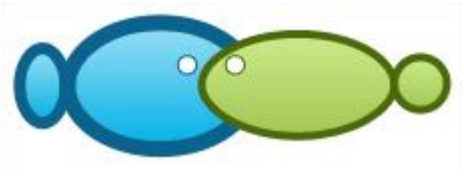


Screening and application of lactic acid bacteria isolated from vanamei shrimp (*Litopenaeus vannamei*) intestine as a probiotic potential for tiger shrimp (*Penaeus monodon*)

Sriwulan, Andi Azwar, Alexander Rantetondok, Hilal Anshary

Laboratory of Fish Parasites and Diseases, Aquaculture Study Program, Department of Fisheries, Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar, Indonesia. Corresponding author: H. Anshary, hilalanshary@gmail.com

Abstract. Bacterial and viral diseases are the main problems in shrimp farming. Vibriosis and white spot disease has been the cause of mass mortality in shrimp in the hatchery and grow-out ponds. One of the possible control measures is by probiotic application of lactic acid bacteria, particularly from indigenous species. The objectives of this study were: 1) to screen lactic acid bacteria (LAB) isolated from vanamei shrimp (*Litopenaeus vannamei*) intestine for their potential antimicrobial activities; 2) characterizing the probiotic candidate and identified molecularly to accurately determine the species; 3) to study the effect of the LAB that was isolated from vanamei shrimp in enhancing the immune system of tiger shrimp (*Penaeus monodon*). Four isolates of LAB were collected, designated as C1, 18K, D3, and 23P. The LAB isolates showed antimicrobial activity against three species of *Vibrio* with an inhibitory zone up to 15 mm, able to live at acidic to basic pH (1.5-7.2), bile salts (3000 ppm), negative catalase, non-motility negative, and Gram-positive. The molecular characterization and phylogenetic tree analysis showed that the isolate of LAB from the gut of vanamei (18K) belongs to *Enterococcus avium*. The application of LAB (18K) in tiger shrimp juvenile through feed could stimulate the cellular immune system of the tiger shrimp after being challenged with *V. harveyi*, indicated by increasing of total haemocyte count (THC) and phagocytosis index, suppressed the population of *Vibrio* in the tiger shrimp and improved survival rate of shrimp. This research proved the importance of indigenous species of lactic acid bacteria to be used as probiotic to combat against bacterial pathogen in shrimp.
Key Words: lactic acid bacteria, probiotic, immune system, *Enterococcus*, tiger shrimp, indigenous.

Introduction. Shrimp culture industry has developed very rapidly in recent years and its role in an economic development has become very important in many countries in the world. Along with these developments, a demand for intensification of cultivation to assure continues production and supply of shrimp are needed. However, impact of shrimp culture intensification also follows, mainly decreasing environmental quality, increasing stress on aquaculture organisms and emergence of various diseases such as bacterial and viral diseases which can reduce shrimp survival and growth, and even mass mortality, which ultimately inhibits shrimp production. Since white spot syndrome virus (WSSV) was discovered in Indonesia, it has caused major problems in the production of tiger shrimp (*Penaeus monodon*) and generally most productive ponds are either left neglected (Sunarto et al 2004) or switched to vannamei shrimp (*Litopenaeus vannamei*, synonym *Penaeus vannamei*) farming. This virus has a very wide range of host mainly crustaceans living in fresh and marine waters, so it can serve as a natural reservoir host (Hossain et al 2001; Vaseeharan et al 2003), consequently it is very difficult to prevent the entry of viruses through the natural host on the cultivation system. Researches on shrimp broodstocks show that the local broodstocks of tiger shrimp in South Sulawesi, Indonesia has been infected with multiple types of viruses: WSSV, MBV, IHHNV, and HPV (Anshary et al 2017). In addition, tiger shrimp seeds and shrimp in grow-out ponds have also been reported to be infected with various types of viruses such as MBV, HPV and IHHNV (Sriwulan & Anshary 2011). Further research shows that the tiger shrimp in South

Sulawesi are infected by infectious and non-infectious type of PstDV-1/IHHNV (Sriwulan & Anshary 2016). This indicates that the virus is still a major obstacle in the development of shrimp farming in this area.

organic matter content in pond's environment can accelerate the development of Bacteria belonging to the genera of *Vibrio*, such as *Vibrio harveyi*, *V. alginolyticus, V. parahaemolyticus, V. ordallii*, and *V. campbelli* which are recognized as the causative agents of vibriosis in hatchery and growth out ponds of shrimp. These bacteria are opportunistic pathogens, which cause problems when their concentrations are abundant. Vibriosis which very often occurs in shrimp hatchery and grow-out ponds can be triggered by various factors. High bacterial content, and on the other hand stress on shrimp due to stressor decrease the host's defense system due to adverse environmental conditions (Lavilla-Pitogo et al 1998; Khamesipour et al 2014). Among *Vibrio* spp. bacteria, *V. harveyi, V. alginolyticus* and *V. parahaemolyticus* are the most commonly found in association with shrimp mortality in both hatcheries and grow-out ponds (Lavilla-Pitogo et al 1990; Lavilla-Pitogo et al 1998; Leano et al 1998; de La Pena et al 2001; Khamesipour et al 2014).

Prevention of diseases occurrence using chemicals and antibiotics have a negative impact on the environment and shrimp. The impact might be in the form of bacterial resistance to antibiotics, accumulation of residues in the environment and muscles of shrimp and disrupting the balance of shrimp intestinal micro flora. Besides, the European Union since 2006 has banned the use of antibiotics (Hernandez-Serrano 2005; Castanon 2007). Therefore, it is necessary to find alternative substitutes for antibiotics that are environmentally friendly and can improve the health of cultivated organisms. Currently the use of probiotic bacteria in improving water quality and in controlling shrimp disease is commonly used (Swain et al 2009; Senthong et al 2012; Reyes-Becerril et al 2014; Sha et al 2016). Lactic acid bacteria (LAB) are normal bacteria that live in the gastrointestinal tract in various aquatic animals. LAB is a Gram positive cocci or rod shaped, which is catalase negative and non motility (Sugimura et al 2011; Senthong et al 2012; Arokiyaraj et al 2014). LAB is a beneficial microbial species for improving survival and improving the immune system (Nair & Surendran 2005). LAB plays an important role in the host digestive tract through enhancing the immune system, modulating bacterial communities and producing antibacterial substances that inhibit the growth of opportunistic pathogens such as lactic acid, acetic acid, and bacteriocin (Ige 2013; Maeda et al 2014; Yousefian & Amiri 2009). Colonization of LAB in the gut serves as a probiotic, which is beneficial to host health, preventing harmful bacteria (Buntin et al 2008).

This research isolated LAB as indigeneous probiotics which could overcome vibriosis and white spot disease in hatchery and ponds of tiger shrimp so that diseases problems commonly occurred in the shrimp farming industry can be overcome. The research objectives were: 1. to isolate and screen of LAB from vanamei shrimp intestine/gut as probiotic candidate, 2. to molecularly identify the LAB, and 3. to study the effect LAB on the immune system and survival rate of tiger shrimp.

Material and Method

Isolation of lactic acid bacteria (LAB) from vanamei shrimp intestine. These researches were conducted on March to September 2016 for LAB screening, and experimental application of the LAB in tiger shrimp was done on June to September 2017. LAB was isolated from intestine of vanamei shrimp that were obtained from shrimp ponds. A total of 30 shrimp that have been cultivated for about three months was obtained from grow out ponds located in Pangkajene Kepulauan Regency (Pangkep), South Sulawesi. The shrimp was transported alive to the Laboratory of Fish Parasites and Diseases, Hasanuddin University. For LAB isolation, shrimp was anesthetized and the surface of the shrimp was wiped with alcohol to clean the shrimp from contaminants, dissected and the intestine was removed and weighed. The intestine was grinded and homogenized in physiological saline solution NaCl 0.85%. The bacteria from the vanamei shrimp intestine was aseptically isolated and grown on deMan Rogosa Sharpe Agar (MRSA) medium, then incubated at 37°C for 24 hours. All colonies grew on the media and showed different morphology were separated, and then re-grown on a medium GYPA

+ $CaCO_3$, the colonies showing clear zones were re-isolated on the MRSA medium, several times to get pure isolate. *Vibrio* spp. was isolated from diseased shrimp larvae obtained from small scale hatchery located in Pinrang District.

