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Modulation of Gut Microbiota, Nutrient Transport Gene Expression, and Growth of Red Hybrid Tilapia (*Oreochromis* sp.) Fed with Black Soldier Fly (*Hermetia illucens*) by Oral Probiotics

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ABSTRACT Maggot Black Soldier Fly (BSF) is a nutritionally rich alternative food for fish. The slow digestibility requires the combination with other technologies to produce fish with excellent growth. This study aims to investigate the impact of probiotic application on the growth, microbiome, and gene expression in the intestines of tilapia-fed black soldier fly (BSF) larvae. Tilapia intestines were examined after two months of rearing with three replications and two treatments: A) 30% maggots and 70% commercial pellets with probiotics, and B) 30% maggots and 70% commercial pellets without probiotics. We discovered that probiotics have a significant impact on the gut microbiomes of fish and their absolute growth. The amplicon sequence variant in the probiotic treatment (A) was 25, with dominance by Cetobacterium, Acinetobacter, Enhydrobacter, and Gemmobacter, while the non-probiotic treatment (B) was 8, with dominance by *Cetobacterium* and *Turicibacter*. The probiotic treatment increased the expression of Ghrelin, Muc-2, IL-1β, and I-FABP genes, but not the CD36 gene. These findings suggest oral probiotics can help boost tilapia production when fed maggot black soldier fly (*Hermetia illucens*).

Keywords: Bacteria; molecular; NGS; RT-qPCR

INTRODUCTION

Tilapia (*Oreochromis* sp.) is a widely farmed freshwater fish that contributes significantly to the global aquaculture industry (Sukkarun *et al*., 2022). Feed is the most crucial aspect in tilapia farming (Lu *et al*., 2023), as it determines fish metabolism, intestinal structure, and growth rate, accounting for 60-70% of overall production costs (Khan *et al*., 2021; Liao *et al*., 2023). The rising price of commercial feed prompts fish farmers to challenge with different types of alternative feeds. BSF maggot (*Hermetia illucens*) is an appropriate substitute meal for fish. Maggots offer several advantages for fish feed, including their high nutrient content, ease of cultivation, and minimal competition with human consumption. (Mokolensang *et al*., 2018; English *et al*., 2021).

Research on the potential of maggots as an alternative fish feed has been conducted by Fahmi et al. (2009), who used maggots as a feed supplement and tested them on tilapia. This research demonstrates that feeding maggots to fish leads to improved growth and survival. However, feeding fresh maggots to fish can produce waste, increasing organic matter in the cultivation air. This will decline water quality and affect the growth of tilapia fish. One way to overcome this is by applying probiotics. Research on the application of probiotics to commercial feed has demonstrated improvements in water quality and tilapia growth. According to research by El-Kady et al. (2022), the application of probiotics has a positive impact on water quality and can improve the growth performance of tilapia.

Maggots can be combined with probiotics for fish food (Murti *et al*., 2023). The effect of probiotics on increasing growth can be observed more clearly by examining the condition of the microbiome and the expression of genes related to lipid and protein transport in the intestines of tilapia. The diversity of the gut microbiome influences growth and the immune system, as the gut microbiota plays a crucial role in nutrient metabolism (Yang *et al*., 2021). The impact of probiotic treatment on immune gene expression in the tilapia gut requires more investigation. In a previous study by Murti et al. (2023), probiotics were found to increase the number of goblet cells in the intestines of tilapia. One way that probiotics work in the gut is by encouraging goblet cells to express the mucin gene (Gou *et al*., 2022). The condition of the microbiome and gene expression in the gut of tilapia, as reported in previous research (Murti *et al*., 2023), remains unknown. Further research is needed to elucidate the mechanisms by which they positively impact tilapia farming.

The gut microbiota is a key factor influencing fish physiology and feed absorption capacity, making it essential for fish health and performance (Viver *et al*., 2023). Interactions between the microbiome and fish can enhance aquaculture management, as a balanced microbiome promotes overall health. The fish gut microbiome influences the metabolism, feeding behaviors, and immune responses (Yukgehnaish *et al*., 2020). Understanding their taxonomic composition is a necessary first step toward unravelling the role of microbes in maintaining host homeostasis. The microbiome composition varies depending on the feed. The microbiome in the intestine plays a crucial role in aiding digestion, food absorption, and immunity; therefore, feed treatment can alter microbiome dynamics (Viver *et al*., 2023). According to Xia *et al*. (2020), the use of probiotic *Lactococcus lactis* JCM5805 can influence the composi-

tion and increase the diversity of the tilapia gut microbiota, altering intestinal metabolism and resulting in enhanced growth and immunity.

Several studies have demonstrated that the application of probiotics influences gene expression in the intestine of tilapia. Probiotics can modulate the expression of genes related to the immune system, particularly those encoding TNF-α, IL-1β, and HSP70, and enhance resistance to stressors (Dawood *et al*., 2020). Research by Hijo *et al*. (2019), Hendam *et al*. (2023), and Marel *et al*. (2012) has demonstrated that probiotics can influence the expression of genes related to intestinal function, including those involved in fatty acid and protein transport, as well as mucus production. Apart from that, according to Kiela and Ghishan (2016), probiotics can also affect the expression of genes related to growth. Gene expression plays a role in determining the physiological function of an organism (Sourav, 2023). Observations regarding the effect of probiotic application on gene expression are crucial for understanding how bacteria in the intestine function and interact with the host.

This is curious to consider employing probiotics to treat the digestive issues with BSF maggot feed. This research aims to utilize probiotics to optimize the use of black soldier fly (BSF) maggots as an alternative diet that can promote growth, bacterial microbiome, and gene expression in the intestines of tilapia fed a maggot-based feed mixture.

MATERIALS AND METHODS

Research design

This research was conducted from November to December 2023 in the Laboratory of Fish Health and Environment, Department of Fisheries, Faculty of Agriculture, Gadjah Mada University. Two treatments were used in this study, with three repetitions of each treatment. The treatment consists of the following options: (A) 30% maggot and 70% commercial pellets with probiotics, and (B) 30% maggot and 70% commercial pellets without probiotics.

Red hybrid tilapia nilasa strain (*Oreochromis* sp.) with an average length of 15 cm and an average weight of 50 g was used in the present study. It was purchased from Mina Raya fish farm, Kalasan, Sleman, Yogyakarta. Tilapia were kept in six fibre tanks with a diameter of 80 cm and a height of 90 cm, with a density of 50 fish per tank. Feeding was carried out with commercial pellets (Hi-Pro Vite 781, containing 31-33% protein) three times a day.

Maggot black soldier fly (BSF) was cultured with oil palm cake by PT Trimitra Bumi Lestari Yogyakarta was used in the present study. We use 4-6 days old fresh maggots, with an average body length of 5-10 mm and a body width of 1-2.5 mm, due to their high protein and low-fat content (Fahmi *et al*., 2009). Maggots were given to the fish once a day in the afternoon. The probiotic bacteria used for oral administration were proteolytic *Bacillus tropicus* PCP1 and lipolytic *Lactococcus formosensis* JAL37, at 10^7 cells/g of feed. Those used for water administration were *Klebsiella variicola* A2 (Aswiyanti et al., 2021) at a concentration of 10^5 cells/mL in water. Probiotic application is done once a week. Water quality checks were conducted on day three after the probiotic application.

Sample collection

Red hybrid ilapia intestine samples were taken after two months of rearing. The intestinal tissue from each fish sample was taken and then rinsed using phosphate-buffered saline (PBS). After that, the intestine was placed into a 1.5 mL microtube, added with 70% ethanol, and stored at -20 °C for DNA extraction and analysis of the gut microbiota. The other portion of the intestinal tissue was stored in RNA later solution at -20 °C for further RNA extraction for gene expression analysis.

