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Antimicrobial activity of characterized *Leuconostoc* spp. as novel probiotics isolated from *Oreochromis* spp. (red tilapia) against fish pathogen *Edwardsiella tarda*

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ABSTRACT

Aims: This study aimed to isolate and characterize putative new probiotic with antimicrobial properties against common fish pathogens from the gut of *Oreochromis* spp. (red tilapia).
Methodology and results: A total of 28 colonies were isolated from gut of *Oreochromis* spp. and characterized phenotypically. Eight isolates were selected for probiotic characterization. Temperature, salinity, pH and bile salt tolerance, antibiotic susceptibility and antimicrobial test against selected fish pathogens (*Aeromonas hydrophila*, *Edwardsiella tarda* and *Staphylococcus aureus* subsp*. aureus* ATCC 25923) were conducted. Characterization studies revealed isolates suited for freshwater environment and exhibited tolerance against wide range of salinity, pH and bile salt. Isolates displayed different antibiotic susceptibility profile, with six exhibited antimicrobial properties against *E. tarda*. Molecular identification based on 16S rRNA gene sequencing showed 99.44%, 98.59% and 91.21% sequence similarity with *Leuconostoc pseudomesenteroides* strain 3832^T, *Leuconostoc lactis* strain KCC202369^T and *Leuconostoc mesenteroides* strain 4332^T, respectively as compared to known sequence in the GenBank. When identified *Leuconostoc* spp. were coated on feed pellets, no major decrease in viability over 21 days of storage at 4 °C were observed, with an average of 8 log CFU/mL.
Conclusion, significance and impact of study: The characterized species allow further application assessment of the probiotic-supplemented tilapia feed. Host-originated *Leuconostoc* displayed potential antimicrobial properties against fish pathogen *E. tarda.* The isolates *Leuconostoc* is expected to provide protective effect for *Oreochromis* spp. against edwardsiellosis and to exert beneficial effects more efficiently as compared to commercial probiotics which are not specifically target for *Oreochromis* spp., thereby indirectly helping fish farmers in achieving economic sustainability and increase affordability of fish.

Keywords: Lactic acid bacteria, probiotics, *Leuconostoc*, antimicrobial, *Oreochromis* spp. (red tilapia)

INTRODUCTION

Food security is one of the current global concerns. According to Food and Agriculture Organization of the United Nations (FAO) (2020b), an estimated 2 billion people around the world were affected by moderate or severe levels of food insecurity. Healthy diets with balanced portions of fruits, vegetables and animal source food are unaffordable for more than 3 billion people worldwide (FAO, 2020b). To increase the affordability of healthy diets, food supply industries should lower production cost, ensuring their supplies are continuous, sustainable and with enhanced efficiencies.

Aquaculture is one of the rising industries worldwide due to its potential in providing continuous supply of fishery products in a sustainable manner, with 7.5% of production growth per year since 1970 (FAO, 2020a). In 2018, global fish production is estimated to have reached approximately 179 million tonnes, with 82 million tonnes (45.81%) of production from the aquaculture industry that valued at United States Dollar (USD) 250 billion. Excluding China as the country with the highest aquaculture production, other Asian countries contributed a significant portion of production which accounted for 34%. In Malaysia, although brackishwater aquaculture is the major aquaculture activity, however, freshwater aquaculture is in development with a production of 101,269.88 tonnes (25.87% of the total production of aquaculture) [Department of Fisheries Malaysia (DoF), 2019].

One of the crucial factors affecting the affordability of fishery products is the production cost. The ever rising feed cost account for up to 70% of the production cost, which has impacted the final market price and the

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sustainability of the farmers (Farahiyah *et al*., 2015). In order to achieve affordability for consumers and maintaining profitability for farmers, fish feed that is cost effective and can improve growth efficacy is more preferred. A few studies have shown that the administration of probiotics can enhance feed conversion ratio in fish (Munir *et al*., 2016; Toutou *et al*., 2016; Jahari *et al*., 2018), further encouraging the use of probiotics in the aquaculture sector.

Prior to the introduction of probiotics in aquaculture, antibiotics and chemicals are often used by farmers to control the outbreak of diseases in livestock. In Thailand, antibiotics such as oxytetracycline (OTC) and enrofloxacin (ENR) are commonly used by farmers in tilapia cage farming and it is believed that the usage of the antibiotics in aquaculture is one of the major sources of antibiotic pollution in the environment (Rico *et al.*, 2014). Vaccines are also used in the aquaculture industry to control disease outbreak. Extensive usage of these substances is not favorable since they might accumulate in the environment, causing pollution and potentially harmful to other organisms (such as human as food consumers) and could result in antibiotic resistance in livestock and the development of antibiotic resistant strains of pathogens. Hence, probiotics have been a promising option to be used as alternatives to these substances.

Probiotics are defined by FAO and World Health Organization (WHO) as “live microorganism that, when administered in adequate amounts, confer a health benefit on host” (FAO, 2016). The administration of adequate amount of probiotics (usually beneficial and harmless bacteria and yeast) can improve the gut microbiota of the livestock by several modes of actions, which is believed to promote health by increasing resistance towards certain gut-associated diseases, as well as improving overall productivity of the livestock. Currently, there are different types of commercial probiotics for aquaculture in the market. In Malaysia, it is easy to purchase probiotics imported from other countries. A probiotic product from India uses multi-strain probiotics isolated from marine environment with red yeasts mixed as active ingredients (Anon, 2021). There are also probiotic products which are formulated with single-strain only or in the form of mixture, with vitamins and enzymes incorporated (Anon, 2020a; Anon, 2020b). In terms of local brand, a probiotic product with mannan-oligosaccharides is commercialized (Anon, 2019). Despite the beneficial effects claimed, the effects of these products to specific fish species are subject for further improvement due to the fact that they are for broad range usage and not specifically formulated for a specific fish species.

The exact probiotics compositions of the commercial probiotic products are not known due to business trade secrets, however, in general, most of the probiotic products utilize a group of bacteria named lactic acid bacteria (LAB). LAB are a group of Gram-positive bacteria. They are fermentative rod or coccus in shape, usually non-motile, aerotolerant, acid tolerant and non-sporulating. The major product synthesized by LAB is short chain fatty acid, for example, lactic acid. They are unable to synthesize porphyrins, cytochromes absent, oxidase and catalase negative (Menconi *et al*., 2014; Islam *et al*., 2016). The use of LAB as probiotics in aquaculture is common due to its beneficial effects. A few studies conducted involving LAB strains such as *Lactobacillus plantarum* (Ren *et al*., 2013; Doan *et al*., 2016; Yu *et al*., 2017; Jahari *et al*., 2018) and *Lactobacillus acidophilus* (Liu *et al*., 2013) had proved the beneficial effects of LAB as probiotics in tilapia.

