DOI: http://dx.doi.org/10.15578/iaj.20.2.2025.209-220 *Indonesian Aquaculture Journal, 20 (2), 2025, 209-220*

Available online at: http://ejournal-balitbang.kkp.go.id/index.php/iaj

CHARACTERZATION OF ENDOGENOUS BACTERIA AS POTENTIAL PROBIOTICS IN LARVAL REARING OF YELLOWFIN TUNA (*Thunnus albacares*)

Ni Wayan Widya Astuti[*)], Ananto Setiadi[**)], Gunawan[**)], Jhon Harianto Hutapea[*)], Ahmad Muzaki[**)] and Haryanti[*)#]

[*)] Research Centre for Marine Aquaculture, National Research and Innovation Agency. Malaka, Kec. Pemenang, Kabupaten Lombok Utara, Nusa Tenggara Barat, 83352, Indonesia

[**)] Research Centre for Marine Aquaculture, National Research and Innovation Agency, Jl Raya Bogor KM 46 Cibinong, Bogor, Indonesia

(Received: October 27, 2025; Final revision: December 18, 2025; Accepted: December 18, 2025)

ABSTRACT

The main objective of this study was to determine the profile of endogenous intestinal bacteria of yellowfin tuna, Thunnus albacares that can function as potential probiotics in the digestive system and used for the maintenance of yellowfin tuna larvae. Wild yellowfin tuna, T. albacares was collected from the waters of Gerokgak, Buleleng, North Bali, Indonesia. Intestinal bacteria were isolated, identified, characterized, and followed by enzymatic hydrolysis activity tests, antagonistic tests and pathogenicity tests to select candidate bacteria as probiotics. The results of the enzymatic hydrolysis activity test showed that there were 4 isolates of candidate probiotic bacteria that were active in enzymatic synthesis for amylase, lipase, casease, lecithinase, gelatinase. Identification through Sanger sequencing resulted in 4 potential bacterial isolates as probiotics, namely Bacillus subtillis strain T-A1, Bacillus amyloliquefaciens strain T-N2, Bacillus subtillis strain T-O4, and Paraclostridium bifermentans strain T-O6. These four bacteria were not antagonistic between varieties and were not pathogenic in Tuna fish. Yellowfin tuna fed probiotic supplements grew faster (5.5002 ± 0.2 mm) than the control (4.9002 ± 0.4 mm). The success of yellowfin tuna seed production is expected to be sustainable and its implementation in the private sector for aquaculture purposes.

KEYWORDS: endogenous bacteria; probiotics; yellowfin tuna; *Thunnus albacares*

INTRODUCTION

Yellowfin tuna (*Thunnus albacares*) is the most important tuna species in Indonesia and a valuable commodity in the global fish market. Tuna aquaculture in Indonesia is relatively rare, if not virtually nonexistent, and research remains limited. Many researchers focus on overfishing, bycatch, climate change, marine pollution, and fisheries management, all of which pose potential risks and challenges to tuna survival (Xie *et al*., 2021). Tuna products are generally wild caught from various species. Currently, pressure on tuna fishing is occurring globally. Tuna stocks are generally declining due to overfishing. In 2016, the IUCN (International Union for Conservation of Nature) added tuna species to the Red List, listing *Thunnus alalunga* (abakora) and *T. albacares* (yellowfin tuna) as threatened (Near Threatened), *T. obesus* (bigeye) and *T. orientalis* (Pacific bluefin tuna) as vulnerable to attack/harvest (Vulnerable), *T. thynnus* (Atlantic bluefin tuna) as endangered (Threatened), and *T. maccoyii* (southern bluefin tuna) as critically endangered (Critically Endangered) (Greenpeace, 2016). This decision was made since some fishing companies catch one or more tuna species. Several countries have started tuna farming, such as Atlantic bluefin tuna (*Thunnus thynnus*) developed through sustainable farming and fattening practices in Croatia (Mrèeliæ *et al*., 2023) and farmed in European Union waters, as well as the sustainability of tuna farming in Mediterranean European countries (Guillen *et al*., 2024). According to Benetti *et al*. (2016), progress in fattening activities has been limited to improved management and reduced mortality rates during the capture, transfer, and feeding stages of tuna in cages. However, tuna aquaculture is now rapidly evolving through advances in closed-system tuna aquaculture technology, including broodstock maturation, spawning, larval rearing, and juvenile production. Japan has successfully pioneered the development of closed-system hatchery technology for Pacific bluefin tuna (PBFT).

Correspondence: Research Centre for Marine Aquaculture, National Research and Innovation Agency. Kabupaten Lombok Utara, Nusa Tenggara Barat, 83352, Indonesia
E-mail: hary016@brin.go.id

In Indonesia and Panama, yellowfin tuna aquaculture has been conducted on a research scale. This process begins with larval development in controlled hatcheries. Broodstock is prepared by cultivating wild-caught juvenile tuna (2 to 3 kg) and domesticating them in large aquariums with fresh feed, vitamins, and environmental and disease control measures. This controlled aquaculture continues until the yellowfin tuna reaches spawning size. (Hutapea *et al*., 2009; Hutapea *et al*., 2010; Margulies *et al*., 2016)

Many issues regarding mortality during yellowfin tuna fry production remain incompletely understood, including the appropriate handling techniques for larvae and fry, the appropriateness of the feed provided, both live and artificial, and other biological and physiological characteristics that still need to be studied (Hutapea *et al*., 2009). Yellowfin tuna is known as endothermic fish with high metabolic rates, cardiac output, ram ventilation, and aerobic performance (Blank *et al*., 2012; Meyer and Emam, 2024). This is related to their migratory nature and fast swimming characteristics. This will influence cultivation needs and methods. Therefore, domesticating these traits under controlled conditions is relatively difficult. Domestication of bluefin tuna (*Thunnus thynnus*) must consider appropriate rearing conditions for larvae. The activity patterns of several digestive enzymes during development are informative indicators of the physiological status of larvae, including nutritional status.

Furthermore, antioxidant enzymes such as catalase (CAT) are known to be indicators of biotic stress (pathology, pollutants, etc.) associated with dietary changes or metamorphosis in fish larvae (Cahu *et al.,* 2011). A physiological approach to reducing high biotic stress is through digestive regulation through the use of active nutritional supplements or specific probiotics (Covès *et al*., 2011). Efforts to identify specific probiotics for larvae and fry will begin with their characterization, then their application to yellowfin tuna larvae, where appropriate active nutritional supplement components are aimed at improving metabolic efficiency, regulating growth, survival, and disease resistance (Cahu *et al*., 2011; Haryanti *et al*., 2021a). The development of a combination of culture methods and the use of probiotics as feed supplements is very promising. The success of this method is expected to improve the survival of yellowfin tuna fry production. The next step is to provide opportunities for the commercial tuna industry to supply yellowfin tuna seeds in floating net cages (Hutapea *et al.,* 2010; Xie *et al.,* 2021). In the future, successful tuna cultivation will enhance the reputation of the Indonesian tuna industry.

