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In vitro assessment on intestinal microflora from commonly farmed fishes for control of the fish pathogen Edwardsiella tarda

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Abstract: This study was aimed to evaluate the microflora isolated from the intestines of tilapia (*Oreochromis niloticus*), freshwater catfish (*Clarias batrachus*), snakehead (*Ophicephalus striatus*), snakeskin gourami (*Trichogaster pectoralis*), climbing perch (*Anabas testudineus*), and river catfish (*Pangasius pangasius*) for their potential probiotic development. Isolates were initially screened for their inhibitory effect against one of the most common fish pathogens, *Edwardsiella tarda*. Positive antagonists were subsequently tested for hemolytic activity, antibiotic susceptibility, and production of bacteriocin-like inhibitory substances (BLISs). Our results revealed 55 of the 405 isolates tested showed inhibitory effects towards the pathogen. Forty isolates were γ-hemolytic bacteria. Only 7 isolates were nonresistant to tested antibiotics. They were identified as *Lactococcus lactis* based on 16S rRNA characterization. Our BLIS data suggest that *L. lactis* subsp. *lactis* CF4MRS at the cell density of 10^6 cfu/mL could be effectively used to control *E. tarda*.

Key words: Aquaculture, *Edwardsiella tarda*, lactic acid bacteria, *Lactococcus lactis*

1. Introduction

Edwardsiella tarda is a common fish pathogen that causes hemorrhagic septicemia (also known as edwardsiellosis) in infected fishes. This pathogen is detrimental to the aquaculture industry worldwide due to the devastating economic losses incurred (1). Infection by *E. tarda* not only leads to mass mortality in a wide range of farmed fish species, but also causes serious health problems in reptiles, shellfish, amphibians, birds, humans, and mammals (2). This is attributed to the ability of *E. tarda* in inducing gastroenteritis and wound infections such as cellulitis, clostridial myonecrosis associated with trauma to mucosal surfaces, septicemia, and meningitis in various species (3,4).

Antibiotics such as aminoglycosides, penicillins, and quinolones were suggested to prevent the outbreak of *E. tarda* (5,6). The overuse of antibiotics, however, can cause the emergence of antibiotic-resistant bacteria. Thus, alternative preventives or control strategies are sought to reduce the usage of antibiotics in fish farming activities (7). One feasible approach to reduce the use of antibiotics in fish farming is the discovery and development of probiotics for sustainable aquaculture (8).

To date, probiotics used to control *E. tarda* were mainly commercial probiotic strains obtained from terrestrial animals found to be beneficial to the target host (7,9). When these probiotics were applied to eel (*Anguilla anguilla* L.) and tilapia (*Oreochromis niloticus*), they enhanced the nonspecific immune system and reduced fish mortality caused by *E. tarda*. However, numerous disadvantages, such as poor intestinal colonization of probiotics and environmental biosafety issues, are emerging during the application of commercial probiotics in fish farms (9,10). Our study explored the advantage of using indigenous intestinal microflora from commonly farmed fishes as potential probiotic candidates. This approach addresses the suitability and efficacy of the potential probiotics to be used to control *E. tarda* infection. The fish specimens used in our study were commonly farmed fishes, which included tilapia (*Oreochromis niloticus*), freshwater catfish (*Clarias batrachus*), snakehead (*Ophicephalus striatus*), snakeskin gourami (*Trichogaster pectoralis*), climbing perch (*Anabas testudineus*), and river catfish (*Pangasius pangasius*). In this study, different parameters of microbiological screening such as antagonistic activities, hemolytic activities, antibiotic susceptibility, and bacteriocin-like inhibitory substance (BLIS) assays were examined to select the beneficial bacteria for probiotic development.

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2. Materials and methods

2.1. Sampling of freshwater fishes

A total of 18 healthy adult freshwater farmed fishes were randomly collected from 4 different fish farms located in Selangor, Malaysia (Table 1). All of these fishes were humanely killed using the pithing method approved by the Monash University Animal Ethics Committee (AEC No.: MARP/2012/117). The fish bodies were then surface-disinfected with ethanol (70%). Incisions were made from the poststomach to the anus of the fish to remove the intestines. The intestinal samples were kept in sterile 50-mL tubes (BD Falcon, USA) and transported to the microbiology laboratory in an ice box within 4 h of sampling.

