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Full Length Research Paper

Isolation, identification and characterization of *Leuconostoc mesenteroides* as a new probiotic from intestine of snakehead fish (*Channa striatus*)

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The intestinal microflora of snakehead (*Channa striatus*) fish was studied to isolate and identify lactic acid bacteria as new probiotic. A total of five lactic acid bacteria were isolated from intestine to evaluate with probiotic properties. *Leuconostoc mesenteroides* sp. *mesenteroides* with more ability to inhibit growth of *Aeromonas hydrophila* was selected and identified by conventional and molecular techniques. This strain was able to survive and grow from pH 3 to 8 with the highest viability and growth rate at neutral conditions (pH 7). In addition, *L. mesenteroides* tolerated 0, 0.15 and 0.3% bile salt concentrations. This bacterium also, showed inhibitory activity against three tested fish pathogens which included; *A. hydrophila, Pseudomonas aeruginosa* and *Shewanella putrefaciens*. Antibiotic sensitivity test indicated that this strain was resistant to Streptomycin, intermediate to Amoxicillin and Kanamycin and sensitive to Gentamycin, Tetracycline, Chloramphenicol, and Ampicillin.

Keywords: Isolation, characterization, probiotic, intestine, *Leuconostoc mesenteroides*, snakehead fish.

INTRODUCTION

Snakehead (*Channa striatus*) is widespread and popular fish in Southeast Asia. This fresh water fish has different beneficial effects, and used as wound healing and remedies (Jais et al., 2002). Since there is no information on the microbiology of this fish; finding the lactic acid bacteria (LAB) as probiotic can be one of the first efforts to develop snakehead culture.

It has been well documented that the use of antibiotics makes drug-resistant microorganisms and keeps antibiotic residues in fish flesh and environment. In addition, antibiotics can affect the normal microflora of digestive tract which is beneficial to host and may be inhibited by treatment (Aly et al., 2008). In this respect, use of probiotic bacteria is a new approach gaining acceptance in aquaculture to control potential pathogens (Gomez-Gil et al., 2000; Aly et al. 2008; Kim and Austin 2008).

Lactic acid bacteria (LAB) are known microorganisms that have probiotic properties. They can produce inhibitory compounds such as lactic acid, hydrogen peroxide, diacetyl, acetaldehyde and bacteriocin. These compounds are able to inhibit the growth of harmful microorganisms (Gatesoupe, 1999; Ringø and Gatesoupe, 1998). According to many reports, lactic acid bacteria are normal flora in gastrointestinal (GI) tract of healthy animals like mammals and aquaculture animals (Nikoskelainen et al., 2001) with no harmful effects (Ringø et al., 1998). Probiotics are products which improve intestinal microflora and support good health for host. In general, probiotics protect against infections, alleviate lactose intolerance, reduce blood cholesterol levels, improve weight gain and feed conversion ratio, and also stimulate the immune system (Salminen et al., 2004; Agrawal, 2005).

Potential probiotic usually performs based on some criteria such as acid and bile salts tolerance, antibiotic

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susceptibility and antibacterial test as antagonistic effect against pathogens (Cebeci and Gurakan, 2003; Pan et al., 2008). The present experiment aimed to isolate and identify lactic acid bacteria as new probiotic from intestine of snakehead fish for the first time. Probiotic properties were also investigated to find out high potential probiotic of selected strain to use in fish production.

MATERIALS AND METHODS

Sampling

A total of 30 live, healthy and wild adult snakehead fish with average weight 350 to 400 g were obtained from the river and was transferred to Aquatic laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). 10 fishes were randomly selected; anesthetized with MS222; then the total body weight and total length measured. The surface of fish bodies were disinfected by alcohol (70%); dissected under antiseptic conditions; intestines taken out and washed three times with normal saline (NaCl 0.85 % w/v). The intestines were then cut in small pieces (1 g) and homogenized (Rengpipat et al., 2008).