Digestive enzymes are found in the digestive tracts of aquatic animals and help break down larger food molecules into particles that are more easily absorbed and utilized by the body. A fish's ability to break down food depends largely on the availability of the appropriate digestive enzymes, which mediate specific degradation pathways based on the physical and chemical properties of the food (Assan *et al.,* 2022). The use of probiotics in aquatic animal cultivation has shown numerous benefits. The role of probiotics can improve water quality, control of pathogenic bacteria and their virulence, avoidance of antibiotic use and consequent reduction of the risk of antibiotic resistance, stimulation of the immune system, improvement of intestinal flora, reduction of disease incidence and improvement of food assimilation (Haryanti *et al*., 2021b; Lein *et al*., 2022; Pascual *et al*., 2022; Chen *et al*., 2023).

The results of the study by Haryanti *et al*., (2021a) showed that the role of probiotics can increase digestive enzyme activity in larvae and juveniles of yellowfin tuna (*T. albacares)*. Treatment with the probiotic *Pediococcus acidilactici* MA18/5M in live feed did not affect the growth of Atlantic bluefin tuna larvae. At 15 days post-hatch (dph), treated larvae had lower expression of genes encoding glutathione peroxidase and interleukin-1β, as well as a less diverse microbiota, compared to the control group, but significantly affects the larval microbiota and the anti-inflammatory/antioxidative status of tuna larvae (Covès *et al.,* 2011).

Maravila *et al.* (2024) stated that several probiotics have been genetically engineered to express and deliver immunomodulatory molecules. This promotes selective therapeutic effects and specific immunization against certain pathogens in fish. Based on this description, improvements to larval culture must be made by adding feed supplements based on beneficial microbes. The aim of this study was to determine the endogenous bacterial profile of the intestines of yellowfin tuna and it is expected to play a role as a potential probiotic in the digestive system and metabolic efficiency in the larval stage of yellowfin tuna.

MATERIALS AND METHODS

This research will be conducted in several stages:

Sample Collection

Bacterial isolates were isolated from the intestines of yellowfin tuna collected from the wild waters of Gerokgak, Buleleng, North Bali, Indonesia. The

intestines were ground in 1 ml of Phosphate Buffered Saline (PBS) and filtered through a 10-µm pore size filter. The resulting filtrate was grown on Marine Agar 2216 (Difco Category 212185, USA) and TCBS Agar (Difco Category 265020, USA) in Petri dishes. After 24 hours of incubation at 25°C, 3-5 bacterial colonies were selected based on their shape, color, and size. These colonies were then purified 3-4 times until a single pure colony was obtained. This culture was stored on Marine Agar slants for further testing.

Identification of Bacterial Isolates

The initial step in identifying bacterial isolates began with DNA extraction from each purified yellowfin tuna intestinal bacterial isolate using the Presto Mini gDNA Bacteria kit (Geneaid, Cat. No. GBB100, Taiwan). PCR amplification of the purified bacterial isolates was performed using the Random Amplification Polymorphism DNA (RAPD) method using universal primers AAM2, L-21, AS-14, and AS-15. However, from preliminary studies of these four primers, only primer 2 AAM2 (5'-CTG CGA CCC AGA GCG G-3') provided the best polymorphism and was used for alignment between the purified bacterial isolates. The PCR amplicons were then electrophoresed using a 1.5% agarose gel in 1x TBE buffer. This screening was used to determine the DNA fragmentation performance of each bacterial isolate. Only one bacterial isolate with the same DNA fragment diversity was selected. Sequencing analysis began with sample preparation of selected bacterial isolates that function as probiotics. Genomic DNA was amplified using 16 Sr RNA PCR with the primer pair 27F (5'-AGA GTT TGA TC (AC) TGG CTC AG-3') and 1492R (5'-ACGG (CT) TAC CTT GTT ACG ACT T-3') with a target region of 1400 bp (Haryanti *et al*., 2024; Sambrook and Russell, 2001). The PCR product was purified and fragmented using an ABI 3730xl Genetic Analyzer sequencer distributed by 1st Best-Malaysia. The resulting nucleotide sequences of the candidate probiotic bacteria were analyzed using Sequence Scanner version 1.0 and alignment via Nucleotide BLAST accessed from the NCBI GenBank.

Characterization of Bacterial Isolates

a. Bacterial Isolate Culture

Molecularly distinct bacterial strains with potential as probiotics were tested for their enzymatic activity. Bacterial strains were cultured in Marine Broth 2216 (Difco Catalog Number 247940, USA) until a density of 10^9-10^{10} CFU mL^{-1} was reached. The culture medium was sterilized at 121°C for 15 minutes. A 15 mL test tube was used for this culture. A single stroke of a sterile toothpick was used to inoculate the bacterial isolate into the culture medium. The culture tube was placed on a rotary shaker, and the culture time was 48 hours at 25°C and a shaker speed of 125 rpm.

b. Enzymatic Hydrolysis Activity Test

Enzymatic hydrolysis activity tests, including lipase, gelatinase, amylase, urease, casease, lecithinase, and chitinase, were conducted on selected bacterial isolates as probiotic candidates. The bacterial isolates were tested for their enzymatic activity through the Extra Cellular Product (ECP) method using a cellophane plate method. Sterile cellophane was placed on the surface of Marine Agar 2216 (Difco Category 212185, USA) in a petri dish and inoculated with bacterial culture (spread) and incubated for 24 hours at 25 ºC. The ECP on the cellophane surface was washed with PBS buffer solution, pH 7.0. The suspension was centrifuged for 10 minutes at 13000 rpm at 4 ºC. The supernatant obtained was sterilely filtered with a 0.22 ìm filter membrane. The enzyme activities of gelatinase, casease, amylase, lecithinase, lipase, urease, and chitinase were tested using a cylindrical tube. A 2% agar medium containing 0.4% gelatin, 0.4% casein, 0.2% starch, 2.5% egg yolk, 1% Tween 80, 2% urea, and 2% phenol red was prepared to test the presence of these enzymatic activities. The test was conducted by placing a paper disc on each agar plate filled with 200 µL of ECP filtrate. The presence of a clear zone around the cylindrical tube indicated the enzymatic activity of the bacterial isolate's probiotic product candidate. The wider the clear zone, the more active the enzyme's hydrolytic activity on the agar substrate with each nutrient content (Haryanti *et al*., 2024)

c. Antagonistic test between bacterial isolates with potential probiotic properties

