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Research Article

Safety, Adherence, Enzymatic Activities, and Application Effects of Oral Probiotic Candidates for Shortfin Eel (*Anguilla bicolor bicolor*)

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Abstract

Aquaculture of the shortfin eel (*Anguilla bicolor bicolor*) has been plagued by low survival and growth due to the low tolerance to water quality and feed. The microbiota and shape of the fish intestinal tract influence the immune and digestive systems. The use of bacterial probiotics is fascinating to enhance the digestion system. This study aimed to characterize bacterial probiotic candidates' safety and potential probiotic features for shortfin eel (*A. bicolor bicolor*) aquaculture. The safety, adherence, and enzymatic activity of three bacterial strains (*Bacillus* sp. PCP1, *Lactococcus* sp. JAL 37, and *Enterobacter* sp. JC05) were investigated. An oral application test was performed on shortfin eel (n=880, 15 g) every four days with 0, $3x10^3$, $3x10^5$, and $3x10^7$ CFU/g diet dosages in quadruplicates for two months. At the end of the experiment, total cultivable bacteria and intestinal morphology were assessed. Based on the hemolytic test and intraperitoneal injection, the bacterial strains were considered harmless. In an in vitro investigation, the bacteria attached to shortfin eel intestinal epithelial cells. An agar diffusion method validated the proteolytic, lipolytic, and cellulolytic activities. In vivo feeding tests with the bacterial cocktail lowered the total viable bacteria in the gut while preserving the gut histology. Bacterial strains of the present study are potential probiotic candidates for shortfin (*A. bicolor bicolor*) aquaculture.

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1. Introduction

Eel is a precious aquaculture product. Eels are not widely consumed in Indonesia, but their potential for export to Japan, Hong Kong, Germany, Italy, Taiwan, and Korea is intriguing (Febrianta and Rawendra, 2019). Indonesia's most frequently cultivated eel is the shortfin eel (*A. bicolor bicolor*) (Taukhid *et al*., 2021). Low feed digestibility, poor water quality, and disease with mortality rates of 80% in glass eels and 30% in elver are all common complaints among Indonesian eel farmers (Soeprijanto *et al*., 2018, Widiantoro, 2020; Shi *et al*., 2020; Wahjuningrum *et al*., 2018). As a result, increasing the efficiency and immunity of the eel digestive system is critical.

Gut morphology has a strong relationship with feed digestibility. How thoroughly nutrients are absorbed in the digestive tract is determined by the length of the intestinal villi. Probiotics are microbial feed additives that benefit the host (Lazado and Caipang, 2014). Probiotics can be in the form of bacteria, bacteriophages, microalgae, or yeasts (Grumezescu and Holban, 2018). Supplementing feed with probiotics has been demonstrated to extend villi in tilapia (*Oreochromis* sp.) (Elsabagh *et al*., 2018) and Japanese eel (*A. japonica*) (Lee *et al*., 2018). Probiotics can produce lytic enzymes to support the digestion system of the host (El-Saadony *et al*., 2021). The application of probiotics is proven to provide benefits to fish farming (De *et al*., 2014) by modification of the host intestine microbial community, feed absorption and nutrient utilization (Yilmaz *et al*., 2022), reducing feed conversion ratio (Nathanailides *et al*., 2021), enhance host resistance to disease (Newaj-Fyzul and Austin, 2015), or improve the water quality (Banerjee and Ray, 2017). Bacterial mixtures are recommended to meet all the desired benefits (Lazado *et al*., 2015; Melo-Bolívar *et al*., 2021).

Probiotics have been used on shortfin eels (*A. bicolor bicolor*) for at least five years, with varying degrees of success (Lee *et al*., 2017, 2018; Muchlisin *et al*., 2020; Soeprijanto *et al*., 2018). Probiotics are still confined to Bacillus and Lactobacillus, with minimal focus on the probiotic's impact on gut microbiota and morphology. The safety, adherence ability, enzymatic activities, and effects of supplementation through feed on total cultivable bacteria and histology of eel intestines were all investigated in this study.

2. Materials and Methods

The Integrated Research and Testing Laboratory ethical clearance commission at Universitas Gadjah Mada, Indonesia, approved this experiment (approval number: 00016/04/LPPT/V/2021).

