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EFFECTIVENESS OF THE FERMENTATIVE EXTRACT OF *Lactobacillus acidophilus* AS ANTIMICROBIALS AGAINST *Aeromonas hydrophila*

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ABSTRACT

The purpose of this study was to evaluate the in vitro and in vivo antimicrobial activity of commercial *Lactobacillus acidophilus* (*L. acidophilus*) cells and cell free extract against *Aeromonas hydrophila (A. hydrophila*). The in vitro method was carried out using well diffusion method. For in vivo evaluation, the effect of *L. acidophilus* on the survival rate of *Pangasianodon hypophthalmus* (*P. hypophthalmus)* infected with *A. hydrophila* was evaluated. The well diffusion method showed a significant inhibition ability of *L. acidophilus* cells against *A. hydrophila* compared to the cell free extract. The inhibition diameters obtained with cells and cell free extract were 17.23 mm and 15.17 mm, respectively. *P. hypophthalmus* injected with *L. acidophilus* cells and cell free extract following challenged with *A. hydrophila* cells showed survival rate of 70% and 60% respectively, at 2-week post challenged. The gas chromatography-mass spectrophotometry (GC-MS) result revealed that a diverse of compounds was detected in both the *L. acidophilus* cells and cell free extract, among them the most abundant component was pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), which showed a promising anticancerous activity and might be played a significant role in the recovery of the infectious *P. hypophthalmus*. The current study revealed that both cells and cell free extract of *L. acidophilus* have antimicrobial activity against *A. hydrophila*.

Key words: *Aeromonas hydrophila*, antimicrobial activity, *Lactobacillus acidophilus, Pangasianodon hypophthalmus*

ABSTRAK

Tujuan penelitian ini adalah mengevaluasi aktivitas antimikrobial dari bakteri asam laktat khususnya Lactobacillus acidophilus (L. acidophilus) cells dan cell free extract terhadap Aeromonas hydrophila (A. hydrophila) secara in vitro dan in vivo. Pemeriksaan secara in vitro dilakukan dengan menggunakan metode well diffusion, sedangkan efek L. acidophilus terhadap tingkat kelangsungan hidup dari ikan Pangasianodon hypophthalmus (P. hypophthalmus) yang diinfeksikan dengan A. hydrophila dievaluasi secara in vivo. Metode well diffusion menunjukkan bahwa L. acidophilus cells lebih mampu menghambat A. hydrophila dibandingkan dengan cell free extract. Diameter zona hambat yang diakibatkan oleh L. acidophilus cells dan cell free extract masing-masing adalah 17.23 mm dan 15.17 mm. Setelah 2 minggu ditantang dengan A. hydrophila cells, ikan P. hypophthalmus yang diinjeksi dengan L. acidophilus cells dan cell free extract menunjukkan tingkat kelangsungan hidup masing-masing sebesar 70% and 60%. Hasil analisis gas chromatography-mass spectrophotometry (GC-MS) menunjukkan adanya beragam senyawa yang terdeteksi pada L. acidophilus cells dan cell free extract. Komponen paling banyak ditemukan adalah pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), yang mempunyai aktivitas antikanker dan diasumsikan memainkan peranan penting dalam proses penyembuhan infeksi pada ikan P. hypophthalmus. Penelitian ini menunjukkan bahwa L. acidophilus cells dan cell free extract mempunyai aktivitas antimikrob terhadap A. hydrophila.

Kata kunci: Aeromonas hydrophila, aktivitas antimikrob, Lactobacillus acidophilus, Pangasianodon hypophthalmus

INTRODUCTION

Aeromonas hydrophila (*A. hydrophila*) is an opportunistic and worldwide available freshwater bacterial species exist in the intestine of various aquatic mammals including fish (Austin and Adams, 1996; Popović *et al*., 2000). *A. hydrophila* is the aetiological agent of ulcer disease, haemorrhagic septicemia, motile aeromonas septicemia (MAS), or red sore disease in a variety of freshwater fish species (Newman, 1993). It is frequently linked with stressed or immunocompromised to hosts (Roberts, 1993). It also has been recognized as the principal infectious agent of fish bacterial septicaemia in freshwater striped catfish.

Diseases of striped catfish are being considered as a prime constraint and may ultimately turn into a limiting factor in the economics of a booming as well as sustainable aquaculture industry (Crumlish *et al*., 2010). Losses of Vietnamese *P. hypophthalmus* production systems due to disease outbreak caused by *A. hydrophila* have been described previously by Subagja *et al*. (1999). Although, disease caused by *Aeromonas* species showed very high resistance capability to antibiotics (Harikrishnan and Balasundaram, 2005), still worldwide many aquaculture industry completely depend on the use of antibiotics and various chemicals for the prevention and control of bacterial diseases (Villamil *et al*., 2014). On the other hand, presently probiotics have been used as an alternative method of controlling ulcerative dermatitis disease caused by *A. hydrophila* by adding them in the feed.

Currently, lactic acid bacteria has been recognized as a most effective probiotic in aquaculture (Al-Dohail, 2010; El-Ezabi *et al*., 2011; Talpur *et al*., 2014) as well as in the dairy farms (Karska-Wysocki *et al*., 2010), due of its antagonistic effect against a wide variety of bacteria (Savadogo *et al*., 2004; Al-Dohail, 2010). *Lactobacillus acidophilus* (*L. acidophilus*) is one of the most significant probiotic candidates among all of the lactic acid bacteria, which has revealed a strong antagonistic effect against a variety of bacteria including *A. hydrophila* (Aly *et al*., 2008; Al-Dohail, 2010); *Staphylococcus xylosus* and *S. agalactiae* (Al-

Dohail, 2010); meticillin-resistant *S. aureus* (Karska-Wysocki *et al*., 2010), and *Chlostridium difficile* (Mkrtchyan *et al*., 2010). Therefore, a preliminary study was design to find out the most abundant bioactive compound that are responsible for the antimicrobial activity of *L. acidophilus* against *A. hydrophila* in order to confirm their potential effect in striped catfish culture.

