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Identification and characterization of the Lactic Acid Bacteria isolated from Malaysian fermented fish (Pekasam)

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Abstract

Recently researchers are interested with the biotherapeutic potential of probiotics in gut disease treatment. The bacteria are generally regarded as a safe, have a stability of usage and originate from the natural resources. The study aims to identify and characterize the potential probiotic Lactic Acid Bacteria (LAB) isolated from Malaysian fermented fish product known as Pekasam. Fourty isolates obtained were firstly screened for their antagonism activities against the common pathogenic bacteria; *Esherichia coli, Staphylococcus aureus* and *Klebsiella* sp. Our study revealed only three (labeled as L8, L20 and S1) of the isolates tested showed broad antimicrobial effects towards the pathogenic bacteria. All of the isolates were also γ-hemolytic and tolerant to various pH (pH 3, 5 and 7.5) and 0.3% (w/v) bile salts. The bacteria isolates of strain L8 and L20 were susceptible to seven antibiotics tested except vancomycin and tetracycline whereas S1 was resistant to all antibiotics. Phenotypic tests revealed that both bacteria isolates of strain L8 and L20 were *Bacillus megaterium* while S1 was *Pediococcus pentosaceus* whereas 16S rRNA gene sequence analysis showed potential bacteria isolates of strain L8 and L20 belonged to the *Lactobacillus plantarum* (99% similarity) and S1 was characterized as *Lactobacillus pentosus* (100% similarity) respectively. Our present study showed that the probiotics of strain L8, L20 and S1 isolated from the fermented fish (Pekasam) exhibited the potential probiotic properties to be developed as biotherapeutic agents.

Introduction

Lactic acid bacteria (LAB) have been isolated from many fermented foods for the use as probiotics and functional food materials (Solieri *et al.*, 2014). Lactic Acid Bacteria have also been used in many Asian fermented foods, especially in non-dairy fermented products such as products from fish, meat and vegetables (Rhee *et al.*, 2011). The main LAB groups are gram-positive, catalase negative organisms and belong to genera *Lactobacillus, Bifidobacterium, Lactococcus, Pediococcus* and *Leuconostoc* (Leroy and de Vuyst, 2004). Lactic acid bacteria are able to produce acids, hydrogen peroxide and bacteriocins and possessed great potential as food bio-preservatives (Aslim *et al.*, 2005). There has been increase attention in the use of diverse strains of LAB as probiotics, mainly *Lacobacilli* and *Bifidobacteria* which are residents of the commensal bacteria in the gut of human and animals, showing good therapeutic functions (Lavanya *et al*., 2011).

Probiotics are live microorganisms and non-pathogenic which give balance and health benefit to the host if they are administered in adequate amounts (Food and Agriculture Organization and World Health Organization, 2001). Discovery of new probiotic to be used as treatment in several medical diseases such as gut discomfort, diarrhea, colorectal cancer, intestinal inflammation and allergic diseases is also important (Minervini *et al.*, 2012). However, there are several important criteria must be fulfilled to select a microorganism as a probiotic, including viability and survival during intestinal passage, production of antimicrobial substances to inhibit pathogenic bacteria, unable to transfer genetic resistance elements to intestinal host and identification at species level for safety purposes are needed to be fulfilled (Saad *et al.*, 2013).

Malaysian fermented fish, also known as Pekasam is usually made from freshwater fish with ground roasted uncooked rice (Ezzat *et al*., 2015). Pekasam is widely consumed in Peninsular Malaysia and used as an additive to improve the taste of foods. According to El Sheikha *et al*. (2013), fermentation of

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fish is an important way of preserving fish especially in situations where drying of fish is not possible. In Ghana, fermented fish is called momone, nuoc-mam of Vietnam and Cambodia, plasom of Thailand, sikhae of Japan, burong-isda of the Philippines and feseekh from Egypt and Sudan. Recent studies showed that the potential probiotic, LAB can be possibly isolated from the fermented fish (Paludan-Muller *et al*., 2002). Ohhira *et al.* (1991) were able to isolate 189 strains of LAB from 16 traditional fermented foods from Southeast Asia including *Lactobacillus, Leuconostoc, Streprococcus* and *Pediococcus*. Since the potential bacteria LAB in Pekasam has never been reported, we aim to discover potential probiotic LAB from local fermented fish.

