



Original Article

## Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics

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### Abstract

One hundred and sixty isolates of lactic acid bacteria (LAB) with inhibitory activity against pathogenic *Escherichia coli* were isolated from gastrointestinal tracts of fish, shrimp and shellfish. One hundred and sixteen isolates were obtained from fish, twenty isolates from shrimp and twenty-four isolates from shellfish. Three strains were selected based on their bile and acid tolerances. All acid-tolerant strains showed inhibitory activity against human pathogens, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* sp. and *Escherichia coli*. However, the antibacterial activities were lost when the culture supernatants were neutralized to pH 6.5-7.0 and treated with catalase, indicating that the inhibition may be contributed by acid and hydrogen-peroxide production of the strains. Nucleotide sequences of their 16s rDNA showed 98% (655/668 bp), 97% (691/712 bp) and 98% (492/501 bp) homology to *Pediococcus pentosaceus* LM2, *Pediococcus pentosaceus* SL4 and *Enterococcus faecium* SF, respectively.

**Keywords:** acid tolerance, antimicrobial activity, bile salt tolerance, marine fish, lactic acid bacteria, probiotic

### 1. Introduction

The increasing consumer awareness of diet and health has stimulated the development of functional foods containing probiotics defined as "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition. Scientific evidence is accumulating to support various health beneficial effects of probiotics including pathogen interference, immunostimulation and immunomodulation, anticarcinogenic and antimutagenic activities, alleviation of symptoms of lactose intolerance, reduction in serum cholesterol, reduction in blood pressure, decrease of incidence and duration of diarrhea, prevention of vaginitis and maintenance of mucosal integrity (Klaen-

hammer and Kullen, 1999; Vaughan *et al.*, 1999). Moreover, probiotic concept has been widely applied for health promoting in farm animals, pets and aquatic animals (Ringø and Gatesoupe, 1998; Nousiainen *et al.*, 2004; Vaughan *et al.*, 2005). The probiotic products traditionally incorporate intestinal species of *Lactobacillus* because of their long history of safe use in the dairy industry and their natural presence in the human intestinal tract, which is known to contain a myriad of microbes, collectively called the microbiota. Many lactic acid bacteria (LAB) are proved to function as probiotics, which are benefit to host health, when ingested in sufficient quantities. The colonization of the gut by probiotic bacteria prevents growth of harmful bacteria by competition exclusion and by the production of organic acid and antimicrobial compounds. The acid and bile tolerance as well are two fundamental properties that indicate the ability of probiotic microorganism to survive the passage though the upper gastrointestinal tract, particularly acidic condition

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in the stomach and the presence of bile in the small intestine (Hyronimus *et al.*, 2000; Erkkila and Petaja, 2000). Promising probiotic strains include the members of genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*. The representative species include *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Enterococcus faecalis* and *Enterococcus faecium* (Kaur *et al.*, 2002).

It is well known that lactic acid bacteria have adapted to grow under widely different environmental conditions, and they are widespread in nature. Apart from food sources, lactic acid bacteria are commonly found in the gastrointestinal tract of various endothermic animals, mice, rats, pigs, fowl and humans (Fuller, 1989; Tannock, 1995; Weese *et al.*, 2004). The role of lactic acid bacteria within the digestive tract of endothermic animals has been extensively investigated and reviewed (Vaughan *et al.*, 2005). Ringø and Gatesoupe (1998) isolated and identified LAB in gastrointestinal tract and feces of fish and reported that LAB normally account for only a marginal part of the intestinal microbiota of fish. Although LAB were not dominant population in fish, it has been well documented in several investigations that lactic acid bacteria are a part of the native microbiota of aquatic animals from temperate regions (Ringø, 2004). In addition to the numerous investigations demonstrating the presence of LAB in the digestive tracts of several different fish species, several studies have reported on the isolation of LAB (carnobacteria, lactobacilli, lactococci, leuconostocs and pediococci) from cold-smoked and fermented fish. All reports on the presence of LAB in fish from both freshwater and marine environments suggest that the fish gut may be a natural reservoir for LAB. Other habitats such as other marine animals, sediments, and water can be possible habitat for LAB. Franzmann *et al.* (1991) isolated *Carnobacterium funditum* and *Carnobacterium alterfunditum* from the water of Ace Lake, Antarctica. A great variation in the frequency of LAB reported in the different studies indicating that real variation exists between fish species as well as geographical location. However, isolations of LAB from fish in a tropical climate are few. The aim of this study was to select potential probiotics, which possessed antibacterial activity against certain pathogens, from gastrointestinal tracts of marine fish available in a tropical marine environment.