Isolation of LAB

Using serial dilution (up to 10^{-4} CFU/ml, NS), 0.1 ml of homogenized intestine samples was spread on tryptic soy agar (TSA) (Merck) followed by 48 h incubation at 30°C to count total colony of bacteria (Paludan-Mu¨ller et al., 1999). The intestine samples were then immersed in de Man Rogosa and Sharp (MRS) (Merck) broth and incubated at 30°C for 24 h. After pipetting, 0.1 ml of the cultured broth was transferred to MRS agar containing bromo-cresol purple (0.17 g/L) (Badis et al., 2004; Rengpipat et al., 2008). The plates were incubated at 30°C for 48 h under anaerobic condition (Oxoid anaerobic gas pack jar). Yellow colonies sub-cultured three times on new MRS agar to obtain single colonies (Rengpipat et al., 2008). Gram staining, catalase reaction by 3% hydrogen peroxide (H_2O_2) and also morphology using phase contrast microscopy were used for primary identification of the isolates (Nguyen et al., 2007; Kopermsub and Yunchalard, 2010).

Antagonistic effect test of the isolated LABs

Antibacterial activity of the strains was tested by disc and well diffusion techniques using cell-free cultured broth of the individual selected colonies (Cappuccino and Sherman, 2002). Briefly, *Aeromonas hydrophila* as freshwater fish pathogen was cultured in TSB broth; incubated at 30°C for 24 h; and then streaked on TSA plates. At the same time, the isolates were cultured in MRS broth for 24 h at 30°C; the bacterial cells were precipitated at 8000 rpm and 4°C for 5 min; the supernatant was used for the assay. Sterile disc were immersed in the supernatant; air dried; and placed on TSA plates containing *A. hydrophila*. The plates were incubated at 30°C for 24 to 48 h to observe inhibition zone (Aly et al., 2008; Lauzon et al., 2008; Rengpipat et al., 2008). 1 of the isolates with the greatest inhibition zone was selected for more potential probiotic evaluation.

Identification of selected isolate

The pattern of carbohydrate fermentation was determined using API kit (50 CH, API 50 CHL medium, bioMerieux, France) according to the manufacturers' instruction to identify the selected strain.

Genotype (16s-rRNA)-based method was also used to confirm and subscribe the conventional identification method (Pond et al., 2006; Thapa et al., 2006; Nguyen et al., 2007). The genomic DNA of the isolates was extracted using DNA extraction kit (Genomic DNA Mini kit, Genaid). Polymerase chain reaction (PCR) was used to amplify the 16s-rRNA of the extracted DNAs using primers pAF 5' AGA GTT TGA TCC TGG CTC AG 3' as forward and phR 5' AAG GAG GTG ATC CAG CCG CA 3' as reverse primers (Rengpipat et al., 2008; Kopermsub and Yunchalard, 2010). The respective fragments were purified from the agarose gel using QIAquick gel extraction kit (QIAGEN, Hilden, Germany). The purification was carried out according to instructions supplied by the manufacturer. The purified products were sequenced by the service of the First BASE Laboratories (Seri Kembangan, Selangor, Malaysia) using the specific primers, pAF and phR. The 16S-rRNA sequencing, around 1500 bp, were analyzed using BIOEDIT nucleotide sequences software and then compared with the published sequences in GenBank using BLAST software (http://www.ncbi.nlm.nih.gov/blast/) from National Center for Biotechnology Information (NCBI) and were used for searching in GenBank as public data.

Probiotic characteristics

pH tolerance

Acid tolerance of the selected bacterium was investigated at different pH. First, MRS broths with different pH including 2, 3, 4, 5, 6, 7 and 8 were prepared using HCl 1% (Sigma) and NaOH 1 N (Sigma) and divided in universal bottles (Samelis et al., 1994). The broths media along with control bottles were autoclaved at 121°C for 15 min and then inoculated with overnight culture of the selected strain in MRS broth followed by incubation at 30°C. Optical density (OD) as growth rate of bacteria was measured by spectrophotometer (Shimadzu, UV-1601, Japan) at 600 nm after 2 h incubation. The viability of the isolates was also controlled by duplicate inoculation on MRS agar (Cebeci and Gurakan, 2003; Balcázar et al., 2008; Kim and Austin, 2008; Lauzon et al., 2008).

