Application of Inulin and *Lactobacillus plantarum* TISTR 050 in *Litopenaeus vannamei* (also called *Penaeus vannamei*)

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Abstract

The aim of this study was undertaken to determine the effects of probiotic and supplementation of inulin on the inhibitory of pathogenic *Vibrio* in *Litopenaeus vannamei*. The result from *in vitro* studies showed that the *Lactobacillus plantarum* TISTR 050 (LP) have strong inhibitory effect on *Vibrio harveyi* and *Vibrio parahaemolyticus* using agar well diffusion and co-culture technique. The results from different salinity medium showed that both *Vibrio* strains grew well on media containing different levels of salinity, i.e., 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%, respectively. Agar well diffusion plate assay indicated that LP can inhibit growth of *V. harveyi* and *V. parahaemolyticus* with largest inhibitory zone and arbitrary unit. Co-culture in seawater supplemented with 2.22% inulin resulted in decrease of *V. harveyi* compared to LP. *In vivo* studies, prebiotic (inulin) was fortified in white shrimp feed for 6-weeks feeding periods. The results showed that inulin had minimal effect on growth of white shrimp. After feeding with LP for 7 days, persistence and growth of LP and *Vibrio* spp. were monitored at 1, 5 and 10 days. The results from FISH technique using DNA gene probes showed increasing of LP and decreasing of *V. harveyi* in intestine and hepatopancreas of white shrimp fed high levels of inulin-supplemented feed (2.22%).

Key words: prebiotic, probiotic, *Litopenaeus vannamei*, shrimp culture

Introduction

Thailand exports cultured shrimps and their products ranking the first globally since 2000 with world market share of about 30%. Major competing countries are China, Vietnam, Indonesia, 'ndia, Ecuador and countries formerly under EU colonies with increased production annually. It is noted that demand for consumption increase as well since meat products carry health risks to the consumers such as mad cow disease, bird flu and hence importing countries are more stringent regarding food safety and traceability for shrimp products. As observed by measures for

certification of products that guarantee standard have played important role in quality assurance recognized in world market for aquaculture industry, e.g. standard production of marine shrimp with no environmental impacts or known as the Code of Conduct for Responsible Shrimp Aquaculture (CoC) and other two measures, e.g. Safety Level (SL) and Good Aquaculture Practice (GAP) (Pongthanapanich and Roth, 2006).

However, during the past decade, production of marine shrimps worldwide decline due to bacteria and virus infections. The problems of bacterial infection particularly *Vibrio* spp. (vibriosis) are common among shrimp aquaculture industry, i.e. *V. harveyi*, that causes luminescence disease in marine shrimp, e.g., *Penaeus monodon*, *P. merguiensis*, and *Litopenaeus vannamei*, viral infection in shrimp e.g. White sport syndrome virus (WSSV), Yellow head virus (YHV), Taura syndrome virus (TSV), Infections hypodermal and hematopoietic necrosis virus (IHHNV) and Infections myonecrosis virus (IMNV), etc. (Cowley *et al.*, 1999; Flegel, 1997b; Hossain *et al.*, 2001b; Karunasagar *et al.*, 1998a; Lightner *et al.*, 1997).

The application of antibiotics to control infectious diseases in aquatic animals can result in the pathogen developing resistance to the antibiotics (Boyd and Massaut, 1999; Tendencia and de la Peña, 2001; Gräslund *et al.*, 2003) and the presence of antibiotic residue in the flesh can adversely affect domestic and export markets and consumer perceptions of food safety for farmed seafood products. Several studies have attempted to develop new, non-antibiotic based techniques to control pathogens. Application of probiotic is one solution to reduce or eliminate antibiotic uses in aquaculture (Vaseeharan and Ramasamy, 2003). Studies have shown that some strains of microbes can reduce the levels of pathogenic bacteria and have a beneficial effect on production in the aquatic environment (Moriarty, 1998).

The term "probiotic" was first used by Parker in 1974 and defined as microbes or substances that maintain the balance of gut microbes. Later, Havenaar and Hius (1992) provided a definition of a probiotic as single or multiple types of microbes with beneficial effects on animals. Probiotics are defined as microbial cells which inhibit the gastrointestinal tract pathogens in doing so, benefit the health of the consumer (Tannock *et al.*, 2000). The application of probiotics in aquaculture has increased greatly in recent years and this trend looks set to continue (Moriarty, 1998; Rengpipat *et al.*, 1998). Research findings indicate that the appropriate application of probiotics can enhance productivity and prevent the occurrence of several diseases, as well as minimizing problems related to dependence on chemicals and antibiotics. The application of the probiotic microbes is normally through supplementation in the feed. Adding probiotics to the feed

can also augment the immune status of *P. monodon*, and can reduce or eliminate dependence on antibiotic treatments that can lead to antibiotic resistant strains of pathogens and residues in shrimp tissue. Appropriate probiotic microbes for aquatic species should be beneficial to the host health, improve feed efficiency and nutritional value, stimulate host immunity, improve innate disease resistance of the host, improve the ambient environment, control proliferation of pathogenic microbes, facilitate digestion and, in some cases, improve water quality (Gram *et al.*, 1999, Chythanya *et al.*, 2002; Sugita *et al.*, 2002; Balcazar *et al.*, 2004; Vine *et al.*, 2006; Gullian *et al.*, 2004; O' Sullivan *et al.*, 2005). This definition of probiotics excludes microbes that serve only as food for host and have no activity or influence on other microbes or the host environment. The trend of using probiotics in aquaculture is increasing due to research results indicated their ability to increase production and prevent disease in farm animals. The development of suitable probiotics for biocontrol in aquaculture would result in less reliance on chemicals and antibiotics and result in a safer environment (Rengpipat *et al.*, 1998; Balcázar *et al.*, 2006). Methods of selecting the probiotic bacteria for application in cultivation of aquatic animals include the following steps: (1) collection of referent information, (2) acquisition of probiotic, (3) evaluation of the ability of probiotic against pathogenic strains, (4) assessment of the pathogenicity of the probiotic, (5) evaluation of the effect of the probiotic in larvae and (6) economic cost benefit analysis according to Gomez-Gil *et al.* (2000). Holzapfel and Schillinger (2002) reported selection of suitable probiotic strains, considering the safety and non-pathogenicity of a new strain. A number of new probiotic strains have been developed in the region that have been demonstrated to have beneficial impacts on health in animal and human trials, including improved protection against intestinal pathogens and modulation of the immune system (Crittenden *et al.*, 2005).

Based on the principle of natural balanced control (Maeda, 1994) using beneficial bacteria or probiotic in controlling pathogens and enhancing the health of aquatic species to increase survival and productivity as reported in several papers, e.g. application of probiotic in feed is expected to use beneficial bacteria to replace pathogenic bacteria, increase immunity and survival from bacterial disease by reducing organic and inorganic that affect shrimp growth. Rengpipat *et al.* (1998) reported the isolation of pure strain of *Bacillus* S11 from intestine of *P. monodon*, showing that it can inhibit *V. harveyi* that cause luminescent disease. Lactic acid bacteria found in digestive tract of many organisms, milk products and fermented foods. Application of lactic acid bacteria (*Carnobacterium divergens*) in aquaculture was carried out in cod (Gilberg *et al.*, 1997). The results showed the cod fingerling fed lactic acid bacteria showed enhanced growth and

survival. After 12 days of feeding with lactic acid bacteria supplemented diet, level of *V. anguillarum* in fish was reduced and mortality was also reduced from 10 to 2.5%. Garcia-de-la-Banda *et al.* (1992) reported the use of *Streptococcus lactis* and *Lactobacillus bulgaricus* in cultured turbot (*Scophthalmus maximus*). The higher survival was obtained in the group fed lactic acid bacteria supplemented diet up to 66% compared to control group with 34% survivability. Similar result was reported by Gatesoupe (1994) using rotifer which has been fed lactic acid bacteria in rearing turbot larvae. The challenge test against vibriosis found that turbot larvae fed lactic acid bacteria showed higher resistance to vibrio infection.

Nikoskelainen *et al.* (2001) studied the prevention of furunculosis in rainbow trout (*Onchorhynchus mykiss*) using *Lactobacillus rhamnosus* (ATCC 53103), isolated from human at 10^9 and 10^{12} CFU/g in the feed. Sixteen days later, test the resistance against *Aeromonas salmonicida* that cause furunculosis and noted that mortality dropped from 52.6% in the control to 18.9% in the group fed feed with 10^9 CFU/g and 46.3% with 10^{12} CFU/g in the feed.

Prebiotic known as a substance used to induce growth of beneficial bacterial communities (Bifidobacteria and Lactobacilli) in the digestive system of animals. Characteristics of the substances with prebiotic properties include indigestibility or unabsorbable in stomach or small intestine, specificity with beneficial bacteria in intestine and in fermented form should benefit the host. Examples of prebiotics studied in humans are fructooligosaccharides (FOS) and inulin, but the information for aquatic animal is limited. FOS and inulin are indigestible by enzyme in stomach and small intestine of humans, however, they are bacterially fermented in large intestine, forming short-chain fatty acid (SCFA) that lower pH and inhibit the growth of pathogenic bacteria. Additionally, butyrate formed help stimulate the cell growth changes in the cell and increase the natural mortality of cells (apoptosis). All three properties can resist the development of cancer. It was also found that FOS help increase the concentration of calcium and magnesium in large intestine, resulting in better absorption of minerals and starting materials for epithelial cells of intestine, forming insoluble bile and fatty acid salts and hence reducing the effect of bile or fatty acid on cell in colon ocytes. Increased cation in large intestine can control cell turnover. Inulin can prevent development of cancer in large intestine (Cummings *et al.*, 2001). Furthermore, other prebiotics that have been reported are oligomannan is a short-chain carbohydrate composed of mannose which is abundantly found in yeast cell wall. Oligomannan has a positive effect on bacterial normal flora in the gut especially anaerobic group and also provides a positive impact on the health of the host. Holanda *et al.*, (2005) demonstrated that mannan can induce lectin

efficiency to inhibit *Bacillus cereus* in humans. Fernandez *et al.* (2002) showed mannan oligosaccharide can induce the increase of lactic acid bacteria and enterococcus in chicken intestine.

The same methods employed for bacterial detection are used to study bacterial colonization. Common techniques include immunoassays, immunocolony blot "ICB" and enzyme-linked immunosorbent assay "ELISA", molecular techniques, random amplification of polymorphic DNA "RAPD", amplified fragment length polymorphism "AFLP", terminal restriction fragment length polymorphism "T-RFLP", immunohistochemical methods and denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA, which was been recently introduced in microbial ecology (Spanggaard *et al.*, 2000; Cunningham, 2002; Temmerman *et al.*, 2003). Fluorescence *in situ* hybridization (FISH) is rapid technique with high specificity in signal detection to efficiently study bacterial colonies, enumerating bacteria and identification. FISH is a part of r-RNA approach hybridization technique that rely on the rRNA giving baseline information conveniently manipulated through computer network, e.g., Ribosomal Database Project (RDP II) which is comfortable for designing oligonucleotide probe, the most commonly used is DNA with approximately 20 nucleotide length fluorochrome labels known as "probe" which is bacteria specific from phylum to species, with target at rRNA on ribosome i.e. approximately 10,000 unit/cell (Amann *et al.*, 1997). When probe is used to detect the target which is reflect in the luminescence and the actual number of bacteria. Currently, FISH can be applied in bacteria detection, e.g. Trebesius *et al.*, (2000) tested *Helicobacter pylori* resistance in human stomach; Jensen *et al.* (2000) studied *Brachyspira* (*Serpulina*) *pilosicoli* infection in swine, bacteria that cause Lyme borreliosis in mite that carry disease to vertebrate animals (Hammer *et al.*, 2001) Tanaka *et al.*, (2004) studied bacterial diversity in intestine of *Haliotis discus hunnai* and found that *Vibrio* sp. is mainly in the group. Application and following of bacteria in intestine of *P. vannamei* has been reported by Viriyapongsutee *et al.* (2005) and Manz *et al.* (2000) detected the bacteria in sponge tissue.

At present, there is concern over the residue that impact consumers as rated by market trends for export of shrimp into the future. The measures regarding hygiene and environment are received to abstract the import. Therefore, producers are required to improve production efficiency of the shrimp products for quality and safety and the products that are environmentally friendly to exporting countries are advantageous in exporting for long-term competition and reducing the export problems.