## 2. Materials and Methods

### 2.1 Marine fish, bacterial media and reagents

Various marine fish, shellfish, shrimp and crab were purchased from fresh markets in Songkhla and directly obtained from fisherman boats docking along Songkhla shoreline. De Man, Rogosa, and Sharpe (MRS), Mueller

Hinton Broth (MHB) and bile salt were supplied by Himedia (Mumbai, India) and Nutrient Broth was from LabScan (Bangkok, Thailand).

### 2.2 Bacterial strains and growth conditions

Bacterial strains including *S. aureus*, *Salmonella* sp. and *L. monocytogenes* were obtained from culture collection of Microbiology Laboratory, Faculty of Agro-Industry, Prince of Songkhla University, and pathogenic *E. coli* (patient isolate) was from Prince of Songkhla University Hospital, Thailand. All bacterial strains were cultivated in 10 ml of MHB. Bacterial cultivation was performed at 37°C for 20 h. Approximately 1 ml of bacterial culture was transferred to 9 ml of liquid medium and incubated at 37°C for another 18 h, cell concentration was then adjusted to obtain final concentration of 10<sup>6</sup> CFU/ml for determination of antibacterial activity. Cultivation of pathogenic *E. coli* for the screening step was performed as above on nutrient broth instead of MHB.

### 2.3 Isolation and screening of lactic acid bacteria inhibiting pathogens

Lactic acid bacteria were isolated from gastrointestinal tracts of fish, shellfish shrimp and crab on MRS agar. Gastrointestinal tracts were aseptically removed and weighed. Twenty-five gram of each sample were ground and diluted in sterilized sea water to obtain the dilutions of 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup>. One milliliter of each dilution was then transferred and mixed with 20 ml of molten MRS agar (45°C) on a sterilized Petri-dish. The plates were incubated aerobically and anaerobically at 37°C for 24 hour. Isolation and selection of LAB colonies with antibacterial activity was performed simultaneously on the agar plates (with approximately 50 LAB colonies) by overlaying with 10 ml of soft agar (0.75% agar in NA) seeded with pathogenic *E. coli* at concentration of 10<sup>5</sup>-10<sup>6</sup> CFU/ml and incubated for another 24 hour at 37°C. Colonies producing inhibition zones were isolated and further purified on MRS agar. All isolates obtained were Gram-stained and tested for catalase reaction. Strains exhibited Gram-positive with catalase negative were selected and stored at -80°C in MRS broth containing 35% glycerol.

### 2.4 Bile Tolerance

The modified method of Arihara *et al.* (1998) was used to determine bile tolerance of selected LAB. Before testing for bile tolerance, LAB strains were grown at 37°C for 24 hour in MRS broth without bile. One ml of the culture broth was poured onto MRS agar with bile salt concentrations of 2000, 3000 and 4000 ppm. Bacterial growth was determined after incubation at 37°C for 48 hour.

## 2.5 Acid Tolerance

The modified method of Erkkila and Petaja (2000) was applied in this study. LAB cells grown in MRS broth at 37°C for 24 hour were collected by centrifugation at 5340 ×g rpm for 15 min. Cell pellet was washed twice and re-suspended into 10 ml of phosphate-buffered saline (PBS) to obtain 10<sup>8</sup> cfu/ml before addition to sterile PBS with the pH values of 1, 2, 2.5 and 3 (adjusted using 5 M HCl) to achieve 10<sup>6</sup> cfu/ml. PBS was prepared by dissolving NaCl (9 g/l), Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O (9 g/l) and KH<sub>2</sub>PO<sub>4</sub> (1.5 g/l) in distilled water. The tubes were incubated at 37°C and the viable organisms were counted after exposure to acidic condition for 0, 1, 2, 3 and 4 hour on MRS agar incubated at 37°C for 48 hour. The survival cell count was calculated according to numbers of colonies grown on MRS agar, compared to the initial bacterial concentration.

