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In vitro Assessment of Multistrain Probiotic on Its Safety, Biofilm Formation Capability, and Antimicrobial Properties Against *Aeromonas hydrophila*

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ABSTRACT

Probiotics have been increasingly considered an alternative to antibiotics in combating disease outbreaks. Combined probiotics have been studied to possibly harbor synergistic effects that could provide better protection for aquatic species. Three potential probiotics, which had shown *in vitro* antagonism towards *Aeromonas hydrophila* in this study, were *Bacillus amyloliquefaciens* (L9, isolated from the blue swimming crab), *Lysinibacillus fusiformis* (A2, isolated from a microalga), and *Enterococcus hirae* (LAB3, isolated from the Asian seabass) were combined into a probiotic mixture. The probiotic mixture produced significantly higher biofilm ($P < 0.05$) (2.441 ± 0.346) than *A. hydrophila* (0.578 ± 0.124) during 24-h and showed a continuous increase in production at 48-h and 72-h time intervals, respectively. Furthermore, no hemolytic action was observed when the probiotic mixture was streaked on sheep blood agar (5%), whereas *A. hydrophila* presented α-hemolysis. The lowest concentration of the probiotic mixture (10^7 CFU mL^{-1}) significantly inhibited ($P < 0.05$) the growth of *A. hydrophila* at 10^6 CFU mL^{-1} after 24 h of incubation, where bacterial count in the treatment was 6.595 ± 0.218 CFU mL^{-1}, which was significantly lower ($P < 0.05$) than the control (7.247 ± 0.061 CFU) mL^{-1}. Significant reduction ($P < 0.05$) in *Aeromonas* count from 7.532 ± 0.026

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CFU mL^{-1} to 6.883 ± 0.015 CFU mL^{-1} was observed at 12 hours of co-incubation. Hence, this research suggests that the probiotic mixture of L9, A2, and LAB3 potentially confers protection against *A. hydrophila* infection due to their characteristics meeting the criteria of probiotics.

Keywords: *Aeromonas hydrophila*, antagonism, biofilm formation, hemolytic activity, probiotic mixture

INTRODUCTION

Aquaculture is a rapidly expanding multibillion-dollar industry with a compound annual growth rate of 5.3% per year (2001–2018) compared to terrestrial meat production, standing at 2.7% (Food and Agriculture Organization of the United Nations [FAO], 2020). Global aquaculture production reached a record of 114.5 million tonnes in 2018, providing >50% of food fish for human consumption (FAO, 2020). Furthermore, with the human population projected to exceed 9 billion by 2050, farmed fish and shellfish production will need to increase by 133% to meet worldwide fish demands (Duarte et al., 2020).

Aquaculture, also known as aquafarming, produces fish, crustaceans, mollusk, aquatic plants, algae, and other organisms in marine, brackish, and freshwater systems under controlled conditions (FAO, 2020; Naylor et al., 2021). One of the main causes of economic loss in cultured fish is bacterial diseases where under stressful conditions, the pre-existing bacteria invade the host and causes a disease outbreak (Fazio, 2019; Morae & Martins, 2004). Aeromonads such as *A. hydrophila* and *A. veronii* spread horizontally, causing hemorrhagic disease, ulcerative syndrome, and Motile *Aeromonas* Septicemia (MAS) in fish, usually resulting in high mortalities, especially in farmed warm-water fishes (Gudmundsdottir & Bjornsdottir, 2017; Janda & Abbott, 2010). Experimental challenge of *Oreochromis aureus* with *A. hydrophila* revealed massive hemocyte aggregation and cellular necrosis of gills, hepatopancreas, and to a lower extent in the digestive system of infected fish (AlYahya et al., 2018).

For decades, animal disease prevention and treatment have revolved around using chemical additives and veterinary medicines, especially antibiotics. However, reports on the detrimental effects caused by the broad spectrum of chemotherapeutics, such as the emergence of drug-resistant bacteria, potential human health hazards, environmental contamination, and elimination of gut microflora beneficial to fishes, have triggered interest in experimentation with biological and eco-friendly approaches (Akanmu, 2018).

The development of alternative therapies to eradicate bacterial pathogens in animal production is indispensable. Several methods have been successfully tested in the aquaculture industry, particularly the application of probiotics (Cavalcante et al., 2020; Munir et al., 2018). Probiotics are live microorganisms beneficial to the host, which alter the microbial community associated with the host or environment, ensuring better feed utilization, health improvement,

and enhanced disease resistance (Tran et al., 2022; Yilmaz et al., 2022). Most probiotics used as biological control agents in aquaculture are lactic acid bacteria (LAB) (*Lactobacillus*, *Enterococcus*, *Lactococcus*, *Micrococcus*, and *Carnobacterium*) (Román et al., 2012). In addition, other genera or species belonging to the genus *Vibrio* (Restrepo et al., 2021), *Bacillus* (Elsabagh et al., 2018), *Pseudomonas* (Qi et al., 2020), and *Aeromonas* (Jinendiran et al., 2021) were also extensively studied. One such example of probiotics application is the diet supplementation of *Bacillus licheniformis* to *Oreochromis mossambicus*, which showed the potential to reduce ammonia toxicity, improve growth performance, general health status as well as resistance to a pathogen (*A. hydrophila*) (Gobi et al., 2018; Gopi et al., 2022). However, many studies only emphasized the different health effects produced by single-strain probiotics, such as *Bacillus subtilis* in the red hybrid tilapia (*Oreochromis* sp.) (Ng et al., 2014) and *Lactobacillus plantarum* in the Nile tilapia (*Oreochromis niloticus*) (Gewaily et al., 2021).

Nevertheless, the supplementation of multi-strain probiotics (MSPs) is relatively new. The combination of probiotics containing *Bacillus subtilis* E20, *Lactobacillus pentosus* BD6, *Saccharomyces cerevisiae* P13, and *Lactobacillus fermentum* LW2 was observed to improve the growth performance and health status of the Asian seabass (*Lates calcarifer*) (Lin et al., 2017). The efficacy of MSPs is due to symbiosis caused by the positive interrelationship between candidate strains (Puvanasundram et al., 2021). A previous study highlighted the benefits of supplementing mixed probiotics (combining *Lysinibacillus fusiformis* SPS11, *Bacillus amyloliquefaciens* L9, and *Enterococcus hirae* LAB3) to improve biofloc production in red hybrid tilapia culture (Zabidi, Yusoff, et al., 2021). Another study showed mixed probiotics (*L. fusiformis* SPS11, *L. fusiformis* A2, and *Bacillus megaterium* I24) protected *Artemia* against *Vibrio alginolyticus* infection (Chean et al., 2021). Thus, the present study aimed to determine whether the probiotic mixture (containing *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3) was selected based on a series of *in vitro* antagonistic assays and could potentially eliminate *A. hydrophila* and can ultimately be categorized as a beneficial probiotic mixture. Additionally, the biofilm formation capability of the probiotic mixture was also assessed in this study.

