The effect of probiotics on digestive enzyme activity during larvae and juvenile stage of Yellow Fin Tuna (*Thunnus albacares*)

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**Abstract.** Yellow fin tuna fry production technology is not successful yet. This was assumed related to the biological and physiological characteristics of the fish. The physiological approach to regulating digestion through probiotics needs to be studied. The aim of this study was to examine the effect of probiotics bacterial strains of the enzymatic activity in digestion system for the larvae to juvenile stages of yellow fin tuna, *Thunnus albacares*. The experiment was initiated by culturing 3 probiotic isolates (*Bacillus subtilis* strain TA-1, *Bacillus amyloliquefaciens* strain TN-2, *Bacillus subtilis* strain TO-4) and apply to the larval rearing of yellow fin tuna. In the experiment the treatments applied were supplemented probiotics and without probiotic, each treatment was held with 2 replications. Fish samples were collected every day and analyze the gene expression profile associated with digestion enzyme synthesis by total RNA isolation and cDNA synthesis followed analysis of its by using the RT –qPCR method. The results showed that the growth response of *T. albacares* tuna larvae reared with probiotics tended to be faster (P<0.05) than the control (without probiotics). The supplementation of probiotic could improved the expressed digestibility of the target genes associated with enzymatic synthesized. Amylase enzymatic synthesized increase 200-1200 times in larvae 10 –17 Day After Hatch (DAH), while in the lipase enzymatic activity started with 13 DAH ( 25 time) and 16 – 21 DAH by 150-300 times. In trypsin the highest enzymatic activity was at 16 DAH, which was 200 times. While in control, the expression of enzymatic synthesis of amylase, lipase, and trypsin was relatively low.

1. **Introduction**

Mortality problems during the yellow fin tuna seed production are still mostly unknown for certain, which include the suitability of larvae – juvenile handling techniques, the suitability of the foods given both living food and artificial food and other biological and physiological characteristics that need to be investigated. It is known that yellow fin tuna is an endothermic fish with a high metabolic rate, heart output rate and aerobic performance ability [1]. This is related to its migration and fast swimmer characteristics, so that its morphological and physiological characters with a high metabolic rate are followed by a high heart output rate. As the result, the effort to domesticate the characteristics with a controlled condition is relative difficult. The physiological effort by reducing the high metabolic rate is made by regulating its digestion through probiotic.
Efforts to obtain specific probiotic candidates for larvae-juveniles have been started by characterizing and identifying probiotic candidates, applying them in larvae culturing up to the juvenile stage and by analysing the enzymatic synthesis responses. The use of suitable nutrient active supplements (probiotic) is meant to make the metabolism more efficient, to regulate the growth, survival rate and disease resistance. The development of the combination of culture method and the use of probiotic as food supplement is very prospective. From the success in that effort it is expected that it can increase growth in tuna fry production. The next impact of this effort is the giving of opportunity to the commercial scale tuna fishery industry to supply tuna fries to floating cages so that in the future success in the culturing will become the image of Indonesia as the biggest fish producer in the world.

Today pressures on the catching of tuna species are occurring globally. The tuna population stock is generally decreasing due to overfishing. In 2016, IUCN (the International Union for Conservation of Nature) put tuna species on the Red List, which made Thunnus alalunga (abacore) and T. albacores (yellow fin tuna) fishes whose populations are being threatened (Near Threatened), T. obesus (big eye) and T. orientalis (Pacific blue fin) susceptible to attacks/catch (Vulnerable), T. thynnus (Atlantic blue fin) a species endangered from extinction (Endangered), and T. maccoyii (Southern blue fin) a species close to extinction (Critically Endangered) [2]. This decision was based on the fact that some fish catching companies caught one or more than one species of the tuna.

