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Isolation and selection of lactic acid bacteria that can antagonize *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in whiteleg shrimp (*Penaeus vannamei)*

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ABSTRACT

This study was conducted from March to June 2015 to select lactic acid bacteria (LAB) strains that can antagonize Vibrio parahaemolyticus for further studies on prevention of acute hepatopancreatic necrosis disease in shrimp. LAB strains were isolated from different sources including (1) gut of whiteleg shrimp (Penaeus vannamei), (2) gut of Nile tilapia (Oreochromis niloticus), and (3) shrimp pond sediment in Tra Vinh, Ben Tre, and Soc Trang. Isolated LAB strains were identified by using morphological, physiological and bio-chemical characteristics, and then their antagonism toward V. parahaemolyticus was determined by using agar well diffusion method. A total of 94 LAB strains were selected for this study (30, 39, 25 isolates from Tra Vinh, Ben Tre, and Soc Trang, respectively). For antimicrobial activity, 13 strains could weakly antagonize V. parahaemolyticus with inhibition diameter smaller than 11.0 mm. However, 81 remaining LAB strains could antagonize V. parahaemolyticus with inhibition diameter around 11.0–18.5 mm. Of the 94 strains above mentioned, 3 strains of RP6.5, RP5.4.1, and RP5.5.1 had the biggest inhibition diameters (17.3±0.58 mm, 18.5±0.289 mm, and 18.00±0.00 mm, respectively). These trains can be used for further studies to evaluate the effect of LAB in prevention acute hepatopancreatic necrosis disease in shrimp.

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1 INTRODUCTION

Shrimp is an aquaculture product having high economic value and becomes the major exported product for many countries like Thailand, India, Vietnam, Malaysia, etc. (FAO, 2013). However, farmers culture shrimp in high density, they are facing with many risks. Especially, acute hepatopancreatic necrosis disease (AHPND) is considered as a dangerous disease that has attacked shrimp farms in Southeast Asia (FAO, 2013). Total annual losses are more than USD1 billion (Zorriehzahra and Banaederakhshan, 2015). This disease was first recorded in China in 2009 and then subsequently confirmed in Vietnam in 2010, Malaysia and Thailand in 2011, and very recently in Mexico in 2013 (Tran Huu Loc *et al.,* 2014). The outbreaks of AHPND naturally occur in the first 30 days after stocking a freshly arranged shrimp pond, and rate of mortality can reach beyond 70% (Zorriehzahra

and Banaederakhshan, 2015). The causative agent of AHPND is a unique strain of *V. parahaemolyticus* that can produce toxins responsible for the primary pathology in affected shrimp (Tran Huu Loc *et al*., 2013).

Nowadays, there are many kinds of proposed methods being used to limit the growth of *V. parahaemolyticus* such as using chemical disinfectants, antibiotics, and biological methods. However, using chemicals and antibiotics not only is ineffective but also causes risk of origination of bacteria resistant strains (Huynh *et al*., 2015). Moreover, remaining of chemicals and antibiotics in the products is a barrier for exporting to many countries around the world (Debaere, 2010). Therefore, applying of useful microorganism is considered as the great method to inhibit the pathogenic bacteria causing AHPND. Using lactic bacteria in aquaculture can not only control the density of bacteria but also enhance food safety, and is eco-friendly because of using useful bacteria (Klayraung *et al.*, 2008).

Lactic acid bacteria (LAB) are widely applied and more popular in producing probiotics. Many studies showed that LAB are able to release inhibitory subtances that inhibit the growth of pathogenic bacteria, it also brings environmental benefits in shrimp pond (Ma *et al*., 2009; Ariole and Nyeche, 2013). Many evidences showed that LAB could inhibit the growth of pathogenic bacteria. For example, *Lactobacillus plantarum* could antagonize *Aeromonas hydrophila* (Ma *et al*., 2009). *L. acidophilus* LA1 could antagonize both gram-positive and gram-negative bacteria (Bernet-Camard *et al.,* 1997; Michetti *et al.,* 1999). *Lactobacillus* sp. could antagonize *Vibrio* sp. (Trinh Hung Cuong, 2011). Nevertheless, no probiotic bacteria are commercially viable for large scale shrimp aquaculture especially against the shrimp pathogen *V. parahaemolyticus.* Therefore, this study was conducted to explore antagonistic properties of LAB toward *V. parahaemolyticus* causing AHPND in shrimp. The objective of this study is to isolate and select suitable LAB strains strongly antagonizing *parahaemolyticus* causing AHPND in shrimp, for further uses in prevention of AHPND in shrimp farms.

