Isolation and screening of lactic acid bacteria producing anti-edwardsiella from the gastrointestinal tract of wild-caught catfish (Clarias gariepinus) for probiotic candidates

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Abstract

Members of lactic acid bacteria (LAB) have been well-known for their antagonistic activities against various bacterial pathogens infecting aquaculture species. Thus, isolation of LAB members from wild aquatic species and screening them for antimicrobial production have been intensively done in the last few decades to replace the indiscriminative use of antibiotics. The present study aimed at isolating LAB members from the intestinal tract of wild-caught catfish, *Clarias gariepinus*, and screening them for antimicrobial production against one of the most common bacterial pathogens, *Edwardsiella ichtaluri*. The result showed that a total of 29 LAB were successfully isolated and further screened for anti-edwarsiella activities. Of the 29, 6 isolates had strong anti-Edwardsiella activity (diameter of inhibition zone > 10mm). Based on their 16s rRNA gene sequences, these LABs were identified as *Lactococcus lactis*, *Enterococcus hirae*, *Weissella confusa*, *Weissella cibaria*, and *Enterococcus faecalis* (2 isolates). Further *in vitro* studies indicated that *E. faecalis, L. lactis, E. confusa* and *E. cibaria* showed the same viability except for *E. hirae* in the intestinal fluid simulation. All LAB had a good adhesion capacity to intestinal mucus between 3,42 – 16,72%. Four LABs were sensitive to erythromycin, oxytetracycline, and novobiocin, while *E. faecalis* and *W. cibaria* were resistant to novobiocin and enrofloxacin, respectively. In vivo assay also indicated that all six LABs were non-pathogenic to catfish. These results suggested that *E. faecalis, L. lactis*, and *W. confusa* are potential probiotic candidates in aquaculture to prevent *enteric septicemia of catfish* (ESC) disease. However, further studies are needed to evaluate the use of probiotics *in vivo*.

Introduction

Aquaculture is growing both conventionally and intensively along with increasing demand for fish consumption. Dumbo catfish (*Clarias gariepinus*) is one of the blooming aquacultures with a high economic value, reaching IDR 19 billion in 2019 (KKP, 2020). In addition, catfish in Indonesia was the 3rd largest production after shrimp and tilapia. *C. gariepinus* is one of the common commodities widely cultivated in freshwater. Different negative impacts, such as the rise of fish disease, reduction in environmental quality, and impediment to fish development, are related to the intensification of the aquaculture system (Zhang et al., 2018).

Fish disease become a significant focus of aquaculture production, especially in intensive aquaculture. Fish diseases arise due to incompatibility between fish as host pathogens (micro-organisms that cause disease) and the environment (Madigan et al., 2018). Pathogens in fish can be a major problem because they can harm aquacultures such as decreasing production, and causing high mortality. Diseases can be caused by several types of pathogens such as viruses, parasites, fungi, and bacteria, one type of bacteria that commonly infects catfish was *Edwardsiella ictaluri*.

Edwardsiella ictaluri, a Gram-negative bacteria from the family Enterobacteriaceae, is the causative agent of *enteric septicaemia of catfish* (ESC) (Hawke et al., 2013). *E. ictaluri* bacteria are highly pathogenic in catfish and cause tissue damage to the liver, kidneys, spleen, nares, brain, and anaemia due to decreased hematocrit and haemoglobin (Hawke et al., 2013; Soto et al., 2013; Suanyuk et al., 2014). This bacterium was initially known to only infect channel catfish (*Ictalurus punctatus*), but then it infects other types of catfish such as tilapia, catfish, eels, and zebrafish (*Danio rerio*) (Hawke et al., 2013). Disease control to overcome this pathogen has been done by the use of antibiotics. Nevertheless, the use of antibiotics has accelerated antibiotic-resistance bacteria (Putra et al., 2021). Therefore, an alternative approach is needed to overcome this problem. An alternative approach to disease prevention in aquaculture is the use of probiotics (Hernández-González et al., 2021; Putra et al., 2021).

Previous studies have shown that probiotic supplementation in aquaculture increases growth feed digestibility, and immunity against pathogens, and improves the quality of water (Putra et al., 2021). Manipulation of gut microbes through fish feed supplementation with LAB has been shown to provide better growth, digestion, immunity, and disease resistance in the host (Maji et al., 2017). The use of host-associated microorganisms as probiotics has increased rapidly in recent years, but it is believed that host-associated microorganisms are more suitable and will provide optimal benefits to the same host compared to commercial probiotics isolated from different species. Certain bacterial species prefer to colonize the digestive tract of fish through water and feed in an aquatic environment which is very different from the terrestrial environment (Van Doan et al., 2018).

Therefore, this study aimed to isolate, identify, and evaluate potential probiotic bacteria from the digestive tract of wild-caught catfish (*C. gariepinus*) regarded their antimicrobial activity against *Edwardsiella ictaluri*, simulation of the gastrointestinal tract, adhesion capacity, antibiotic resistance, and biosafety assay.

Materials And Methods

Gut sampling dan bacterial isolation

Fish gut sampling was conducted according to a modified protocol of Amin et al. (2022). In brief, a total of nine wild catfish, *Clarias gariepinus*, with an average weight of 139.2 ± 53.5 g and an average length of 24.5 ± 4.1 cm) were caught at the Brantas River, East-Java Indonesia (-7.445550, 112.467981) using a cast net. Then, the fish were killed by spiking their brain using a dissecting needle. Dead catfish were disinfected using 70% ethanol by spraying on the entire surface of the fish to kill bacteria on the fish's body surface. Afterwards, the fish were rinsed with sterile distilled water. The fish belly was sliced ventrally from the anus to interbrancial membrane, and the digestive tract was taken out and placed on a sterile plate. The digestive tract was then weighed and mixed with a sterile phosphate-buffered saline (PBS, pH 7.2) solution to make a 10-fold dilution rate in a stomacher bag, followed by homogenization using a stomacher (Laboratory Paddle Blender/Stomacher 3 ~ 400 mL/ 500W, Model SC11L). 100 uL of homogenate was then spread on a Man Rogosa Sharpe Agar (MRSA, Merck 10661) previously added 10 g/L calcium carbonate as an indicator and incubated anaerobically for 7 days at 30°C. Colonies that produced clear zone were selected and further purified. Pure isolates were then tested for Gram staining, catalase assay, oxidase assay, and capacity to ferment glucose. Those isolate with Gram-positive, catalase-negative, and oxidase negative and were able to ferment glucose were considered LAB members and chosen for further assay.