Fish Growth and Survival Rate

The survival rate and growth of the red hybrid tilapia (Oreochromis sp.) were recorded at the end of the experiment. Growth performance was measured based on the absolute weight gain and absolute length gain.

Gut microbiota analysis

DNA extraction of the anterior intestine of tilapia was conducted by using the PureLink Genomic DNA Mini Kit (Invitrogen, Cat. No. K1820- 01). The intestinal tissue lysis procedure was performed using the PureLinkTM Genomic DNA Mini Kit (Invitrogen, Cat No. K1820-01. A total of ≤ 25 mg of intestinal tissue in a tube is added with 180 µL of Digestion Buffer and 20 µL of Proteinase K. The mixture is then incubated at 55 °C for 4 hours. Afterward, 20 µL of RNase A is added, and the mixture is incubated at room temperature for 2 minutes, followed by centrifugation for 5 minutes. The supernatant was transferred to a new microcentrifuge tube, supplemented with 200 µL Lysis/Binding Buffer, and vortexed. Then, 200 µL 90-100% ethanol was added, vortexed for 5 seconds, and the mixture was centrifuged for 5 minutes. The column was then added with 500 µl of Wash Buffer 2 and further centrifuged at 10.000 x g for 3 min. The DNA in the collection tube was eluted with Elution Buffer, incubated at room temperature for 1 minute, and then centrifuged for 1 minute. The collected DNA was visualized using agarose gel electrophoresis (Mupid Exu) and a UV Transilluminator (Viber Lourmat). The DNA quality (quantity and purity) was measured by using a Nanodrop spectrophotometer (Nanodrop 1000, Thermo Scientific, USA). The nanodrop yield of a suitable DNA genome is at least 100 ng/µl. and

The gut microbiota of the red hybrid tilapia was analyzed using the next-generation sequencing (NGS) method with V3-V4 16S rRNA amplicon sequencing. The extracted DNA samples were subjected to PCR for the 16S rRNA gene using a set of primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), and a PCR master mix (Promega) with 30 cycles of temperatures for denaturation (95°C), annealing (60°C) and elongation (72 °C). The PCR product then subjected for emperature of, and next-generation sequencing (NGS), starting from library preparation and sequencing. The V3-V4 area of the 16S rRNA gene was amplified, purified, sequenced, and then identified based on sequence similarity to references in the database. Amplification was carried out with a fragment length of 470 bp using primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (3'-GGACTACNNGGGTATTCTAAT-5') (Li *et al*., 2022). Sequencing was performed using the MGI DNBSEQ-G400 platform. Amplification and sequencing were performed by NovogeneAIT in Singapore. The gDNA samples were amplified using target-specific primers (16S V3-V4). Library preparation was carried out using the final PCR product. The final library was sequenced on the MGI platform to generate paired-end raw reads.

Data analysis was done using adapter primer sequences, and paired-end reads were removed using Cutadapt. DADA2 is used to correct sequencing errors and remove low-quality sequences and chimaera errors. The resulting Amplicon Sequence Variants (ASV) data was used for taxonomic classification against SILVA (silva_nr99_v138.1). Downstream analysis and visualisation were performed using packages in Rstudio (R version 4.2.3) (https://www.R-project.org/), Krona Tools (https://github.com/marbl/Krona), PICRUSt2 (https://github.com/picrust/picrust2).

Analysis of gene expressions in the intestine

Isolation of total RNA

RNA isolation was performed using Direct-zol™ RNA Miniprep Plus R2073 (Zymo Research Corporation) according to the specified protocol. The sample was lysed by mixing it with 800 µl of TRI Reagent®, centrifuged at 14.000 xg for 1 min, and then the supernatant was taken. Next, 800 µl of ethanol was added and centrifuged at 10.000-16.000 xg, 30 s. Then, the column was transferred to a new collection tube, and the supernatant was discarded. Next, 400 µl of RNA Wash Buffer was added to the column and centrifuged at 10.000-16.000 xg for 30 s. Then, the liquid was discarded. Add a mixture of 5 µl DNase I and 75 µl DNA Digestion Buffer to the column. Then, incubate at room temperature for 15 min. Add 400 µl RNA Pre Wash to the column, centrifuge 10.000-16.000 xg, 30 s. Then, the liquid is discarded, and the step is repeated. After that, 700 µl of RNA Wash Buffer was added to the column, centrifuged at 10.000-16.000 xg, 1 min, and then the liquid was discarded. The column was carefully transferred to the new microtube. Next, to elute RNA, add 100 µl DNase/RNase-Free Water to the column and centrifuge 10.000-16.000 xg, 30 s. Then, the supernatant (which already contained pure RNA) was taken.

RNA purity and concentration can be evaluated using a nanodrop spectrophotometer (Nanodrop 1000, Thermo Scientific, USA).

Reverse transcription-qPCR

Gene expression was analyzed using Reverse transcription-qPCR using PCR mix SensiFAST™ SYBR® Lo-ROX One-Step Kit (Bioline) according to a predetermined protocol. PCR mix consists of 10 µL SensiFAST™ SYBR® Lo-ROX One-Step Kit Mix (2x), 0.8 µL forward primer (10 µM), 0.8 µL forward primer (10 µM), 0.2 µL Reverse transcriptase, 0.4 µl RiboSafe RNase Inhibitor, >16 µL NFW, and 4 µl RNA template for a total final volume of 20 µL. The PCR mix was put into a PCR tube covered with optical strips and spun down. Next, it was entered into the reverse transcription-qPCR tool according to the maps prepared to go through the stages of the gene expression analysis process. RT-qPCR conditions were adjusted based on the instrument from the SensiFAST SYBR® Lo-ROX One Step Kit, namely reverse transcription at 45 °C for 10 min, polymerase activation at 95 °C for 2 min, denaturation for 5 s, annealing at 56.1 °C for 10 s, and extension at 72 °C for 5 s with 40 cycles. The primers used in this study are listed in Table 3.

Table 1. List of genes used in research.

Function	Name	Sequence	Reference
Energy balance (feed intake & metabolisme)	Ghrelin	F; GTGGTGCAAGTCAACCAGTG R: CATGGCTTGGCGACCAATTC	Amin et al. (2019)
Lipid and protein transport	CD-36	F: GCTAAATGAGACTGGGACCAT R: CACCACTCCAATCCCAAGTAA	Kiela & Ghishan (2016)
Lipid and protein transport	I-FABP	F: ACCGCCACCATGACTTTCAA R: GGTCCACGCACCTGATAGTT	Qiang et al. (2019)
Mucin genes	Muc-2	F: CAGCASTGGGGAACTTCCAC R: CATCGATGTTGTGTTCCTCAC	Marel et al. (2012)
Immune genes	IL- 1β	F: AAGGATGACGACAAGCCAAC R: CGCTGTGCTGATGTACCAGT	Shater et al. (2022)
Housekeeping	β-actin	F: GGCTGTGCTGTCCCTGTA R: GGGCATAACCCTCGTAGAT	Aditama (2022)

Measurement of gene expression levels

Gene expression quantities were calculated based on RT-PCR quantification's melting curve (Ct) results. Calculation of gene expression values is based on Schmittgen & Livak (2001):

$$\text{Gene expression} = 2^{-\Delta\Delta Ct}$$

Data analysis

Analysis of bacterial microbiome data in the intestine is explained descriptively by comparing the literature. Analysis of microbiome data on adapter primer sequences and PCR of paired-end deletion reads using Cutadapt (Bellemain *et al*., 2010). Furthermore, DADA2 is used to correct sequencing errors, remove low-quality sequences, and chimera errors (Martin, 2011). The resulting ASV data were used for taxonomic classification against SILVA (silva_nr99_v138.1). Final analysis and visualization were performed using RStudio (version 4.2.3) (https://www.R-project.org/), Krona Tools (https://github.com/marbl/Krona), and PICRUSt2 (https:// github.com/picrust/picrust2). The qPCR results will be processed using Microsoft Excel software for calculation of gene expression using the Schmittgen & Livak (2001) method. Relative gene expression parameters were explained using t-test.