2 MATERIALS AND METHODS

2.1 LAB isolation from different sources

2.1.1 Sample collection and storage

Whiteleg shrimp, Nile tilapia and sediment samples were collected from Tra Vinh, Ben Tre and Soc Trang from March to June, 2015. In each province, 5 ponds were selected, and, from each pond, 5 samples were collected. However, just one sediment sample was collected from each shrimp pond. Healthy whiteleg shrimps were collected from intensive farming or semi-intensive farming ponds. The average size was 20 grams per individual. Nile tilapias were collected in settling ponds or in integrated Nile tilapia - shrimp farming systems. The selected individual was healthy and weighed about 100 grams per individual. Sediment samples were collected from 3 sites (near inlet, outlet, and center of each pond) then mixed together (Somsiri *et al*., 2006). The collected samples were stored on ice (4^0C) and carried to laboratory for immediate isolation

V. parahaemolyticus causing AHPND in shrimp (Nguyen Trong Nghia *et al*., 2015) was provided from Department of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University.

2.1.2 LAB isolation from gut of whiteleg shrimp, Nile tilapia and sediment samples

All LAB strains were isolated from gut of whiteleg shrimp and Nile tilapia (Noordiana *et al.,*2013). In which, 75 whiteleg shrimp and 75 tilapia samples were operated to cut an interval of intestinal tracts (foregut for tilapia, entire gut for shrimp) and put in tubes containing 5 mL sterile 0.85% NaCl solution. Next, the samples were crushed by glass rod in sterile 0.85% NaCl solution and left to settle.

LAB strains were also isolated from sediment collected in 15 shrimp ponds (Alessandro *et al.,* 2015). In detail, one gram of each sample was placed in each tube containing 9 mL of 0.85% NaCl solution, with 3 replications for each sample. The sample was regularly shaken and settled solution.

Then, 1mL supernatant of each shrimp, tilapia and sediment samples were put into separate tube containing 5 mL de Man - Rogosa - Sharpe (MRS, Darmstadt, Merck) broth containing 1.5% NaCl) and incubated at 28°C for 48 hours. After 48 hours, the solution in test tubes was diluted at 10^{-1}, 10^{-2},10^{-3} times in steriled 0.85% NaCl solution. Then, 50µL incubated solution from the diluted tubes was spread on MRS agar containing 1,5% NaCl and 1% $CaCO_3$. Each sample was repeated 3 times and incubated at 28^0C for 48 hours. After 48 hours, white, yellow or colorless colonies that could resolve $CaCO_3$ were selected and purified for further study.

2.2 LAB screening via morphological, physiological and biochemical characteristics

A total of 94 isolated LAB colonies were purified and identified by biochemical test. Morphological, physiological and biochemical characteristics were tested including gram stain, spore stain, oxidase, catalase (Kandler and Weiss, 1986), and oxidation fermentation (O/F) test (Parvathy and Puthuvallil, 2005).

2.3 Antibacterial activity assay

The antimicrobial activity of LAB was determined by agar well diffusion method (Noordiana *et al.,* 2013).

V. parahaemolyticus causing AHPND was recovered on thiosulphate-citrate-bile-sucrose (TCBS, Darmstadt, Merck) agar, checked for purification, and then proliferated in nutrient broth (NB, Merk) containing 1.5% NaCl at 28ºC for 24 hours. The bacteria solution was spread onto NA plate containing 1.5% NaCl by sterilized swab and put in cool store at 4^0C for 1 hour. Finally, the plate was made wells of 6 mm in diameter for further study.