Various parameters of probiotic screening such as antagonistic activity, hemolytic activity, pH and bile tolerance, antibiotic susceptibility were examined and also characterization of selected isolates using biochemical test and 16S rRNA to select the potential probiotic.

Materials and Methods

Isolation of lactic acid bacteria (LAB) from fermented fish

Two types of fermented fish (Pekasam) used in the study, marine fish *(Johnius Belangerii)* and freshwater fish *(Thynnichthys thynnoides)* obtained from the industrial Pekasam Warisan Utara, at Kampung Pengkalan Ikan, Kuak, Perak. The fermented fish sampels were weighed and blended in 250 ml sterile phosphate buffer saline (PBS), pH 7. The samples were suspended appropriately and serial dilutions were subsequently prepared in sterile PBS and inoculated onto : De Mann, Rogosa Sharpe (MRS) medium (Difco BD, USA) followed by incubation at 37°C for 48 h. Pure isolates were preserved in MRS broth medium (Difco BD, USA) containing 20% (v/v) glycerol and stored at -80°C.

Detection of antagonistic activity

All LAB isolates were screened for their antagonistic activities against three bacterial pathogens (*S. aureus, E. coli* and *Klebsiella* sp.) using the agar well diffusion assay as previously described by Ashwani and Dinesh (2015). 200 µl of bacterial pathogens (10^6 CFU/ml) were overlaid on 7 ml of MHA soft agar. After solidification of the agar, wells were punched using sterilized cork-borer. 30 µl of isolates (10^9 CFU/ml) was added in each well. All MHA plates were incubated at 37°C for 24 h and the zone of inhibition was measured. Ampicillin (10 µg) was used as a positive control while *Lactobacillus casei* strain Shirota (Yakult, Japan), a commercial strain was used as a reference strain. The inhibition zone diameter of 10 mm and above was considered as positive antagonism effect (Ashwani and Dinesh, 2015).

Hemolytic activity assay

Positive antagonistic isolates were then subjected to the hemolytic activity assay to determine their pathogenic potentials. The assay was performed by streaking the bacterial isolates onto 5% blood agar (Scharlau, Spain) followed by incubation at 37°C for 24 h. The hemolytic zones formed were observed and classified based on lysis activities of red blood cells in the media around and under the colonies as α-hemolysis (green zones around colonies), ß-hemolysis (clear zones around colonies), and γ-haemolysis (no clear zones around colonies). The experiment was conducted in triplicates. Only bacterial isolates with γ-haemolysis were selected for further analysis.

Acid tolerance test

Acid tolerance of the cultures was evaluated in different pH according to the method described by Bassyouni *et al.* (2012). The pH was adjusted with 1 N HCl and 1 N NaOH to of 3, 5 and 7.5. 200 µl cell suspensions of isolates with the concentration of 10^8 CFU/ml was added in the broth with different pH (test cultures) followed by incubation at 37°C for 3 h. Growth of test cultures were monitored at 0, 3 and 24 h of incubation by measuring the absorbance at 600 nm.

Bile salts tolerance test

The tolerance against bile was carried out as described earlier by Bassyouni *et al.* (2012) with slight modification based on the intestinal bile concentration, 0.3% (w/v). Isolates were grown in 15 ml of MRS broth at 37°C for 24 h. 200 µl of the cultures were inoculated into 20 ml of MRS broth prepared with 0.3% bile salts. The initial inoculums concentration was 108 CFU/ml and all samples were incubated at 37°C for 4 h. Growth were monitored after 4 h of incubation by measuring the absorbance at 600 nm.