Bile salt tolerance

Bile salt tolerance was further tested in MRS broth which included 0.0, 0.15 and 0.3% (w/v) Oxgall bile salt (Sigma Chemical Co.St. Louis, MO, USA). Duplicate bottles of MRS broth containing filtered different concentrations of bile salt were inoculated by 30 µl of cultured strain and incubated at 30°C. Growth rate was assessed by measuring the optical density by spectrophotometer (Shimadzu, UV-1601, Japan) at 600 nm after 0, 2, 4 and 8 h incubation (Cebeci and Gurakan, 2003; Balcázar, et al., 2008; Kim and Austin, 2008).

Antibacterial Activity of the isolated LAB

Three freshwater fish pathogens, *A. hydrophila, Pseudomonas aeruginosa* and *Shewanella putrefaciens* were used to determine the antibacterial effect of the candidate strain by disc diffusion and well diffusion techniques. The pathogenic bacteria were cultured in TSB and incubated at 30°C for 24 h. Thereafter, 30 µl of the cultures with 10^3 CFU/ml was spread on TSA by swab. At the same time, the selected strain was cultured in MRS broth at 30°C for 24 h. The bacterium cells were harvested by centrifugation at 8000 rpm and 4°C (Eppendorf, Centrifuge 5810R, Germany) for 5 min and their supernatants were used for antibacterial test using disc and well diffusion methods (Cappuccino and Sherman, 2002; Balcázar et al., 2008).

Antibiotic sensitivity test

Antibiotic sensitivity test was carried out for selected strain on the most common antibiotics in aquaculture by disc diffusion technique (Akinjogunla et al., 2010).
They included Gentamycin (GM, 10 µg), Streptomycin (S, 10 µg), Amoxicillin (AMX, 25 µg), Tetracycline (TE, 30 µg), Chloramphenicol (C, 30 µg), Ampicillin (AM, 10 µg), Erythromycin (E, 15 µg) and Kanamycin (K, 30 µg). 50 µl of the 24 h broth culture of the strain was spread on MRS agar and, antibiotic Bio-discs (BioMerieux, France) were subsequently placed on plates by Oxoid Disc Dispenser System. Finally, the plates were incubated at 30°C for 24 to 48 h to observe and measure the inhibition zone (Cebeci and Gurakan, 2003; Kim and Austin, 2008). The interpretations and zone sizes were illustrated based on table of Kirby-Bauer test (Bauer et al., 1966).

Statistical analysis

Statistical analysis was conducted to compare the quantitative results of treatments using one-way analyses of variance (ANOVA) by the SAS program. Duncan's Multiple Range Test and Least Square Difference (LSD) were performed to determine difference between treatments (SAS, 1988).

RESULTS AND DISCUSSION

Total colony count of bacteria in intestine

Total plate counts of bacteria in fish intestine were determined on TSA agar medium using serial dilution (up to 10^{-4}). Total bacterial count showed 1.5×10^5 CFU/g population in intestine. This result is supported by some reports. Austin and Al-Zahrani (1988) reported 2×10^4 to 4×10^5 bacterial population levels in stomach of rainbow trout (*Salmo gaidneri*). Ringø et al. (2006) determined the population levels of adherent bacteria in foregut, midgut and hindgut of Atlantic cod with different diet. The bacterial population level varied between $7 \times 10^{3-4}$, 4×10^3 and $4.5 \times 10^{4-5}$ in foregut, midgut and hindgut, respectively. Also, Hovda et al. (2007) determined the average bacterial counts in foregut, midgut and hindgut of Atlantic salmon which were log 3.9, log 3.7 and log 5.6 CFU/g, respectively.