Tilapia is selected as the research target due to its economic status. According to FAO (2002; 2020c), nearly 80% of total world tilapia aquaculture production took place in Asia in 2002 and Asia remained as the largest tilapia producing region worldwide in 2018; with the total production of 4.15 million tonnes (68.83%). In 2018, China was the largest tilapia production country by contributing 26.93% of the total world production, followed by Indonesia (20.27%) (FAO, 2020c). In Malaysia, red tilapia is the most cultured tilapia species. According to DoF (2019), as in 2018, red tilapia remained as the second most produced freshwater fish in Malaysia, with the production of 25,199.89 tonnes (24.88% of total freshwater aquaculture production).

This research aims to isolate and characterize LAB with antimicrobial properties against common fish pathogens from the gut of local red tilapia as a putative new probiotic that specifically target for red tilapia. Temperature, salinity, pH and bile salt tolerance, antibiotic susceptibility and antimicrobial test against common fish pathogens were evaluated. Species identification of the isolated strains were carried out based on 16S ribosomal ribonucleic acid (rRNA) gene sequencing and the effect of storage on viability upon top coating of probiotic supplementation on commercial fish feed was assessed.

MATERIALS AND METHODS

Isolation of LAB strains

The LAB strains were isolated based on Wamala *et al*. (2018) with some modification. A total of six live red tilapia obtained from local market located in Wangsa Maju, Kuala Lumpur, Malaysia were sacrificed and the surface of the fish was sterilized with 70% ethanol prior to gut dissection. The gut was dissected and the extensive adipose tissue surrounding the gut was removed. The gut was separated into four equal parts, followed by grinding and homogenization by sterile pestle and mortar in the presence of 3.0 mL sterile phosphate-buffered saline (PBS) (pH 7.4). A total of four stock solutions were prepared and homogenized. Serial dilutions were performed using De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom) and spread plate method was carried out. The plates were incubated at 37 °C for 48 h anaerobically. Colonies were randomly selected and subsequently cultured for further characterization.

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Phenotypic characterization was proceeded to screen for the LAB based on the characteristics of LAB described in Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986). Catalase test and Gram staining were conducted. For catalase test, a few drops of 3% hydrogen peroxide (H_2O_2) were applied to the colony spread. Distilled water was used as negative control. Isolates with no effervescence observed were selected and proceeded with Gram staining as LAB are catalase negative. Isolates that were Gram-positive and rod or coccus in shape were selected. The stock culture samples were stored at –80 °C.

Probiotic characterization of LAB strains

Isolates were selected from the screened LAB and subjected to probiotic characterization test; temperature, salinity, pH and bile salt tolerance, antibiotic susceptibility and antimicrobial test to select the isolates with probiotic properties. The methods were adapted from Muthukumar and Kandeepan (2015).

For the temperature tolerance test, the isolates were subjected to four different temperatures [25, 30, 37 (control group) and 45 °C]. The optical density (OD) at 600 nm (OD_{600}) of the cultures were measured and recorded at 24 and 48 h of incubation. In the salinity (NaCl) tolerance test, all isolates were subjected to six different salt concentration adjusted MRS broth [0.00% (unadjusted, control group), 0.05%, 0.10%, 0.20%, 2.00% and 4.00%]. OD_{600} of the cultures were measured and recorded at 48 h after incubation at 37 °C anaerobically. Five different pH adjusted MRS broth [pH 3, 5, 6.25 (unadjusted, control), 7 and 9] were selected for the pH tolerance test. In the bile tolerance test, isolates were subjected to three different bile salt concentration [0.0% (unadjusted, control group), 0.3% and 0.5%]. OD_{600} of the respective cultures were measured and recorded at 2 and 5 h after incubation at 37 °C under anaerobic condition. Each treatment was performed in triplicates. The percentage of growth/survival were calculated by applying the following formula:

Percentage of growth/survival (%) = X / Control × 100%

Where X = OD_{600} of the treatment (temperature/salinity adjusted medium/pH/bile); Control = OD_{600} of the control group (temperature/salinity adjusted medium/pH/bile)

Antibiotic susceptibility test

Antibiotic susceptibility test was carried out by applying Kirby-Bauer disk diffusion method (Bauer *et al*., 1966). The method was in accordance with the latest European Committee on Antimicrobial Susceptibility Test (EUCAST) disk diffusion method (EUCAST, 2020). The 15-15-15 min rule was applied. A total of seven antibiotics (Oxoid, Basingstoke, United Kingdom) were used: ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin G (10 µg), tetracycline (30 µg) and vancomycin (30 µg). The test was performed in triplicates. As the standard breakpoint of disk diffusion method for Gram-positive anaerobes had yet to be established by EUCAST, thus, no susceptibility categories (S, I and R) were assigned for all isolates.

Antimicrobial test

Antimicrobial test was carried out by applying Kirby-Bauer disk diffusion method (Bauer *et al*., 1966). The antimicrobial properties of LAB against two different types of common fish pathogens, *Aeromonas hydrophila* and *Edwardsiella tarda*, and a common human pathogen, *Staphylococcus aureus* subsp. *aureus* ATCC 25923 were determined. Similar to antibiotic susceptibility test, a 15-15-15 min rule was applied. Gentamicin and sterile distilled water were used as positive and negative control, respectively. The cultures were incubated anaerobically at 30 °C (for *A. hydrophila* and *E. tarda*) and at 37 °C (for *S. aureus*) for 16 h. At 16 h, the zone of inhibition for each plate was measured and recorded. Each treatment was performed in triplicates for all LAB isolates.

Identification of bacteria

Genomic deoxyribonucleic acid (DNA) were extracted from overnight cultures of three potential isolates by using Geneaid Presto™ Mini gDNA Bacteria Kit (Geneaid, New Taipei City, Taiwan). The method applied was according to the kit manufacturer's instruction. The purity of the extracted DNA were determined using a Nanodrop spectrophotometer (Thermo Scientific™, Massachusetts, United States of America). The 16S rRNA gene sequence coding region were amplified by polymerase chain reaction (PCR) using the prokaryotic 16S rRNA universal primers (27F, 1429R). A master mix containing 0.0002 mol L^{-1} dNTP, 2 × 10^{-7} mol L^{-1} universal forward and reverse primer, 1.5 U Taq polymerase, 1× PCR buffer and sterile water was initially prepared. DNA samples were added with the master mix solutions respectively. The final volume for all PCR reactions was 20 µL. The thermal cycling condition was as follows; initial denaturation (94 °C, 5 min), denaturation (40 cycles, 94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min). Gel electrophoresis was used to separate the amplicons of the isolates and the PCR products were subjected to purification and Sanger 16S rRNA gene sequencing (Bio Basic, Singapore). The isolates were identified by comparing the sequence similarity with the known sequences in National Center for Biotechnology Information (NCBI) GenBank using nucleotide BLAST program (NCBI). The highest maximum identity score (%) obtained by sequence comparison using the GenBank database revealed the bacterial identity of the isolated sequences.