Antibiotic Susceptibility Testing

Potential isolates were also assessed for their antibiotic susceptibilities by disc diffusion method as described by Estifanos (2014) using antibiotics discs. Seven different types of antibiotic discs (Thermo Scientific, USA) including Ampicillin (25 µg), Streptomycin (10 µg), Tetracyclin (10 µg),

Vancomycin (30 µg), Bacitracin (10 µg), Penicilin (2 units), and Chloramphenicol (10 µg) were used. One ml of cultures (10^8 CFU/ml) was mixed with 10 ml of MRS soft agar and poured into a petri dish. After solidification, the antibiotic discs were placed on the solidified agar surface, and the plates were left over for 30 min at 4°C for diffusion of antibiotics followed by incubation at 37°C for 48 h.

Phenotypic characterization of probionts

The potential of three bacterial isolates were further analysed for their phenotypic characteristics. These probionts were firstly identified using Gram-staining and catalase test. Further characterization was done using biochemical test, BBL CrystalTM Identification Systems, Gram-positive ID Kit (BD Diagnostic Systems, Sparks,MD). This method is intended for the identification of aerobic gram-positive bacteria (Murray *et al*., 1995). A single colony of fresh culture isolates was picked and suspended in a tube of BBL Crystal ANR, GP, RGP, N/H ID inoculums fluid followed by incubation at 37°C for 24 h. The resulting profile number was entered to the BBL Crystal Mind Software (BD Diagnostic Systems, Sparks, MD) to identify each of the tested bacteria.

Molecular characterization of probionts

DNA extraction was done using One-Tube Bacterial Genomic DNA Extraction Kit (Bio Basic, Canada) following the manufacturer's instruction with minor modifications. Amplification of the 16S rRNA gene was performed using universal primers, fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rp2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Allen *et al*., 2001). The PCR mixture consisted of 50 ng of 1 µl template DNA, 10 µl of 5X Green Go Taq Flexi buffer, 2 µl of 10 mM dNTPs, 1 µl of 5U Taq DNA polymerase (Promega, USA), 4 µl of 25 mM $MgCl_2$, 2 µl of 20 pmol of each primer. The total volume was brought up to 50 µl with sterile pure water. The PCR was carried out according to Ravi *et al.* (2007): pre-denaturation at 95°C for 5 min, followed by 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min then were terminated by final extension at 72°C for 5 min. The PCR products were purified using GeneJet Gel Extraction Kit (Thermo Scientific, USA) according to the manufacturer's instruction. The purified PCR products were sent for sequencing (1st Base Laboratories, Malaysia).

DNA sequence analysis and nucleotide submission

The nucleotide sequences obtained were compared to 16S rRNA gene sequences available in the GenBank database *(http://www.ncbi.nlm.nih.gov/genbank)* using the nBLAST program. Multiple sequence alignment of probionts with closely related genus was performed using BioEdit 7.1 program and phylogenetic trees were constructed by the Maximum Likelihood method (Fisher, 1922) using MEGA 6.0 software (Tamura *et al.*, 2013).

Statistical analysis

The results were expressed as the mean and standard deviation (S.D) using Microsoft Excel 2013 and were run in triplicate.

Results and Discussion

Isolation of LAB

Our present study revealed that fermented fish product (Pekasam) has many bacterial growths. After 48 h of incubation on MRS agar at 37°C, 40 single colonies were obtained and were labeled; (fermented marine fish) and S1-S16 (fermented freshwater fish). The probiotics would have the ability to interact directly with the disease-causing microbes thus can strengthen the immune system and help to prevent disease at the gut (Deepa and Mehta, 2009).