MATERIALS AND METHODS

Isolation and Identification of *A. hydrophila*

Fresh water pathogenic species of *A. hydrophila* collected from the National Fish Health Research Center Penang, Malaysia was applied in this current study. *A. hydrophila* was re-isolated from the experimentally infected kidney of striped catfish and incubated on tryptic soy agar (TSA, Himedia, India) for about 24 hours at 30° C (Figure 1A). Morphologically different and well-shaped colonies were separately selected and streaked to a new TSA plates until pure colonies were gained (Figure 1B).

Gram Staining Method

A Gram staining technique was conducted in order to confirm whether the bacterium is Gram negative or positive. A pure single colony of newly cultured *A. hydrophila* was inoculated into 10 ml of triptic soy broth (TSB) for 24 hours in a constant shaking incubator (INFORS HT electron, 180 rpm) at 30° C. A loop full of the broth culture was taken and placed on a sterile slide in order to prepare a thin smear. Then the inoculating loop was spread by means of rounded motion to create 1 cm in diameter. After air dried, the slide including sample was fixed by moving quickly the entire slide over the flame of a Bunsen burner two to three times. The heat-fixed smear slide was then Gram stained following the method described by Bhattacharyya *et al*. (2015).

Molecular Identification Method

For molecular identification and confirmation of *A. hydrophila*, a single colony from a 24 hours culture of isolated bacterium in TSA was transferred to 10 ml of sterile TSB and incubated for 24 hours in a constant shaking incubator (INFORS HT electron, 180 rpm) at 30° C. The bacterial deoxyribonucleic acid (DNA) was isolated according to the protocol of genomic DNA isolation from Gram positive and Gram negative bacteria as described in the Wizard Genomic DNA Purification kit (Promega, USA) and stored the isolated DNA at 2-8° C until used.

Amplifications of *A. hydrophila* DNA were performed with the MyCycler™ Thermal Cycler (BioRad, USA). The universal primers (68F 5'TNANACATGCAAGTCGAKCG'3, Tm 52.7° C and 1392R5'ACGGGCGGTGTGTRC'3, Tm 51.4° C) were used in this study (Mashayekhan, 2002). A total reaction volume of 50 µL was used. Each reaction contained 1x reaction buffer, 1 µL of 0.2 mM dNTP, 4 µL of 2.0 mM $MgCl_2$, 2.5 µL of 0.5 µM each primers, 0.25 µL of Taq DNA polymerase, 2 µL of extracted DNA as template and 27.75 µL ddH2O. PCR was performed according to a simplified hot start protocol. Briefly, the reaction tubes were directly heated to 95° C and the temperature kept at 95° C for 5 min. Cycles were: for initial denaturation 2 min at 95° C, 30 sec at 95° C for denaturation, 30 sec at 47° C for annealing, 90 sec at 72° C for extension, and 5 min at 72° C for final extension.

The amplified DNA was detected through agarose gel electrophoresis. Briefly, the gel was prepared by adding 1 g agarose powder in 100 mL of Tris-acetate-EDTA (TAE) buffer (0.5x). The solution was heated in a small conical flask for 3-4 minutes until dissolved completely in microwave. Then, the gel was poured into a gel-tray which contained a comb in one end and left to solidify at room temperature. The gel was placed in the electrophoresis tank containing 0.5x TAE buffer. Then a ratio of 1:5 loading dye to sample were gently mixed by pipetting and loaded into the well. About 2 µL of the 1kb DNA ladder (Fermentas, USA) was also loaded to determine the sizes of the DNA fragments. The gel electrophoresis was then run at 60 volts for 40 minutes. The gel was then stained in ethidium bromide (EtBr) solution in order to check the appearance of bands. Gel doc imaging system (VersaDoc™ Imaging System Bio-Rad, USA) was used to take the image of the band in the gel.

The purification of the DNA was done by following the protocol described in the QIAquick gel extraction kit (Qiagen, USA). The gel containing the target DNA fragments were carefully removed using a sharp and clean scalpel. The gel slices were transferred into eppendorf tubes and weighed. The QG buffer were then added to the tube at a ratio of 1:3 gel to QC buffer and

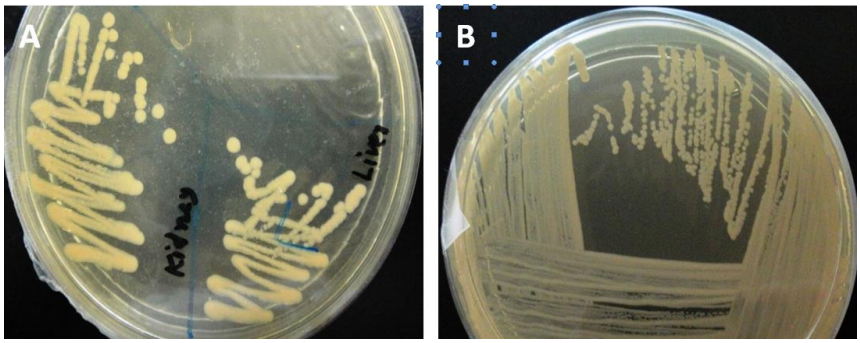

Figure 1. Cultures of *Aeromonas hydrophila*. A= *Aeromonas hydrophila* isolated from infected striped catfish, *Pangasianodon hypophthalmus* kidney and liver; B= Pure colonies of *Aeromonas hydrophila* re-isolated from infected kidney of striped catfish