In Thailand, probiotics have to be registered at the Feed Quality Control Unit of the Department of Livestock Development, Ministry of Agriculture and Co-operatives. Various forms are available, ranging from products with single microbe species or strain (e.g., yeast or bacteria) such as *Streptococcus faecium* and *Bacillus subtilis*) and those containing a mixture of 2 or more strains (e.g. mixtures of *L. acidophilus* and *S. faecium*, or mixtures of yeast, *Lactobacillus* spp. and *Streptococcus* spp.). The commercial products can be in liquid or powder form with a carrier such as ground yellow corn, corn gluten, paraffin oil, peptone, dextrose, rice bran, calcium silicate, wheat bran, sucrose, lactose and others. The Animal Feed Control Act of 1982 requires that probiotics supplemented in animal feeds contain no less than $1x10^5$ CFU/kg.

The current research tested the bacteria with probiotic properties and select the best strain that inhibit *V. harveyi* and *V. parahaemolyticus* by the method of agar well diffusion, co-culture that can tolerate salinity (*in vitro*). Subsequently, prebiotics is fortified in feed for white shrimp for a 6-weeks feeding period. Record shrimp growth, bacterial counts in hepatopancreas and intestine. Six weeks later feed is given with fresh cell of selected probiotics in 1.5% NaCl solution. Continue feeding for 7 days to monitor the stability and efficiency of probiotics on growth of *Vibrio* spp. Record the data on the monitoring of probiotic after 1, 5 and 10 days of probiotics feeding, including the test on resistance against luminescent bacteria in marine shrimp.

Materials and methods

1. *In vitro* study

1.1 Preparation of microorganisms

1.1.1 Isolation of lactic acid bacteria from fermented foods

Lactic acid bacteria from fermented foods, e.g. fermented pork, fish silage, fermented cabbage and fermented garlic (Figure 1) were cultured on De Man Rogosa Sharpe agar (MRS agar; Merck, Germany), supplemented with 1.5% NaCl and incubated at $37^{\circ}C$ for 48 h. Purified bacterial cell were stored at $4^{\circ}C$ and maintained by subculture monthly.

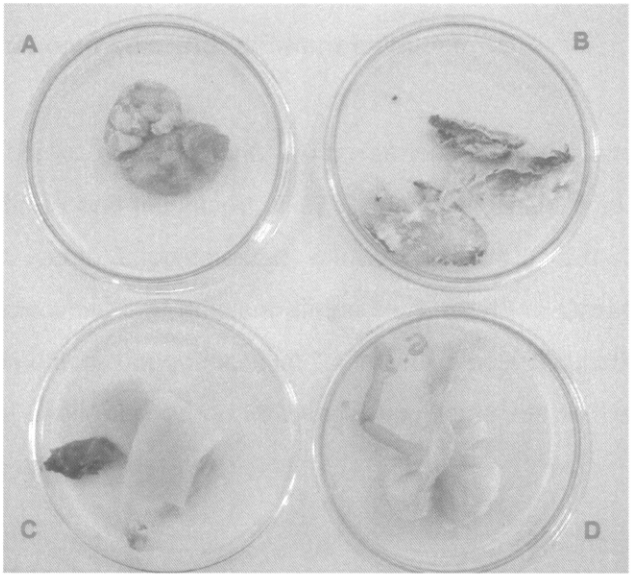

Figure 1 Fermented food items, (A) fermented pork, (B) fish silage, (C) fermented cabbage, and (D) fermented garlic.

1.1.2 Preparation of *Lactobacillus*

Four strains of *Lactobacillus*, i.e., *L. acidophilus* TISTR 1034, *L. brevis* TISTR 855, *L. casei* TISTR 1304 and *L. plantarum* TISTR 050 [Thailand Institute of Scientific and Technological Research (TISTR)] were used in this studied. Bacteria were grown on MRS agar supplemented with 1.5% NaCl and incubated at 37°C for 48 h. Cultures were stored at 4°C and maintained by subculture monthly.

1.1.3 Preparation of *V. harveyi* and *V. parahaemolyticus*

V. harveyi and *V.parahaemolyticus* were isolated from infected shrimp, culture in commercial farm which was submitted to Aquatic Animal Health Research Center, Faculty of Natural Resources, Prince of Songkla University for disease examination. Bacteria were isolated from hepatopancreas and cultured on Thiosulfate Citrate Bile Salts Sucrose (TCBS; Merck) and purified on Tripticase soy agar (TSA; Merck, Germany) supplemented with 1.5% NaCl. Purified bacterial cells were confirmed to genus and species by polymerase chain reaction (PCR) assay (Maeda *et al.*, 2002).

1.2 Growth of *Lactobacillus* on media with different salinities

Single colony of Lactic acid bacteria from fermented foods and *Lactobacillus* were inoculated into 5 ml MRS broth and incubated at 37°C for 48 h. Then, 5 µl of each sample was transferred into 5 ml MRS broth supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% NaCl, respectively. Samples were incubated at ambient temperature for 48 h. Growth was determined by observation of the development of opaqueness at 24 to 48 h post incubation. Growth was also confirmed by drop plating of each microbe on MRS agar supplemented with same concentration of NaCl (modified from Cheng and Chen, 1999).

1.3 Agar well diffusion assay

Inhibitory effect of Lactic acid bacteria from fermented foods and *Lactobacillus* against *V. harveyi* and *V. parahaemolyticus* was performed using agar well diffusion assay which was modified from Schillinger and Lucke (1989). The starter of *Lactobacillus* was inoculated into 5 ml MRS broth supplemented with 1.5% NaCl and incubated at 37°C for 48 h. *V. harveyi* and *V. parahaemolyticus* were pre-cultured in TSB supplemented with 1.5% NaCl and incubated at 37°C for 18-24 h. Three hundred milliliters of Mueller-Hinton-Agar (MHA; Merck, Germany) supplemented with 1.5% NaCl was heated and cooled to 40 to 50°C and then 300 µl of *V. harveyi* (OD_{610}= 0.1) was added into the media. Thirty milliliters of media was poured into the plate and leaved for solidification. The diameter of well on agar plate was 0.6 cm which was applied by sterile pasture pipette tip. The same method was used to *V. parahaemolyticus* too. The control depression is filled with sterile NaCl solution, with the remaining were filled with each saline-diluted bacteria at 50 µl (3 replications) and incubated at 37°C for 24-48 h. Antibacterial activity was define as the diameter (mm) of the clear inhibitory zone formed around the well and calculation of Arbitary Unit (AU/ml).

1.4 Co – culture method

The experiments were divided into two sets consist of experiment cultured *V. harveyi* (VH) and *L. plantarum* (LP) in 15 ppt seawater and cultured VH and LP in 15 ppt sterile seawater supplemented with 2.22% inulin. Shrimp feed as organic substances were added with 50 ppm

concentration in 15 ppt seawater (modified from Kiriratnikom *et al.*, 2000). An initial level of *V. harveyi* was 10^3 CFU.ml^{-1} and LP were 10^2, 10^3 and 10^4 CFU.ml^{-1}, respectively. All combinations were performed in triplicate. The other experiment was supplementation with 2.22% inulin in 15 ppt sterile seawater and repeated to test the growth of both bacteria.

1.4.1 Sample collection

Sample from each flask was collected at two-day interval for ten days. *L. plantarum* was enumerated by plate diluting technique on MRS agar supplemented with 1.5% NaCl and incubated at 37°C for 24-48 h. *V. harveyi* was enumerated by plate diluting technique on TCBS and incubated at 37°C for 18-24 h.

1.4.2 Statistical analysis

Data were analyzed using the analysis of variance (ANOVA) and Duncan's multiple range tests (DMRT) according to the statistical package for the social sciences (SPSS). Differences were considered significant at $p<0.05$.

2. *In vivo* study

This study was carried out to evaluate inulin in shrimp feed in order to determine the effect on growth performance as well as growth enhancement of normal flora in shrimp's gut.

2.1 Test animals

White shrimp with an average of 2-3 g were purchased from CoC standard farm. Test shrimp were subjected to examine for pathogens free (i.e., *Vibrio* spp., WSSV and TSV) prior to transport to the laboratory. Twenty shrimps were stocked in 200-L glass aquarium equipped with air stone and seawater flow through system. Shrimps were fed with commercial feed 4 times daily for one week prior to the experimental test.

2.2 Test diet

Four iso-nitrogenous and iso-caloric diets were formulated. All diets have the same composition except the level of inulin added to achieve different concentrations. Composition of the test diets was showed in table 1. All diets were mixed well and processed using Hobart mixer. Mixtures were stabilized with approx. 30% moisture by adding water and re-mixed for 20 min. Then the spaghetti-like feed was pelletized. This process was followed by four hours of drying in an air flow oven at 60°C until the moisture content was lower than 10%. The dry pellet was kept in two layer plastic bags in a refrigerator until use.

Table 1 Composition of the test diet.

Ingredients	T-1	T-2	T-3	T-4
Fish meal	28.75	28.75	28.75	28.75
Soybean meal	19	19	19	19
Corn gluten	3	3	3	3
Wheat four	20	20	20	20
Rice four	20.60	20.38	20.05	18.38
Fish oil	2	2	2	2
Lecithin	1.5	1.5	1.5	1.5
Cholesterol	0.15	0.15	0.15	0.15
Vitamin and mineral premix[1]	2.5	2.5	2.5	2.5
Choline chloride	0.3	0.3	0.3	0.3
Vitamin E	0.08	0.08	0.08	0.08
Vitamin C	0.1	0.1	0.1	0.1
Wheat gluten	0.5	0.5	0.5	0.5
BHT	0.02	0.02	0.02	0.02
Zeolite	1.5	1.5	1.5	1.5
Inulin	**0**	**0.22**	**0.55**	**2.22**
Total	100	100	100	100

[1] Vitamin and Mineral premix(g/kg diet) : Thiamine (B_1) 10 mg; Riboflavin (B_2) 20 mg; Pyridoxine (B_6) 10 mg; Cyanocobalamin (B_{12}) 2 mg; Retinal (A) 4,000 IU, Cholecalciferol (D_3) 2,000 IU; Menadione sodium bisulfite (K_3) 80 mg; Folic acid 5 mg; Calcium pantothenate 40 mg; Inositol 400 mg; Niacin 150 mg; DL-alpha-tocopherol (E) 50 IU; Choline chloride 6,000 mg; Ascorbic acid (C) 500 mg; Biotin 1 mg; NaCl 0.25 g; $MgCO_3$ 3.75 g; $FeSO_4$ 0.72 g; $(CH_3COO)_2$ $Ca.5H_2O$ 0.88 g; $ZnSO_4.7H_2O$ 0.088 g; $MnSO_4.4H_2O$ 0.040 g; $CuSO_4.5H_2O$ 0.008 g; $CoCl_2.6H_2O$ 0.00025 g; $KIO_3.6H_2O$ 0.00075 g

2.3 Growth performance

Shrimps with an initial weight of 2-3 g were stocked in a 200-L glass tank. Each experiment was conducted in 5 replications with 20 shrimps per system. Shrimps were fed experimental diets four times a day at different levels to satiation according to water temperature and molting cycle. Feeding was carried out for 6 weeks in a flow through seawater system with a flow rate of 0.8-1 L/min. Growth performance i.e., weight gain, FCR, survival and feed consumption were recorded.

2.4 Composition of gut normal flora

After feeding with test diet (mixed with fresh cell of LP in 1.5% NaCl) for 1 week, 5 shrimps were randomly sampled from each treatment on the day 1, 5 and 10 after stop feeding with test diet. Weight of each shrimp was recorded and the whole intestinal tract was dissected and separated into 2 parts; hepatopancreas and intestine. Each part was weighted and grinded in sterile mortar. After dilution in sterile 1.5% NaCl, total bacteria, total *Vibrio*, in green and yellow appearance were counted by spread plating technique. Serial dilution was performed for each sample and 100 µl of each dilution was dropped and spread on TCBS media. Culture plate was incubated at 35°C and colony forming unit (CFU) was counted after 24 h.

2.4.1 Sample preparation for FISH technique

The same sample was fixed with 10% buffered formalin at 4°C for 12 h and transfer to 70% ethanol for further process. Sample are ground in mortar and smear on 1% gelatin coated slide and dehydrated with ethanol series for 3 min. Dry samples are ready for FISH technique processing (Viriyapongsutee *et al.*, 2005).