RESULTS AND DISCUSSION

Growth performance and survival rate

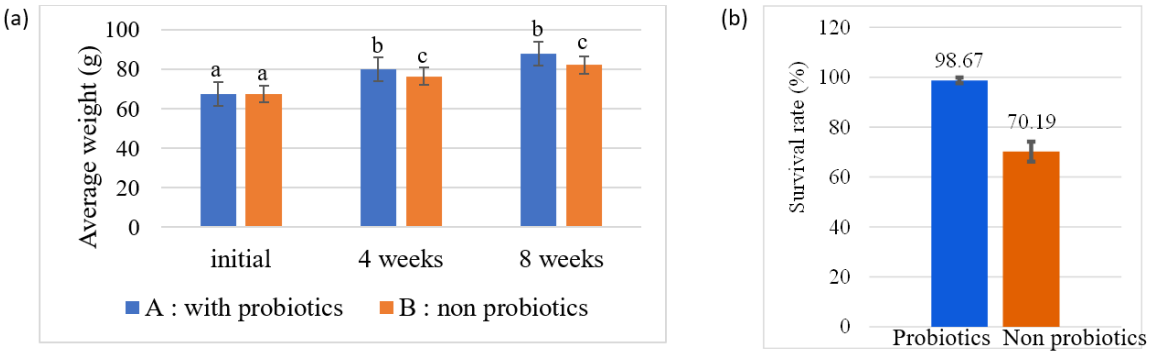


Figure 1. Average weight (g) of tilapia treated with probiotics and not given probiotics (a), survival rate of tilapia treated with probiotics and not given probiotics (b).

The results of observations of the weight growth of tilapia treated with probiotic application and without probiotic application can be seen in Figure 1[a]. Based on Figure 1[a], the highest average growth based on weight in the fourth and eighth weeks was in treatment A (with probiotics), while the lowest average weight growth was in treatment B (without probiotics). The data obtained met the requirements, namely, standard and homogeneous distribution. Based on the results of the T test, it is determined that T count > T table. Therefore, Ho (probiotic application has no effect) is rejected, so it can be concluded that probiotic application influences increasing weight growth in tilapia. There was a significant difference in weight growth between probiotic treatment and without probiotic treatment.

The survival rate results in Figure 1[b] show that treatment A has the highest average SR value, 98.67%, while treatment B has the lowest, 70.19%.

The gut bacterial microbiome

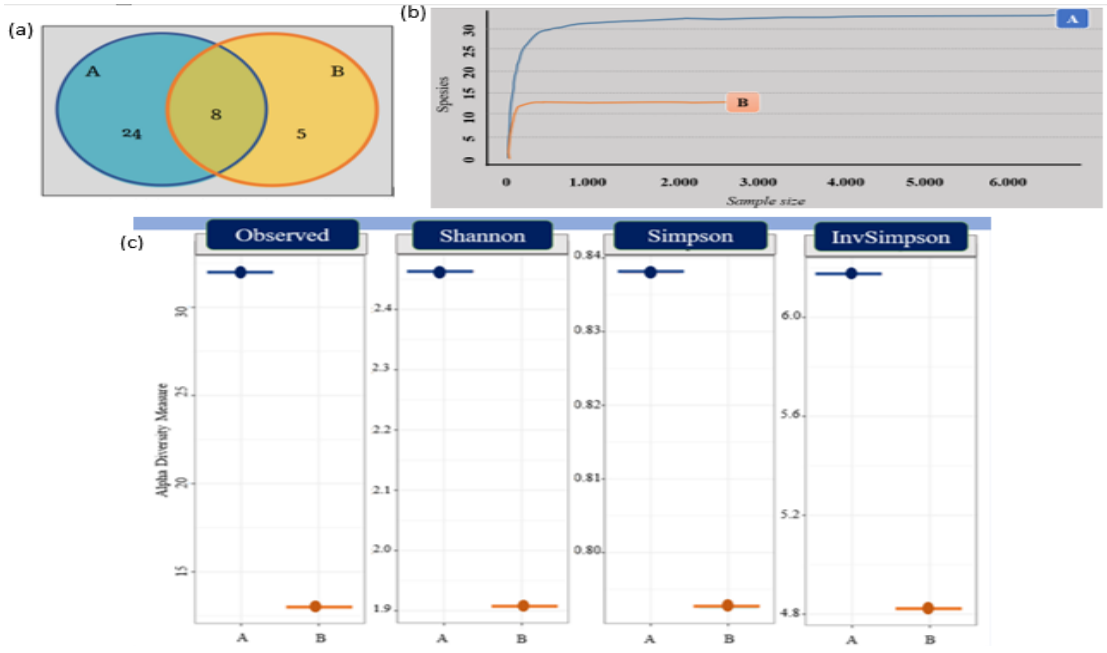


Figure 2. (a) Venn diagram of the number of intestinal microbial communities of tilapia, (b) rarefaction curve of the gut microbiome of tilapia, and (c) results of alpha diversity measurements in intestinal samples treated with probiotics (A) and those not treated with probiotics (B).

The diversity of the microbiome in the tilapia intestine can be seen using a Venn diagram created from the Amplicon Sequence Variant (ASV) distribution pattern between treatments (Figure 2a). According to Plessis *et al*. (2023), the Amplicon Sequence Variant (ASV) is one of the single DNA sequences obtained from marker gene sequencing analysis with high yield. Because the sequences in ASV are created after the deletion of erroneous sequences during PCR and sequencing, ASV can be used to distinguish sequence variations by single nucleotide changes, classify species groups by species based on DNA sequences, and discover microbes of a species.

Eight common ASVs were identified in the two treatments, so 8 ASVs were found in the probiotic (A) and no probiotic (B) treatments. Unique ASVs in each treatment varied in number. A total of 24 unique ASVs were found in treatment A (probiotic treatment), and five unique ASVs were identified in treatment B (without probiotics). These results show that the microbial diversity of tilapia given probiotics (A) is higher than the microbes in the intestines of tilapia without probiotics (B).

Rarefaction curves can be used to analyses the sequence distribution of each sample. Rarefaction curves can be a factor in the suitability of sequencing data and indirectly reflect the abundance and diversity of bacterial communities (Kong *et al*., 2023). All samples showed a stable curve (Figure 2[b]), which indicated that the sequencing results met the requirements. The shape of the curve reflects the even distribution of bacteria in each community. The rarefaction curve above shows that species diversity was highest in the treatment with probiotics (A) and lower in the treatment without probiotics (B).

Based on (Figure 2[c]), it is known that, based on the results of alpha diversity analysis, in intestinal samples treated with probiotics, the microbial diversity was higher than in those without probiotics. In the probiotic treatment (A), the alpha diversity value was 32, while in the treatment without probiotics (B), it was 13. Furthermore, microbiota diversity can be measured using the Shannon index. In their statement, Yin *et al*. (2019) said that "The Shannon Index is a quantitative indicator of species diversity by considering the number of species and the abundance of each species and considering the uniformity of species distribution in a community". The increasing number of species and evenness can increase the diversity index value. The higher the value of the Shannon index, the higher the value of bacterial microbial diversity (Yin *et al*., 2019). The Shannon index value in the probiotic treatment (A) was 2.463, while in the treatment without probiotics it was 1.907.