MATERIALS AND METHODS

Probiotic Strains

The agar and broth media used in this study were Trypto-casein soy agar (TSA) (Biokar Diagnostics, France) and Trypto-casein soy broth (TSB) (Biokar Diagnostics, France), respectively. In addition, potential probiotics used in this study were isolated and identified through earlier research at the Laboratory of Fish Health, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia (Table 1).

Table 1

Potential probiotics isolated from different hosts

Probiotic	Strain	Genbank accession number	Host	Reference
Bacillus amyloliquefaciens	L9	MN096656	Blue swimming crab (*Portunus pelagicus*)	Azrin et al. (2019)
Enterococcus hirae	LAB3	MK757970	Asian seabass (*Lates calcarifer*)	Masduki et al. (2020)
Lysinibacillus fusiformis	A2	MK764895	Microalga (*Amphora* sp.)	Rosland et al. (2021)

All isolates were cultured overnight in the TSB medium before any screening assays. *Aeromonas hydrophila*, provided by Laboratory of Aquatic Animal Health and Therapeutics, Institute of Biosciences, Universiti Putra Malaysia, was previously isolated from diseased *Oreochromis* sp. A single colony from each potential isolate and *A. hydrophila* was picked and inoculated separately into the TSB medium and incubated at 30 °C for 24 h.

Antagonistic Screening of Potential Probiotics and Mixture

All potential probiotics were screened *in vitro* on TSA agar to test for inhibitory effects against *A. hydrophila*. Overnight cultures of probiotics and the pathogen were centrifuged at 1,957 × *g* for 10 min. The supernatant was discarded, and cells were resuspended with distilled water (ddH_2O). The absorbance of the resuspended overnight culture of *A. hydrophila* was measured using the UV Spectrophotometer (Eppendorf, Germany) at 550 nm to adjust the concentration of the bacterial culture.

The agar well diffusion assay was used as a primary screening step to determine the antagonism of potential probiotics towards *A. hydrophila*. The assay was conducted on TSA agar using isolates cultured overnight in TSB following Rengpipat et al. (2008), with some modifications. After 24 h, the pathogenic *A. hydrophila* was diluted to the concentration of 10^6 CFU mL^{-1} and swabbed onto the TSA agar using a sterile cotton swab. Next, the agar was punched with a 5 mm sterile cork borer, and 10 µL of the overnight culture of potential probiotics (10^9CFU mL^{-1}) was inoculated into the well. The plate was then incubated at 30 °C for 24 h, and the inhibition zones were observed while measuring the diameter in mm. Furthermore, an equal volume (1:1:1) of the probiotics was added to a 1.5 mL microcentrifuge tube to produce a probiotic mixture. The probiotic mixture was then incubated for 20 min at 30 °C. The antagonistic activity of the probiotic mixture against *A. hydrophila* was also evaluated as described above.

Compatibility Assay of the Selected Potential Probiotics

The compatibility among selected probiotics was also evaluated using the agar well diffusion assay (Rengpipat et al., 2008). The probiotics combined into a probiotic mixture were cultured overnight in TSB prior to the assay. A sterile cotton swab was used for each probiotic to obtain the culture before spreading it onto the TSA. Afterward, a 5 mm cork borer was used to punch two wells on the swabbed agar to inoculate the other two probiotics. These steps were repeated for each probiotic in triplicate. Finally, the plates were incubated for 24 h at 30 °C, and the presence of any inhibitory zones was duly recorded.

In vitro Hemolysis Assessment

Sheep blood agar plates (5%) (Thermo Scientific Microbiology Sdn. Bhd., Malaysia) were used to detect the presence of hemolysin in the hemolysis test to rule out potential pathogenicity. This assay determines whether the probiotic mixture (comprising *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3) can produce enzymes that destroy red blood cells if supplemented with a host. Overnight cultures of the potential probiotic mixture and *A. hydrophila* (positive control) were streaked onto two separate blood agar plates and incubated overnight for 30 °C. After 24 h, the results of both blood agars were compared, and the presence or absence of clearing zones around the colonies was observed to interpret the hemolytic activity. Isolates that caused no change in the agar around the colonies were considered non-hemolytic (γ-hemolysis), and isolates showing a clean zone around colonies were considered to be hemolytic (β-hemolysis) (Foulquié Moreno et al., 2003).

Biofilm Production Assay

With some modifications, the biofilm production assay was conducted following (Bruhn et al., 2007). Before conducting the assay, the potential probiotics and *A. hydrophila* were cultured overnight in the TSB medium on an orbital incubator shaker (BioSan Laboratories Inc., Latvia). The biofilm production assay was performed in triplicate for each sample: the control (TSB only), *A. hydrophila* only, probiotic mixture only, and the three selected single probiotics during 6-, 12-, 24-, 48-, and 72-h, respectively. Initially, 2 mL of TSB media was added into separate glass vials, followed by 200 µL of each bacterial culture at a concentration of 10^9 CFU mL^{-1}. The glass vials were left to incubate at room temperature without shaking, and the biofilm formation was observed over the specified period. At every sampling interval, the contents in the glass vials were discarded and thoroughly rinsed with distilled water to remove poorly adhered cells. Next, the crystal violet (200 µL, 0.2%) dye was aliquoted into the glass vials to stain the contents. Finally, the glass vials were rinsed thoroughly with ddH_2O to remove excess crystal violet stain. Subsequently, the crystal violet stain was eluted with 95% ethanol (Systerm, Malaysia)), and the absorbance level was measured using the UV Spectrophotometer (Eppendorf, Germany) at 550 nm for each respective sample. The biofilm formation of a probiotic mixture, constituent single strain probiotics, and *A. hydrophila* was recorded and compared.