2.1 Bacterial Strain and Culture Condition

Bacillus sp. PCP1, *Lactococcus* sp. JAL 37, and *Enterobacter* sp. JC05 were isolated from the water and digestive tracts of marine fish for this study (Atitus, 2018; Rohman *et al*., 2021). The bacteria were purified and pre-tested for enzyme activity before being stored in the freezer (-80°C). The isolates were cultured in a tryptone soy broth (TSB, Oxoid) for 24 hours, then inoculated on a Tryptone soy agar (TSA, Oxoid) plate to test the colony shape and purity. Pure isolates were given daily stock on TSA medium, kept in the fridge and replenished every four days.

2.2 Enzymes Activity Confirmation

Each bacterial isolates were first cultured on tryptic soy broth (TSB) medium for 24 hours for enzyme activity test at 10^8 cells/mL. To test duplicate proteolytic, lipolytic, and cellulolytic capabilities, skim milk agar, tween 80 agar, and carboxymethyl cellulose (CMC) agar were employed (Istiqomah *et al*., 2019; Soleha and Retnaningrum, 2020). PBS was utilized as a negative control. The cells were incubated at 37°C for 24 hours. Proteolytic and lipolytic activities were discovered during incubation. Dripping a 1% congo red solution onto the CMC plate revealed cellulolytic action. According to Rohman *et al*. (2021), the enzymatic activity index was determined.

2.3 Hemolytic Activity Test

Blood agar produced from pure sheep blood was used to conduct a hemolytic activity test. The bacterial strains were inoculated on blood agar and incubated at room temperature for 24 hours. The hemolytic activity test was double-checked. Bacteria were classified as α-hemolysis (greenish halo), β-hemolysis (clear halo), or γ-hemolysis (no halo) (Ghosh *et al*., 2021). The type γ -hemolysis is the most excellent probiotic candidate.

2.4 Infection Test

Safety assay was done by injection of the probiotic candidates to eel. For one week, a healthy eel population (average weight 30.4 g) was acclimated in a fish aquarium. For each treatment, three eels were chosen at random (non-injected, injected with physiological saline, and injected with each bacterial isolate). Each bac-

teria was cultivated for 24 hours in a TSB medium before being injected intraperitoneally into fish at 10^6 cells per fish. Eelr was kept in an enclosed tub with a shelter, aeration, and a running water system. For 10 days, illness signs and fish mortality were observed daily. It was established that the bacterium group that did not cause disease symptoms or fish mortality was safe.

2.5 Adherence Assay to Intestinal Epithelial Cells

Adherence test was carried out using shortfin intestinal epithelial cells according to Yin *et al.* (2020) and Suryaningsih *et al.* (2021). Eels (100 g) were starved for two days to clean the gut. Eels were anaesthetized with ice, and blood was taken from the caudal vein with a 21G syringe. The blood was centrifuged at 3,000 rpm for 5 minutes and filtered with a 200 nm filter for serum isolation. The eels were dissected to remove the intestine. Two ends of the eel intestines were knotted, then disinfected for 5 seconds in an alcohol solution before being dipped in PBS solution to remove residual alcohol. Scissors were used to open the intestine to isolate epithelial cells.

Individual bacterial cultures were created at three concentrations, 10^5, 10^6, and 10^7 cells/mL, then inoculated with 100 uL of epithelial cell culture. After a two-hour incubation period, the non-adherent cells were washed away with PBS. Adhered cells were fixed at 60°C for 30 minutes using a dry block heater, stained with crystal violet solution, and steeped in 100 uL citrate buffer (20 mmol/L; pH 4) for 45 minutes. Absorbance was measured using a microplate reader (625 nm). The commercial probiotic *Lactobacillus casei* Shirota was utilized as a positive control. Negative control was employed, which consisted of epithelial cells that had not been exposed to bacteria. Each therapy was carried out twice more. According to Iorizzo *et al.* (2022), bacterial adherence was measured.