A total of 94 LAB strains were cultured in 5 mL MRS broth containing NaCl 1.5%, incubated at 28ºC for 48 hours. Next, 1 mL cultured bacteria were put into eppendorf tube and centrifuged at 10,000 rpm at 4ºC for 20 minutes. Next, 50 µL supernatant of LAB was put into each agar well with replication 3 times for each LAB strain. Then, the plates were incubated at 28^0C for 24 hours. Based on the zone of inhibition surrounding the wells, the effectiveness of antimicrobial activity was determined. According to Ngo Thi Phuong Dung *et al*. (2011), antibacterial activities were divided into 3 types, including type (+) with the inhibition zones<11mm; type (++) with the inhibition zones from 11 to 16 mm and type (+++) with the inhition zones > 16mm.

3 RESULTS AND DISCUSSION

3.1 LAB isolation from different sources

A total of 94 LAB strains were isolated from the gut of whiteleg shrimp, gut of Nile tilapia and sediment samples in Tra Vinh, Ben Tre, and Soc Trang. Among them, there were 51 (54.25%), 41 (43.62%), and 2 (2.13%) strains from gut of whiteleg shrimp (*Penaeus vannamei*), gut of Nile tilapia (*Oreochromis niloticus*), and sediment samples, respectively. The study revealed that gut of whiteleg shrimp and gut of Nile tilapia were good sources for LAB inhabitation.

The largest amount of LAB isolated from gut of whiteleg shrimp (51/94 strains) may be originated from probiotics mixed with feed. This can be described as in the process of shrimp farming, farmers add probiotics such as *Lactobacillus, Bacillus* to the feed. Therefore, the presence of these bacteria in shrimp's gut is completely normal. This result is also matched with Khuat Huu Thanh's (2009), 60 LAB strains in shrimp's gut were found. The result in Parvathy's research (2005) has pointed out that 65 LAB strains were isolated in culture shrimp.

Slightly lower number of LAB obtained from gut of tilapia (41/94 strains). Similar results obtained by Nirunya *et al*. (2008), 81 out of 106 *Lactobacillus* strains were isolated from the gut of seawater fish, shrimps and mollusks. Nguyen Van Thanh and Nguyen Ngoc Trai (2012) were also isolated 45 strains of *Lactobacillus* in gut and stomach of Nile tilapia in intensive farming ponds. Noordiana *et al.* (2013) isolated 64 strains of *Lactobacillus* from gut of striped catfish and Nile tilapia collected from the market and farming ponds.

The lowest number of LAB were obtained from sediment samples (2/94 strains). Similar result was obtained by Alessandro *et al.* (2015), the lowest number of LAB was isolated in sediment samples compared with the number of LAB strains isolated from water and shrimp gut. In short, in sediment samples, many kinds of bacteria grew and inhibited the development of LAB; therefore, the frequency of occurrence of LAB is low.

3.2 LAB screening via morphological, physiological and biochemical characteristics

All colonies releasing substances which were able to resolve $CaCO_3$ were selected to identify morphological, physiological and biochemical characteristics. The result was shown in Table 1.

For morphological characteristics, after growing for 48 hours on MRS agar, colonies of these strains showed opaque, creamy, smooth round, protrusion, sized from 1 to 2 mm and could resolve $CaCO_3$. This research was also similar to the study of Nguyen Van Thanh and Nguyen Ngoc Trai (2012) about morphological characteristics. All colonies that were identified as *Lactobacillus* are round, creamy, smooth, raise, and opaque, size varies from 1 to 2 mm; positive Gram, negative oxidase and catalase and are able to dissolve $CaCO_3$ Ponce *et al.* (2008) also explained that organic acids were excreted from these colonies led to reduce in pH levels, and then $CaCO_3$ was resolved. For physiological characteristics, under light microscope,

cells of these LAB represented as gram positive, non-spore forming and cocci shape (56 strains) or rod shape (38 strains). For biochemical characteristics, all LAB illustrated negative reactions for oxidase, catalase, but positive reaction for O/F test.

