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Lactic acid bacteria, *Enterococcus faecalis* Y17 and *Pediococcus pentosaceus* G11, improved growth performance, and immunity of mud crab (*Scylla paramamosain*)

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ABSTRACT

Mud crabs (*Scylla paramamosain*), a commercially important cultured species in the southeastern region of China, is usually infected by *Vibriosis* or parasites, causing great economic losses in cultured farms. Previous studies have demonstrated that probiotics benefited in enhancing the immune response against invading pathogens in aquatic animals. In this study, the effects of dietary administration of lactic acid bacteria (LAB) (*Enterococcus faecalis* Y17 and *Pediococcus pentosaceus* G11) on growth performance and immune responses of mud crab were assessed. Both strains (Y17 and G11) showed an inhibitory activity against bacterial pathogens (*Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Staphylococcus aureus*, and β *Streptococcus*), and a wide pH tolerance range of 2–10. *In vivo*, mud crabs were fed a control diet and experimental diets supplemented with 10^9 *cfu* g^{-1} diet either Y17 or G11 for 6 weeks before subjecting to a challenge test with *V. parahaemolyticus* for 12 h. The probiotic-supplemented diets had significant effects on weight gain and specific growth rate during the feeding trial. Increased serum enzyme activities of phenoloxidase, lysozyme, and SOD were observed in the hemolymph of mud crab in Y17 and G11-supplemented groups compared to that in the controls ($P < 0.01$). The significantly up-regulated expression of gene CAT, LYS, proPO, and SOD could be seen in hepatopancreas in G11-supplemented groups. After the pathogenicity test, the survival rate of Y17 + and G11 + *V. parahaemolyticus* groups was 66.67% and 80.00%, respectively, compared with 53.33% for the control groups. Taken together, dietary supplementation of Y17 and G11 strains were beneficial in mud crab, which could increase growth performance, modulate immune system and protect the host against *V. parahaemolyticus* infection.

1. Introduction

Mud crabs (*S. paramamosain*) is widely distributed throughout tropical, subtropical and warm areas in the Indo-Pacific region, contributing the important inshore fisheries in many countries [1]. Production of mud crab in China reached up to 148,977 tons in 2016, accounting for 50.87% of all crab aquaculture production [2,3]. Under culture conditions, mud crab aquaculture is facing disease outbreaks that cause increasing economic losses and affect the sustainable development of the mariculture [4]. In the last decades, control of diseases has been carrying out with the use of chemical drugs, mainly antibiotics, which have been considered a serious risk due to the persistence of antibiotic residues in animal tissues and the increase of antimicrobial resistance [5–7]. The problems, including altering gut microbiota, damaging living environment, and inducing the emergence of resistant bacterial populations, have been reported to be involved in the overuse of antibiotics that lead to unpredictable long-term effects on public health [8,9]. An alternative to antibiotics is urgently required for prevention of and/or intervention in disease outbreaks in cultured aquatic animals. Therefore, probiotic is currently believed to be a reliably prospective choice [10].

Probiotics are live microbial or culture product feed supplements that have beneficial effects on the health of the host when consumed in adequate doses [11]. Probiotics are known to be beneficial in enhancing

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immune response, competing for nutrition and energy with pathogens, secreting inhibitory and antibacterial substances (i.e. bacteriocins), and providing nutrients and enzymatic contributions on their hosts, as well as improving water quality in an aquaculture system [12,13]. Within the wide range of probiotics studied, lactic acid bacteria (LAB) have aroused to be important in recent years. In addition to their historical role in the preservation of food by their uses as starter cultures under controlled conditions for food fermentations [7,14], LAB is reported to be present in the gut microbiota of fishes [15] and shrimps [16]. Several LAB strains, including *Lactobacillus* spp. [17,18], *Leuconostoc mesenteroides* [7,17], *Lactococcus lactis* [19], *Enterococcus* spp. [20], *Pediococcus acidilactici* [21], and *Pediococcus pentosaceus* [22] have been used as probiotics in aquaculture to increase the immune enhancement, disease resistance, modulate the gut microbiota and competitive exclusion of pathogens through the production of inhibitory compounds.

Although much has been performed to investigate the roles of probiotics in growth performance, nutrient digestibility of feedstuff, and disease resistance of shrimp and fish [7,15,21], little is known for the application of LAB on mud crab *S. paramamosain*. The aim of this study was to evaluate the potential roles of both LAB (*E. faecalis* Y17 and *P. pentosaceus* G11) in the growth performance and disease resistance of mud crab. The results of this study indicated that the dietary supplementation of Y17 and G11 improved the growth rate and immune response of mud crab, which could provide the basic information for the uses of these LAB as probiotics in mud crab aquaculture.

2. Materials and methods

2.1. Microbial isolations

All animal handling procedures were reviewed and approved by the ethics committee of the “Regulations for the Administration of Affairs Concerning Experimental Animals”. The Institutional Animal Care and Use Committee of Shantou University, China, approved the experiments.

A total of 20 pond-raised healthy mud crabs (average weight 100 ± 3 g) were collected from Shantou, China [23]. The mud crabs were injected with an LD_{50} dose of *V. parahaemolyticus* [4]. After 12 h immersion infection, the survived mud crabs were euthanized and used for collecting the whole intestinal tracts. The samples were immediately homogenized in sterile physiological saline, serially diluted, cultured on MRS plates and incubated at 28 °C for 2–3 days. Besides, a 10-mL volume of water from culture pond was collected, serially diluted, cultured on MRS plates, and incubated at 28 °C for 2–3 days. Single colonies with a high frequency of similar appearance were selected and re-streaked onto MRS plates to obtain pure strains after incubation at 28 °C for 48 h.

2.2. Antimicrobial activity assay

To assess the growth inhibition of the pathogens, a total of 68 isolated strains from the intestine of mud crab were screened. Antimicrobial activity was determined using the agar-well diffusion method as described previously [24] with some modifications. A volume of 100 μL of bacterial suspension was poured into each well of 2216 E plates, previously coated with an overnight culture of *A. hydrophila*, *V. alginolyticus*, *V. parahaemolyticus*, *S. aureus*, and β *Streptococcus*. Wells filled with 100 μL MRS liquid medium was used as controls. After incubation at 28 °C for 24 h, the diameter of the inhibitory zone around the wells was measured. The bacteria with stable antimicrobial activity were stored at −80 °C containing 15% glycerol.

LAB strains (Y17 and G11) were selected to characterize their antibacterial compounds. An overnight suspension culture of strains was filtered through 0.2 mm pore-size filters. The cell-free culture supernatants were neutralized (pH 7.0 with sterile 5 M NaOH) and used for determination of the antimicrobial activity using the above-described method [24].

2.3. Microbial identification

The identification of both strains (Y17 and G11) was based on both traditional and molecular methods. The morphological, physiological, and biochemical characteristics were characterized using Bergy's manual of systematic Bacteriology [25,26], and identified by VITEK® 2 compact (BioMérieux, S.A. France) microbial automatic identification system, and the genotypic identification was conducted using 16S rRNA gene sequence analysis [27]. A phylogenetic tree of the bacteria was made using N-J method in MEGA 4.1 package (http://www.megasoftware.net) [4].

2.4. pH tolerance and sensitivity to antibiotics

Y17 and G11 strains were used for the determination of pH tolerance [28]. LB broth was adjusted to pH 2, 3, 4, 5, 7, 9 or 10, using 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH before autoclaving. The media (100 mL) were inoculated with 1 mL of an overnight bacterial culture (containing 10^8 *cfu* mL^{-1}) and incubated at 28 °C for 12 h, viable bacteria were counted with plate count method on LB agar (at 28 °C for 24 h) in triplicate.

For susceptibility testing, the disc diffusion method according to Kirby-Bauer was used. Susceptibility testing was done on 2216 E agar plates from overnight cultures followed by incubation at 37 °C for 48 h. Inhibition zone diameters were measured and interpreted according to CLSI 2009-11 guidelines [29]. The antibiotics sensitivity discs included ampicillin (10 μg), amikacin (30 μg), amoxicillin acid (30 μg), cefazolin (30 μg), ceftazidime (30 μg), cefuroxime (30 μg), chloramphenicol (10 μg), enrofloxacin (5 μg), erythromycin (10 μg), gentamicin (10 μg), midecamycin (30 μg), minocycline (10 μg), neomycin (5 μg), oxacillin (1 μg), penicillin (10 μg), polymyxin B (300 μg), compound sulfamethoxazde tablets (23.75/1.25 μg), tetracycline (30 μg), vancomycin (30 μg).