Antagonistic activity against pathogens

One of the major criteria for an isolate to be used as probiotic is its ability to inhibit or prevent the growth of bacterial pathogens. The probiotics must have the ability to interact directly with the disease-causing microbes strengthen the immune system and help prevent disease at the gut (Deepa and Mehta, 2009). Among the 40 pure isolates from the fermented fish, only 3 isolates (L8, L20 and S1) were selected for further characterization base on their positive antagonism towards pathogenic bacteria (*E. coli, S. aureus* and *Klebsiella* sp.). Table 1 indicates the diameter of inhibition zone (mm) after 24 h incubation. We showed, the highest zone of inhibition (15.83±0.29 mm) was observed with isolate L20 followed by L8 with the inhibition zone of 14.83±0.28 mm. Both inhibition zones showed antagonism activity against *E. coli.* Probionts strain L8 and L20 showed broad spectrum antimicrobial activities, suppressing the growth of *E. coli, S. aureus* and *Klebsiella* sp. while probionts strain S1 could only inhibit the growth of *S. aureus*. Our results are in agreement with report by Paulraj *et al.* (2010) which revealed *Enterococcus faecium* MC13, *Streptococcus phocae* P180, and *Carnobacterium divergens* were able to inhibit more than 18 pathogenic strains but failed to show inhibitory activity against both *E.*

Table 1. The mean ± SE of the inhibition zone (mm) of isolates (10^9 CFU/ml) against pathogenic bacteria (10^6 CFU/ml) after 24 h incubation period at 37°C

Isolates	Inhibition zone (mm)		
	E. coli	*S. aureus*	*Klebsiella sp.*
L8	+++	+++	+++
L20	+++	+++	++
S1	-	+++	-
Ampicillin (10 µg)	+++	++++	++
L. casei strain Shirota	++	++	++

Symbols for diameter of zone inhibition: ++++,>20 mm; +++,>14mm; ++,>9mm; +,>5mm; - no activity (Guo *et al.*, 2010)

Table 2. Susceptibility of isolates towards antibiotics using disc diffusion method with MHA after 24 h of incubation at 37°C

Antibiotic	S	B	A	P	E	T	V
Conc. of antibiotic	10 µg	10 µg	25 µg	2 units	10 µg	10 µg	30 µg
Isolates	Inhibition zone (mm)						
L8	S	S	S	S	S	R	R
L20	S	S	S	S	S	R	R
S1	R	R	R	R	R	R	R

S-Streptomycin, B-Bacitracin, A-Amplcillin, P-Penicillin, E-Erythromycin, T-Tetracycline, V-Vancomycin
Susceptible (S): annular radius ≥ 6mm; Resistance (R): annular radius < 6mm (Bell *et al.*, 2013)

coli strain CSH57 and *E. coli* strain SK39. This is because *E. coli* is a gram-negative bacteria which has a complex cell wall structure. The outer layer has three-layered in the form of lipoproteins, and also consists of middle layer of lipopolysaccharide which has a system of selection against foreign substances and in the form of layer of peptidoglycan (Pelzcar and Chan, 1986). In our case, a density of isolates used was 10^9 CFU/ml. This is based on Nurhidayu *et al.* (2012) study, used of 10^9 CFU/ml of probionts had shown the highest inhibitory activity against pathogenic bacteria; *V. parahaemolyticus* and *A. hydrophila.* Moreover, Strus *et al.* (2001) reported that LAB also produces bacteriocin and peptides with some inhibitory properties which can inhibit the growth of pathogenic bacteria. Antimicrobial action of LAB is also due to the production of inhibitory compunds such as lactic acid, organic acid, hydrogen peroxide, aldehydes and other metabolites (Jacobsen *et al*., 1999).