Minimum Inhibitory Concentration (MIC) of the Potential Probiotic Mixture against *Aeromonas hydrophila*

The probiotic mixture of *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3 was further analyzed for its inhibitory potential against *A. hydrophila*. Minimum inhibitory concentration is done to determine the lowest concentration of the potential probiotic mixture that can inhibit the growth of pathogenic *A. hydrophila* in a liquid medium (Andrews, 2001). Before the assay, selected single strain probiotics and *A. hydrophila* were cultured overnight in the TSB medium. The next day, the potential probiotics were combined to produce a probiotic mixture and allowed to incubate for 20 min. Absorbance levels of both probiotic mixture and pathogen were measured using the UV Spectrophotometer (Eppendorf, Germany) at 550 nm. The pathogen was adjusted to the concentration of 10^6 CFU mL^{-1}. Next, each concentration of the potential probiotics (10^1 CFU mL^{-1} to 10^9 CFU mL^{-1}) was inoculated and cultured individually with *A. hydrophila* at 10^6 CFU mL^{-1} in Falcon tubes. The tubes were allowed to incubate overnight on an orbital incubator shaker (BioSan Laboratories Inc., Latvia) at 30 °C. The following day, 1 mL from each tube containing different concentrations of the probiotic mixture co-cultured with the pathogen was transferred into 1.5 mL Eppendorf tubes for serial dilution to ease the counting of colonies. The serially diluted cultures (100 uL) were plated on *Aeromonas* Isolation Medium Base (HiMedia, India). The plates were incubated overnight at 30 °C, and afterward, the colonies of *A. hydrophila* were counted as colony-forming units per mL (CFU mL^{-1}) using the following formula:

$$\text{CFU mL}^{-1} = \frac{\text{(Number of colonies) x (Dilution factor)}}{\text{Volume of culture plate (mL)}}$$

Co-culture Assay of the Potential Probiotic Mixture with *Aeromonas hydrophila*

The co-culture assay is a liquid medium used to observe and quantify the interaction between the potential probiotic mixture and pathogen over time (Vaseeharan & Ramasamy, 2003). As determined from the minimum inhibitory concentration assay, the concentration of the potential probiotic mixture used was 10^7 CFU mL^{-1}, whereas the concentration of pathogen, *A. hydrophila*, was 106 CFU mL^{-1}. Samples were taken at 0-, 6-, 12-, 24-, 48-, and 72-h. At each sampling interval, 100 µL of the co-culture treatment was serially diluted for the ease of counting the colonies and proceeded to plate on *Aeromonas* Isolation Medium Base (HiMedia, India). Then, suitable dilution was aliquoted onto the medium, and colonies of *A. hydrophila* were counted as CFU mL^{-1} using the formula below:

$$\text{CFU mL}^{-1} = \frac{\text{(Number of colonies) x (Dilution factor)}}{\text{Volume of culture plate (mL)}}$$

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 20 software. All data collected were analyzed using a one-way analysis of variance (ANOVA). In addition, Tukey's test was applied for pairwise comparison of the means. Data

were expressed as mean ± standard error of the mean (SEM) at a significant level of $P < 0.05$.

RESULTS

Antagonistic Screening of Potential Probiotics and Mixture

All three potential probiotics had an inhibitory effect against *A. hydrophila*, as indicated by the clear zone around the well (Figure 1). The inhibitory zone recorded by *B. amyloliquefaciens* L9 was 14.5 ± 0.3 mm (Table 2), whereas *L. fusiformis* A2 has the highest inhibitory zone size with an average of 15.5 ± 0 mm. Contrarily, *E. hirae* LAB3 only showed the presence of inhibitory activity, but the inhibition zone could not be quantified. Therefore, a well diffusion assay was conducted using the mixture to ensure that the antagonistic activity of the probiotic mixture against *A. hydrophila* was conserved. Zones of inhibition were measured and recorded accordingly in Table 2. The probiotic mixture (L9 + LAB3 + A2) inhibited the growth of *A. hydrophila* with an inhibition zone size of (15 ± 0) mm.

Table 2

Antagonistic screening of potential probiotics *against* Aeromonas hydrophila

Potential probiotic	Strain	Diameter of inhibition zone (mm) ± SEM
Bacillus amyloliquefaciens	L9	14.5 ± 0.3
Enterococcus hirae	LAB3	+
Lysinibacillus fusiformis	A2	15.5 ± 0
Probiotic mixture	L9 + LAB3 + A2	15 ± 0

Note. Size of inhibition zone ± SEM, n = 3; + = The presence of inhibitory zone but not measurable

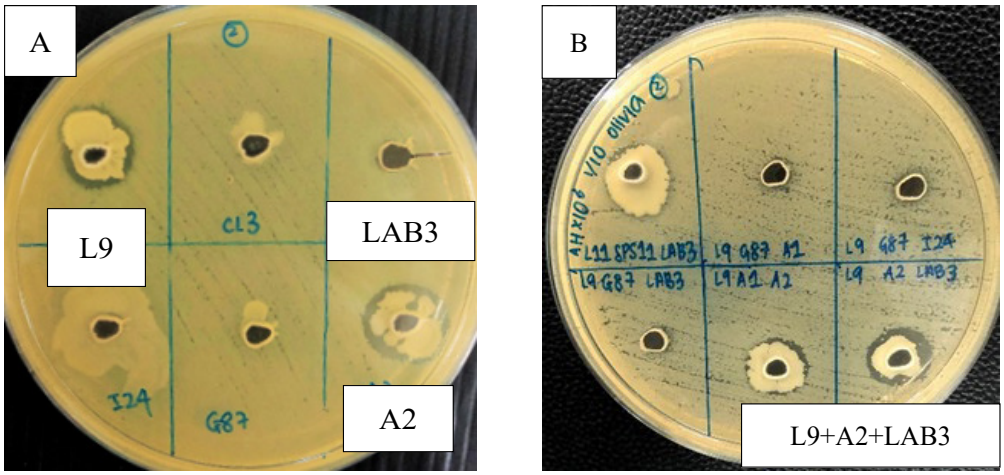

Figure 1. Inhibition zones of potential probiotic strains against *Aeromonas hydrophila* at a concentration of 10^6 CFU mL^{-1} were produced in *in vitro* well diffusion assay. Clear zones indicate the inhibitory activity by the respective probiotic mixture (A) = *Bacillus amyloliquefaciens* (L9), *Enterococcus hirae* (LAB3), *Lysinibacillus fusiformis* (A2); (B) = L9 + LAB3 + A2 (Probiotic mixture)

Compatibility Assay of Selected Probiotics

The selected probiotics, consisting of *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3, were tested for their compatibility. All probiotics showed no presence of inhibition when tested against each other. It indicates that the functionality of each probiotic will not be affected when combined into a mixture.

In vitro Hemolysis Assessment

The probiotic mixture showed a lack of hemolysis activity in the area surrounding the bacterial colony and was classified as γ- hemolysis (Figure 2). In contrast, *A. hydrophila* exhibited a clear zone of hemolysis representing the complete breakdown of the hemoglobin of the red blood cells in the vicinity of the bacterial colony and was classified as β-hemolysis.