2.2. Isolation of bacteria from fish intestines

The fish intestines (1 g) were cut into smaller pieces with sterile scissors and forceps, and were homogenized in 10 mL of sterile saline solution (NaCl, 0.85%) using an electric homogenizer (LabGEN 125, Cole-Parmar, USA). Serial dilution was performed (10^{-1} to 10^{-8}), and 100-µL aliquots were spread-plated onto 3 different culture media: nutrient agar (NA; Merck, Germany), tryptone soy agar (TSA; LAB, USA), and de Man Rogosa and Sharp agar (MRS; Difco BD, USA). The inoculated plates were incubated at room temperature (26 ± 2 °C) for 24 h. Normal room temperature was used due to the fact that bacteria in the gut corresponded to body temperatures of fishes. Pure cultures were subsequently picked from single colonies and established on corresponding fresh agar plates.

2.3. In vitro screening for antagonistic bacteria

Bacterial isolates were screened for their antagonistic activity against *E. tarda* using the cross-streak method as previously described (11). Briefly, the isolates were streaked onto Mueller-Hinton agar (MHA; Oxoid, UK) in a single line (60 mm), followed by incubation at 26 ± 2 °C for 24 h. After incubation, an overnight culture of *E. tarda* BCRC 16703 at 10^6 cfu/mL was streaked perpendicularly (50 mm) to the line of the bacterial isolate. This was followed by incubation at 26 ± 2 °C for 24 h. After 24 h, the length of the *E. tarda* was measured. Levels of inhibition were scored as 0 (no inhibition), 1 (1–5 mm inhibited), 2 (6–10 mm), 3 (11–15 mm), 4 (16–20 mm), 5 (21–25 mm), 6 (26–30 mm), 7 (31–35 mm), or 8 (36–40 mm) (12). The experiment was performed with 4 replicates.

2.4. Hemolytic activity assay

Positive antagonistic isolates were subjected to hemolytic activity assay to determine their pathogenic potential (12). The assay was performed by streaking the bacterial isolates onto horse blood agar (Thermo Scientific, Malaysia) with incubation at 26 ± 2 °C for 24 h. The hemolytic zones formed were measured (cm) and classified as α-, β-, and γ-hemolysis based on lysis activities of red blood cells in the media around and under the colonies. The experiment was conducted with 4 replicates. Only bacterial isolates with γ-hemolysis were selected for the antibiotic susceptibility assay.

2.5. Antibiotic susceptibility assay

The antibiotics that are commonly used in Malaysian fish farms were selected for this assay. These include tetracycline (30 µg), chloramphenicol (30 µg), kanamycin (30 µg), streptomycin (25 µg), gentamicin (10 µg), and ampicillin (10 µg) (Oxoid, UK). Bacterial isolates were first seeded on MHA and incubated at 26 ± 2 °C for 24 h. The corresponding antibiotic disks were then placed on the seeded plates at approximately 3-cm radial distances from one another (13). A blank bio-disk impregnated with sterile distilled water was used as the control. All plates were incubated at 26 ± 2 °C for 48 h. The assay was performed with triplicates. Clearing zones (halo zones formed on agar) were measured and recorded. Annular radius of ≥6 mm was accepted as a susceptible response, while <6 mm was recorded as the resistant response to the particular antibiotic (13). Only bacterial isolates with positive susceptible responses to all 6 antibiotics were selected for molecular identification based on 16S rRNA gene sequences.

Table 1. Fish specimens and sampling locations.