Table 1: Characteristics of LAB isolated from 3 provinces

Province	Samples	Total isolates	Latic acid production ($CaCO_3$)	Morphological characteristics	Physiological characteristics				Biochemical characteristics		
				Colonial diameter (mm)	Colonial shape	Bacterial shape	Gram-staining	Spore-forming	Oxi	Cat	O/F
Tra Vinh	Shrimp	15	15	1-1.5	//	*cocci, rod*	+	-	-	-	+/+
	Tilapia	15	15	1-2	//	*cocci, rod*	+	-	-	-	+/+
	Sediment	0	0	ND	ND	ND	ND	ND	ND	ND	ND
Soc Trang	Shrimp	20	20	1-2	//	*cocci, rod*	+	-	-	-	+/+
	Tilapia	3	3	1-2	//	*rod*	+	-	-	-	+/+
	Sediment	2	2	1-1.5	//	*rod*	+	-	-	-	+/+
Ben Tre	Shrimp	16	16	1	//	*Cocci, rod*	+	-	-	-	+/+
	Tilapia	23	23	1-1.5	//	*Cocci, rod*	+	-	-	-	+/+
	Sediment	0	0	ND	ND	ND	ND	ND	ND	ND	ND

Note: //: opaque, creamy, smooth round, raised; +: positive; -: negative;
oxi: oxidase; cat: catalase; ND: not determined

3.3 Antibacterial activity assay

The LAB antibacterial activity was determined by the agar-well diffusion method. Generally, 96.81% LAB strains isolated from gut of shrimp, gut of tilapia, and sediment samples in 3 provinces had antagonistic activity against *V. parahaemolyticus*.

The result of LAB antibacterial activity toward *V. parahaemolyticus* in Tra Vinh was shown in Figure 1.

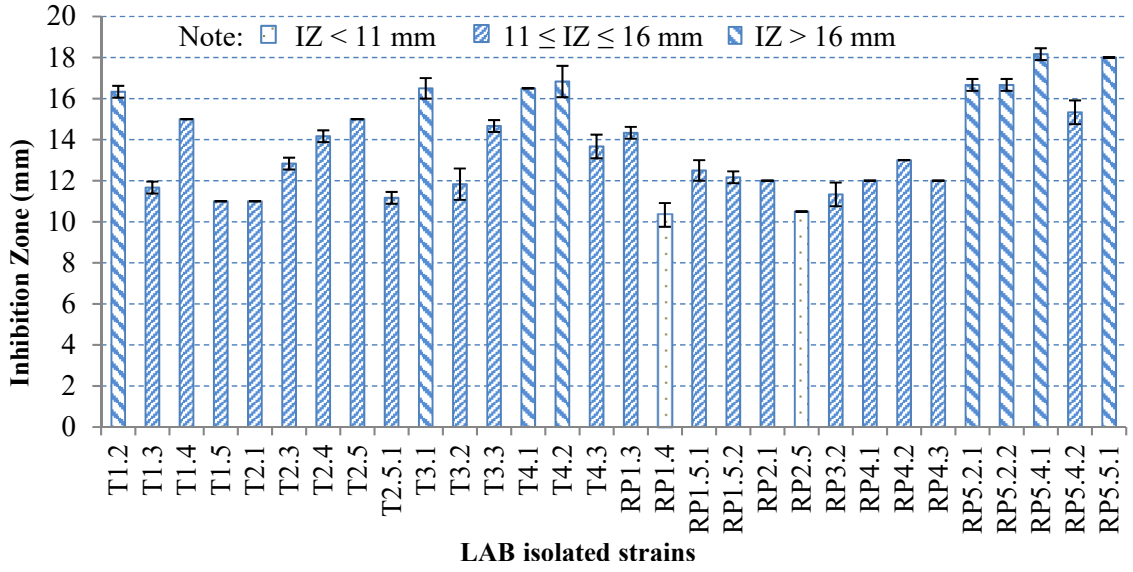

Fig. 1: Inhibition zones of LAB isolated from Tra Vinh province against *V. parahaemolyticus;* T (LAB were isolated from gut of whiteleg shrimp); RP (LAB were isolated from gut of tilapia); IZ: Inhibition Zone