2.6 In Vivo Application of Probiotics in Elver

Shortfin eels (*A. bicolor bicolor*) were acquired from PT Iroha Sidat Indonesia in Denpasar, Bali (n=1500, average weight 15 g). Eels were grown for seven days in six fibre tanks (100x100x100 cm^3) with aeration and a flow-through system. In this experiment, the fish were fed commercial fish pellets ad libitum twice a day. The fish were then placed in 16 fibre tanks with a density of 440 fish per m^3 and aerated by air stones with a flow-through system after acclimatization. Each tank was protected by a PVC pipe and sterilized with potassium permanganate (KMnO4).

A commercial eel feed (Japfa Aqua Feed powder, minimum protein: 50-52%, minimum fat: 5%, maximum crude fibre: 2%, maximum ash: 15%, and maximum water: 10%) was employed. Feeding was done at 5% of the fish biomass (at 8:00 am and 04:00 pm). These eels were fed a paste prepared from a 1:2.2 powdered feed to water ratio. Based on earlier studies (Aisyah *et al.*, 2020; Apún-Molina *et al.*, 2015), a new culture of *Bacillus* sp. PCP1, *Lactococcus* sp. JAL 37, and *Enterobacter* sp. JC05 was mixed to the feed every four days at 0 (K), $3x10^3$ (A), $3x10^5$ (B), and $3x10^7$ (C) CFU/g diet as a mixture of three strains.

Five eels from each tank were randomly selected at the end of the feeding treatment and anaesthetized with ice cubes. The bacterial gut count was performed on an eel from each group. The gut was opened, cleaned with sterile PBS, and calculated the weight. The inner lining of the gut was scraped with a sterile spatula, and the cells were collected in a 1.5 ml microtube. TSA agar for total bacterial count, GSP agar for Aeromonas and Pseudomonas count, and TCBS agar for Vibrio count were used. The bacterial cultures were cultured for 48 hours at 37°C (Hikmawati *et al.*, 2019). A histology examination was performed following the methods described by Lee *et al.* (2018).

2.7 Data Analysis

Data on safety and enzymatic activity were reviewed in a detailed manner. In the meantime, the data on adherence, total intestine viable bacterial count, and histological morphometry were reported as mean ± standard deviation (SD). The data were analyzed using the SPSS program for normality and homogeneity of variance, followed by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). The statistical significance level was set at $p<0.05$.

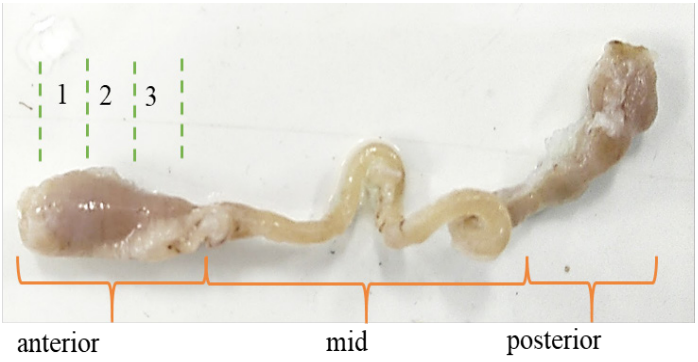

Figure 1. Intestinal morphology of *Anguilla bicolor bicolor*. Histology analysis was made on the front (1), mid (2), and posterior (3) parts of the anterior intestines.