incubated at 50° C for 10 minutes and mixed by vortex the tube every 2-3 minutes until the gel completely dissolved. One mL isopropanol was added to the sample and mixed well. The samples were then transferred into QIAquick columns (with collection tubes at bottom) and centrifuged at 10,000 g for 1 minute. The solution in collection tubes was discarded and about 500 µL of QG buffer was added to QIAquick column and centrifuged with same speed. Then, 750 µL of PE buffer was added to each QIAquick column and centrifuged for 1 minute. Centrifugation was once again conducted to remove residual ethanol from PE buffer. In the final step, QIAquick column was placed into sterile 1.5 mL microcentrifuge tube and 50 µL of EB buffer (10 mM Tris-CI, pH 8.5) was added to QIAquick column, and then centrifuged for 1 minute. The tubes were then kept at -20° C. The purified sample was sent to the service provider, First Base, Malaysia for sequencing.

Antimicrobial Activity of *L. acidophilus* Against *A. hydrophila*

Aeromonas hydrophila was grown-up in a similar approach as mentioned previously. Bacterial cell free extract was then removed from the cells by centrifuging at 3000 *g* for 10 minutes at 4° C and the cells were then washed two times with phosphate buffered saline (PBS) (pH 7.4) and re-suspended in the same buffer (Zheng *et al.,* 2011). The turbidity of the washed samples was measured to achieve an OD 600 nm value of 1, which corresponded to $1x10^8$ CFU mL^{-1} of bacterial suspension resulted from the plate counting.

Commercial *L. acidophilus* (International Food Grade, Laboratory of USA) was used as a probiotic bacterial strain in this current study. For seed culture, 1 g of commercial *L. acidophilus* (LAB) was incubated into 50 mL of MRS broth (2% w/v) (De Mann *et al*., 1960; Wang, 2011) for 12 hours at 37° C in a shaking incubator (INFORS HT electron, 180 rpm). Thereafter, 1 mL of those cultured bacterial suspension was transferred to 99 mL of MRS broth for mass culture and incubated for 36 hours (Al-Dohail, 2010). The bacterial cells were then harvested by centrifugation at 3000 *g* for 10 minutes and washed twice with PBS (pH 7.4) and re-suspended in the same buffer (Villamil *et al*., 2014). The cell free extract was transferred and filtered through a filter (ministart, 0.20 µm) and kept in a sterile tube for further use. Colony-forming units (CFU) were determined by a decimal dilution method in the same buffered saline. 100 µL of the diluted bacterial cells were plated in petri dishes containing MRS agar (Himedia, India) and after 48 h incubation at 37° C (Drago *et al*., 1997; Andani *et al*., 2012) counted manually. Concurrently, one mL of bacterial suspension contains $1x10^9$ CFU, which also corresponded to 1.0 OD at 600 nm wavelength.

In vitro Antimicrobial Activity of *L. acidophilus* Against *A. hydrophila*

The well diffusion method was conducted to assess the antimicrobial activity of LAB against *A. hydrophila*. About 0.2 mL of cultured *A. hydrophila* cells in PBS prepared following the method as described earlier was mixed to 15 mL of cool molten TSA (0.2%). Well mixed samples were then transferred to a new sterile petri dish and left to solidify for about 3 hours at room temperature. Then five holes (6 mm) were made using a sterilized cork tool on each petri dish. Among five holes, two holes filled with 20 µL of the cells of LAB (A), another two holes were filled with 20 µL of the cell free extract of LAB (B) and the last hole filled with 20 µL sterile PBS to serve as control (C). Three replicate petri dishes were prepared in the similar way and incubated for 24 hours at 37° C (Das *et al*., 2006). The inhibition zone around the hole was measured for the determination of antimicrobial activity.

Effect of *L. acidophilus* on Survival of *P. hypophthalmus* infected to *A. hydrophila*

In order to verify the in vitro antimicrobial activity of LAB against *A. hydrophila* a second in vivo experiment was conducted. Ninety *P. hypophthalmus* (50.23±1.34 g) were selected randomly and distributed into 9 aquariums (each of 10 fish). *A. hydrophila* and LAB were prepared in the similar way as mentioned before. The first group of fish was intraperitoneally (IP) injected with 0.2 mL of *A. hydrophila* cells (AM) suspension containing $1x10^8$ CFU mL^{-1} in PBS (considered as a lethal dose as observed in the pathogenicity test), which served as a control. While, a second group of fish was injected with 0.2 mL of LAB cells (LABP) suspension containing $1x10^9$ CFU mL^{-1} and the third group was injected with 0.2 mL of LAB cell free extract (LABS). The next day, all fish from the second and third groups were injected with the similar concentration of *A. hydrophila* cells (that injected in the first group) and the mortality was recorded for 2-week.

Identification of Bioactive Compounds from *L. acidophilus* Cells and Cell Free Extract

The extraction of bacterial metabolized was performed by following the method of Mithun and Rao (2012) with some modification. Twenty mL of the cultured bacterial samples (OD_{600}= 1.0) were harvested by centrifuging at 3000 *g* for 10 minutes. Then the cell free extract was separated and filtered using Ministart (20 µm mesh size) to remove almost all the cells. Bacterial cells re-suspended in 20 mL of broth and 20 mL of cell free extract were used for the extraction of metabolized after mixing with another 20 mL of mixture (methanol:chloroform:distilled water= 2:2:1) separately and transferred to a separating funnel and vertically left for the two phases. The lower phase was then collected after 1 hour and the upper phase was re-suspended with the similar volume of the mixture (methanol:chloroform:distilled water= 2:2:1). Similar steps were followed twice in order to extract almost all the metabolized. The extract was then evaporated using a rotary vacuum evaporator. The bacterial extract was dissolved in 1 mL of 100% methanol and mixed well

by pipetting. After filtering, the bacterial extract was transferred to a sterile 2 mL capacity vial and stored at -20° C until GC-MS analysis.