Preparation of cell-free supernatant (CFSn)

Screening for anti-edwardsiella activity was carried out according to a protocol previously described by Amin et al. (2016) with slight modification. Briefly, each LAB was subcultured in 10 ml of MRS broth (MRSB, Merck 10661) and then incubated at room temperature anaerobically for 24 hours. Bacterial cells were then harvested by centrifugation at 15,000 *g* for 10 min at 4°C. The supernatant was taken out using a 1000 µl micropipette pH was adjusted to 6.6–6.8 with the addition of 1 M sodium hydroxide (NaOH, Merck 106498) to remove the antimicrobial effect of the organic acids. Both supernatants were then sterilized by filtering through a syringe filter size 0.22 um (Merck Millex™-GS Sterile Syringe Filter) and stored at 4°C until further analysis.

Preparation of indicator pathogen

An indicator bacterial pathogen used for antimicrobial activity was *Edwardsiella ictaluri* NCIMB 13272 which was obtained from a collection of Fish Quarantine Standard Laboratory (**Fish Quarantine** and Inspection Agency,

Surabaya Indonesia). The bacterial isolate was firstly inoculated into brain heart infusion (BHI) broth and incubated aerobically at 30°C for 48 h. Thereafter, the bacterium solvent was subcultured by streaking on Tryptic Soy Agar (TSA, *Merck* 105458) at 30°C for 48 h. A single colony was picked up and diluted in phosphate buffer saline (PBS) to reach ~ 1 x 10^8 CFU/mL or 0,5 McFarland. Thereafter, 100 µL *E. ictaluri* dilution was spread onto a Mueler Hinton (MH) agar (Merck 1.05437.0500) and air dried for 5 min.

Screening for anti-edwardsiella activity

Six mm wells were punctured in each MH agar plate previously impregnated with *E. ictaluri* with a Pasteur pipette. Subsequently, 5 µL of CFSn was added to the wells and incubated aerobically at 30°C. in addition, sterile MRS broth was used as a substitute to CFSn as negative control and each treatment had two replicates. After 48 h incubation, a clear zone formed around the wells was observed and measured for antimicrobial activity (Plumb et al., 1995).

Phenotypic and Molecular Identification

The selected LAB was then identified based on phenotypical and molecular methods. The phenotype assay was carried out using a conventional method as described by Cowan (2003). The assay consisted of Gram stain, catalase, oxidase, carbohydrate fermentation, motility, Voges Proskauer (VP), indole, H_2S production, OF test, the ability to grow at 5°C, 15°C, and 6.5% NaCl medium.

Furthermore, a molecular assay was performed based on bacterial partial 16s rRNA gene sequences. Firstly, each bacterial DNA was extracted using a Bacterial DNA isolation kit (Geneaid Biotech Ltd) according to the manufacturer's instructions. Subsequently, the bacterial DNA was used as a template for polymerase chain reaction (PCR) by targeting the bacterial 16S rRNA gene sequence. The primers used for the target DNA amplification were 27F (5'-AGA GTT TTG ATC CTG GCT CAG – 3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T -3'). The PCR was performed using SENSOQUEST Labcycler according to the protocol developed by Loh et al. (2014). The amplified PCR product was then sent for DNA sequencing. Partial 16s rRNA sequences were analyzed by alignment using Bioedit software and identified by comparing them to existing data at the NCBI (National Center for Biotechnology Information) using the BLAST (http://blast.ncbi.nlm.nih.gov) to find out the closest species from these bacteria (Zhang et al., 2000). The phylogeny tree was created using MEGA X software using the statistical method of Neighbour-joining with 1000 replication bootstraps (Kumariya et al., 2019).

Viability in the gastrointestinal conditions

Simulation of the survival rate of LAB to tolerate and pass through the fish stomach was assessed with the method performed by Geraylou et al. (2014). Each LAB was subcultured on MRS broth and incubated aerobically at 30 ± 2°C for 24 h. LAB was harvested by centrifugation at 6000 rpm for 15 min at 4°C. The supernatant was removed and then the pellet was rinsed with sterile PBS twice. The last centrifuged pellet was resuspended in sterile PBS and the concentration of LAB was adjusted to 10^8 CFU/mL. 50 µL of LAB supernatants were added to 5 mL of a simulated gastric juice. A simulated gastric juice was prepared by mixing 3 mg/mL pepsin (Merck 7197) in 0.85% NaCl (Merck 6404) (w/v) and the pH was adjusted to 4 (according to the pH in catfish stomach) with the addition of 1 M HCl. After 3-h incubation at 30 ± 2°C, the viability of bacteria was assessed by total plate count. Each LAB had two replicates.

The probiotic candidates were then exposed to a simulation of the intestinal fish according to a protocol of Geraylou et al. (2014) with slight modifications. LAB in the gastric juice simulation was then adjusted to pH 7.4 with the addition of 1 M NaOH. Thereafter, 1 mg/mL of pancreatin (Merck 7130) and 0.3% (w/v) bile salt from

catfish were added according to the last volume. Bile salt was taken from catfish by piercing the gallbladder of catfish and taking its liquid with a syringe then stored at -20°C (Nikoskelainen et al., 2001) until further used. The samples were then incubated at 30°C ± 2°C for 4 h. LAB viability was determined using total plate count after 4 h of incubation.