Hemolytic activity

The absence of hemolytic activity is one of the important safety precautions for a candidate probiotic (Schulze *et al*., 2006). Isolates with the positive antagonism activity (L8, L20 and S1) were further characterized for their hemolytic activity. Hemolytic activity is the ability of the isolates to cause lysis of red blood cells in the host. Results showed all isolates were γ-hemolytic (non-hemolysis), no red blood cell lysis activity observed on the blood agar. In contrast with hemolytic bacteria, they are able to break down the epithelial layer of the host cells and would cause invasive diseases in the host (Nurhidayu *et al.*, 2012).

Acid tolerance

In order to select a potential probiotic, the isolates must have an ability to survive in the stomach with acidic environment and high bile concentrations (Klaenhammer and Kullen, 1999). One of the most important characteristics of probiotic is the viability and survival in acidic condition (Boke *et al*., 2010) where they have to pass through stressful conditions of the stomach with the time taken during the digestion in the stomach is 3 h (Cakir *et al*., 2003). Isolates L8, L20 and S1 were assessed for their viabilities in the different pH (3, 5 and 7.5) during 3 h of incubation. Based on the Optical Density (OD) value at 600 nm, all isolates tested were able to survive in all different pH during 3 h of incubation. Each isolates (Figure 1a) is able to grow in all pH tested. Highest resistance was also observed in isolate L20 at all ranges of pH. In our study, all isolates were also acid tolerant and among the three isolates, strain L8 and L20 were having better pH tolerance compared to strain S1. Previous study by Argyri *et al.* (2013), 9 strains of LAB belonging to *Lactobacillus* species such as *L. plantarum, L. pentosus*, and *L. paracasei* showed very high viability and survival at low pH. Our result was also strongly supported by Bassyouni *et al.* (2012) which reported 8 of the 11 tested *Lactobacillus* LAB isolates were resistant to pH 3 during 3 h of incubation period. Survivals at pH 3 is important as ingestion with food raises the pH in stomach to 3 or higher (Martini *et al*., 1987).

Table 3. Identification of Gram-positive bacteria using BBL CrystalTM Identification System, Gram-positive ID Kit after 24 h incubation

Substrate	Probionts			Substrate	Probionts		
	L8	L20	S1		L8	L20	S1
Fluorescent negative control	–	–	–	4MU-N-acetyl-ß-D-glucosaminide	+	+	–
4 MU-ß-D-glucoside	+	+	+	4MU-phosphate	+	+	–
L-valine-AMC	–	–	+	4MU-ß-D-glucuronide	–	–	–
L-phenylalanine-AMC	+	+	+	L-isoleucine-AMC	+	+	+
4MU-α-D-glucosied	–	–	–	Trehalose	+	+	–
L-pyroglutamic acid-AMC	–	–	–	Lactose	+	+	–
L-tryptophan-AMC	+	+	+	Methyl-α and ß glucoside	+	+	–
L-arginine-AMC	+	+	+	Sucrose	+	+	–
Mannitol	+	+	–	Proline and Leucine-p-nitroanilide	+	+	+
Maltotroise	+	+	–	p-n-p-phosphate	+	+	–
Arabinose	+	+	–	p-n-p-α-D-maltoside	+	+	–
Glycerol	+	+	–	ONPG and p-n-p-α-D-galactoside	+	+	–
Fructose	+	+	–	Urea	–	–	–
p-n-p-ß-D-glucoside	+	+	–	Esculin	+	+	+
p-n-p-ß-D-cellobioside	+	+	–	Arginine	–	–	–

+ : positive reaction; - : negative reaction

Bile salts tolerance

The presence of bile in the gastrointestinal affects the viability of probiotic. In this study, the survival of isolates at 0.3% bile salts was examined. All isolates showed tolerance to 0.3% bile salts up to 24 h incubation period (Figure 1b). Isolate L8 showed the highest resistance against 0.3% bile salts compared to isolates L20 and S1. Bile salts can inhibit the growth of microorganism even at low concentration (Fuller, 1992). Kumar and Murugalatha (2012) suggested the mean intestinal bile salts concentration was 0.3% (w/v) and the staying time of food in small intestine was 4 h. Meanwhile, 0.3% (w/v) of bile salts is considered as critical and high enough to screen for resistant strain (Gilliland *et al*., 1984). Ramos *et al.* (2013) revealed that *L. plantarum* CH3, *L. plantarum* CH41 and *L. brevis* FFC199 had the highest bile tolerance compared to *L. fermentum* and *L. brevis* which had the lowest tolerance. This study showed that all these isolates can survive in the low pH of the stomach and also capable to grow and colonize in the high bile environment of the intestine.