The derivative extracts separated from the cultured bacterial samples were analyzed using GCMS-QP2010 Ultra, SHIMADZU. Approximately, 1 µL of the aliquot of the extracts were injected into a BPX5 capillary column (L. 30m; I.D. 0.25 mm; film thickness 0.25 µm; max. temp. 360/370° C) using auto injector (AOC-20i, SHIMADZU). The initial gas chromatography oven temperature was 70° C, 5 min after injection the GC oven temperature was increased from 5° C/min to 320° C and held for 5 min at 320° C. Helium gas was used as a carrier gas, and pressure programmed such that the helium flow was kept constant at a flow rate of 1.7 mL/min. Detection was achieved using MS detection in electron-ionization mode and full scan monitoring mode (m/z 35-500). The ion source temperature was set at 200° C and interface temperature was set at 320° C.

Data Analysis

The results were analysed statistically using one-way analysis of variance (ANOVA) and the mean differences among the three different treatments were tested with a significance level of $P<0.05$ using a Duncan's multiple range test (Duncan, 1955). The data were presented as mean ± SD. An independent-samples T test was also performed in order to determine the mean differences within the two different groups.

RESULTS AND DISCUSSION

Gram Staining

After Gram staining, the slide was examined under a light microscope and the result revealed that the isolated bacteria showed pink colour colonies which indicated that the bacterium was Gram negative (Figure 2).

Molecular Identification

Based on morphological and biochemical characteristics, a number of colonies representing all recovered aeromonads in this study were amplified using PCR. The agarose gel (1%) stained with ethidium bromide (EtBr) of PCR product is shown in Figure 3. The obtained sequences were then aligned using the Basic Local Alignment Search Tool (BLAST) for the identification of bacteria species. The 16S rRNA gene sequences showed 100% similarity with *A. hydrophila* in the existing NCBI database (Accesion no KR067615.1) (Table 1)

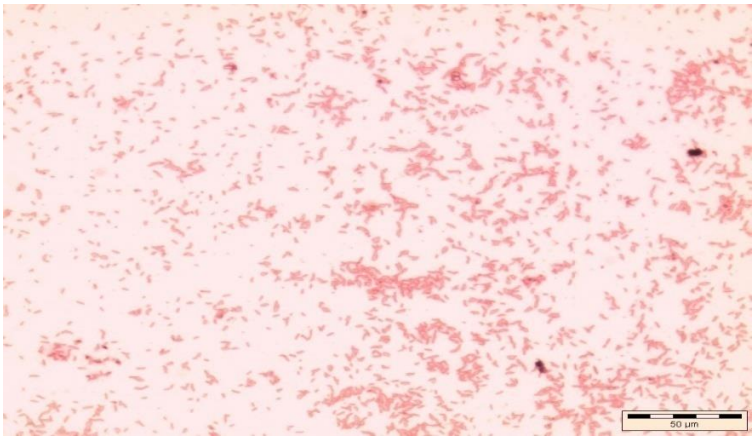

Figure 2. Gram staining of the isolated bacteria **(**pink to red stain indicate Gram-negative bacteria)

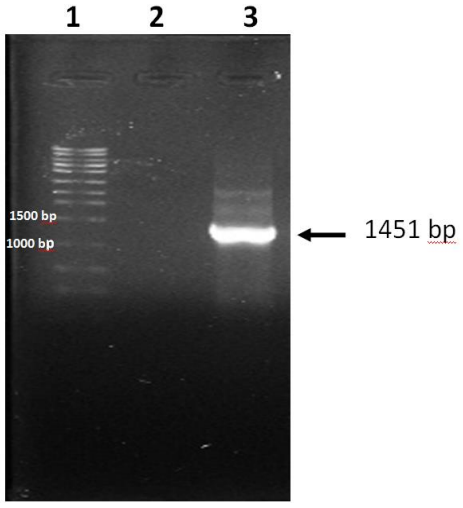

Figure 3. Agarose gel (1%) stained ethdium bromide (EtBr). Lane 1 shows a 1kb DNA ladder, lane 2 shows control and lane 3 shows PCR for *Aeromonas hydrophila*

Table 1. Identification of strain using BLAST analysis

Strain identified	E value	Identity	Accession number
Aeromonas hydrophila	0.0	98%	KR006248.1

Table 2. Inhibition zone diameter (mm) of cells and cell free extract of *Lactobacillus acidophilus* against *Aeromonas hydrophila*

Aeromonas hydrophila	*Lactobacillus acidophilus*	
	Cells	Cell free extract
Well diffusion method	17.23±0.25[a]	15.17±0.15[b]

[a,b]Different superscripts within the same row indicate significant difference (P<0.05)

Table 3. Survival of *P. hypophthalmus* injected with *A. hydrophila* cells (AM), *Lactobacillus acidophilus* cell (LABP), and *Lactobacillus acidophilus* cell free extract (LABS) following challenged with *A. hydrophila*

Survival (%)		
AM	LABP	LABS
0.00±0.00[a]	70.00±10.00[b]	60.00±10.00[b]

[a,b]Different superscripts within the same row indicate significant difference (P<0.05)

Table 4. Bioactive compounds identified from the *Lactobacillus acidophilus* cells (LABP) and cell free extract (LABS) by GC-MS