According to Poulsen *et al*. (2021), the Simpson index can reflect the diversity of the microbial community, which is measured based on species dominance and has a value range between 0 and 1. The higher the Simpson index, the more the species contained in the sample are evenly distributed and not dominated by one or several particular species. Therefore, an increasing Simpson index value indicates high species diversity in the sample. The Simpson index value in the probiotic treatment (A) was 0.838, while in the treatment without probiotics (B), it was 0.793. Based on this statement, the microbial diversity in the intestines of tilapia given probiotics is higher than without probiotics. The InvSimpson value in the probiotic treatment (A) was 6.175, while in the treatment without probiotics (B), it was 4.822.

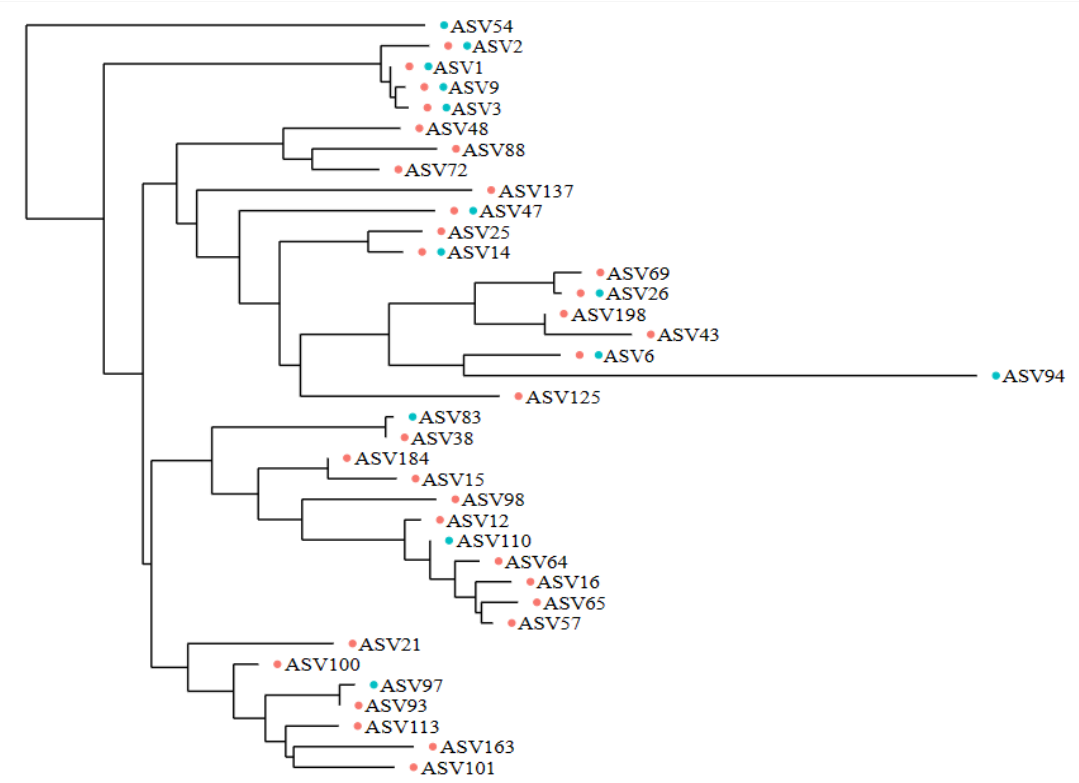


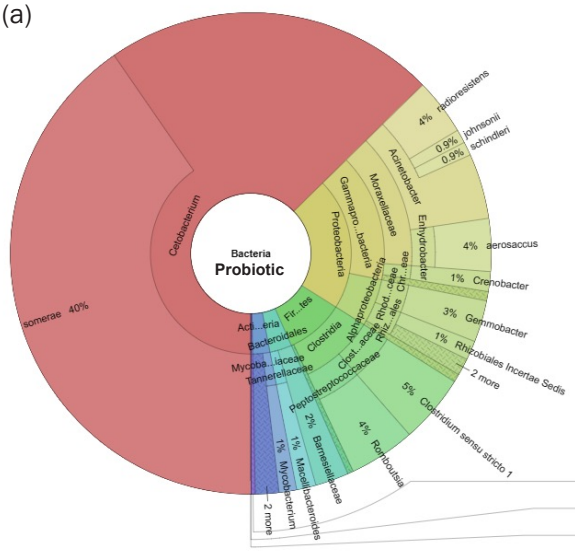
Figure 3. Phylogenetic tree based on the neighbour-joining (NJ) algorithm in treatments given probiotics (A, red) and without probiotics (B, blue).

The species taxonomic tree in Figure 3 is arranged based on the neighbour-joining (NJ) algorithm. Based on the phylogenetic tree above, the most abundant bacterial diversity was detected in the probiotic treatment (A). The species detected in the treatment with probiotics (A) were more diverse when compared to the treatment without probiotics (B).

There were eight common ASVs identified in the two treatments, both treated with probiotics (A) and without probiotics (B), namely *Somerset* Species (ASV1), Cetobacterium Genus (ASV2), *Somerae* Species (ASV3), Romboutsia Genus (ASV6), Genus Cetobacterium (ASV9), Genus Clostridium sensu stricto 1 (ASV14), Family Barnesiellaceae (ASV26), and Genus Turicibacter (ASV47). There were five unique ASVs in the treatment without probiotics, including the Isosphaeraceae family (ASV54), the Crenobacter genus (ASV83), the Vermiphilaceae family (ASV94), the Rhizobiales Incertae Sedis family (ASV97), the *Acinetobacter* genus (ASV110).

There were 24 unique ASVs in treatment A (which were only found in the treatment with probiotics), consisting of Genus Acinetobacter (ASV12), Genus Enhydrobacter (ASV15), Genus Acinetobacter (ASV16), Genus Gemmobacter (ASV21), Genus Clostridium sensu stricto 1 (ASV25), Genus Crenobacter (ASV38), Genus Macellibacteroides (ASV43), Genus Mycobacterium (ASV48), Family Moraxellaceae/ Genus Acinetobacter (ASV57), Family Moraxellaceae/ Genus Acinetobacter/ Species *johnsonii* (ASV64), Family Moraxellaceae/ Genus Acinetobacter/ Species *schindleri* (ASV65), Order Bacteroidales/ Family Barnesiellaceae (ASV69), Family Microbacteriaceae/ Genus Aurantimicrobium/ Species *minutum* (ASV72), Family Propionibacteriaceae (ASV88), Family Rhizobiales Incertae Sedis (ASV93), Genus Plesiomonas/ Species *shigelloides* (ASV98), Family Rhizobiales Incertae Sedis (ASV100), and the Genus Macellibacteroides (ASV198).