## 2.6 Determination of antibacterial activity

Antimicrobial effects of the selected LAB on *S. aureus*, *L. monocytogenes*, *Salmonella* sp. and pathogenic *E. coli* were determined by agar diffusion method. LAB were grown in MRS broth for 24 hour at 37°C. The LAB cultures were centrifuged at 5340×g for 15 min. The clear supernatants obtained were treated as follows: (i) clear supernatants without any treatment (CFF) (ii) clear supernatants adjusted to pH 6.5-7.0 with 1M NaOH (CFBH) (iii) clear supernatants adjusted to pH 6.5-7.0 with 1 M NaOH and treated with 200 unit/ml of catalase (CFB). Both treated and non-treated supernatants were filter sterilized by membrane filtration (0.2µm) before being subjected to the antibacterial assay. Petri-dishes filled with 20 ml of nutrient agar (1.5% agar) were overlaid with 10 ml of MHA (0.75% agar) which was inoculated with 10<sup>5</sup>-10<sup>6</sup> CFU/ml of indicator microorganism. Five wells (5.8 mm of diameter) were made and filled with 80 µl of supernatants treated as above. Control wells filled with sterile MRS broth and standard antibiotic solution of 100µg/ml chloramphenicol were performed as negative control, and positive control, respectively. The inoculated plates were incubated for 24 hour at 37°C. The diameter of the inhibition zone was measured with calipers (Aslim *et al.*, 2005).

## 2.7 Analysis of 16S rDNA sequences

### 1) DNA extraction

Bacterial cells grown in MRS broth at 37°C 24 h were collected by centrifugation at 10,000 rpm 5 min and washed twice with TE-buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA). Washed cells resuspended in 300 ml of TE-buffer were boiled at 100°C for 10 min, removed the supernatant by centrifugation at 7392×g for 5 min to obtain DNA solution for DNA template.

### 2) Amplification of 16S rDNA, nucleotide sequencing and sequence analysis

The prokaryotic 16S rDNA gene was amplified by PCR with a thermal cycler (GeneAmp PCR system 9700, Applied Biosystem, Forster City, CA, USA). The PCR reaction contained 20 ml of final solution consisting of 2.5 ml 10xPCR buffer pH 8.8 (10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCl, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100) for Taq DNA polymerase (BioLab), 2 mM of each deoxynucleoside triphosphate (dNTP), 0.4 mM of each primer and 1 unit of Taq polymerase. The oligonucleotide primers used for the bacterial 16S rRNA gene were: UFUL and URUL primers. The thermocycle program was as follows: 95°C for 5 min; 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 3 min. After cycling, the PCR products were detected by electrophoresis on a 1% agarose gel, staining with ethidium bromide and visualizing under UV light.

The bacterial 16S rRNA gene amplicons were purified by ethanol precipitation (80 ml of DNA solution, 14.5 ml of water, 62.5 ml of absolute ethanol and 3.0 ml of 3 M sodium acetate pH 4.6, left at room temperature for 15 min). The supernatant was removed by centrifugation at 10645×g 15 min, washed the precipitant with 70% ethanol, centrifuged to remove the ethanol and dried the precipitant at 95°C for 2-3 min. The dried precipitant was dissolved in 10 l of Hi-Di Formamide, and then boiled at 95°C for 2 min and cooled with ice for 5 min. DNA sequencing PCR reaction was performed using BigDye Terminator v 3.1 Cycle Sequencing Kit with the primer sets used previously. The thermocycle program was as follows: 95°C for 5 min; 30 cycles of 95°C for 30 sec, 50°C for 10 sec, and 60°C for 4 min; and a final extension step at 60°C for 4 min. Nucleotide sequencing was carried out with an automated DNA sequencer (Applied Biosystems GeneScan 3700, Foster City, CA). Nucleotide sequences of 16S rDNA were aligned to nr database using BLAST program (Altschul *et al.*, 1990)

## 3. Results and Discussion

### 3.1 Isolation of LAB from gastrointestinal tracts (GI) of marine fish

LAB were isolated from 24 marine fish, including shellfish, crabs, shrimps, and fish. The selection for the colonies exhibiting antibacterial activity against pathogenic *E. coli* was performed simultaneously on MRS agar plate overlaid with pathogenic *E. coli* containing NA. The colonies surrounded by clear zone as shown in Figure 1 were carefully picked and purified by re-streaking on MRS agar to obtain pure cultures of LAB. Such technique yielded 160 isolates of LAB, which inhibited the growth of the indicator microorganism. One hundred and sixteen strains were isolated from GI-tracts of fish, 20 strains were isolated from