Common name	Scientific name	Weight range (kg/fish)	Sampling sites	Coordinates
Tilapia	*Oreochromis niloticus*	0.3–0.45	Sekinchan	3°27′N, 101°08′E
Freshwater catfish	*Clarias batrachus*	0.3–0.45	Rawang	3°20′N, 101°14′E
Snakehead	*Ophicephalus striatus*	0.3–0.45	Kuala Selangor	3°22′N, 101°28′E
Snakeskin gourami	*Trichogaster pectoralis*	0.03–0.05		
Climbing perch	*Anabas testudineus*	0.04–0.06	Bestari Jaya	3°24′N ,101°22′E
River catfish	*Pangasius pangasius*	1.10–1.60		

Three fish specimens of each species were collected from the respective sampling sites.

2.6. 16S rRNA characterization of selected bacterial isolates

Selected bacterial isolates were cultured in NB at 26 ± 2 °C for 24 h prior to the extraction of DNA. Bacterial cells were collected by centrifugation at 8000 rpm and 24 °C for 3 min. DNA extraction was performed using a GF-1 Bacterial DNA extraction kit (Vivantis Tech, Malaysia). The 16S rRNA gene was amplified using primers 27f (5' – AGA GTT TGA TCC TGG CTC AG – 3') forward primer and 1492r (5' – GGT TAC CTT GTT ACG ACT T – 3') reverse primer, able to recover nearly the full length of the 16S rRNA. The polymerase chain reaction (PCR) process was performed on 50-µL reaction mixtures containing 5 µL of extracted DNA, 5 µL of 10 µM 27f primer, 5 µL of 10 µM 1492r primer, 25 µL of PCR master mix (GoTaq Green Master Mix, Promega, USA), and 10 µL of nuclease-free water (Promega, USA). PCR reaction was carried out using the MJ Mini Personal Thermal Cycler (Bio-Rad, Singapore) with the following requirements: predenaturation at 94 °C for 5.00 min, followed by 30 cycles of denaturation at 94 °C for 0.30 min, annealing at 57 °C for 0.45 min, and extension at 72 °C for 1.30 min, with a final extension at 72 °C for 7.00 min. PCR products were outsourced for sequencing (FIRST BASE, Malaysia). The results of gene sequences were viewed using BioEdit 7.0.5.3, and a query search was performed for homologous sequences in the BLAST (NCBI) database.

2.7. BLIS assay

The BLIS assay determines the effects of initial density (cfu/mL) and incubation period (h) of potential antagonists on *E. tarda*. Selected bacterial isolates were first cultured in 30 mL of NB and incubated for 24 h at 26 ± 2 °C. The bacterial cultures were then adjusted to the densities of 10^6, 10^7, 10^8, and 10^9 cfu/mL using a microplate reader (Infinite 200 PRO NanoQuant, Switzerland) based on their respective growth curves (OD_{540}). The bacterial isolates with various cell densities were streaked onto MHA in a single line (60 mm) using a sterile cotton swab, followed by incubation at 26 ± 2 °C for 24 h. After incubation, an overnight culture of *E. tarda* at 10^6 cfu/mL was streaked perpendicularly (50 mm) to the line of the test bacterial isolate of each density. The inoculated plates were further incubated for 24, 48, and 72 h at 26 ± 2 °C. The experiment was performed in triplicates. Inhibitory lengths were measured and compared with the controls, and percentage of inhibition was expressed based on the following formula:

$$\text{percentage of inhibition (\%)} = (A_o - A_1) / A_o \times 100,$$

where A_o is the length of streak of control (autoclaved water) and A_1 is the length of *E. tarda* exposed to different bacterial antagonists at different cell densities and incubation times. The results of the BLIS assay (% of inhibition) were subjected to square root transformation prior to the statistical analysis. ANOVA and post hoc analysis (Tukey's test) were used to analyze the significance of the values and interaction between time and initial bacterial cell density. Statistical significance was accepted at $P < 0.05$. Statistical analysis was performed using SPSS 20.