(a)



(b)

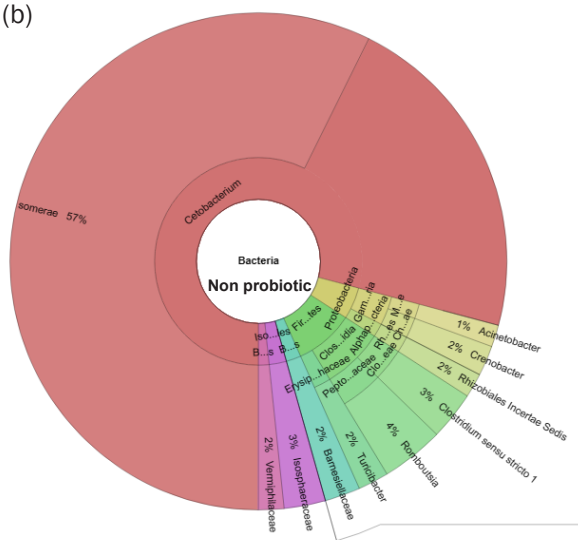


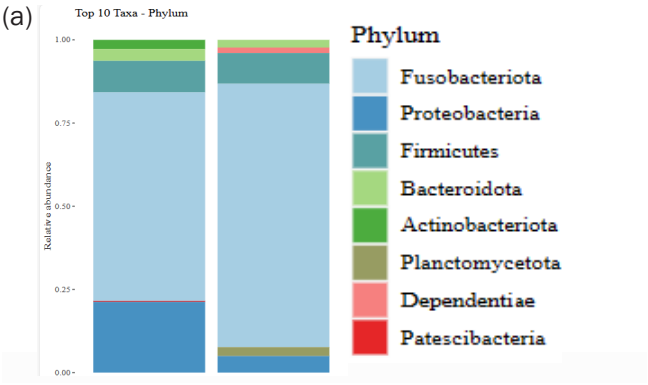
Figure 4. Chronic chart representing the taxonomic composition of all bacteria in the intestines of tilapia treated with probiotics (a) and non-probiotics (b).

Based on the chrona analysis in (Figure 4[a]) above, it is known that the total bacteria detected in the treatment with probiotics was higher than in the treatment without probiotics, namely 6.442 (Figure 4[a]). The intestines of tilapia treated with probiotics were dominated by Cetobacterium at 63%, which included *Somerae* species at 40%. Furthermore, the Proteobacteria phylum dominates at 21%, the phylum Firmicutes at 9%, the order Bacteroidales at 4%, and the phylum Actinobacteria at 3%.

Based on the chronic analysis (Figure 4[b]), it is known that the total bacteria detected in the treatment without probiotics was less than in the treatment with probiotics. The number of known bacteria in the treatment without probiotics was 2.599. The intestines of tilapia that were not given probiotics were dominated by Cetobacterium at 78%, which included *Somerae* species at 57%. Furthermore, Proteobacteria phylum Proteobacteria dominates it at 6%, the phylum Firmicutes at 9%, the Order Bacteroidales at 2%, Isosphaerales at 3%, and Babeliales at 2%.

Based on the results of the analysis of the top ten taxa of tilapia gut bacteria at the phylum level (Figure 5[a]), the Fusobacteriota phylum was found in the probiotic treatment and without probiotics. According to Bereded et

(a)



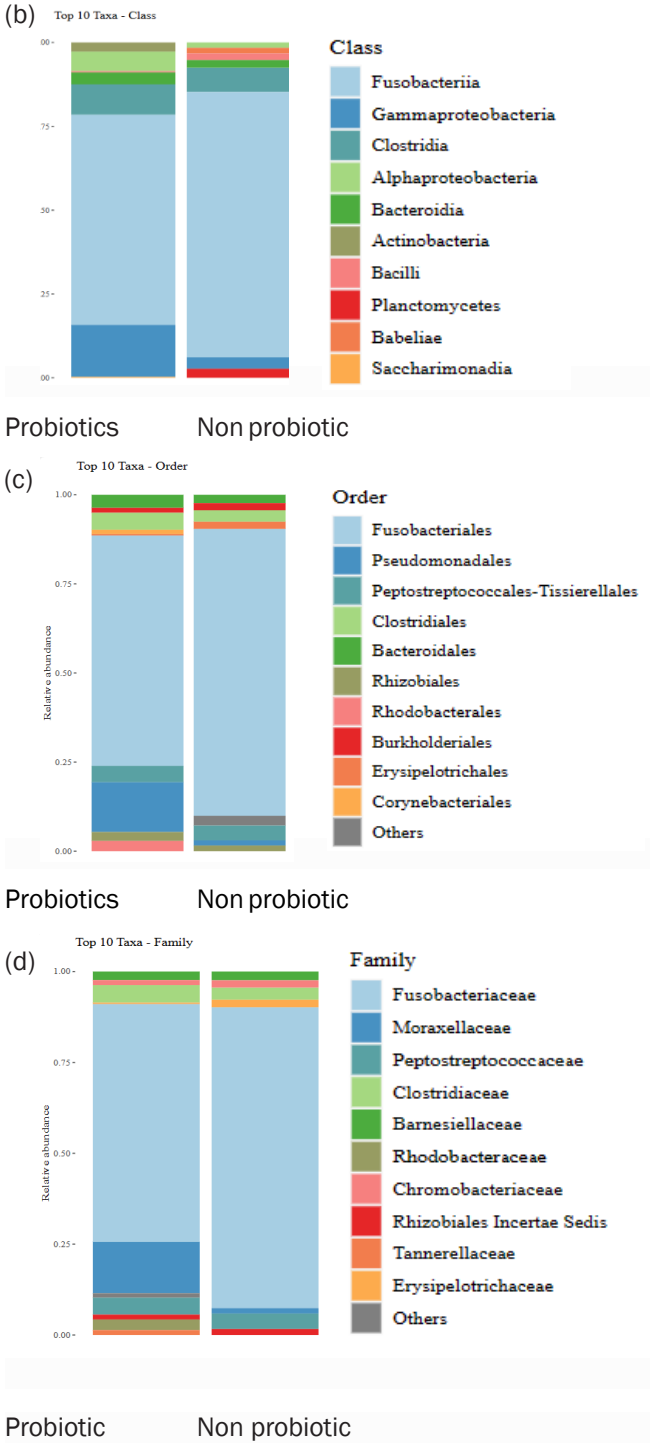


Figure 5. Top ten taxa of tilapia gut bacteria at phylum (a), class (b), order (c), and family (d) level.

al. (2022), the number of Fusobacteriota is inversely proportional to the abundance of Actinobacteriota and Firmicutes. This statement is also in line with Bi *et al*. (2023) that when Firmicutes increase, the number of Fusobacteria will decrease. According to the research results obtained in the probiotic treatment, the number of Fusobacteria decreased compared to the treatment without probiotics. However, the number of Actinobacteria and Firmicutes in the probiotic treatment increased. According to Mao *et al*. (2024), Fusobacteriota can indirectly increase mucus secretion and produce butyrate as an anti-inflammatory. Fusobacteria can reduce the risk of intestinal inflammation and damage to intestinal tissue.

The Proteobacteria in the probiotic treatment were increased. The high number of Proteobacteria plays an essential role in the mucosal microbial barrier. It contains various opportunistic pathogens so that it can stimulate the improvement of the immune system and maintain immune function (Wu *et al*., 2021). According to Xia *et al*. (2020), Proteobacteria play a crucial role in differential metabolite production.

The probiotic treatment had a higher number of Firmicutes. According to Zheng *et al*. (2018), Firmicutes is a phylum that is abundant in the digestive tract of tilapia after Proteobacteria. The Firmicutes phylum can provide nutrition for intestinal mucosal cells through the production of short-chain fatty acids and can regulate the intestinal microecological environment (Wu *et al*., 2021). According to Bereded *et al*. (2022), Firmicutes can produce several enzymes for the degradation of food nutrients so that they can help the host in the digestion and absorption of nutrients. High numbers of Firmicutes in the gut microbiota are positively correlated with efficient absorption of dietary energy. Tilapia, with a high number of Firmicutes, are also more efficient in collecting energy, thus helping them adapt to the environment (Bereded *et al*., 2022).

Based on the research results obtained, the Actinobacteria phylum was only found in the intestines of tilapia treated with probiotics. According to Euanorasetr *et al*. (2020), based on microbiome analysis, Actinobacteria were identified in the intestines of tilapia fish. The relative abundance of Actinobacteria in the intestine is higher than elsewhere. Actinobacteria can produce antibiotics that can inhibit the growth of intestinal pathogenic bacteria. Therefore, the abundance of Actinobacteria can improve gut health, thereby having a positive impact on the fish's immune system.