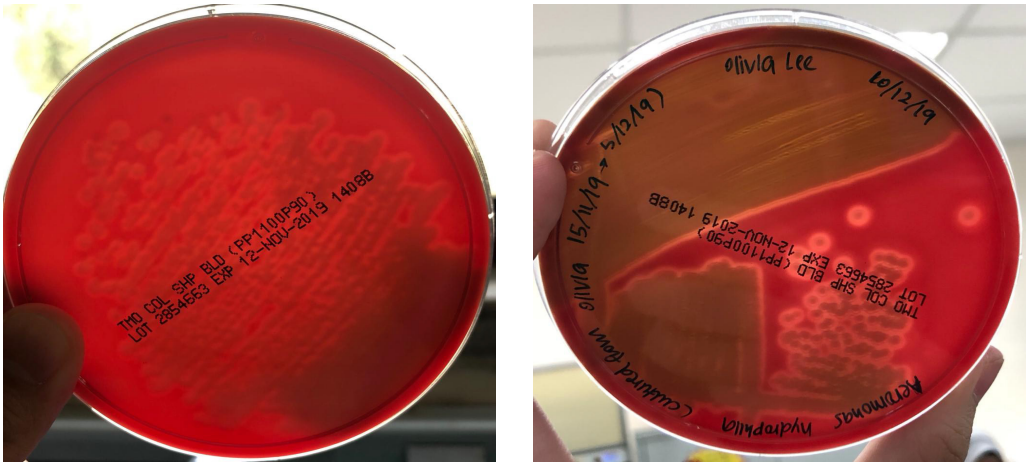

Figure 2. Potential probiotic mixture (containing *Bacillus amyloliquefaciens* L9, *Lysinibacillus fusiformis* A2, and *Enterococcus hirae* LAB3) (Left) and *Aeromonas hydrophila* (Right) streaked on the blood agar

Biofilm Production Assay

Biofilm assay determines the probiotic mixture's ability to produce biofilm to confer protection against *A. hydrophila*. During 6-, 12-, 24-, 48-, and 72-h, the absorbance of the stained-biofilms produced by *A. hydrophila*, probiotic mixture (*B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3), and its single probiotics were shown in Figure 3.

The biofilm formation of the probiotic mixture showed an increasing trend from 6- to 72-h. The absorbance reading for the probiotic mixture was significantly higher ($P > 0.05$) (2.441 ± 0.346) than the pathogen *A. hydrophila* (0.578 ± 0.124) at 24-h interval. Moreover, the biofilm production of single-strain probiotics showed no significant results. Hence, the probiotic mixture harbors a synergistic effect to produce a higher amount of biofilm.

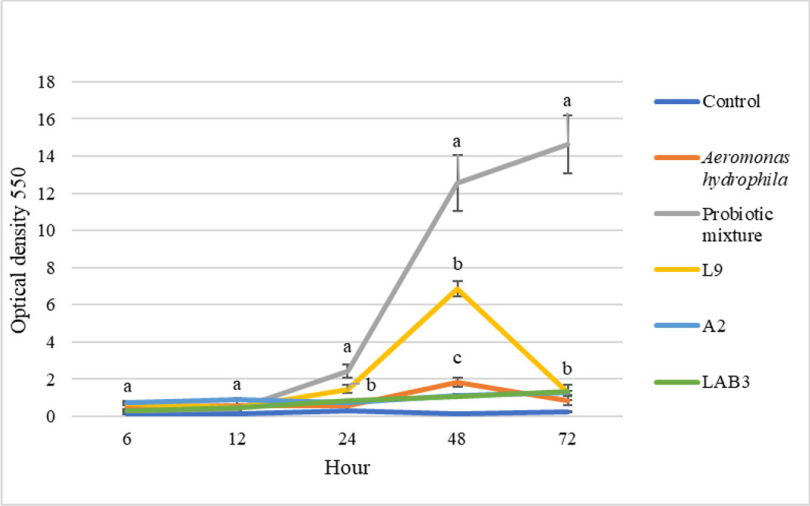

Figure 3. The biofilm production by *Aeromonas hydrophila*, probiotic mixture (L9 + A2 + LAB3), and single-strains probiotics (L9, A2, and LAB3) during 6-, 12-, 24-, 48-, and 72-h. Different alphabets indicate significant differences among treatments ($P < 0.05$)

Note. L9 = *Bacillus amyloliquefaciens*; A2 = *Lysinibacillus fusiformis*; LAB3 = *Enterococcus hirae*

Minimum Inhibitory Concentration of the Potential Probiotic Mixture Against *Aeromonas hydrophila*

The assay was carried out to determine the lowest concentration of probiotic mixture that could inhibit the growth of *A. hydrophila* in liquid mode when they were cultured together in TSB. The *A. hydrophila* count reduced significantly ($P < 0.05$) when treated with 10^7, 10^8, and 10^9 CFU mL^{-1} of the probiotic mixture (Figure 4). Nevertheless, the probiotic mixture at concentrations ranging from 10^1 to 10^6 CFU mL^{-1} showed no significant effect ($P > 0.05$) in inhibiting the growth of *A. hydrophila*. Hence, the lowest concentration of probiotic mixture required to inhibit the growth of *A. hydrophila* was 10^7 CFU mL^{-1}.

Co-Culture Assay of the Potential Probiotic Mixture with *Aeromonas hydrophila*

Based on the minimum inhibitory concentration assay, the lowest concentration of probiotic mixture that significantly reduced ($P < 0.05$) the growth of *A. hydrophila* was 10^7 CFU mL^{-1}. The MSP concentration at 10^7 CFUmL^{-1} was further used to analyze the interaction between the probiotic mixture and *A. hydrophila* during 0-, 6-, 12-, 24-, 48-, and 72-h (Figure 5). The co-culture treatment (T2) (10^7 CFU mL^{-1} probiotic mixture + 10^6 CFU mL^{-1} *A. hydrophila*) demonstrated a gradual significant decrement ($P < 0.05$) in pathogen count at all time intervals. The most significant reduction ($P > 0.05$) observed was at 72-h, where the probiotic mixture reduced the *Aeromonas* count from 8.91 ± 0.02 CFU mL^{-1} to 5.781 ± 0.01 CFU mL^{-1}.