3. Results

3.1. In vitro screening for antagonistic bacteria

A total of 405 bacterial isolates were isolated from the intestinal samples of the fish specimens. The highest number of intestinal microflora isolates was recovered from snakeskin gourami (107 isolates), followed by freshwater catfish (104 isolates), river catfish (88 isolates), climbing perch (41 isolates), snakehead (35 isolates), and tilapia (30 isolates). Of these, only 55 isolates (13.6% of total isolates) showed positive antagonistic activity towards *E. tarda* (Figure 1), with the highest number of potential antagonists isolated from river catfish (28 isolates), followed by tilapia (11 isolates) and snakehead and freshwater catfish (8 isolates each, respectively) (Figure 1). None of the intestinal microflora from the climbing perch and snakeskin gourami showed any positive inhibition towards *E. tarda*. Most of the potential antagonists had a weak inhibitory effect towards *E. tarda* with inhibitory levels of 1 to 3 (31 isolates, 56.4%). They were predominantly isolated from the remaining 24 isolates, which showed stronger inhibitory effect at inhibitory levels of 4 and above (43.6%) (Figure 1). Among the 24 isolates, 12 bacteria performed at level 4, and 6 bacteria at level 5, of inhibition. They were all obtained from the intestinal samples of various fishes, except for climbing perch and snakeskin gourami. The other 6 isolates were obtained from freshwater catfish (1 isolate at level 6 and 2 isolates at level 8), river catfish (1 isolate at level 6), tilapia (1 isolate at level 6), and snakehead (1 isolate at level 8).

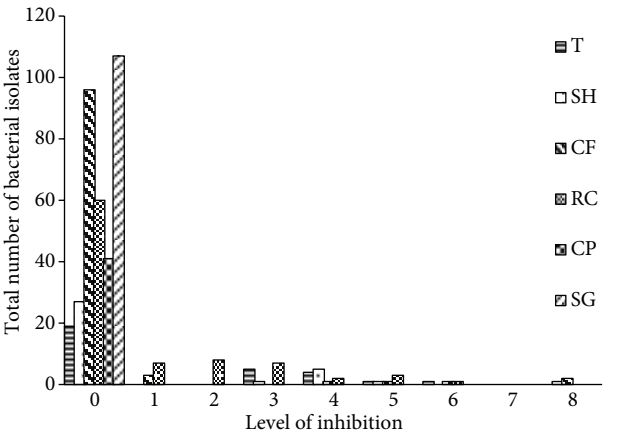

Figure 1. Antagonistic activities of intestinal bacteria isolated from tilapia (T), freshwater catfish (CF), snakehead (SH), climbing perch (CP), snakeskin gourami (SG), and river catfish (RC) against *E. tarda*.

3.2. Hemolytic activity assay

Of the 55 bacterial isolates tested for hemolytic activity assay, 15 isolates showed either α- or β-hemolytic activities, suggesting a potential pathogenic effect in causing lysis of red blood cells in the host. Only γ-hemolytic strains were selected for subsequent antibiotic susceptibility assay. When observed based on host species, it is interesting to note that the highest number of isolates showing γ-hemolysis was found in the river catfish (19 isolates), followed by tilapia (11 isolates) and snakehead and freshwater catfish (5 isolates each, respectively) (Figure 2).

3.3. Antibiotic susceptibility assay

Results revealed that 7 isolates (SH6MRS, T5TSA, CF4MRS, CF2MRS, RC7MRS, RC6MRS, and RC5MRS) were susceptible to all antibiotics tested. Susceptibility to ampicillin was the most severe, ranging from 9.2 to 20.8 mm, followed by kanamycin, 7.5–15.0 mm; gentamicin, 7.0–11.0 mm; streptomycin, 6.2–10.8 mm; tetracycline, 6.0–14.7 mm; and chloramphenicol, 6.0–18.0 mm (Figure 3). Of these 7 isolates, 3 isolates were indigenous intestinal microflora of the river catfish (RC5MRS, RC6MRS, and RC7MRS), 2 isolates were from the freshwater catfish (CF2MRS and CF4MRS), and 1 isolate each was from from tilapia (T5TSA) and snakehead (SH6MRS) (Figure 3).