Antagonistic testing is a method for measuring the ability of a bacterial isolate to grow together with another bacterial isolate without inhibiting each other (Goulden *et al*., 2012). The test was conducted in vitro using a dual culture method, where both bacterial isolates are grown together in a single medium. The growth medium used in this test was Marine 2216 Agar (Difco Cat No. 212185, USA). Two potential probiotic bacterial isolates were streaked in the petri dish, with a distance of 3 cm between streaks and a height of 3 cm for each streak. The streaks were combined in such a way that each bacterial isolate had the opportunity to grow together. Each bacterial isolate tested had a control, where a single streak was present in each petri dish. After the incubation period (25ºC and 48 hours), the growth of each bacterial isolate was measured by the width of the streak in each petri dish.

d. Pathogenicity tests of bacterial isolates that have potential as probiotics in yellowfin tuna (*T. albacares*)

In the pathogenicity test, 10 yellowfin tuna (*T. albacares*) fries with a body length of 2.5±0.2 cm were used, with 3 replications. The test was carried out by immersion of bacterial isolate as a potential probiotic with density of 10^8 CFU mL^{-1} for 96 hours. Observations survival rate were made every 24 hours. The survival rate obtained is evidence that the bacterial isolate as a probiotic candidate is not pathogenic and toxic.

Larval rearing of yellowfin tuna

In this study, larval rearing was supplemented with (A) probiotics from the test results (candidate probiotics) and (B) without probiotics (control). Yellowfish tuna larvae were rearing begins with the spreading of eggs that have been checked for virus infection (VNN and Iridovirus), hatching them in a larval tank and carrying out reared until the fry. During larval rearing, natural food was given namely microalgae, *Nannochloropsis oculata*, rotifers *Brachionus* sp. that have been enriched with commercial enrichment materials, Copepoda, Brine shrimp , *Artemia salina* , live food in the form of milkfish fry (Prihadi *et al*., 2025) and the use of 4 probiotic strains (isolated results of probiotic candidates). In the control no probiotics were used.

The larval rearing container used a 200 L volume tank. The tank was placed in a water bath system equipped with a heater at 30ºC and a temperature control thermostat, to maintain a stable temperature of the rearing water. Each tank contained eggs with an initial density of 20 eggs/L. The study was designed with a T-test with 6 replications so that there were 12 rearing tanks. The parameters observed in this larval rearing were growth and survival rate.

Data Analysis

The results of this study are presented as mean ± standard deviation. The design used was an experimental design with a T-test. The analysis was conducted to evaluate the characteristics of bacteria as potential probiotic candidates and their effects on growth and survival in tuna larvae culture. The differences were considered statistically significant at the 95% confidence level ($p<0.05$).

RESULTS AND DISCUSSION

Intestinal sampling of wild-caught yellowfin tuna (*T. albacares)*

Bacterial isolation results from the intestines and stomachs of wild-caught yellowfin tuna weighing 2 kg (I) and 5 kg (II) showed predominantly white and yellow colonies on Marine E2216 Agar (Difco Category No. 212185, USA) and yellow and green colonies on TCBS Agar (Difco Category No. 265020, USA). The number of colonies is shown in Table 1.

Table 1. Results of bacterial isolation from the intestines and stomachs of wild-caught yellowfin tuna *T. albacares*

Code	Number of colony (CFU mL^{-1})	Remark (colony color)	Code	Number of colony (CFU mL^{-1})	Remark (colony color)
intestine			Stomach		
U-1	29 x10^2	white	L-1	14 x10^2	white
U-2	37 x 10^2	White	L-2	8 x 10^2	White
	2 x10^2	yellow		1 x10^2	yellow
U-3	38 x 10^2	white	L-3	27 x 10^2	white
U-4	4 x 10^2	white	L-4	43 x 10^2	white
U-5	3 x10^2	white	L-5	5 x10^2	white

Overall screening results from the two tunas yielded 29 bacterial isolates from the intestines and 27 isolates from the stomachs. Further screening to determine which isolates were *Vibrio* using TCBS growth medium yielded 11 Vibrio isolates (38%) from the intestines and 11 isolates (41%) from the stomachs. Thus, the non-Vibrio bacterial population in the intestines totaled 18 isolates (62%), while the stomach population totaled 16 isolates (59%). Furthermore, 34 bacterial isolates showed similar morphological performance after three purifications, resulting in only 18 isolates.

Identification of endogenous bacteria isolate

Isolate the purified bacteria, then culture it with Marine 2216 Broth (Difco Cat.No. 247940, USA) media in test tubes (15 mL). The bacterial culture was then subjected to DNA isolation using the Presto Mini gDNA Bacteria kit (Geneaid, Cat. No. GBB100. Taiwan). In all strains the isolates indicated to be different after homology between strains was carried out - RAPD method. The diversity profile of potential probiotic bacterial isolates from the intestines and stomachs of wild-caught yellowfin tuna is as shown in Figure 1. However, the results of stable PCR amplification showed differences in 16 bacterial isolates. These 16 isolates were then tested for their ability to hydrolyze carbohydrates, lipids and proteins.

Figure 1 shows that two bacterial isolates had similar DNA fragmentation profiles, except for the brightness of the DNA bands. This left 16 bacterial isolates of interest for further analysis. Figure 1 shows that the diversity of bacterial microflora in the tuna digestive system exhibited distinct strains after filtration. Although it is possible that the same strains may exist within these species, the reasons for the similarities and differences in bacterial strains between species are understandable. According to Gadoin *et al.* (2021); Astuti *et al*. (2023); and Jääskeläinen *et al*. (2019), in addition to phylogeny, several other endogenous factors can explain the differences and similarities between microbes, including size, which is a key determinant of the tuna gut bacteriome. Significant changes in the gut microflora have been observed during growth in yellowfin and bigeye tuna, but skipjack tuna is particularly interesting due to the stability of its microbiota and its unique composition.

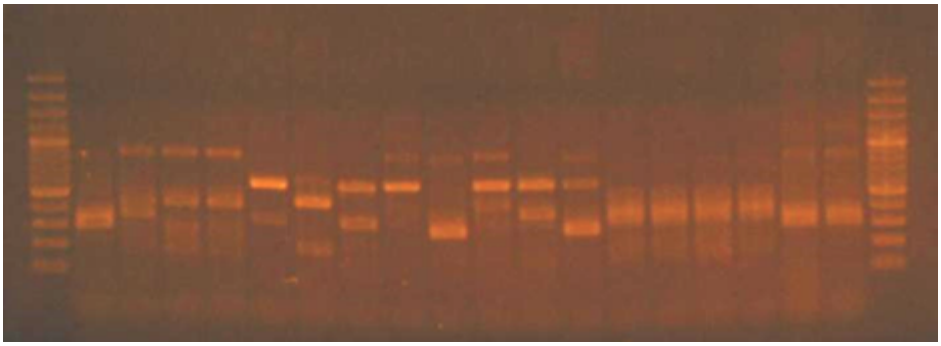


Figure 1. Diversity profile of potential probiotic bacterial isolates from the intestines and stomachs of wild yellowfin tuna through PCR amplification using RAPD method (M= Marker 100 bp DNA ladder, Line 1-9 bacteria isolate from intestine with code of A-1, A-6, B-1, B-3, H-1, H-6, B-6, L-1 , L-5 and line 10-18 isolate from stomach with code of K-1, N-2, O-2, O-4, O-6, P-1, S-1, S-3, M-1.