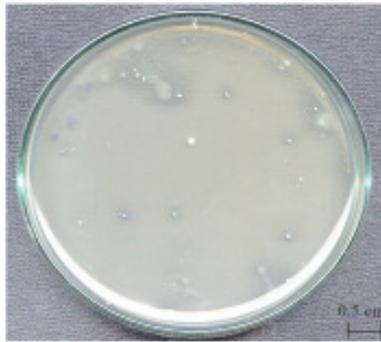


Figure 1. LAB colonies on MRS agar overlaid with pathogenic *E. coli* containing NA agar layer. The colonies that expressed inhibitory activity with clear zone were selected.

GI-tracts of shrimp and 24 isolated from shellfish. *Dascyllus aruanus*, *Perna viridis*, *Plotosus canius* and *Parupeneus cinnabarinus* provided high number of LAB isolates of 28, 19, 19 and 15 strains, respectively (Table 1). All 160 isolates were Gram-positive and catalase negative and showed anti-bacterial activity against pathogenic *E. coli*. Total bacteria grown on MRS indicated bacterial population level of  $4-5 \times 10^4$  to  $10^5$  cfu/g wet weight of GI tracts from all samples. The bacterial population was correlated to the number reported from stomach of salmonid fish, which the bacterial population level is in the range of  $2 \times 10^4$  to  $10^5$  (Austin and Al-Zahrani, 1988; Ringø, 1993). However, it is well known that LAB are not under normal circumstances numerically dominant in the digestive tract of fish (Ringø and Gatesoupe, 1998). Ringø (1993) reported that LAB from the stomach of Arctic charr, *Salvelinus alpinus* (L) were only a minor part of the microbiota approximately 10%. On the contrary, the results from this study indicated high population of LAB in all fish samples. Most colonies were Gram-positive bacteria with catalase negative, indicating majority population was LAB. The reason could be due to the variation of fish species and geographical location. Our research was conducted with warm-water fish whereas most researches regarding LAB in aquatic animals were conducted in cold-water fish, so scientists encountered certain obstacles in isolation of LAB. Ringø and Gatesoupe (1998) raised three factors of prime importance when isolating LAB from fish, which are nutrient medium, incubation temperature and incubation time. In most investigations, trypticase soy agar (TSA), Marine-medium or brain-heart infusion agar (BHIA)

Table 1. Numbers of strains isolated from various sources of marine fish.

Sources	Strain numbers	
	Aerobe	Anaerobe
<i>Lycothrissa crocodiles</i>	0	3
<i>E. areolatus</i> (Areolated Grouper)	1	
<i>Penaeus monodon</i> (giant tiger prawn)		7
<i>Dascyllus aruanus</i> (Damsel fish)	14	14
<i>Perna viridis</i> (Green mussel)	19	
<i>Parupeneus cinnabarinus</i> (Red Mullet)	10	5
<i>Rastrelliger brachysoma</i> (Short mackerel)	4	6
<i>Leiognathus eguulus</i> (Pla pan)	10	4
<i>Nibea soldado</i> (Soldier croaker)	2	0
Pla cock	1	0
<i>Metapenaeus ensis</i> (Greasy back shrimp)	0	3
<i>Siganus canaliculatus</i> (Whitespotted spinefoot)	0	13
Pla Tong-Taew	8	0
<i>Miyakea neap</i> (mantis shrimp)	6	4
<i>Plotosus canius</i> (Catfish)	13	6
<i>Arca granulosa</i> (Cockle)	3	2

have been used for recovery of LAB from intestines and internal organs such as kidney, liver, spleen, etc. However, Shotts and Teska (1989) reported that 5% (v/v) bovine or rabbit blood agar or tomato juice agar were also very useful for initial isolation. One of the limiting factors for initial isolation of LAB from fish intestines may have been very high incubation temperature. With respect to incubation time, it is worthwhile to notice that LAB from the gastrointestinal tracts of coldwater fish are slow-growing. In addition, as for the human gastrointestinal tract, it can also be expected that a number of LAB representatives are not culturable by existing methods.