Figure 1 illustrated that 2 isolates produced inhibition zones (+) smaller than 11.00 mm and 20 strains could antagonize *V. parahaemolyticus* at medium level (++) (11.00-16.00 mm). However, 8 LAB strains strongly antagonize *V. parahaemolyticus* with inhibition zones (+++) greater than 16.00mm. Especially, RP5.4.1 and RP5.5.1 (Figure 2) showed the biggest inhibition zones (18.17±0.29 mm, 18.00±00 mm, respectively). It was confirmed that 2 LAB strains of RP5.4.1 and RP5.5.1 isolated from gut of healthy Nile tilapia can be used for producing probiotics. This result was also compatible with the research carried out by Nguyen *et al.* (2014), *Bacillus polyfermenticus F27* produced largest inhibition zone (18.50 mm) against *V. parahaemolyticus* and can be used as probiotics.

The result of LAB antibacterial activity toward *V. parahaemolyticus* in Ben Tre was shown in Figure 3.

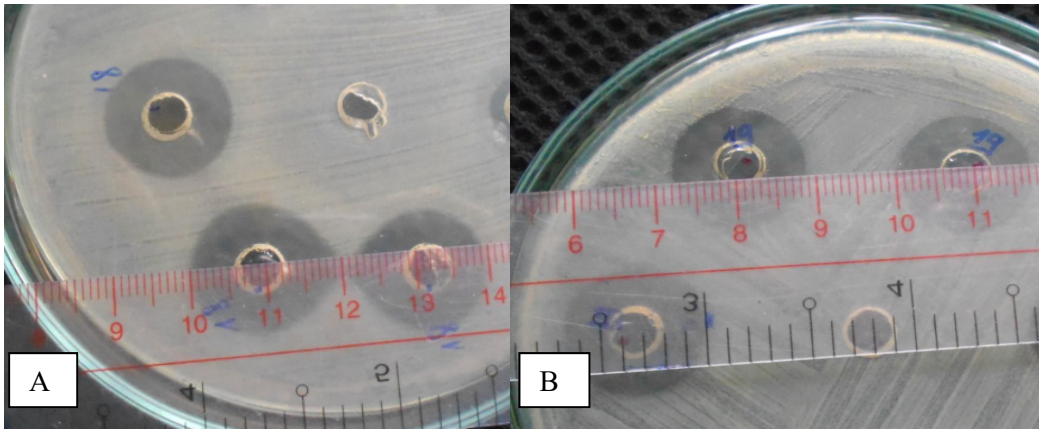

Fig. 2: Antimicrobial activity of RP5.4.1 (Figure 2A) and RP5.5.1 (Figure 2B) against *V. parahaemolyticus* in Tra Vinh