Characteristics of endogenous bacterial isolates in yellowfin tuna

a. Enzymatic hydrolysis activity of bacterial isolates with potential probiotic properties in yellowfin tuna (*T. albacares*)

The results of the enzymatic activity test indicated that 16 selected bacterial isolates have the potential for enzymatic hydrolysis. The enzymatic hydrolysis results are shown in Figure 2 and Table 2. All isolates appear to be able to enzymatically hydrolyze to produce gelatinase, casease, amylase, and lipase, while only one strain, O-6, can hydrolyze urea to urease.

Overall, all 16 bacterial isolates were able to hydrolyze casein (protein) into casease, although 5 strains (A-6, B-2, B-3, H-6, and S-1) had relatively low hydrolysis ability. Most bacterial isolates were able to convert starch into amylase, but 7 bacterial isolates had low starch hydrolysis ability. The same condition was observed in the ability to hydrolyze fat into lipase, which showed that 9 bacterial isolates had high hydrolysis ability, while the other 7 bacterial isolates had relatively low hydrolysis ability. The results of nucleotide sequence analysis of 16 isolates showed that only 4 isolates showed the ability to enzymatically hydrolyze carbohydrate, protein, and lipid sources (Extra Cellular Products). carbohydrate, protein and lipid sources.

The results of in vitro analysis using Extracellular Products (ECP) observations showed that of the 16 isolates, only four showed the ability to enzymatically hydrolyze carbohydrates, proteins, and fats. This is evident from the scoring results obtained for each isolate, which indicated a high ability to enzymatically hydrolyze carbohydrates, fats, and proteins. There were four isolates, coded A-1, N-2, O-4, and O-6 (Table 2). These bacterial isolates were then used for testing yellowfin tuna larval cultures.

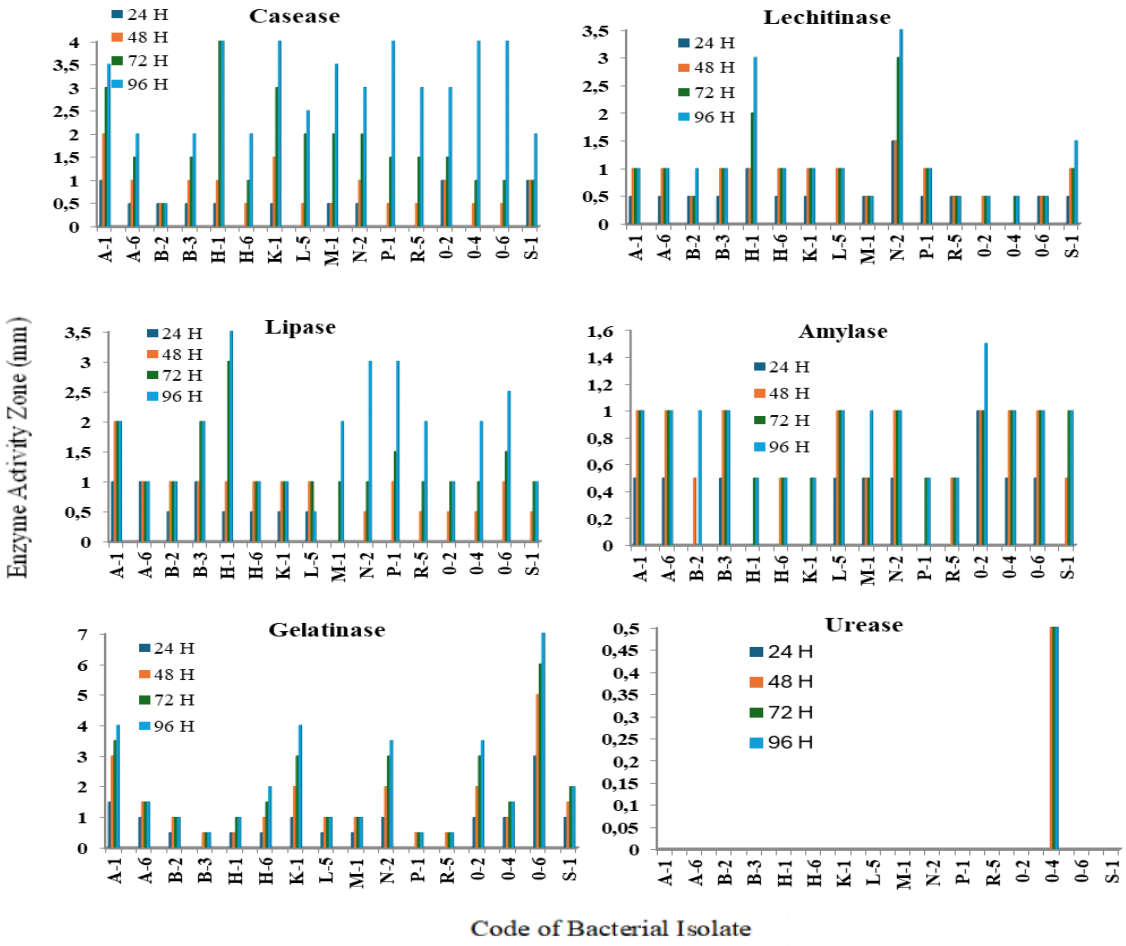


Figure 2. Results of enzymatic hydrolysis activity tests on 16 strains of bacterial isolates in yellowfin tuna *L. albacares.*

Table 2. Scoring results of 16 bacterial isolates as potential probiotics with enzymatic hydrolysis capabilities in the digestive system of yellowfin tuna *L. albacares*

Isolat	Gelatinase	Casease	Amylase	Lecithinase	Lipase	Urease	Score
A-1	+	+	+	—	+	—	4
A-6	—	—	+	—	—	—	1
B-2	—	—	—	—	—	—	0
B-3	—	—	+	—	+	—	2
H-1	—	+	—	+	+	—	3
H-6	—	—	—	—	—	—	0
K-1	+	+	—	—	—	—	2
L-5	—	+	+	—	—	—	2
M-1	—	+	—	—	+	—	2
N-2	+	+	+	+	+	—	5
P-1	—	+	—	—	+	—	2
R-5	—	+	—	—	+	—	2
0-2	+	+	+	—	—	—	3
0-4	—	+	+	—	+	+	4
0-6	+	+	+	—	+	—	4
S-1	—	—	+	+	—	—	2