**3.2 Bile salt and acid tolerance**

One hundred and sixty strains of LAB isolated from gastrointestinal tracts of marine fish were tested for their abilities to grow at the bile salt levels of 2000, 3000 and 4000 ppm in order to select bile-tolerant strains, and 69, 32

Table 2. Bile salt tolerance of lactic acid bacteria isolated from marine fish.

Bile Salt Concentration (ppm)	Number of bile-tolerant strains				Percentage
	Shrimp	Shellfish	Fish	Mantis Shrimp	
2000	1	8	57	3	43.12
3000	1	5	24	2	20.00
4000	1	1	15	2	11.85

and 19 isolates were selected (Table 2). This is similar to the result obtained by Erkkila and Petaja (2000) with the strains of *Pediococcus acidilactici* (P2), *Lactobacillus curvatus* (RM 10) and *Lactobacillus sake* (L2) were the most resistant to 3000 ppm bile salt at pH 6. Pennacchia *et al.* (2004) reported that the bile salt tolerance of the *Lactobacillus* strains were able to grow in MRS agar supplemented with 3000 ppm bile salt. In the human GI-tract, the mean bile salt concentration is believed to be 3000 ppm, which is considered as critical and high enough to screen for resistant strains (Gilliland *et al.*, 1984; Goldin and Gorbach, 1992). However, *Lac. rhamnosus* strains isolated from Parmigiano Reggiano cheese were able to survive at bile salt concentration of 10,000, 15,000 and 20,000 ppm after 48 hour of incubation at 37°C (Succi *et al.*, 2005). It has been reported that certain strains of *Lactobacillus* are able to reduce this detergent effect by their ability to hydrolyze bile salt by bile

salt hydrolase enzyme (BSH) (Erkkila and Petaja, 2000), which are then readily excreted from the GI-tract (Maragkoudakis *et al.*, 2006). This particular enzyme decreases bile solubility and thus weakening its detergent effect.

Acid tolerant strains were selected from nineteen isolates, which were resistant to the bile salt concentration of 4000 ppm, by exposing LAB cells to PBS adjusted to pH 2.5 with 5 M HCl. After 1 hour of incubation at 37°C, only 6 strains including strains APa4, AIa1, APa5, AEa3, ARA1, AEa2 survived the exposure. They were further tested for the ability to tolerate acidic condition at pH 1.0, 2.0, 2.5 and 3.0 and number of cells survived after 0, 1, 2, 3 and 4 hours exposure were determined. Only strains APa4 and ARA1 could tolerate to pH 2.0 with 4.29 and 6.19 log CFU/ml reduction (from 9.01 and 9.78 to 4.72 and 3.59 log CFU/ml survival) after 1 hour of exposure, respectively (Figure 2). Strain ARA1 showed no survival after 2 hours of exposure, whereas APa4 exhibited 2.60 log CFU/ml survival (6.41 log CFU/ml reduction) at the same condition indicating that it was the most acid resistant. Four isolates (APa4, AIa1, APa5 and ARA1) showed a survival at pH 2.5 for 2 hour with survival of 3.80, 3.49, 2.87 and 3.83 log CFU/ml, respectively and they were only strains, which survived exposure to pH 4.0 for 4 hours. These results are in agreement with Succi *et al.* (2005), who selected *Lac. rhamnosus* strain isolated from Parmigiano Reggiano cheese based on their survivals after 2 and 4 hours of incubation at pH 3.0, which was similar to the study performed by Chou and Weimer (1999) on *Lac. acidophilus* strain from the American Type Culture Collection. At 90 min of incubation in buffer pH 3.5, all test strains showed maximum survival, but no colony was observed at 96 hour. Pennacchia *et al.* (2004) reported that LAB showed 60-80% survival in PBS buffer pH 2.5 for 3 hour at 37°C. Some *Lactobacillus* strains retained their viability, at pH 1 for 1 hour (Maragkoudakis *et al.*, 2006). In the present study, strains of APa4, AIa1, and ARA1 were selected based on their survival at pH 2.5 for 1 hour (53, 41 and 37% survival, respectively) (Figure 3). None of the strain was able to survive the exposure to pH 1 for 1 hour. Acid-tolerance is a fundamental property that indicates the ability of probiotic microorganisms to survive passage through the stomach. Prasad *et al.* (1998) obtained four acid tolerant strains from 200 LAB isolates based on their 80% survival after exposure to pH 3 for 3 hours.