2.5. Feeding trial and challenge test

2.5.1. Diets preparation

A commercial pelleted feed (43% crude protein, 6% crude lipid) (Yuehai, Guangdong, China) was used as basal diet. The selected strains Y17 and G11 were cultivated in MRS broth at 28 °C for 24 h, harvested by centrifugation, washed twice with sterile saline, adjusted to obtained suitable concentration, which were then sprayed homogeneously with commercial feed pellets (set as experimental diets, including Y17- and G11-supplemented groups, respectively) [30]. The control diets (set as controls) was also sprayed with the same volume of sterile saline and stored under the same condition as the supplemented one. All feeds were dried at 40 °C for 24 h, separately sealed in vacuum-packed bags, and stored at 4 °C prior to use in the feeding trial [1]. The viability of supplemented bacteria in the feeds was confirmed each 3 days [31].

2.5.2. The indoor aquaculture and cumulative mortality assay

A total of 180 juvenile mud crabs were acclimatized under the laboratory conditions (temperature 27.0 °C, pH 7.95 and salinity 30.0‰) for 7 days before starting the experiment. Mud crab was fed a commercial diet twice a day. After the time of acclimatization, mud crabs (initial average weight 28.22 ± 0.60 g, carapace width 5.40 ± 0.07 cm) were individually introduced into polypropylene tanks of size 40 × 25 × 15 cm^3 (Xiehou, Xiamen, China) collected to the same water system. The tanks were supplied with an intermittent supply at 0.2-L·min^{-1} of sand-filtered brackish water from a depth of 5 cm for 8 h every day. Twenty tanks each were randomly allocated for Y17, G11 and control groups, respectively. Mud crabs in three groups were fed on the corresponding diet twice (07:00 and 17:00) a day for 6 weeks. During the experimental period, water parameters were

modulated: temperature 26.8–28.8 °C, pH 7.95–8.16, NH_3 below 0.05 mg·L-1, and salinity 30.0–32.0‰. Feed residues and feces were removed every day. The exuviate and dead mud crabs were removed, weighed and recorded daily. After 6 weeks of feeding, 15 crabs of each treatment were randomly distributed into 3 tanks ($80 \times 60 \times 50\ cm^3$). All mud crabs were exposed to *V. parahaemolyticus* (1.38×10^6 *cfu* mL^{-1}) for 12 h. After the immersion infection, the crabs were kept under the initial experimental conditions. The cumulative mortality rate and the survival rate were recorded for 14 days.

2.5.3. Sample collection and immune enzyme analysis

Mud crabs from each experimental group were randomly sampled at the third/six week point of feeding period, and the 0, 36 and 60 h time point after immersion infection with *V. parahaemolyticus*. Mud crabs were weighed, chilled on ice, and dissected. The hepatopancreas were quickly collected and put into the liquid nitrogen immediately, and then stored at −80 °C for subsequent RNA extraction. The hemolymph (1 mL) was withdrawn from the arthropodial membrane at the base of the third walking legs of each mud crab with a 1 mL disposable sterile syringe needle, and collected in tube containing an equal volume of precooled sterile anticoagulant solution (30 mM trisodium citrate, 0.34 M NaCl and 10 mM EDTA at pH 7.5 with the osmolality adjusted with 0.115 M glucose to 780 mOsm·lg^{-1}). The hemolymph sample with an equal of anticoagulant was divided into two aliquots: a part of diluted sample was placed in a hemocytometer to measure the total hemocyte cells (THCs) using an inverted phase-contrast microscope (Carl Zeiss, Germany) [4], and the other sample was centrifuged at 800 ×*g* at 4 °C for 20 min to collect the hemocytes sediment, which then used for RNA extraction using the TRIzol® Reagent (Ambion, USA), the remaining supernatant was transferred into an new centrifuge tube, overnight, and centrifuged at 12,000 ×*g* at 4 °C for 20 min. The serum was collected for assaying the catalase (CAT), lysozyme (LYS), prophenoloxidase (PPO), and total superoxide dismutase (T-SOD) activities using commercial kits (Jiancheng, Nanjing, China) and analyzed using a multi-functional microporous plate detector (Infinite® M200 Pro, Tecan, Switzerland).

2.5.4. Quantification of immune-related gene expression

Samples of hemocytes and hepatopancreas were used to extract total RNA using a Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First strand cDNAs were synthesized using PrimeScript ® RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocols. Four immune-related genes, including catalase (CAT, GenBank accession number: FJ774660.1), lysozyme (LYS, 304441008), prophenoloxidase (proPO, DQ435606.1) and copper/zinc superoxide dismutase (Cu/Zn-SOD, EF100890.1), were selected to evaluate the immune response of mud crab upon *V. parahaemolyticus* stimulation [32]. The real-time PCR analysis was performed using an SYBR® Premix Ex Taq™ II Kit (Takara, Dalian, China) on an ABI 7300 Real-time Detection System (Applied Biosystems, FosterCity, CA) with β-actin as an internal control [4]. The real-time PCR program was 95 °C for 30s, followed by 40 cycles of 95 °C for 5s, and 60 °C for 30s. Relative expression of genes was determined using the $2^{-\Delta\Delta Ct}$ method [23].

2.6. Calculations and statistical analysis

The parameters were calculated as follows [33]:

Survival (%) = $100 \times N_t / N_0$

Weight gain (WG, g) = $W_t - W_0$

Specific growth rate (SGR, %·d^{-1}) = $100 \times (\ln W_t - \ln W_0) / t$

Where N_t is the final amount of crabs. N_0 is the initial amount of crabs. W_t is the final body weight (g). W_0 is the initial body weight (g). Results were presented as mean ± SE.

The statistical analyses were performed using the Statistical Package for Social Science software version 17.0 for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism (GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego, CA, USA). All data were examined by one-way analysis of variance (ANOVA). When the significant differences were found ($P < 0.05$), Duncan's multiple range tests were used to identify the differences among groups.

2.7. Key resources table

Resource key resources table	Source	Identifier
Chemical		
amikacin		
amoxicillin		
ampicillin		
cefazolin		
ceftazidime		
cefuroxime		
chloramphenicol		
EDTA		
enrofloxacin		
erythromycin		
gentamicin		
glycerol		
HCl		
midecamycin		
minocycline		
NaCl		
NaOH		
neomycin		
NH3		
oxacillin		
penicillin		
polymyxin B		
SOD		
tetracycline		
trisodium citrate		
vancomycin		
ProteinPeptide		
catalase		
copper/zinc superoxide dismutase		
LYS		
lysozyme		
prophenoloxidase		

3. Results

3.1. Isolation and identification of bacterial strains

Out of 20 *V. parahaemolyticus*-injected mud crabs, 13 mud crabs were survived after 12 h post-infection. Totally, 46 and 22 bacterial strains were isolated from the intestine of the survived mud crabs and pond water, respectively. Two out of the 68 bacterial strains were coded as Y17 and G11 exhibited a strong growth inhibition of *A. hydrophila*, *V. alginolyticus*, *S. aureus*, and β *Streptococcus*, causing a clear zone of > 20 mm in the agar-well diffusion assay (Table 1, Fig. 1). The inhibitory activity of cell-free culture supernatants was not found to act against *V. parahemolyticus* (Table 1). Both Y17 and G11 exhibited no hemolytic activity (γ hemolytic) on MRS agar. The results revealed that both Y17 and G11 showed a high percentage of hydrophobicity (66.09% and 56.37%, respectively, Table 2). These results confirmed that both Y17 and G11 strains may be used safely as potential probiotics in mud crab.