Screening of LAB isolate as probiotic candidate. Screening of LAB isolate as probiotic candidate was based on Gram staining, catalase and motility, antibacterial activitiy, tolerance test to pH 1.5, 3, and 7.2, and bile salt tolerance tests at 3000 ppm. Antibacterial activity test was performed using agar diffusion method. About 100 µL of *Vibrio* sp. were inoculated into solid TSA media in a petri dish and spread evenly using a cotton bud. LAB isolates were grown in MRSB and were incubated for 48 hours in the incubator shaker, then centrifuged for 15 minutes at 6000 rpm, the supernatant was filtered using Syringe Filters Sterile 0.2 µm, and rinsed with saline solution twice. Then 50 µL supernatant was dropped on a paper disc and left to air dry for 15 minutes. After drying, it is then placed on solid TSA media that has previously grown *Vibrio* bacteria on its surface. The media was incubated at 37^{o}C for 24 hours. Fifty (50) µL of Ampicillin antibiotic was used as a positive control. LAB which produces antibacterial compounds and inhibit the growth of *Vibrio* sp. will appear as a clear zone around the paper disc.

The resistance of LAB to acidic conditions was measured as follows: LAB isolates were grown on MRSB media for 24 hours at 37^{o}C in the incubator shaker. Then as much as 1 mL of the grown bacteria was distributed to each test tube containing 9 mL PBS with different pH: 1.50; 3.0; and 7.2, then incubated for 3 hours. To measure the concentration of LAB, serial dilution technique was performed and 100 µL the dilution was poured and spread on MRSA media. The number of colonies grew on the MRSA media was counted after 24 hours incubation at 37^{o}C.

LAB resistance test on bile salts was carried out by growing the LAB isolates on MRSB medium containing bile salt with a concentration of 3000 ppm, incubated for 24 hours at 37^{o}C, and the number of bacterial colonies that grew on the medium was calculated after 24 hours incubation. LAB growth was carried out by growing isolates on TSB media. Then the culture is incubated for 24, 48, and 72 hours at 37^{o}C. The isolate growth was measured by OD (optical density) at λ = 600 nm.

Molecular identification of LAB (polymerase chain reaction and sequencing). The LAB isolates selected as probiotic candidates were identified molecularly to determine its species. DNA extraction of the LAB bacteria was performed using GENAID DNA Extraction Kit, following the procedure of the kit. Polymerase chain reaction (PCR) of the LAB was performed using the Universal Primers 16S rRNA which amplifies about 1.5 kb length of nucleotides. PCR product of the bacterial DNA was sent to 1st BASE (a commercial company) through PT Genetica Science Indonesia for sequencing. The results of sequencing were analyzed using Bioedit software. The nucleotides of LAB were analyzed using the Basic Local Alignment Search Tool (BLAST) to determine the homology of the LAB nucleotides with data available in the Gen Bank. A phylogenetic tree based on 16S rRNA genes was constructed to determinate the closest bacterial species with the present isolate by the neighbour-joining method (Saitou & Nei 1987), using MEGA version 6 (Tamura et al 2013).

Effect of LAB on tiger shrimp immune system. Experimental animals were juvenile tiger shrimp with an average weight of 5 grams obtained from a nursery pond. The number of bucket used for the experiment was 12 pieces, which consist of 4 treatments with 3 replications; with a volume capacity of each bucket was 50 L. The buckets were filled with 30 L of treated sea water with a salinity of 30 ppt, supplemented with aeration in each bucket. Shrimp juveniles were maintained with a stocking density of 6 shrimp in each bucket, and maintained for 14 days.

The treatments in this study were: treatment A (control - without probiotics); treatment B (lactic acid bacterium (18K) - isolated from vannamei shrimp intestine in this study), treatment C (lactic acid bacterium (UW7) - isolated previously from tiger shrimp intestine) and treatment D (commercial probiotic - *Bacillus* sp.). The feed used was commercial feed (Charoen Pokphand) applied with a dose of 5% of the weight of biomass

with the frequency of feeding 4 times a day, administered at 6:00 a.m., 10:00 a.m., 2:00 p.m. and 14:00 p.m. LAB was applied to the feed (5 mL g^{-1} feed) by mixing the feed and the LAB using vegetable oil (0.01 mL oil g^{-1} feed) as an adhesive, dried in a sterile room for 24 hours. Preparation of feeds containing probiotics was done every 5 days. After 14 days application of the feed containing probiotics in the shrimp, the shrimp was challenged with *Vibrio harveyi* by intramuscular injection. A total of 0.1 mL of bacteria with a density of $1x10^7$ CFU mL^{-1} was injected into shrimp. Ten hours after the challenge test, an analysis of total haemocytes (THC), phagocytic activity, *Vibrio* and LAB density, and survival of tiger shrimp were carried out.

Total haemocyte count (THC). About 1 mL of the shrimp haemolymph was taken at the base of the pleopod in the abdominal segment using a 1 mL syringe. Fifty (50) µL of the haemolymph was added to a sterile micro tube which contained 50 µL of 0.45 M NaCl and mixed gently using micropipette. Subsequently, 10 µL of the mixture was placed on haemocytometer to calculate the total haemocyte count (THC).

Phagocytic index. Measurement of phagocytosis index in haemocytes refers to Amlacher (1970). Briefly, *V. harveyi* cultured on TSA and incubated at 30^{o}C for 24 hours was harvested, and then killed with 2% formalin for 24 hours. *V. harveyi* was washed in PBS 3 times. The density of *V. harveyi* was estimated by the total plate count (TPC). Micro tube containing 0.45 M NaCl was filled with haemocytes and centrifuged. Then, 100 µL of the haemocytes was put in a new micro tube, and added with *V. harveyi* (density of 10^8 cells mL^{-1}) with the same volume. *V. harveyi* was mixed gently with the haemocytes by pipetting and incubated for 20 minutes. About 5 µL of the mixture was taken and placed on an object glass to make smear preparation, and left to air dry. The smear was fixed with methanol for 5 minutes and allowed to air dry. The preparations were stained with Giemsa staining solution for 10 minutes and observed under a light microscope.

Density of Vibrio and LAB. The density of *Vibrio* and LAB was calculated using TPC method. Hepatopancreas and intestine of tiger shrimp sample was first grinded in a micro tube. About 0.1 g of the sample was taken and added to a tube containing 0.9 mL sterile phosphate buffer saline (PBS). Then a serial dilution was carried out until 10^3 series dilutions. A volume of 0.1 mL of the sample from the dilution was spread on TCBSA and MRSA media. The number of colonies grew on the media was calculated after incubation for 24 hours at 37^{o}C. The density of *Vibrio* and LAB was calculated using the formula of Buller (2004).

Survival rate (SR). The survival rate of tiger juvenile tiger shrimp was calculated after 10 hours of challenging tests using the formula as follows:

Survival rate (%) = no of live shrimp at harvest x 100 / no of shrimp at initial stocking.

Statistical analysis. Data on several parameters of the LAB such as antimicrobial activity, tolerance to acidic pH and bile salt, and PCR-sequencing were analyzed descriptively. Data on THC, phagocytosis, *Vibrio* and LAB density and survival rate of shrimp were analyzed using ANOVA.