Three strains of APa4, AIa1 and ARA1 were selected and identified based on their nucleotide sequences of 16S rDNA as *P. pentosaceus*, *P. pentosaceus* and *Ent. faecium*, respectively. Alignment by the nr database using the BLAST program showed that the 16S rDNA sequences of strains APa4, AIa1 and ARA1 are significantly similar to those of 16S rDNA from *P. pentosaceus* LM2 (98% identical over 655 bp; GenBank accession number AY675245), *P. pentosaceus* SL4 (97% identical over 691 bp; GenBank accession number AY675243), and *Ent. faecium* SF (98% identical over 492 bp; GenBank accession number AY 675247), respectively.

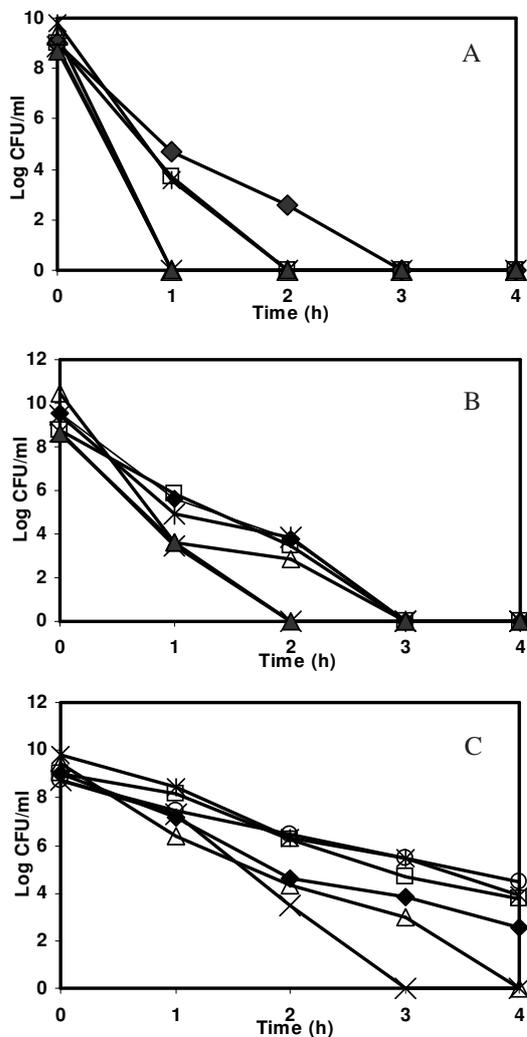


Figure 2. Survival of LAB strains: APa 4 (◆), AIa1 (◻), APa5 (Δ), AEa3 (×), ARA1 (∗) and AEa2 (◊) under acidic conditions at A) pH 2; B) pH 2.5 and C) pH 3.

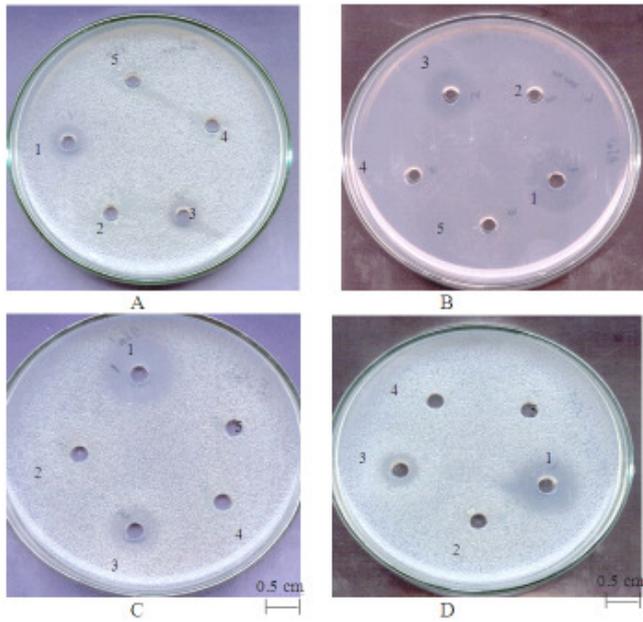


Figure 3. Inhibition zones from antimicrobial activity of *P. pentosaceus* AIA1 on A) *Salmonella* sp.; B) *L. monocytogenes*; C) pathogenic *E. coli*; D) *S. aureus*, were shown. 1: Positive control (100 µg/ml chloramphenical), 2: negative control (MRS broth), 3: culture supernatant (CFF), 4: neutralized culture supernatant (CFBH), 5: catalase-treated supernatant (CFB)

