M.H., & Hing, H.W.Y. (2024). Dietary lacto-sacc stimulates the immune response of gravid mud crab (*Scylla olivacea*). *Comparative Immunology Reports*, *7*, 200156.

Hoseinifar, S.H., Roosta, Z., Hajimoradloo, A. & Vakili, F. (2015). The effects of *Lactobacillus acidophilus* as feed supplement on skin mucosal immune parameters, intestinal microbiota, stress resistance and growth performance of black swordtail (*Xiphophorus helleri*). *Fish & Shellfish Immunology*, 42(2): 533-538. DOI: 10.1016/j.fsi.2014.12.003

Hossain, M.K., Ishak, S.D., Ambok-Bolong, A.M., Noordin, N.M., Iehata, S. & Kader, M.A. (2022). Effect of intestinal autochthonous *Enterococcus faecalis* on the growth performance, gut morphology of Malaysian mahseer (*Tor tambroides*) and protection against *Aeromonas hydrophila*. *International Aquatic Research*, 14(1): 1-12.

Hosseini, M., Miandare, H.K., Hoseinifar, S.H. & Yarahmadi, P. (2016). Dietary *Lactobacillus acidophilus* modulated skin mucus protein profile, immune and appetite genes expression in gold fish (*Carassius auratus gibelio*). *Fish & Shellfish Immunology*, 59: 149-154. DOI: 10.1016/j.fsi.2016.10.026

Iehata, S., Nosi, M.Z., Danish-Daniel, M. & Sharifah, N.E. (2021). Gut microbiome associated with cultured Malaysian mahseer *Tor tambroides*. *Aquaculture, Aquarium, Conservation & Legislation*, 14(1): 46-58.

Ige, B.A. (2013). Probiotics use in intensive fish farming. *African Journal of Microbiology Research*, 7(22): 2701-2711. DOI: 10.5897/AJMRx12.021

Kong, Y., Liao, Z., Ma, X., Liang, M., Xu, H., Mai, K. & Zhang, Y. (2023). Response of intestinal microbiota of tiger puffer (*Takifugu rubripes*) to the fish oil finishing strategy. *Microorganisms*, 11(1): 208. DOI: doi.org/10.3390/microorganisms11010208

Kottelat, M., Pinder, A. & Harrison, A. (2018). *Tor Tambroides*. Cambridge, UK: International Union for Conservation of Nature. https://www.iucnredlist.org/species/187939/91076554

Lau, M.M.L., Lim, L.W.K., Ishak, S.D., Abol-Munafi, A.B. & Chung, H.H. (2021a). A review on the emerging asian aquaculture fish, the Malaysian Mahseer (*Tor tambroides*): Current status and the way forward. *Proceedings of the Zoological Society*, 74(2): 227-237. DOI: 10.1007/s12595-021-00368-4

Lau, M.M.L., Kho, C.J.Y., Lim, L.W.K., Sia, S.C., Chung, H.H., Lihan, S. & Apun, K. (2021b). Microbiome analysis of gut bacterial communities of healthy and diseased Malaysian mahseer (*Tor tambroides*). *bioRxiv*, 2021-12. DOI: 10.1101/2021.12.08.471852

Mahajan, P., Sahoo, J. & Panda, P. (2000). Effect of probiotic (Lacto-Sacc) feeding and seasons on the different quality characteristics of poultry meat. *Indian Journal of Poultry Science*, 35(3): 297-301.