Efforts in aquaculture and hatchery are needed to produce yellow fin tuna larvae and juveniles. The metabolic efficiency rate can be expressed by using probiotic during larvae and juveniles culture. The use of probiotic can improve physical growth, enzyme, digestion, resistance to diseases, immune gene expression and reduce infection prevalence in fish or shrimps [3], [4], [5]. The antimicrobial protein response transcription and immunity gene expression in the larvae - juveniles can increase some fold [6], [7], [8].

Probiotic functions include to indirectly increase growth performance and digestive intestine, to stimulate nonspecific immunity mechanism and to protect pathogenic microorganisms by producing compounds that have bacteriocin-like activities [9], [10].

Other probiotic roles that have been proven in aquatic animal cultivation in aquaculture are to increase water quality so that it increases health, controls pathogenic bacteria and controls their virulence, eliminates the use of antibiotic, so that it can minimize antibiotic resistance, stimulates immune system, increases intestine flora, reduces disease incidences and increases food assimilation [11], [12], [13], [14].

The role of probiotic bacterial isolates produced from isolating yellow fin tuna intestines may be positive for the digestive system and metabolism efficiency of yellow fin tuna at larvae to juvenile stage. With the result of the analysis of the effect of probiotic bacterial isolate on digestion enzymatic activities of yellow fin tuna (Thunnus albacares) at larvae to juvenile stadia, it is expected that yellow fin tuna controlled production will become an industrial endeavour in Indonesia. In the future yellow fin tuna commercial hatchery business will grow faster in the society.

2. Material and method

2.1. Culture of Yellow fin tuna larvae to juvenile
In this study yellow fin tuna larvae and juvenile stage rearing with supplemented of specific probiotic (A) was used as the experiment group and yellow fin tuna larvae and juvenile rearing without probiotic (B) as the control one. The rearing of tuna larvae was started by stocking eggs that had been screened for virus infections free (VNN and Iridovirus), which hatched in a larval rearing tank until they became juveniles. During the larvae rearing, Nannochloropsis oculata natural feed, rotifer enriched with commercial enrichment, artemia, living feeds in the form of milkfish larvae were added with 3 probiotic strains (strains of bacteria as the result of isolation from yellow fin tuna intestine from nature) for metabolism efficiency, an increase in growth, and health of the tuna larvae. In the control treatment no probiotic was used. The experiment was initiated by culturing 3 probiotic isolates...
(Bacillus subtilis strain TA-1, Bacillus amyloliquefaciens strain TN-2, Bacillus subtilis strain TO-4) and apply to the larval rearing of yellow fin tuna. Probiotic isolates were cultured in a Marine 2216 Broth until they attained peak density of 10^9-10^10 CFU/mL. The culture media were sterilized at temperature of 121 °C for 15 minutes. As aerobic bacteria require oxygen, aeration was provided to accelerate growth. Duration of incubation was 48 hours.

The concrete tanks were used for larvae rearing with volume of 5m^3. The initial egg density was 15 eggs/L. The study was designed using T-test with 2 replications so there were 4 larvae tanks.

2.2. Analysis of gene expression profile related to digestion enzymatic synthesis

2.2.1. Isolation of RNA total and cDNA synthesis. Yellow fin tuna larvae that had been collected were 30 - 40, then they were centrifuged at 10,000 rpm at 4°C for 5 minutes to separate the water that was unintentionally brought in micro tube. The total RNA of the larvae obtained was extracted by RNA extraction solution using the IQ-2000 method. The cDNA (complementary DNA) was done by using SensiFastcDNA Synthesis kit (Bioline) according to the existing procedures. 20 µL reaction volume which consisted of 4.0 µL master mix, 1 uL Reverse Transcript and 20.0 µg of total RNA. The solution in the micro tube was incubated respectively at 25ºC for 5 minutes, 42 ºC (15 minutes) and 85 ºC for 5 minutes. cDNA was then put into ice to stop synthesis reaction and was kept at -20°C for the next analysis.

