Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from Fermented Fish

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Abstract

The present study describes the potential probiotic lactic acid bacteria isolated from fermented fish. A total of 23 isolates of lactic acid bacteria were purified using MRS medium. All the isolates were assessed for tolerance to low pH. It was observed that only 4 isolates (UP2, UP9, UP16, and UP20) could survive at pH 2. These isolates further evaluated in vitro for bile salts tolerance, NaCl tolerance, phenol tolerance, antagonistic activity, β-Galactosidase production, and cell surface hydrophobicity. Based on results, all selected isolates possessed a β-galactosidase and showed an ability to tolerate in bile salt, NaCl, and phenol. Among 4 selected LABs, isolates UP 20 and UP2 showed the higher cell hydrophobicity as 81% and 68.8%, respectively. These results indicate that isolate UP20 and UP2 might be useful as probiotics. However, further studies may be performed to confirm their potential health benefits and applications.

Keywords: probiotic properties, lactic acid bacteria, fermented fish

Introduction

Probiotics are living microorganisms that exert a health benefit to the host when administered in sufficient amount (FAO/WHO, 2002). Probiotics show diverse health benefits including, immunomodulation and improvement of inflammatory bowel disease and diarrhea (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). Moreover, its revealed beneficial effects include improving lactose intolerance, lowering serum cholesterol level, and decreasing the use of antibiotics (Guo, Kim, Nam, Park, & Kim, 2010). The effectiveness of the probiotic preparations is species or strain-dependent; in consequence, each candidate probiotic strain should meet some requirements, including safety, functional, and beneficial characteristics (FAO/WHO, 2002). To be classified as a probiotic microorganism, bacteria must have the ability to survive in the acidic condition of the stomach and within bile acid at the gastrointestinal tract, as well as adhesion ability is also an important property. (Rubio, Jofre, Martin, Aymerich, & Garriga, 2016). Some of the bacteria used in probiotic preparations have been isolated from fermented food to maximize the compatibility with the human gut microflora and improve the chances of survival. Microorganisms that have been most reported for use as probiotics are lactic acid bacteria (LAB) (Tamura et al., 2011). LAB are Gram-positive, rod-or coccus-shape, non-spore forming, catalase-negative and strictly fermentative bacteria, with the major metabolic end-product of carbohydrate fermentation as lactic acid. (Holzapfel, Haberer, Geisen, Bjorkroth, & Schillinger, 2001). They can be found in various sources such as soil, water, animal, and human gastrointestinal tract, as well as in food and fermented products; (Zacharof & Lovitt, 2012). LABs isolated from fermented food product are commonly used as starter cultures and may provide potential probiotic microorganisms (Abriouel et al., 2012). In the past few decades,

LABs are most commonly studied for probiotic properties as there is dominant microflora of fermentation product and generally regarded as safe (Tannock, 1997).

Traditional fermented fish has been an essential part of food culture in Thailand. Fermented fish found in all part of the country, sold in both traditional market and supermarket. It is made from fish, sugar, salt, and roasted rice and is fermented with natural microbial flora (Hwanhlem et al., 2011). Fish fermentation has many benefits and could be used as a convenient, low-cost technique for the preservation of fish muscle, improving its organoleptic qualities and increasing the nutritional value and/or digestibility of the raw material (Adams, 2009). Lactic acid bacteria are the dominant microorganisms in many fermented fish products where their primary role is to ferment carbohydrates and reduce pH. The combination of low pH (below 4.5) and organic acids is the main preservation factor in this fermented fish products (Kose & Hall, 2011). LAB starter cultures for fermented food are developed mainly by design and/or screening. The design principles are based on knowledge of bacterial metabolism and physiology as well as on the interaction with the food product (Ammor & Mayo, 2007). Nowadays, a trend in food microbiology is the use of starter microorganisms, capable of performing another function different from the whole acidification (e.g., probiotic activity) (Bevilacqua, Corbo, & Sinigaglia, 2012). This tendency was an answer to the diffuse interest towards foods added with probiotics in recent time that people around the world consume probiotic daily to maintain their health. Thus, specific strains of Lactobacillus and Bifidobacterium already introduced as probiotics in food products due to their health-promoting effects (Collado, Meriluoto, & Salminen, 2008). The use of starter cultures in fish products is still limited, and few pieces of evidence are still present about the use of probiotic/starter in these products.