Based on (Figure 5[a]), the research results show that the Phylum Patescibacteria was found in the probiotic treatment and was not found in the treatment without probiotics. According to Yang *et al*. (2023), Patescibacteria in the intestine are associated with genes involved in glucose and lipid metabolism and can ameliorate the unfavorable adverse effects of a high-fat diet. Planctomycetota was only detected in the treatment without probiotics and was not found in the probiotic treatment. Based on Zhang *et al*. (2023), Planctomycetota will increase along with a decrease in the number of Firmicutes and Bacteroidetes. This is following the results of this study that in the treatment without probiotics, the abundance of Firmicutes and Bacteroidetes was lower, so in the treatment without probiotics, the presence of Planctomycetota was detected. Menurut M. Wang *et al*. (2022), Planctomycetes is the main phylum in tilapia which has a negative effect on tilapia survival and intestinal development.

Based on the results of the analysis of the top ten taxa of tilapia gut bacteria at the class level (Figure 5[b]), the results showed that the abundance of Fusobacteriia in the treatment without probiotics was higher than in the probiotic treatment. Increasing the number of Fusobacteria can reduce the relative abundance of Clostridia (Peng *et al*., 2019). Fusobacteria have an elongated shape, so they are classified as bacilli bacteria. Fusobacteria, under optimal environmental conditions and sufficient energy sources, can live in harmony with their hosts without causing disease.

Gammaproteobacteria were more abundant in the pro-

biotic treatment than in the treatment without probiotics. According to Lan & Love (2012), the abundance of Gammaproteobacteria is inversely proportional to the abundance of Bacilli. If Gammaproteobacteria increases, Bacilli tend to decrease. This is following the results of research conducted that showed that in the treatment of probiotics, the number of Gammaproteobacteria increased while the number of Bacilli decreased. In this study, the results showed that Bacilli were found to be more numerous in the treatment without probiotics than in the probiotic treatment.

Clostridia were more abundant in the probiotic treatment than in the treatment without probiotics. Clostridia include gram-positive bacteria as an important and essential part of the total bacteria in the intestinal microbiota. Clostridia play an important role in physiological, metabolic, and immune processes in the intestine. Clostridia are involved in maintaining intestinal function and improving intestinal health (Lopetuso *et al*., 2013). The abundance of Alphaproteobacteria was higher in the probiotic treatment compared to the treatment without probiotics. Alphaproteobacteria have a negative correlation with Vibrio (Hu *et al*., 2024). The high abundance of Alphaproteobacteria causes the number of vibrios to decrease. According to Egerton *et al*. (2018), pathogenic vibrios can infect fish and cause sudden and significant death.

There were more Bacteroidia in the probiotic treatment than in the treatment without probiotics. According to Bi *et al*. (2023), Bacteroides is the most common and dominant bacterium in the intestines and habitat of tilapia fish. Habitat will influence the number of bacteria in the intestines of tilapia. Bacteroides are anaerobic bacteria that play a role in the fermentation process of intestinal contents and the metabolic production of short chain short-chain fatty acids and can fight host enteritis (Liu *et al*., 2022).

Saccharimonadia was only detected in probiotic treatment and is a bacterium found in the intestines of fish. These bacteria can improve fish health by breaking down cellulose and producing short-chain fatty acids as a source of fish energy (Liu *et al*., 2021). Planctomycetes were detected in the treatment without probiotics and not detected in the probiotic treatment. Planctomycetes are included in the fish gut microbiota, which maintains intestinal health (Wang *et al*., 2022). Planctomycetes can use carbohydrate fermentation for growth and survival. An increase in microbial communities that can ferment digestible carbohydrates under anaerobic conditions in the intestine was found in tilapia that were not given probiotics (Wang *et al*., 2022).

Barbeliae were only found in the intestinal tissue of tilapia, which was not treated with probiotics. In the probiotic treatment, no Barbeliae were found. Barbeliae is a potential fish pathogen that causes death in infected fish. Fish infected with Barbeliae show symptoms of decreased appetite, causing decreased growth performance (Liu *et al*., 2021). The negative impacts of Barbeliae infection in fish intestines are not entirely known, but several studies have shown that bacterial infections in fish intestines affect fish health and reduce fish growth (Wang *et al*., 2023; Sutarni *et al*., 2021). Based on the results of the analysis of the top ten taxa of tilapia gut bacteria at the order level (Figure 5[c]), the abundance of the Order Pseudomonadales in the probiotic treatment was higher than in the treatment without probiotics. Pseudomonadales are known to dominate the intestines of freshwater fish species. Pseudomonadales have a role in the microbiome that is beneficial to the host in areas such as nutrient absorption, digestion, and the formation of immune responses (Talwar *et al*., 2018). The treatment without probiotics had a higher abundance of the Erysipelotrichales. Erysipelotrichales have high abundance in the intestinal tract but can cause host metabolic disorders and inflammatory diseases (Wu *et al*., 2021).

The abundance of the Clostridiales order in the probiotic treatment was higher. The abundance of Bacteroidales in the probiotic treatment was higher than without probiotics. Bacteroidales, Clostridiales, and Fusobacteriales will increase when the animal protein contained in the feed is high (Michl *et al*., 2017). The treatment without probiotics had a higher abundance of the Burkholderiales. The order Burkholderiales consists of several species of environmental and pathogenic bacteria (Meng *et al*., 2021).

Based on the results of the analysis of the top ten taxa of tilapia gut bacteria at the family level (Figure 5[d]), it was found that the Barnesiellaceae family was found to be more abundant in the treatment without probiotics. Barnesiellaceae constitutes a large proportion of water pollution and is a common pathogen in fish (Zhang *et al*., 2022). The Caulobacteraceae family was only found in probiotic treatment. According to Caulobacteraceae, it has a mutualistic relationship with fish hosts by protecting fish against opportunistic infections (Dvergedal *et al*., 2020).

The Chromobacteriaceae family was found most frequently in the treatment without probiotics. Chromobacteriaceae is a family of bacteria commonly found in aquatic environments. It has been isolated from fish and can act as pathogens and commensals. The Clostridiaceae family was more abundant in the treatment with probiotics. These bacteria can ferment carbohydrates and produce short-chain fatty acids (Guo *et al*., 2020).

The number of Erysipelotrichaceae was higher in the treatment with probiotics compared to the treatment without probiotics. Erysipelotrichaceae is an indicator that is abundant in herbivorous/omnivorous fish, which plays a role in forming the gut microbial community. Erysipelotrichaceae can also contribute to producing cellulase and amylase (Huang *et al*., 2020). The number of Moraxellaceae was higher in the treatment without probiotics. Moraxellaceae It is known that many species in this family can cause infections in humans and animals, including fish.