Phylogenetic analysis

Nucleotide sequences were aligned with other nucleotide sequences of identified bacteria retrieved from NCBI GenBank database using Clustal W. Phylogenetic tree

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was constructed with a bootstrap value of 1000 by Neighbor-joining method using MEGA-X software (Version 10.2.2) (Felsenstein, 1985; Saitou and Nei, 1987; Kumar *et al*., 2018). *Escherichia coli* strain BDS3^T (GU065251.1) was used as the outgroup.

Nucleotide sequence accession number

Nucleotide sequences obtained in this study have been deposited in the NCBI GenBank database under the accession numbers MW362782, MW362783 and MW362784 for the isolates TARicum AI1, TARicum AI2 and TARicum AI3, respectively.

Diet preparation and LAB viability upon storage

Method applied was modified from Wanka *et al*. (2018). Probiotic suspension was prepared by harvesting 15 mL of overnight LAB liquid culture (OD_{600} 1.5–2.0) through centrifugation at 6026× *g* for 20 min. The commercial fish pellets (Dindings 920 Starter Fish Pellet 2 mm, Dindings, Kuala Lumpur, Malaysia) were washed twice with 0.9% saline prior usage. A total of 2 mL harvested cell mass was diluted with 75 mL saline solution. Then, 3 g of the treated pellets were coated for a few times with 3 sets of 25 mL of the probiotic-saline suspension. To coat the pellets equally, the probiotic-saline suspension was mixed with the treated pellets and placed in a shaking incubator at 4× *g* for 10 min. The probiotic coated pellets were dried for 48 h until the initial weight of the pellets were obtained. The original structural stability was ensured to be conserved. Then, the dried pellets were stored in sterile plastic bag at 4 °C. The viability of the probiotics were monitored weekly through plate counting at day 7, 14 and 21 by mixing 1 g of pellets with 10 mL of 0.9% saline solution for 2 min, followed by homogenization using pestle and mortar. Serial dilution of the solutions up to 10^{-8} were prepared and spread on MRS agar media, followed by incubating anaerobically at 37 °C for 48 h in triplicates. The colony forming unit per mL (log CFU/mL) for each isolate were recorded for day 0, 7, 14 and 21 of storage.

Statistical analysis

Data for temperature, salinity, pH and bile salt tolerance test were expressed as percentage of growth or survival while data for viability test were expressed as log CFU/mL. All data were analyzed by IBM SPSS Statistics 22 (2013) and expressed as mean. Significance was determined at $P<0.05$ by ANOVA followed by Duncan's multiple-comparison tests.

RESULTS

Isolation and phenotypic characterization of LAB from gut of red tilapia

A total of four stock solutions prepared from different portion of gut of red tilapia were serially diluted up to 10^{-8} and individually cultured on MRS agar media anaerobically. Based on plate morphology, a total of 30 isolates were selected for phenotypic characterization; two out of 30 isolates failed to grow after subcultured from stock plates to MRS broth, thus, no characterization was performed for the two isolates. All the 28 successfully isolated LAB were coccus or coccobacilli in morphology, Gram-positive and catalase negative (Table 1).

Table 1: Phenotypic characterization of isolated lactic acid bacteria.

Isolate	Microscopic morphology	Gram stain	Catalase
A1	Coccus	+	-
A3	Coccus	+	-
AM1	Coccobacilli	+	-
AM2	Coccus	+	-
AM3	Coccus	+	-
AM4	Coccus	+	-
AM5	Coccus	+	-
AM6	Coccus	+	-
TARicum AI1	Coccobacilli	+	-
TARicum AI2	Coccus	+	-
TARicum AI3	Coccus	+	-
AI4	Coccus	+	-
AI5	Coccus	+	-
AI6	Coccus	+	-
AII1	Coccus	+	-
AII2	Coccus	+	-
AII3	Coccus	+	-
AII4	Coccobacilli	+	-
CI1	Coccus	+	-
CI2	Coccus	+	-
CII1	Coccus	+	-
CII2	Coccus	+	-
CII3	Coccus	+	-
DI1	Coccus	+	-
DI2	Coccus	+	-
DI3	Coccus	+	-
DII1	Coccus	+	-
DII3	Coccus	+	-

Probiotic characterization of selected LAB

Probiotic characterization tests conducted were temperature, salinity, pH and bile salt tolerance, antibiotic susceptibility test as well as antimicrobial test. From the successfully screened LAB, eight isolates were randomly selected for probiotic characterization. The isolates selected were designated as TARicum AI1, TARicum AI2, TARicum AI3, AI5, AII2, AII4, A3 and AM5, respectively.

Temperature tolerance test

All isolates were able to tolerate all temperature tested. All isolates showed lower percentage of growth at 45 °C as compared to 25 and 30 °C for both 24 and 48 h. Isolate TARicum AI1 exhibited significantly higher ($P<0.05$) percentage of growth at 45 °C as compared to other isolates (Table 2). Isolate A3 showed

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Table 2: Percentage of growth of lactic acid bacteria in different growth temperature (25 °C, 30 °C and 45 °C) as compared to control (37 °C) at 24 h and 48 h.

Isolate	Percentage of growth (%)					
	24 h			48 h		
	25 °C	30 °C	45 °C	25 °C	30 °C	45 °C
TARicum AI1	91.88 ± 4.19^{a}	150.75 ± 7.31^{de}	108.95 ± 2.01^{f}	133.23 ± 22.85^{b}	172.19 ± 28.24^{d}	103.61 ± 16.83^{e}
TARicum AI2	110.32 ± 5.23^{cd}	155.79 ± 8.45^{e}	94.06 ± 3.99^{e}	134.69 ± 2.15^{b}	139.46 ± 1.81^{c}	94.03 ± 3.87^{d}
TARicum AI3	109.26 ± 1.17^{bcd}	99.07 ± 2.98^{ab}	35.84 ± 0.28^{b}	128.09 ± 0.88^{b}	120.74 ± 3.15^{abc}	37.50 ± 0.58^{b}
AI5	100.64 ± 4.42^{abcd}	94.60 ± 3.98^{a}	74.30 ± 2.07^{c}	126.49 ± 4.24^{b}	123.65 ± 1.60^{abc}	77.44 ± 1.99^{c}
AII2	99.86 ± 0.34^{abc}	102.58 ± 1.99^{ab}	19.28 ± 10.14^{a}	107.23 ± 1.25^{a}	106.01 ± 1.95^{a}	29.78 ± 17.27^{ab}
AII4	115.41 ± 14.93^{d}	141.01 ± 12.04^{d}	84.61 ± 6.35^{d}	120.08 ± 2.60^{ab}	126.30 ± 0.87^{bc}	73.87 ± 0.36^{c}
A3	119.53 ± 12.02^{e}	108.57 ± 4.89^{b}	21.25 ± 2.19^{a}	119.30 ± 2.62^{ab}	118.64 ± 4.31^{ab}	18.80 ± 0.57^{a}
AM5	94.70 ± 8.61^{ab}	124.05 ± 5.22^{c}	77.80 ± 3.77^{cd}	121.61 ± 2.85^{ab}	128.94 ± 3.85^{bc}	80.25 ± 1.83^{cd}

Values were means ± standard deviation of three replicates (n=3). Values in the same column with different superscripts (a-f) were significantly different (P<0.05).

higher percentage of growth at 25 °C at 24 h. All isolates showed more than 91% of growth in 25 and 30 °C.