The potential probiotics properties of LAB isolated from traditional Thai fermentation fish have rarely been reported. The present study, therefore, we are aiming at isolating LAB from fermented fish, a local product of Phayao province. Further probiotic properties of the isolated LAB strains were tested, including in vitro tolerance to low pH and bile salt. Additionally, antimicrobial activity was investigated with the aim of their potential probiotic application.

Methods and Materials

Isolation of lactic acid bacteria

Fermented fish were purchased from the local market in Phayao province, Thailand. All fermented fish products were made by traditional method or homemade product and the fermentation time between 3-5 days. Five grams of each sample was homogenized, with 45 ml of 0.85 % (w/v) saline and serially diluted. One hundred microliters of sample suspension were spread on MRS agar media supplemented with 125 ppm bromocresol purple (Sriphannam et al., 2012). The plates were incubated anaerobically at 37° C for 48 h. Single colony surrounded by the yellow zone was isolated by observing their colony morphology and some biochemical tests including Gram staining and catalase test. Only Gram-positive, catalase-negative strains were select and the selected strains were maintained on MRS broth at -80° C.

Catalase Test

Slide method is used to perform the catalase test. Bacterial colonies were emulsified to make a suspension. Hydrogen peroxide solution was dropped over the test suspension and the other drop on control part. The fluid over the suspension was observed for the appearance of gas bubbles.

Evaluation of probiotic properties of lactic acid bacteria

Acid tolerance test (Mishra & Prasad, 2005)

Acid sensitivity of LAB isolates were conducted according to the method of Tigu, Assefa, Mehari, and Ashenafi (2016) with some modification. Ten milliliters of PBS (adjusted to pH 2.0) was separately inoculated with an overnight culture of the LAB isolates to give a final population of ~10^8 cfu/ml. LABs were incubated at 37° C for 3 h. An inoculated without pH adjustment served as a control. Survival was investigated by pour plate method using MRS medium supplemented with 0.02% bromocresol purple as an indicator. The growth was observed after 48 h of incubation, isolates, which grew and turned the indicator to yellow on the agar media were considered as acid-tolerant and selected for further studies.

NaCl tolerance test

MRS broth were adjusted with different concentration (1-10%) of NaCl and supplemented with bromocresol purple as an indicator. Then 5% (v/v) fresh overnight culture of selected strains were inoculated and incubated at 37° C for 24 h. After 24 h of incubation, their growth was measured by observing the ability to change the indicator from purple to yellow. Maximum growth which seen as completely yellow were indicated as the double-positive sign (+ +), normal growth as a single positive sign (+) and no growth which seen as purple was indicated as the negative sign (-).

Bile resistance test

The selected strains of lactic acid bacteria were investigated for bile salt tolerance according to the method described by Rahman (2015). Overnight culture (5% v/v) of selected lactic acid bacteria was cultivated in MRS broth containing 0.15 and 0.3% (w/v) Oxgall (Difco) and incubated anaerobically at 37° C for 24 h. Then the survival rates of the isolates were monitored by measuring optical density (OD) at 600 nm. The cultivation medium without bile salts was served as control.

Tolerance to phenol

The ability of the isolates to survive in the presence of phenol solution was investigated according to the method of Rahman (2015). Briefly, bacterial culture (10^7 cfu/ml) were incubated in MRS broth containing 0.4% phenol and incubated for 24 h at 37° C. The growth was determined by observing the cell concentration at OD 600 by spectrophotometer.

Antagonistic activity

The antagonistic activity of the selected LAB isolates against *Staphylococcus aureus* and *Escherichia coli* were assayed by the agar spot test. The isolated colony of lactic acid bacteria was suspended in MRS broth and adjusted the turbidity to 0.5 McFarland standard. LAB suspension then spotted (3 µl) on MRS agar and incubated anaerobically for 12 h. Molten MHA agar was overlaid on the LAB plate after that *S. aureus* or *E. coli* was swab over the surface of MHA agar. The plate was incubated at 37° C for 24 h, and the inhibition zone around the spot of LAB was observed.