Both Y17 and G11 were Gram-positive, globular shaped, and facultatively anaerobic, which showed 0.5–1.0 μm in diameter, occurring in pairs or short chains (Fig. 2). Colonies of Y17 and G11 on MRS agar appeared round, translucent, raised, moist, and white, consisting entire margins, and grow rapidly, attaining a diameter of 1.0–2.0 mm and 2.0–3.0 mm, respectively, in 3 days at 28 °C. The strain Y17 was positive in tests for mannitol, sorbitol, sucrose, lactose, D-mannitol, D-

Table 1
Antagonistic activity and identification of Y17 and G11 strains isolated from the intestine of mud crab.

Strain		Y17	G11
Isolated source		IS	IS
Isolated medium		MRS	MRS
Strain identification		*Enterococcus faecalis*	*Pediococcus pentosaceus*
Pathogen inhibition[#]	*Aeromonas hydrophila*	21.00 ± 1.00	25.33 ± 0.58
	Vibrio alginolyticus	20.66 ± 0.58	27.33 ± 0.58
	Staphylococcus aureus	15.00 ± 1.00	19.67 ± 1.50
	Beta *Streptococcus*	14.66 ± 2.31	20.33 ± 1.15
	V. parahaemolyticus[a]	20.66 ± 1.15	24.00 ± 2.00
	V. parahaemolyticus[b]	17.67 ± 2.52	23.67 ± 1.15
	V. parahaemolyticus[c]	ND	ND

IS: Intestine of surviving mud crab challenged with *Vp*.
MRS medium: *Lactobacillus* Agar acc. to De Man, Rogosa and Sharpe.
ND: no inhibitory zone.
[#].Data are presented as mean ± SE (n = 3).
[a] Overnight suspension culture tested for the antibacterial activity using an agar-well diffusion method.
[b] Overnight suspension culture was filtered through 0.2 mm pore-size filters and used for testing the antibacterial activity using an agar-well diffusion method.
[c] Overnight suspension culture filtered through 0.2 mm pore-size filters and neutralized to pH 7.0 with sterile 5 M NaOH, was used for testing the antibacterial activity using an agar-well diffusion method.

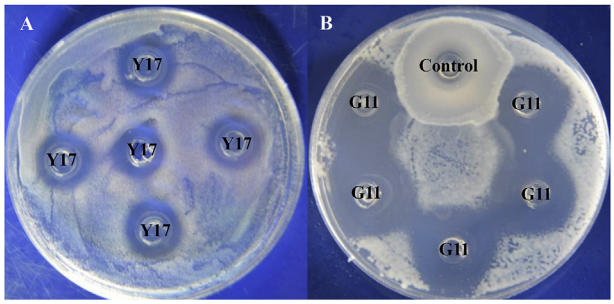

Fig. 1. The antibacterial activity against *V. parahaemolyticus* of Y17 and G11. Results of the antibacterial activity against *V. parahaemolyticus* of Y17 (A) and G11 (B) strains using an agar-well diffusion method.

ribose, cyclodextrin, tyrosine arylamidase, O/129 resistance, L-aspartic acid arylaminase, D-galactose, D-sorbitol and D-maltose, which was distinguished from the strain G11 (Table 2). The results of VITEK® 2 compact system showed that both Y17 and G11 depicted 99% similitude with *Enterococcus faecalis* and *Pediococcus pentosaceus*, respectively, reported in the GenBank database, and grouped together to form a cluster with their own homologs (*E. faecalis* ATCC 19,433, Genbank accession number: NR_115,765.1 and *P. pentosaceus* DSM 20,336, NR_042,058.1, respectively) (Fig. 3). The results indicated that Y17 was identified as *E. faecalis* Y17 and G11 was *P. pentosaceus* G11 (Table 1).

3.2. pH tolerance and sensitivity to antibiotics

Both Y17 and G11 strains showed a relatively strong resistance to low pH, which remained viable after a 24-h exposure to pH 2.0 (with a survival rate of 17.10 ± 0.01% and 2.45 ± 0.01%, respectively) (Table 3). G11 was not resistant to most of the antibiotics, excepting ceftazidime and compound sulfamethoxazde tablets, whereas Y17 was resistant to amikacin, ceftazidime, cefuroxime, gentamicin, midecamycin, neomycin, oxacillin, polymyxin B, and compound sulfamethoxazde tablets (Table 4).

3.3. Probiotics affecting the growth and immune response of mud crabs

The effects of probiotics on the growth performance of mud crab was evaluated after feeding for a 6-week period of time. At the end of the feeding trial, a significantly enhanced weight gain was found in mud crabs fed a G11-supplemented diet ($P < 0.05$), but not in that fed with Y17-supplemented diet ($P > 0.05$), compared to the controls. Mud crabs in the G11-supplemented group had the highest specific growth rate (SGR), followed by that in Y17 and control groups. There was no significant difference in survival rate among the groups ($P > 0.05$) (Table 5).

After 3 weeks of feeding, the serum prophenoloxidase (PPO) activity in both Y17- and G11-supplemented groups was significantly increased compared to the control groups ($P < 0.01$). However, the remaining enzyme activities (CAT, LYS and T-SOD) showed no significant difference between the probiotic-supplemented groups and the control ($P > 0.05$) (Fig. 5A). After 6 weeks of feeding, the lysozyme (LYS) and PPO activities of serum in both Y17- and G11-supplemented groups were significantly higher than the control groups ($P < 0.01$). The total superoxide dismutase (T-SOD) activity of Y17-supplemented group significantly increased compared to the control groups ($P < 0.05$) (Fig. 5A).

After challenged with *V. parahaemolyticus*, an increase in catalase (CAT) and T-SOD activities found in Y17 + and G11 + *V. parahaemolyticus* groups were higher than those in controls ($P < 0.01$). The increase in serum lysozyme activity was significant for mud crabs in the G11 + *V. parahaemolyticus* group ($P < 0.01$), but not in the Y17 + *V. parahaemolyticus* group ($P > 0.05$), with respect to the control groups. The PPO concentrations were not significantly different between the probiotic-supplemented groups and the control after challenged with *V. parahaemolyticus* ($P > 0.05$) (Fig. 5B).

The total hemocyte cell counts (THC) in the hemolymph significantly increased in Y17 + *V. parahaemolyticus* group compared to the control groups after 36 h immersion infection with *V. parahaemolyticus* ($P < 0.01$) (Fig. 4). Expression levels of the immune-related genes of mud crabs at 36 h after immersion infection with *V. parahaemolyticus* were shown in Fig. 6. An up-expression in Cu/Zn-SOD (Y17 + *V. parahaemolyticus* group: 2.9-fold and G11 + *V. parahaemolyticus* group: 2.2-fold) and a down-expression in CAT (Y17 + *V. parahaemolyticus* group: 0.4-fold) in the hemocyte of mud crabs were found compared to the controls ($P < 0.05$) (Fig. 6A). In hepatopancreas, a significantly increased expression of CAT (17.3-fold), LYS (10.3-fold), proPO (6.8-fold) and Cu/Zn-SOD (3.8-fold) in the G11 + *V. parahaemolyticus* groups was found, compared to those in controls ($P < 0.01$). In hepatopancreas of Y17 + *V. parahaemolyticus* groups, with the exception of CAT (2.7-fold), which was significantly higher than in the control groups ($P < 0.01$), the expression of all remaining genes was not significantly different compared to the control groups ($P > 0.05$) (Fig. 6B).

3.4. The survival rate

The survival of mud crabs after challenge with *V. parahaemolyticus* was shown in Fig. 7. After the pathogenicity test, the survival rate of Y17 + and G11 + *V. parahaemolyticus* groups was 66.67% and 80.00%, respectively, compared with 53.33% for the control groups. The results showed that the survival rate of mud crabs in G11 + *V. parahaemolyticus* groups were significantly ($P = 0.028$) ($P < 0.05$) higher than that in the controls.

4. Discussions

A previous study has documented the important roles of probiotics in aquatic animals, including controlling and inhibiting infectious pathogens, improving growth performance and activities of digestive enzymes, and enhancing immune responses of the host against

Table 2
Physiological and biochemical characteristics of Y17 and G11 strains.