Based on the results of the analysis of the top ten taxa of tilapia gut bacteria at the genus level (Figure 6[a] & 6[b]), it was found that although the *Cetobacterium* genus was found in both treatments, based on the results of the analysis, it was found that the probiotic treatment could increase the number of *Cetobacterium* in the intestinal tract. According to Zhang *et al*. (2022), *Cetobacterium* is a group of intestinal bacteria that dominate freshwater fish and play a role in producing amino acids. *Cetobacterium* can use starch, sucrose, maltose, glucose, and mannose and can synthesise and utilize glycogen. *Cetobacterium* can catabolise various vitamins and all the genes involved in folate synthesis. According to Liu et al. (2023), *Cetobacterium* can produce vitamin B12 in the

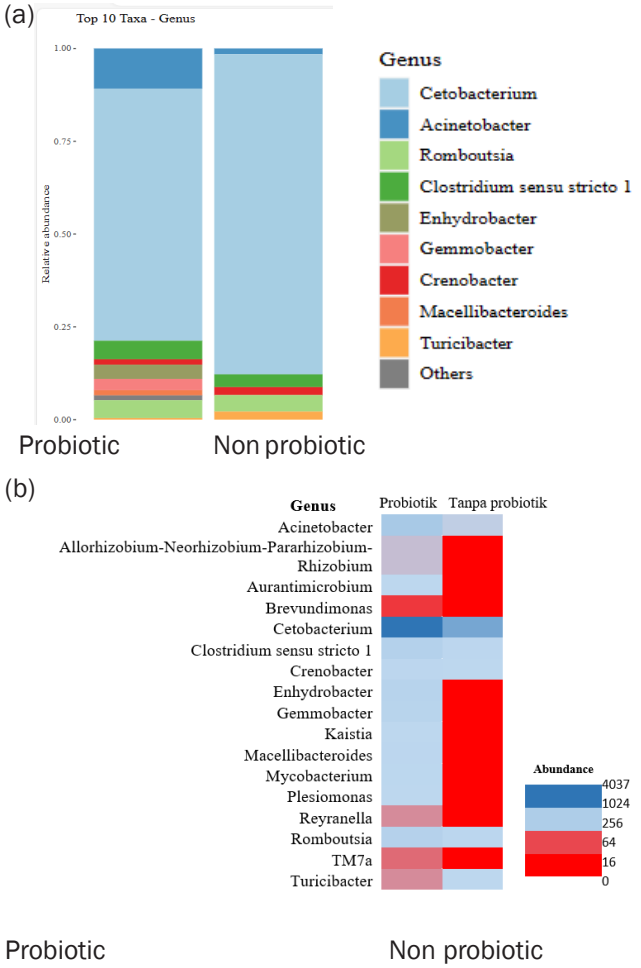


Figure 6. Top ten taxa (a) and cluster heatmap (b) of tilapia gut bacteria at the genus level treated with probiotic and non-probiotic.

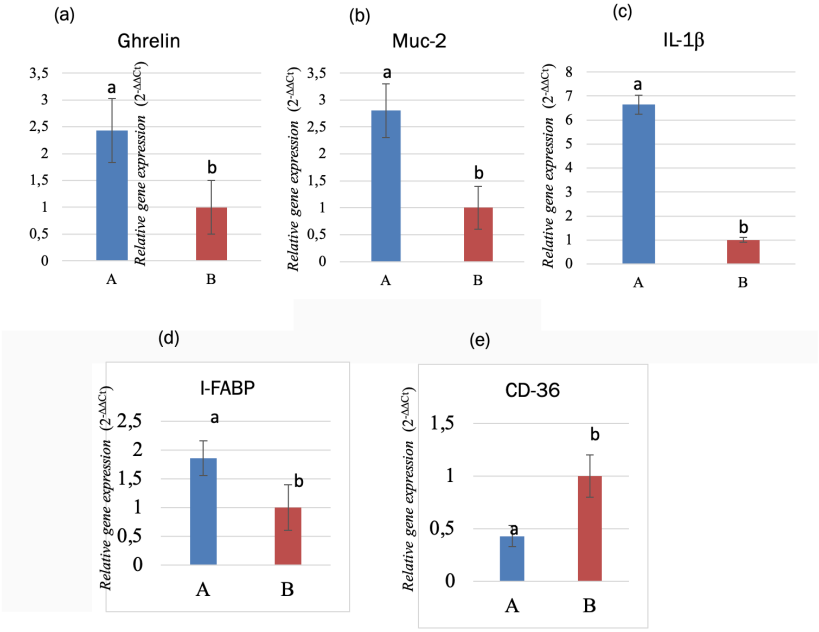
carbohydrate fermentation process and plays a vital role in nutrient metabolism. According to Liu *et al*. (2023), *Cetobacterium* can improve intestinal health through reducing intestinal inflammation and lipid deposition. In addition, *Cetobacterium* metabolites such as short-chain fatty acids are dominant in the fish intestine and are beneficial for the fish intestine. The presence of *Cetobacterium* will increase the expression of genes involved in lipid metabolism (Liu *et al*., 2023).

Romboutsia increased more in the treatment with probiotics than without probiotics. According to Fan *et al*. (2024), the abundance of the genus *Romboutsia* is positively correlated with food sugar content. *Romboutsia* strains are often found in the intestines of the tilapia fish, which can convert carbohydrates into SCFA during fermentation. *Crenobacter* increased more in treatment without probiotics. *Crenobacter* is a gram-negative, facultative anaerobic bacterium, often found as a pathogen in fish (Das *et al*., 2021).

Gene expression in the fish intestine

Based on Figure 7[a], it is known that on observation during the four weeks of maintenance, it was discovered that the Ghrelin gene in the probiotic treatment (A) increased more than in the treatment without probiotics (B). The effect of giving probiotics is known to stimulate the expression of genes related to growth, namely the Ghrelin gene. According to Ghalwash *et al*. (2021), the addition of probiotics can induce upregulation of ghrelin gene expression. Therefore, based on Ghalwash *et al*. (2021), the application of probiotics will increase the expression of the ghrelin gene, thereby allowing increased growth of tilapia fish. According to (Schalla & Stengel, 2020), changes in the microbiome are associated with changes in ghrelin expression, secretion, activation, and signaling.

The expression of the Muc-2 gene in the treatment given probiotics (A) was higher than in the treatment with-



A : Probiotics B : Non probiotics

Figure 7. Expression of Ghrelin (a), Muc-2 (b), IL-1β (c), I-FABP (d), and CD-36 (e) genes in the intestines of tilapia treated with probiotics (A) and non-probiotics (B).

out probiotics (B) (Figure 7[b]). According to Midhun *et al*. (2019), mucin acts as the main barrier that consistently selects and transports essential materials through the intestinal epithelium and eliminates pathogens. One of the mucin genes expressed in the intestinal tract is Muc-2. Based on research by Midhun *et al*. (2019), it is known that the application of probiotics can increase the regulation of the Muc-2 gene in fish intestinal tissue. Mucin has a protective layer that plays a role in the immune response against microbial invaders.

The expression of the IL-1β gene in the treatment given probiotics (A) was higher than in the treatment without probiotics (B) (Figure 7[c]). According to Wang *et al*. (2021), interleukin-1β (IL-1β) is the main pro-inflammatory cytokine that functions in the inflammatory response to bacterial and viral infections. Based on research conducted by Wang *et al*. (2021), IL-1β functions in immune regulation and increases fish resistance to bacterial infections so that it can increase a higher survival percentage. The results showed that the expression of the I-FABP gene in the probiotic treatment was higher than in the treatment without probiotics (Figure 7[d]). According to Debnath & Saikia (2021), intestinal fatty acid-binding protein (I-FABP) plays a role in binding peptides or proteins before endocytosis. Gut microbes can facilitate protein absorption. Therefore, the application of probiotics can increase the expression of genes related to protein transport, so that it will help with protein absorption, which will increase the growth of fish. This is in accordance with the results of previous research, that fish growth with probiotic application was higher than without probiotics (Latifah, 2023). The expression of the CD-36 gene in the probiotic treatment (A) was lower than in the treatment without probiotics (B) (Figure 7[e]). This result is related to the results of microbiome analysis in the intestine, which shows that the number of Cetobacterium in the probiotic treatment (A) is lower than in the treatment without probiotics (B). This is following Y. Liu *et al*. (2023) that when the number of Cetobacterium is low, it will be associated with a decrease in the expression of genes involved in lipid metabolism. This situation in the research carried out occurred in the treatment given probiotics (A), where in this treatment, from the results of microbiome analysis, it was found that the number of Cetobacterium was lower than in the treatment without probiotics (B). This correlates with the lower CD-36 gene expression in the probiotic treatment (A) than in the treatment without probiotics (B).