Salinity tolerance test

All isolates survived in all NaCl concentration tested. In general, the percentage of growth decreased as the salinity increased. All isolates showed the highest percentage of growth in 0.20% NaCl and showed the lowest percentage of growth in 4.00% NaCl (Table 3). The percentage of growth for isolate TARicum AI3 was significantly higher (P<0.05) than other isolates in 0.20% NaCl, however, the percentage of growth was significantly lower (P<0.05) as compared to isolate TARicum AI2 as the salinity increased.

pH tolerance test

The viability of all isolates remained more than 89% up to 5 h in all pH tested (Table 4). All isolates can survive in pH as low as pH 3 and showed favorable growth in less acidic pH, with higher percentage of survival at pH 9 as compared to other pH tested at 5 h. The percentage of survival of isolate TARicum AI2 was significantly higher (P<0.05) than other isolates in pH 5 at 2 h. Isolate TARicum AI3 showed significantly higher (P<0.05) rate of survival in pH 9 at 5 h as compared to other isolates while isolate AII4 showed the best survival in unadjusted MRS medium (pH 6.25) at both 2 and 5 h.

Bile salt tolerance test

All isolates survived in all bile concentration tested with the percentage of survival more than 92% at both 2 and 5 h (Table 5). The percentage of survival of the isolates was lower in the presence of bile as compared to the unadjusted MRS medium without bile. Isolate AI5 showed significantly lower (P<0.05) percentage of survival as compared to other isolates in 0.3% bile for both 2 and 5 h. No isolate exhibited significantly higher (P<0.05) percentage of survival in the presence of bile at both 2 and 5 h due to overlapping of subset.

Antibiotic susceptibility test

All isolates exhibited different antibiotic susceptibility profile (Table 6). Isolates TARicum AI1, TARicum AI2 and AM5 showed no zone of inhibition when erythromycin was used. Isolate TARicum AI3 showed total resistance against chloramphenicol, while isolate A3 showed total resistance against vancomycin, but was significantly susceptible (P<0.05) to penicillin G as compared to other isolates.

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Table 3: Percentage of growth of lactic acid bacteria in different salinity adjusted MRS broth (0.05%, 0.10%, 0.20%, 2.00% and 4.00%) at 48 h as compared to control (0.00%).

Isolate	Percentage of growth (%)				
	NaCl concentration				
	0.05%	0.10%	0.20%	2.00%	4.00%
TARicum AI1	107.74 ± 3.20^{b}	95.53 ± 7.28^{bc}	118.17 ± 8.38^{ab}	101.14 ± 6.15^{bc}	59.25 ± 3.44^{a}
TARicum AI2	116.87 ± 2.05^{c}	94.63 ± 3.52^{bc}	120.94 ± 5.20^{b}	107.08 ± 1.62^{c}	70.13 ± 4.27^{bc}
TARicum AI3	119.19 ± 5.11^{c}	101.13 ± 6.71^{c}	139.00 ± 6.86^{c}	95.45 ± 0.45^{ab}	61.06 ± 3.83^{a}
AI5	97.70 ± 0.54^{a}	84.64 ± 5.50^{ab}	118.21 ± 2.39^{ab}	87.86 ± 3.77^{a}	64.99 ± 1.46^{ab}
AII2	94.45 ± 4.08^{a}	83.06 ± 3.20^{ab}	101.95 ± 14.19^{a}	89.12 ± 1.23^{a}	60.97 ± 2.50^{a}
AII4	100.22 ± 3.16^{ab}	95.25 ± 8.71^{bc}	118.12 ± 2.00^{ab}	89.77 ± 2.01^{a}	60.65 ± 1.62^{a}
A3	102.17 ± 9.84^{ab}	90.07 ± 15.23^{abc}	120.85 ± 16.15^{b}	99.51 ± 9.93^{bc}	74.78 ± 8.21^{c}
AM5	98.20 ± 2.99^{a}	79.05 ± 2.07^{a}	114.32 ± 5.57^{ab}	87.73 ± 2.52^{a}	58.96 ± 5.98^{a}

Values were means ± standard deviation of three replicates (n=3). Values in the same column with different superscripts (a-c) were significantly different (P<0.05).
MRS: De Man, Rogosa and Sharpe.

Table 4: Percentage of survival of isolated lactic acid bacteria in different pH (pH 3, pH 5, pH 6.25, pH 7 and pH 9) of MRS broth at 2 h and 5 h.

Isolate	Percentage of survival (%)									
	2 h					5 h				
	pH 3	pH 5	pH 6.25	pH 7	pH 9	pH 3	pH 5	pH 6.25	pH 7	pH 9
TARicum AI1	92.50 ± 2.75^{a}	144.14 ± 14.55^{a}	266.12 ± 12.45^{b}	346.27 ± 34.97^{c}	394.39 ± 23.20^{b}	95.40 ± 3.65^{ab}	326.97 ± 22.86^{bc}	507.86 ± 14.91^{b}	588.57 ± 18.41^{d}	589.13 ± 17.33^{c}
TARicum AI2	107.09 ± 0.68^{b}	177.60 ± 42.99^{b}	269.21 ± 17.51^{b}	344.53 ± 19.82^{c}	452.70 ± 42.71^{c}	110.98 ± 0.95^{d}	349.71 ± 67.67^{c}	505.06 ± 38.49^{b}	546.49 ± 21.72^{c}	650.06 ± 45.22d
TARicum AI3	105.40 ± 1.13^{b}	126.58 ± 8.98^{a}	161.42 ± 16.90^{a}	185.55 ± 3.01^{a}	221.43 ± 5.47^{a}	108.65 ± 0.60^{d}	250.75 ± 14.44^{a}	492.35 ± 24.53^{b}	578.75 ± 2.44^{cd}	768.14 ± 18.25^{e}
AI5	101.24 ± 4.42^{b}	120.68 ± 8.89^{a}	147.15 ± 17.76^{a}	161.17 ± 15.65^{a}	188.33 ± 27.21^{a}	102.39 ± 6.22^{bcd}	248.35 ± 12.66^{a}	387.12 ± 34.82^{a}	416.23 ± 30.88^{a}	516.63 ± 40.83ab
AII2	104.32 ± 4.72^{b}	120.84 ± 13.66^{a}	158.42 ± 8.72^{a}	178.12 ± 14.11^{a}	211.27 ± 5.78^{a}	105.29 ± 5.92^{cd}	241.80 ± 32.34^{a}	410.30 ± 20.92^{a}	470.60 ± 25.48^{b}	564.79 ± 7.15^{bc}
AII4	89.73 ± 7.62^{a}	136.78 ± 10.12^{a}	296.41 ± 5.54^{c}	260.25 ± 26.68^{b}	599.67 ± 57.62^{d}	89.44 ± 10.23^{a}	293.49 ± 14.52^{abc}	600.34 ± 17.17^{c}	559.44 ± 32.59^{cd}	697.21 ± 47.65^{d}
A3	106.39 ± 5.81^{b}	136.72 ± 3.29^{a}	163.58 ± 3.50^{a}	176.97 ± 9.20^{a}	168.85 ± 6.61^{a}	103.60 ± 2.12^{bcd}	279.67 ± 23.32^{ab}	414.57 ± 4.58^{a}	447.50 ± 14.24^{ab}	498.04 ± 23.14^{a}
AM5	101.37 ± 2.78^{b}	129.95 ± 8.18^{a}	155.93 ± 8.06^{a}	193.91 ± 5.06^{a}	198.75 ± 17.24^{a}	97.31 ± 2.68^{abc}	250.75 ± 21.79^{a}	397.04 ± 21.98^{a}	487.57 ± 19.18^{b}	552.74 ± 30.82^{abc}