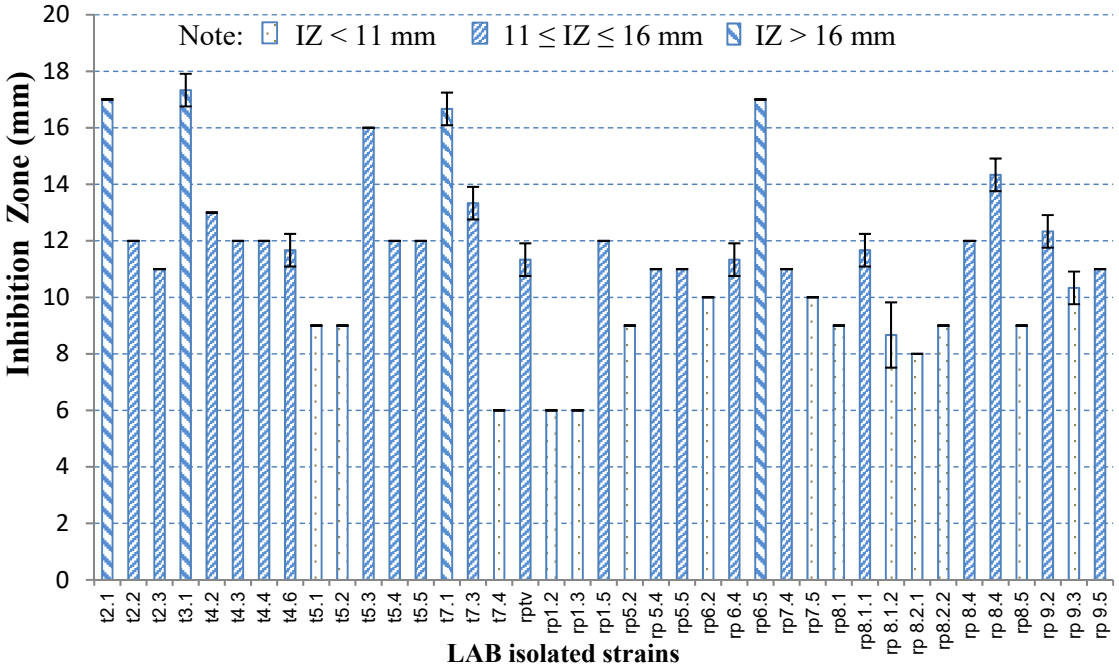

Fig. 3: Inhibition zones of LAB isolated from Ben Tre province against *V. parahaemolyticus*; T (LAB were isolated from gut of whiteleg shrimp); RP (LAB were isolated from gut of tilapia); IZ: Inhibition Zone

Figure 3 showed that 14 strains got weak antagonism with inhibition zone (+) smaller than 11.00 mm. Twenty-one strains produced medium inhibition zone (++) from 11.00 to 16.00 mm. Four remaining strains strongly antagonized against *V. parahaemolyticus* (+++) with inhibition zone greater than 16.00 mm. The strongest one RP6.5 was isolated from gut of Nile tilapia with inhibition zone was 17.3±0.58 mm (Figure 4). It was confirmed that RP6.5 would be a potential source of probiotics to inhibit the growth of *V. parahaemolyticus.*

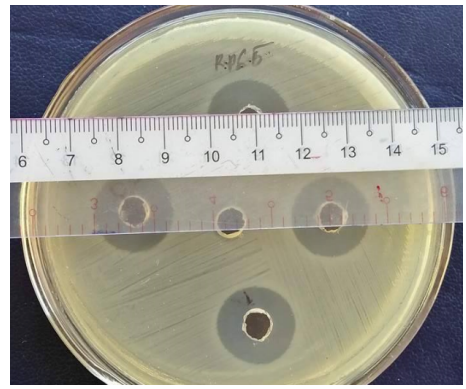

Fig. 4: Antimicrobial activity of RP6.5 against *V. parahaemolyticus* in Ben Tre

The result of LAB antibacterial activity against *V. parahaemolyticus* in Soc Trang was shown in Figure 5.

Figure 5 showed that 20 isolates (100%) had medium antagonism (++) against *V. parahaemolyticus* (11.00-14.30 mm).