3.4. Characterization of selected bacterial isolates based on 16S rRNA

Intestinal isolates with γ-hemolytic activity and susceptibility to antibiotics were characterized using a molecular approach to determine their species. The 6 isolates (SH6MRS, T5TSA, CF4MRS, RC7MRS, RC6MRS, and RC5MRS) showed a 96%–99% similarity to *Lactococcus lactis* subsp. *lactis*. Only one isolate, CF2MRS, was identified to the species level as *L. lactis*. *L. lactis* subsp. *lactis* was noticeably most frequently isolated from the fish specimens, i.e. river catfish, tilapia, snakehead, and freshwater catfish (Table 2). *L. lactis* subsp. *lactis* CF4MRS was selected for subsequent tests in the study.

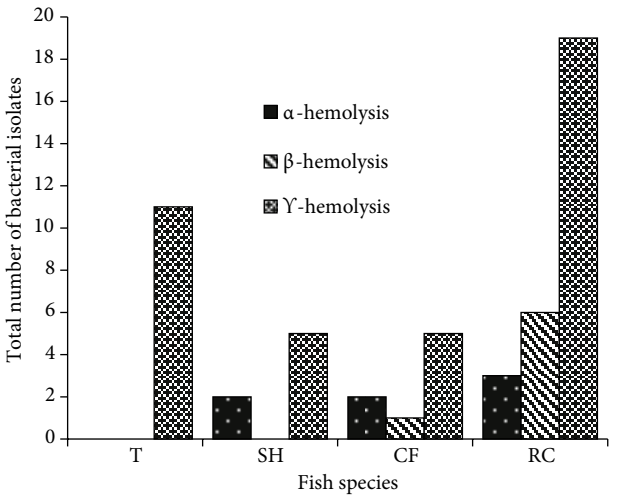

Figure 2. Hemolytic activities of intestinal bacteria isolated from tilapia (T), freshwater catfish (CF), snakehead (SH), and river catfish (RC). Isolates from climbing perch and snakeskin gourami were not tested as they showed no inhibitory effect against *E. tarda*.

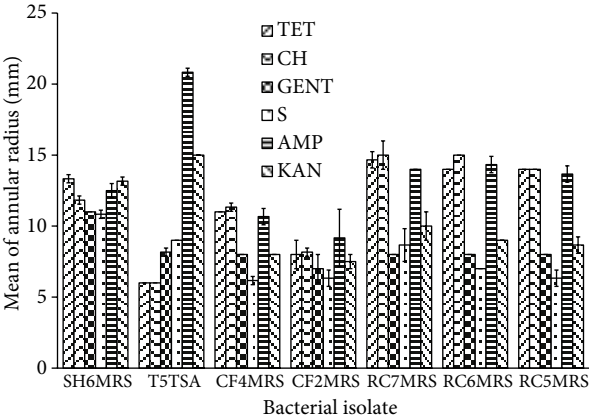

Figure 3. The mean of annular radius showed by different bacterial isolates exposed to tetracycline (TET), chloramphenicol (CH), gentamicin (GENT), streptomycin (S), ampicillin (AMP), and kanamycin (KAN). Vertical bars indicate standard deviation of means.

3.5. BLIS assay

BLIS assay revealed that the growth of pathogen *E. tarda* was inhibited by *L. lactis* subsp. *lactis* CF4MRS at the cell density of 10^9 cfu/mL (42.2% inhibition), followed by 10^8 (38.0%), 10^7 (35.6%), and 10^6 (31.6%) cfu/mL at 24 h of incubation (Figure 4). However, there was no significant difference ($P > 0.05$) in the percentage of inhibition regardless of the cell density within 24 h of incubation (Figure 4a). The bacterial inhibition trend was not observed in the prolonged incubation times of 48 and 72 h (Figure 4b). The data show that prolonged incubation time of more than 24 h does not seem to promote a better and/or consistent inhibitory effect towards *E. tarda*. The effective cell density of *L. lactis* subsp. *lactis* was suggested to be between 10^6 to 10^9 cfu/mL. However, a higher cell density such as 10^9 cfu/mL of *L. lactis* subsp. *lactis* CF4MRS was recommended for the inhibition of *E. tarda*.