Lecithinase enzyme activity testing revealed only three bacterial isolate strains capable of hydrolysis. This is understandable, as lecithin-hydrolyzing bacteria can act as a virulence factor by destroying host cell membranes and tissues, making them unbeneficial. Meanwhile, urease enzyme activity was only demonstrated in one isolate capable of hydrolysis. Urease plays an important role in both beneficial processes, such as nutrient recycling. Fish also use the internal urea cycle to convert ammonia (a waste product) into urea, but this is for osmoregulation, not for creating foul odors, and fish have much lower internal urease activity compared to bacteria (Mohammed *et al.*, 2024).

b. Antagonist test between bacterial isolates with potential as probiotics

Four isolates proven to have enzymatic hydrolysis capabilities were then tested for their antagonistic characteristics between bacterial isolates. This was intended to ensure that the isolates obtained as probiotics did not inhibit each other in their use for the maintenance of yellowfin tuna larvae. The inhibitory characteristics between isolates will affect the work or probiotic ability of each isolate against yellowfin tuna larvae. The results of the in vitro antagonist test did not appear to show antagonistic results or growth inhibition between bacterial isolates as potential probiotics. This is shown in Figure 3.

The results of the antagonistic test between bacterial isolates show that the colony width range of strains A-1 (3.0 mm - 5.5 mm) and N-2 (2.5 mm to 5.5 mm) from the beginning of incubation to 120 hours did not differ. This also occurs in other bacterial isolates, especially those in the same genus, namely *Bacillus*. The absence of inhibitory power against bacterial colony growth indicates that these probiotic bacterial isolates can be used together (mixed) as supplementary nutrition in the maintenance of yellowfin tuna larvae *T. albacares*. According to Nurikhsanti *et al.* (2024) stated that the antagonistic protease enzyme production test showed that all bacterial isolates produced protease enzymes, but isolate RKT9 had the highest proteolytic index. Both isolates showed different inhibition test results, with isolate RKT2 having high inhibition power, while isolate RKT9 had low inhibition power. This indicates that antagonistic characteristics are determined by the ability of bacteria to produce certain compounds.

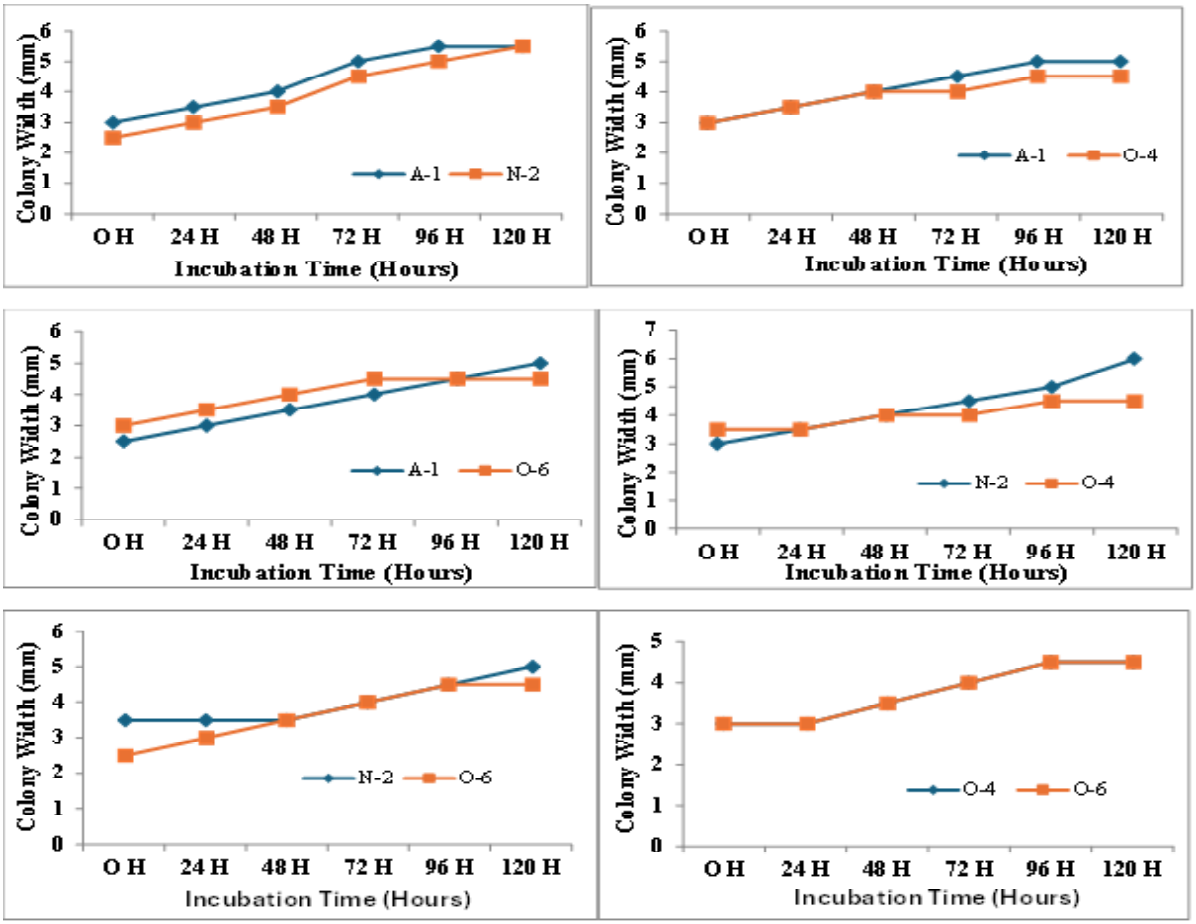


Figure 3. Results of antagonistic tests on four strains of bacterial isolates as potential probiotic on yellowfin tuna T. *albacares.*

c. Pathogenicity test of probiotic isolates on yellowfin tuna fry *L. albacares*

The pathogenicity test of bacterial isolates as potential probiotics showed that immersion of these bacterial isolates in tuna fry measuring 2.5 ± 0.2 cm in length for 96 hours at a density of 10^8 CFU mL-1 did not cause mortality. Observations every 24 hours for 96 hours showed that 70–90% of the tuna fry remained viable. This indicates that the four bacterial isolates were neither toxic nor pathogenic to yellowfin tuna fry (Table 3).