Adhesion capacity of LAB to intestinal mucus

The adhesive of LAB isolates to the intestinal mucus of fish was examined using the method used by Geraylou et al. (2014). The preparation of fish intestinal mucus was carried out according to Nikoskelainen et al. (2001). Briefly, catfish were fasted for 2 days and then killed as previously protocol. Subsequently, the catfish was cut in the ventral part using scissors from the anus to the head, and the intestine (pylorus to rectal of the intestine) was removed using a scalpel and placed on a sterile plate. The inner surface of the intestine was rinsed with sterile PBS (pH 7.2) and scraped the mucus using a rubber spatula. The intestinal mucus was transferred to a sterile tube and then centrifuged at 13,000 g for 30 min at 4°C to remove particulates and cellular material. The supernatant was filtered out with a 0.22 µm syringe filter. The protein of mucus was adjusted to 0.5 mg/mL in PBS using the Bradford method (1976) and stored at -70°C until further tests were carried out. Thereafter, prepare a 96-well microplate then coated with 150 µL of catfish gut mucus and left for one night at 4°C with plastic covered. The mucus was removed and washed twice with 100 µL PBS sterile and then discarded, this was carried out to remove mucus that was not attached to the microplate.

One hundred µL LAB with a concentration of ~ 1.0 x 10^8 CFU/mL were measured using a microplate reader (Microplate Reader and Washer AMR 100) with a wavelength of 600 nm and the results were recorded (A_0). Another well added 100 µL each LAB then incubate for 1 hour. LAB that did not stick after 1 hour was discarded while the attached bacteria were fixed by incubation at 60°C for 20 minutes then stained with 0.1% crystal violet (100 µL/mL) for 45 min. Crystal violet was then removed and washed with sterile PBS three times to remove excess dye. The dye bound to the bacteria was removed by adding 100 µL of 20 mM acetate buffer, pH 4.3 to the well. The absorbance of bacteria adhesion on catfish intestinal mucus was determined using a microplate reader at a wavelength of 600 nm and recorded (A_1). A microplate filled only with catfish intestinal mucus was used as a negative control ($A_{control}$). Bacterial adhesion was determined with 2 replications for each LAB and each test was carried out on eight repetitions to correct the variations. The percentage of bacterial adhesion was calculated using a formula previously described by Geraylou et al. (2014).

$$AC\,(\%) = \frac{(A_1 - A_{Ctrl}}{A_0} x100$$

Where:

AC

Adhesion capacity (%)

A_{Ctrl}

The absorbance values of control (stained intestinal mucus)

A_0

The absorbance values of the tested LAB used in the assay (10^8 CFU mL^{-1})

A_1

The absorbance values of LAB attached to intestinal mucus

Antibiotic Resistance Assay

This assay was conducted to determine the resistance of LAB to certain antibiotics to ensure it does not carry antibiotic-resistant plasmids that can infect the surrounding bacteria. The standard Kirby-Bauer method was carried out to examine each LAB. Each LAB was subcultured on MRSA and incubated at 30°C for 24 hours and subsequently diluted in PBS until it had turbidity to a 0.5 McFarland standard or equivalent to 10^8 CFU/mL. Within 15 min after adjustment, dip a sterile cotton swab into the adjusted suspension, rotate several times and press firmly on the inside wall of the tube above the fluid level to remove excess fluid from the swab. Inoculate the bacteria to MHA by streaking the swab over the entire sterile agar surface. Repeat this procedure two more times and rotate the plate to ensure an even distribution of inoculum. Leave the lid jar for three to five minutes at room temperature to dry. Three antibiotic disks containing enrofloxacin (CT0639B), oxytetracycline, and novobiocin (CT0038B) were pressed on the surface of MH agar and incubated at 35 ^oC for 16–18 h. LAB which was sensitive to these antibiotics will produce a clear zone around the disc.

Safety evaluation of probiotic candidate

To confirm that the candidate probiotics are not harmful to the host, each LAB was given to catfish fingerling by *in vivo* experiment according to Nayak and Mukherjee (2011). One hundred and forty catfish fingerlings (7–9 cm) were divided into seven groups where six groups for each LAB and one for negative control with two replicates. Before treatment, fish were kept in the tank (25 L with an aerator) and fed with a commercial pellet for a week. All LAB was inoculated on MRSA and then incubated for 24 h at 37°C. LAB colonies were suspended in PBS at a concentration of 10^8 CFU/mL. The experiment was carried out by injecting 0.1 mL of each LAB intraperitoneally (IP) into the previously prepared catfish. The negative control group of fish was injected with 0.1 mL of sterile PBS. All fish were observed for survival rate for 7 days and the mortality of each treatment was recorded.

Data analysis

The results of the experiment including survival in gastrointestinal conditions, adhesion capacity in catfish intestinal mucus, and safety evaluation of LAB were analyzed either using Paired T Test or one-way analysis of variants (ANOVA). Differences within the experiment were assessed with the Tukey post-hoc test. All statistic was performed using SPSS 22. Antimicrobial activity and antibiotic resistance were evaluated with descriptive analysis.

Result

Isolation and LAB screening

A total of 29 isolates of LAB showing a clearance zone on the MRS agar were isolated from the intestinal tract of nine wild-caught catfish. Preliminary phenotypical assay indicated that all these 29 isolates showed to be: Gram-positive, catalase-negative, oxidase negative and were able to ferment glucose. Six of these 29 isolates (6,5%) had antagonistic activity against *E. ictaluri*, indicated by the formation of a clearance zone > 10mm (Table 1). The largest diameter of inhibitory activity was found for isolates ZMW55 and ZMW102 as these isolates inhibited more

than 19 mm diameter clearance zone. While the lowest diameter of the inhibition zone was observed from isolate ZMW150

Table 1
Inhibitory activity of six CFSn LAB isolated from gastrointestinal tract of *C. gariepinus* against *E. ictaluri* screened from 29 isolates

LAB isolates	Diameter of inhibition zone (mm)[a]
ZMW50	10.1 ± 0.2
ZMW55	19.0 ± 0.5
ZMW94	14.6 ± 0.5
ZMW96	12.9 ± 0.6
ZMW101	13.7 ± 0.5
ZMW102	19.0 ± 0.4

[a] Values are means with standard deviations of two replicate

Phenotypic and genotypic identification of LAB

The result from phenotypic tests for the six LAB were presented in Table 2. The cell morphology of 4 LABs was spherical-shaped while the remaining two have rod-shaped. All LAB were Gram-positive and grew in aerobic or anaerobic conditions. Negative results were obtained for the motility test, catalase, oxidase, and indole test, as well as were unable to produce hydrogen sulfate (H_2S). The isolates were also able to utilize glucose and maltose. Meanwhile, only three isolates were able to utilize arabinose (ZMW50, ZMW96, ZMW102). Two isolates were able to utilize salicin. None of these LAB isolates grew at 5°C, producing indole activity as well as gas production. But only a single isolate (ZMW102) was able to grow at 15°C. From these phenotype assays, ZMW50, ZMW94, and ZMW96 were identified as *Enterococcus* sp. ZMW55 which did not grow in NaCl 6,5% was identified as *Lactococcus* sp. and ZMW101 and ZMW102 were identified as *Weissella* sp., Table 2.