2.2.2. Analysis of yellow fin tuna enzymatic activity (T. albacares) with RT-qPCR. The analysis of digestion enzymatic synthesis gene expression profile related to digestive enzymes (lipase, amylase and trypsin) was done by using quantitative RT-qPCR with specific primary. RT-qPCR done with ABI PRISM 7500 sequence detection system with 5x Hot FirepolEvagreenqPCR mix (ROX). The reaction volume for cDNA amplification was 20 µl with 1x hot Master mix (Rox) final concentration, Primer F/R 10 pmol respectively, NFW (Nucleic Free Water) added to a volume of 20 µl and cDNA (0.01ng/ul). The cycling temperature condition for RT-qPCR consisted of the holding temperature of 50 °C for 20 minutes, the initial denaturation, extension 72 temperature was at 95 °C (15 minutes) followed by 95 °C (15 seconds) and the annealing temperature at 60 °C (30 seconds), for 40 cycles and the final extension temperature at 72 °C for 20 seconds. The counting of ∆Ct of the threshold PCR (Ct) genes tested was normalized relative to Ct β actine (internal control) in the same sample. The ∆∆Ct value was calculated from ∆Ct (the sample groups tested) ─ ∆Ct (initial expression). The relatively different multiplication representation from the initial expression can be counted with 2 ─∆∆Ct.

Relative Change of enzymatic activity was calculated with:

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RC(\%) = \frac{At - At-1}{At-1} \times 100\% (I)
\]

Where RC = Relative Change, At = Enzyme activity at time t and At-1 = Enzyme activity at time t-1

| Digestive enzyme | Target gene | Name | Primer F/R | Sequence (5”-3”) |
|------------------|-------------|------|------------|------------------|
| Amylase Activity | Amylase     | Amy  | F: TT (CT) gAg Tgg Cg CTgg R: (Ag) gg CCA CA Tg Tg CTT |
| Lipase Activity  | Lipase      | Lipase | F: TGG ATG GCA TGA TGG AGA R: CTG CAG CAG GTG GGC TAT |
| Trypsin Activity | Trypsin     | Trypsin | F: GAG AGC ATG ATA TCT ACC GC R: CGC AGA ACA TGG CAT CGG TG |
| Internal control | β-actine    | β-actine | F: GGA ATC CAC GAG ACC ACC TA R: GCT GGA AGG TGG ACA GAG AG |
3. Results and discussion

3.1. Larvae and juvenile growth of yellow fin tuna T. albacares

The result of observation during the larvae rearing of yellow fin tuna showed that the growth was relatively faster in the treatment with probiotic compared with the control treatment. The growth on 2 Day After Hatch (DAH) to 9 DAH in the probiotic treatment and that in the control were relatively the same, however, on 10 DAH, the growth of the length of the tuna larvae in the probiotic treatment tended to be faster than that in the control group. This was made possible by the role of probiotic that contributed to the regulation of larvae intestinal micro flora, which increased digestive enzyme useful for the digestibility and supported the health condition of the tuna larvae. The probiotic functions include to increase indirectly the growth performance of intestinal micro biota, to stimulate nonspecific immunity mechanism and to protect fish from microorganism pathogens by producing compounds that have bacteriocin-like activities [9], [10].

![Figure 1](image1.png)

**Figure 1.** Growth pattern of total length (mm) of yellow fin tuna *T. albacores* on 2 to 23 DAH with supplemented probiotic and without probiotic (control)

![Figure 2](image2.png)

**Figure 2.** Growth pattern of body weight (g) of yellow fin tuna *T. albacores* on 2 to 23 DAH with supplemented probiotic and without probiotic (control)

3.2. Gene Expression related to digestive enzyme in larvae and juvenile of yellow fin tuna, T. albacares

The results of gene expression related to digestive system in yellow fin tuna larvae after being cultivated by supplemented of probiotic treatment and without probiotic treatment showed that there was an increase in the ability to synthesize feed enzymatically. Yellow fin tuna that was supplemented probiotic produced multiplication in amylase enzyme synthesis activity from 10 DAH (200 times), 17 DAH (1200 times) up to 23 DAH (400 times). This enzymatic activity was higher than that in the control group. In the control group, the highest amylase enzyme activity was reached on 22 DAH showing a multiplication of 200 times and at the previous stages the enzyme activity was low.