Discussion

In general, the average growth of tilapia treated with probiotic application was higher than that without probiotic application (Figure 1a). After 60 days of maintenance, the tilapia fish given probiotics weighed an average of 88 g, while the non-probiotics weighed 80 g. In addition, after 60 days of maintenance, tilapia fish given probiotics had a specific growth rate of 0.967%, while non-probiotics had an absolute weight of 0.612%. Based on research by Aisyah *et al*. (2020), tilapia fish given probiotics (*Saccharomyces* sp., *Enterobacter* sp. JC10, *Aeromonas* sp. JC33, and *Lactococcus* sp. JAL12) on 100% pelleted feed produced a specific weight growth of 0.97% (probiotics daily), 0.96% (probiotics three-day interval), while for those not given probiotics it was 0.85. Tilapia (*Oreochromis niloticus)* with *Metschnikowia* sp. GXUS03 on pellet with a dose of 10^7 has a specific weight of 0,96% (Liao *et al*., 2023). This is because, according to Abdel-Latief *et al.* (2023), the application of probiotics to feed can improve the digestive process so that fish can grow well. Dewanti *et al*. (2022) reported that *Bacillus* sp. PCP1, *Enterobacter* sp. JC05, and *Lactococcus* sp. JAL37 in the intestine has proteolytic, cellulolytic, and lipolytic activity. The three probiotic strains are safe because it does not produce changes in behavior or death in eel fish, but these three bacteria have low adherence to intestinal epithelial cells shortfin eel (*A. bicolor bicolor*). A picture of the number of tilapias' gut microbial communities can be seen in Figure 4. Based on (Figure 4), the number of intestinal microbial communities in tilapia treated with probiotics (A) is higher than those treated without probiotics (B). This shows that the application of probiotics increases the diversity of the intestinal microbial community.

The level of diversity of the good bacterial community in the intestine will have a good effect on fish. It has been found that several bacteria from the intestines of tilapia treated with probiotics have been found to play a role in feed digestion, nutrient absorption, improving the immune system, and inhibiting pathogens. Therefore, because of the diversity of bacteria in the intestines of fish treated with probiotics, the growth of fish in the probiotic treatment was higher than in the treatment without probiotics. Yin *et al*. (2019) stated that healthy and intact microbiota can increase disease resistance, so that loss of microbial diversity can cause an increase in mortality rates. This statement is in accordance with the results of research that has been carried out previously, which found that the survival rate of tilapia treated with probiotics was higher than without probiotics. The phylogenetic tree (Figure 3) was created based on the Neighbor Joining (NJ) algorithm. According to (Saitou & Nei, 1987), the Neighbor Joining (NJ) method is a grouping method used to create phylogenetic trees based on DNA or protein sequence data. The Neighbor Joining (NJ) method uses a distance matrix to build a tree by determining which members are "neighbors" through an iterative grouping process. Based on the phylogenetic tree (Figure 4[a]), ASVs were grouped into three groups, namely general ASVs, ASVs unique to the probiotic treatment, and ASVs unique to the treatment without probiotics. Eight common ASVs were identified in the two treatments, both given and without probiotics, including bacteria that often dominate in freshwater fish and play a role in anaerobic metabolism (Zhang *et al*., 2022). There were five unique ASVs in the treatment without probiotics, including bacteria often found as pathogens in fish (Das *et al*., 2021). The unique ASVs of treatment A (only found in the probiotic treatment) numbered 24, including bacteria that often dominate in the intestines of healthy fish with a role in improving fish health, feed digestibility and host growth (Bereded *et al*., 2022) and (Wang *et al*., 2022).

A chronic chart representing the taxonomic composition of all bacteria in the intestines of tilapia treated with probiotics can be seen in Figure 4[a]. The intestines of tilapia that were given probiotics were dominated by Cetobacterium, which included the Somerae species, Phylum Proteobacteria, Phylum Firmicutes, Order Bacteroidales, and Phylum Actinobacteria. Based on the chron analysis (Figure 4[b]), it is known that the total bacteria detected in the treatment without probiotics was less than in the treatment with probiotics. In both probiotic and non-probiotic treatments, the percentage of some species was

the highest compared to the others. According to Zhang *et al*. (2023), *somerae* are anaerobic bacteria that occupy an important ecological niche in the intestinal tract of freshwater fish. However, the potential function of somerae is still unknown. *Some species of bacteria include those* with connections to fish, can thrive in diverse environments, and contribute to vitamin production (Merrifield & Ringo, 2014).

Based on the gene expression analysis results (Figure 6), it is known that the expression of the Ghrelin gene in the probiotic treatment is higher than in the treatment without probiotics. This correlates positively with the growth of fish treated with probiotics, which was better than that of those treated without probiotics. Therefore, the growth of fish treated with probiotics has been confirmed to increase the expression of genes related to growth, namely Ghrelin. Based on the results of Muc-2 gene expression (Figure 6), it is known that the Muc-2 gene in the probiotic treatment was higher than in the treatment without probiotics. This is related to previous research (Murti *et al.*, 2023) that found that the number of goblet cells in probiotic treatment was higher than in treatment without probiotics. Because probiotics can stimulate goblet cells to activate mucin gene expression, probiotics can increase mucin gene expression in the intestine. This is due to the results of research conducted that showed that mucin gene expression was higher in the probiotic application treatment than in the treatment without probiotics.

Based on (Figure 6), the expression of genes related to immunity (IL-1β) is higher in probiotic treatment than in treatment without probiotics. This positively correlates with the results of previous research that applying probiotics can increase tilapia's survival (Latifah, 2023). Survival is related to immunity, so the expression of immune genes in the intestine needs to be observed. Good survival is proof that probiotics have a function in improving immunity, so research is needed to check whether improved survival is related to improved immunity. Based on the results of the research carried out, the expression of genes related to immunity increased, so this was related to improved survival. The increase in survival in probiotic treatment proves that, based on the analysis results, the expression of genes related to immunity (IL-1β) also increased. Therefore, the application of probiotics has a good effect on increasing immune gene expression, which can increase survival.

The bacteria applied in this research were *Bacillus* sp. PCP1, which has lipolytic activity. Based on the gene expression analysis related to fat transport (CD-36), CD-36 was lower in the probiotic application treatment than in the treatment without probiotics. This is because in the gut microbiome of tilapia that were given probiotics, high levels of Cetobacterium bacteria were found, causing low expression of genes related to fat transport, apart from *Bacillus* sp. PCP1, the bacteria applied orally in this study, is *Lactococcus* sp. JAL37, which has proteolytic activity. Therefore, to see the effect of probiotic application, an analysis of the expression of genes related to protein transport (I-FABP) was carried out in this study. The results showed that the expression of the I-FABP gene in the probiotic treatment was higher than in the treatment without probiotics. Therefore, applying probiotics can increase the expression of genes related to protein transport, which will help with protein absorption and increase fish growth.

CONCLUSION AND RECOMMENDATION

Conclusion

The application of probiotics can increase the growth performance of bacteria in the intestines, increase the expression of the Ghrelin, Muc-2, IL-1β, and I-FABP genes, but does not increase the expression of the CD-36 gene in tilapia (*Oreochromis* sp.) fed Maggot (*Hermetia illucens*).

Recommendation

These findings suggest oral probiotics can help boost tilapia production when fed maggot black soldier fly (*Hermetia illucens*).

AUTHOR'S CONTRIBUTION

MNL, II, and AI designed the study. MNL and FAUM carried out laboratory work. MNL, SH, and II analysed the data. MNL and II wrote the manuscript. MM and AI review the manuscript. All authors read and approved the final version of the manuscript.

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