SN	Identified Compounds	Chemical Formula	Molecular Weight	LABP (%)	LABS (%)
1	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	5.551	ND
2	3-Methyl-2-pyrazinylmethanol	$C_6H_8N_2O$	124	0.326	1.094
3	1-Dodecanol	$C_{12}H_{26}O$	186	0.318	ND
4	2-Piperidinone	C_5H_9NO	99	0.459	ND
5	1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$	134	2.155	ND
6	Pyrazine, 2-methyl-5-propyle-	$C_8H_{12}N_2$	136	ND	1.306
7	1-Tetradecene	$C_{14}H_{28}$	196	0.499	1.770
8	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	16.736	27.003
9	2-Undecene, 3-methyl-, (Z)-	$C_{12}H_{24}$	168	ND	0.227
10	1-Heptadecene	$C_{17}H_{34}$	238	0.417	1.856
11	Acetic acid, [(3,5,6-trichloro-2-pyridinyl)oxy]-, methyl ester	$C_8H_6C_{l3}NO_3$	269	0.420	0.230
12	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.174	ND
13	Uric acid	$C_5H_4N_4O_3$	168	1.646	2.217
14	2-Methylhexadec-1-ene	$C_{17}H_{34}$	238	ND	0.360
16	dl-Alanyl-l-leucine	$C_9H_{18}N_2O_3$	202	0.454	0.856
17	dl-Alanyl-l-leucine	$C_9H_{18}N_2O_3$	202	0.307	0.581
18	Undecane, 3-methylene-	$C_{12}H_{24}$	168	ND	0.274
19	1-Octadecene	$C_{18}H_{36}$	252	0.452	1.469
20	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	1.141	2.213
21	Isopropyl Myristate	$C_{17}H_{34}O_2$	270	ND	0.206
22	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	47.457	33.368
23	Dotriacontane	$C_{32}H_{66}$	450	ND	0.199
24	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	5.128	4.965
SN	Identified Compounds	Chemical Formula	Molecular Weight	LABP (%)	LABS (%)
25	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, m	$C_{18}H_{28}O_3$	292	4.586	8.035
26	Eicosyl pentafluoropropionate	$C_{23}H_{41}F_5O_2$	444	ND	0.137
27	Behenic alcohol	$C_{22}H_{46}O$	326	0.297	0.698
28	Isopropyl Palmitate	$C_{19}H_{38}O_2$	298	0.098	0.234
29	1,3-Diaminobenzo[f]quinazoline	$C_{12}H_{10}N_4$	210	0.174	ND
30	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294	0.817	0.316
31	9-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	296	2.803	1.179
32	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	0.353	0.677
33	1-Heptacosanol	$C_{27}H5_6O$	396	N.D.(Ref)	0.355
34	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	$C_{12}H_{22}N_2O_2$	226	0.681	0.594
35	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	$C_{14}H_{18}N_2O_2$	246	0.141	ND
36	14-Pentadecanoic acid	$C_{15}H_{28}O_2$	240	0.102	ND
37	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244	5.152	6.333
38	Cyclo-(l-leucyl-l-phenylalanyl)	$C_{15}H_{20}N_2O_2$	260	ND	0.225
39	Total (%)			98.848	98.977

In vitro Antimicrobial Activity of *L. acidophilus* against *A. hydrophila*

The inhibition ability of the commercial *Lactobacillus acidophilus* (International Food Grade, Laboratory of USA) cells and cell free extracts against the growth of *A. hydrophila* is presented in Table 2 and Figure 4.

In the well diffusion method, the significantly higher inhibition zone (mm) was observed, in the cells of LAB (17.23±0.25) to *A. hydrophila* compared to the cell free extract (15.17±0.15). The evidence of these clear zones produced by the inhibition of pathogens revealed that LAB successfully inhibited the growth of

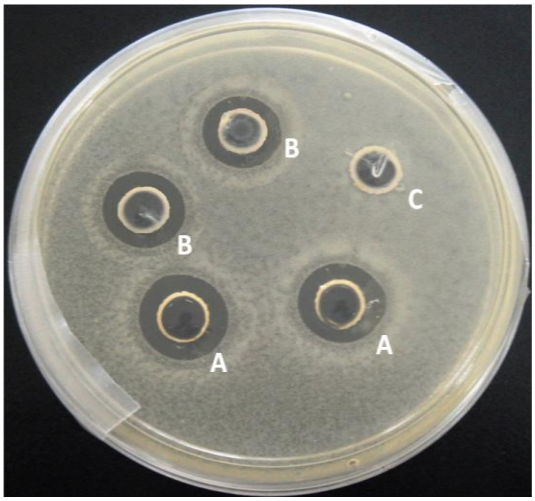

Figure 4. Zone of antimicrobial inhibition of *Lactobacillus acidophilus* against *Aeromonas hydrophila* used well diffusion method. A= Indicates cells of *Lactobacillus acidophilus*; B= Cell free extract of *Lactobacillus acidophilus*, C= Control

the *A. hydrophila*. However, the actual mechanisms involved in inhibiting growth are not completely understood yet. This inhibition zone afforded by the cells of LAB might be due to antagonism to the pathogen and/or the competition between the probiotic and the pathogenic bacteria for their adhesion sites in agar (Al-Dohail, 2010), or in the mucous membrane (Olsson *et al*., 1992) or for their nutrition (Enany *et al*., 2012). It has been well recommended that the LAB may exert their antimicrobial activities through the production of bioactive compounds, such as bactericins, organic acids and hydrogen peroxide during their metabolism (Drago *et al*., 1997). Similar to the present study, Ajitha *et al*. (2004) also reported the positive influence of four strains of lactic acid bacteria (LAB) such as *Lactobacillus acidophilus*, *Streptococcus cremoris*, *Lactobacillus bulgaricus*-56 and *Lactobacillus bulgaricus*-57 in vitro against some bacterial pathogens and observed cell free extracts of those probiotics are effective in inhibiting the growth of *Vibrio alginolyticus*. In contrast with the present study, no inhibition was observed in the well diffusion method in the case of cell free extract of lactobacilli cultures incubated for 48 hours in MRS broth (Drago *et al*., 1997).