**3.3 Antibacterial activity against foodborne pathogens**

Three strains of APa4, AIA1 and ARA1 identified as *P. pentosaceus* APa4, *P. pentosaceus* AIA1 and *Ent. faecium* ARA1, respectively, were selected based on their bile salt and acid tolerances, and subjected to assay for the ability to inhibit growth of *S. aureus*, *Salmonella* sp., pathogenic *E. coli* and *L. monocytogenes* by agar well diffusion method. All LAB strains selected showed antibacterial activity against all indicator microorganisms. Culture supernatants (CFF) obtained from *P. pentosaceus* APa4, *P. pentosaceus* AIA1 and *Ent. faecium* ARA1 exhibited varying degree of inhibitory activity against strains of *S. aureus*, *Salmonella* sp., pathogenic *E. coli* and *L. monocytogenes*. *P. pentosaceus* APa4, *P. pentosaceus* AIA1 exhibited stronger antibacterial activity

than *Ent. faecium* ARA1, and showed the highest inhibition against *L. monocytogenes*. Neutralized culture supernatants (CFBH) and catalase treated supernatants (CFB) of all strains exhibited no inhibition (Table 3). The inhibitory effect may be due to acid or the bacteriocin-like substances or combination of both (Aslim et al., 2005). Aroutcheva et al. (2001) have revealed that no correlation was found between bacteriocin activity, lactic acid and hydrogen peroxide production. They reported that *Lactobacillus* strains 228, 345, and 431 produced H<sub>2</sub>O<sub>2</sub> but did not demonstrate any inhibitory effect. Similar results were obtained in this study; all strains may produce H<sub>2</sub>O<sub>2</sub> but did not show any inhibitory effect. All the Gram positive and Gram negative indicators were inhibited by the culture supernatants (CFF). The highest antimicrobial activity was against *L. monocytogenes* and *E. coli*, respectively (Figure 3). Similar results were reported by Gurira and Buy (2005) who showed that *Pediococcus* sp. was highly inhibitory against *L. monocytogenes* ATCC 7644 but low inhibitory against *B. cereus* ATCC 1178. LAB may exert their antibacterial activity through the production of lactic acid and other metabolites such as hydrogen peroxide and short chain fatty acids. Also specific antibacterial compounds such as bacteriocins or antibiotics have been identified in the culture medium of several lactic acid producing bacteria.

**4. Conclusions**

One hundred and sixty strains of LAB were isolated from gastrointestinal tracts of marine fish. Strains APa4, AIA1 and ARA1 identified as *P. pentosaceus* APa4, *P. pentosaceus* AIA1 and *Ent. faecium* ARA1, respectively were selected based on their survival at low pH and high concentration of bile salt indicating their high potential to be able to survive in the human upper gastrointestinal tract. Besides, they also showed inhibitory activity against human pathogens including *S. aureus*, *Salmonella* sp., pathogenic *E. coli* and *L. monocytogenes*.

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Table 3. Diameter of inhibition zone (mm) caused by antimicrobial activity of LAB strains against test microorganisms.

LAB strains	<i>S. aureus</i>			<i>Salmonella</i> sp.			<i>L. monocytogenes</i>			Pathogenic <i>E. Coli</i>		
	CFF	CFBH	CFB	CFF	CFBH	CFB	CFF	CFBH	CFB	CFF	CFBH	CFB
<i>P. pentosaceus</i> APa4	12.23±0.40	NI*	NI	10.80±0.20	NI	NI	17.80±0.35	NI	NI	14.13±1.10	NI	NI
<i>P. pentosaceus</i> AIA1	10.53±0.31	NI	NI	10.0±0.17	NI	NI	17.80±0.6	NI	NI	15.80±0.60	NI	NI
<i>Ent. faecium</i> ARA1	11.27±0.50	NI	NI	10.67±0.12	NI	NI	14.80±0.2	NI	NI	12.47±0.58	NI	NI

\*No inhibition

Note: CFF = culture supernatant, CFBH = culture supernatant adjusted to pH 6.5-7.0 with 1M NaOH, CFB = culture supernatant adjusted to pH 6.5-7.0 with 1 M NaOH and treated with 200 unit/ml of catalase (CFB).

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