Results. A number of 27 isolates of lactic acid bacteria from vanamei shrimp intestine that grew on MRSA media and appeared as white colonies was selected (Table 1 and Figure 1). All of the isolates were then re-grown on GYPA + $CaCO_3$ media. Ten out of the 27 isolates that showed clear zones on the GYPA+$CaCO_3$ medium were selected (Table 2). The 10 isolates were re-selected based on their colonies and finally 4 isolates were chosen (Figure 2). The four isolates were coded C1, D3, 18K, and 23P.

Table 1

The morphological of LAB from vanamei shrimp intestine on MRSA media

Sample code	*Color*	*Forms*	*Elevation*
A1,2,3,4	White milk	Round	Convex
B1,2,3	White milk	Round	Convex
C1,2	White milk	Round	Convex
D1,2,3	White milk	Round	Convex
E1,2,3	White milk	Round	Convex, flat
F1	White milk	Round	Flat
G1	White milk	Round	Convex
H1	White milk	Round	Convex
I1	White milk	Round	Flat
J1	White milk	Round	Convex
18K	White milk	Round	Flat
L1	White milk	Round	Flat
M1	White milk	Round	Convex
N1	White milk	Round	Convex
O1	White milk	Round	Convex
23P	White milk	Round	Convex
Q1	White milk	Round	Convex

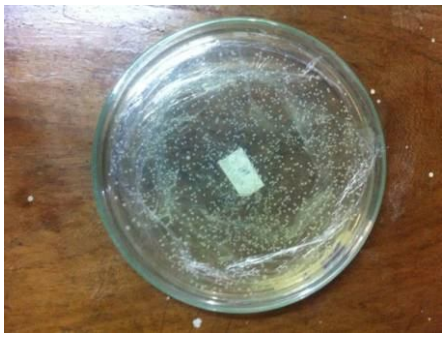


Figure 1. LAB colony on MRSA medium.

Table 2

Clear zone observation on GYPA + $CaCO_3$ media

Code sample	*Clear zone*	*Code sample*	*Clear zone*
A1,2,3,4	-	J1	+
B1,2,3	-	18K	+
C1	+	L1	-
D3	+	M1	-
E1	+	N1	-
F1	-	O1	+
G1	+	23P	+
H1	+	Q1	-
I1	+		

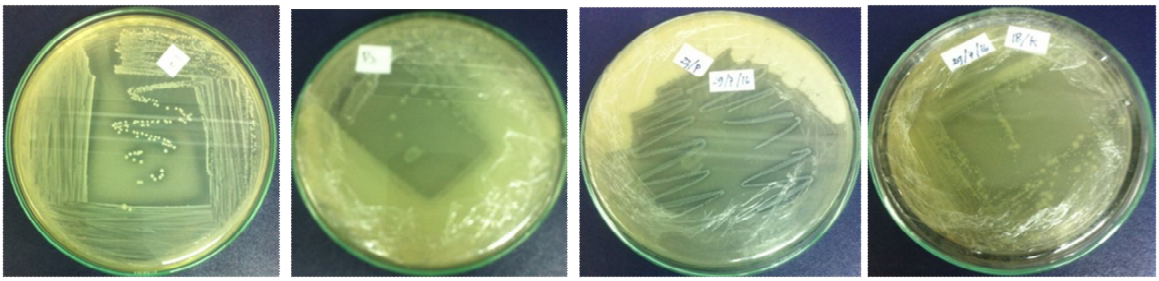


Figure 2. Colony of LAB from vaname intestine (C1, D3, 18K, 23P) on GYPA + $CaCO_3$ medium showed clear zone around the colony.

Selection of LAB as a probiotic candidate

The pH tolerance test. Gram staining on the LAB C1, D3, 18K, and 23P isolates showed that all of them were Gram positive (Figure 3). pH tolerance test is one of the tests for selection of LAB as a probiotic candidate. The results of the resistance test against pH 1.5, 3, and 7.2 (Table 3) showed that the LAB isolate was able to grow at pH 1.5 - 7.2 after 24 hours incubation. One of the characteristics commonly used to select for probiotic candidates was that the isolates must be able to withstand acid conditions in the stomach of shrimp, when applied through feed. Bacterial candidates used as probiotics should be able to survive under low pH conditions and under high or alkaline pH conditions (Minh 2014).

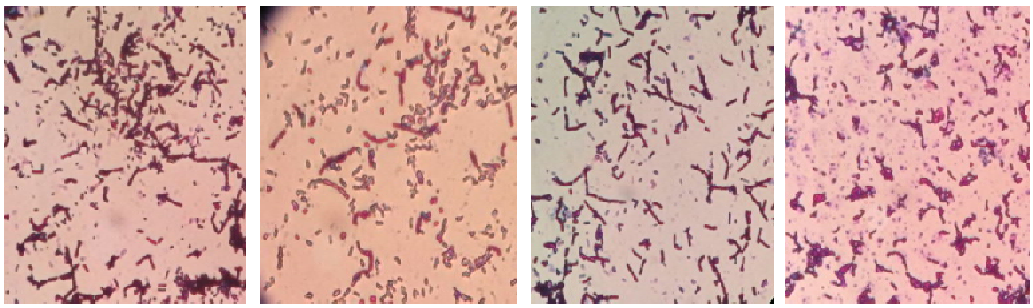


Figure 3. Gram staining of the LABs shows that all are Gram positive (100 X).

Table 3

Density of LAB isolate on tolerance pH test

No.	*Isolate code*	*pH 1.5 ± SD*	*pH 3 ± SD*	*pH 7.2 ± SD*
1	C1	$0.3 \times 10^3 \pm 0.6 \times 10^3$	$4.3 \times 10^3 \pm 3.9 \times 10^3$	$\geq 3.0 \times 10^5 \pm 0$
2	18K	0 ± 0	$5.4 \times 10^4 \pm 3.6 \times 10^4$	$\geq 3.0 \times 10^5 \pm 0$
3	D3	$0.3 \times 10^3 \pm 0.6 \times 10^3$	$3.3 \times 10^4 \pm 1.9 \times 10^4$	$\geq 3.0 \times 10^5 \pm 0$
4	23P	0 ± 0	$4.3 \times 10^3 \pm 3.6 \times 10^3$	$\geq 3.0 \times 10^5 \pm 0$

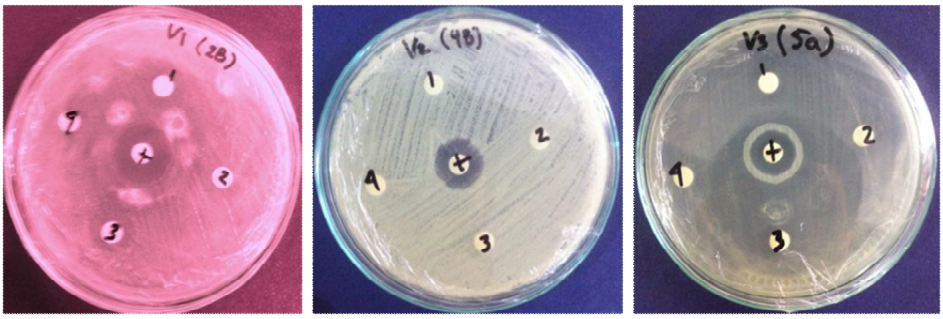
Antimicrobial activity test of LAB. The ability of LAB to inhibit the growth of *Vibrio* was determined through inhibitory test. The results showed that the LAB had antimicrobial substances indicated by the clear zone around the discs of about 7 mm to 15 mm length (Table 4), which indicated inhibition of the growth of *Vibrio* spp. Positive control used was antibiotic Ampicillin (Figure 4).