Table 2

Phenotypic test results of six lactic acid bacterial members isolated from intestinal tracts of wild-caught catfish.

Observed Characteristics	LAB isolates					
	ZMW50	ZMW55	ZMW94	ZMW96	ZMW101	ZMW102
Morphology	S	S	S	S	R	R
Motile	-	-	-	-	-	-
Growth in:						
– Aerobe	+	+	+	+	+	+
– Anaerobe	+	+	+	+	+	+
– 5°C	-	-	-	-	-	
– 15°C	-	-	-	-	-	+
– NaCl 6.5%	+	-	+	+	-	+
Catalase	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
OF	F	F	F	F	F	F
Gas produce	-	-	-	-	+	+
H_2S produce	-	-	-	-	-	-
Indole	-	-	-	-	-	-
VP	+	-	-	-	-	-
Fermentation on:						
– Glucose	+	+	+	+	+	+
– Arabinose	+	-	-	+	-	+
– Maltose	+	+	+	+	+	+
– Salicin	+	+	+	+	-	-
Results	*Enterococcus* sp.	*Lactococcus* sp.	*Enterococcus* sp.	*Enterococcus* sp.	*Weissella* sp.	*Weissella* sp.

OF: oxidative fermentative; VP: Voges-proskaeur; S (spherical); R (Rod); F (Fermentation); + (positive reaction); - (negative reaction).

Based on partial 16s rRNA gene sequences, isolate ZMW50, ZMW55, ZMW94, ZMW96, ZMW101, and ZMW102 were identified as *Enterococcus faecalis* (99,89%), *Lactococcus Lactis* ssp. *lactis* (99,93%), *Enterococcus hirae* (100%), *Enterococcus faecalis* (99,93%), *Weissella confusa* (100%) and *Weissella cibaria* (99,79%), respectively. This result is consistent with the phenotypic test. All six LAB had been submitted to Genbank NCBI. The phylogenetic tree of six LAB isolated indigenous catfish with 16s rRNA sequences is shown in Fig. 1.

Viability in the stomach and intestinal conditions

Each LAB was firstly exposed to the simulation stomach Juice for 3h, followed by intestinal juice for 4h. The results all tested LAB had good viability after passing through the gastrointestinal tract juice simulation, ranging from 5.07 log CFU to 6.38 log CFU. The viability of all LABs was firstly decreased after being exposed to stomach juice for 3h. *E. faecalis* (isolate ZMW50, ZMW96), *E. hirae*, *W. confusa* and *W. cibaria* decreased significantly, except for *Lactococcus lactis* which still showed high viability after 3 h incubation (Fig. 2). Although all tested bacterial strains survived the simulation of the stomach, all LAB isolates showed a decrease after being exposed to the simulated stomach juice which consisted of pepsin 3 mg/mL, 0,85% NaCl and pH 4.

Further simulation was performed by incubating each LAB in the simulated intestinal juice for 4 h. The result showed that there was a different result in survival for each LAB. *E. hirae* showed a significant difference in increasing the viability after 3h incubation. However, viable cells of two LAB (*E. faecalis* ZMW50, and ZMW96) were increasing after being exposed to simulated intestinal juice for 4h, and the number of viable cells was not a significant difference compared to the initial concentration of both LAB, $p > 0.05$. While viable cells of three LAB (*L. lactis*, *W. confusa* and *W. cibaria*) counted at the end of stomach exposure were not significantly different before and after 4 h exposure to the simulated intestinal juice, Fig. 2.

Adhesion capacity to intestinal mucus

All six LAB were able to adhere to the mucus of catfish gastrointestinal tracts, ranging from 3,42% to 16,72%. However, the adherence capacity was significantly different among the LAB isolates, $p < 0.05$. The highest adhesion capacity was obtained from *Weisella ciberia* (16.72%), followed by *Weisella confusa* (9.00%), *Enterococcus faecalis* (5.59%), and *Lactococcus lactis* (4.56%). Meanwhile, the lowest adhesion capacity was observed from *Enterococcus faecalis* (3.57%), though there were no significant differences between *L. lactis*, *E. hirae*, and *E. Faecalis* (ZMW96) (Fig. 3).

Susceptibility to antibiotics

The six LAB species showed inhibition zones (11–32 mm) after being challenged with enrofloxacin, oxytetracycline, and novobiocin. Diameters of the inhibition zones were interpreted based on CLSI. The six LABs were highly susceptible to oxytetracycline indicated by an inhibition zone > 20mm. Five LAB were susceptible to enrofloxacin (*Lactococcus Lactis* ZMW55, *Enterococcus hirae* ZMW94, and *Weissella confusa* ZMW101 were categorized as highly susceptible with a diameter of inhibition zone > 20mm; 2 intermediate susceptible in diameter of inhibition zone ranging from 15mm-19mm). meanwhile, one LAB (*Weissella cibaria* ZMW102) was found to be resistant to enrofloxacin indicated by a diameter of inhibition zone < 14mm. The result showed also that 5 LAB were intermediately susceptible to Novobiocin, but one LAB (*Enterococcus faecalis* ZMW96) was resistant to Novobiocin. Based on these results, two LAB (*E. faecalis* (ZMW96) and *W. cibaria*) were excluded from probiotics candidates (Table 3).