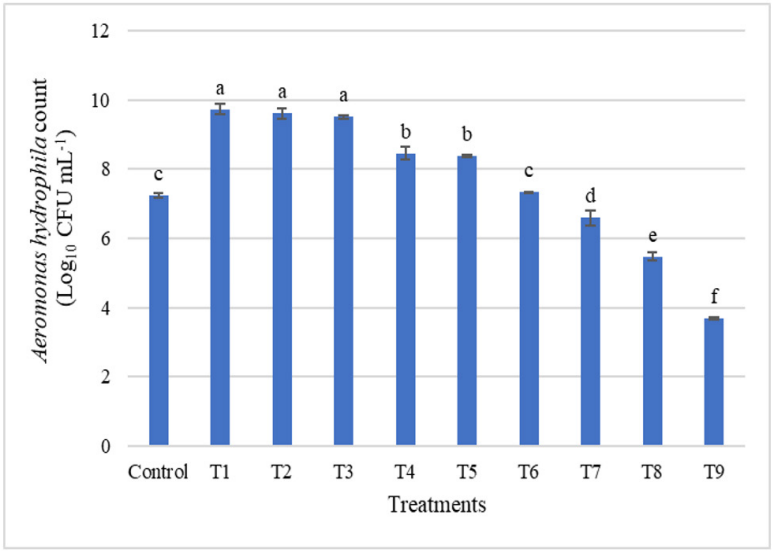

Figure 4. The inhibition of *Aeromonas hydrophila* by the probiotic mixture (L9 + A2 + LAB3) at different concentrations after 24 h incubation at 30 °C. Different alphabets indicate significant differences among treatments ($P < 0.05$)

Note.
Control = *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T1 = Probiotic mixture 10^1 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T2 = Probiotic mixture 10^2 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T3 = Probiotic mixture 10^3 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T4 = Probiotic mixture 10^4 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T5 = Probiotic mixture 10^5 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T6 = Probiotic mixture 10^6 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T7 = Probiotic mixture 10^7 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T8 = Probiotic mixture 10^8 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T9 = Probiotic mixture 10^9 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}

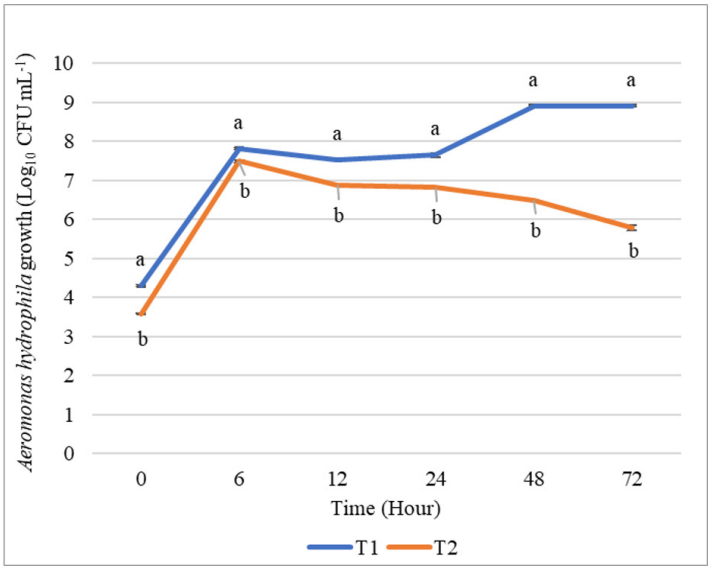

Figure 5. The growth of *Aeromonas hydrophila* (10^6 CFU mL^{-1}) co-cultured with the probiotic mixture (L9 + A2 + LAB3) (10^7 CFU mL^{-1}) during 0-, 6-, 12-, 24-, 48-, and 72-h. Different alphabets indicate significant differences among treatments

Note.
T1 = *Aeromonas hydrophila* 10^6 CFU mL^{-1}
T2 = Probiotic mixture 10^7 CFU mL^{-1} + *Aeromonas hydrophila* 10^6 CFU mL^{-1}

DISCUSSION

Aquaculture has been increasingly viewed as an important sector for food security with the growing global human population. The industry has drastically developed due to the intensification of cultivation methods. However, disease outbreaks are the most significant constraint that causes damaging effects on the economic development of the aquaculture sector worldwide (Hai, 2015). Moreover, antimicrobials have put pressure on developing a more sustainable alternative. Therefore, the screening of beneficial probiotics through *in vivo* and *in vitro* assessments are necessary for supplementing aquatic animals to obtain favorable results (Van Doan et al., 2021). For instance, in this study, a potential probiotic mixture (L9 + LAB3 + A2), which showed no hemolytic action and showed high biofilm formation, significantly inhibited the growth of *A. hydrophila* when tested *in vitro*.

The efficiency of antagonistic activity is one of the modes of action of probiotics (Yi et al., 2019). Thus, it is a critical prerequisite when screening for potential probiotics. Furthermore, the higher growth rate of probiotics compared to pathogenic microbes causes the exclusion of pathogenic microbes due to competition for adhesion sites (Kuebutornye et al., 2020). Thus, the present study indicated that the inhibitory activities of the potential probiotics against *A. hydrophila* suggest them as promising candidates for a probiotic mixture.

The formulation of multi-species probiotics is vital as they confer beneficial synergistic effects to the host (Puvanasundram et al., 2021). In this study, the antagonism between selected single strain probiotics and *A. hydrophila* reflects the antagonistic activities of the multi-species probiotic mixture. The compatibility between *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3 indicated that these probiotic strains could be used in a probiotic mixture without affecting each other's functionality in the host (Toscano et al., 2014).

A safety assessment test is important for the evaluation of probiotics. The absence of hemolytic activity by the potential probiotics suggested that the probiotic mixture is non-virulent and lacks hemolysin (Yasmin et al., 2020). Hemolysis is the breakdown of the membrane of red blood cells by a bacterial protein known as hemolysin, which catalyzes the release of hemoglobin from the red blood cells. Many researchers reported that probiotics should not show any hemolytic activity (Kaktcham et al., 2018; Nayak & Mukherjee, 2011).

Biofilms play a major role in bacterial proliferation and persistence by assisting in the tolerance to external impacts, including antimicrobials (Flemming et al., 2016). The mechanism of biofilm production by probiotic strains is to outcompete pathogenic bacteria for the source of nutrients and habitat colonization (Bhandary et al., 2021). The current results demonstrated that the potential probiotic mixture, when supplemented with a host, may provide functionality in competing against *A. hydrophila* in the gut, thus protecting the

host. The present findings also suggested that the potential probiotics synergistically complement each other when combined into a probiotic mixture and could be more effective in producing biofilm than its constituent single-strain probiotics.