Index	Y17	G11	Index	Y17	G11
Morphology	Globularity	Globularity	D-amygdalin	+	+
Gram staining	+	+	Alanine-phenylalanine-valine arylaminase	–	–
Motility	–	–	Leucine arylaminase	–	–
Hemolytic activity	γ	γ	Alanine arylaminase	+	+
Starch hydrolysis	–	–	D-ribose	+	–
Protein hydrolysis	–	–	Novobiocin resistance	+	+
Egg Yolk Lecithin hydrolysis	+	+	D-raffinose	–	–
Catalase	–	–	Optochin resistance	+	+
Mannitol	+	–	Phosphatidylinositol phospholipase C	–	–
D(+)-Cellobiose	+	+	Cyclodextrin	+	–
Maltose	+	+	L-Proline arylaminase	–	–
D(−)-Salicin	+	+	Tyrosine arylaminase	+	–
Sorbitol	+	–	L-lactate alkalization	–	–
Sucrose	+	–	Growing in 6.5% NaCl	+	–
Raffinose	–	–	O/129 resistance	+	–
Escalin hydrolysis	+	+	D-xylose	–	–
Inulin	+	(+)	L-aspartic acid arylaminase	+	–
Lactose	+	–	Phosphatase	–	–
D-maltose	+	–	D-galactose	+	–
D-mannitol	+	–	Bacitracin resistance	+	+
Salicin	+	+	Amylopectin	–	–
Arginine dihydrolase 1	+	+	Arginine dihydrolase 2	+	+
β-galactopyranosidase	–	–	β- glucuronidase	–	–
α-galactosidase	–	–	D-sorbitol	+	–
Urease	–	–	α-glucosidase	–	–
N-acetyl-D-glucosamine	+	+	D-trehalose	+	+
D-mannose	+	+	methyl-B-D-glucopyranoside	+	+
β-galactosidase	–	–	Polymyxin B	+	–
α-mannosidase	–	–	L-pyrrolidone arylaminase	+	–
Hydrophobicity (%)[a]	66.09 ± 3.08	56.37 ± 0.21			

γ = Growth, but not hemolytic.
[a] Data are presented as mean ± SE (n = 3).

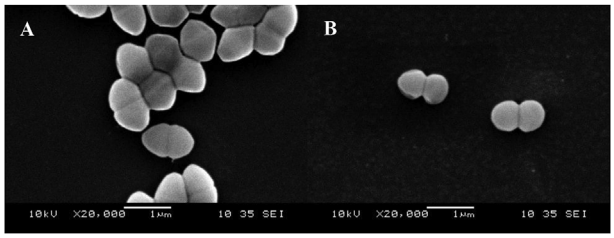

Fig. 2. Scanning electron micrograph of Y17 (A) and G11 (B).

pathogens or stresses [34]. LAB with antagonistic activity has been commonly applied as probiotics for human, mammals and aquatic animals [22,35–39], but the influences of LAB in growth performance and immune responses of mud crabs are still limited. In the present study, a total of 68 bacterial strains were isolated from the intestine of survived mud crab challenged with *V. parahemolyticus* and from the pond water using a selective medium for LAB, MRS. Based on the antimicrobial activity assay, two strains identified as *E. faecalis* Y17 and *P. pentosaceus* G11 were selected for further studies.

Bacteria could be able to produce variety of antibacterial substances with a wide range of activities. The antagonistic activity of lactic acid bacteria (LAB) is due to antimicrobial compounds produced by the bacteria such as lactic acid, hydrogen peroxide (H_2O_2), diacetyl, reuterin, and bacteriocin [40,41]. Lactic acid can disrupt the out membrane of bacteria that causes lysis, whereas bacteriocins are antimicrobial proteins or peptides synthesized by ribosomes [42] that initiate pore formation on the bacterial membrane [43]. In this study, the neutralized-cell free supernatant of both Y17 and G11 did not exhibit a clear zone around the well against *V. parahaemolyticus* (Table 1). This finding indicated that the neutralized-cell free supernatant did not contain an antimicrobial substance such as bacteriocin, which was consistent with the results of stain *Aerococcus* NJ-20 [43]. Therefore, lactic or acetic acid inhibitory substances [41] might be responsible for controlling or inhibiting the mud crab pathogen bacteria, *V. parahaemolyticus*. However, the detailed inhibition mechanism remains unclear and needs further investigations.

According to FAO/WHO, probiotic microorganisms must be non-pathogenic and non-toxic and survived in the target niche where it exerts beneficial effects [44]. The absence of hemolytic activity in a bacterial strain that is considered as a safety prerequisite for the selection of a potential probiotic strain [45]. In this study, the hemolytic activities were negative for both Y17 and G11 strains, indicating that these strains were suitable as probiotics for application in mud crab, which was consistent with the results of a previous study [46]. It is reported that most of the antibiotic-resistant genes are plasmid mediated, which could be horizontally transferred from probiotic organisms to the pathogens [47,48]. Our findings revealed that the G11 strain was not resistant to most antibiotics tested, while Y17 was resistant to 9/19 kinds of tested antibiotics, indicating that G11 is more effective than Y17. The results showed that both Y17 and G11 strains were not pathogens causing neither clinical syndrome nor mortality in the mud crabs exposed to a concentration of 10^7 *cfu* mL^{-1} by immersion compared to the controls (data not shown). Taken together, both Y17 and G11 strains may be used as potential probiotics in mud crab aquaculture.

It is known that the ability to adhere to the epithelial cells is one of the main criteria for selecting probiotic strains [49]. In this regard, the isolated strains (Y17 and G11) displayed better hydrophobicity values in comparison to that in the results of previous reports [28], indicating the potential adhere capacity of both Y17 and G11 to the intestinal epithelial cells. Furthermore, the tolerance to acid is also important for the colonization and growth of microorganisms in the gut of the host [50]. However, there is still no consensus about the precise concentration that is accordant for the selected strains [51]. The results of this study showed that both LAB strains were tolerant of high acidic (*e.g.*, at pH 2.0), which was consistent with the case of *Lactococcus lactis*, isolated from the intestinal tract of marine fish (*Paralichthys olivaceus*

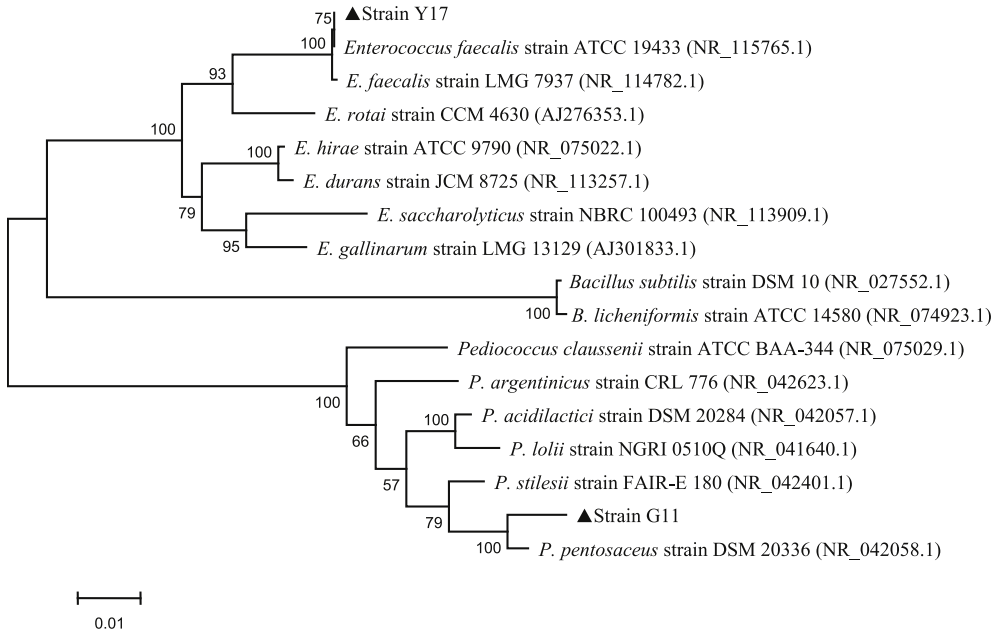

Fig. 3. Phylogenetic analysis using the Neighbor-Joining method based on 16S rRNA gene sequences of Y17 and G11.