Table 3
Antibiotic resistance assay of six LAB isolated from the gastrointestinal tracts of wild-caught catfish.

Strains	Antibiotic		
	Enrofloxacin	Oxytetracycline	Novobiocin
Enterococcus faecalis ZMW50	I	S	I
Lactococcus Lactis ZMW55	S	S	I
Enterococcus hirae ZMW94	S	S	I
Enterococcus faecalis ZMW96	I	S	R
Weissella confusa ZMW101	S	S	I
Weissella cibaria ZMW102	R	S	I

Note: the diameter of bacteriostatic zone includes the outer diameter of drug sensitive tablets (6 mm). S: sensitivity (diameter ≥ 20 mm) I: intermediate sensitivity (15 mm ≤ diameter ≤ 19 mm) R: resistance (diameter ≤ 14 mm).

Safety evaluation of LAB

Pathogenicity of the six LAB was assessed by i*n vivo* trials in which each LAB was intraperitoneally injected into 20 catfish juvenile and reared for 7 days. The results showed that no mortality was observed, except in *E. faecalis* (ZMW50 and ZMW96) which caused 5.2% mortality (one of 19 fish). Similarly, no mortality was observed in 20 fish injected with PBS (negative control), which were similar to those in catfish injected with *L. lactis*, *E. hirae*, *W. confusa* and *W. cibaria* (Fig. 4).

Discussion

Intestinal tracts of wild catfish can be a potential source of antimicrobials-producing bacteria as probiotic candidates to replace the indiscriminative use of antibiotics in aquaculture industries (Sahoo et al., 2016). Among the available bacterial strains, lactic acid bacteria have been commonly targeted by many researchers due to their ability to produce a variety of antimicrobial compounds, and are generally regarded as safe (GRAS) microorganisms (Amin et al., 2020; Hernández-González et al., 2021). In addition, LAB members are well known for their ability to cope with a wide range of environmental conditions including fish skin, gills, and intestinal tracts (Merrifield et al., 2014; Araujo et al., 2015; Amin et al., 2020; Li et al., 2020), which were considered as common entry points for many bacterial pathogens. The present study isolated six LABs having the capacity to produce anti-erdwadsiella compounds, identified as *L. lactis* ZMW55, *E. hirae* ZMW94, *W. confusa* ZMW101, *W. cibaria* ZMW102 and two strains of *E. faecalis* ZMW50 and *E. faecalis* ZMW96. Of these, four LABs (*Enterococcus hirae* ZMW94, *E. faecalis* ZMW50, *Lactococcus lactis* ZMW55, and *Weissella confusa* ZMW101) appeared to be potential probionts (having anti-erdwasiella activity, able to tolerate harsh environmental conditions of simulated stomach and intestina juice, susceptible to antibiotics, and non pathogens. While the other two LAB strains (E. *faecalis* ZMW96 and *W. cibaria* ZMW102) were excluded due to carrying antibiotic resistance genes to Novobiocin and Enrofloxacin respectively.

Three enterococci (2 strains *E. faecalis*, and one strain of *E. hirae*) isolated in the present study seemed to be quite common reported in previous studies. A study by Muñoz-Atienza et al. (2013), for instance, reported *E. faecalis* AP45 isolated from the intestinal tract of Cod (*Gadus morhua*), produced antimicrobial compounds against

Clostridium perfringens, and *L. monocytogenes*. Additionally, *E. faecalis* has also been reported to have inhibitory activity against several fish pathogens such as *Aeromonas hydrophila, Pseudomonas aureginosa*, *Shewanella putrefaciens* (Allameh et al., 2017). Another by Muñoz-Atienza et al. (2013) showed *E. faecalis* inhibited several fish pathogens including *Lactococcus garvieae, Streptococcus iniae*, and *Listonella anguillarum*. Similarly, *E. faecium* NRW-2 was reported to inhibit the growth of *Vibrio harveyi* and *Vibrio parahaemolyticus* in white shrimp, *Litopenaesu vannamei* (Hernández-González et al., 2021).

Another potential species isolated in the present study was identified as *L. lactis* ZMW55. This species was also reported from many freshwater species such as tilapia (*Oreochromis niloticus*) and catfish (*Clarias batrachus*) (Loh et al., 2014; Kaktcham et al., 2019). In addition, *L. lactis* subsp. *lactis* CF4MRS isolated from gastrointestinal *Clarias batrachus* had a capcity to produce various antimicrobial compounds that can potentially be useful in controlling bacterial pathogen such as *Edwardsiella tarda, Pseudomonas fluorescens* and *Serratia marcescens* (Loh et al., 2014). Furthermore, Muñoz-Atienza et al. (2013) reported that *Lactococcus* strains isolated from fish were reported to be sensitive to ciprofloxacin, erythromycin, gentamicin, nitrofurantoin, tetracycline, and vancomycin.

The other potential species was identified as *Weissella confusa* ZMW101. This species was also generally isolated from fish and shellfish in saltwater fish (Muñoz-Atienza et al., 2013), and synthesize diverse antibacterial compounds toward different fish bacterial pathogens (Ringø et al., 2018). A study by Prachom et al. (2020) showed that *Weisella* sp. isolated from the digestive tract of tilapia was found to be able to suppress the growth of *B. subtilis, M. morganii*, and *E. coli*. Similar studies by Bukhori and Sartini (2020) also reported that *Weisella* sp. isolated from the intestinal tract of Nile Tilapia was able to inhibit two common pathogens (*Staphilococcus aureus* and *Shigella* sp). The present study showed also that *W. confusa* was safe for catfish. These results are consistent with previous studies where W. confusa-supplemented diet and fed on rainbow trout (*Oncorhynchus mykiss*) increased growth performance, serum immune parameters, immune gene expression, and gut microbiota. These results confirm the benefits of using *W. confusa* as a probiotic in rainbow trout (Kahyani et al., 2021).