Identification results indicate that four bacterial isolates have potential as probiotics for yellowfin tuna. Nucleotide sequencing analysis using Blast-N showed a high similarity (98-99%) between the analyzed samples and the DNA sequences of their respective accession numbers in GenBank. The potential probiotic bacterial isolates are *Bacillus subtilis* strain TA-1, *Bacillus amyloliquefaciens* strain TN-2, *Bacillus subtilis* strain TO-4, and *Paraclostridium bifermentans* strain O-6. The DNA sequence similarity of these bacterial strains to those in GenBank is presented in Table 4.

Table 3. Results of pathogenicity test of four bacterial isolate strains as potential probiotics on yellowfin tuna fry *T. albacares*

Tank/Survival Rate -SR (%)	Treatment	Fry (pcs) initial	Fry mortality (pcs) 24 H	48 H	72 H	96 H
1	Strain A-1	10	0	1	2	0
SR (%)			100%	90%	70%	70%
2	Strain N-2	10	0	0	1	0
SR (%)			100%	100%	90%	90%
3	Strain O-4	10	0	0	1	0
SR (%)			100%	100%	90%	90%
4	Strain O-6	10	0	0	1	0
SR (%)			100%	100%	90%	90%
5	Mixed strain	10	0	1	0	0
SR (%)			100%	90%	90%	90%
6	Control	10	0	1	0	0
SR (%)			100%	90%	90%	90%

Table 4. DNA sequence similarity of bacterial isolates with potential probiotics compared to the total gene sequences in GenBank for each accession number

Code Sample	Description Strain	Scientific name	Percentage similarity	Accession
T-A-1	*Bacillus subtilis*, subtilis str.168 complete genome	*Bacillus subtillis*	99.22%	NC_000964.3
T-N-2	*Bacillus amyloliquefaciens* DSM7 complete genome	*Bacillus amyloliquefaciens*	98.85%	NC_014551.1
T-O-4	*Bacillus subtilis*, subtilis str.168 complete genome	*Bacillus subtilis*	99.21%	NC_000964.3
T-O-6	*Paraclostridium bifermentans* ATCC 638 gcdATCC38 contig), whole genome shotgun sequence	*Paraclostridium bifermentans*	86.71%	NZ_AVNC01000001.1

Larval rearing of Yellowfin Tuna with Probiotic Supplementation

a. Total Body Length Growth of Yellowfin Tuna Larvae *L. albacares*

Observations during the cultivation of yellowfin tuna larvae showed that larval growth was relatively faster with probiotic supplementation compared to the control. Growth from day 2 (DPH) to day-6 (DPH) between probiotics and the control was relatively similar, but at day 7 (DPH), yellowfin tuna larvae supplemented with probiotics tended to grow faster until day 10 (DPH) compared to the control (Figure 4). This is likely due to the role of probiotics in contributing to the regulation of the larvae's intestinal microflora, increasing digestive enzymes beneficial for digestibility, and supporting healthy conditions for yellowfin tuna larvae. Probiotics function includes improving growth performance, indirectly affecting the gut microbiota, stimulating non-specific immune mechanisms, and protecting fish from infection by pathogenic microorganisms by producing compounds with bacteriocin-like activity (Daniels *et al., 2013*, Ringo *et al.*, 2020)

Meanwhile, the combination of endogenous probiotics isolated from the intestines of yellowfin tuna is known to produce the enzymes lecithinase, casease, lipase, gelatinase, and amylase, which stimulate the digestive system of yellowfin tuna, thus facilitating nutrient absorption for growth. Research by Nababan *et al.* (2022) showed that probiotic supplementation in shrimp culture can increase the growth rate of *L. vannamei*. Assan *et al.* (2022) stated that probiotics can be a source of macronutrients and micronutrients in feed, and their activity in the digestive tract can stimulate the activity of digestive enzymes, such as amylase, protease, and lipase, thereby increasing and improving feed digestibility and efficient utilization of nutrient supplies. Therefore, yellowfin tuna larvae supplemented with probiotics showed significantly different growth ($P < 0.05$) compared to the control (without probiotics).

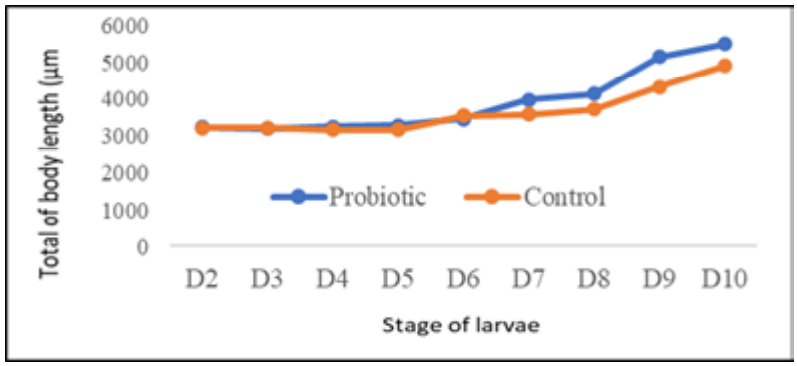


Figure 4. Total body length growth of yellowfin tuna larvae, *L. albacares*, from D-2 to D-10 with potential probiotic supplementation and without probiotics (control).

b. Development of yellowfin tuna larvae, *T. albacares*

Observations of the larvae during larval rearing showed no clear differences from the early stages of D-2 to D-6. However, from D-7 to D-10, the digestive systems of larvae fed probiotic supplements appeared to contain denser food and their metabolisms appeared active, producing feces (Figure 5).

CONCLUSION

Endogenous bacterial isolates from the intestines and stomachs of yellowfin tuna, *L. albacares*, have been successfully isolated, and four of them have potential probiotic properties: *Bacillus subtillis* strain T-A1, *Bacillus amyloliquefaciens* strain T-N2, *Bacillus subtillis* strain T-O4, and *Paraclostridium bifermentans* strain T-O6. Digestive activity began to increase, particularly in DPH-6, progressing to the next stage. The bacterial isolates, acting as probiotics, were not antagonistic, pathogenic, or toxic. Therefore, probiotic technology is environmentally friendly and can prevent yellowfin tuna larval disease infections. Going forward, the successful production of yellowfin tuna fry is expected to be sustainable, and its application in the private sector can be utilized for aquaculture businesses.

ACKNOWLEDGMENTS

The authors would like to thank all the technicians in the Biotechnology Laboratory and the Tuna Laboratory for their assistance in conducting this research. Thanks, are also due to the Mariculture Research and Fisheries Extension Center, Gondol, Ministry of Maritime Affairs and Fisheries.