Table 4

Antimicrobial activity of the LAB to *Vibrio* after 48 hours incubation

LAB isolate	*Inhibition zone V1 (mm)*	*Inhibition zone V2 (mm)*	*Inhibition zone V3 (mm)*
C1	14	7.5	13
18K	15	7	12
23P	15	7.5	11
D3	15	7.5	10
Ampicillin (Control +)	20	17	15

Note: V1, V2, V3 are *Vibrio* spp. bacteria.



A B C

Figure 4. Antimicrobial activity test of the 4 LAB and Ampicillin (+) against 3 isolates of *Vibrio* spp. (V1, V2, and V3).

The results showed different clear zone length of the LAB against 3 species of *Vibrio.* The largest clear zone was found on the LAB D3, 18K, and 23P which have 15 mm clear zone length against *Vibrio* sp1. This difference is probably due to the resistance levels of the *Vibrio* species to the LAB and the potential inhibitory effect of the LAB. Pelczar & Chan (1998) stated that secondary metabolites (antimicrobials) are produced by microorganisms at the end of the stationary phase of growth. This is because secondary metabolites are usually synthesized at the end of the cell growth cycle, i.e. in the stationary phase when the population remains constant because the number of cells that grow is equal to the number of dead cells. The secondary metabolites in question might be bacteriocins that are able to inhibit the growth of Gram-positive and/or Gram-negative bacteria. Generally, bacterial inhibition zones are usually caused by several factors, either single or combination factors such as antibiotic production, bacteriocin, siderophores, lysozyme, proteases and/or hydrogen peroxide (Verschuere et al 2000).

Test of resistance to bile salts. The result of LAB resistance test against bile salt at the concentration of 3000 ppm after 24 hours incubation showed that all isolates of the LAB were able to survive on bile salt (Table 5). This indicates that LAB can be utilized as probiotic with oral application through feed.

Table 5

LABs isolate resistance against bile salt 3000 ppm

Isolate code	*Colony number (mean±SD*
C1	$2.3x10^2 \pm 1.3x10^2$
18K	$1.6x10^3 \pm 0.6x10^3$
D3	$1.4x10^3 \pm 0.3x10^3$
23P	$1.4x10^3 \pm 0.3x10^3$

Total haemocyte count. Total haemocyte in tiger shrimp after administration of LAB showed that feed containing LAB as in treatment B (18K probiotics) and treatment D (commercial probiotics) was significantly higher than treatment C (probiotics UW7) and treatment A (control), while treatment C was not different from treatment A, and treatment B was not different with treatment D (Figure 5). This shows that administration of the LAB probiotics, especially 18K, was able to modulate THC increase in tiger shrimp after being challenged with *V. harveyi*, while it was no seen in the treatments without probiotics supplement.

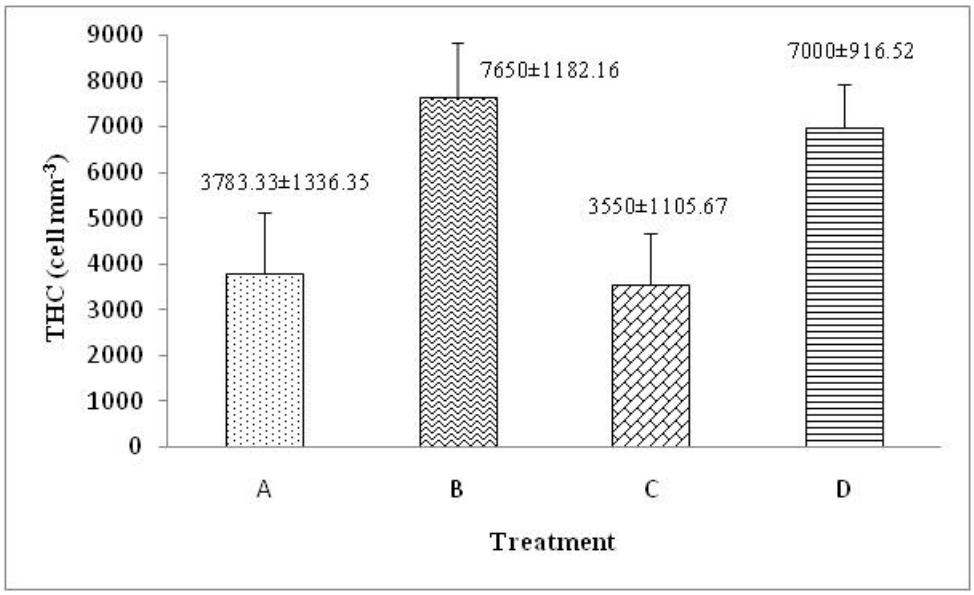


Figure 5. Total haemocyt count in hemolymph of tiger shrimp treated with probiotics after challenge test with *V. harveyi*.

Phagocytosis index. The effect of probiotics on tiger shrimp hemolymph phagocytosis index showed that treatments B (18K probiotics), C (UW7 probiotics), and D (commercial probiotics) were significantly higher than that seen in the treatment A (control) (Figure 6), indicating that the administration of probiotics could increase the phagocytosis index of tiger shrimp.

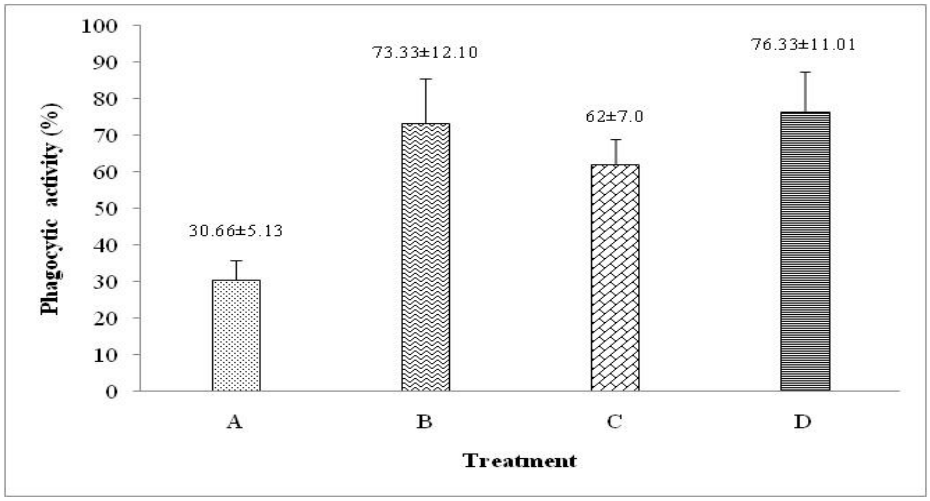


Figure 6. Phagocytosis index in hemolymph of tiger shrimp treated with probiotics after challenge test with *V. harveyi*.

Survival rate. Shrimp survival of shrimp after being challenged with the bacterium *V. harveyi* showed that treatment A (control) was the same as treatment C (UW7 probiotics) in terms that they have lower survival rate, whereas the treatment B (18K probiotics) and D (commercial probiotics) have significantly higher survival rate than the treatments A and C (Figure 7). This finding shows that the 18K probiotics improve the survival of tiger shrimp.