Besides having antagonistic activity against bacterial pathogens, probiotic candidates should be able to survive under gastrointestinal conditions (Geraylou et al., 2014; Amin et al., 2016). The result of our evaluation suggests that six LAB were able to survive in stomach conditions, although their viability slightly decreased except for *L. lactis*. However, after the simulation of intestinal juice, the viability of bacteria increased. These results suggest that the growth of LAB was affected by low pH and bile salt. A similar outcome has been reported for LAB in an acidic environment (Ramos et al., 2013) (Ramos et al., 2013; Tokatli *et al*., 2015; Mubarak dan Soraya, 2018). Another criterion when selecting probiotics is to survive and adhere to intestinal so that they can colonize and compete with pathogens for adhesion sites and nutrients (Amin et al., 2017). A different strain of bacteria could have a different ability to tolerate bile salt and adhere to the intestinal mucosa (Tokatlı et al., 2015). The ability to tolerate bile salt might be because these bacteria produce bile salt hydrolase (Ru et al., 2019). The adhesion capacity of six LAB varied from 3,42% to 16,72%. These variations are consistent with the variation in the mucus-adhering capacity of eight probiotic candidates isolated from *Acipenser baeri* ranging from 0.86% − 10.09% ((Geraylou et al., 2014). While the percentage of LAB attachment to the intestinal mucus of Atlantic salmon was 8.8%, 24%, and 36.1% for *L. farraginis, P. acidilactici*, and *P. pentosaceus*, respectively (Amin et al., 2016). Differences in adhesion capacity between bacterial strains might due to the presence of specific receptors on bacteria in intestinal mucus or specific carbohydrate molecules on the surface of bacterial components that act as mediators for adherence to mucus (Geraylou et al., 2014; Muscariello et al., 2020).

The antagonistic activity of LABs identified in the present study can be due to several compounds such as hydrogen peroxide, fatty acids, organic acids, ethanol, antibiotics, and bacteriocin-like inhibitory substances (BLIS) (Madigan et al., 2018). The antimicrobial activity of cell-free supernatant (CFS) from six LAB was observed after pH neutralization which indicated that these LAB strains produced other antimicrobial compounds besides the organic acids such as BLIS (Amin et al., 2016). Bacteriocins are ribosomal-synthesized antimicrobial peptides (Sahoo et al., 2016). Enterocin is commonly bacteriocin produced by *Enterococcus*, lacticin, and nisin by *Lactococcus*, and plantaricin by *Lactobacillus* (Sahoo et al., 2016; Hernández-González et al., 2021). Another antimicrobial peptide from the genus *Weissella* was Weissellicin and Weisselin (Fusco et al., 2015). However, further studies are still required to identify bacteriocin-like inhibitory substances produced by each isolated LAB.

In conclusion, the present study isolated three lactic acid bacteria (*L. lactis, E. hirae* and *W. confusa*) and showed potential characteristics for probiotic candidates: antagonistic to-*Edwardsiella ictaluri*, able to tolerate harsh environmental conditions of the stomach and intestinal conditions, good adhesion capacity to intestinal mucus, susceptible to antibiotics, and safe for catfish. However, these study was based solely on in vivo studies; therefore i*n vivo* trials on how these LABs might protect catfish from *enteric septicaemia of catfish* disease should be further investigated.

Declarations

Ethical Approval

No applicable since fish sample was obtained from a recreational fishing

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

Awik PDH: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Enny Zulaika**: Writing - original draft, Writing - review & editing. **Edwin Setiawan**: Writing - original draft, Writing - review & editing. **Zaki MW**: Writing - original draft, Investigation. **Muhamad Amin**: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Resources.