Table 3
pH tolerance of Y17 and G11 strains.

pH	Survival rate (%)[a]	
	Y17	G11
2.0	17.10 ± 0.01	2.45 ± 0.01
3.0	20.26 ± 0.01	7.16 ± 0.01
4.0	20.13 ± 0.01	11.24 ± 0.02
5.0	17.95 ± 0.01	94.55 ± 0.02
7.0	89.39 ± 0.01	100.00 ± 0.01
9.0	100.00 ± 0.01	49.89 ± 0.03
10.0	71.74 ± 0.04	17.08 ± 0.01

[a] Data are presented as mean ± SE (n = 3).

Table 4
Antibiotic susceptibilities of Y17 and G11 strains.

Antibiotic	Y17		G11	
	Inhibition zone diameter (mm)[a]	Susceptibility	Inhibition zone diameter (mm)[a]	Susceptibility
Ampicillin	29.00	S	33.33	S
Amikacin	0	R	24.00	S
Amoxicillin acid	15.67	S	20.33	S
Cefazolin	13.67	I	24.67	S
Ceftazidime	0	R	13.00	R
Cefuroxime	0	R	29.00	S
Chloramphenicol	21.67	S	25.33	S
Enrofloxacin	15.33	S	25.67	S
Erythromycin	11.00	I	24.67	S
Gentamicin	0	R	23.00	S
Midecamycin	0	R	21.00	S
Minocycline	29.33	S	37.33	S
Neomycin	0	R	20.00	S
Oxacillin	0	R	20.00	S
Penicillin	10.33	I	28.33	S
Polymyxin B	0	R	16.67	S
Sulfamethoxazde tablets	0	R	0	R
Tetracycline	25.00	S	27.00	S
Vancomycin	17.67	S	16.00	S

S: sensitive; I: moderately sensitive; R: resistance.
[a] Average number of three measurements (n = 3).

and *Takifugu niphobles*) [19,52]. Previously, it has been discussed that the potential probiotic strains with high hydrophobicity and less sensitive to acid seems to be survived through the gastrointestinal tract and potentially colonized in the intestinal surfaces [50]. Therefore, both Y17 and G11 strains may be considered to be candidate probiotic supplements in mud crabs. Moreover, dietary supplementation with G11 improved the weight gain and SGR of mud crab, which was consistent with the case of giant river prawn (*Macrobrachium rosenbergii*) inoculated with *Lactobacillus* [53]. An application of LAB as probiotics effectively involved in the growth enhancement has been previously reported in several finfish and shellfish species [22,54–56]. The previous studies have showed that LAB was capable of increasing the activities of digestive enzymes and voluntary feed intake, changing the production of metabolites, stimulating hydrolysis of non-digestible substrates, as well as inducing adaptive responses in the intestinal morphology [54,57].

Both Y17 and G11 strains increased the disease resistance in mud crabs against a challenge by *V. parahemolyticus*, showing a survival rate of 66.67% and 80.00% in Y17 + and G11 + *V. parahaemolyticus* groups, respectively. The role of LAB in protecting mud crabs against bacterial infection is possibly explained by the strengthening the immunity system along with the enhancing growth performance, as well as the better regulation of immune responses antagonizing the pathogens in the LAB-fed mud crabs. Previous studies have shown that a probiotic-supplemented diet is able to regulate both cellular and humoral immune functions of the immunized animals enhancing resistance against invading pathogens [58,59]. In this study, the serum enzyme activities and immune-related gene expression profiles in the hemocytes and hepatopancreas of LAB-fed mud crabs were investigated in order to evaluate the immune responses of mud crabs after *V. parahaemolyticus* infection. T-SOD catalyzes the dismutation of the highly reactive O_2^- to the less reactive H_2O_2 and belongs to the main antioxidant defense pathways in response to oxidative stresses [60]. It has been reported that the CAT/SOD activity or their gene expression increases parallel to immune-stimulant challenges or pathogen infections in crustaceans [61]. In this study, LAB significantly improved serum T-SOD activities and Cu/Zn-SOD gene expression in both hemocytes and hepatopancreas of mud crab after an infection with *V.*

Table 5
Growth performance of mud crab in Y17- and G11-supplemented groups.

Index	Controls	Y17-supplemented groups	G11-supplemented groups
Final carapace width (cm)	6.07 ± 0.07[a]	6.47 ± 0.14[ab]	6.83 ± 0.17[b]
Final body weight (g)	43.63 ± 1.07[a]	51.60 ± 3.64[ab]	54.23 ± 2.21[b]
Weight gain (g)	37.57 ± 1.01[a]	45.13 ± 3.49[ab]	47.40 ± 2.15[b]
Specific growth rate (%·d^{-1})	1.04 ± 0.06[a]	1.43 ± 0.17[ab]	1.55 ± 0.10[b]

Values (mean ± SE) were from three groups of the crabs, with different superscripts in rows denoting significantly different ($P < 0.05$).

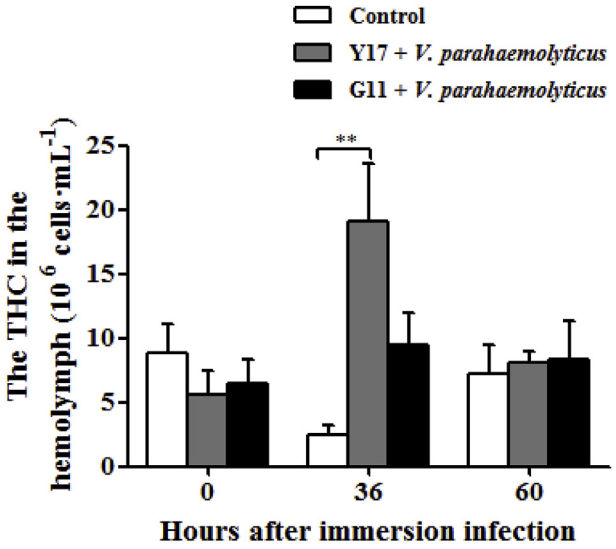

Fig. 4. The total haemocyte cell counts (THC) in the hemolymph. The total haemocyte cell counts in the hemolymph after 0, 36, 60 h after *V. parahaemolyticus* immersion infection ($n = 3$). Asterisks indicate the significant differences (**$P < 0.01$).

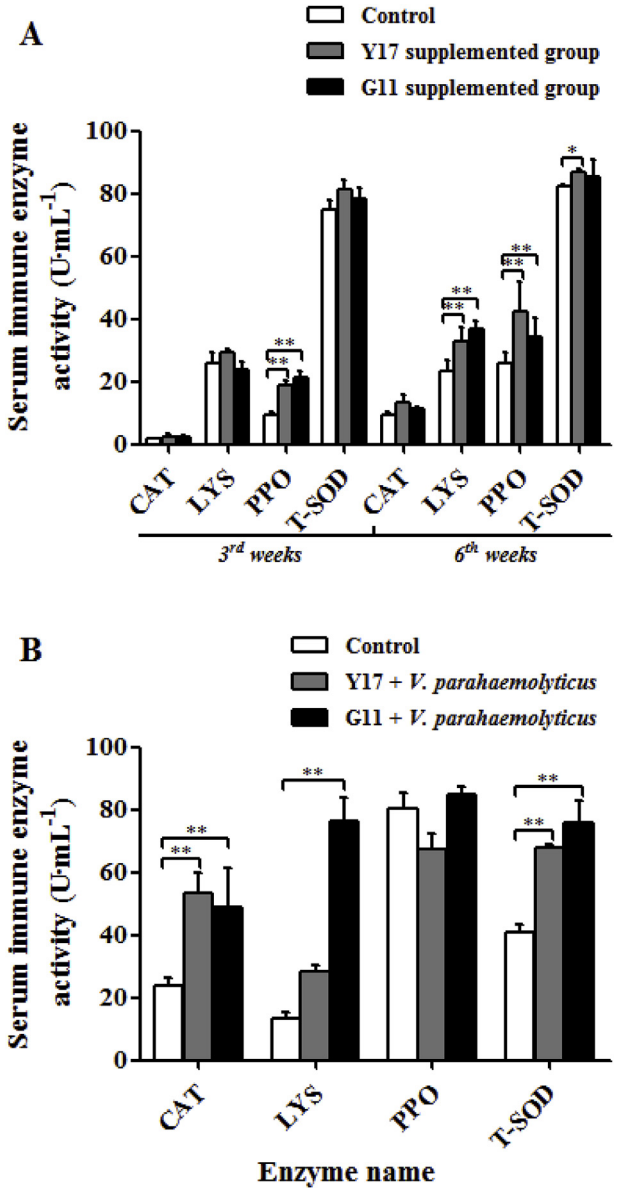

Fig. 5. Serum enzyme activities in the hemolymph. Serum enzyme activities in mud crabs in LAB-fed period of a third/sixth week (A) and after 36 h after *V. parahaemolyticus* immersion infection (B) ($n = 3$). Asterisks indicate the significant differences (*$P < 0.05$ and **$P < 0.01$).

parahaemolyticus.