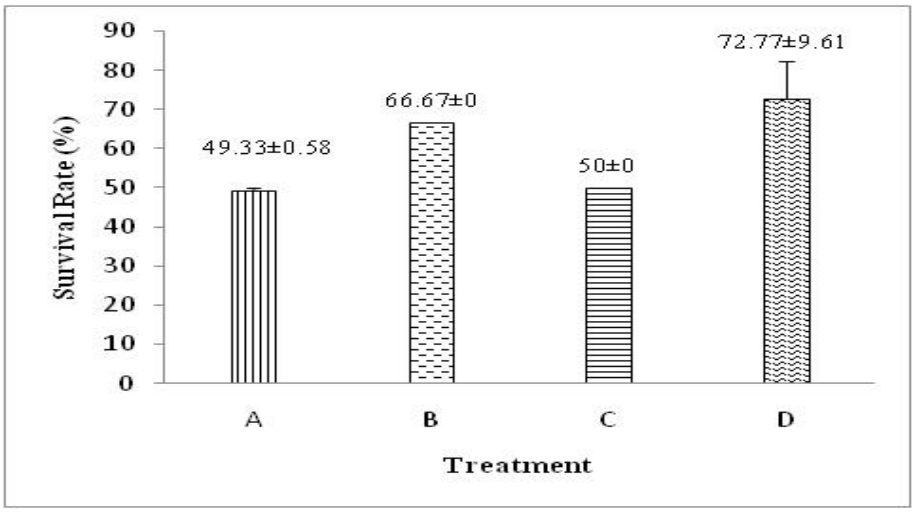


Figure 7. Survival rate of tiger shrimp treated with probiotics after challenge test with *V. harveyi.*

Density of Vibrio and LAB. *Vibrio* density in tiger shrimp showed that in treatment A (control), bacterial density increased after being challenged with *V. harveyi,* in contrast, the treatment of LAB and commercial probiotics *Vibrio* density decreased (Figure 8). This showed that probiotics could reduce the population of *Vibrio* bacteria in tiger shrimp. LAB density in the control treatment was lower compared to probiotic treatment. Treatment B (18K) showed an increase in LAB density from the start of culture (0 hpi = hour post infection) to 10 hours after the challenge test (10 hpi) (Figure 9). This showed that feeding with LAB (18K) increased the density of LAB in the gut of tiger shrimp so that it was thought that the LAB might have played significant role to suppress *Vibrio*'s population.

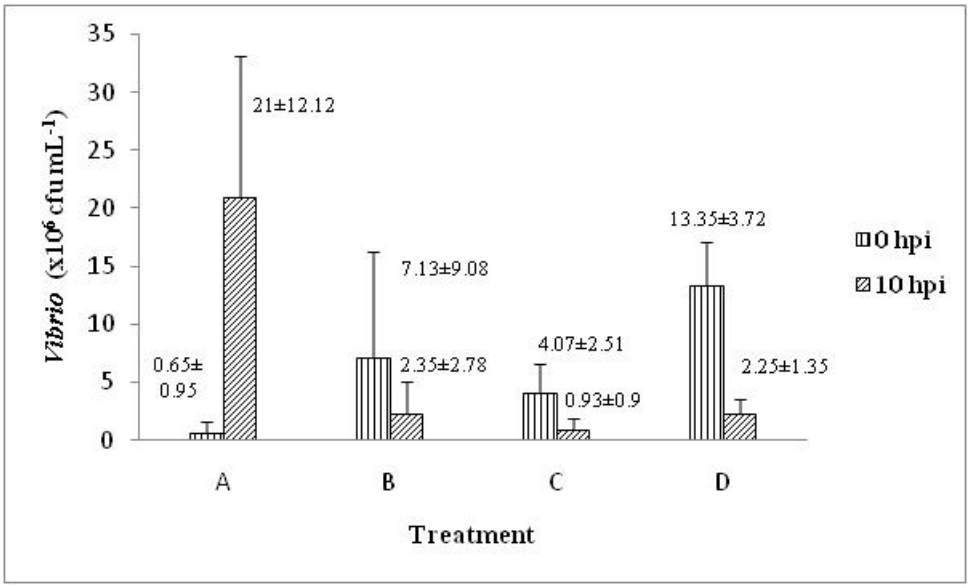


Figure 8. Density of *Vibrio* in tiger shrimp treated with probiotics after challenge test with *V. harveyi.*

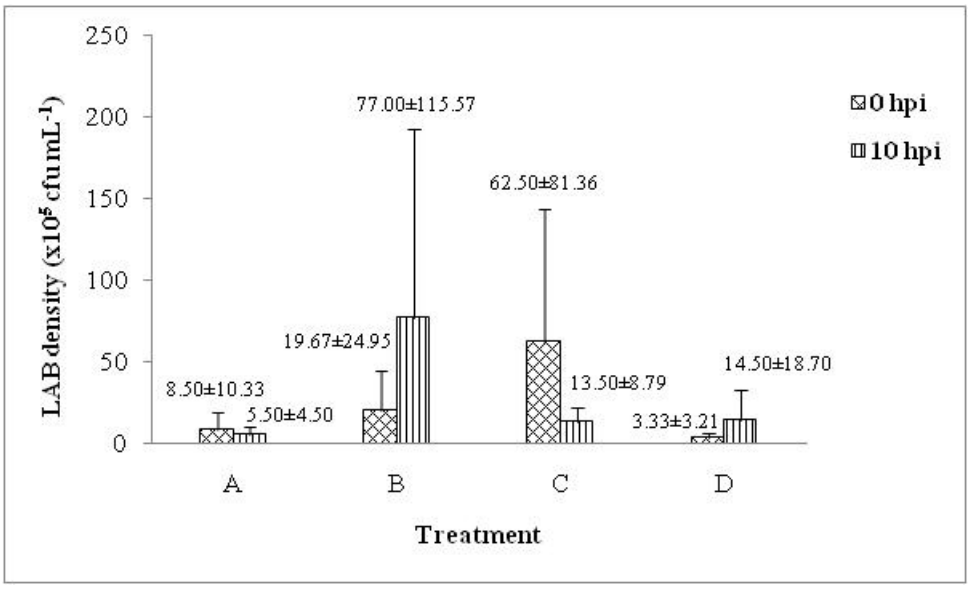


Figure 9. Density of LAB in the intestine of tiger shrimp.

Polymerase chain reaction and sequencing of LAB. Amplification of the LABs DNA through PCR showed that the 4 LABs vanamei isolates DNA could be amplified using universal primers 16S rRNA. The size of PCR product was seen at about 1500 bp (Figure 10). Only 18K isolates were pursued for sequencing and applied in shrimp feed because this isolates showed better performances compared to the other probiotic candidates. Sequencing and BLAST analysis to the GenBank showed that LAB 18K has 97% homology with the LAB of *Enterococcus avium* species. Phylogenetic tree analysis showed that the isolate LAB 18K was in the same cluster as the bacterium *E. avium* LT223664, indicating that the isolate LAB 18K might be the same species as *E. avium* (Figure 11).

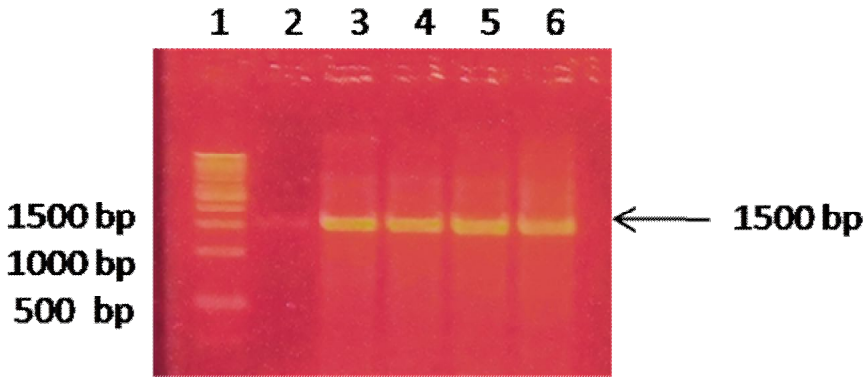


Figure 10. PCR product of isolate LAB using 16S rRNA primer. 1. (Marker 500 bp), 2. (negative control), 3-6 (LAB isolates vanamei: C1, 23P, 18K, and D1). Clear band are read at 1500 bp.