Isolation, selection and identification of selected LAB

The bromo-cresol purple in MRS agar as an indicator causes yellow colonies in color for lactic acid bacteria (Badis et al., 2004; Rengpipat et al., 2008). A total of five yellow colonies of lactic acid bacteria designed as LAB-1 to LAB-5 were isolated from adult snakehead fish intestine. The isolates were gram-positive, catalase-negative and short rod or coccobasilli shaped. According to the antibacterial test, LAB-4 showed more inhibition zone than other LABs. Therefore, this strain was selected for identification and further probiotic characteristics analysis.

Results obtained from the pattern of carbohydrate fermentation using API kit and 16s-rRNA as molecular technique identified LAB-4 as *Leuconostoc mesenteroides* sp. mesenteroides. Figure 1 shows the electron microscopic image of the strain that was taken by scanning electron microscopy (SEM).

Isolation step showed that lactic acid bacteria were a minor part of microflora in snakehead gut. The same result has been reported by Ringo (1993). He found that 10% population level of gut microbiota in Arctic charr (Salvelinus alpinus L.) was lactic acid bacteria. Though, Ringo et al. (2005) reported the gut microbiota of fish was less diverse than in terrestrial animals, the gastrointestinal tract of fish is not as simple as believed. Some reports confirmed the presence of Leuconostoc species in Arctic charr (Salminen et al., 2004), rainbow trout (Lyhs et al., 2002) and sea foods (Mauguin and Novel,1994).

pH tolerance

Statistical analysis showed that the growth rate (optical density) of L. mesenteroides sp. mesenteroides changed significantly ($p< 0.05$) from pH 2 to 8. This LAB did not have any activity and viability at pH 2 after 2 h incubation but, presented viability and growth at pH 3 and more. The growth rate of L. mesenteroides increased from pH 3 to 7; then decreased at pH 8 significantly ($p<0.05$) (Figure 2).

The results show that the pH could significantly affect viability and growth activity of L. mesenteroides sp. mesenteroides. The lowest viability and growth was obtained at pH 2 and the highest at pH 7; since L. mesenteroides sp. mesenteroides was isolated from intestine that has neutral condition and the highest activity was observed at pH 7. In addition, the disability of this strain to survive and grow at pH 2 can be considered as a criterion for differentiation of this LAB from other species.

According to reports, one of the most important criteria for selection of LAB as probiotic is potential viability at low pH (Nguyen, et al., 2007; Kim and Austin, 2008). Cebeci and Gurakan (2003) declared that Lactobacillus plantarum could survive at pH 4. L. plantarum PH04 was able to grow at pH between 6 and 10 while tested for pH ranged from 2 to 10 values (Nguyen, et al., 2007). Kim and Austin (2008) reported growth of probiotic Carnobacterial strains that had been isolated from rainbow trout intestine which occurred at pH 5 to 10. In the recent study, survival and growth at low pH confirm that this strain can transit through stomach.