Effect of *L. acidophilus* on Survival of *P. hypophthalmus* infected to *A. hydrophila*

The fish in all the three groups started to be evidence for clinical signs 1 day after injection with *A. hydrophila*. Typical clinical sings of this haemorrhagic septicaemia disease caused by this pathogenic bacterium includes lesions of haemorrhages at the base of the pelvic fins, on the ventral surface of the body and abdominal distension (Figure 5B, 5C) when the bacteria and their toxins are exist within various organs of the fish, and severe ulcers of the fish abdominal skin (Figure 5A). Interestingly, the fish injected with either LABP or LABS following challenge using *A. hydrophila* showed recovery from severe infection (Figure 6).

The cumulative survival of striped catfish after being infected with *A. hydrophila* is presented in Figure 7 and Table 3. The result revealed that *A. hydrophila* cells (AM) injected fish started to give evidence of its infection sign from the first day after injection and all the fish were died within the first four days. Whereas, the fish injected with LABP and LABS following challenged with *A. hydrophila* cells showed 70% and 60% survival rate respectively, at 2-week post challenged, but did not differ significantly (P>0.05) when compared between these two groups.

Bioactive Compounds Identified from *L. acidophilus* Cells and Cell Free Extract

The Gas Chromatography-Mass spectrometry (GC-MS) study on the extract of LAB cells (LABP) reveals 28 peaks, yielding an acceptable 98.848%, while, LAB cell free extract (LABS) represents 29 peaks with 98.977% yield (Table 4). The most abundant metabolite present in both LABP and LABS is pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- which account to 47.457 and 33.368% respectively.

The GC-MS result revealed that a diverse of compounds was detected in both the LAB cells and cell free extract. The most abundant component is pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl). This component was also detected in the metabolites of *Micrococcus luteas* previously, which showed a promising anti-cancerous activity (Mithun and Rao, 2012). The recovery from severe infection of fish previously injected with LAB cells and cell free extract might be due to the presence of this anti-cancerous component. Beside this component, a large number of components includes antimicrobials, organic acids and alcohols were also detected in both the LAB cells and cell free extract, which might be a reason of inhibition the growth of *A. hydrophila* as well as higher survival of previously injected striped catfish using LABP and LABS. Several studies also reported the ability of lactic acid bacteria in producing antimicrobial compounds, organic acid and alcohols (De Keersmaecker *et al*., 2010; Rattanachaikunsopon and Phumkhachorn 2010).

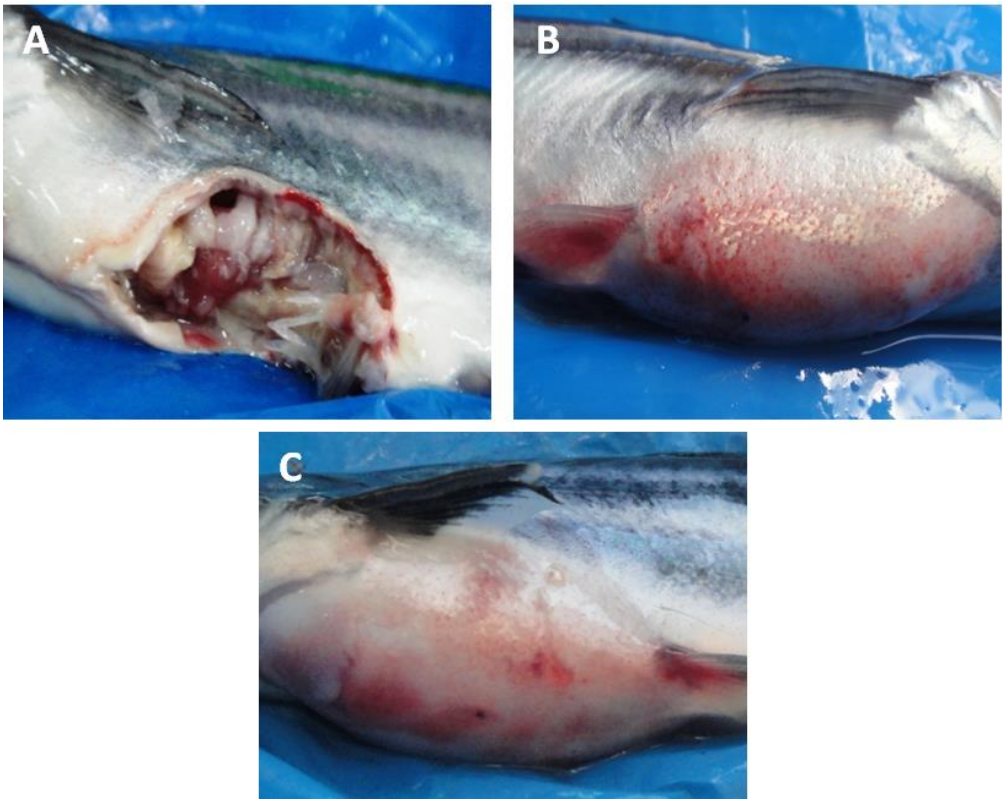

Figure 5. Clinical signs of infected *P. hypophthalmus* with *Aeromonas hydrophila*. A= First group fish (AM) showed severe abdominal skin ulceration, B= Second group (LABP), C= Third group of fish (LABS) showed haemorrhages on the ventral surface of the body and at the base of the pelvic fins and abdominal distension