It has been showed that the host is invaded by a pathogen, an immediate response in the cellular defense is probably caused by melanization, which is the stimulation result of proPO system. Therefore, the proPO-activating system is an important defense mechanism in invertebrates against diverse pathogens [62]. The previous studies reported that up-regulated transcription of proPO resulted in increased PO activity in shrimp fed with *Lactobacillus plantarum* supplemented diet, which enhanced the resistance against the pathogenic *V. alginolyticus* [63], it is also reported increased expression of proPO in mud crab with *Bacillus subtilis* or *Bacillus pumilus* supplemented diets after infection with *V. parahemolyticus* [28], similar resulted was founded in our research, the expression levels of proPO is significantly increased fed with supplement G11 after immersion infection with *V. parahemolyticus*.

The absence of lysozyme led to a decrease in hemocyte counts, increased bacterial counts in the hemolymph, and loss of lytic activity, the bacterial profile in lysozyme-deficient shrimp showed a proliferation of Gram-negative bacteria [64]. In this study, the serum LYS enzyme activity with feed added Y17 or G11 is more higher than the control group, and the expression levels of LYS is significantly increased fed with supplement G11 after immersion infection with *V. parahemolyticus,* the serum activity and expression levels of CAT, LYS and proPO in the LAB-fed mud crabs were higher than that in the control groups after a bacterial challenge, it seem that with added probiotics to the feed would produce a better immune response against pathogens infection, it suggests the major roles of probiotics in strengthening mud crab immune system, although most of these underlying mechanisms remain unclear, the findings could provide a sounding ground for future application of probiotics against *V. parahaemolyticus* infection in crab aquaculture.

5. Conclusions

In conclusion, the two LAB strains, *E. faecalis* Y17 and *P. pentosaceus* G11, isolated from the intestine of survived mud crab after challenged with *V. parahaemolyticus*, may be used as potential probiotics in mud crab. The results of this study revealed that the dietary supplementation of Y17 and G11 improved the growth rate and the immune response of

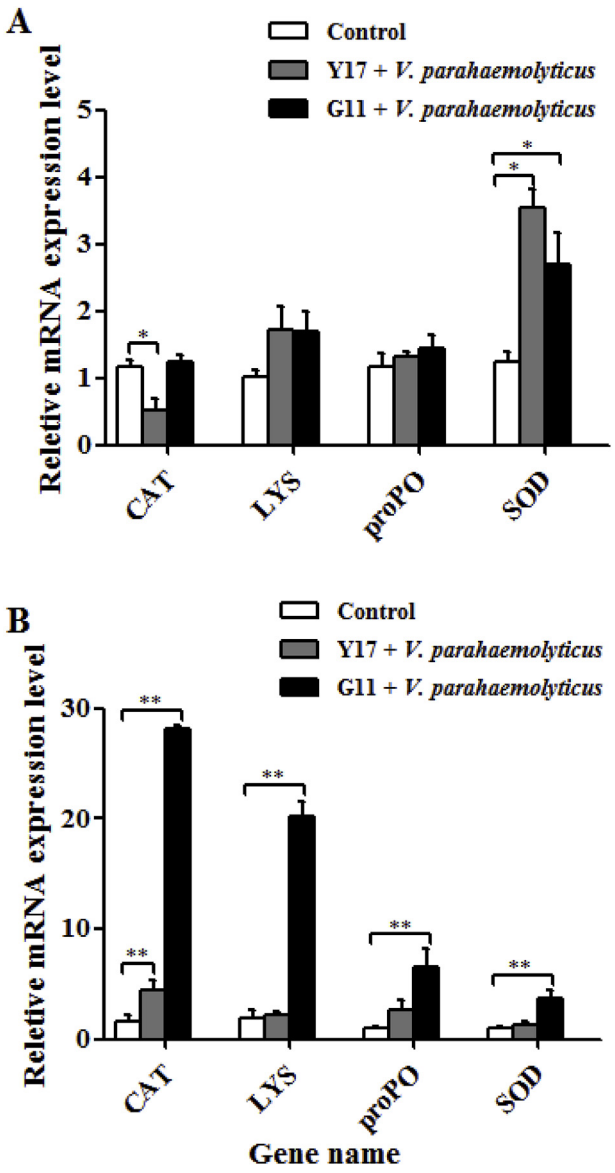

Fig. 6. Relative expressions of immune-related genes. Expressions of immune-related genes of hemocytes (A) and hepatopancreas (B) in Y17 and G11-supplemented groups after 36 h after *V. parahaemolyticus* immersion infection ($n = 3$). Asterisks indicate the significant differences ($^{*}P < 0.05$ and $^{**}P < 0.01$).

mud crab. To our knowledge, this is the first report demonstrating an increased protection against *V. parahaemolyticus* infection by indigenous intestinal LAB bacteria in mud crab. The findings of this study provide a better information of the future application of the LAB strains as probiotics in mud crab aquaculture.

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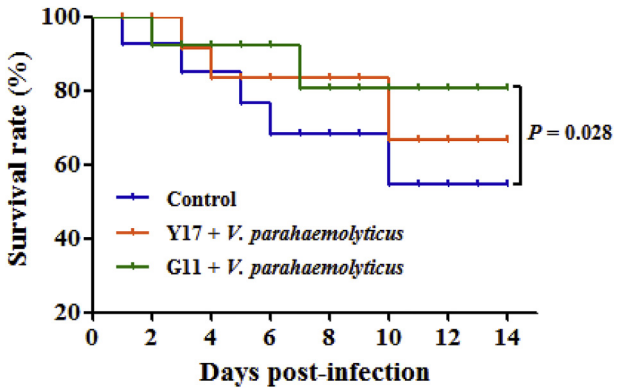

Fig. 7. The survival rates with probiotic-supplemented diets. After the pathogenicity test (exposure to *V. parahaemolyticus* (1.38×10^6 *cfu* mL^{-1}) for 12 h), the survival rate of Y17 + and G11 + *V. parahaemolyticus* groups was 66.67% and 80.00%, respectively, compared with 53.33% for the control groups.