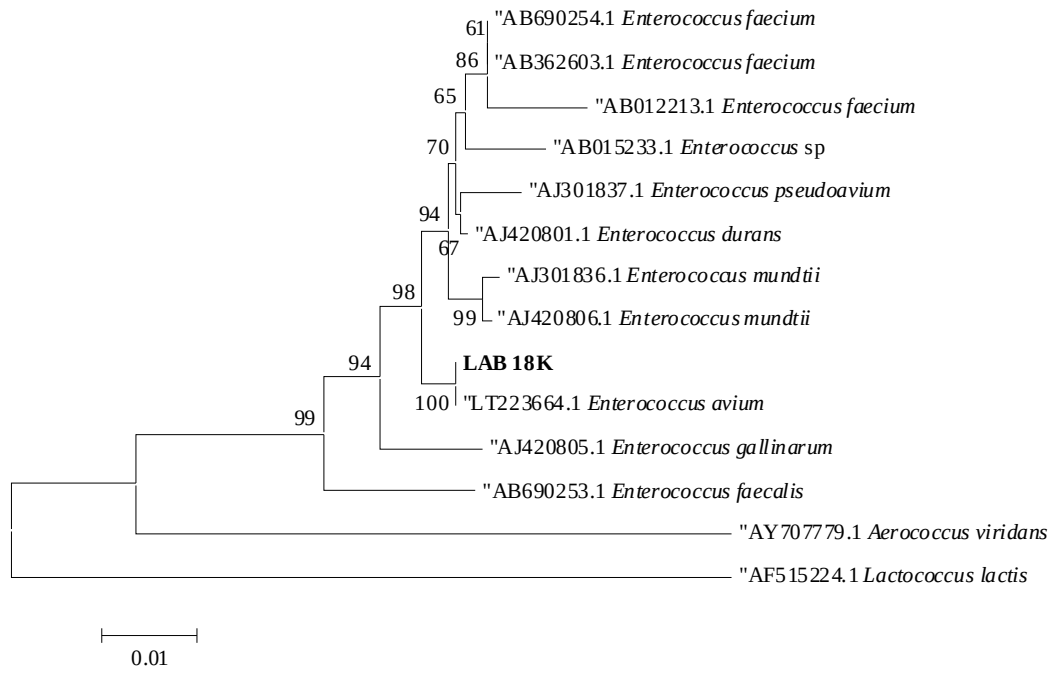


Figure 11. Phylogenetic tree of *Enterococcus* spp. and present samples of lactic acid bacteria from intestine of vannamei/18K based on 16S rRNA gene sequences. Neighbour-Joining tree was constructed using software MEGA version 6.

Discussion. The 4 isolates of LAB isolated from vanamei shrimp intestine produced clear zones on the medium GYPA + $CaCO_3$, tolerance to acidic to alkaline pH condition, resistant to bile salts and have an inhibitory effect on *Vibrio* spp. and thus they could be used as candidates for probiotics. Using indigenous isolates as probiotics have advantages over exogenous ones of being able to adapt easily and survive in the intestine because the host environment conditions of animals are the same as the original host, which may not be found in exogenous probiotics from different hosts (Maheshwari et al 2012).

Tolerance to acid and bile salts condition showed that the 4 isolates of LAB will be able to survive the acid condition in the stomach and in the presence of bile salts. The application of LAB as a probiotic is expected to reach intestine/gut of shrimp and form colonies on it to be able to improve the normal intestinal micro biota which can further inhibit the growth of pathogenic bacteria. The LAB (18K) isolated from vanamei intestine has antimicrobial activity against *Vibrio* bacteria. The presence of LAB antimicrobial properties against *Vibrio* might be due to presence of lactic acid, H_2O_2 or bacteriocin, which are toxic to opportunistic bacteria (Verschuere et al 2000). LAB, besides being able to have antimicrobial effect on pathogenic bacteria, it can also enhance the natural immune system, thereby increasing resistance of shrimp to disease after application of the probiotics (Ige 2013; Maeda et al 2014). Application of LAB as a probiotic in shrimp culture industry is very useful for dealing with disease problems, especially vibriosis and viral diseases.

The application of LAB 18K in feed increased THC and phagocytic index, and could suppress the development of *Vibrio* population, as well as increased LAB density in the intestines and survival of tiger shrimp. The total haemocytes number of probiotic-treated samples in this study was higher than those found in the normal shrimp without probiotic application. This increase could be due to the presence of peptidoglycan in probiotic bacterial cells that can stimulate the non-specific immune response of tiger shrimp. Non-self polysaccharide molecules such as peptidoglycan are pathogen-associated molecular patterns (PAMPs) which are commonly used as immunostimulants to enhance the immune response in fish and shellfish (Ringo et al 2012). Shi et al (2018) shows that

shrimp haemocytes that receive exogenous polysaccharides will degranulate and release endogenous molecules that activate innate immunity and synergize the immune response in shrimp. This is also because shrimp haemocytes are blood cells that have a very important role in the shrimp immune response system, and increase rapidly when an infection occurs. Haemocytes are one component of blood in shrimp that functions as a non-specific defense which acts to localize and eliminate pathogens through phagocytosis (Anderson 1992). Effendi et al (2004) reported that haemocytes increased due to the attack of pathogens so that haemocyte cells will play a role in the process of degranulation, cytotoxicity and lysis of the pathogen, so that the presence of pathogens stimulates the production of blood cells as a form of resistance to pathogens. The increased production of haemocytes is to achieve a homeostatic state after administration of probiotics. High THC levels in probiotic treatments appear to be related to the inflammatory response of haemocytes that leave the blood circulation and migrate to the site of infection (Van de Braak et al 2002). In addition, haemocytes can aggregate to form haemocyte nodules in which adhesion of cell molecules, such as peroxynectin, capture microorganisms between them (Jiravanichpaisal et al 2006) and are then mechanically removed from circulation through the gills (Martin et al 2000). Haemocyte consists of 3 types, *i.e.* hyaline, semi-granular and granular which have different roles and functions. Haemocyte cells that play a role in phagocytosis are mainly semi-granular and hyaline cells. Phagocytosis activity as a form of non-specific cellular defense in shrimp will increase when exposed to antigens and extreme environmental conditions (Supamattaya et al 2000). In the process of phagocytosis, phagocytic cells destroy organisms internalized through two routes: 1) aerobic processes that use NADPH or NADH as electron donors, and reduce oxygen electrons to form superoxide ions or called respiratory burst and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (^{-}OH), singlet oxygen ($^{1}O_2$), and nitric oxide (NO) which play an important role in microbiocidal activity; 2) anaerobic processes that show the action of various microbicide enzymes, such as lysozyme and antimicrobial peptide (AMP) that have low molecular weight (Munoz et al 2000; Aguirre-Guzman et al 2009).

Molecular identification of LAB isolates (18K) showed that the bacterium belongs to *Enterococcus avium*. Enterococci are a group of bacteria that are tolerant to low pH, temperatures, and high salt concentration (Fisher & Phillips 2009). Enterococci bacteria are normal intestinal micro biota in humans and animals, and is used as indicators of faecal contaminants in recreational water, but can also be isolated from water which is free of faecal contaminants (Roberts et al 2009) and their presence in fish and fish environments has been widely studied. Ringo & Gatesoupe (1998) has stated that *Entrococcus* is not an indigenous fish intestinal flora, however, subsequent studies prove that Enterococci bacteria are normal fish intestinal flora as evidenced by their high prevalence and predominate bacteria in fish intestines. Cai et al (1999) isolated LAB from the intestines of carp (*Cyprinus carpio*) and giant prawns (*Macrobrachium rosenbergii*) and found 3 types of LAB which were dominant in the gut of carp and giant prawns, namely *Lactococcus garvieae*, *Pediococcus acidilactici*, and *Enterococcus faecium*. Bourouni et al (2015) reported that in the gastrointestinal tract of sea bream (*Sparus aurata*) was found to be the predominance of lactic acid bacteria from the species *E. faecium* and *E. faecalis*. Other species, such as *E. sanguinicola*, *E. casseliflavus*, *E. gallinarum*, *Carnobacterium* sp., *Aerococcus viridans* and *Vagococcus carniphilus* were also identified. *E. avium* has also been found by Najjari et al (2008) in grey mullet (*Mugil cephalus*). *E. avium/devriesei* members was first described in the oral cavity of horses and then observed associated to animals (Collins et al 2004; Svec et al 2005). Al Bulushi et al (2010) stated that *E. avium* is a Gram positive bacterium found in subtropical marine fish in the muscles, gills and intestines.