Values were means ± standard deviation of three replicates (n=3). Values in the same column with different superscripts (a-e) were significantly different (P<0.05).
MRS: De Man, Rogosa and Sharpe.

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Table 5: Percentage of survival of lactic acid bacteria in different bile salt concentration MRS broth (0.0%, 0.3% and 0.5%) at 2 h and 5 h.

Isolate	Percentage of survival (%)					
	2 h			5 h		
	0.0% Bile	0.3% Bile	0.5% Bile	0.0% Bile	0.3% Bile	0.5% Bile
TARicum AI1	134.61 ± 21.85^{b}	101.14 ± 2.48^{bc}	106.43 ± 1.83^{ab}	539.24 ± 73.33^{d}	111.33 ± 0.80^{b}	116.58 ± 1.88^{ab}
TARicum AI2	203.04 ± 11.43^{d}	101.66 ± 1.97^{bc}	104.50 ± 2.20^{a}	448.40 ± 14.21^{c}	110.66 ± 4.91^{b}	112.46 ± 2.85^{a}
TARicum AI3	149.25 ± 9.02^{bc}	97.84 ± 1.76^{b}	110.31 ± 2.37^{b}	466.94 ± 13.43^{c}	109.92 ± 2.68^{b}	117.37 ± 3.48^{b}
AI5	75.97 ± 4.75^{a}	92.59 ± 2.05^{a}	105.71 ± 3.58^{a}	247.25 ± 21.05^{a}	102.00 ± 3.62^{a}	116.09 ± 2.84^{ab}
AII2	151.41 ± 16.93^{bc}	100.16 ± 2.64^{b}	106.45 ± 1.14^{ab}	420.58 ± 36.07^{bc}	110.39 ± 2.28^{b}	116.31 ± 1.42^{ab}
AII4	128.08 ± 8.79^{b}	99.39 ± 3.03^{b}	106.97 ± 2.34^{ab}	365.67 ± 11.33^{b}	108.06 ± 1.88^{b}	119.08 ± 1.66^{b}
A3	162.33 ± 13.85^{c}	102.53 ± 5.88^{bc}	107.96 ± 2.64^{ab}	456.07 ± 6.81^{c}	109.31 ± 2.32^{b}	118.98 ± 0.18^{b}
AM5	169.15 ± 8.23^{c}	106.14 ± 1.86^{c}	106.08 ± 1.84^{ab}	532.77 ± 33.38^{d}	113.08 ± 5.52^{b}	117.90 ± 1.78^{b}

Values were means ± standard deviation of three replicates (n=3). Values in the same column with different superscripts (a-d) were significantly different (P<0.05).
MRS: De Man, Rogosa and Sharpe.

Table 6: Antibiotic susceptibility profile of isolated lactic acid bacteria.

Isolate	Zone of inhibition (mm)						
	Ampicillin (10 µg)	Chloramphenicol (30 µg)	Erythromycin (15 µg)	Gentamicin (10 µg)	Penicillin G (10 µg)	Tetracycline (30 µg)	Vancomycin (30 µg)
TARicum AI1	36.00 ± 2.00^{b}	10.00 ± 0.00^{c}	-^{a}	9.67 ± 0.58^{a}	23.67 ± 0.76^{ab}	7.83 ± 0.29^{ab}	15.50 ± 0.50^{b}
TARicum AI2	37.33 ± 1.53^{b}	9.00 ± 0.00^{bc}	-^{a}	9.67 ± 0.58^{a}	24.33 ± 0.76^{b}	7.50 ± 0.00^{a}	15.33 ± 0.29^{b}
TARicum AI3	37.67 ± 1.53^{b}	-^{a}	8.33 ± 0.58^{c}	10.00 ± 0.00^{a}	21.67 ± 0.58^{a}	8.50 ± 0.50^{ab}	16.00 ± 0.00^{b}
AI5	29.67 ± 2.08^{a}	7.67 ± 0.29b	4.50 ± 3.97^{b}	8.17 ± 0.29^{a}	23.67 ± 2.08^{ab}	9.00 ± 0.00^{b}	17.83 ± 0.76^{b}
AII2	43.33 ± 4.16^{c}	28.00 ± 2.65^{de}	35.33 ± 1.23^{d}	32.00 ± 0.87^{bc}	34.50 ± 0.87^{c}	31.33 ± 1.53^{c}	2.67 ± 4.62^{a}
AII4	29.00 ± 0.00^{a}	8.00 ± 0.00^{b}	7.33 ± 0.76^{bc}	7.83 ± 0.29^{a}	25.50 ± 0.50^{b}	9.00 ± 0.50^{b}	17.67 ± 0.76^{b}
A3	43.33 ± 1.15^{c}	28.67 ± 1.15^{e}	35.00 ± 2.00^{d}	29.00 ± 1.73^{b}	41.00 ± 2.65^{d}	32.33 ± 0.58^{e}	-^{a}
AM5	45.33 ± 1.53^{c}	26.00 ± 1.73^{d}	-^{a}	34.76 ± 5.03^{c}	25.50 ± 0.50^{b}	9.00 ± 0.50^{b}	17.67 ± 0.76^{b}

Values were means of three replicates ± standard deviation (n=3). Values in the same column with different superscripts (a-e) were significantly different (P<0.05).

Antimicrobial test

No zone of inhibition was detected for *S. aureus* and *A. hydrophila* (Table 7). A total of six out of eight LAB isolates exhibited antimicrobial properties against *E. tarda*, with zone of inhibition ranging from 8.00 mm to 9.67 mm. Isolates TARicum AI3 and AII2 showed no antimicrobial properties against *E. tarda*. No strains showed significantly higher (P<0.05) of antimicrobial properties among the isolates that showed inhibition due to overlapping of subset.