References

[1] J. Zhao, X.B. Wen, S.K. Li, D.S. Zhu, Y.Y. Li, Effects of dietary lipid levels on growth, feed utilization, body composition and antioxidants of juvenile mud crab *Scylla paramamosain* (Estampador), Aquaculture 435 (2015) 200–206.
[2] Bureau of Fisheries, Ministry of Agriculture of the People's Republic of China, China Fishery Statistical Yearbook, China agriculture press, 2017, p. P22.
[3] Y.Y. Li, X.A. Xia, Q.Y. Wu, W.H. Liu, Y.S. Lin, Infection with *Hematodinium* sp. in mud crabs *Scylla serrata* cultured in low salinity water in southern China, Dis. Aquat. Org. 82 (2008) 145–150.
[4] Z. Zhang, S.K. Li, C.P. Xie, L.Z. Zhou, C.B. Li, W.H. Liu, X.B. Wen, Innate immune response and gene expression of *Scylla paramamosain* under *Vibrio parahaemolyticus* infection, Aquacult. Res. 46 (2015) 462–471.
[5] R.M. McPhearson, A. DePaola, S.R. Zywno, M.L. Motes Jr., A.M. Guarino, Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds, Aquaculture 99 (1991) 203–211.
[6] J.L. Balcázar, I.D. Blas, I. Ruiz-Zarzuela, D. Cunningham, D. Vendrell, J.L. Múzquiz, The role of probiotics in aquaculture, Vet. Microbiol. 114 (2006) 173–186.
[7] S. Alonso, M.C. Castro, M. Berdasco, I.G. Banda, X. Moreno-Ventas, A.H. Rojas, Isolation and partial characterization of lactic acid bacteria from the gut microbiota of marine fishes for potential application as probiotics in aquaculture, Probiotics & Antimicro. Prot. (2018), https://doi.org/10.1007/s12602-018-9439- 2.
[8] F.C. Cabello, Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment, Environ. Microbiol. 8 (2006) 1137–1144.
[9] L. Verschuere, G. Rombout, P. Sorgeloos, W. Verstraete, Probiotic bacteria as biological control agents in aquaculture, Microbiol. Mol. Biol. Rev. 64 (2000) 655–671.
[10] D. Kim, B.R. Beck, S.B. Heo, J. Kim, H.D. Kim, S.M. Lee, Y. Kim, S.Y. Oh, K. Lee, H. Do, K. Lee, W.H. Holzapfel, S.K. Song, *Lactococcus lactis* BFE920 activates the innate immune system of olive flounder (*Paralichthys olivaceus*), resulting in protection against *Streptococcus iniae* infection and enhancing feed efficiency and weight gain in large-scale field studies, Fish Shellfish Immunol. 35 (2013) 1585–1590.
[11] Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, (2001).
[12] G. Reid, Safe and efficacious probiotics: what are they? Trends Microbiol. 14 (2006) 348–352.
[13] Z.Z. Qi, X.H. Zhang, N. Boon, P. Bossier, Probiotics in aquaculture of China - currents state, problems and prospect, Aquaculture 290 (2009) 15–21.
[14] S. Saelao, S. Maneerat, K. Thongruck, N. Watthanasakphuban, S. Wiriyagulopas, J.M. Chobert, T. Haertlé, Reduction of tyramine accumulation in Thai fermented shrimp (*kungsom*) by nisin Z-producing *Lactococcus lactis* KTH0-1S as starter culture, Food Control 90 (2018) 249–258.
[15] S. Mohapatra, T. Chakraborty, A.K. Prusty, P. Das, K. Paniprasad, K.N. Mohanta, Use of different microbial probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora, Aquacult. Nutr. 18 (2011) 1–11.
[16] N. Noordiana, A. Fatimah, A.S. Mun, Antibacterial agents produced by lactic acid bacteria isolated from Threadfin salmon and grass shrimps, Int Food Res J 20 (2013) 117–124.
[17] F. Askarian, A. Kousha, W. Salma, E. Ringø, The effect of lactic acid bacteria administration on growth, digestive enzyme activity and gut microbiota in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) fry, Aquacult. Nutr. 17 (2011) 488–497.
[18] A.D. Talpur, A.J. Memon, M.I. Khan, M. Ikhwanuddin, M.M.D. Daniel, A.B. Abol-Munafi, Control of *Vibrio harveyi* infection in blue swimming crab, *Portunus pelagicus*