The *Enterococcus* species has been found in the digestive tract of freshwater fish, shrimp and seawater, and has the properties as seen in other LAB in terms of being able to maintain normal micro biota balance in the gastrointestinal tract, have antimicrobial activity, and enhance the host immune system. The genus *Enterococcus* has been extensively studied for use as probiotics (Araujo & Ferreira 2013). This genus has desirable characteristics as probiotics particularly its resistance to gastric fluid and bile

salts and produces antimicrobial compounds such as enterocin (Araujo & Ferreira 2013). While Buntin et al (2008) that isolate and screen of LAB from fish and sea shrimp digestion tract found that LAB which could be used as a probiotic are *Pediococcus pentosaceus*, and *E. faecium*. Sha et al (2016) studied that *E. faecium* as a probiotic for vanamei shrimp showed that the bacterium does not have haemolytic activity, and has antagonist effect against the pathogenic bacteria *V. harveyi* and *V. parahaemolyticus*, shows high adhesion to intestine mucus, and enhance the immune system of vanamei shrimp. Hanchi et al (2018) in their study found that *E. faecium* and *E. faecalis* are the main producers of enterocins, whereas other species such as *E. mundtii, E. avium, E. hirae,* and *E. durans* only produce small amount of the substance.

The next study will be focused on analyzing the ability of LAB probiotic to combat against vibriosis and WSSV disease, immune response, growth and survival of tiger shrimp in hatchery facilities and grow-out ponds.

Conclusions. Four isolates of lactic acid bacteria from vanamei shrimp intestine (C1, D3, 18K, and 23P) were successfully isolated. The isolates are Gram positive cocci, catalase and motility negative, tolerance to acidic to alkaline pH, tolerance to bile salts and shows antibacterial activity to *Vibrio* spp. The 18K isolate tested stimulates immune system of tiger shrimp through increasing of THC and phagocytosis index, suppress the development of *Vibrio* and increase the survival of tiger shrimp. Phylogenetic tree analysis of the LAB solates (18K) shows that the LAB was in the same cluster as *Enterococcus avium.*

Acknowledgements. This research was financially supported by Indonesian Government through directorate of higher education (DIKTI) for the research grant (MP3EI).

References

Aguirre-Guzman G., Sanchez-Martinez J. G., Campa-Cordova A. I., Luna-Gonzalez A., Ascencio F., 2009 Penaeid shrimp immune system. Thai Journal of Veterinary Medicine 39(3):205-215.

Al Bulushi I. M., Poole S. E., Barlow R., Deeth H. C., Dykes G. A., 2010 Speciation of Gram-positive bacteria in fresh and ambient-stored sub-tropical marine fish. International Journal of Food Microbiology 138(1-2):32-38.

Amlacher E., 1970 Taschenbuch der Fischkrankheiten. Fischer Verlag, Jena (Translated by Conray D. A., Herman R. L.: Textbook of fish diseases. TFH Publications, Hong Kong, 302 pp.

Anderson D. P., 1992 Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Annual Review of Fish Diseases 2:281-307.

Anshary H., Sriwulan, Sukenda, Baxa D. V., 2017 Multiple viral pathogens occurrence in tiger shrimp (*Penaeus monodon*) broodstock from Sulawesi coastal waters. AACL Bioflux 10(4):936-950.

Araujo T. F., Ferreira C. L. L. F., 2013 The genus *Enterococcus* as probiotic: safety concerns. Brazilian Archives of Biology and Technology 56(3):457-466.

Arokiyaraj S., Hairul Islam V. I., Bharanidharan R., Raveendar S., Lee J., Kim D. H., Oh Y. K., Kim E. K., Kim K. H., 2014 Antibacterial, anti-inflammatory and probiotic potential of *Enterococcus hirae* isolated from the rumen of *Bos primigenius*. World Journal of Microbiology and Biotechnology 30(7):2111-2118.

Bourouni O. C., Barros-Velazquez J., Calo-Mata P., El Bour M., 2015 Lactic acid bacteria associated with the digestive tract and skin of sea bream (*Sparus aurata*) cultured in Tunisia. African Journal of Microbiology Research 9(31):1853-1865.

Buller N. B., 2004 Bacteria from fish and other aquatic animals: a practical identification manual. CABI Publishing, Oxfordshire, UK, 361 pp.

Buntin N., Chanthachum S., Hongpattarakere T., 2008 Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics. Songklanakarin Journal of Science and Technology 30(1):141-148.

Cai Y., Suyanandana P., Saman P., Benno Y., 1999 Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. Journal of General and Applied Microbiology 45(4):177-184.
Castanon J. I. R., 2007 History of the use of antibiotic as growth promoters in European poultry feeds. Poultry Science 86(11):2466-2471.
Collins M. D., Lundstrom T., Welinder-Olsson C., Hansson I., Wattle O., Hudson R. A., Falsen E., 2004 *Streptococcus devriesei* sp. nov., from equine teeth. Systematic and Applied Microbiology 27(2):146-150.
de la Pena L. D., Lavilla-Pitogo C. R., Paner M. G., 2001 Luminescent vibriosis associated with mortality in pond-cultured shrimp *Penaeus monodon* in the Philippines: species composition. Fish Pathology 36:133-138.
Effendi S., Alexander R., Akbar T., 2004 [Enhance of shrimp seed haemocytes (*Penaeus monodon* Fabricius) post immersion with bread yeast extract (*Saccharomyces cerevisiae*) at different concentrations] Jurnal Sains dan Teknologi 14(2):46-53. [in Indonesian]
Fisher K., Phillips C., 2009 The ecology, epidemiology and virulence of *Enterococcus*. Microbiology 155(6):1749-1757.
Hanchi H., Mottawea W., Sebei K., Hammami R., 2018 The Genus *Enterococcus*: between probiotic potential and safety concerns - an update. Frontiers in Microbiology 9: 1791.
Hernandez-Serrano P., 2005 Responsible use of antibiotics in aquaculture. FAO Fisheries Technical Paper No. 469, FAO, Rome, 97 pp.
Hossain M., Otta S. K., Karunasagar I., Karunasagar I., 2001 Detection of white spot syndrome virus (WSSV) in wild captured shrimp and in non-cultured crustaceans from shrimp ponds in Bangladesh by polymerase chain reaction. Fish Pathology 36(2):93-95.
Ige B. A., 2013 Probiotics use in intensive fish farming. African Journal of Microbiology Research 7(22):2701-2711.
Jiravanichpaisal P., Lee B. L., Soderhall K., 2006 Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211(4):213-236.
Khamesipour F., Noshadi E., Moradi M., Raissy M., 2014 Detection of *Vibrio* spp. in shrimp from aquaculture sites in Iran using polymerase chain reaction (PCR). AACL Bioflux 7(1):1-7.
Lavilla-Pitogo C. R., Baticados M. C. L., Cruz-Lacierda E. R., de la Pena L. D., 1990 Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philiphines. Aquaculture 91(1-2):1-13.
Lavilla-Pitogo C. R., Leano E. M., Paner M. G., 1998 Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent *Vibrios* in the rearing environment. Aquaculture 164(1-4):337-349.
Leano E. M., Lavilla-Pitogo C. R., Paner M. G., 1998 Bacterial flora in the hepatopancreas of pond-reared *Penaeus monodon* juveniles with luminous vibriosis. Aquaculture 164:367-374.
Maeda M., Shibata A., Biswas G., Korenaga H., Kono T., Itami T., Sakai M., 2014 Isolation of lactic acid bacteria from kuruma shrimp (*Marsupenaeus japonicus*) intestine and assessment of immunomodulatory role of a selected strain as probiotic. Marine Biotechnology 16(2):181-192.
Maheshwari R., Rani B., Verma D., Yadav R. K., 2012 Indigenous probiotics and health benefits. Bulletin of Environment, Pharmacology and Life Sciences 2(1):83-86.
Martin G. G., Quintero M., Quigley M., Khosrovian H., 2000 Elimination of sequestered material from the gills of decapod crustaceans. Journal of Crustacean Biology 20(2):209-217.
Minh N. P., 2014 Isolation, identification and characterization of *Lactobacillus* on black tiger shrimp. International Journal of Multidisciplinary Research and Development 1(6):153-158.