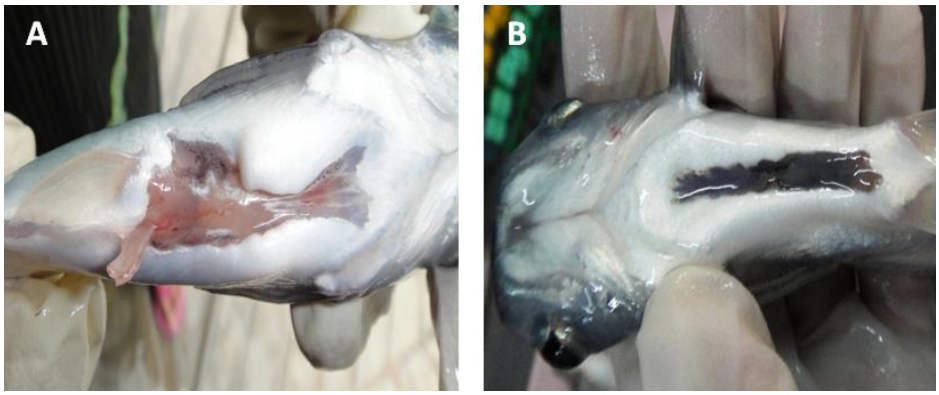

Figure 6. *P. hypophthalmus* followed challenged with *Aeromonas hydrophila* cells showed a healing from severe infection. A= Injected with *Lactobacillus acidophilus* cells (LABP), B= Injected with *Lactobacillus acidophilus* cell free extract (LABS)

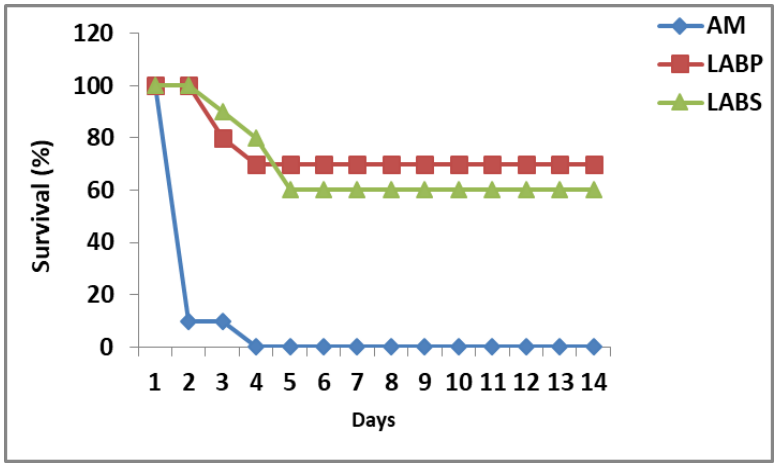

Figure 7. Striped catfish survival after 2-week of the challenged. AM= *Aeromonas hydrophila* cells; LABP= *Lactobacillus acidophilus* cells; LABS= *Lactobacillus acidophilus* cell free extract

CONCLUSION

The current study revealed that both the cells and cell free extract of *L. acidophilus* have antimicrobial activity against *A. hydrophila*, which is being recognized as one of the most significant causative agent of striped catfish disease.

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REFERENCES

Ajitha, S.M., Sridhar, N. Sridhar, I.S.B. Singh, and V. Varghese. 2004. Probiotic effects of lactic acid bacteria against *Vibrio alginolyticus* in *Penaeus (Fenneropenaeus) indicus* (H. Milne Edwards). **Asian Fish. Sci**. 17:71-80.

Al-Dohail, M.A. 2010. Effect of Probiotic, *Lactobacillus acidophilus* on Pathogenic Bacteria, Growth, Haematological Parameters and Histopathology of African Catfish, *Clarias gariepinus*. **Ph.D. Thesis**. Universiti Sains Malaysia. Malaysia.

Aly, S.M., Y.A.G. Ahmed, A.A.A. Ghareeb, and M.F. Mohamed. 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica* (*Oreochromis nilotica*) to challenge infections. **Fish Shellfish Immunol.** 25:128-136.

Andani, H., R.R., A. Tukmechi, S. Meshkini, and N. Sheikhzadeh. 2012. Antagonistic activity of two potential probiotic bacteria from fish intestines and investigation of their effects on growth performance and immune response in rainbow trout (*Oncorhynchus mykiss*). **J. Appl. Ichthyol.** 28(5):728-734.

Austin, B. and C. Adams . 1996. Fish Pathogens. In **The Genus Aeromonas.** Austin, B., M. Altiweg, P.J. Gosling, and S.W. Joseph (Eds.). John Wiley & Sons, Chichester, U.K.

Bhattacharyya, S., A. Prasad, A. Sarfraz, N.K. Jaiswal, and R. Kumar. 2015. Evaluation of a new method for Gram staining of bacteria. **Med. Sci**. 18(73):16-17.

Crumlish, M., P.C. Thanh, J. Koesling, V.T. Tung, and K. Gravningen. 2010. Experimental challenge studies in Vietnamese catfish, *Pangasianodon hypophthalmus* (Sauvage), exposed to *Edwardsiella ictaluri* and *Aeromonas hydrophila*. **J. Fish Dis.** 33(9):717-722.