2.4.2 FISH technique

Groups of bacteria such as *Vibrio* and specific probiotics were detected and quantified by FISH technique using various DNA-probes (Table 2). Probes were labeled with reddish luminescence (Cy-3) while each probe had different formamide concentration. Samples and probe

were incubated in hybridizer (TECHNE, UK) at 46°C filled with vapour of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 0.01% SDS and formamide concentration was designed from each probe) for 2 h and washed in washing buffer once at 48°C for 15 min, dropped with anti-fading solution (*p*-phenylenediamine 0.1% in mixture of Na_2CO_3 and glycerol on slides to delay the fading of luminescence and cover with cover glass before examining under epifluorescence microscope (Olympus AX 70) and photographed with cooled CCD (Olympus DP 71).

Table 2 List of DNA probes used in FISH technique.

No.	Probe name	Sequence (5'-3')	Specificity	Reference
1	EUB mixed		Eubacteria	
	EUB I	GCT GCC TCC CGT AGG AGT	Most bacteria	Amann *et al.*, 1999
	EUB II	GCA GCC ACC CGT AGG TGT	Planctomycetales	Daims *et al.*, 1999
	EUB III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	Daims *et al.*, 1999
2	GV	AGG CCA CAA CCT CCA AGT AG	*Vibrio* spp.	Eilers *et al.*, 2000
3	Lab158	GGT ATT AGC A(T/C)C TGT TTC CA	*Lactobacillus* spp., *Enterococcus* spp.	Harmsen *et al.*, 1999
4	Enc131	CCC CTT CTG ATG GGC AGG	*Enterococcus* spp.	Behr *et al.*, 2000

2.5 Challenge test

Twenty shrimps from each treatment were challenged with pathogenic vibrio (*Vibrio harveyi*) by immersion method. Suspension of *V. harveyi* was prepared in 1.5% NaCl and added to the culture tank to attain the final concentration of 3×10^5 CFU/ml. Test shrimp was exposed to bacterial suspension for 1 h before moved to normal condition. Survival rate was recorded for 10 days post infection.

Results

1. *In vitro* study

1.1 Test for selected bacteria growth on media with varying salinities

The study on tolerance of selected bacteria in varying salinities showed that all bacterial strains were grown in both MRS broth and MRS agar supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% NaCl, respectively (Table 3).

Table 3 Microbial growth in MRS broth and MRS agar at the varying concentrations of NaCl.

No.	Bacteria	0.5%	1.0%	1.5%	2.0%	2.5%	3.0%
1	LA	+	+	+	+	+	+
2	LB	+	+	+	+	+	+
3	LC	+	+	+	+	+	+
4	LD	+	+	+	+	+	+
5	*L. acidophilus* TISTR 1034	+	+	+	+	+	+
6	*L. brevis* TISTR 855	+	+	+	+	+	+
7	*L. casei* TISTR 1304	+	+	+	+	+	+
8	*L. plantarum* TISTR 050	+	+	+	+	+	+

\+ Bacteria can grow on MRS broth and MRS agar

\- Bacteria can not grow on MRS broth and MRS agar

LA = Lactic acid bacteria from fermented pork

LB = Lactic acid bacteria from fish silage

LC = Lactic acid bacteria from fermented cabbage

LD = Lactic acid bacteria from fermented garlic

1.2 Test for inhibitory effect on *V. harveyi* and *V. parahaemolyticus* by agar well diffusion assay

Inhibitory effect of each bacterial strain against *V. harveyi* and *V. parahaemolyticus* was performed using agar well diffusion assay which was modified from Schillinger and Lucke (1989). Bacteria were conducted to study from 4 fermented materials and 4 strains of *Lactobacillus*. The results showed that all 8 bacterial strains were capable of inhibiting *V. harveyi* (Table 4) and *V. parahaemolyticus* (Table 5) comparing the diameter of inhibitory zone and arbitary unit. The result indicated that the capability of *L. plantarum* that inhibited *V. harveyi* with largest diameter of inhibitory zone and arbitary unit. Diameter of inhibitory zone was 12.50±0.50 mm and arbitary unit was 50.00±0.00 AU/ml which were significantly different (p<0.05) (Table 4). And also, *L. plantarum* inhibited growth of *V. parahaemolyticus* with largest inhibitory zone and arbitary unit of 12.83±0.29 mm and 55.53±9.58 AU/ml, respectively, which were significantly different (p<0.05) (Table 5).

Table 4 Inhibitory activity of selected microbial against *V. harveyi* by agar well diffusion method.

No.	Bacteria	Diameter of inhibitory zone (mm)	Arbitary Unit (AU/ml)
1	LA	9.67 ± 0.15^{cd}	40.00 ± 10.00^{abc}
2	LB	9.83 ± 0.25^{cd}	43.33 ± 5.77^{abc}
3	LC	8.73 ± 0.25^{e}	33.33 ± 5.77^{c}
4	LD	9.23 ± 0.15^{de}	36.67 ± 5.77^{bc}
5	*L. acidophilus* TISTR 1034	9.50 ± 0.50^{cde}	40.00 ± 0.00^{abc}
6	*L. brevis* TISTR 855	10.83 ± 0.76^{b}	46.60 ± 5.77^{ab}
7	*L. casei* TISTR 1304	10.33 ± 0.58^{bc}	43.40 ± 1.96^{abc}
8	*L. plantarum* TISTR 050	12.50 ± 0.50^{a}	50.00 ± 0.00^{a}

Data are mean values of triplicate determination ± standard deviation

Means within columns not sharing the same superscript are significantly different (p < 0.05).

LA = Lactic acid bacteria from fermented pork

LB = Lactic acid bacteria from fish silage

LC = Lactic acid bacteria from fermented cabbage

LD = Lactic acid bacteria from fermented garlic

Table 5 Inhibitory activity of selected microbial against *V. parahaemolyticus* by agar well diffusion method.

No.	Bacteria	Diameter of inhibitory zone (mm)	Arbitary Unit (AU/ml)
1	LA	9.57 ± 0.12^{d}	40.00 ± 10.00^{de}
2	LB	9.83 ± 0.29^{d}	43.33 ± 5.77^{cd}
3	LC	8.87 ± 0.12^{e}	33.33 ± 5.77^{e}
4	LD	9.47 ± 0.25^{d}	36.67 ± 5.77^{de}
5	*L. acidophilus* TISTR 1034	9.83 ± 0.29^{d}	40.00 ± 0.00^{de}
6	*L. brevis* TISTR 855	11.67 ± 0.58^{b}	46.67 ± 5.77^{cd}
7	*L. casei* TISTR 1304	10.50 ± 0.50^{c}	43.33 ± 5.77^{cde}
8	*L. plantarum* TISTR 050	12.83 ± 0.29^{a}	55.53 ± 9.58^{c}

Data are mean values of triplicate determination ± standard deviation

Means within columns not sharing the same superscript are significantly different ($p < 0.05$).

LA = Lactic acid bacteria from fermented pork

LB = Lactic acid bacteria from fish silage

LC = Lactic acid bacteria from fermented cabbage

LD = Lactic acid bacteria from fermented garlic

1.3 Co-Culture method

1.3.1 Culture of VH and LP in seawater

Results of inhibiting effect on *V. harveyi* and *V. parahaemolyticus* by agar well diffusion method revealed the primary efficiency of LP. LP were at 10^2, 10^3 and 10^4 CFU.ml^{-1} that co-cultured with *V. harveyi* at 10^3 CFU.ml^{-1} in 15 ppt sterile seawater and added to shrimp feed (representing organic material) with concentration 50 ppm for 10 day. It noted that day-2, treatment 1 (control without LP), treatment 2 (with 10^2 CFU.ml^{-1} LP), and treatment 3 (10^3 CFU.ml^{-1} LP) caused probiotic development of *V. harveyi* as compared to treatment 4 (10^4 CFU.ml^{-1} LP) and further noted that on 8th and 10th day, growth of *V. harveyi* in treatment 4 showed a trend of decline (Table 6) which were significantly different ($p<0.05$). While growth of LP co-cultured with *V. harveyi* showed on 2nd, 4th and 6th day of treatment 4 (addition of 10^4 CFU.ml^{-1} LP) the highest growth compared to other treatments with significant difference ($p<0.05$) and on 8th and 10th day, detected the growth of LP in treatment 4 as well (Table 7).

Hence, comparing all treatments, treatment 4 with (10^4 CFU.ml^{-1} LP) showed a trend of inhibiting growth of *V. harveyi* at 10^3 CFU.ml^{-1}, while treatments 2 and 3 with LP at 10^2 and 10^3 CFU.ml^{-1}, respectively; were incapable of inhibiting growth of *V. harveyi* at 10^3 CFU.ml^{-1}.

Table 6 The growth of *V. harveyi* were co-cultured with *L. plantarum* TISTR 050 (LP).

Treatments	*V. harveyi* (Log CFU.ml^{-1})					
	Day-0	Day-2	Day-4	Day-6	Day-8	Day-10
T1 (VH 10^3)	3.00	6.59 ± 0.04^{a}	5.95 ± 0.03^{b}	5.00 ± 0.13^{c}	5.55 ± 0.02^{a}	5.32 ± 0.06^{a}
T2 (LP 10^2:VH 10^3)	3.00	6.60 ± 0.08^{a}	5.56 ± 0.05^{c}	4.30 ± 0.02^{d}	5.21 ± 0.01^{b}	5.17 ± 0.15^{ab}
T3 (LP 10^3:VH 10^3)	3.00	6.64 ± 0.04^{a}	5.95 ± 0.05^{b}	5.38 ± 0.01^{c}	5.34 ± 0.01^{b}	5.20 ± 0.20^{ab}
T4 (LP 10^4:VH 10^3)	3.00	6.17 ± 0.01^{b}	6.32 ± 0.16^{a}	5.56 ± 0.01^{a}	5.20 ± 0.16^{b}	5.01 ± 0.02^{b}

Means within columns not sharing the same superscript are significantly different ($p < 0.05$).

Table 7 The growth of *L. plantarum* TISTR 050 (LP) were co-cultured with *V. harveyi*.

Treatments	*L. plantarum* TISTR 050 (Log CFU.ml^{-1})					
	Day-0	Day-2	Day-4	Day-6	Day-8	Day-10
T2 (LP 10^2:VH 10^3)	2	3.90 ± 0.01^{b}	2.90 ± 0.01^{b}	2.31 ± 0.03^{b}	nd	nd
T3 (LP 10^3:VH 10^3)	3	3.97 ± 0.02^{b}	3.74 ± 0.05^{b}	3.18 ± 0.09^{ab}	2.62 ± 0.08	2.50 ± 0.02
T4 (LP 10^4:VH 10^3)	4	5.03 ± 0.02^{a}	4.74 ± 0.08^{a}	3.45 ± 0.22^{a}	2.96 ± 0.06	2.72 ± 0.06

Means within columns not sharing the same superscript are significantly different ($p < 0.05$)

nd = not detected

1.3.2 Culture of VH and LP in substrate with inulin at varying concentrations

Prior to co-culture with addition of inulin in TSA for the culture of VH and addition of MRS for the culture of LP at 0.02, 0.05, and 2.22% as presented in Table 8. Results showed at 18th and 24th h during the log phase of VH in treatment with inulin. However, at 72 h, growth of VH in treatment with inulin declined as compared to the treatment without inulin addition significantly (p<0.05) (Table 9). The growth of LP in treatment with inulin increased markedly from 48th, 96th and 120th h. respectively; which were significantly different (p<0.05) as presented in Table 10.

Table 8 Experimental design for the effect of inulin on growth enhancement of bacteria.

Treatments	Bacteria species	
	V. harveyi (VH)	*L. plantarum* (LP)
1	0	0
2	Beneo 0.02%	Beneo 0.02%
3	Beneo 0.05%	Beneo 0.05%
4	Beneo 2.22%	Beneo 2.22%

Table 9 The growth of *V. harveyi* in the different concentration of inulin (Log CFU.ml^{-1}).