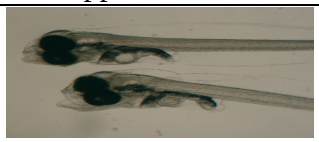
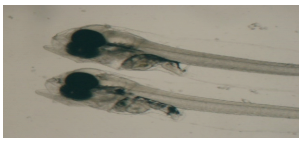
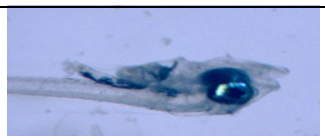
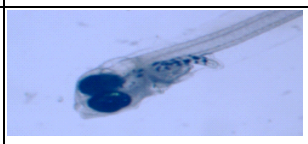
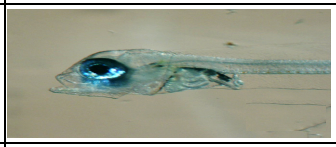
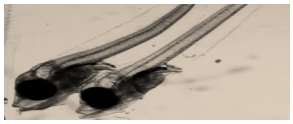
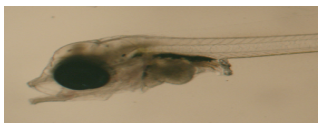
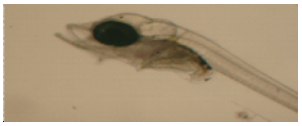
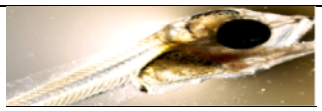
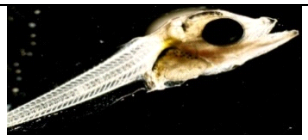
Stage	Probiotic supplementation	Without probiotic-Control	Total length
D-3 (DPH)			P=3199,23 µm C= 3220,19 µm
D-4 (DPH)			P= 3254,25 µm C= 3171,04 µm
D-5 (DPH)			P= 3279,92 µm C= 3161,95 µm
D-6 (DPH)			P= 3467,83 µm C= 3549,71 µm
D-7 (DPH)			P= 3974,84 µm C= 3585,02 µm
D-8 (DPH)			P= 4140,46 µm C= 3729,14 µm
D-9 (DPH)			P= 5140,47 µm C= 4329,17 µm
D-10 (DPH)			P= 5500,18 µm C= 4900,12 µm

Figure 5. Development profile of tuna larvae cultured with probiotic supplementation and without probiotics (Control) (P: Probiotic, C: Control/ without probiotic).

REFERENCES

Assan, D., Kuebutornye, F. K. A., Hlordzi, V. , Chen, H., Mraz , J., Mustapha, U. F., & Abarike, E. D. (2022). Effects of probiotics on digestive enzymes of fish (finfish and shellfish); status and prospects: a mini review, *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 257, 110653. https://doi.org/10.1016/j.cbpb.2021.110653.

Astuti, S. D., Muhamad, A. B., Rahmatillah, A., Yaqubi, A. K., & Susilo, Y. (2023). Electronic Nose (E-Nose) for Quality Detection of Tuna (*Thunnus thynnus*) Contaminated Bacteria. *Indonesian Journal of Tropical and Infectious Disease* (*IJTID*),11(1), 52–65. https://doi.org/10.20473/ijtid.v11i1.39206.

Benetti, D. D., Partridge, G. J., & Stieglitz, J. (2016). Overview on Status and Technological Advances in Tuna Aquaculture Around the World. *In* Book Advances in Tuna Aquaculture, From Hatchery to Market (Eds: Benetti, D.D.,Partridge, G.J. and Buentell, A.), 1-19, https://doi.org/10.1016/B978-0-12-411459-3.00001-1.

Blank, J. M., Morrissettle, J. M., Davie, P. S., & Block B.A. (2012). Effect of temperature, epinephrine and Ca^{2+} on the hearts of yellowfin tuna (*Thunus albacores*). *The Journal of Environmental Biology* 205, 1881-1888.

Cahu, C.L., Papandroulakis, N., Ortega, A., Covès, D., Le Gall, M.M., Desbruyères, E., de la Gándara, F., Zambonino, J.L., D. & Mazurais, D. (2011). Onset of selected digestive enzymes in bluefin tuna (*Thunnus thynnus*) larvae reared in intensive or mesocosm system. Conference: World aquaculture Society Symposium at Rhodes.

Covès, D., de Vogué, B., Desbruyères, E., Dhormes, B., Fievet, J., Huelvan, C., Lallement,S., ... Gatesoupe, F. J. (2011). Probiotic treatment of live food organisms for Atlantic Bluefin tuna larvae : Microbiological & Immunological Criteria, Conference: World aquaculture Society Symposium at Rhodes.

Chen, H., Pan, J., Wang, Y., Qi, Y., Han, F., Xu, C., Farhadi, A., & Li, E. (2023). Growth, health status and gut microbiota of the scalloped spiny lobster (*Panulirus homarus*) at different salinities. *Aquaculture,* 562, 738779.

Daniels, C. L., Merrifield D. L., Ringø, E., & Davies S. J. (2013). Probiotic, prebiotic and synbiotic applications for the improvement of larval European lobster (*Homarus gammarus*) culture. *Aquaculture*, 416-417, 396-406.

Gadoin, E., Durand, L., Guillou, A., Crochemore, S., Bouvier, T., d'Orbcastel,E.R. , Dagorn, L., Auguet, J.C, Adingra, A., Christelle Desnues,C. and Bettarel,Y. (2021). Does the Composition of the Gut Bacteriome Change during the Growth of Tuna?. *Microorganisms*, 9(6), 1157. https://doi.org/10.3390/microorganisms9061157.

Goulden, E. F., Hall, M. R, Pereg, L.L., & Høj L. (2012). Identification of an antagonistic probiotic combination protecting ornate spiny lobster (*Panulirus ornatus*) larvae against *Vibrio owensii* infection. PLoS One 7(7):e39667. https://doi.org/10.1371/journal.pone.0039667.

Greenpeace, (2016). From Sea to Can: (2016). Southeast Asia canned Tuna Ranking, Greenpeace November 2016.

Guillen, J., Asche, F., Carvalho, N., Druon, J.N., Llorente, I., Sciberras, A., Novakoviæ, S.V., & Vukov, I. (2024). How sustainable is tuna aquaculture? A methodology to assess the sustainability of seafood production systems. *Frontiers in Aquaculture*. https://doi.org/10.3389/faquc.2024.1422372.

Haryanti, H., Fahrudin, F., Sembiring, S. B. M., Permana, I. G. N., Koesharyani, I., Muzaki, A., Giri, N. A., & Sugama, S. (2024). The potential of endogenous bacteria as probiotics to enhance survival, growth, and immunity of cultured spiny lobster *Panulirus homarus* (Linnaeus 1758), *AACL Bioflux*, 17(6). http://www.bioflux.com.ro/aacl.