β-Galactosidase activity

For β-galactosidase activity, bacterial cultures were streaked on MRS agar plates containing 60 µl X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 10 µl of IPTG (iso-propyl-thiob-D galactopyranoside) solution as an inducer (Angmo, Kumari, Savitri, & Bhalla, 2016). The bacterial colony that possesses β-galactosidase will be shown as a blue colony.

Cell surface hydrophobicity

Bacterial cell surface hydrophobicity was assessed by measuring microbial adhesion to hydrocarbons (MATS: Microbial Adhesion to Solvents) as described by Kotzamanidis, Kourelis, Litopoulou-Tzanetaki, Tzanetakis, and Yiangou (2010). Cells cultivated at 37 °C for 24 h were washed twice in phosphate buffer saline and finally re-suspended in 3 mL of 0.1 M KNO_3 containing about 10^8 cfu/ml of bacteria and the absorbance was measured at 600 nm (A0). One milliliter of toluene was then added to the cell suspension to form a two-phase system. After a 10 minute pre-incubation at room temperature, the two-phase system was mixed by vortexing for 2 min. Then, the water and toluene phases were allowed to separate by incubating for 20 min at room temperature. The aqueous phase was carefully removed, and its absorbance at 600 nm (A1) was measured. The percentage of cell surface hydrophobicity (H%) was calculated using the following formula: H% = (1- A1/A0) × 100.

Results

Isolation of lactic acid bacteria

A total of 23 acid-producing bacterial strains were isolated from 6 samples of fermented fish. Of these, three isolates were grouped as Gram's positive cocci, one isolates was Gram's positive short-rod, and 19 isolates were Gram's positive bacilli. All isolates possess catalase- negative, colony morphology, microscopic observation, and catalase test were summarized in Table 1.

Table 1 Cultural, morphological and characteristics of bacterial isolates

Isolates	Colony morphology					Microscopic observation	Catalase test
	Form	Color	Elevation	Margin	Surface	Gram stain	
UP1	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP2	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP3	circular	white	convex	entire	smooth	positive cocci in cluster	negative
UP4	circular	white	convex	entire	smooth	positive cocci in cluster	negative
UP5	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP6	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP7	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP8	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP9	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP10	circular	light yellow	flat	undulate	smooth	positive bacilli	negative
UP11	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP12	circular	deep yellow	raised	entire	smooth	positive, short rod	negative
UP13	circular	light yellow	raised	entire	smooth	positive bacilli	negative
UP14	circular	white	convex	entire	smooth	positive bacilli	negative
UP15	circular	white	convex	entire	smooth	positive bacilli	negative
UP16	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP17	circular	white	convex	entire	smooth	positive bacilli	negative
UP18	circular	white	convex	entire	smooth	positive bacilli	negative
UP19	circular	light yellow	convex	entire	smooth	positive bacilli	negative
UP20	circular	white	convex	entire	smooth	positive bacilli	negative
UP21	circular	deep yellow	raised	entire	smooth	positive bacilli	negative
UP22	circular	light yellow	raised	entire	smooth	positive bacilli	negative
UP23	circular	white	raised	entire	smooth	positive cocci in cluster	negative

Evaluation of probiotic properties of lactic acid bacteria

Acid tolerance test

LABs isolated from fermented fish were tested for the ability to survive in the acidic condition. It was found that only 4 isolates were showed the capability to survive in pH 2, which formed the colony and turn the indicator from purple to yellow around the colony. According to this result, lactic acid bacteria isolates UP2, UP8, UP16, and UP20 were selected to investigate in the next experiments.

NaCl tolerance test

All of 4 selected isolates of lactic acid bacteria were able to survive and grow in 4-9% NaCl. Isolate UP 2 and UP 9 could not tolerate to NaCl at a concentration higher than 6%, whereas UP 16 and UP20 showed better growth rate in the higher salt concentration. Interestingly, UP20 could tolerate to the maximum level at 9% NaCl (Table 2).