Bile salt tolerance

Three concentrations (0.0, 0.15 and 0.3%) of bile salt

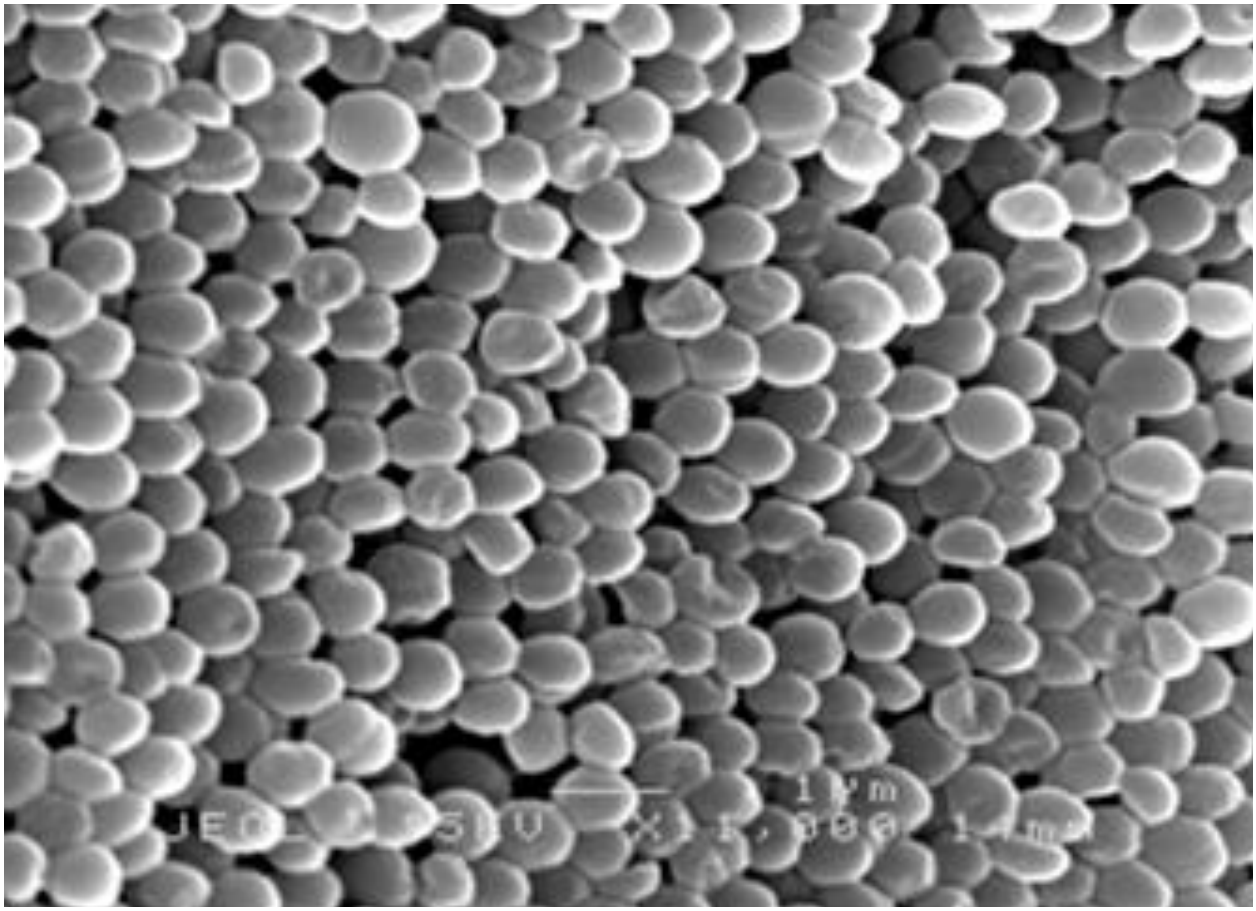

Figure 1. Image of *Leuconostoc mesenteroides* sp. *mesenteroides* (LAB-4) by electron microscope (SEM method), isolated from adult snakehead intestine.