Mathai, P.P., Byappanahalli, M.N., Johnson, N.S. & Sadowsky, M.J. (2021). Gut microbiota associated with different sea lamprey (*Petromyzon marinus*) life stages. *Frontiers in Microbiology*, 12: 706683. DOI: 10.3389/fmicb.2021.706683

Mohammadian, T., Nasirpour, M., Tabandeh, M.R., Heidary, A.A., Ghanei-Motlagh, R. & Hosseini, S.S. (2019). Administrations of autochthonous probiotics altered juvenile rainbow trout *Oncorhynchus mykiss* health status, growth performance and resistance to *Lactococcus garvieae*, an experimental infection. *Fish & Shellfish Immunology*, 86: 269-279. DOI: 10.1016/j.fsi.2018.11.052

Mohapatra, S., Chakraborty, T., Prusty, A., Das, P., Paniprasad, K. & Mohanta, K. (2012). Use of different microbial probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora. *Aquaculture Nutrition*, 18(1): 1-11. DOI: 10.1111/j.1365-2095.2011.00866.x

Munir, M.B., Hashim, R., Chai, Y.H., Marsh, T.L. & Nor, S.A.M. (2016). Dietary prebiotics and probiotics influence growth performance, nutrient digestibility and the expression of immune regulatory genes in snakehead (*Channa striata*) fingerlings. *Aquaculture*, 460: 59-68. DOI: 10.1016/j.aquaculture.2016.03.041

Nikoskelainen, S., Ouwehand, A., Salminen, S. & Bylund, G. (2001). Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. *Aquaculture*, 198 (3-4): 229-236. DOI: 10.1016/S0044-8486(01)00593-2

Ofek, T., Lalzar, M., Izhaki, I. & Halpern, M. (2022). Intestine and spleen microbiota composition in healthy and diseased tilapia. *Animal Microbiome*, 4(1): 50. DOI: 10.1186/s42523-022-00201-z

Pooramini, M., Kamali, A., Hajimoradloo, A., Alizadeh, M. & Ghorbani, R. (2009). Effect of using yeast (*Saccharomyces cerevisiae*) as probiotic on growth parameters, survival and carcass quality in rainbow trout *Oncorhynchus mykiss* fry. *International Aquatic Research*, 1(1): 39.

Ray, C., Bujan, N., Tarnecki, A., Davis, A.D., Browdy, C. & Arias, C.R. (2017). Analysis of the gut microbiome of Nile tilapia *Oreochromis niloticus* L. fed diets supplemented with Previda® and Saponin. *Journal of Fisheriessciences.com*, 11(2): 36-45. DOI: 10.21767/1307-234x.1000116

Redhwan, A.I., Rao, S.N., Mohamad-Zuki, N.A., Kari, A., Kamarudin, A.S., Ismail, N., Nguang, S.I., Ha, H.C., Yong, W.S., Yong, F.H. & Komilus, C.F. (2022). Mahsheers in Malaysia: A Review of Feed for Cultured *Tor tambroides* and *Tor tambra*. *Bioscience Research*, 19(1): 349-359.

Risjani, Y., Mutmainnah, N., Manurung, P. & Wulan, S.N. (2021). Exopolysaccharide from

Porphyridium cruentum (purpureum) is not toxic and stimulates immune response against vibriosis: The assessment using zebrafish and white shrimp *Litopenaeus vannamei*. *Marine drugs*, 19 (3): 133. DOI: 10.3390/md19030133

Siddik, M.A., Foysal, M.J., Fotedar, R., Francis, D.S. & Gupta, S.K. (2022). Probiotic yeast *Saccharomyces cerevisiae* coupled with *Lactobacillus casei* modulates physiological performance and promotes gut microbiota in juvenile barramundi, *Lates calcarifer*. *Aquaculture*, 546: 737346. DOI: 10.1016/j.aquaculture.2021.737346

Soltani, M., Abdy, E., Alishahi, M., Mirghaed, A.T. & Hosseini-Shekarabi, P. (2017). Growth performance, immune-physiological variables and disease resistance of common carp (*Cyprinus carpio*) orally subjected to different concentrations of *Lactobacillus plantarum*. *Aquaculture International*, 25: 1913-1933. DOI: 10.1007/s10499-017-0164-8

Sotirov, L., Denev, S., Tsachev, I., Lalev, M., Oblakova, M. & Porfirova, Z. (2001). Effect of different growth promoters on lysozyme and complement activity. II. Studing in turkeys. *Revue de medecine veterinaire*, 152(1): 67-70.