Treatments	The growth of *V. harveyi* (Log CFU.ml^{-1})					
	h-0	h-18	h-24	h-30	h-48	h-72
VH1	3	7.38±0.03^{c}	7.65±0.17^{b}	8.53±0.08^{a}	7.81±0.29^{a}	7.92±0.04^{a}
VH2	3	7.46±0.03^{a}	8.61±0.02^{a}	8.22±0.07^{b}	7.69±0.21^{a}	7.76±0.04^{b}
VH3	3	7.45±0.02^{ab}	8.66±0.04^{a}	8.44±0.12^{a}	7.79±0.10^{a}	7.82±0.04^{b}
VH4	3	7.39±0.05^{bc}	7.94±0.31^{b}	8.54±0.07^{a}	7.98±0.34^{a}	7.81±0.07^{b}

Means within columns not sharing the same superscript are significantly different ($p < 0.05$).

VH1 = *V. harveyi* cultured in TSA without inulin

VH2 = *V. harveyi* cultured in TSA supplemented with 0.02% inulin

VH3 = *V. harveyi* cultured in TSA supplemented with 0.05% inulin

VH4 = *V. harveyi* cultured in TSA supplemented with 2.22% inulin

Table 10 The growth of *L. plantarum* in the different concentration of inulin (Log CFU.ml^{-1}).

Treatments	The growth of *L. plantarum* (Log CFU.ml^{-1})				
	h-0	h-48	h-72	h-96	h-120
LP1	3	5.10±0.17^{c}	5.25±0.06^{c}	5.53±0.08^{d}	5.59±0.04^{c}
LP2	3	5.26±0.45^{c}	6.41±0.05^{c}	6.68±0.03^{c}	7.34±0.01^{b}
LP3	3	6.03±0.05^{b}	6.96±0.16^{b}	7.21±0.18^{b}	7.78±0.05^{a}
LP4	3	7.03±0.02^{a}	7.53±0.02^{a}	7.75±0.03^{a}	7.79±0.07^{a}

Means within columns not sharing the same superscript are significantly different ($p < 0.05$).

LP1 = *L. plantarum* TISTR 050 cultured in MRS without inulin

LP2 = *L. plantarum* TISTR 050 cultured in MRS supplemented with 0.02% inulin

LP3 = *L. plantarum* TISTR 050 cultured in MRS supplemented with 0.05% inulin

LP4 = *L. plantarum* TISTR 050 cultured in MRS supplemented with 2.22% inulin

1.3.3 Culture of VH and LP in seawater with 2.22% inulin prebiotic

Results of co-culture in 15 ppt sterile seawater with 2.22% inulin showed treatment 4 with gradual decline in growth of VH as compared to the control and were significantly different (p<0.05) as presented in table 11. For growth of LP in treatment with prebiotic increased compared to control, which was significantly different (p<0.05) as presented table 12.

Table 11 The growth of *V. harveyi* (VH) in co-culture experiment with inulin supplementation.

Treatments	Growth of VH (Log CFU.ml^{-1})							
	Day-0	Day-2	Day-4	Day-6	Day-8	Day-10	Day-12	Day-14
PV	3.00	5.79±0.26^{a}	6.32±0.11^{a}	6.33±0.17^{a}	6.49±0.03^{a}	6.13±0.07^{a}	5.72±0.09^{a}	5.92±0.04^{a}
PVL2	3.00	5.26±0.12^{b}	6.33±0.23^{a}	6.23±0.09^{a}	6.02±0.05^{ab}	6.08±0.22^{a}	5.67±0.03^{a}	5.69±0.05^{b}
PVL3	3.00	5.10±0.17^{bc}	5.67±0.12^{b}	5.73±0.02^{b}	5.91±0.06^{b}	5.60±0.02^{b}	5.60±0.11^{ab}	5.59±0.02^{b}
PVL4	3.00	4.69±0.39^{c}	5.08±0.16^{c}	5.30±0.16^{c}	5.39±0.52^{c}	5.37±0.11^{b}	5.48±0.02^{b}	4.87±0.15^{c}

PV : VH culture alone

PVL2 : VH (10^{3}) cultured with LP (10^{2})

PVL3 : VH (10^{3}) cultured with LP (10^{3})

PVL4 : VH (10^{3}) cultured with LP (10^{4})

Table 12 The growth of *L. plantarum* TISTR 050 in co-culture experiment with inulin supplementation.

Treatments	Growth of LP (Log CFU.ml^{-1})						
	Day-0	Day-2	Day-4	Day-6	Day-8	Day-10	Day-12
PVL2	2.36±0.06^{c}	3.67±0.04^{c}	3.77±0.04^{c}	4.14±0.14^{c}	4.12±0.07^{c}	4.12±0.05^{b}	4.12±0.05^{b}
PVL3	3.36±0.04^{b}	4.65±0.02^{b}	4.73±0.06^{b}	4.62±0.02^{b}	4.56±0.06^{b}	4.32±0.21^{b}	4.32±0.21^{b}
PVL4	4.47±0.02^{a}	5.63±0.06^{a}	5.67±0.02^{a}	5.62±0.02^{a}	5.42±0.09^{a}	5.06±0.10^{a}	5.06±0.10^{a}

PVL2 : VH (10^{3}) cultured with *L. plantarum* (10^{2})

PVL3 : VH (10^{3}) cultured with *L. plantarum* (10^{3})

PVL4 : VH (10^{3}) cultured with *L. plantarum* (10^{4})

2. *In vivo* study

2.1 Total bacteria in white shrimp before starting experiment

The experiment initially enumerated bacteria in hepatopancreas and intestine of white shrimp from ten samples. The results showed that average of total bacteria and *Vibrio* spp. in hepatopancreas were 6.06±0.51 and 2.66±0.31 Log CFU.g^{-1}, respectively. Total bacteria and *Vibrio* spp. in the intestine were 8.48±0.42 and 5.72±0.59 Log CFU.g^{-1}, respectively. (Figure 2)

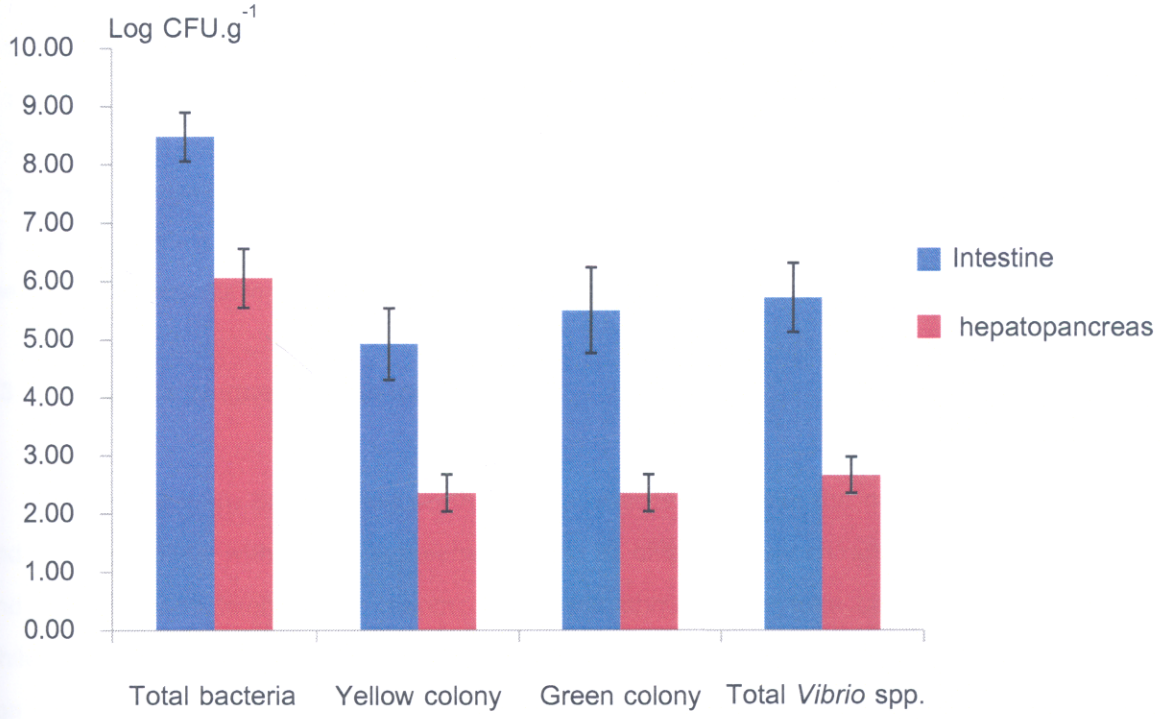

Figure 2 Enumeration of bacteria in intestine and hepatopancreas of white shrimp before starting experiment (Log CFU.g^{-1}).

2.2 Growth performance

After feeding trial for 6 weeks, the results showed that inulin had minimal effect and non significant on growth of white shrimp (Table 13). Supplementation of inulin at 2.2 g/kg in treatment 2 showed a little improvement on growth while higher level of inulin at 22.2 g/kg in treatment 4 had

no effect on growth. It was noticed that the diet containing highest level of inulin (22.2 g/kg) was harder in texture than the other group.

Table 13 Growth performance, survival rate, FCR, rate of feed intake (RF) of shrimp fed experimental diets for a 6-week period.

Treatment	Initial weight (g)	Final weight (g)	Survival rate (%)	Weight gain (%)	Specific growth rate (%/day)	FCR	RF (%/shrimp /day)
T1	4.71±0.00	11.06±0.30	98.75±2.5	134.72±6.24	2.03±0.06	1.35±0.09	2.55±0.09[a]
T2	4.72±0.01	11.25±0.40	98.75±2.5	138.50±9.07	2.07±0.09	1.31±0.08	2.49±0.05[ab]
T3	4.73±0.01	11.06±0.39	100.00±0.00	133.69±8.14	2.02±0.08	1.29±0.07	2.45±0.07[ab]
T4	4.72±0.02	10.70±0.47	100.00±0.00	126.77±10.08	1.95±0.11	1.38±0.11	2.54±0.08[a]

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

The different superscript in the same column indicated statistical different at $p<0.05$

2.3 Bacteria enumerated on media

Bacteria were enumerated on the media after feeding with the control feed with no inulin, and the test diets with inulin supplementation, in different concentrations of inulin, 0.22, 0.55 and 2.22%, respectively, for 1 month. The result showed that total bacteria in the intestine of white shrimp fed inulin-supplemented feeds, tended to increase over the control although non significantly ($p>0.05$). *Vibrio* spp. and *Enterococcus* spp. in the intestine of white shrimp of experiment 2 (0.22% inulin) was lowest ($p>0.05$) (Table 14). Total bacteria in the hepatopancreas of white shrimps of experiment 2 showed the quantity was lower than other groups ($p>0.05$). *Vibrio* spp. in the hepatopancreas of experiment 4 was highest, when compared with other groups ($p<0.05$) and *Enterococcus* spp. in the hepatopancreas of white shrimp of control group was lowest when compared with other groups ($p<0.05$) (Table 15).

Table 14 Enumeration of bacteria in intestine of white shrimp after feeding with test diets for 6 weeks.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria (cultured on TSA)	Total *Vibrio* spp. (cultured on TCBS)	*Enterococcus* spp. (cultured on KMA)	*Lactobacillus* spp. (cultured on MRS)
T1	8.04±0.61	6.12±0.49	7.48±0.92	0
T2	8.81±0.78	5.44±0.65	6.37±1.74	0
T3	8.93±0.64	6.20±1.00	7.29±1.22	0
T4	8.57±0.19	5.77±0.90	7.08±0.44	0
	NS	NS	NS	

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

NS = not significant

Table 15 Enumeration of bacteria in hepatopancreas of white shrimp after feeding with test diets for 6 weeks.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria (cultured on TSA)	Total *Vibrio* spp. (cultured on TCBS)	*Enterococcus* spp. (cultured on KMA)	*Lactobacillus* spp. (cultured on MRS)
T1	6.25 ± 0.90^{a}	2.58 ± 0.27^{b}	2.43 ± 0.38^{c}	0
T2	5.19 ± 0.62^{a}	2.20 ± 0.17^{b}	4.79 ± 0.07^{a}	0
T3	6.64 ± 0.84^{a}	2.36 ± 0.10^{b}	4.34 ± 0.03^{b}	0
T4	5.58 ± 0.27^{a}	3.07 ± 0.51^{a}	4.57 ± 0.05^{ab}	0

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

The different superscript in the same column indicated statistical different at $p<0.05$.