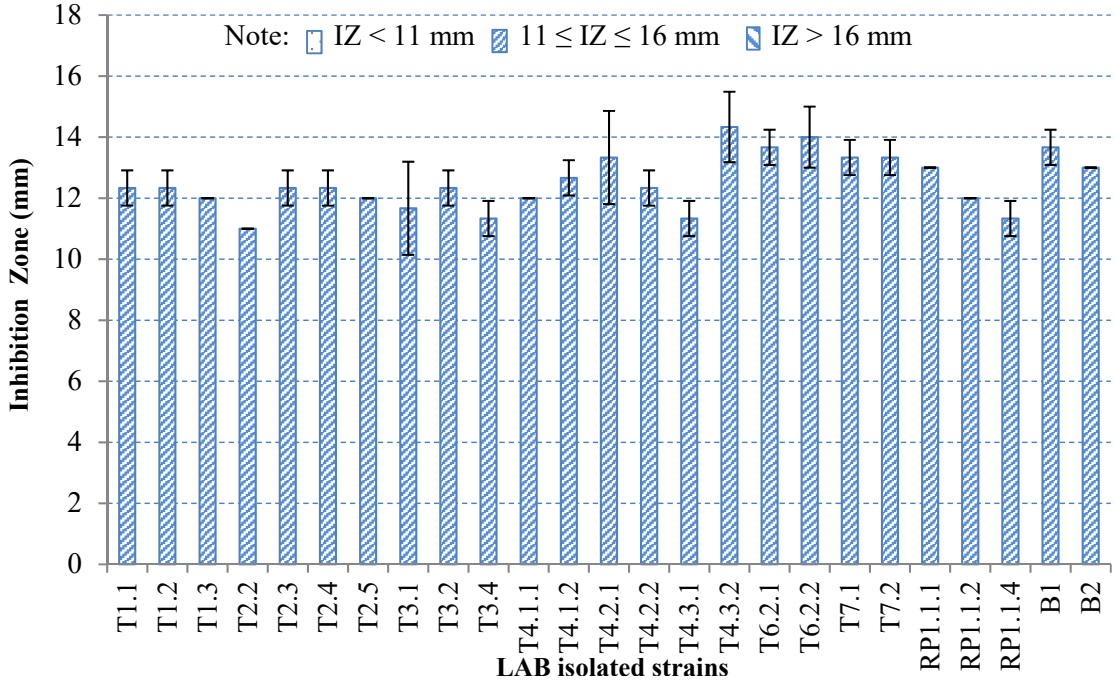

Fig. 5: Inhibition zones of LAB isolated from Soc Trang against *V. parahaemolyticus*; T (LAB were isolated from gut of whiteleg shrimp); RP (LAB were isolated from gut of tilapia); IZ: Inhibition Zone

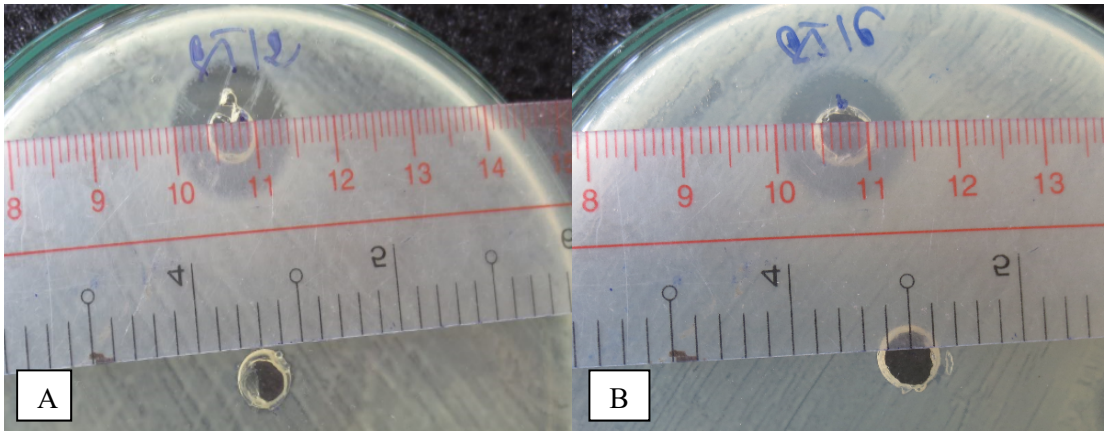

Fig. 6: Antimicrobial activity of LAB against *V. parahaemolyticus* in Soc Trang province

In this study, antimicrobial substances produced by LAB strains were not determined. However, many studies revealed that the antagonistic activity may have been due to the presence of organic acid such as lactic and acetic acids (Ma *et al*., 2009); hydrogen peroxide, carbon dioxide, diacetyl and bacteriocin (Ammor *et al.,* 2006); competition for nutrients and naturally prevented the colonization by many bacteria (Tambekar *et al.,* 2009). These substances may be excreted by isolated LAB strains and could inhibit the growth of *V. parahaemolyticus* causing AHPND in shrimp *in-vitro* condition.

In short, 3 LAB strains (RP6.5, RP5.4.1, and RP5.5.1) could be used as a potential source of probiotics to prevent AHPND in shrimp.

4 CONCLUSIONS

In conclusion, 94 LAB strains were isolated from the gut of whiteleg shrimp, gut of Nile tilapia, and sediment samples in 3 provinces of Tra Vinh, Ben Tre, and Soc Trang, Vietnam. Among those, three isolates of RP6.5, RP5.4.1, and RP5.5.1 exhibited a strong antagonism with *V. parahaemolyticus* bacteria caused AHPND in shrimp.

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