4. Discussion

This study revealed that farmed fishes harbor many intestinal microflora, with snakeskin gourami, *T. pectoralis*, having the most isolates, followed by freshwater catfish, *C. batrachus*. Tilapia, *O. niloticus*, has the least number of intestinal microflora. This is presumably due to different cultivation practices, such as application of prophylactic antibiotics and therapeutic chemicals to prevent and/or

Table 2. Identification of selected bacterial strains isolated from different fishes with partial sequences of 16S rRNA genes.

No.	Isolate	Host	Bacterial species	Strain	Max. identity (%)
1.	RC5MRS	*P. pangasius*	*Lactococcus lactis* subsp. *lactis*	4001C2	96%
2.	RC6MRS	*P. pangasius*	*Lactococcus lactis* subsp. *lactis*	Fmb112-4	98%
3.	RC7MRS	*P. pangasius*	*Lactococcus lactis* subsp. *lactis*	CE-2	99%
4.	T5TSA	*O. niloticus*	*Lactococcus lactis* subsp. *lactis*	4001C2	98%
5.	SH6MRS	*O. striatus*	*Lactococcus lactis* subsp. *lactis*	GYL32	97%
6.	CF2MRS	*C. batrachus*	*Lactococcus lactis**	N2	99%
7.	CF4MRS	*C. batrachus*	*Lactococcus lactis* subsp. *lactis*	4001C2	99%

*: NCBI matching only to the species level.

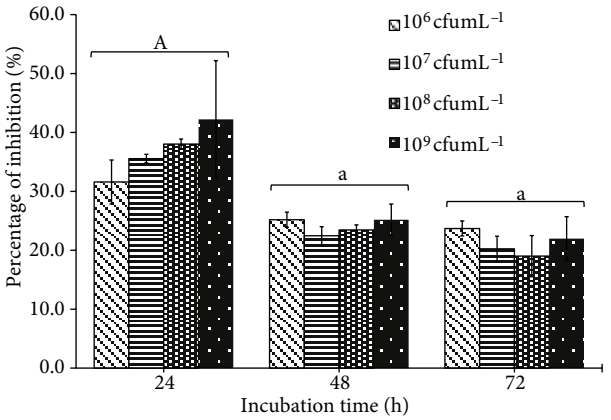

Figure 4A. The percentage of inhibition (%) against *E. tarda* by various cell densities (10^6, 10^7, 10^8, and 10^9 cfu/mL) of *L. lactis* subsp. *lactis* CF4MRS at 24, 48, and 72 h. Mean values with same letters within incubation times are not significantly different ($HSD_{0.05}$). Vertical bars indicate standard deviation of means.

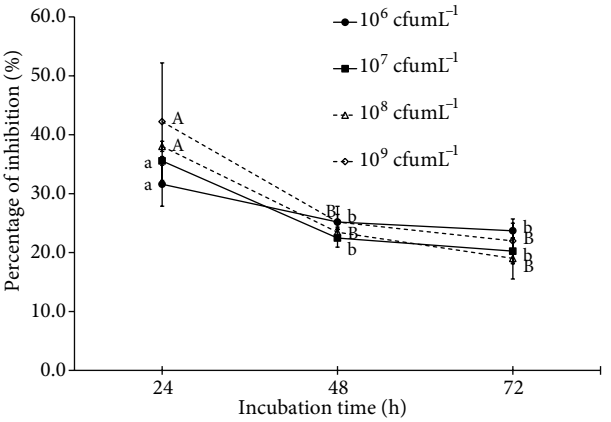

Figure 4B. The percentage of inhibition (%) against *E. tarda* by various cell densities (10^6, 10^7, 10^8, and 10^9 cfu/mL) of *L. lactis* subsp. *lactis* CF4MRS at 24, 48, and 72 h. Mean values with same letters within cell densities are not significantly different ($HSD_{0.05}$). Vertical bars indicate standard deviation of means.

control fish disease in commercial fish farms (14). Apart from this, bacteria naturally present in the fish culture environment might also play an important role in the diversity of intestinal microflora in some species (15). In spite of having large numbers of intestinal microflora in *T. pectoralis* and *A. testudineus*, these bacterial isolates mostly have no antibacterial activity towards *E. tarda*. On the contrary, *C. batrachus* and *P. pangasius* in this study possess higher numbers of bacterial isolates with antibacterial activity towards the pathogen.