Antibiotic susceptibility

To investigate the safety of candidate probiotic against antibiotic-resistance genes, antibiotic susceptibility assays were carried out. Bacteria carrying these genes can spread the antibiotic-resistance genes using mobile genetic elements such as plasmids and transposable elements to bacterial residents in the gut system (Romero *et al*., 2012). All isolates with positive characteristic of probiotic were tested for their susceptibilities to seven antibiotics using disc diffusion method. L8 and L20 showed

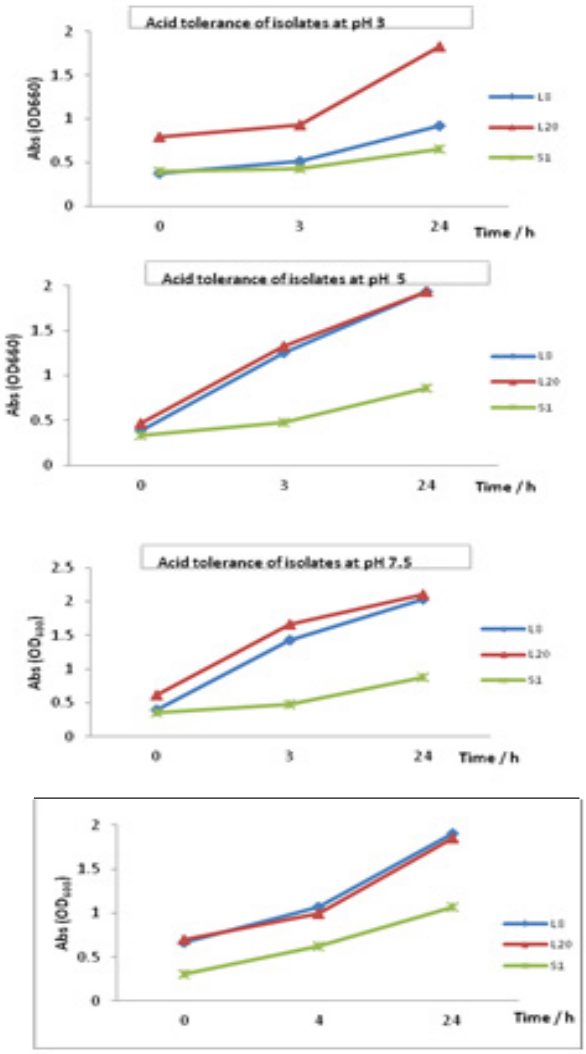

Figure 1. (a) Growth of isolates at pH 3, pH 5 and pH 7.5 for 0 h, 3 h and 24 h incubation at 37°C in MRS broth. (b) Growth of isolates at 0.3% bile salts for 0 h, 3 h and 24 h incubation at 37°C in MRS broth

susceptibilities to all antibiotics except tetracycline and vancomycin (Table 2). Isolate S1 was found to be resistance to all antibiotics tested (Table 2). All isolates were also tetracycline and vancomycin-resistant. Nelson (1999) reported LAB belonging to genus *Lactobacillus, Pediococcus* and *Leuconostoc* had high levels of resistance to vancomycin. Liasi *et al.* (2009) reported LAB is associated with their natural and intrinsic resistance due to membrane impermeability, most likely complemented by potential efflux mechanism resistance. Mathur and Singh (2005) reported intrinsic resistance is not transferrable and possess no risk in LAB because they are non-pathogenic bacteria. Therefore, the resistant genes might be present in probiotic strains but are silent.