The current study showed that the co-culture between the probiotic mixture (10^7 CFU mL^{-1}) and *A. hydrophila* caused a reduction in bacterial count on the *Aeromonas* selective media. Furthermore, a higher concentration of probiotic strain *L. fusiformis* SPS11 (10^8 CFU mL^{-1}) reduced the growth of *Vibrio parahaemolyticus* after 6 hours of incubation (Zabidi, Rosland, et al., 2021). The results from the co-culture assay suggested that the potential probiotic mixture may confer protection against *A. hydrophila* infection when supplemented with a host. Similarly, *Oreochromis mossambicus* fed with a diet containing *Bacillus licheniformis* at 10^7 cfu g^{-1} improved the health status and resistance of the host against *A. hydrophila,* with the highest value of relative percentage of survival (RPS) of 71.2% (Gobi et al., 2018).

However, other than competitive exclusion as a probable mechanism of action of a probiotic mixture, the production of inhibitory substances could be the facilitator in antagonizing pathogenic *A. hydrophila*. Generally, microbial populations may release chemical substances with bactericidal or bacteriostatic properties. These substances halt the proliferation of pathogenic bacteria (El-Saadony et al., 2021).

Bacillus amyloliquefaciens L9 and *L. fusiformis* A2 are Gram-positive bacteria from the genus of *Bacillus* known as spore-forming bacteria resistant to aggressive physical and chemical conditions (Soltani et al., 2019). *Bacillus* species can produce many antimicrobial metabolites and bioactive peptides, such as bacteriocins, bacteriocin-like substances, and lipopeptides (Stein, 2005). Some major probiotics consist of LAB (Alonso et al., 2019). The genus from LAB includes *Enterococcus*, which are Gram-positive microorganisms. Furthermore, LAB is known to produce compounds, such as bacteriocins, that inhibit the growth of other microorganisms (Vandenbergh, 1993).

Conclusively, probiotics have many mechanisms of action yet to be elucidated. However, in this study, it could be speculated that competitive exclusion and production of inhibitory substances are the main mechanisms. However, future *in vivo* studies should confirm probiotics' potential in protecting the host from disease caused by *Aeromonas*. It is because *in vitro* testing is insufficient in selecting probiotics for use in aquaculture, as some studies indicated that positive *in vitro* results may not be reflected in the *in vivo* trials towards the host (Kesarcodi-Watson et al., 2008).

CONCLUSION

The *in vitro* assessment of the potential probiotic mixture (consisting of *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3) had shown promising prospects in substituting antibiotics as an eco-friendlier approach. This study serves as a platform for future research into other

properties and mechanisms of action of the probiotic mixture. Further *in vivo* challenge with *A. hydrophila* on freshwater aquatic animals treated with the probiotic mixture is necessary. Hence, the *in vivo* test shall determine if the probiotic mixture has the potential to protect aquatic animals against a bacterial pathogen.

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REFERENCES

Akanmu, O. A. (2018). Probiotics, an alternative measure to chemotherapy in fish production. In S. Enany (Ed.), *Probiotics: Current knowledge and future prospects*. Intech Open. https://doi.org/10.5772/intechopen.72923

Alonso, S., Carmen Castro, M., Berdasco, M., de la Banda, I. G., Moreno-Ventas, X., & de Rojas, A. H. (2019). Isolation and partial characterization of lactic acid bacteria from the gut microbiota of marine fishes for potential application as probiotics in aquaculture. *Probiotics and Antimicrobial Proteins*, *11*(2), 569-579. https://doi.org/10.1007/s12602-018-9439-2

AlYahya, S. A., Ameen, F., Al-Niaeem, K. S., Al-Sa'adi, B. A., Hadi, S., & Mostafa, A. A. (2018). Histopathological studies of experimental *Aeromonas hydrophila* infection in blue tilapia, *Oreochromis aureus*. *Saudi Journal of Biological Sciences*, *25*(1), 182–185. https://doi.org/10.1016/j.sjbs.2017.10.019

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48*(Supplement 1), 5-16. https://doi.org/10.1093/jac/48.suppl_1.5

Azrin, N. A. R., Yuzine, E., Ina-Salwany, M. Y., & Karim, M. (2019). The efficacy of potential probiont *Bacillus amyloliquefaciens* strain L11 in protecting *Artemia* nauplii and blue crab juveniles against *Vibrio harveyi* infection. *Journal of Pure and Applied Microbiology*, *13*(2), 923-932. https://dx.doi.org/10.22207/JPAM.13.2.29

Bhandary, T., Riyaz, A. L., & Paari, K. A. (2021). Probiotic properties of *Bacillus subtilis* isolated from dried anchovies (*Stolephorus indicus*) and evaluating its antimicrobial, antibiofilm and growth-enhancing potential in *Danio rerio*. *Journal of Animal Health and Production*, *9*(3), 205-212. https://doi.org/10.17582/journal.jahp/2021/9.3.205.212

Bruhn, J. B., Gram, L., & Belas, R. (2007). Production of antibacterial compounds and biofilm formation by *Roseobacter* species are influenced by culture conditions. *Applied and Environmental Microbiology*, *73*(2), 442–450. https://doi.org/10.1128/AEM.02238-06

Cavalcante, R. B., Telli, G. S., Tachibana, L., de Carla Dias, D., Oshiro, E., Natori, M. M., da Silva, M. M., & Ranzani-Paiva, M. J. (2020). Probiotics, prebiotics and synbiotics for Nile tilapia: Growth performance and protection against *Aeromonas hydrophila* infection. *Aquaculture Reports*, *17*, 100343. https://doi.org/10.1016/j.aqrep.2020.100343

Chean, M. Y. B., Puvanasundram, P., Yaminudin, J., & Karim, M. (2021). Evaluation of antagonism activity and control of *Vibrio alginolyticus* in *Artemia* culture using mixed probiotic. *Pertanika*

Journal of Tropical Agricultural Science, *44*(1), 117-137. https://doi.org/10.47836/pjtas.44.1.07

Duarte, C. M., Agusti, S., Barbier, E., Britten, G. L., Castilla, J. C., Gattuso, J., Fulweiler, R. W., Hughes, T. P., Knowlton, N., Lovelock, C. E., Lotze, H. K., Predragovic, M., Poloczanska, E., Roberts, C., & Worm, B. (2020). Rebuilding marine life. *Nature*, *580*, 39–51. https://doi.org/10.1038/s41586-020-2146-7

El-Saadony, M. T., Alagawany, M., Patra, A. K., Kar, I., Tiwari, R., Dawood, M., Dhama, K., & Abdel-Latif, H. (2021). The functionality of probiotics in aquaculture: An overview. *Fish and Shellfish Immunology*, *117*, 36–52. https://doi.org/10.1016/j.fsi.2021.07.007

Elsabagh, M., Mohamed, R., Moustafa, E. M., Hamza, A., Farrag, F., Decamp, O., Dawood, M. A. O., & Eltholth, M. (2018). Assessing the impact of *Bacillus* strains mixture probiotic on water quality, growth performance, blood profile and intestinal morphology of Nile tilapia, *Oreochromis niloticus*. *Aquaculture Nutrition*, *24*(6), 1613-1622. https://doi.org/10.1111/anu.12797