larvae by the gut isolated lactic acid bacteria under challenge bioassay, Pak. Vet. J. 32 (2012) 408–411.
[19] T.L. Nguyen, C. Park, D. Kim, Improved growth rate and disease resistance in olive flounder, *Paralichthys olivaceus*, by probiotic *Lactococcus lactis* WFLU12 isolated from wild marine fish, Aquaculture 471 (2017) 113–120.
[20] O.B. Braïek, H. Ghomrassi, P. Cremonesi, S. Morandi, Y. Fleury, P.L. Chevalier, K. Hani, O.B. Hadj, T. Ghrairi, Isolation and characterisation of an enterocin P-producing *Enterococcus lactis* strain from a fresh shrimp *Penaeus vannamei*, Antonie Leeuwenhoek 110 (2017) 1–16.
[21] M. Castex, L. Chim, D. Pham, P. Lemaire, N. Wabete, J.L. Nicolas, P. Schmidely, C. Mariojouls, Probiotic *P. acidilactici* application in shrimp *Litopenaeus stylirostris* culture subject to vibriosis in New Caledonia, Aquaculture 275 (2008) 182–193.
[22] J.B. Huang, Y.C. Wu, S.C. Chi, Dietary supplementation of *Pediococcus pentosaceus* enhances innate immunity, physiological health and resistance to *Vibrio anguillarum* in orange-spotted grouper (*Epinephelus coioides*), Fish Shellfish Immunol. 39 (2014) 196–205.
[23] S.K. Li, Z. Zhang, C.B. Li, L.Z. Zhou, W.H. Liu, Y.Y. Li, Molecular cloning and expression profiles of nitric oxide synthase (NOS) in mud crab S*cylla paramamosain*, Fish Shellfish Immunol. 32 (2012) 945–946.
[24] C.M. Ruiz, G. Román, J.L. Sánchez, A marine bacterial strain effective in producing antagonisms of other bacteria, Aquacult. Int. 4 (1996) 289–291.
[25] W. Whitman, M. Goodfellow, P. Kämpfer, H.J. Busse, M. Trujillo, W. Ludwig, K. Suzuki, A. Parte, Bergey's Manual of Systematic Bacteriology, The Actinobacteria, vol. 5, Springer, Berlin, 2011.
[26] J.G. Holt, N.R. Krieg, P.H.A. Snealth, J.T. Staley, Bergey's Manual of Determinative Bacteriology, (1993) Baltimore.
[27] W. Weisburg, S.M. Barns, D.A. Pelletier, D. Lane, 16S ribosomal DNA amplification forphylogenetic study, J. Bacteriol. 173 (1991) 697–703.
[28] H.J. Wu, L.B. Sun, C.B. Li, Z.Z. Li, Z. Zhang, X.B. Wen, Z. Hu, Y.L. Zhang, S.K. Li, Enhancement of the immune response and protection against *Vibrio parahaemolyticus* by indigenous probiotic *Bacillus* strains in mud crab (*Scylla paramamosain*), Fish Shellfish Immunol. 41 (2014) 156–162.
[29] M. Hombach, G.V. Bloemberg, E.C. Böttger, Effects of clinical breakpoint changes in CLSI guidelines 2010/2011 and EuCAST guidelines 2011 on antibiotic susceptibility test reporting of Gram-negative bacilli, J. Antimicrob. Chemother. 67 (2012) 622–632.
[30] V.D. Nguyen, T.T. Pham, T.H. Nguyen, T.T. Nguyen, L. Hoj, Screening of marine bacteria with bacteriocin-like activities and probiotic potential for ornate spiny lobster (*Panulirus ornatus*) juveniles, Fish Shellfish Immunol. 40 (2014) 49–60.
[31] K. Kongnum, T. Hongpattarakere, Effect of *Lactobacillus plantarum* isolated from digestive tract of wild shrimp on growth and survival of white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi*, Fish Shellfish Immunol. 32 (2012) 170–177.
[32] M.S. Kim, E. Min, J.H. Kim, J.K. Koo, J.C. Kang, Growth performance and immunological and antioxidant status of Chinese shrimp, *Fennerpenaeus chinensis* reared in bio-floc culture system using probiotics, Fish Shellfish Immunol. 47 (2015) 141–146.
[33] Y.Z. Sun, H.L. Yang, R.L. Ma, W.Y. Lin, Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*, Fish Shellfish Immunol. 29 (2010) 803–809.
[34] T. Pérez, J.L. Balcázar, I. Ruiz-Zarzuela, N. Halaihel, D. Vendrell, I. Blas, J.L. Múzquiz, Host-microbiota interactions within the fish intestinal ecosystem, Mucosal Immunol. 3 (2010) 355–360.
[35] J.Y. Kim, E.G. Awji, N.H. Park, J.Y. Park, J.C. Kim, S.P. Lee, J.W. Suh, S.C. Park, Probiotic properties and adsorption of *Entercococcus faecalis* PSCT3-7 to vermiculite, J. Equine Vet. Sci. 18 (2017) 95–99.
[36] G. Ladha, K. Jeevaratnam, Probiotic potential of *Pediococcus pentosaceus* LJR1, a bacteriocinogenic strain isolated from rumen liquor of goat (*Capra aegagrus* hircus), Food Biotechnol. 32 (2018) 60–77.
[37] A.K. Mishra, A.R. Ghosh, Characterization of functional, safety, and probiotic properties of *Enterococcus faecalis* AG5 isolated from wistar rat, demonstrating adherence to HCT 116 cells and gastrointestinal survivability, Probiotics Antimicr O 10 (2018) 435–445.
[38] B.R. Beck, D. Kim, J. Jeon, S.M. Lee, H.K. Kim, O.J. Kim, J. Il Lee, B.S. Suh, H.K. Do, K.H. Lee, W.H. Holzapfel, J.Y. Hwang, M.G. Kwon, S.K. Song, The effects of combined dietary probiotics *Lactococcus lactis* BFE920 and *Lactobacillus plantarum* FGL0001 on innate immunity and disease resistance in olive flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 42 (2015) 177–183.
[39] E. Munoz-Atienza, C. Araujo, S. Magadan, P.E. Hernandez, C. Herranz, Y. Santos, L.M. Cintas, *In vitro* and *in vivo* evaluation of lactic acid bacteria of aquatic origin as probiotics for turbot (*Scophthalmus maximus* L.) farming, Fish Shellfish Immunol. 41 (2014) 570–580.
[40] D. Cizeikiene, J. Gražina, A. Paškevičius, E. Bartkiene, Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread, Food Control 31 (2013) 539–545.
[41] J.A. Vázquez, M.P. González, M.A. Murado, Effects of lactic acid bacteria cultures on pathogenic microbiota from fishes, Aquaculture 245 (2005) 1–32.
[42] N. Hwanhlem, J.M. Chobert, A.H. Kittikun, Bacteriocin-producing lactic acid bacteria isolated from mangrove forests in southern Thailand as potential biocontrol agents in food: isolation, screening and optimization, Food Control 41 (2014) 202–211.
[43] T.F. Putra, H. Suprapto, W. Tjahjaningsih, H. Pramono, The antagonistic activity of lactic acid bacteria isolated from *peda*, an Indonesian traditional fermented fish, IOP Conf. Ser. Earth Environ. Sci. 137 (2018) 012060.
[44] L. Morelli, L. Capurso, FAO/WHO guidelines on probiotics, J. Clin. Gastroenterol. 46 (2012) S1–S2.
[45] Food and Agricultural Organization/World Health Organization (FAO/WHO), Guidelines for the evaluation of probiotics in food, Food Agri Organ Ontario (2002) 1–11.
[46] Y. Gao, D. Li, Y. Liu, Probiotic potential of *L. sake* C2 isolated from traditional Chinese fermented cabbage, Eur. Food Res. Technol. 234 (2012) 45–51.
[47] M. Jamuna, R. Kolanchiammal, K. Jeevaratnam, Plasmid-associated bacteriocin production in *Lactobacillus* strains isolated from some traditional fermented foods, Glob. J. Biotechnol. Biochem. 5 (2010) 175–181.
[48] T. Pérez-Sánchez, J.L. Balcázar, Y. García, N. Halaihel, D. Vendrell, I. Blas, D.L. Merrifield, I. Ruiz-Zarzuela, Identification and characterization of lactic acid bacteria isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), with inhibitory activity against *Lactococcus garvieae*, J. Fish Dis. 34 (2011) 499–507.
[49] S. Blum, R. Reniero, E. Schiffrin, R. Crittenden, T. Mattila-Sandholmb, A.C. Ouwehand, S. Salminen, A. von Wright, M. Saarela, M. Saxelin, K. Collins, L. Morelli, Adhesion studies for probiotic: need for validation and refinement, Trends Food Sci. Technol. 10 (1999) 405–410.
[50] S. Nikoskelainen, S. Salminen, G. Bylund, A. Ouwehand, Characterization of the properties of human and dairy-derived probiotics for prevention of infectious diseases in fish, Appl. Environ. Microbiol. 67 (2001) 2430–2435.
[51] C.H. Liu, C.H. Chiu, S.W. Wang, W. Cheng, Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*, Fish Shellfish Immunol. 33 (2012) 699–706.
[52] S. Takanashi, A. Miura, K. Abe, J. Uchida, S. Itoi, H. Sugita, Variations in bile tolerance among *Lactococcus lactis* strains derived from different sources, Folia Microbiol. 59 (2014) 289–293.
[53] H.K. Venkat, N.P. Sahu, K.K. Jain, Effect of feeding *Lactobacillus* based probiotics on the gut microflora, growth and survival of postlarvae of *Macrobrachium rosenbergii* (de Man), Aquacult. Res. 35 (2004) 501–507.
[54] O. Safari, M. Paolucci, H.A. Motlagh, Effects of synbiotics on immunity and disease resistance of narrow-clawed crayfish, *Astacus leptodactylus leptodatylus* (Eschscholtz, 1823), Fish Shellfish Immunol. 64 (2017) 392–400.
[55] A. Neissi, G. Rafiee, M. Nematollahi, O. Safari, The effect of *Pediococcus acidilactici* bacteria used as probiotic supplement on the growth and non-specific immune responses of green terror, *Aequidens rivulatus*, Fish Shellfish Immunol. 35 (2013) 1976–1980.
[56] S.K. Allameh, E. Ringø, F.M. Yusoff, H.M. Daud, A. Ideris, Dietary supplement of *Enterococcous faecalis* on digestive enzyme activities, short-chain fatty acid production, immune system response and disease resistance of Javaneses carp (*Puntinus gonionotus*, Bleeker 1850), Aquacult. Nutr. 23 (2015) 331–338.
[57] S.H. Hoseinifar, A. Mirvaghefi, M.A. Amoozegar, D.L. Merrifield, E. Ringø, In vitro selection of a synbiotic and in vivo evaluation on intestinal microbiota, performance and physiological response of rainbow trout (*Oncorhynchus mykiss*) fingerlings, Aquacult. Nutr. 23 (2017) 111–118.
[58] T. Perez-Sanchez, J.L. Balcazar, D.L. Merrifield, O. Carnevali, G. Gioacchini, I. Blas, I. Ruiz-Zarzuela, Expression of immune-related genes in rain bow trout (*Oncorhynchus mykiss*) indeced by probiotic bacteria during *Lactococcus garvieae* infection, Fish Shellfish Immunl 31 (2011) 196–201.
[59] W.S. Liu, P.F. Ren, S.X. He, L. Xu, Y.L. Yang, Z.M. Gu, Z.G. Zhou, Comparison of adhesive gut bacteria composition, immunity, and disease resistance in juvenile hybrid tilapia fed two different *Lactobacillus* strains, Fish Shellfish Immunl 35 (2013) 54–62.
[60] I. Fridovich, Supperoxide radical and superoxide dismutases, Annu. Rev. Biochem. 64 (1995) 97–112.
[61] K.F. Liu, C.S. Chiu, Y.L. Shiu, W. Cheng, C.H. Liu, Effects of the probiotic, *Bacillus subtilis* E20, on the suivival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannameri* larvae, Fish Shellfish Immunl 28 (2010) 837–844.
[62] K. Soderhall, L. Cerenius, Role of the prophenoloxidase-activating system in invertebrate immunity, Curr. Opin. Immunol. 10 (1998) 23–28.
[63] C.H. Chiu, Y.K. Guu, C.H. Liu, T.M. Pan, W. Cheng, Immune responses and gene expression in white shrimp, Litopenaeus vannamei, induced by Lactobacillus plantarum, Fish Shellfish Immunol. 23 (2007) 364–377.
[64] A. Kaizu, F.F. Fagutao, H. Kondo, T. Aoki, I. Hirono, Functional analysis of C-type lysozyme in Penaeid shrimp, J. Biol. Chem. 286 (2011) 44344–44349.