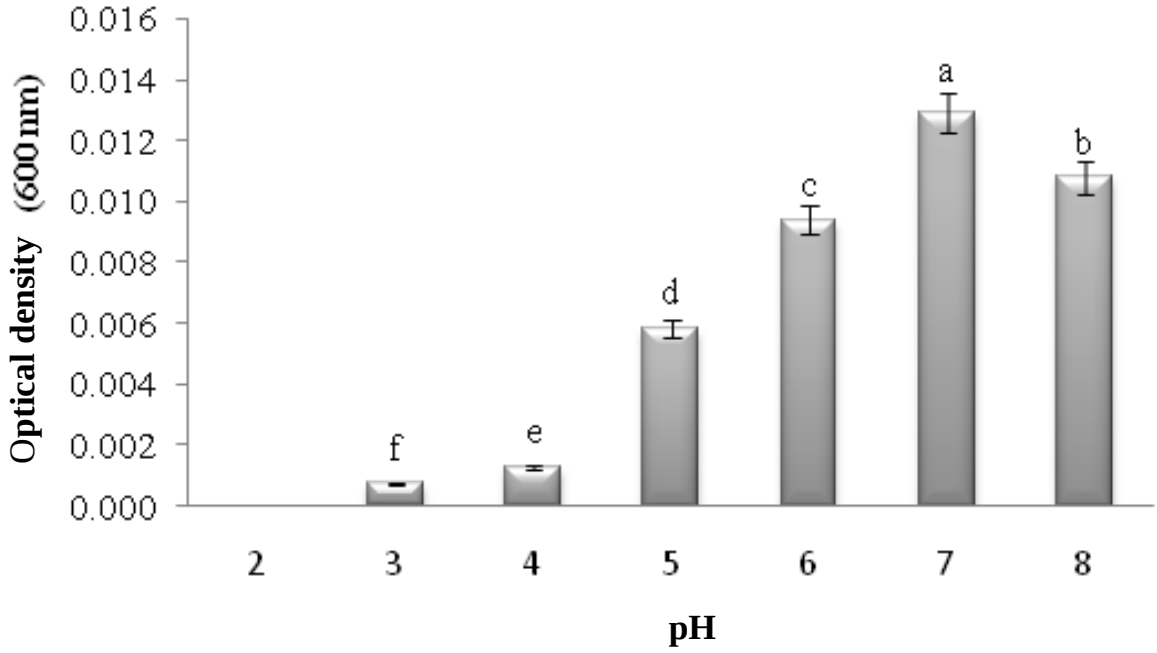

Figure 2. pH tolerance of *L. mesenteroides* in MRS broth after 2 h incubation.

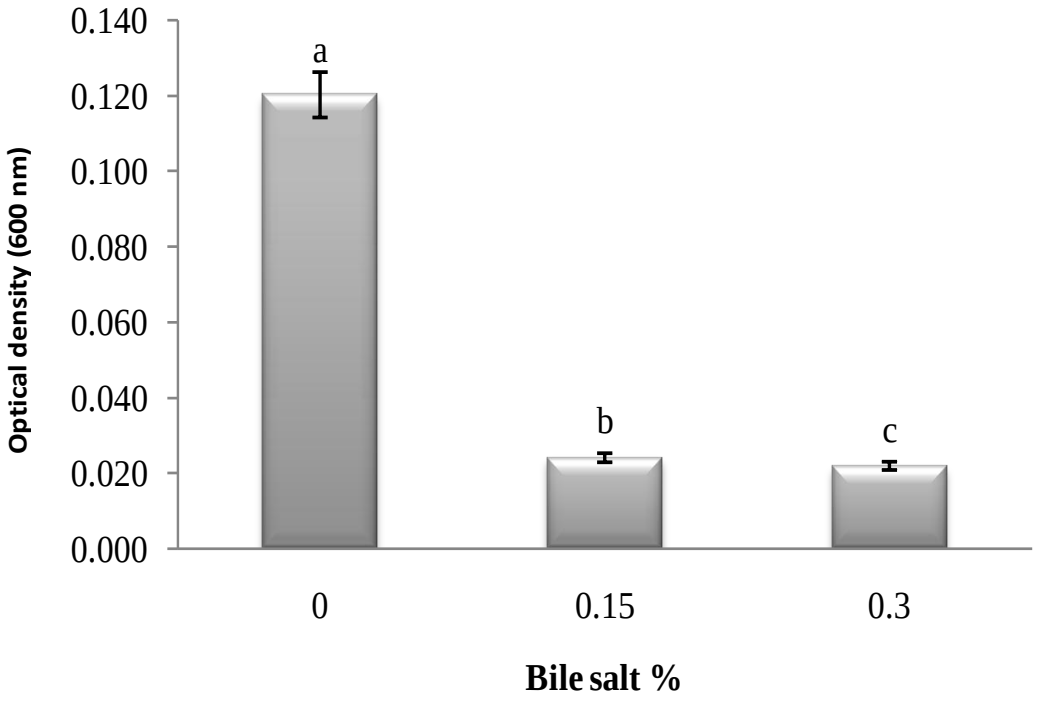

Figure 3. Bile salt tolerance of *L. mesenteroides* sp. *mesenteroides* in MRS broth after 2 h incubation.