Phenotypic characterization of probionts

Colony morphological analysis of all probionts showed bacterial isolates strain L8 and L20 shared the same morphological appearance; tiny, flat, round, smooth, and whitish in colour while bacterial isolate strain S1 is small, smooth, raised, round and grayish-white in colour. In addition, gram staining proved that all probionts were identified as Gram-positive bacteria. The probionts strain L8 and L20 were rods and arranged in clusters while S1 was cocci-bacilli shaped bacteria. However, all probionts were catalase negative and shared the same morphology; non-spore forming and non-motile. Probiont strain L8 and L20 were identified as Bacillus megaterium while probiont strain S1 was identified as Pediococcus pentosaceus based on biochemical test, BBL CrystalTM Identification System, Gram-positive ID kit (Table 3). This finding was in accordance with previous study done by Rhee *et al.* (2011) which reported in Sikhae, another example of fermented fish product from Korea contained *Leuconostoc mesenteroides* and *Lactobacillus plantarum* while Burong-isda from Philipines contains *Lactobacillus brevis* and *Streptococcus* sp. Noraphat *et al*. (2011) revealed that from 133 bacterial isolates from Thai traditional fermented fish called Plasom, 25 isolates were cocci, 75 isolates were short rods and 33 isolates were rods.

Molecular characterization of probionts using 16S rRNA sequence analysis

It is crucial to ensure that probiotics are correctly identified. Therefore, probionts strain L8, L20 and S1 were characterized using 16S rRNA analyses to determine their species. Two probionts; strain L8 and L20 showed 99% similarity to *Lactobacillus plantarum* while probiont strain S1 was identified as *Lactobacillus pentosus* with 100% similarity

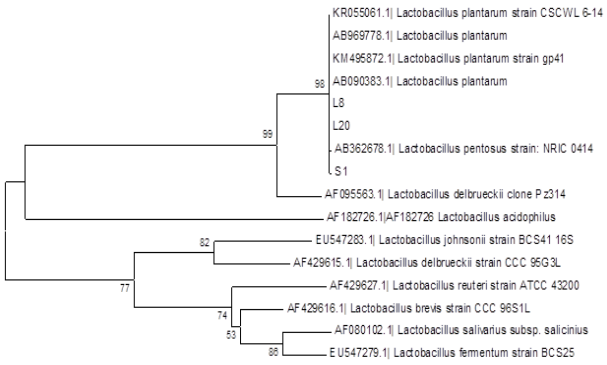

Figure 2. Phylogenetic tree by maximum-likelihood method of probionts strain L8, L20 and S1 based on 16S rRNA sequence analysis

based on GenBank database. All of the probionts strain, L8, L20, and S1 were deposited into GenBank with accession numbers KT591874, KT591875 and KT920464, respectively. Analysis using 16S rRNA is important for reliability, quality assurance and organism safety which able to identify accurately between non-pathogenic and pathogenic bacteria. The requirement of safety aspect includes organism identification to their species and strain levels. According to Janda and Abbott (2007), the function of 16S rRNA gene over time has been consistent and sufficient for informative purposes. The gene can also distribute commonly and widely in almost bacteria (Weisburg *et al*., 1991). The phylogenetic relations of the probionts with other closely-related bacteria were displayed in the dendrograms (Figure 2).

Conclusion

Forty pure colonies from fermented fish were isolated and identified according to their physiological and biochemical characteristic. In summary, 3 probionts, *Lactobacillus plantarum* strain L8, *L. plantarum* strain L20 and *Lactobacillus pentosus* strain S1 possessed probiotic potential and safe for human consumption. Further analysis will be required to evaluate the attachment to the intestinal cells, immunomodulatory activity and gene expression effects of the probionts against intestinal host.

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