Das, B.K., S.K. Samal, B.R. Samantaray, S. Sethi, P. Pattnaik, and B.K. Mishra. 2006. Antagonistic activity of cellular components of *Pseudomonas* species against *Aeromonas hydrophila*. **Aquaculture**. 253(1):17-24.

De Keersmaecker, S.C.J., T.L.A. Verhoeven, J. Desair, K. Marchal, J. Vanderleyden, and I. Nagy. 2006. Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. **FEMS Microbiol. Lett.** 259(1):89-96.

De Mann, J.C., M. Rogosa, and M.E. Sharp. 1960. A medium for the cultivation of lactobacilli. J**. Appl. Microbiol**. 23(1):130-135.

Drago, L., M.R. Gismondo, A. Lombardi, C. de Haën, and L. Gozzini.1997. Inhibition of in vitro growth of entero pathogens by new *Lactobacillus* isolates of human intestinal origin. **FEMS Microbiol. Lett.** 153(2): 455-463.

El-Ezabi, M.M., S.S. El-Serafy, M.A. Essa, S. Lall, S.M. Daboor, and N.A. Esmael. 2011. The viability of probiotics as a factor influencing the immune response in the Nile tilapia, *Oreochromis niloticus.* **Egypt. J. Aquat. Res.** 15(1):105-124.

Enany, M.E., M.E.A. El-Atta, and M.M.E. Tantawy. 2012. In vitro effects of *Lactobacillus acidophilus* cell free extract and crab haemolymph serum as antagonizing *Aeromonas hydrophila* and *Vibrio alginolyticus.* **Egypt. J. Aquat. Res.** 2(2): 63-72.

Harikrishnan, R. and C. Balasundaram. 2005. Modern trends in *Aeromonas hydrophila* disease management with fish. **Rev. Fish. Sci.** 13(4):281-320.

Karska-Wysocki, B., M. Bazo, and W. Smoragiewicz. 2010. Antibacterial activity of *Lactobacillus acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus* (MRSA). **Microbiol. Res**. 165**:**674-686.

Mashayekhan, S. 2002. Isolation And Characterization of Capable of Forming Biofilms on Metal Surfaces. **Master thesis**. Osaka University, Japan.

Mithun, S.L.V. and C.S.V.R. Rao. 2012. Isolation and molecular characterization of anti cancerous compound producing marine bacteria by using 16S rRNA sequencing and GC-MS techniques. **IJMER.** 2(6):4510-4515.

Mkrtchyan, H., S. Gibbons, S. Heidelberger, M. Zloh, and H.K Limaki. 2010. Purification, characterisation and identification of acidocin LCHV, an antimicrobial peptide produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine. **Int. J. Antimicrob. Agents.** 35(3):255-260.

Newman, S.G. 1993. Bacterial vaccines for fish. **Annu. Rev. Fish Dis.** 3:145-185.

Olsson, J.C., A. Westerdahl, P.L. Conway, and S. Kjelleberg S. 1992. Intestinal colonization potencial of turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*) associated bacteria with inhibitory effects against *Vibrio anguillarum*. **Appl. Environ. Microbiol.** 58(2):551-556.

Popović, N.T., E. Teskeredžić, I. Strunjak-Perovic, and R. Čož-Rakovac. 2000. *Aeromonas hydrophila* isolated from wild freshwater fish in Croatia. **Vet. Res. Commun.** 24**:**371-377.

Rattanachaikunsopon, P. and P. Phumkhachorn. 2010. Lactic acid bacteria: Their antimicrobial compounds and their uses in food production. **Ann. Biol. Res.** 1(4):218-228.

Roberts, R.J. 1993. Motile Aeromonad Septisemia. In **Bacterial Diseases of Fish.** Inglis, V., R.J. Roberts, and N. R. Bromage (Eds.). Blackwell Scientific Publications, New Jersey, USA.

Savadogo, A., C.A.T. Ouattara, I.H.N. Bassole, and A.S. Traore. 2004. Antimicrobial activities of lactic acid bacteria strains isolated from Burkina Faso fermented milk**. Pakistan J. Nutr**. 3(3):174-179.

Subagja, J., J. Slembrouck, L.T. Hung, and M. Legendre. 1999. Larval rearing of an Asian catfish *Pangasius hypophthalmus* (Siluroidei Pangasiidae): Analysis of precocious mortality and proposition of appropriate treatments. **Aquat. Living Resour.** 12(1):37-44.

Talpur, A.D., M.B. Munir, A. Mary, and R. Hashim. 2014. Dietary probiotics and prebiotics improved food acceptability, growth performance, haematology and immunological parameters and disease resistance against *Aeromonas hydrophila* in snakehead (*Channa sriata*) fingerlings. **Aquaculture**. 426-427:14-20.

Villamil, L., C. Reyes, and M.A. Martínez-Silva. 2014. In vivo and in vitro assessment of *Lactobacillus acidophilus* as probiotic for tilapia (*Oreochromis niloticus*, Perciformes: Cichlidae) culture improvement. **Aquacult. Res.** 45(7):1116-1125.

Wang, Y. 2011. Use of probiotics *Bacillus coagulans*, *Rhodopseudomonas palustris* and *Lactobacillus acidophilus*as as growth promoters in grass carp (*Ctenopharyngodon idella*) fingerlings. **Aquacult. Nutr.** 17(2):372-378.

Zheng Z.L., K.Y. Wang, D.M. Gatlin III, and J.M. Ye. 2011. Evaluation of the ability of GroBiotic®-A to enhance growth, muscle composition, immune responses, and resistance against *Aeromonas hydrophila* in Nile tilapia, *Oreochromis niloticus*. **J. World Aquacult. Soc.** 42(4):549-557.