The control and experiment groups were fed with different concentrations of inulin, 0, 0.22, 0.55 and 2.22%, respectively. The *L. plantarum* suspension was diluted in 1.5% saline solution and mixed in the feed by spaying and air drying. The *L. plantarum* in feed were enumerated for following 7 days. The mean of *L. plantarum* in experiments 1, 2, 3 and 4 were $1.63x10^{6}\pm3.09x10^{5}$, $1.64x10^{6}\pm2.89x10^{5}$, $1.63x10^{6}\pm2.63x10^{5}$ and $1.63x10^{6}\pm2.72x10^{5}$ CFU.g^{-1}, respectively. *L. plantarum* the intestine and hepatopancreas of white shrimp were monitored on days 1, 5 and 10, respectively and enumerated by culturing on the media and non-culturing using the FISH technique.

In condition, feeding with *L. plantarum* supplemented feed. Bacteria in the white shrimp's intestine were enumerated on day 1 by culturing on media. The results showed that total bacteria and *Enterococcus* spp. of the experiment 4 and total *Vibrio* spp. of the experiment 2 had the lower numbers than other groups ($p>0.05$). *L. plantarum* had the quantity marginally in all experiment groups although non significantly ($p>0.05$) (Table 16). Total *Vibrio* spp. in the hepatopancreas of white shrimp of the experiment 4 showed the lowest number, but not different from the control group and *L. plantarum* in the control group showed the lowest, which was significantly different ($p<0.05$) (Table 17).

Table 16 Enumeration of bacteria in the intestine of white shrimp after feeding with test diets supplemented with probiotic at day 1.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria (cultured on TSA)	**Total *Vibrio* spp. (cultured on TCBS)**	***Enterococcus* spp. (cultured on KMA)**	***Lactobacillus* spp. (cultured on MRS)**
T1	8.47±0.37	5.78±0.21	6.97±0.48	4.46±0.08
T2	8.46±0.43	5.20±0.35	7.06±0.67	4.49±0.14
T3	8.32±0.37	6.07±0.65	6.75±6.35	4.44±0.12
T4	8.14±0.62	5.44±0.36	6.35±0.18	4.49±0.11
	NS	NS	NS	NS

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

NS = not significant

Table 17 Enumeration of bacteria in the hepatopancreas of white shrimp after feeding with test diets supplemented with probiotic at day 1.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria (cultured on TSA)	**Total *Vibrio* spp. (cultured on TCBS)**	***Enterococcus* spp. (cultured on KMA)**	***Lactobacillus* spp. (cultured on MRS)**
T1	4.46 ± 0.32^{ab}	2.42 ± 0.10^{b}	2.10 ± 0.17^{a}	3.31 ± 0.03^{b}
T2	4.81 ± 0.50^{a}	2.89 ± 0.19^{a}	3.58 ± 0.38^{a}	3.47 ± 0.06^{a}
T3	3.70 ± 0.64^{b}	2.65 ± 0.33^{ab}	2.99 ± 0.57^{a}	3.51 ± 0.10^{a}
T4	4.07 ± 0.52^{b}	2.36 ± 0.32^{b}	3.94 ± 1.26^{a}	3.54 ± 0.03^{a}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. The different superscript in the same column indicates statistical difference at $p<0.05$.

After feeding with *L. plantarum*, bacteria in the intestine were enumerated on day 5 by culturing on media. The results showed that total bacteria and *Enterococcus* spp. of the control group and total *Vibrio* spp. of the experiment 3 had lower numbers than other group, but not significant ($p>0.05$). *L. plantarum* of the experiment 2 had the lowest, which was significantly different ($p<0.05$) (Table 18). *Vibrio* spp. and *Enterococcus* spp. in the hepatopancreas of experiment 4 had the lowest and significantly different ($p<0.05$) when compared with the control group and *L. plantarum* of the control group showing the lowest, which was significantly different ($p<0.05$) (Table 19).

Table 18 Enumeration of bacteria in the intestine of white shrimp after feeding with test diets supplemented with probiotic at day 5.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria (cultured on TSA)	**Total *Vibrio* spp. (cultured on TCBS)**	***Enterococcus* spp. (cultured on KMA)**	***Lactobacillus* spp. (cultured on MRS)**
T1	7.82 ± 0.52^{a}	5.83 ± 0.84^{a}	6.65 ± 0.27^{a}	4.14 ± 0.12^{ab}
T2	8.08 ± 0.44^{a}	5.98 ± 0.21^{a}	7.39 ± 0.73^{a}	4.05 ± 0.02^{b}
T3	8.18 ± 0.22^{a}	5.52 ± 0.56^{a}	6.84 ± 0.32^{a}	4.18 ± 0.10^{ab}
T4	8.24 ± 0.21^{a}	5.60 ± 0.39^{a}	7.27 ± 0.56^{a}	4.27 ± 0.08^{a}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. The different superscript in the same column indicates statistical difference at $p<0.05$

Table 19 Enumeration of bacteria in the hepatopancreas of white shrimps after feeding with test diets supplemented with probiotic at day 5.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria (cultured on TSA)	**Total *Vibrio* spp. (cultured on TCBS)**	***Enterococcus* spp. (cultured on KMA)**	***Lactobacillus* spp. (cultured on MRS)**
T1	4.65±0.43[a]	3.98±0.47[a]	3.45±0.34[a]	3.16±0.08[b]
T2	4.20±0.65[a]	2.53±0.07[c]	2.84±0.12[b]	3.36±0.05[a]
T3	5.06±0.99[a]	3.05±0.20[b]	2.55±0.13[b]	3.36±0.08[a]
T4	5.16±0.42[a]	2.46±0.15[c]	2.32±0.28[b]	3.40±0.08[a]

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. The different superscript in the same column indicates statistical difference at $p<0.05$

After feeding with *L. plantarum*, bacteria in the intestine of white shrimp were enumerated on day 10 by culturing on media. The results showed that total bacteria of the experiment 4, *Vibrio* spp. of the experiment 2 and *Enterococcus* spp. of the control group tended to be lower than other groups, without significance ($p>0.05$) and *L. plantarum* of the control group tended to be lower than other groups with significant difference ($p<0.05$) (Table 20). Total bacteria and *Vibrio* spp in the hepatopancreas of the control group was lower than the experiment groups, without significant difference ($p>0.05$). *L. plantarum* of experiment 3 was higher than the control group with significant difference ($p<0.05$) (Table 21).

Table 20 Enumeration of bacteria in the intestine of white shrimp after feeding with test diets supplemented with probiotic at day 10.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria (cultured on TSA)	**Total *Vibrio* spp. (cultured on TCBS)**	***Enterococcus* spp. (cultured on KMA)**	***Lactobacillus* spp. (cultured on MRS)**
T1	8.30±0.15[a]	6.53±0.43[a]	6.40±0.08[a]	3.83±0.05[c]
T2	8.50±0.11[a]	6.20±0.06[a]	7.01±0.97[a]	3.89±0.02[ab]
T3	8.64±0.26[a]	6.48±0.29[a]	7.41±0.58[a]	3.96±0.01[b]
T4	8.24±0.28[a]	6.26±0.27[a]	7.20±0.41[a]	3.97±0.01[a]

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. The different superscript in the same column indicates statistical difference at $p<0.05$

Table 21 Enumeration of bacteria in the hepatopancreas of white shrimp after feeding with test diets supplemented with probiotic at day 10.

Treatment	Enumeration of bacteria [Log (CFU/g)]			
	Total bacteria	**Total *Vibrio* spp.**	***Enterococcus* spp.**	***Lactobacillus* spp.**
	(cultured on TSA)	**(cultured on TCBS)**	**(cultured on KMA)**	**(cultured on MRS)**
T1	3.48±0.31[a]	2.54±0.28[a]	2.91±0.51[a]	2.32±0.28[b]
T2	4.54±0.37[a]	2.79±0.43[a]	2.84±0.47[a]	2.62±0.15[ab]
T3	4.33±0.90[a]	2.54±0.28[a]	2.92±0.53[a]	2.82±0.11[a]
T4	3.70±0.35[a]	2.76±0.15[a]	2.54±0.28[a]	2.69±0.21[ab]

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. The different superscript in the same column indicates statistical difference at $p<0.05$

2.4 FISH technique

After feeding with the control feed and the test feeds in different concentrations of inulin, 0.22, 0.55 and 2.22%, respectively. Thereafter, the feed were mixed with fresh cell of *L. plantarum* in 1.5% saline solution and fed for next 7 days. Bacteria in intestine and hepatopancreas of white shrimp were enumerated by FISH technique using DNA probe, such as, EUB-mixed with Cy-3 labeled probe to detect all bacteria, GV with Cy-3 labeled probe for *Vibrio* spp., ENC131 with Cy-3 labeled probe for *Enterococcus* spp. and Lab158 with Cy-3 labeled probe for *Lactobacillus* spp. Each group of bacteria was monitored on day 1, 5 and 10, respectively.

The results showed that total bacteria in the intestine of white shrimp of experiment 4 before feeding with *L. plantarum* (Day-0) and experiment 2 after day 1 of feeding with *L. plantarum* had the lowest numbers, with significance ($p<0.05$) when compared to the control group (Table 22). Total bacteria in the hepatopancreas of white shrimp of experiment 3 that were the lowest before feeding with *L. plantarum* (Day-0) but they become the highest after feeding with *L. plantarum* on day 10 (Table 23). Epifluorescence micrographs of microbes (arrows) in various treatments were detected by FISH technique using EUB-mixed Cy3-labeled gene probe before started feeding with *L. plantaurm* supplemented feed as presented in Fig 3.

Table 22 Total bacteria in intestine of white shrimp were enumerated by FISH technique using EUB -mixed Cy3-labeled gene probe on different days.

Treatment	Total bacteria in intestine of white shrimps [Log (cell/g)]			
	Day-0	Day-1	Day-5	Day-10
T1	9.69±0.14^{ab}	9.90±0.05^{a}	9.84±0.17^{a}	9.84±0.11^{a}
T2	9.90±0.12^{a}	9.66±0.03^{b}	9.95±0.03^{a}	9.79±0.08^{a}
T3	9.53±0.21^{b}	9.80±0.15^{ab}	9.78±0.24^{a}	9.84±0.15^{a}
T4	9.49±0.24^{b}	9.72±0.08^{b}	9.88±0.35^{a}	9.74±0.09^{a}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. Values in the same column sharing a common superscript are not statistically difference (p>0.05).

Day-0 : Before started feeding with *L. plantarum* supplemented feed

Day-1 : After stopped feeding with mixed *L. plantarum* supplemented feed at 1 day

Day-5 : After stopped feeding with mixed *L. plantarum* supplemented feed at 5 day

Day-10 : After stopped feeding with mixed *L. plantarum* supplemented feed at 10 day

Table 23 Total bacteria in hepatopancreas of white shrimp were enumerated by FISH technique using EUB-mixed Cy3-labeled gene probe on different days.

Treatment	Total bacteria in hepatopancreas of white shrimps [Log (cell/g)]			
	Day-0	Day-1	Day-5	Day-10
T1	7.26±0.11^{a}	7.46±0.22^{a}	7.38±0.09^{a}	7.29±0.20^{a}
T2	7.32±0.02^{a}	7.62±0.08^{a}	7.51±0.11^{a}	7.30±0.23^{a}
T3	6.82±0.29^{b}	7.44±0.20^{a}	7.44±0.08^{a}	7.45±0.09^{a}
T4	6.99±0.19^{ac}	7.67±0.14^{a}	7.39±0.21^{a}	7.37±0.04^{a}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. Values in the same column sharing a common superscript are not statistically difference (p>0.05).