were studied to find out tolerance of L. mesenteroides sp. mesenteroides after 2, 4 and 8 h incubation periods, respectively. It showed not only viability but also proliferation in all three concentrations for all the incubation periods. As bile salt concentration increased, the growth rate of LAB decreased significantly ($p<0.5$) (Figure 3). Similar trend with more proliferation was observed at 4 and 8 h incubation periods.

In the recent study, the bile salt affected the growth rate of isolated *L. mesenteroides* sp. *mesenteroides* and limited its ability. Furthermore, this strain showed different ability to survive and grow in bile salt. Bile salt tolerance is required for probiotic bacterial to grow and survive in fish intestine (Salminen et al., 2004). Cebeci and Gurakan (2003) determined that *L. plantarum* as a probiotic could survive in 0.3% of bile salt. Nguyen et al. (2007) reported growth of *L. plantarum* PH04 for bile salt ranging from 0 to 0.4%. Also, *Lactobacillus fermentum* and *L. plantarum* isolated from intestine of rainbow trout were examined for growth at 2.5 to 10% extracted bile from fish gall bladder. They tolerated bile concentration for 1.5 h and no significant changes in viable counts were observed (Balcázar et al., 2008). The probiotics that can tolerate low pH and bile salt means they not only can transit through stomach and be active in intestine but also are able to be alive and survive in stress conditions (Cebeci and Gurakan, 2003). In the present study, *L. mesenteroides* sp. *mesenteroides* had bile and acid-tolerant and may appear to have high potential to adhere to fish mucus as a desirable probiotic.

Antibacterial test

Antagonistic effect test of *L. mesenteroides* sp. *mesenteroides* against three freshwater fish pathogens with two approaches was studied. Results obtained from disc diffusion technique determined the significant ($P<0.05$) inhibitory effect against *A. hydrophila* and *S. putrefaciens* compared to *P. aeruginosa.* This LAB also showed significant ($P<0.05$) inhibition zone against three tested fish pathogens in well diffusion technique. The inhibitory activity in well diffusion technique against pathogens was significantly ($P<0.05$) greater than disc diffusion method (Figure 4).

In general, results obtained from antibacterial test indicated that well diffusion method was more effective than disc diffusion method. Aly et al. (2008) reported that the growth of *A. hydrophila* was inhibited by three species of *Bacillus* bacteria that used as probiotic and also, Rengpipat et al. (2008) confirmed growth inhibition on *A. hydrophila* using a cell-free cultured broth of five LAB. Kim and Austin (2008) determined the antibacterial ability of two probiotic strains that were isolated from rainbow trout intestine against *A. hydrophila* and *A. salmonicida*. These strains inhibited the growth of both *A. hydrophila and A. salmonicida*. Moreover, similar results for *Lactobacillus delbrueckii* were reported by Pan et al. (2008).

Since intestinal tract and feces can serve as an enrichment site for pathogenic bacteria such as *Aeromonas* and *Vibrio* species, the use of probiotics with