16S rRNA gene sequencing and phylogenetic analysis

Bacteria identification of isolates TARicum AI1, TARicum AI2 and TARicum AI3 were performed. These strains were selected after their overall probiotic

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Table 7: Antimicrobial test of isolated lactic acid bacteria against *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Edwardsiella tarda* and *Aeromonas hydrophila*.

Isolate	Zone of inhibition (mm)		
	Staphylococcus aureus subsp. *aureus* ATCC 25923	*Edwardsiella tarda*	*Aeromonas hydrophila*
TARicum AI1	-	9.00 ± 0.00^{bc}	-
TARicum AI2	-	9.67 ± 1.53^{c}	-
TARicum AI3	-	-[a]	-
AI5	-	9.00 ± 1.00^{bc}	-
AM5	-	8.00 ± 0.00^{b}	-
AII2	-	-[a]	-
A3	-	8.33 ± 0.58^{bc}	-
AII4	-	9.00 ± 1.00^{bc}	-
Gentamicin (Positive control)	21.17 ± 1.04	16.00 ± 0.00^{d}	12.67 ± 1.15

Values were means ± standard deviation of three replicates (n=3).
Values in the same column with different superscripts (a-d) were significantly different (P<0.05).
Symbol (-): no zone of inhibition detected.

Table 8: Sequence similarity based on 16S rRNA gene as compared with sequences in NCBI GenBank for isolates TARicum AI1, TARicum AI2 and TARicum AI3.

16S rRNA phylotype	% sequence similarity	Nearest valid taxon	Accession number
TARicum AI1	99.44	*Leuconostoc pseudomesenteroides* strain 3832^{T}	MT538679.1
TARicum AI2	98.59	*Leuconostoc lactis* strain $KCC202369^{T}$	MF992235.1
TARicum AI3	91.21	*Leuconostoc mesenteroides* strain 4332^{T}	MT544871.1

Table 9: Viability of lactic acid bacteria isolates TARicum AI1, TARicum AI2 and TARicum AI3 upon storage at 4 °C.

Isolate	Viability (log CFU/mL)			
	Day 0 (control)	Day 7	Day 14	Day 21
TARicum AI1	9.43 ± 0.13^{c}	8.87 ± 0.02^{b}	8.60 ± 0.02^{a}	8.56 ± 0.03^{a}
TARicum AI2	8.85 ± 0.03^{c}	8.61 ± 0.04^{b}	8.61 ± 0.08^{b}	8.29 ± 0.10^{a}
TARicum AI3	9.19 ± 0.06^{d}	8.51 ± 0.03^{c}	8.40 ± 0.06^{b}	8.29 ± 0.06^{a}

Values were means ± standard deviation of three replicates (n=3). Values within the same row with different superscripts (a-d) were significantly different (P<0.05).

potential were evaluated based on the characterization tests conducted. The length of the PCR product was approximately 1,400 bp. Sequential alignment sequence analysis using Bioedit software and comparison with resources from the GenBank using NCBI nucleotide BLAST revealed that isolate TARicum AI1 showed 99.44% similarity with *Leuconostoc pseudomesenteroides* strain 3832^{T}, isolate TARicum AI2 showed 98.59% similarity with *Leuconostoc lactis* strain $KCCM202369^{T}$ while isolate TARicum AI3 showed 91.21% similarity with *Leuconostoc mesenteroides* strain 4332^{T} (Table 8). Phylogenetic tree generated showed isolates TARicum AI1 and TARicum AI3 were closely related to each other, while isolate TARicum AI2 showed closest relation with *Leuconostoc lactis* strain FWA-$ZB1^{T}$ (Figure 1).

Viability test of LAB coated feed upon storage

All isolates tested (isolates TARicum AI1, TARicum AI2 and TARicum AI3) showed decrease in viability as storage duration progressed at 4 °C (Table 9). Isolates TARicum AI1 and TARicum AI3 showed similar pattern in the decrease of viability upon storage; a significant difference (P<0.05) of log CFU/mL from day 0 to 7 and from day 7 to 14 upon storage was observed. Isolate TARicum AI2 showed a significant decrease (P<0.05) in viability in accordance to log CFU/mL from day 0 to 7 and from day 14 to 21 when stored at 4 °C.

DISCUSSION

The current study focused on the isolation and characterization of LAB as potential probiotics for freshwater red tilapia. Red tilapia is selected due to its economic value. In 2018, the total market price from all tilapia products in the international market is 1,499,516 thousand USD, with frozen tilapia fillet as the most demanding product (51.6%) (FAO, 2020c). In Malaysia, red tilapia is selling in wholesale markets in live form, fresh or as fillet. As for export, the products can be in live form but mainly as frozen whole fish and fillet. Some of the tilapia products importers of Malaysia are Saudi Arabia, Dubai and Singapore (Hashim, 2015). In 2018, Malaysia was one of the top 10 tilapia exporters to

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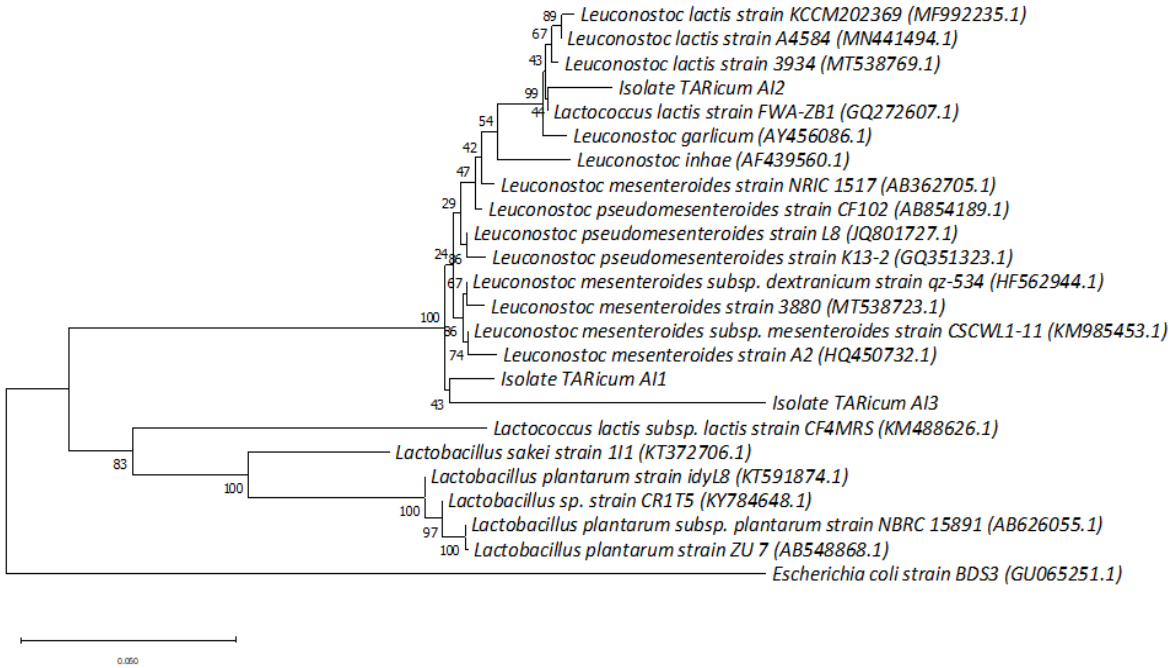