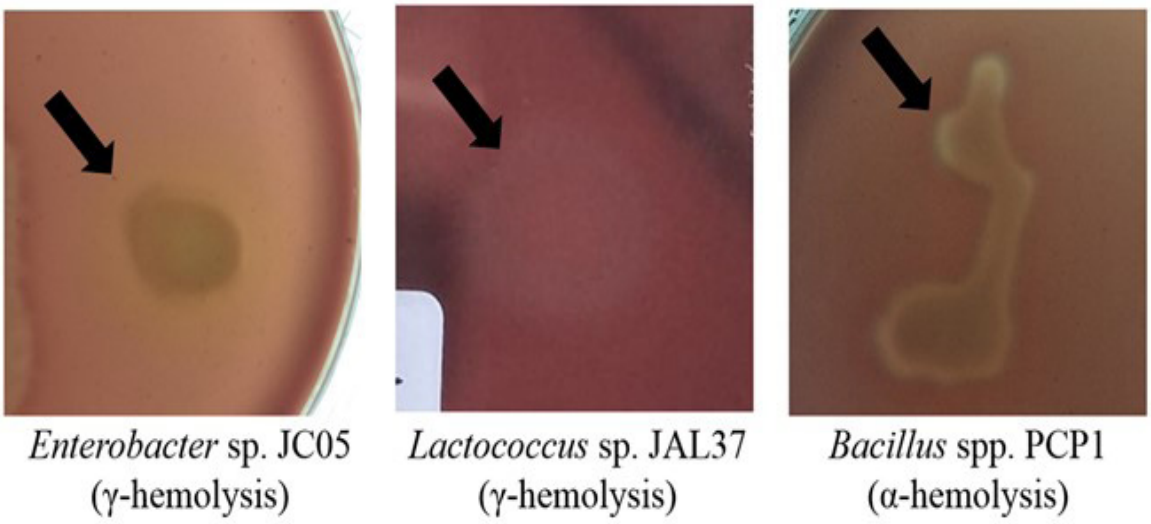

Figure 2. Hemolytic activity test of *Enterobacter* sp. JC05, *Lactococcus* sp. JAL37, and *Bacillus* sp. PCP1 on a blood agar produced from pure sheep blood. The presence of a greenish halo indicated an α-hemolysis, whereas the absence of a halo indicated a γ-hemolysis (Ghosh *et al*., 2021)

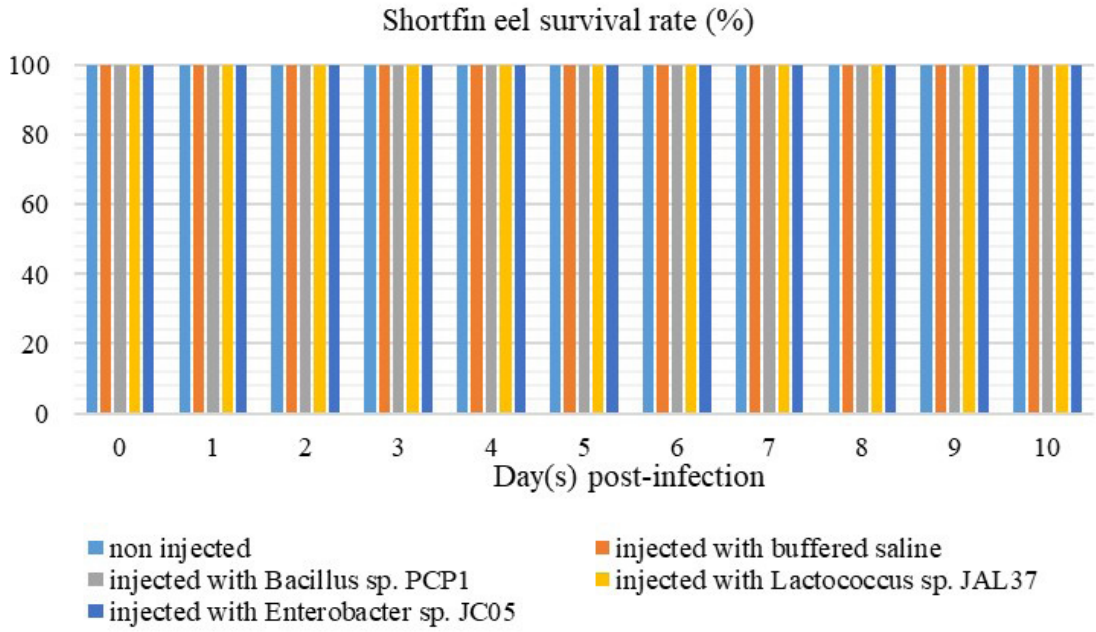

Figure 3. Daily survival rate (%) of shortfin eel elver post-intraperitoneal injection of bacterial probiotic candidates, *Bacillus* sp, PCP1, *Enterobacter* sp. JC05, and *Lactococcus* sp. JAL37. The experimental fish had a 100% survival rate, demonstrating that shortfin eel elver is safe

Table 1. Enzymatic index of *Lactococcus* sp. JAL37, *Bacillus* sp. PCP1, and *Enterobacter* sp. JC05