Gene expression in Lipase enzyme activity of the yellow fin tuna that was given probiotic occurred on 13 DAH with the multiplication of activity of 25 times, and increased on 6 DAH (150 times), 18 DAH (200 times) and 21 DAH (300 times), while in the control group, the activity started to occur
only on 16 DAH, however, it only reached an activity increase of 10 - 15 times and the highest activity occurred on 18 DAH (100 times). On the other hand, the trypsin activity of the yellow fin tuna larvae with the probiotic treatment showed an increase of 200 times on 6 DAH and decreased up to 23 DAH, while in the control group, the trypsin activity showed that there was no increase in enzymatic activity with the value lower than those of the 3 to 23 DAH.

This shows that the probiotic role in increasing enzymatic activity system in yellow fin tuna larvae was proven, and that it could increase the intestinal flora and reduce disease incidences and stimulate an increase in food assimilation [11, 12, 13, 14].

**Figure 3.** Expression gene of Amylase of yellow fin tuna *T. albacares* larvae and juvenile from 2 to 23 DAH with supplemented of probiotics and without probiotics (Control)

**Figure 4.** Expression gene of Lipase of yellow fin tuna *T. albacares* larvae and juvenile from 2 to 23 DAH with supplemented of probiotics and without probiotics (Control)

**Figure 5.** Expression gene of Trypsin of yellow fin tuna *T. albacares* larvae and juvenile from 2 to 23 DAH with supplemented of probiotics and without probiotics (Control)
3.3. Relative change in percentage of digestive enzyme of larvae and juvenile of yellow fin tuna T. albacares

The results of gene expressions related to enzymatic activity in the larvae of yellow fin tuna T. albacares showed a relative percentage change pattern in each coding gene. On overall, the relative change pattern and digestive enzymatic activity is related to the change in food composition given and is assumed that there is also a morpho-functional change at the time of the larvae development. It seems that on 2-10 DAH the larvae of the yellow fin tuna that was supplemented of probiotic showed a relatively fast and high digestive change in enzymatic gene expression and enzymatic synthesis especially in lipase, trypsin and amylase. This was an indicator that there was digestive activity that started to increase especially on 4-6 DAH up to the next stage. The relative change in the control showed a low value and even it showed a negative value.

**Figure 6.** Pattern of relative change of Amylase activities (%) on yellow fin tuna T. albacares larvae and juvenile with supplemented of probiotic and Control (without probiotics)

**Figure 7.** Pattern of relative change of Lipase activities (%) on yellow fin tuna T. albacares larvae and juvenile with supplemented of probiotic and Control (without probiotics)
Figure 8. Pattern of relative change of Trypsin activities (%) on yellow fin tuna T. albacares larvae and juvenile with supplemented of probiotic and without probiotics (Control)

The growth profile of yellow fin tuna from larvae to juvenile in the group which was supplemented of probiotic was relatively faster than that in the control. It often happened in the larvae that was supplemented of probiotic the stomach was seen to contain more food than that of the control group. This observation was made every morning before the larvae got foods (rotifer, artemia and living food the form of fish larvae). It was this that might make larvae more efficient in digesting food in its digestive system with the probiotic treatment.

4. Conclusion

The growth response in the form of the length of yellow fin tuna T. albacares supplemented of probiotic (Bacillus subtilis strain TA-1, Bacillus amyloliquefaciens strain TN-2, Bacillus subtilis strain TO-4) is significantly different (P<0.05) and tends to be faster than that of the control group (without probiotic treatment). The probiotic supplementation in the cultivation of larvae up to juveniles of yellow fin tuna can increase its digestibility expression expressed in the gene target that is related to amylase, lipase and trypsin enzymatic synthesis. The relative changes rate of enzymatic synthesis shows to be faster and higher especially at the beginning of the larvae growth.

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