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Figures

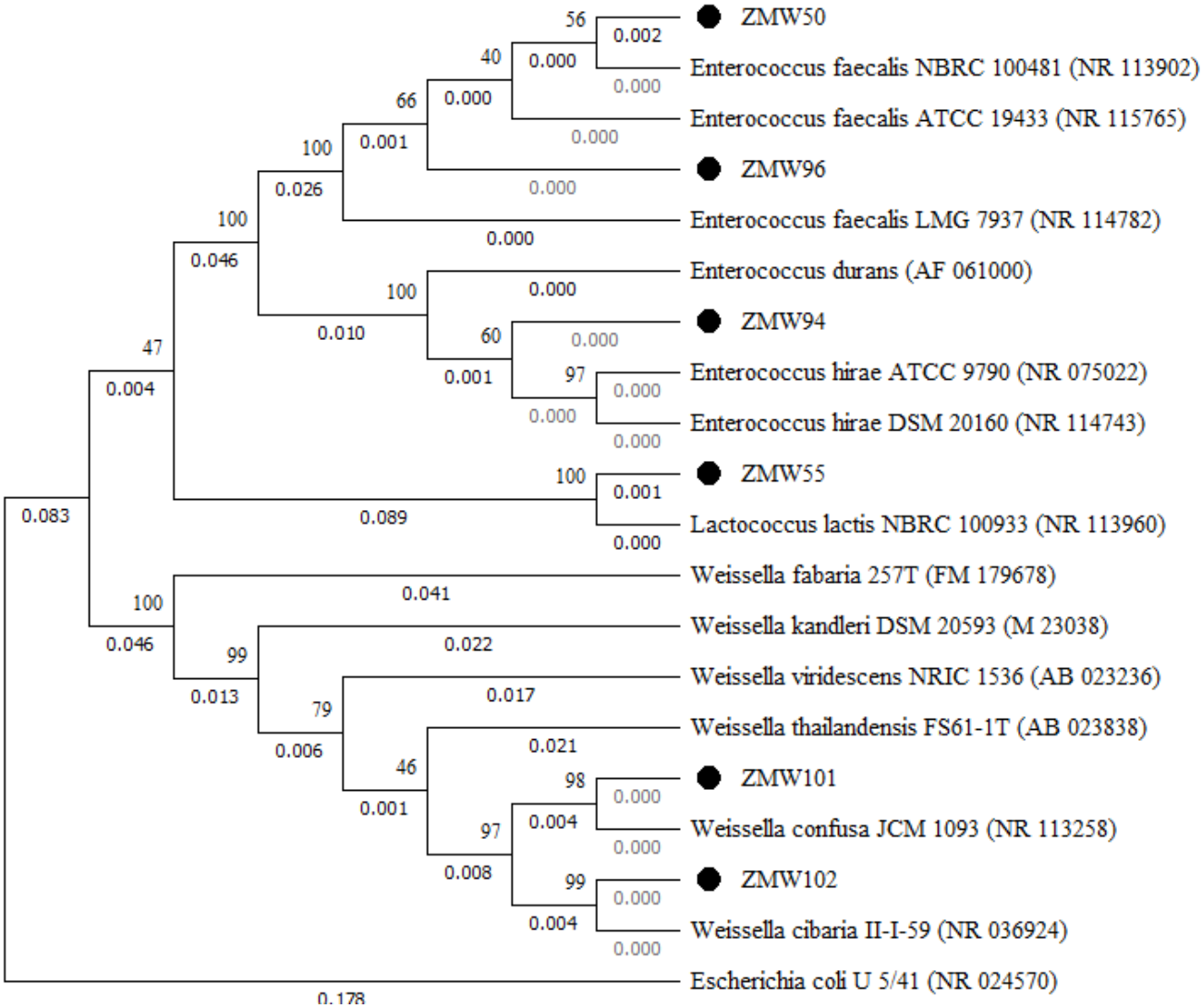

Figure 1

Phylogenetic tree of LAB isolates based on 16s rRNA gene sequence. Dots (•) is LAB isolates from gastrointestinal tracts of wild-caught catfish in the present study. The numbers on the lines indicate the distance between the branches. The bootstrap value for each node is displayed at the base of the branch. The neighbour-joining method was used as a model with Maximum Composite Likelihood Substitution; *E. coli* is used as the outgroup.

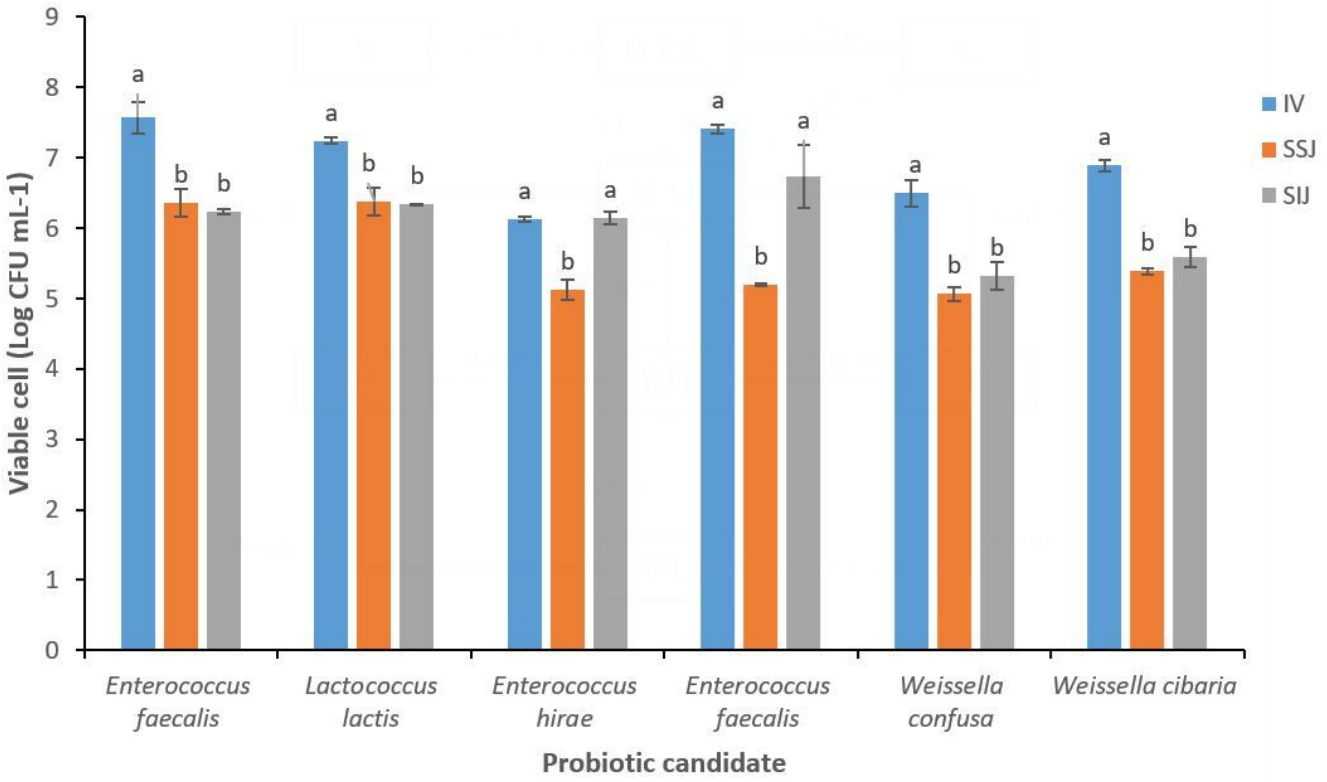

Figure 2

Viability of six LAB in simulated gastrointestinal conditions. IV (initial bacterial viability), SSJ (simulatied stomach juice), SIJ (simulated intestinal juice). All values are means with standard deviations represented by vertical bars of two replicates. Blue bars represent initial viability; orange bars represent viable cell number after simulation of stomach juice; green bars represent viable cell number after exposure simulation intestinal juice. Different letters indicate significant differences ($p < 0,05$).