Bacterial strain	**Enzymatic index**		
	Proteolytic	**Cellulolytic**	**Lipolytic**
Enterobacter sp. JC05	1.2	**1.3**	1.1
Bacillus sp. PCP1	1.2	1.1	**1.3**
Lactococcus sp. JAL 37	**1.3**	1.2	1.1

3. Results and Discussion

3.1 Results

3.1.1 Enzymatic activity

The bacteria have proteolytic, cellulolytic and lipolytic activity, with varying enzymatic indexes (Table 1). *Enterobacter* sp. JC05 has the highest cellulolytic activity. *Lactococcus* sp. JAL 37 showed the highest proteolytic activity and *Bacillus* sp. PCP1 have the highest lipolytic activity.

3.1.2 Safety

Lactococcus sp. JAL 37 and *Enterobacter* sp. JC05 did not lyse blood and was grouped as the γ-hemolytic type, while *Bacillus* sp. PCP1 was α-hemolytic (Figure 2). However, intraperitoneal injection of the three isolates into shortfin eel elvers did not cause disease symptoms and fish mortality up to 10 days after infection (Figure 3). Hence, the three strains are considered safe to shortfin eel elver.

3.1.3 Adherence to shortfin eel intestine epithelial cells

Epithelial cells produced an optical absorption of 0.23 ± 0.03, while epithelial cells with *Enterobacter* sp. JC05, *Lactococcus* sp. JAL 37, *Bacillus* sp. PCP1, and or the positive control resulted in higher optical absorptions, i.e,. 0.27 - 0.32, 0.25 - 0.29, 0.29 - 0.33, 0.28 - 0.32, respectively (Figure 4). Although each bacteria was added in concentrations of 10^5, 10^6 and 10^7 cells/ml to the epithelial cell culture, the number of adherent bacterial cells was the same.

3.1.4 Total number of viable intestinal bacteria

The amount of Aeromonas, Pseudomonas, and Vibrio in the intestine of A. bicolor bicolor was sampled in the mid part of anterior segment (Figure 1). The amount of the bacterial genera did not differ significantly between treatments ($p > 0.05$) (Table 2). However, total bacteria were substantially higher in the non-probiotic group (K) than in the probiotic treatments (A, B, and C) (p 0.05).

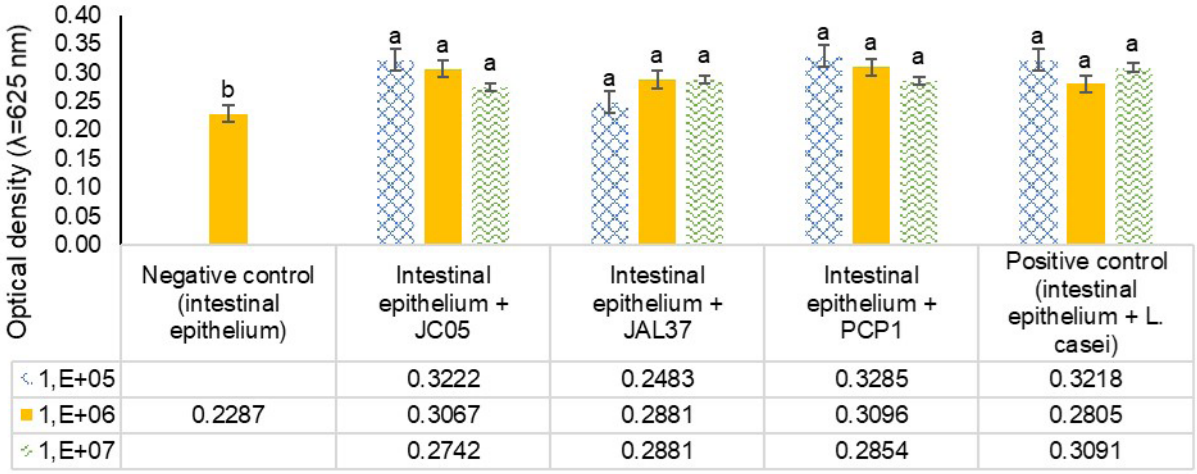

Figure 4. The absorbance of eel intestinal epithelial cells with or without bacterial cells adherence by spectrophotometry detection. Results are expressed as means ± SD (n = 2). Mean values with different letter differ significantly (P < 0.05)