Fazio, F. (2019). Fish hematology analysis as an important tool of aquaculture: A review. *Aquaculture*, *500*, 237-242. https://doi.org/10.1016/j.aquaculture.2018.10.030

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563–575. https://doi.org/10.1038/nrmicro.2016.94

Food and Agriculture Organization of the United Nations. (2020). *The state of world fisheries and aquaculture: Sustainability in action*. FAO. https://doi.org/10.4060/ca9229en

Foulquié Moreno, M. R., Callewaert, R., Devreese, B., Van Beeumen, J., & De Vuyst, L. (2003). Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. *Journal of Applied Microbiology*, *94*(2), 214–229. https://doi.org/10.1046/j.1365-2672.2003.01823.x

Gewaily, M. S., Shukry, M., Abdel-Kader, M. F., Alkafafy, M., Farrag, F. A., Moustafa, E. M., Van Doan, H., Abd-Elghany, M. F., Abdelhamid, A. F., Eltanahy, A., & Dawood, M. A. O. (2021). Dietary *Lactobacillus plantarum* relieves Nile Tilapia (*Oreochromis niloticus*) juvenile from oxidative stress, immunosuppression, and inflammation induced by Deltamethrin and *Aeromonas hydrophila*. *Frontiers in Marine Science*, *8*, 621588. https://doi.org/10.3389/fmars.2021.621558

Gobi, N., Vaseeharan, B., Chen, J. C., Rekha, R., Vijayakumar, S., Anjugam, M., & Iswarya, A. (2018). Dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 improves growth performance, mucus and serum immune parameters, antioxidant enzyme activity as well as resistance against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*. *Fish and Shellfish Immunology*, *74*, 501-508. https://doi.org/10.1016/j.fsi.2017.12.066

Gopi, N., Iswarya, A., Vijayakumar, S., Jayanthi, S., Nor, S. A. M., Velusamy, P., & Vaseeharan, B. (2022). Protective effects of dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 against ammonia induced immunotoxicity and oxidative stress in *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, *259*, 109379. https://doi.org/10.1016/j.cbpc.2022.109379

Gudmundsdottir, B. K., & Bjornsdottir, B. (2017). *Aeromonas salmonicida* and *A. hydrophila*. In P. T. K. Woo & R. C. Cipriano (Eds.), *Fish viruses and bacteria: Pathobiology and protection* (pp. 173-189). CABI. https://doi.org/10.1079/9781780647784.0000

Hai, N. (2015). The use of probiotics in aquaculture. *Journal of Applied*

Microbiology, *119*(4), 917-935. https://doi.org/10.1111/jam.12886

Janda, J. M., & Abbott, S. L. (2010). The genus *Aeromonas*: Taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, *23*(1), 35-73. https://doi.org/10.1128%2FCMR.00039-09

Jinendiran, S., Archana, R., Sathishkumar, R., Kannan, R., Selvakumar, G., & Sivakumar, N. (2021). Dietary administration of probiotic *Aeromonas veronii* V03 on the modulation of innate immunity, expression of immune-related genes and disease resistance against *Aeromonas hydrophila* infection in common carp (*Cyprinus carpio*). *Probiotics and Antimicrobial Proteins*, *13*, 1709-1722. https://doi.org/10.1007/s12602-021-09784-6

Kaktcham, P. M., Temgoua, J. B., Zambou, F. N., Diaz-Ruiz, G., Wacher, C., & Pérez-Chabela, M. D. L. (2018). *In vitro* evaluation of the probiotic and safety properties of bacteriocinogenic and non-bacteriocinogenic lactic acid bacteria from the intestines of Nile tilapia and common carp for their use as probiotics in aquaculture. *Probiotics and Antimicrobial Proteins*, *10*(1), 98-109. https://doi.org/10.1007/s12602-017-9312-8

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., & Gibson, L. (2008). Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture*, *274*(1), 1-14. https://doi.org/10.1016/j.aquaculture.2007.11.019

Kuebutornye, F. K., Abarike, E. D., Lu, Y., Hlordzi, V., Sakyi, M. E., Afriyie, G., Wang, Z., Li, Y., & Xie, C. X. (2020). Mechanisms and the role of probiotic *Bacillus* in mitigating fish pathogens in aquaculture. *Fish Physiology and Biochemistry*, *46*(3), 819-841. https://doi.org/10.1007/s10695-019-00754-y

Lin, H. L., Shiu, Y. L., Chiu, C. S., Huang, S. L., & Liu, C. H. (2017). Screening probiotic candidates for a mixture of probiotics to enhance the growth performance, immunity, and disease resistance of Asian seabass, *Lates calcarifer* (Bloch), against *Aeromonas hydrophila*. *Fish and Shellfish Immunology*, *60*, 474-482. https://doi.org/10.1016/j.fsi.2016.11.026

Masduki, F., Zakaria, T., Min, C. C., & Karim, M. (2020). Evaluation of *Enterococcus hirae* LAB3 as potential probiont against *Vibrio harveyi* in *Artemia* nauplii and Asian seabass larvae (*Lates calcarifer*) cultures. *Journal of Environmental Biology*, *41*, 1153-1159. https://doi.org/10.22438/jeb/41/5(SI)/MS_06

Moraes, F. R., & Martins, M. L. (2004). Favourable conditions and principal teleostean diseases in intensive fish farming. In J. E. P. Cyrino, E. C. Urbinati, D. M. Fracalossi, N. Castagnolli (Eds.), *Especial topics in tropical intensive freshwater fish farming* (pp. 343-383). TecArt.