Tachibana, L., Telli, G.S., Dias, D.D.C., Goncalves, G.S., Guimaraes, M.C., Ishikawa, C.M., Cavalcante, R.B., Natori, M.M., Fernandez Alarcon, M.F. & Tapia-Paniagua, S. (2021). *Bacillus subtilis* and *Bacillus licheniformis* in diets for Nile tilapia *(Oreochromis niloticus*): Effects on growth performance, gut microbiota modulation and innate immunology. *Aquaculture Research*, 52(4): 1630-1642. DOI: 10.1111/are.15016

Tan, C.K., Natrah, I., Suyub, I.B., Edward, M.J., Kaman, N. & Samsudin, A.A. (2019). Comparative study of gut microbiota in wild and captive Malaysian Mahseer (*Tor tambroides*). *Microbiology Open,* 8(5): e00734. DOI: 10.1002/mbo3.734

Vallejos-Vidal, E., Reyes-López, F., Teles, M. & MacKenzie, S. (2016). The response of fish to immunostimulant diets. *Fish & Shellfish Immunology*, 56: 34-69. DOI: 10.1016/j.fsi.2016.06.028

Van Kessel, M.A., Dutilh, B.E., Neveling, K., Kwint, M.P., Veltman, J.A., Flik, G., Jetten, M. S., Klaren, P.H. & Op den Camp, H.J. (2011). Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.). *AMB Express*, 1(1): 1-9. DOI: 10.1186/2191-0855-1-41

Wan Alias, F.S.L., Munir, M.B., Asdari, R., Hannan, A. & Hasan, J. (2023). Dietary lacto-sacc improved growth performance, food acceptability, body indices, and basic hematological parameters in empurau (*Tor tambroides*) fries reared in the aquaponics system. *Journal of Applied Aquaculture*, 35(4): 1131-1153. DOI: 10.1080/10454438.2022.2095239

Xie, M., Xie, Y., Li, Y., Zhou, W., Zhang, Z., Yang, Y., Olsen, R.E., Ringo, E., Ran, C. & Zhou, Z. (2022). Stabilized fermentation product of *Cetobacterium somerae* improves gut and liver health and antiviral immunity of zebrafish. *Fish & Shellfish Immunology*, 120: 56-66. DOI: 10.1016/j.fsi.2021.11.017

Xiong, J.B., Nie, L. & Chen, J. (2019). Current understanding on the roles of gut microbiota in fish disease and immunity. *Zoological Research*, 40(2): 70-76.

Xu, Y., Li, Y., Xue, M., Yang, T., Luo, X., Fan, Y., Meng, Y., Liu, W., Lin, G., Li, B., Zeng, L. & Zhou, Y. (2021). Effects of dietary *Saccharomyces cerevisiae* YFI-SC2 on the growth performance, intestinal morphology, immune parameters, intestinal microbiota, and disease resistance of Crayfish (*Procambarus clarkia*). *Animals*, 11(7): 1963. DOI: 10.3390/ani11071963

Yang, J., Lin, Y., Wei, Z., Wu, Z., Zhang, Q., Hao, J., W.S. & Li, A. (2023). *Edwardsiella ictaluri* almost completely occupies the gut microbiota of fish suffering from Enteric septicemia of catfish (Esc). *Fishes*, 8(1): 30. DOI: 10.3390/fishes8010030

Zhang, Y., Wen, B., Meng, L.J., Gao, J.Z. & Chen, Z.Z. (2021). Dynamic changes of gut microbiota of discus fish (*Symphysodon haraldi*) at different feeding stages. *Aquaculture*, 531: 735912. DOI: 10.1016/j.aquaculture.2020.735912

Zomorni, M.S., Hai, C.T., Redhwan, A.I. & Komilus, C.F. (2022). Efficacy of black soldier fly (*Hermetia illucens*) larvae meal as feed on growth performance for juvenile Javan Mahseer (*Tor tambra*). *Journal of Agrobiotechnology*, 13(1): 118-130. DOI: 10.37231/jab.2022.13.1s.321