Table 2. Total intestinal viable bacterial count (mean ± SD) of Indonesian short-fin eel (*A. bicolor bicolor*) fed with various doses of probiotic

Parameter	Probiotic doses (CFU/g diet)			
	K(0)	A (3×10^3)	B (3×10^5)	C (3×10^7)
Total bacteria (log10 CFU/g intestine)	8.23 ± 7.14^b	7.13 ± 6.39^a	7.46 ± 6.51^a	6.41 ± 5.39^a
Aeromonas (log10 CFU/g intestine)	3.18 ± 2.28^a	2.70 ± 1.76^a	2.88 ± 1.70^a	4.44 ± 3.51^a
Pseudomonas (log10 CFU/g intestine)	5.78 ± 4.98^a	6.52 ± 5.70^a	7.45 ± 6.73^a	6.22 ± 5.42^a
Vibrio (log10 CFU/g intestine)	5.95 ± 4.86^a	5.73 ± 4.52^a	6.25 ± 5.47^a	4.91 ± 3.84^a

Description: Different superscripts in the same column shows significant differences ($p \leq 0.05$).

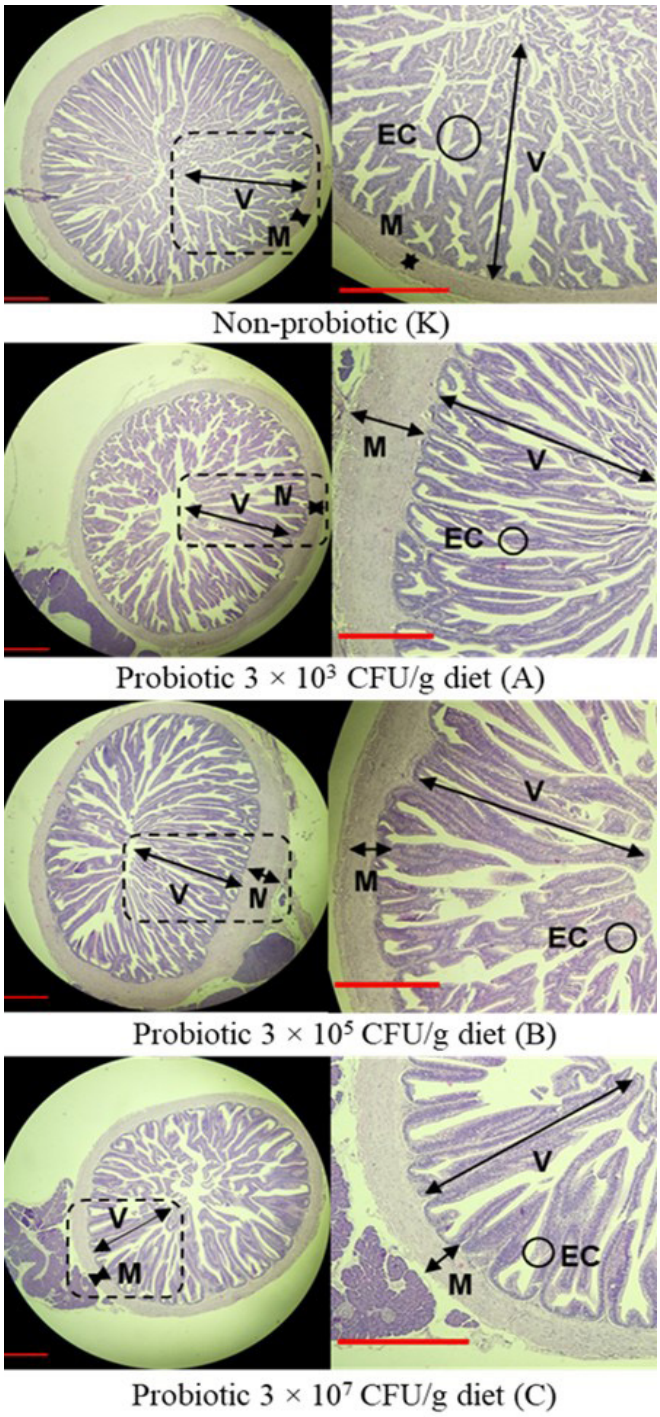

Figure 5. Intestinal histological structure of shortfin eel, *A. bicolor bicolor* fed with various doses of probiotics. The intestinal villi (V), muscular layer (M), and epithelial cells (EC) could be identified as similar in each sample. Bar=100 µm