Figure 1: Phylogenetic tree of 16S rRNA partial sequences of isolated strains and identified bacteria retrieved from NCBI GenBank database. The evolutionary history was inferred using Neighbor-joining method, with a bootstrap value of 1000 (Felsenstein, 1985; Saitou and Nei, 1987). The evolutionary distances were computed using Maximum Composite Likelihood method and are in the units of number base substitutions per site (Tamura *et al*., 2004). *Escherichia coli* strain BDS3^{T} (GU065251.1) was used as the outgroup. The evolutionary length is shown by the bar = 0.050.

members of South Asian Association for Regional Cooperation (SAARC), such as Afghanistan and Maldives (FAO, 2020c). To ensure the steady growth of red tilapia aquaculture, red tilapia produced should be disease-free, of economic sustainable, profitable and affordable.

The use of probiotics in aquaculture is common for improving growth efficacy of livestock. Despite various types of commercial probiotics available, there is no probiotic that target specifically for local red tilapia. Host-originated bacteria is expected to exert beneficial effects more efficiently as compared to others since the bacteria are originated from the host, therefore they are adapted to the inner environment of host which allow them to have higher opportunity of colonization against pathogens. Based on results of phenotypic characterization, it was proven that the bacteria isolated were LAB. Similar tests were performed in other studies to prove that the isolated bacteria belong to LAB (Chen *et al*., 2013; Giri *et al*., 2019). The anaerobic incubation of isolated bacteria in MRS media were also said to be selective. Therefore, the tests carried out were sufficient to classify the isolated bacteria as LAB.

The results of probiotic characterization tests revealed the tolerance of isolated LAB towards wide range of temperature, salinity, pH and bile salt. Temperatures tested were designed in accordance to the recommended temperatures (25 to 32 °C) for freshwater aquaculture (DoF, n.d.). Besides that, potential probiotics should remain viable with promising stability for long periods during processing and storage (Fuller, 1989; FAO, 2016). The results obtained was in contrast with two other studies where their isolated LAB were found to grow well at high temperatures (45 to 48 °C) (Chen *et al*., 2013; Huang *et al*., 2014). However, the origin of isolation in these studies were plant silages and food, thus, the adaptability to high temperature range was possibly due to the original growth environment where the LAB were isolated, possibly strain-dependent.

There was no specific range of salinity requirements for fishery III group (common and of economic value and tolerant species) or freshwater aquaculture (DoE, 2019; DoF, n.d.), however, many species of tilapia are euryhaline and their cultures were not limited to freshwater, but also in salt or brackish water. This was further supported by the evidence provided by DoF as brackish water production of red tilapia was recorded in Malaysia, although with a low production value (775.48 tonnes) as compared to freshwater production (25,199.89 tonnes) (DoF, 2019). This study aimed to isolate potential probiotics for freshwater red tilapia species, thus the range of NaCl concentration selected mainly focused on concentration lower than seawater salt concentration (approximately 3.5%) (The Malaysia Water Association, 2015). Result obtained was in agreement with several studies conducted, where growth decreased as salinity increased (Itoi *et al*., 2009; Jini *et al*., 2011; Islam *et al*.,

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2016). This result proved that isolated LAB were suitable to grow in freshwater if they were to be administrated directly into water as water quality enhancer.

One of the probiotic characteristics is the ability to survive, tolerate and metabolize in low pH environment, such as the gut of livestock (Fuller, 1989; FAO, 2016). Effects of both acidic and alkalinity on the isolated LAB will affect its method of administration as probiotics; LAB that can tolerate acidic pH can be potentially administrated as feed additives while those that can tolerate alkaline environment can be potentially administrated into water as the optimum pH of rearing water in freshwater aquaculture is pH 6.5 to 8.5 (DoF, n.d.). Thus, a broad range of pH was tested. All isolates showed higher percentage of survival at pH 9 as compared to other pH tested at 5h was in agreement with *Enterococcus* sp. isolated by Alonso and colleagues (2019), indicating the pH tolerance of LAB is strain-dependent. The underlying mechanisms of alkali tolerance in LAB remained unclear as not many studies had been conducted. However, it was believed to have a relation with the origin of the LAB isolated and it was proposed that a transmembrane proton gradient was generated in a opposite direction when LAB was exposed to alkaline environment (pH 8.5 to 8.9) as compared to acidic environment (Sawatari and Yokota, 2007). A more recent study revealed the reduction of cytoplasmic pH by sodium and potassium proton anti-porters with the involvement of alkaline shock protein 23 (Asp23) in LAB growing in alkaline environment (Zhang *et al*., 2017).

Bile salt tolerance was conducted because isolated bacteria must exhibit the ability to tolerate the exposure to bile as the administration of probiotics as feed additives require the bacteria to survive through the condition of gut during digestion of lipids where bile will be released. The results obtained in this study was in accordance with several studies conducted (Islam *et al*., 2016; Rajoka *et al*., 2018; Alonso *et al*., 2019).

Antibiotic susceptibility test results showed all strains displayed different antibiotic susceptibility profile. Bacteria can show different susceptibility profile despite isolated from same origin, possibly arise due to the presence or absence of related resistance gene in their DNA (usually in their plasmid), giving rise to a variety of pangenomes, even though they might be of the same genus or species. It was possible that the isolated LAB could obtain their resistance through horizontal gene transfer. Thus, there is no standard definition or method in predicting antibiotic susceptibility profile of certain specific group of bacteria unless they are intrinsically resistance to specific antibiotic. The importance of antibiotic susceptibility profiling should not be neglected as one of the purposes of using probiotics is to reduce or to eliminate the usage of antibiotics. However, the possibility of horizontal gene transfer by potential probiotics to other bacteria when administrated as feed additives or water quality enhancer should be noted.