Haryanti, Gunawan, Setiadi, A., Sembiring, S. B. M, Permana, I. G. N., Hutapea, J. H. & Sugama, K. (2021a) The effect of probiotics on digestive enzyme activity during larvae and juvenile stage of Yellow Fin Tuna (*Thunnus albacares*), *IOP Conf. Series: Earth and Environmental Science*, 012019. doi:10.1088/1755-1315/890/1/012019.

Haryanti., Widiastuti, Z., Fahrudin,, Sembiring, S. B. M., Permana, I. G. N., & Sugama K. (2021b). The effect of probiotic and prebiotic-MOS on the survival, growth and immune competence on spiny lobster *Panulirus homarus* culture. *IOP Conf. Series: Earth and Environmental Science* 919:012050.

Hutapea, J. H., Permana, I. G. N., & Giri, I. N. A. (2009). Achievements and bottlenecks for yellowfin tuna, *Thunnus albacares* propagations at the Gondol Research Institute for Mariculture, Bali,Indonesia, *In* Sustainable Aquaculture of the Bluefin and Yellowfin Tuna - Closing the Life Cycle for Commercial Production (eds: Allan, G., Booth, M., Mair, G., Clarke, S., Biswas, A.,) Proceedings of The 2nd Global COE Program Symposium of Kinki University, ISBN 978 1 74256 021 2.

Hutapea, J. H., Permana, G. N., & Setiadi, A. (2010). Rearing of yellowfin tuna, *Thunnus albacares* in controlled tank. *J. Ris. Aquaculture*, 5(1), 79-90.

Jääskeläinen, E., Jakobsen, L. M. A., Hultman, J., Eggers, N., Bertram, H. C., & Björkroth, J. (2019). Metabolomics and bacterial diversity of packaged yellowfin tuna (*Thunnus albacares)* and salmon (*Salmo salar*) show fish species-specific spoilage development during chilled storage. *International Journal of Food Microbiology*, *293*, 44–52. https://doi.org/10.1016/j.ijfoodmicro.2018.12.021.

Lein, Y. E., Lal, M. T. M, Maran, V. A. B., Ch'ng, L. C., Hamasaki, K., Sano, M., & Tuza, D. A. (2022). Gastrointestinal microbiota of spiny lobster: a review. Fishes, 7, 108.

Margulies, D., Scholey, V. P., Wexler, J. B., & Stein, M. S. (2016). Research on the Reproductive Biology and Early Life History of Yellowfin Tuna *Thunnus albacares* in Panama. *In* Book Advances in Tuna Aquaculture, From Hatchery to Market (Eds: Benetti, D. D., Partridge, G. J., & Buentell, A.), 77-114 https://doi.org/10.1016/B978-0-12-411459-3.00004-7.

Maravilla, E.T. , Parra, M., Maisey, K.,Vargas, R.A., Cabezas-Cruz, A. , Gonzalez, A., Tello, M., & Humarán, L.G.B. (2024). Importance of Probiotics in Fish Aquaculture: Towards the Identification

and Design of Novel Probiotics. *Microorganisms*, 12, 626. https://doi.org/10.3390/microorganisms12030626.

Meyer, B., & Wasseem Emam, W. (2024). Welfare implications of closed cycle farming of Atlantic bluefin tuna (*Thunnus thynnus*), *Front. Anim. Sci*. 5, 1445306 https://doi.org/10.3389/fanim.2024.1445306.

Mohammed, S. O., Ashry, E. S. H., Khalid, A., Mohamed R., Amer, M. R., Metwaly, A. M., Ibrahim Eissa, I. H., ... Hafez, E. E. (2022). Expression, Purification, and Comparative Inhibition of Helicobacter pylori Urease by Regio-Selectively Alkylated Benzimidazole 2-Thione Derivatives, *Molecules* , 27, 865. https://doi.org/10.3390/molecules27030865.

Mrèeliæ ,G. J., Nerloviæ, V., Sliškoviæ, M., & Èižmek, I. Z. (2023). An Overview of Atlantic Bluefin Tuna Farming Sustainability in the Mediterranean with Special Regards to the Republic of Croatia. *Sustainability*, 15, 2976 https://doi.org/10.3390/su15042976

Nababan, Y. I., Yuhana, M., Penataseputro, T., Nasrullah, H., Alimuddin, A., & Widanarni, W. (2022). Dietary supplementation of *Pseudoalteromonas piscicida* 1UB and fructooligo saccharide enhance growth performance and protect the whiteleg shrimp (*Litopenaeus vannamei*) against WSSV and Vibrio harveyi coinfection. *Fish and Shellfish Immunology*, 131, 746-756. https://doi.org/10.1016/j.fsi.2022.10.047.

Nurikhsanti, M., Zulkifli, L., Rasmi, D. A. C., & Sedijani, P. (2024). Antagonistic Test of Bacteria Producing Siderophore and Protease Enzymes from The Rhizosfer of Peanut Plants on The Growth of Pathogenic Fungus *Colletotrichum gloeosporioides*. *Jurnal Biologi Tropis*, 24(1), DOI: 10.29303/jbt.v24i1.6459.

Pascual, C., Rodríguez-Canul, R., Huchin-Mian, P. J., Mascaró M., Briones-Fourzán P., Lozano-Álvarez E., Sánchez A., & Escalant K. (2022). Immune response to natural and experimental infection of *Panulirus argus* Virus 1 (PaV1) in juveniles of Caribbean spiny lobster. *Animals (Basel)* 12(15), 1951.

Prihadi, T. H., Gunawan, G., Hutapea, J. H., Permana, I. G. N., Radona, D., Hadie, L. E., Saputra, A., ... Kusmini, I. I. (2025) Study on eggs through larval development of yellowfin tuna, *Thunnus albacares*, based on tropical temperature condition. *AACL Bioflux*, 18(1). http://www.bioflux.com.ro/aacl.

Ringo, E., Doan, H. V., Lee, S. H., Soltani, M., Hoseinifar, S. H., Harikrisnan, R., & Song, S. K. (2020). Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. *Journal of Applied Microbiology*, 129(1), 116-136. https://doi.org/10.1111/jam.14628.

Sambrook, J., & Russell D.W. 2001 Molecular Cloning: A Laboratory Manual, Third Edition (3 volume set) Lab Manual Edition. Cold Spring Harbor Laboratory Pr, 2344 pp. https://www.amazon.com/Molecular-Cloning-Laboratory-Manual-Third/dp/0879695773.

Xie, J., Bian, Z., Wu, Q., Tao,L., Wu, F., & Lin, T. (2021). Global knowledge domain and prospects in tuna research: A bibliometric analysis. *Aquaculture and Fisheries,* 8, 202–210. https://doi.org/10.1016/j.aaf.2021.09.005.