Munoz M., Cedeno R., Rodriguez J., van der Knaap W. P. W., Mialhe E., Bachere E., 2000 Measurement of reactive oxygen intermediate production in haemocyte of the penaeid shrimp *Penaeus vannamei*. Aquaculture 191:89-107.
Najjari A., Ouzari H., Boudabous A., Zagorec M., 2008 Method for reliable isolation of *Lactobacillus sakei* strains originating from Tunisian seafood and meat products. International Journal of Food Microbiology 121(3):342-351.
Nair P. S., Surendran P. K., 2005 Biochemical characterization of lactic acid bacteria isolated from fish and prawn. Journal of Culture Collections 4:48-52.
Pelczar M. J., Chan E. C. S., 1998 Dasar-dasar mikrobiologi 1. Universitas Indonesia, Jakarta, 576 pp. [in Indonesian]
Reyes-Becerril M., Ascencio F., Gracia-Lopez V., Macias M. E., Roa M. C., Esteban M. A., 2014 Single or combined effects of *Lactobacillus sakei* and inulin on growth, nonspecific immunity and IgM expression in leopard grouper (*Mycteroperca rosacea*). Fish Physiology and Biochemistry 40(4):1169-1180.
Ringo E., Gatesoupe F. J., 1998 Lactic acid bacteria in fish: a review. Aquaculture 160(3-4):177-203.
Ringo E., Olsen R. E., Vecino J. L. G., Wadsworth S., Song S. K., 2012 Use of immunostimulants and nucleotides in aquaculture: a review. Journal of Marine Science: Research and Development 2(1):1000104.
Roberts M. C., Soge O. O., Giardino M. A., Mazengia E., Ma G., Meschke J. S., 2009 Vancomycin-resistant *Enterococcus* spp. in marine environments from the West Coast of the USA. Journal of Applied Microbiology 107(1):300-307.
Saitou N., Nei M., 1987 The neighbour-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4):406-425.
Senthong R., Chanthachum S., Sumpavapol P., 2012 Screening and identification of probiotic lactic acid bacteria isolated from *Poo-Khem*, a traditional salted crab. International Conference on Nutrition and Food Sciences 39:111-115.
Sha Y., Wang L., Liu M., Jiang K., Xin F., Wang B., 2016 Effects of lactic acid bacteria and the corresponding supernatant on the survival, growth performance, immune response and disease resistance of *Litopenaeus vannamei.* Aquaculture 452:28-36.
Shi Y. Z., Chen J. C., Chen Y. Y., Kuo Y. H., Li H. F., 2018 Endogenous molecules released by haemocytes receiving *Sargassum oligocystum* extract lead to downstream activation and synergize innate immunity in white shrimp *Litopenaeus vannamei*. Fish and Shellfish Immunology 76:1-12.
Sriwulan, Anshary H., 2011 [Detection of viruses causative agents of monodon slow growth syndrome of tiger shrimp (*Penaeus monodon*) post larvae in Indonesian using multiplex PCR]. Jurnal Perikanan 13(1):1-7. [in Indonesian]
Sriwulan, Anshary H., 2016 Occurrence of infectious and non infectious Decapod Penstyldensovirus 1 (PstDV-1) from tiger shrimp (*Penaeus monodon*) in South Sulawesi, Indonesia. AACL Bioflux 9(4):790-801.
Sugimura Y., Hagi T., Hoshino T., 2011 Correlation between in vitro mucus adhesion and the in vivo colonization ability of lactic acid bacteria: screening of new candidate carp probiotics. Bioscience, Biotechnology and Biochemistry 75(3):511-515.
Sunarto A., Widodo, Taukhid, Koesharyani I., Supriyadi H., Gardenia L., Sugianti B., Rukmono D., 2004 Transboundary fish diseases in Indonesia: occurrence, surveillance, research and training. In: Transboundary fish diseases in southeast Asia: occurrence, surveillance, research and training. Lavilla-Pitogo C. R., Nagasawa K. (eds), SEAFDEC Aquaculture Department, Tigbauan, Iloilo, Pilippines, pp. 91-121.
Supamattaya K., Pongmaneerat J., Klowklieng T., 2000 The effect of β–glucan on growth performance, immune response and disease resistance in black tiger shrimp, *Penaeus monodon* Fabricius. Songklanakarin Journal of Science and Technology 22:677-688.
Svec P., Vancanneyt M., Koort J., Naser S. M., Hoste B., Vihavainen E., Vandamme P., Swings J., Bjorkroth J., 2005 *Enterococcus devriesei* sp. nov., associated with animal sources. International Journal of Systematic and Evolutionary Microbiology 55(6):2479-2484.

Swain S. M., Singh C., Arul V., 2009 Inhibitory activity of probiotics *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13 against vibriosis in shrimp *Penaeus monodon*. World Journal of Microbiology and Biotechnology 25(4):697-703.
Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., 2013 MEGA 6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30(12): 2725-2729.
Van De Braak C. B. T., Botterblom M. H. A., Taverne N., Van Muiswinkel W. B., Rombout J. H. W. M., van der Knaap W. P. W., 2002 The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. Fish and Shellfish Immunology 13(4):293-309.
Vaseeharan B., Jayakumar R., Ramasamy P., 2003 PCR-based detection of white spot syndrome virus in cultured and captured crustaceans in India. Letters in Applied Microbiology 37:443-447.
Verschuere L., Rombaut G., Sorgeloos P., Verstraete W., 2000 Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64(4):655-671.
Yousefian M., Amiri M. S., 2009 A review of the use of prebiotic in aquaculture for fish and shrimp. African Journal of Biotechnology 8:7313-7318.

Received: 12 April 2019. Accepted: 23 August 2019. Published online: 29 October 2019.
Authors:
Sriwulan, Hasanuddin University, Faculty of Marine Science and Fisheries, Department of Fisheries, Jl. Perintis Kemerdekaan KM 10, Makassar 90245, Indonesia, e-mail: sriwulancinga@mail.com
Andi Azwar, Hasanuddin University, Faculty of Marine Science and Fisheries, Department of Fisheries, Jl. Perintis Kemerdekaan KM 10, Makassar 90245, Indonesia, e-mail: andimuhazwar27@gmail.com
Alexander Rantetondok, Hasanuddin University, Faculty of Marine Science and Fisheries, Department of Fisheries, Jl. Perintis Kemerdekaan KM 10, Makassar 90245, Indonesia, e-mail: alexander_tondok@yahoo.com
Hilal Anshary, Hasanuddin University, Faculty of Marine Science and Fisheries, Department of Fisheries, Jl. Perintis Kemerdekaan KM 10, Makassar 90245, Indonesia, e-mail: hilalanshary@gmail.com

How to cite this article:
Sriwulan, Azwar A., Rantetondok A., Anshary H., 2019 Screening and application of lactic acid bacteria isolated from vanamei shrimp (*Litopenaeus vannamei*) intestine as a probiotic potential for tiger shrimp (*Penaeus monodon*). AACL Bioflux 12(5):1866-1881.