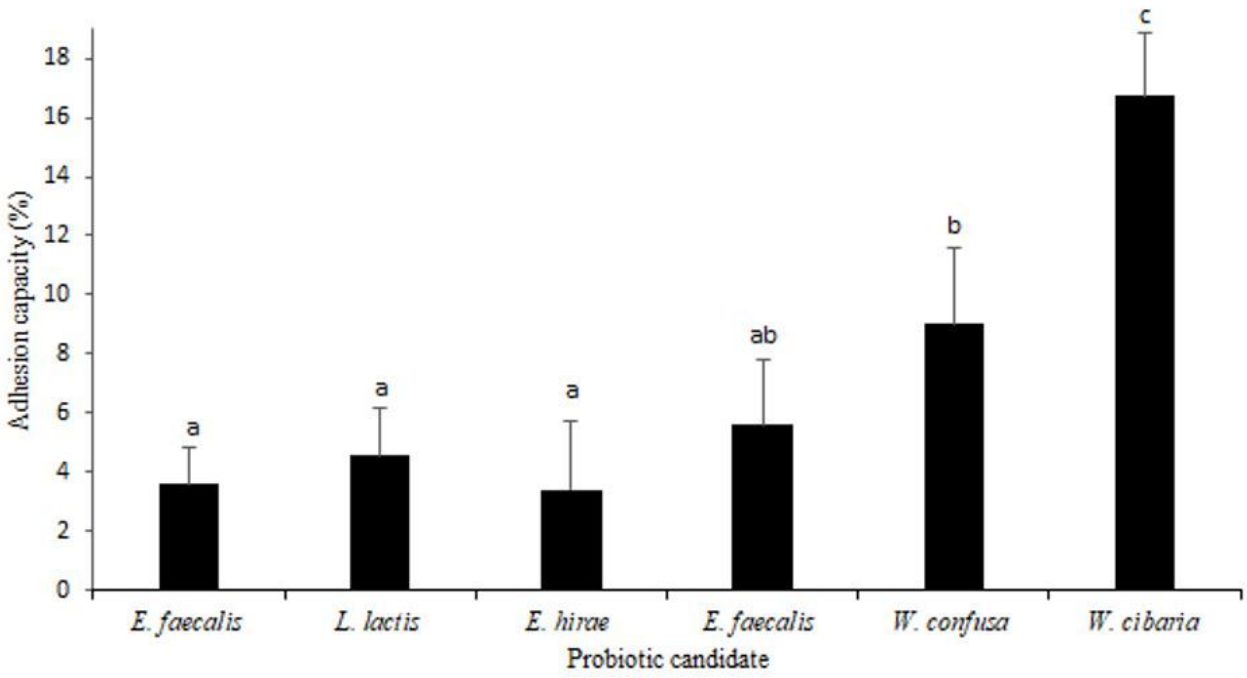

Figure 3

Adhesion capacity of six LAB isolates expressed in percentage. Bars represented average values and standard deviation of eight replicates. Different letters indicate significant differences in adhesive capacity at $p < 0.05$.

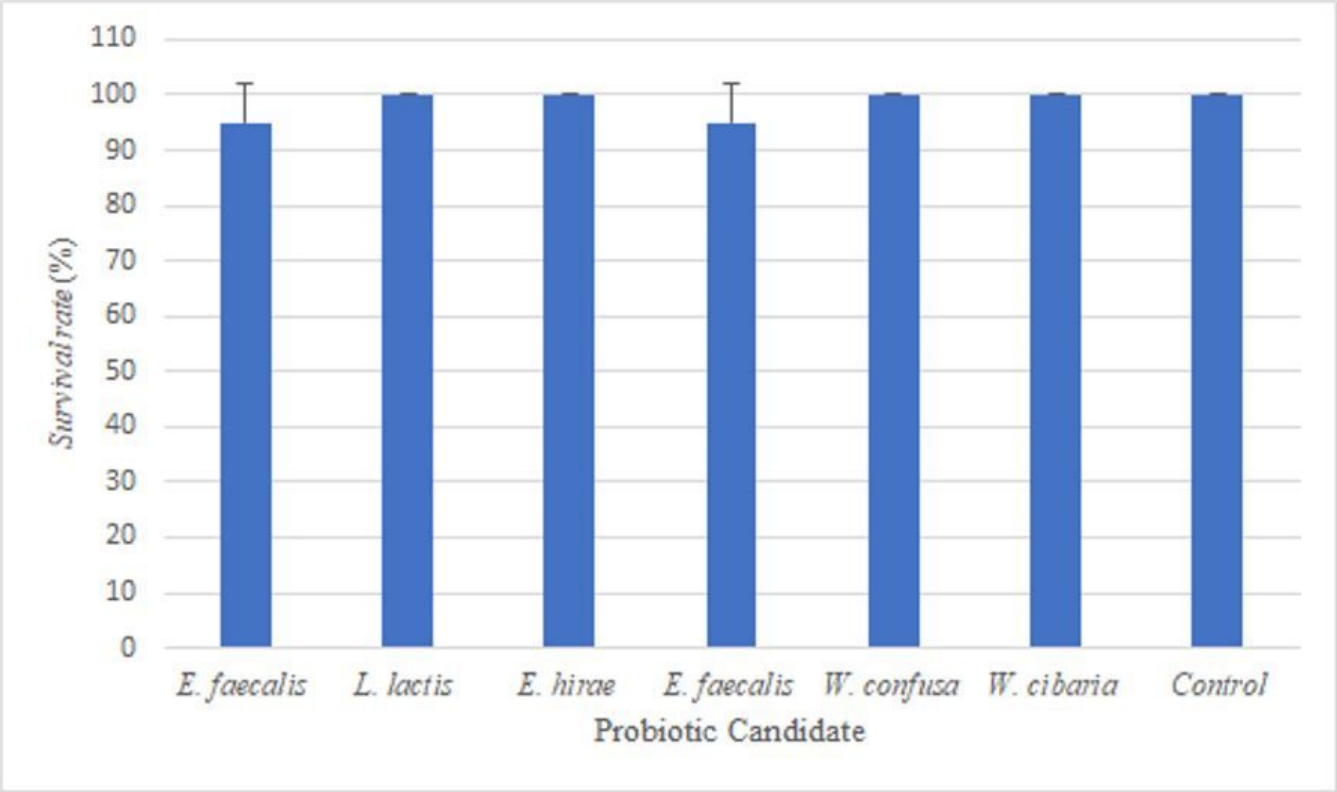

Figure 4

Survival rate of catfish on pathogenicity test by intraperitoneal injection (IP) method. The error bar shows the standard deviation (n=2). There was no significant difference in all treatments with control ($p > 0.05$)