Munir, M. B., Hashim, R., Mohd Nor, S. A., & Marsh, T. L. (2018). Effect of dietary prebiotics and probiotics on snakehead (*Channa striata*) health: Haematology and disease resistance parameters against *Aeromonas hydrophila*. *Fish and Shellfish Immunology*, *75*, 99-108. https://doi.org/10.1016/j.fsi.2018.02.005

Nayak, S. K., & Mukherjee, S. C. (2011). Screening of gastrointestinal bacteria of Indian major carps for a candidate probiotic species for aquaculture practices. *Aquaculture Research*, *42*(7), 1034-1041. https://doi.org/10.1111/j.1365-2109.2010.02686.x

Naylor, R. L., Hardy, R. W., Buschmann, A. H., Bush, S. R., Cao, L., Klinger, D. H., Little, D. C., Lubchenco, J., Shumway, S. E., & Troell, M. (2021). A 20-year retrospective review of global aquaculture. *Nature*, *591*, 551–563. https://doi.org/10.1038/s41586-021-03308-6

Ng, W. K., Kim, Y. C., Romano, N., Koh, C. B., & Yang, S. Y. (2014). Effects of dietary probiotics on the growth and feeding efficiency of red

hybrid tilapia, *Oreochromis* sp., and subsequent resistance to *Streptococcus agalactiae*. *Journal of Applied Aquaculture*, *26*(1), 22-31. https://doi.org/10.1080/10454438.2013.874961

Puvanasundram, P., Chong, C. M., Sabri, S., Yusoff, M. S., & Karim, M. (2021). Multi-strain probiotics: Functions, effectiveness and formulations for aquaculture applications. *Aquaculture Reports*, *21*, 100905. https://doi.org/10.1016/j.aqrep.2021.100905

Qi, X., Xue, M., Cui, H., Yang, K., Song, K., Zha, J., Wang, G., & Ling, F. (2020). Antimicrobial activity of *Pseudomonas monteilii* JK-1 isolated from fish gut and its major metabolite, 1-hydroxyphenazine, against *Aeromonas hydrophila*. *Aquaculture*, *526*, 735366. https://doi.org/10.1016/j.aquaculture.2020.735366

Rengpipat, S., Rueangruklikhit, T., & Piyatiratitivorakul, S. (2008). Evaluations of lactic acid bacteria as probiotics for juvenile seabass *Lates calcarifer*. *Aquaculture Research*, *39*(2), 134-143. https://doi.org/10.1111/j.1365-2109.2007.01864.x

Restrepo, L., Domínguez-Borbor, C., Bajaña, L., Betancourt, I., Rodríguez, J., Bayot, B., & Reyes, A. (2021). Microbial community characterization of shrimp survivors to AHPND challenge test treated with an effective shrimp probiotic (*Vibrio diabolicus*). *Microbiome*, *9*, 88. https://doi.org/10.1186/s40168-021-01043-8

Román, L., Real, F., Sorroza, L., Padilla, D., Acosta, B., Grasso, V., & Acosta, F. (2012). The *in vitro* effect of probiotic *Vagococcus fluvialis* on the innate immune parameters of *Sparus aurata* and *Dicentrarchus labrax*. *Fish and Shellfish Immunology*, *33*(5), 1071–1075. https://doi.org/10.1016/j.fsi.2012.06.028

Rosland, N. A., Ikhsan, N., Min, C. C., Yusoff, F. M., & Karim, M. (2021). Influence of symbiotic probiont strains on the growth of *Amphora* and *Chlorella* and its potential protections against *Vibrio* spp. in *Artemia*. *Current Microbiology*, *78*(11), 3901-3912. https://doi.org/10.1007/s00284-021-02642-2

Soltani, M., Ghosh, K., Hoseinifar, S. H., Kumar, V., Lymbery, A. J., Roy, S., & Ringø, E. (2019). Genus *Bacillus*, promising probiotics in aquaculture: aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. *Reviews in Fisheries Science and Aquaculture*, *27*(3), 331-379. https://doi.org/10.1080/23308249.2019.1597010

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology*, *56*(4), 845-857. https://doi.org/10.1111/j.1365-2958.2005.04587.x

Toscano, M., De Vecchi, E., Gabrieli, A., Zuccotti, G. V., & Drago, L. (2014). Probiotic characteristics and *in vitro* compatibility of a combination of *Bifidobacterium breve* M-16 V, *Bifidobacterium longum* subsp. *infantis* M-63 and *Bifidobacterium longum* subsp. *longum* BB536. *Annals of Microbiology*, *65*(2), 1079-1086. https://doi.org/10.1007/s13213-014-0953-5

Tran, N. T., Yang, W., Nguyen, X. T., Zhang, M., Ma, H., Zheng, H., Zhang, Y., Chang, K., & Li, S. (2022). Application of heat-killed probiotics in aquaculture. *Aquaculture*, *548*(Part 2), 737700. https://doi.org/10.1016/j.aquaculture.2021.737700

Van Doan, H., Soltani, M., & Ringø, E. (2021). *In vitro* antagonistic effect and *in vivo* protective efficacy of Gram-positive probiotics versus Gram-negative bacterial pathogens in finfish and shellfish. *Aquaculture*, *540*, 736581. https://doi.org/10.1016/j.aquaculture.2021.736581

Vandenbergh, P. A. (1993). Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiology Reviews*, *12*(1-3), 221-237. https://doi.org/10.1111/j.1574-6976.1993.tb00020.x

Vaseeharan, B., and Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Letters in Applied Microbiology*, *36*(2), 83-87. https://doi.org/10.1046/j.1472-765x.2003.01255.x

Yasmin, I., Saeed, M., Khan, W. A., Khaliq, A., Chughtai, M. F. J., Iqbal, R., Tehseen, S., Naz, S., Liaqat, A., Mehmood, T., & Ahsan, S. (2020). *In vitro* probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of *Bifidobacterium* strains isolated from raw camel milk. *Microorganisms*, *8*(3), 354. https://doi.org/10.3390/microorganisms8030354

Yi, C. C., Liu, C. H., Chuang, K. P., Chang, Y. T., & Hu, S. Y. (2019). A potential probiotic *Chromobacterium aquaticum* with bacteriocin-like activity enhances the expression of indicator genes associated with nutrient metabolism, growth performance and innate immunity against pathogen infections in zebrafish (*Danio rerio*). *Fish and Shellfish Immunology*, *93*, 124-134. https://doi.org/10.1016/j.fsi.2019.07.042

Yilmaz, S., Yilmaz, E., Dawood, M. A. O., Ringø, E., Ahmadifar, E., Abdel-Latif, H. M. R. (2022). Probiotics, prebiotics, and synbiotics used to control vibriosis in fish: A review. *Aquaculture*, *547*, 737514. https://doi.org/10.1016/j.aquaculture.2021.737514

Zabidi, A., Rosland, N. A., Yaminudin, J., & Karim, M. (2021). *In vitro* assessment of bacterial strains associated with microalgae as potential probiotics. *Pertanika Journal of Tropical Agricultural Science*, *44*(1), 205-220. https://doi.org/10.47836/pjtas.44.1.12

Zabidi, A., Yusoff, F. M., Amin, S. M., Yaminudin, N. J. M., Puvanasundram, P., & Karim, M. M. A. (2021). Effects of probiotics on growth, survival, water quality and disease resistance of red hybrid Tilapia (*Oreochromis* spp.) fingerlings in a biofloc system. *Animals*, *11*(12), 3514. https://doi.org/10.3390/ani11123514