3.1.5 Intestinal morphology

Intestinal morphology of Anguilla bicolor bicolor was examined on the front, mid, and posterior parts of the anterior intestines (Figure 1). The muscular layer (M) and villi are visible in this cross-section of the intestine (V). Epithelial cells appear to cover the entire surface of the villi (EC) (Figure 5). Intestinal histology revealed no significant difference in *A. bicolor bicolor* across treatments ($P > 0.05$) (Table 3).

3.2 Discussion

In vitro profiling of major oral probiotic characteristics and application effect testing are required (Nandi *et al*., 2017). A variety of tests can be used to establish whether an oral probiotic candidate is safe, able to survive and attach to the intestinal lumen of cultured fish, and has certain beneficial activities (Kavitha *et al*., 2018). The bacteria *Enterobacter* sp. JC05 and *Lactococcus* sp. JAL37, as well as *Bacillus* sp. PCP1 from water (Atitus, 2018; Rohman *et al*., 2021) was tested in this investigation.

Oral probiotics' potential to create digestive enzymes is an appealing concern. Intestinal microbiota (Faturrahman *et al*., 2021) can naturally aid fish digestion, which is critical in establishing accessible nutrients for biological processes in fish (Hani *et al*., 2018). Including probiotics with high digestive enzyme activity improved feed efficiency and growth of a Japanese eel, *A. japonica* (Lee *et al*., 2018, 2013). Proteolytic, cellulolytic, and lipolytic enzymes were found in the bacterial strain used in this study, which might be used to digest the primary macromolecules in fish feed. It also has the potential to be used in the shortfin eel *A. bicolor bicolor*.

The host must be safe when using probiotics. Bacteria were deemed safe in this study due to the lack of clinical signs or fish mortality following bacterial infection at high concentrations in elver. The safety was consistent with *Enterobacter* sp. JC05 and *Lactococcus* sp. JAL37's non-hemolytic nature. Meanwhile, *Bacillus* sp. PCP1 demonstrated alpha hemolysis against sheep's blood, which was similar to the character of *Streptococcus salivarius* probiotic in a prior investigation (Li *et al*., 2021).

The capacity of bacteria to adhere is the first step in their colonization of the host (Istiqomah *et al*., 2015; El-Saadony *et al*., 2021). Probiotics must adhere to the intestinal epithelium and mucus to grow correctly and avoid gaining a competitive edge in their ecosystem at the host target location (Zhao *et al*., 2020; Sarojini *et al*., 2020) even though they were classed as low adhesion bacteria, *Enterobacter* sp. JC05, *Lactococcus* sp. JAL37, and *Bacillus* spp. PCP1 were able to stick to shortfin eel intestinal epithelial cells, similar to the positive control (Slizewska *et al*., 2020). We believe this is

Table 3. The histology (mean ± SD) of Indonesian short-fin eel (*A. bicolor bicolor*) fed with various doses of probiotics

Intestinal area	Parameter	Probiotic doses (CFU/g diet)			
		0	**3×10^3**	**3×10^5**	**3×10^7**
Front anterior	Diameter of intestine (µm)	1679 ± 444[a]	1795 ± 176[a]	1718 ± 279[a]	1683 ± 79[a]
	Villi length (µm)	565 ± 162[a]	725 ± 138[a]	571 ± 125[a]	643 ± 181[a]
	Muscular layer thickness (µm)	122 ± 67[a]	162 ± 80[a]	145 ± 16[a]	178 ± 62[a]
Mid anterior	Diameter of intestine (µm)	1926 ± 301[a]	2019 ± 295[a]	1773 ± 262[a]	1932 ± 122[a]
	Villi length (µm)	681 ± 324[a]	717 ± 217[a]	585 ± 160[a]	615 ± 178[a]
	Muscular layer thickness (µm)	192 ± 98[a]	142 ± 77[a]	199 ± 15[a]	238 ± 69[a]
Posterior anterior	Diameter of intestine (µm)	1609 ± 358[a]	1536 ± 321[a]	1421 ± 359[a]	1616 ± 197[a]
	Villi length (µm)	633 ± 178[a]	603 ± 175[a]	571 ± 138[a]	611 ± 48[a]
	Muscular layer thickness (µm)	182 ± 47[a]	209 ± 80[a]	185 ± 28[a]	193 ± 45[a]