In this study, the pathogens selected were *A. hydrophila*, *E. tarda* and *S. aureus* subsp*. aureus* ATCC 25923. *A. hydrophila* is an opportunistic pathogen affecting tilapia, usually causes the occurrence of hemorrhagic septicemia and its infection was ranked as one of the most economically significant diseases (Hamid *et al*., 2016; Dong, 2018). *E. tarda* is a fish pathogen with zoonotic potential that cause edwardsiellosis in various organisms, which included fish and amphibians and cause high mortality in aquaculture (Haenen, 2017; Aznan *et al*., 2018; Li *et al*., 2019; Sherif *et al*., 2021). Although *S. aureus* is not a fish pathogen but a human pathogen, however, it was selected as part of the target because there were studies showed successful isolation of *S. aureus* from tilapia aquaculture (Kato *et al*., 2016; Hardi *et al*., 2018). The presence of *S. aureus* in tilapia might potentially contribute to zoonotic transmission, thus served its importance of study. No zone of inhibition was detected for *S. aureus* and *A. hydrophila*, indicating all isolated LAB did not possess any antimicrobial activities against both pathogens. This is possible as probiotic properties including antimicrobial activity is strain-specific. The result was in contrast with several studies where inhibition or decreased growth of both pathogens by LAB were reported (Liu *et al*., 2013; Ren *et al*., 2013; Bagunu *et al*., 2018). Despite showing no antimicrobial properties against *S. aureus* and *A. hydrophila*, isolates TARicum AI1, TARicum AI2, AI5, AM5, A3 and AII4 were potential to inhibit the growth of *E. tarda*. This result was in accordance with two studies where LAB was found to exhibit antimicrobial effects against *E. tarda,* but *in vivo* as compared to current study (Pirarat *et al*., 2006; Sherif *et al.*, 2021). Although treatments such as antibiotics, chemotherapy and vaccination are available to tackle *E. tarda* infection, however, the use of chemicals might cause adverse effects to the environment in terms of pollution and potential health hazards to consumers. Also, Xu and Zhang (2014) reported that lots of vaccines are under development and the complete pathogenicity mechanisms of *E. tarda* are still unknown. No commercial vaccines were recorded available in market of Malaysia for *E. tarda*, and yet *E. tarda* with multi-resistant to antibiotics and heavy metals was reported in river and farms where red hybrid tilapia cultures were present (Ismail *et al*., 2016; Lee and Wendy, 2017). Thus, this finding serves its value in tackling *E. tarda* infections.

Molecular identification based on 16S rRNA gene sequencing revealed identities of three potential isolates (isolates TARicum AI1, TARicum AI2 and TARicum AI3). All isolates belonged to the genus *Leuconostoc*. *Leuconostoc* spp. are Gram-positive mesophiles with irregular coccoid morphology, and are facultative anaerobes, non-motile and catalase-negative (Feiner, 2006; McSweeney, 2007). They are native to plants but widespread in the environment (Issa and Tahergorabi, 2019). *Leuconostoc* spp. are identified as one of the indigenous gut species in warm water fish species such as tilapia (Merrifield and Ringø, 2014). Also, *Leuconostoc.* spp. are intrinsically resistant to vancomycin, although, in this study, the isolated *Leuconostoc* spp. (*L. pseudomesenteroides* strain TARicum AI1*, L. lactis* strain TARicum AI2 and *L. mesenteroides* strain TARicum AI3) showed susceptibility towards vancomycin. One of the

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possible explanations is the use of non-fastidious Mueller-Hinton agar media, which might not fulfill the specific growth requirements of the bacteria.

A few studies had demonstrated the isolation of novel species from *Leuconostoc* genus, their probiotic potential and pharmaceutical significance. *Leuconostoc* spp. were previously successfully isolated from Nile tilapia (Lara-Flores and Olvera-Novoa, 2013; Zapata and Lara-Flores, 2013; Paray *et al*., 2018). Corresponding to the identification of isolate TARicum AI2, *L. lactis* was also isolated from intestine of black porgy fish (Zhang *et al*., 2013). Their study showed the ability of the bacteria to tolerate bile and low pH, with autoaggregation properties *in vitro* and antimicrobial properties. Corresponding to the identification of isolate TARicum AI3, a number of studies had demonstrated the isolation of *L. mesenteroides* with probiotic and/or pharmaceutical properties (Allameh *et al*., 2012; Zapata and Lara-Flores, 2013; Allameh *et al*., 2015; Paray *et al*., 2018; Selim *et al*., 2019). However, none of studies that successfully isolated *L. mesenteroides* were tested to display any antimicrobial effects against *E. tarda.* The results of pH tolerance of this study were in agreement with two studies involving *L. mesenteroides*, where highest bacterial growth was recorded in pH 7 (Allameh *et al*., 2012; Selim *et al*., 2019). Interestingly, a study showed higher bacterial growth in the presence of bile as compared to media without bile, which is in agreement with this study (Selim *et al*., 2019).

Commercializable probiotics should remain stable and viable for long periods under storage and field conditions (Fuller, 1992). To exert its beneficial effects, viable cells should be maintained at 10^6–10^8 CFU g^{-1} (Wirunpan *et al*., 2016). However, viability of probiotics can be affected during industrial processes, by environmental factors and by its own product properties, such as moisture content, water activity (A_w), pH and whether the product is made of single or mixed strains (Vesterlund *et al*., 2012; Dianawati *et al*., 2016; Wirunpan *et al*., 2016). Thus, the viability of the probiotics should be studied in order to maintain its effectiveness.

In this study, the viability of LAB coated feed upon storage was investigated. The results were in accordance with two studies where there was no major decrease in viability of probiotics fortified feed although with a decreasing trend in viability over storage time was obtained (Wanka *et al*., 2018; Akter *et al*., 2019). To improve viability, adjustment on the product properties can be made. A moisture content below 11% and A_w at 0.07–0.11 are proved to be optimum to ensure minimum viable loss (Vesterlund *et al*., 2012; Wirunpan *et al*., 2016). Probiotics should be harvested at the end of the logarithmic growth phase before the production of unwanted secondary metabolites that could lead to self-inhibition (Wanka *et al.*, 2018). Although the results of some studies were in accordance with this study that a low storage temperature (4 °C) can maintain overall viability of the probiotics without major loss (Allameh *et al*., 2015; Wirunpan *et al*., 2016; Wanka *et al.*, 2018), however, the capability of storing probiotic products at room temperature is preferable for minimizing costs of handling, transportation and storage (Dianawati *et al.*, 2016). Plus, chemical damage of feed can occur when probiotics fortified feed stored at 0 °C although high viability remained (Amstrong *et al*., 2016). Therefore, it is important to strike a balance by manipulating probiotic product properties if the product is to maintain at room temperature. Further exploration on maintaining viability at room temperature and over a longer storage period can be conducted.

CONCLUSION

The current study presented three *Leuconostoc* spp. strains (*L. pseudomesenteroides* strain TARicum AI1, *L. lactis* strain TARicum AI2 and *L. mesenteroides* strain TARicum AI3) as new probiotic candidates with potential antimicrobial activities against common fish pathogen *E. tarda*. The screening of the host-originated native strains based on phenotypic and molecular characterization, and subsequent characterization study for growth condition suited for freshwater environment, pathogen antagonism as well as storage viability study presented the value of putative new probiotic strains to translate potential protective effect against edwardsiellosis and to deliver promising effects more effectively as compared to commercially available probiotics particularly for *Oreochromis* spp. Application assessment of the *Leuconostoc* spp. strains fortified feed is suggested prior to further usage of the probiotic candidates in tilapia aquaculture.

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