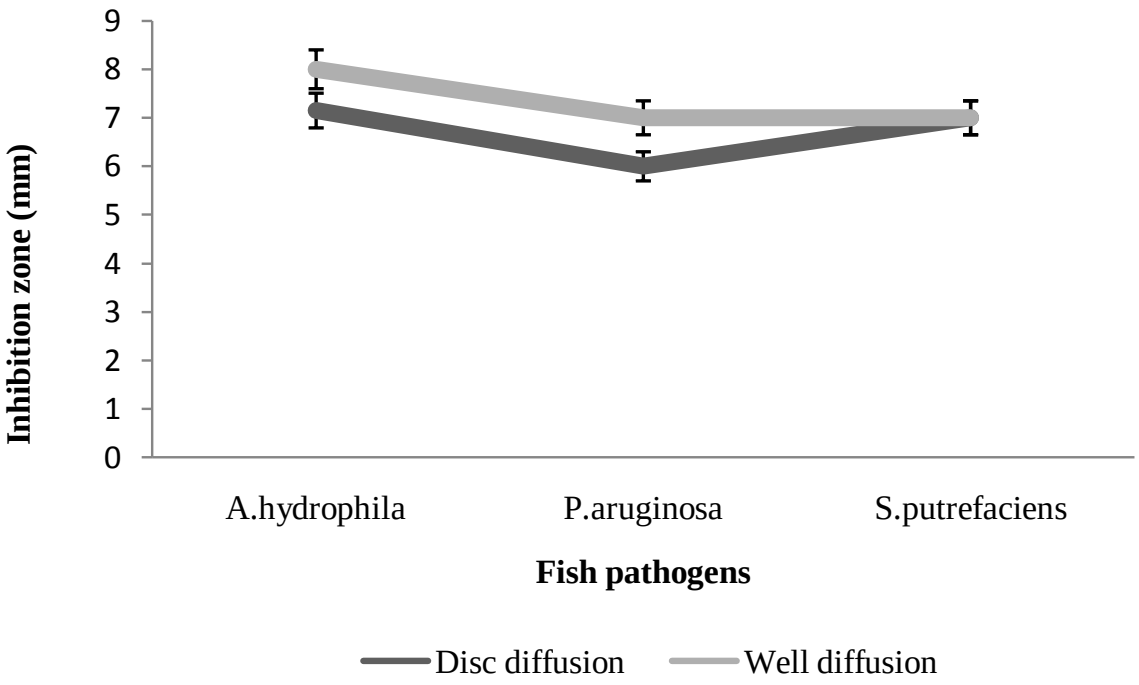

Figure 4. Antagonistic effect test of *L. Mesenteroides* sp. *mesenteroides* by disc and well diffusion techniques.

antagonistic activity may be used to reduce or inhibit pathogens activities (Balcázar et al., 2008). In general, lactic acid bacteria species have different ability to inhibit growth of pathogenic bacteria. Therefore, the findings in this study suggest that *L. mesenteroides* sp. *mesenteroides* may have high potential probiotic and anti adhesion effect against pathogens.

Antibiotic sensitivity test

Susceptibility testing was studied out to find antibiotic sensitivity of *L. mesenteroides* sp. *mesenteroides*. The interpretations of inhibition zone were determined according to zone size of chart of Kirby-Bauer test results (Bauer et al., 1966). Antibiotic susceptibility profiles showed that this strain was resistant (R) to Streptomycin, intermediate (I) to Amoxicillin and Kanamycin and sensitive (S) to Gentamycin, Tetracycline, Chloramphenicol, and Ampicillin.

Resistance to specific antibiotic means that, the probiotic can be given at the same time when antibiotic treatment is required. Secondly, microflora of intestine can recover more quickly (Cebeci and Gurakan, 2003; Kim and Austin, 2008). Kim and Austin (2008) determined the antibiotic susceptibility of *Carnobacterium* strains. They reported resistance to Ampicillin, Gentamycin, Kanamycin, Streptomycin and Penicillin G but sensitivity to Chloramphenicol, Tetracycline and Cotrimaxazole. They also believe that antibiotic-resistant probiotic may be advantageous in the case of administration of antibiotics to fish and the establishment of the beneficial microorganisms in the intestine for prolonged periods.

Conclusion

The present study concluded that *L. mesenteroides* sp. *mesenteroides* was normal in microflora in snakehead gut. This LAB also was strongly bile and acid-tolerant and may have high potential to adhere to fish mucus. In addition, this LAB showed high ability to inhibit growth of freshwater fish pathogens particularly *A. hydrophila*. Therefore, it seems that *L. mesenteroides* sp. *mesenteroides* has high potential probiotic needed in aquaculture systems for development of sustainable fish production.

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