Day-0 : Before started feeding with *L. plantarum* supplemented feed

Day-1 : After stopped feeding with mixed *L. plantarum* supplemented feed at 1 day

Day-5 : After stopped feeding with mixed *L. plantarum* supplemented feed at 5 day

Day-10 : After stopped feeding with mixed *L. plantarum* supplemented feed at 10 day

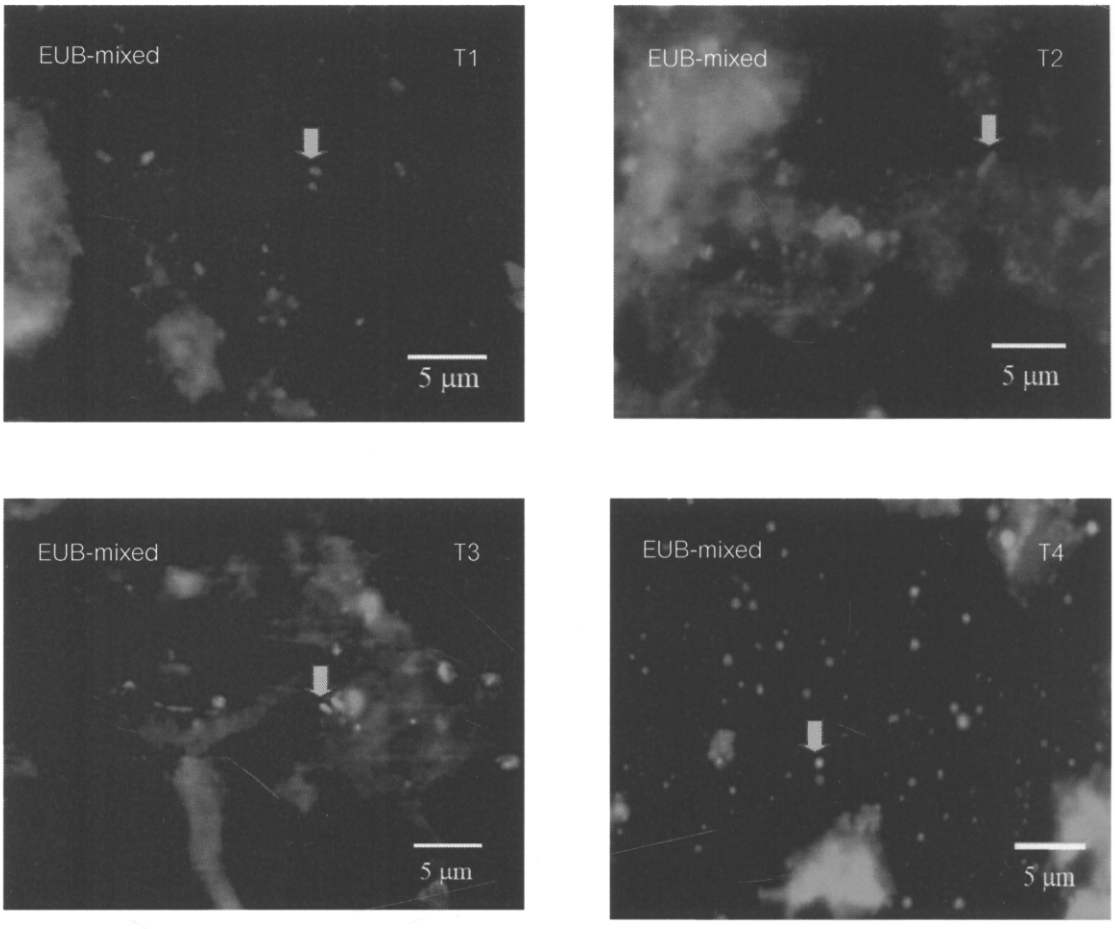

Figure 3 Epifluorescence micrographs of microbes (arrows) in various treatments were detected by FISH technique using EUB-mixed Cy3-labeled gene probe before started feeding with *L. plantaurm* supplemented feed.

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Vibrio spp. in intestine of experiment 3 detected with GV probe, which was rather stable through monitoring on day 1, 5 and 10. *Vibrio* spp. detected in the control group, which was increased over the experiment groups on day 5 and 10 (Table 24). Epifluorescent micrograghs of *Vibrio* spp. detected in the intestine of each experiment group before feeding with *L. plantarum* (Fig. 4) and after stop feeding with *L. plantarum* for 5 days (Fig. 5). *Vibrio* spp. in intestine and hepatopancreas of experiment groups (inulin supplemented) which decreased numbers on day 5 and 10.

Table 24 Total *Vibrio* spp. in intestine of white shrimp were enumerated by FISH technique using GV Cy3-labeled gene probe on different days.

Treatment	Total *Vibrio* spp. in intestine of white shrimps [Log (cell/g)]			
	Day-0	**Day-1**	**Day-5**	**Day-10**
T1	7.59 ± 0.05^{a}	7.44 ± 0.17^{b}	7.86 ± 0.08^{a}	7.50 ± 0.07^{a}
T2	7.47 ± 0.03^{a}	7.24 ± 0.05^{b}	7.61 ± 0.13^{ab}	7.37 ± 0.26^{a}
T3	7.54 ± 0.11^{a}	7.49 ± 0.24^{ab}	7.55 ± 0.21^{b}	7.50 ± 0.13^{a}
T4	7.53 ± 0.15^{a}	7.75 ± 0.07^{a}	7.72 ± 0.15^{ab}	7.43 ± 0.08^{a}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean$\pm$SD. Values in the same column sharing a common superscript are not statistically difference (p>0.05).

Day-0 : Before started feeding with *L. plantarum* supplemented feed

Day-1 : After stopped feeding with mixed *L. plantarum* supplemented feed at 1 day

Day-5 : After stopped feeding with mixed *L. plantarum* supplemented feed at 5 day

Day-10 : After stopped feeding with mixed *L. plantarum* supplemented feed at 10 day

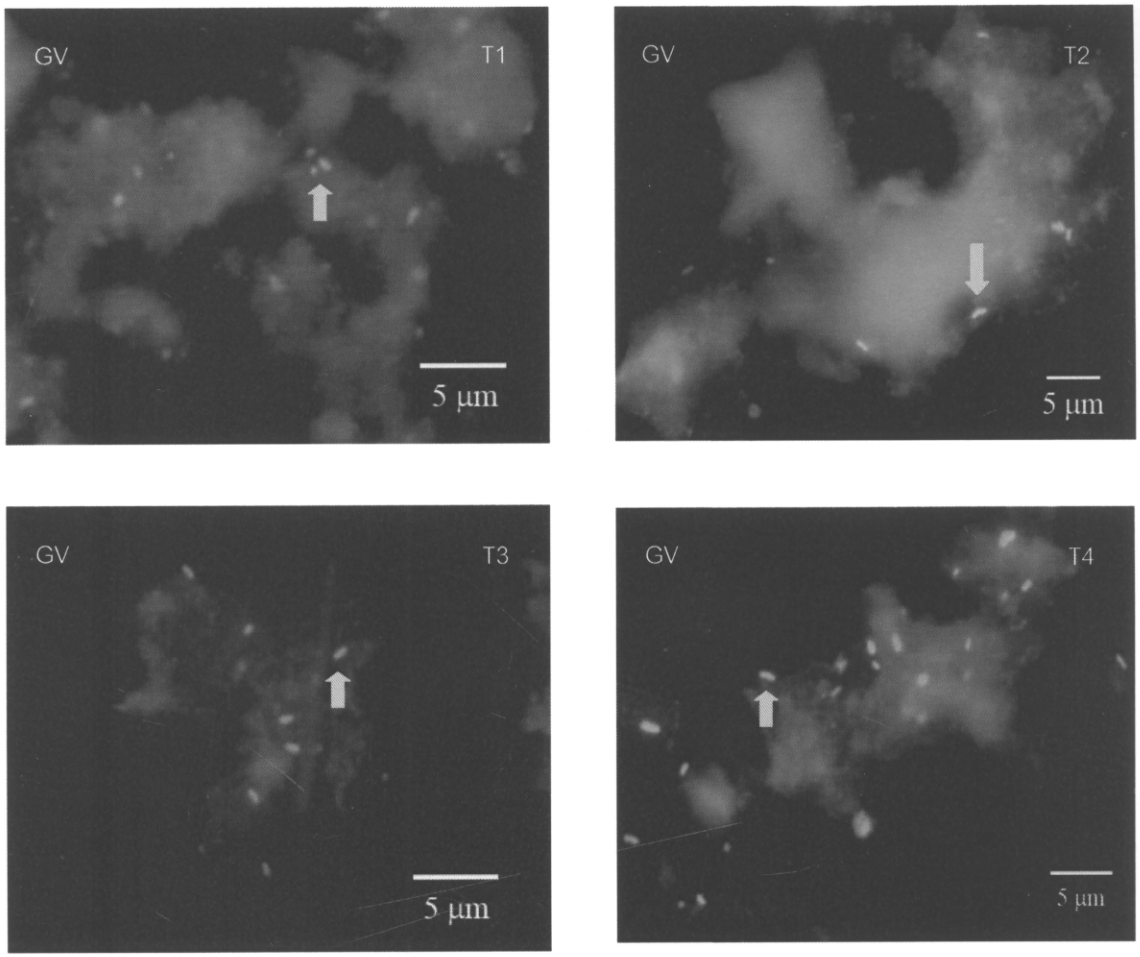

Figure 4 Epifluorescence micrographs of *Vibrio* spp. (arrows) in various treatments taken by FISH technique using GV Cy3-labeled gene probe before started feeding with *L. plantaurm*.

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

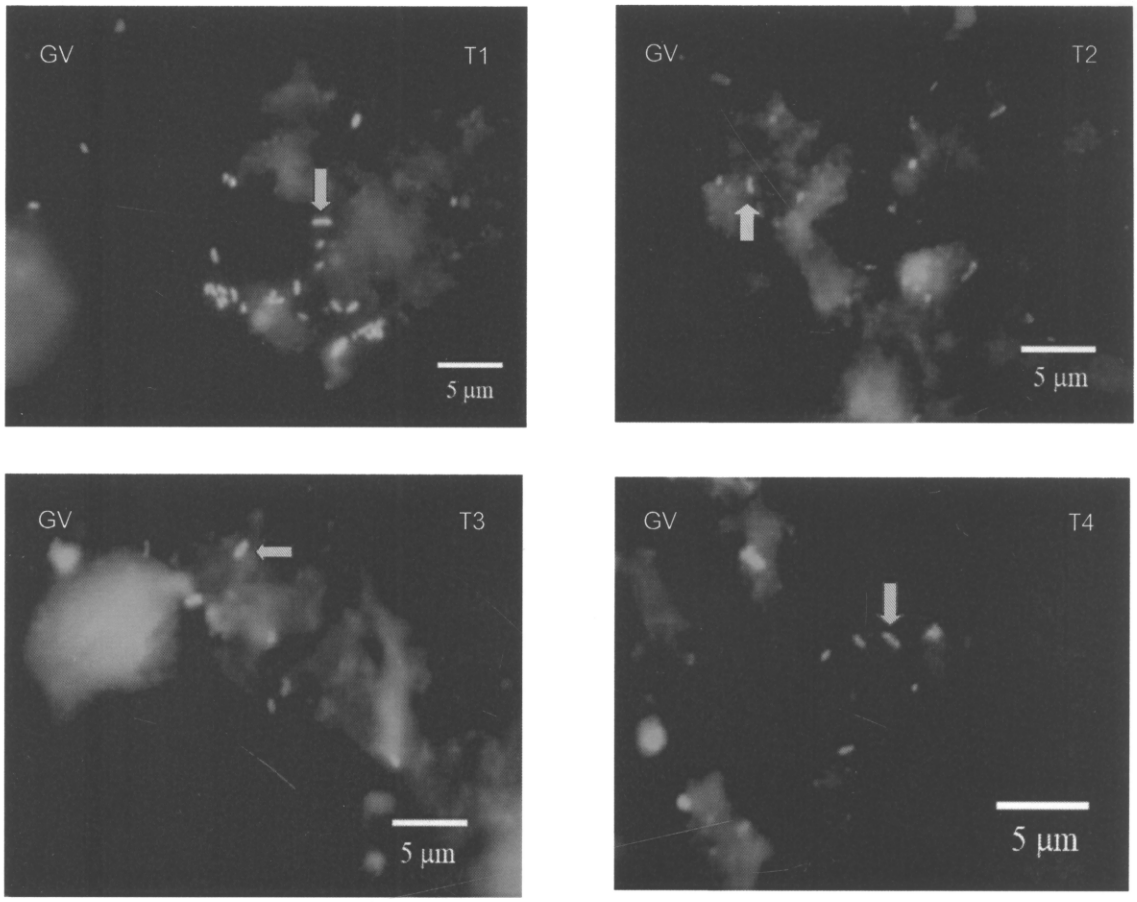

Figure 5 Epifluorescence micrographs of *Vibrio* spp. (arrow) in various treatments taken by FISH technique using GV Cy3-labeled gene probe after stopped feeding *L. plantarum* supplemented feed on day 5.