Data represented as means ± SD (n = 4). Different superscripts in the same column show significant differences ($p \leq 0.05$).

impacted in part by the bacteria's origin, which is not from an eel's digestive tract.

The capacity of bacteria to attach to the intestinal epithelium of the shortfin eel has been proven in the present study. Every four days, we gave probiotics in various doses. Probiotics stabilized the total number of viable bacteria in the intestines of eels but did not affect the viable number of potentially harmful species such as Aeromonas, Pseudomonas, and Vibrio. The results are different from the application of commercial probiotics that reduces the number of pathogenic bacteria in shortfin eel aquaculture water (Triyatmo and Isnansetyo, 2020). There was also no difference in the overall viable bacterial count in the fish gut between the low, medium, and high probiotic dose groups. A similar outcome is demonstrated in human probiotics (Ouwehand, 2017). We believe it has something to do with the surface. According to the findings, raising probiotic doses to more than 10^5 CFU/g of feed will have the same effect as the 10^5 CFU/g diet. This finding differs from earlier research in that probiotics applied to Japanese eels at 10^7 CFU/g outperformed 10^6 and 10^8 CFU/g diets (Lee *et al*., 2017). Due to the use of procedures that are limited to live bacteria, the current study has limitations in demonstrating the actual condition of the eel's intestines. Because only around 1% of bacteria in environmental samples can be cultivated, the findings of this study must be validated molecularly (Stepanauskas, 2013). Other techniques that are not culture-dependent, such as Next-generation Sequencing of DNA, must be used.

The length of the intestinal villi is linked to nutritional absorption in the gastrointestinal system. Increased villi length can increase the intestines' ability to absorb digested nutrients, resulting in better fish growth. In tilapia, Prussian carp, and Japanese eel, probiotics have extended villi (Lee *et al*., 2018). Nonetheless, we discovered that the histological features of shortfin

eels in control groups were similar to those in probiotic-treated groups in the current study. All of the fish in this study had the same gut diameter, villi length, and muscle layer thickness as the fish in the prior study given probiotics (Lee *et al*., 2017). As a result, it's thought that the current result is attributable to the ideal circumstance. The impact of probiotic treatments on shortfin eel growth has been documented (Soeprijanto *et al*., 2018). As a result, more research into the effects of the current probiotic application on shortfin eel digestion, growth, immune system, disease resistance, and other factors is needed.

4. Conclusion

Bacillus sp. PCP1 from water and *Enterobacter* sp. JC05 and *Lactococcus* sp. JAL37 from the fish intestine complements proteolytic, cellulolytic, and lipolytic activities. Three probiotic strains were safe since they did not produce behavioural changes or mortality in shortfin eel elvers. These three bacteria were rather weakly adherent to the intestinal epithelial cells of shortfin eels (*A. bicolor bicolor*). The gut microbiota was controlled by lowering the total viable bacterial count by using the bacterial mixture in a shortfin eel elver diet every four days at a minimum $3x10^3$ CFU/g diet for two months. The use of these probiotics did not affect the histological state of the intestine, including its diameter, villi length, and muscular layer thickness. These bacteria could be a good match for shortfin eel elvers. Further research with the probiotic application at lower or higher doses than those utilized in the current study is needed to see if they perform better as shortfin eel elver probiotics.

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Authors' Contributions

All authors have contributed to the final manuscript. Each author's contribution is as follows, ARD; designed and built the installation, prepared the probiotic, monitored and controlled the experiment, collected and analyzed the data, and drafted the manuscript. II and AI; devised the main conceptual ideas and critical revision of the article. AOP; designed and built the installation, prepared the probiotic, monitored and controlled the experiment, collected the secondary data. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors have no conflict of interest.

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