The hemolytic assay further revealed the suitability of intestinal microflora for further antibiotic susceptibility assay. The γ-hemolytic bacteria isolated from the fishes revealed no red blood cell lysis activity on the blood agars. This safety precaution is relatively important in the screening process, as hemolytic bacteria would break down the epithelial layer of host cells and cause malfunction in the defense system. Failure of this defense mechanism would cause invasive diseases in the host (12,16). Therefore, the absence of hemolytic activity is one of the selection criteria for probiotic strains to signify that these bacteria are nonvirulent.

Antibiotic susceptibility assays were also established in the study to investigate the suitability of these bacterial isolates for further probiotic development. This safety consideration is of particular importance to exclude bacteria with antibiotic-resistance genes, as bacteria carrying these genes can take advantage of mobile genetic elements (e.g., plasmids and transposable elements), which would allow the bacteria to access a large pool of itinerant genes and subsequently spread the antibiotic-resistance genes to the entire bacterial population in the gut system (17). Therefore, strains resistant to any of the antibiotics should not be considered as probiotics. From

the antibiotic susceptibility results, we found that most of the γ-hemolytic bacteria isolated from *O. niloticus*, *P. pangasius*, *C. batrachus*, and *O. striatus* were not susceptible to the antibiotics tested in the study. This might imply overuse of antibiotics in the fish farms, which led to development of antibiotic resistance genes in the fish intestinal microflora (18). This scenario is particularly distinct for the tetracycline and ampicillin, where bacterial isolates of the farmed fishes were found to be less susceptible to these antibiotics. In our case, *P. pangasius* was found to have more isolates susceptible towards various antibiotics used, especially tetracycline, chloramphenicol, and ampicillin. However, it is also interesting to note that most of the bacterial isolates from *P. pangasius* were resistant to antibiotics as compared to other fishes, as the proportion of isolates successfully screened through antibiotic susceptible assay was only 15.8%. We speculate that this could be due to the acquisition of antibiotic resistance genes either from the fish hosts or from their living environment.

The species identification revealed that all bacterial isolates were of the species *L. lactis*, with 6 isolates were identified to subspecies level. These lactic acid bacteria (LAB) are commonly found in animal intestines, which constitute the major part of the adherent intestinal microflora in most healthy marine or freshwater fishes (19). In recent years, use of LAB as probiotics in aquaculture has been well documented in many studies. However, no report has been found particularly on the application of *L. lactis* subsp. *lactis* in *E. tarda* control and prevention. Our study showed that fish-originated *L. lactic* subsp. *lactis* CF4MRS could be used to inhibit the growth of *E. tarda*, apart from commercially available probiotic strains. *L. lactis* subsp. *lactis* is thought to be able to attach and colonize better on the intestinal tract compared to nonindigenous bacteria, as this bacterium was reported to be acid-tolerant, and it adapts extremely well in low pH conditions, similar to those reported for enteric bacteria in the gut system (20). The proliferation capability of putative probiotics in the intestinal tract system is crucial, as only these bacteria that can survive and flourish in the gut system are able to confer their beneficial effects to the host (9).

Our BLIS results further revealed that *L. lactic* subsp. *lactis* CF4MRS could suppress the growth of pathogen *E. tarda* with a cell density of as low as 10^6 cfu/mL at 24 h of application. These results could provide a basic guideline for determination of the bacterial dosage for future probiotic development. In conclusion, the microbiological screening assays in this study revealed that *L. lactis* subsp. *lactis* CF4MRS could be the most potential candidate for probiotic development to offer antibiotic-free alternatives and prophylaxis against edwardsiellosis infection in aquaculture.

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