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

The result showed that *Enterococcus* spp. in the intestine of experiment groups were higher than control group on day 0, 1 and 10, without significant difference ($p>0.05$). *Enterococcus* spp. in the intestine of experiment 2 had lowest on day 5 when compared with other groups ($p<0.05$) as presented in table 25. *Enterococcus* spp. in the hepatopancreas of experiment 3 on day 5 and experiment 2 on day 10 had lowest when compared with other groups, without significant difference ($p>0.05$) as presented in table 26. Epifluorescence micrographs of *Enterococcus* spp. (arrows) in various treatments were detected by FISH technique using ENC131

Cy3-labeled gene probe after stopped feeding *L. plantarum* supplemented feed on day 5 as presented in Fig. 6.

Table 25 Total *Enterococcus* spp. in intestine of white shrimp were enumerated by FISH technique using ENC131 Cy3-labeled gene probe on different days.

Treatment	Total *Enterococcus* spp. in intestine of white shrimps [Log (cell/g)]			
	Day-0	Day-1	Day-5	Day-10
T1	7.74±0.28^{a}	8.05±0.30^{a}	9.31±0.03^{bc}	9.21±0.05^{a}
T2	8.12±0.31^{a}	8.13±0.29^{a}	9.17±0.07^{c}	9.29±0.09^{a}
T3	8.11±0.33^{a}	8.39±0.23^{a}	9.51±0.13^{a}	9.23±0.07^{a}
T4	7.94±0.33^{a}	8.45±0.09^{a}	9.45±0.03^{ab}	9.30±0.06^{a}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. Values in the same column sharing a common superscript are not statistically difference (p>0.05).

Day-0 : Before started feeding with *L. plantarum* supplemented feed

Day-1 : After stopped feeding with mixed *L. plantarum* supplemented feed at 1 day

Day-5 : After stopped feeding with mixed *L. plantarum* supplemented feed at 5 day

Day-10 : After stopped feeding with mixed *L. plantarum* supplemented feed at 10 day

Table 26 Total *Enterococcus* spp. in hepatopancreas of white shrimp were enumerated by FISH technique using ENC131 Cy3-labeled gene probe on different days.

Treatment	Total *Enterococcus* spp. in hepatopancreas of white shrimps [Log (cell/g)]			
	Day-0	Day-1	Day-5	Day-10
T1	5.75±0.12^{c}	5.68±0.15^{c}	5.92±0.07^{a}	5.99±0.25^{a}
T2	6.20±0.10^{ab}	6.19±0.02^{b}	6.03±0.13^{a}	5.87±0.13^{a}
T3	5.99±0.07^{b}	6.20±0.33^{b}	5.69±0.52^{a}	6.09±0.07^{a}
T4	6.24±0.16^{a}	6.61±0.19^{a}	6.11±0.21^{a}	6.01±0.18^{a}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. Values in the same column sharing a common superscript are not statistically difference (p>0.05).

Day-0 : Before started feeding with *L. plantarum* supplemented feed

Day-1 : After stopped feeding with mixed *L. plantarum* supplemented feed at 1 day

Day-5 : After stopped feeding with mixed *L. plantarum* supplemented feed at 5 day

Day-10 : After stopped feeding with mixed *L. plantarum* supplemented feed at 10 day

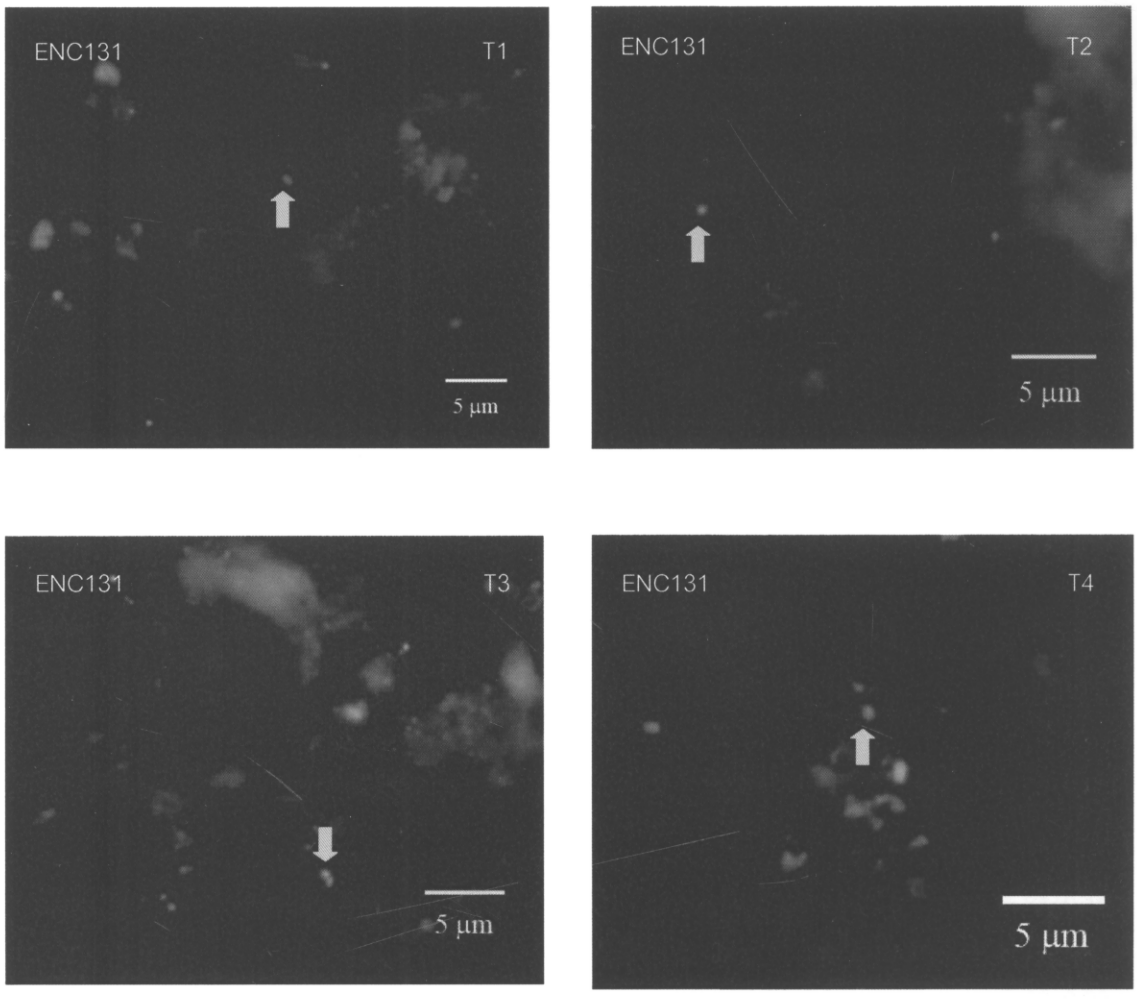

Figure 6 Epifluorescence micrographs of *Enterococcus* spp. (arrow) in various treatments were detected by FISH technique by using ENC131 Cy3-labeled gene probe after stopped feeding *L. plantarum* supplemented feed on day 5.

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

The result showed that *L. plantarum* were not detected in the intestine and hepatopancreas before feeding with *L. plantarum* (day 0). *L. plantarum* in the intestine of experiment groups were higher than control group on day 1, without significant difference ($p>0.05$). *L. plantarum* in the intestine of experiment 3 and 4 on day 5 were higher than experiment 1 and 2, with significant difference ($p<0.05$). *L. plantarum* in the intestine of experiment groups were higher than control group on day 10 and significant difference ($p<0.05$) as presented table 27. *L. plantarum* in the hepatopancreas of experiment 4 had highest when compared with other groups and significant

difference (p<0.05). *L. plantarum* in the hepatopancreas of experiment 3 and 4 on day 5 were higher than experiment 1 and 2, with significant difference (p<0.05). *L. plantarum* in the hepatopancreas of experiment groups were higher than control group on day 10 and significant difference (p<0.05) as presented table 28. Epifluorescence micrographs from FISH technique by using Lab158 Cy3-labeled gene probe showed *Lactobacillus* spp. (arrows) in various treatments after stopped feeding the diet supplemented with *L. plantarum* (Fig. 7). In this study, it found that inulin had effected to growth of *L. plantarum*. The results of the interaction with *L. plantarum* confirmed that it is possible to decrease the colonization of *Vibrio* spp. in the shrimp intestine and hepatopancreas.

Table 27 Total *Lactobacillus* spp. in intestine of white shrimp were enumerated by FISH technique using Lab158 Cy3-labeled gene probe on different days.

Treatment	Total *Lactobacillus* spp. in intestine of white shrimps [Log (cell/g)]			
	Day-0	Day-1	Day-5	Day-10
T1		5.87±0.07^{a}	5.91±0.15^{a}	5.72±0.24^{a}
T2		5.98±0.21^{a}	5.99±0.11^{a}	6.21±0.15^{b}
T3		5.99±0.33^{a}	6.54±0.06^{b}	6.46±0.10^{b}
T4		6.19±0.11^{a}	6.60±0.04^{b}	6.32±0.09^{b}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. Values in the same column sharing a common superscript are not statistically difference (p>0.05).

Day-0 : Before started feeding with *L. plantarum* supplemented feed

Day-1 : After stopped feeding with mixed *L. plantarum* supplemented feed at 1 day

Day-5 : After stopped feeding with mixed *L. plantarum* supplemented feed at 5 day

Day-10 : After stopped feeding with mixed *L. plantarum* supplemented feed at 10 day

Table 28 Total *Lactobacillus* spp. in hepatopancreas of white shrimp were enumerated by FISH technique using Lab158 Cy3-labeled gene probe on different days.

Treatment	Total *Lactobacillus* spp. in **hepatopancreas** of white shrimps [Log (cell/g)]			
	Day-0	**Day-1**	**Day-5**	**Day-10**
T1		5.46 ± 0.17^{A}	5.25 ± 0.17^{a}	5.33 ± 0.17^{a}
T2		5.61 ± 0.07^{ab}	5.19 ± 0.28^{a}	5.66 ± 0.10^{b}
T3		5.46 ± 0.10^{A}	5.51 ± 0.10^{ab}	5.69 ± 0.15^{b}
T4		5.83 ± 0.13^{b}	5.80 ± 0.06^{b}	5.77 ± 0.07^{b}

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Mean±SD. Values in the same column sharing a common superscript are not statistically difference (p>0.05).

Day-0 : Before started feeding with *L. plantarum* supplemented feed

Day-1 : After stopped feeding with mixed *L. plantarum* supplemented feed at 1 day

Day-5 : After stopped feeding with mixed *L. plantarum* supplemented feed at 5 day

Day-10 : After stopped feeding with mixed *L. plantarum* supplemented feed at 10 day

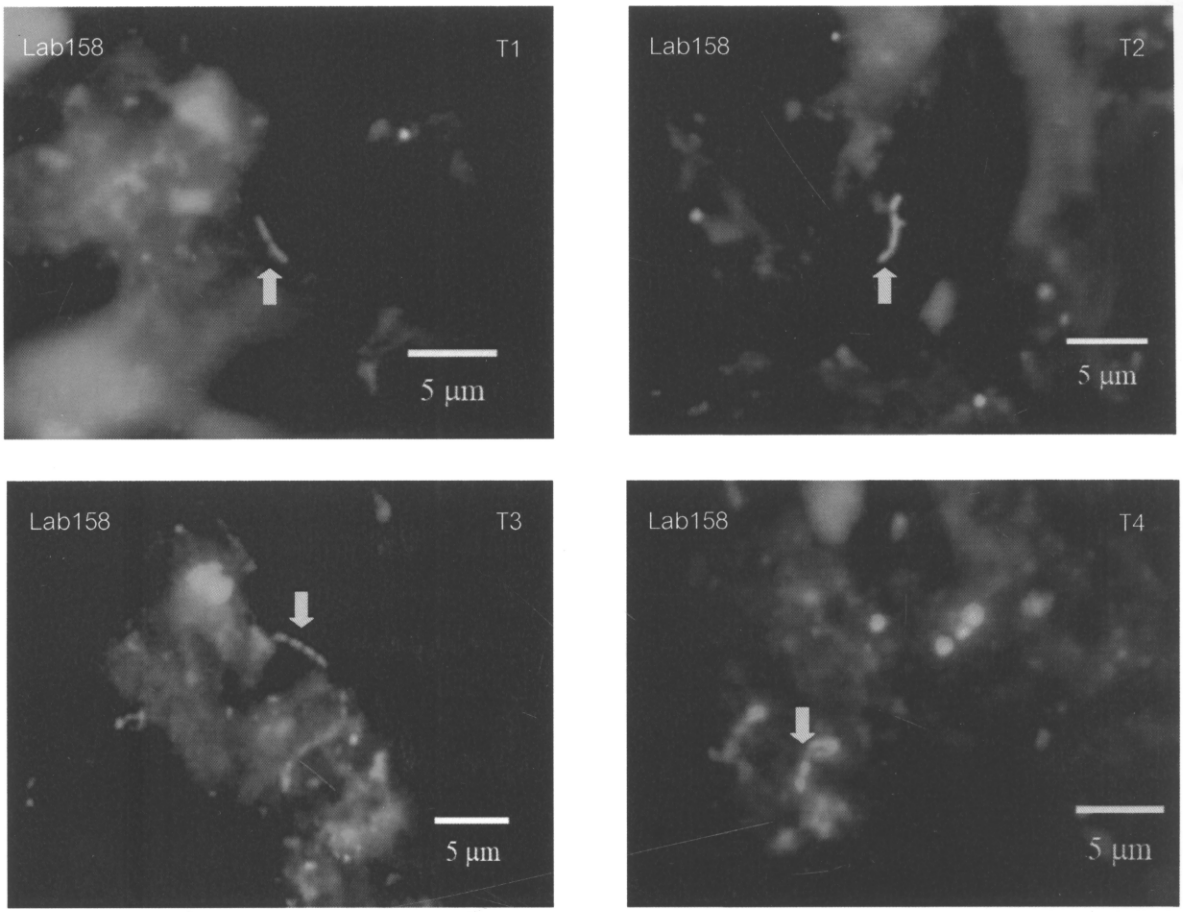

Figure 7 Epifluorescence micrographs of *Lactobacillus* spp. (arrows) in various treatments detected by FISH technique using Lab158 Cy3-labeled gene probe after stopped feeding *L. plantarum* supplemented feed on day 5.

T1 = no inulin; T2 = 0.22% inulin; T3 = 0.55% inulin and T4 = 2.22% inulin

Disease resistance

After injection with *V. harveyi* suspension, it was found that survival rate of shrimp in treatment groups (T1-T4) showed higher resistance to pathogenic bacteria than control group (C) (Figure 8). This is an indication of more healthy shrimp in treatment groups.

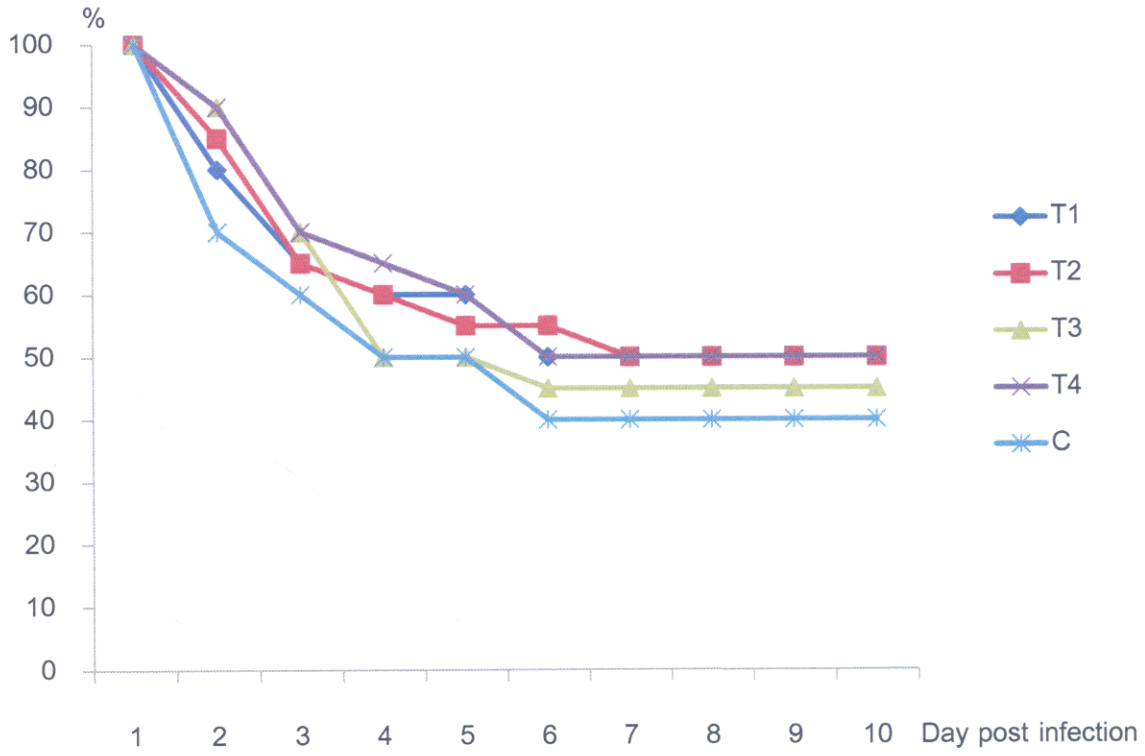

Figure 8 Survival rate of white shrimp after challenge with pathogenic *Vibrio* (*V. harveyi*).

T1 = no inulin + feeding *L. plantarum* supplemented feed

T2 = 0.22% inulin + feeding *L. plantarum* supplemented feed

T3 = 0.55% inulin + feeding *L. plantarum* supplemented feed

T4 = 2.22% inulin + feeding *L. plantarum* supplemented feed

C = no inulin and no feeding *L. plantarum* supplemented feed

Discussion

In this study we demonstrated that the candidate bacteria from fermented foods and 4 strains of *Lactobacillus* i.e., *L. acidophilus* TISTR 1034, *L. brevis* TISTR 855, *L. casei* TISTR 1304 and *L. plantarum* TISTR 050 are potential probiotic and high competitors of pathogenic bacteria. The results of *in vitro* showed that all candidate bacteria grew well on media containing different levels of salinity, i.e., 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%, respectively. Agar well diffusion plate assay indicated that *Lactobacillus plantarum* can inhibit growth of *V. harveyi* and *V. parahaemolyticus* with largest inhibitory zone and arbitory unit. Similar results were reported by Mahasawasde *et al.*, (2003) for the efficiency of *Lactobacillus* spp. against *V. harveyi*, which cause the luminescent disease in black tiger shrimp. It was found that, the filtrate of *Lactobacillus* spp. can inhibit growth of *V. harveyi* and the filtrate of *L. plantarum* can inhibit growth of *V. parahaemolyticus*. Results of co-culture in seawater supplemented with 2.22% inulin probiotic showed decreased *V. harveyi* growth compared to increase of *L. plantarum*. This result supported the origin of the concept of prebiotics, which aimed towards stimulation of the growth of potentially beneficial bacteria (Roberfroid, 1998).

Thus, *in vivo* study inulin supplementation were used in white shrimp feed, in different concentrations of inulin, 0.22, 0.55 and 2.22%, respectively, for a 6-weeks feeding period. The result indicated that supplementation of inulin at 2.2 g/kg in treatment 2 showed a little improvement on growth while higher level of inulin at 22.2 g/kg in treatment 4 had no effect on growth of white shrimp. *Vibrio* spp. was enumerated on TCBS after feeding with inulin supplementation for 1 month. The number of *Vibrio* spp. in intestine of experiments groups were not significant difference ($p>0.05$) when compared with control group. However, the number of *Vibrio* spp. in hepatopancreas of experiments 4 was significant difference ($p<0.05$) when compared with control group. Although, the number of *Vibrio* spp. in intestine and hepatopancreas each groups were different. After mixing with *L. plantarum* as probiotic, the number of *Vibrio* spp. in hepatopancreas of experiments 4 reduced from 3.07 ± 0.51 to 2.36 ± 0.32 Log (CFU/g). This result supported the concept of prebiotic which can reduce the risk of pathogeny. It has been hypothesized that the gut microflora must remain a 'balanced microflora'. The gut microflora must be composed predominantly (in numbers) of bacteria recognized as potentially health promoting (like *L. plantarum*) to prevent, impair or control the proliferation of the potentially pathogenic/harmful bacteria (like some *Vibrio* spp.). Inulin is the most studied and well-established

prebiotics. As previously mentioned they escape digestion in the upper gastrointestinal tract and reach the large intestine virtually intact, they are quantitatively fermented and act as prebiotics. Indeed, in the many studies that investigated the effects of inulin on the human gut microbiota both *in vitro* and *in vivo*, a selective stimulation of growth of the beneficial flora, namely bifidobacteria, to lesser extent lactobacilli and possibly other species like Clostridium coccoides–Eubacterium rectal cluster known to be a butyrate producer has been reported (Kleessen *et al.*, 2001; Apajalahti *et al.*, 2002).

Following feeding with *L. plantarum* for 7 days, persistence and efficiency of *L. plantarum* on growth of *Vibrio* spp. was monitored at 1, 5 and 10 days by enumerated with FISH technique using DNA gene probe. The result indicated that high levels of inulin-supplemented feed resulted in increased growth of *L. plantarum* and decreased *V. harveyi* in intestine and hepatopancreas. *L. plantarum* can survived in intestine for 10 days in this condition. After injection with *Vibrio harveyi* suspension, it found that survival rate of shrimp in treatment groups (T1-T4) showed higher resistance to pathogenic bacteria than control group (C). This is an indication of more healthy shrimp in treatment groups. Possibly these bacteria colonized the gut, therefore, they help in improve the immune system of shrimp. It is known that colonization with specific bacteria in the gut may play a role in balancing the intestinal mucosal immune system, which may contribute to the induction and maintenance of immunological tolerance or to the inhibition of disregulated response induced by pathogens in host. Balcazar *et al.*, (2006) suggested that the use of probiotics is important management tool, but its efficiency depends on understanding the nature of competition between species or strains. Gibson and Roberfroid (1995) suggested that combining a prebiotic with a probiotic in a 'synbiotic' approach could open new perspectives. Indeed, *in vitro* experiments designed to test the inhibitory effect of probiotics on the growth of human intestinal pathogens (*E. coli*, *Campylobacter jejuni*, *Salmonella enteritidis*). Previous reports had objective to use probiotic for improved the growth and survival rates of host or reduced pathogeny i.e., Phianphak *et al.*, (1999) reported that a mixture of *Lactobacillus* spp. isolated from chicken gastrointestinal tracts has improved the growth and survival rates of juvenile *P. monodon* when fed these strains for 100 days. The probiotic effect in *L. vannamei* has been reported using three strains isolated from the hepatopancreas of shrimp. These strains were identified as *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 and achieved inhibition percentage against *V. harveyi* S2 under *in vivo* conditions of 83, 60 and 58%, respectively. Histological analyses after the colonization and

interaction experiment confirmed that the probiotic strains had no pathogenic effect on the host (Gullian *et al.*, 2004).

Conclusion

1. All candidate bacteria can grow on media containing 3% NaCl. This can be applied in shrimp pond condition.
2. *L. plantarum* can inhibit *V. harveyi* and *V. parahaemolyticus* with largest diameter of inhibitory zone and arbitory unit.
3. A significant growth of *V. harveyi* decrease was observed in the co-culture in 15 ppt sterile seawater with 2.22% inulin while a significant growth of *L. plantarum* increase.
4. Supplementation of inulin at 2.2 g/kg in treatment 2 showed a little improvement on growth while higher level of inulin at 22.2 g/kg in treatment 4 had no effect on growth of white shrimp.
5. Inulin supplementation for a 6 week period result in a non significant growth of *Vibrio* spp. difference in intestine of experiment groups, when compared with the control.
6. Inulin supplementation and *L. plantarum* mixing fed for next 7 days. The result indicated that the growth of *Vibrio* spp. decreased while a significant growth of *L. plantarum* increased in intestine and hepatopancreas by enumerated with FISH technique using DNA gene probe.
7. The survival rate of shrimp in treatment groups (T1-T4) showed higher resistance to pathogenic bacteria than control group (C).

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