Table 2 Tolerance to NaCl of isolated lactic acid bacteria

Isolates	Concentration of NaCl (%)						
	4	5	6	7	8	9	10
UP2	++	++	+	-	-	-	-
UP9	++	++	+	-	-	-	-
UP16	++	++	++	+	-	-	-
UP20	++	++	++	++	++	+	-

Legend: ++, good growth, +, fair growth, -, no growth

Bile resistance test

The selected strains were screened for their ability to tolerate bile salt in MRS broth containing 0.15-0.3% (w/v) oxgall (Difco). The results indicated that the selected isolates were able to grow at 0.15% and 0.3% of bile salt as shown by the relative growth (%) compared with control (without bile salt). Isolate UP2 and UP9 showed a better capability to grow in bile salt than isolate UP16 and UP 20. Their capacity to tolerate bile salt in different concentration-time interval showed in Figure 1.

Tolerance to phenol

The ability of the selected isolates to survive in the presence of phenol solution was investigated. It was found that all selected lactic acid bacteria capable of growing in the presence of 0.2% phenol. However, at 0.3% phenol, lactic acid bacteria could be survived but could not be propagated compare to the present of 0.2% phenol.

Antagonistic activity

The antimicrobial activity of the selected LAB isolates against *S. aureus* and *E. coli* were shown in Table 3. The agar spot test showed that all of the selected LAB could inhibit the growth of both *S. aureus* and *E. coli*. The inhibition zone was variable depended on LAB isolate and pathogenic bacteria strain. The results revealed that the LABs slightly inhibit Gram's positive bacteria better than Gram's negative bacteria.

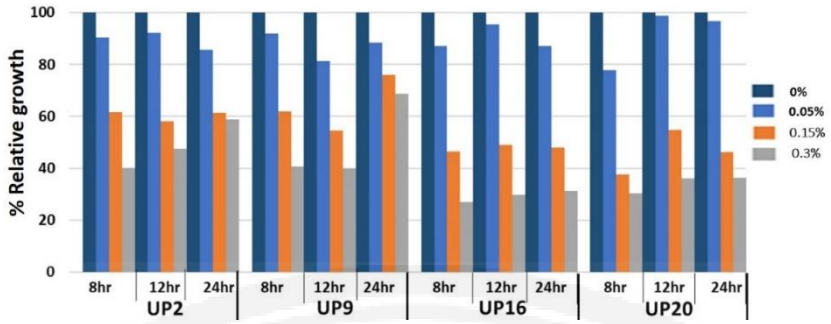

Figure 1 Tolerance against bile salt (0.15%, 0.3%), the result represent as % relative growth compared with control (without bile salt)

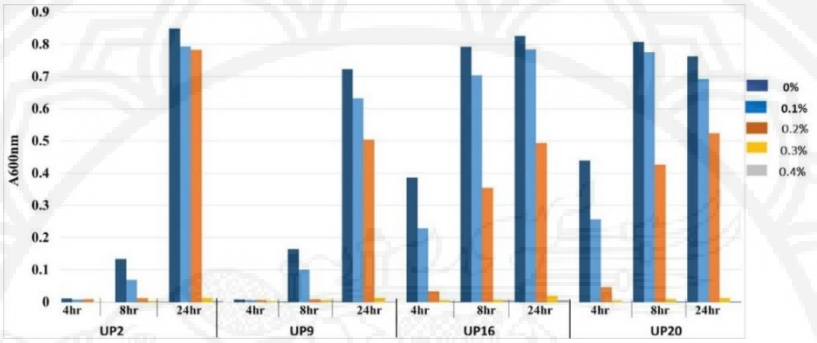

Figure 2 Tolerance against phenol (0.1- 0.4%), the result represent as the cell concentration by spectrophotomically at 600 nm

Antagonistic activity

The antimicrobial activity of the selected LAB isolates *against S. aureus* and *E. coli* were shown in Table 3. The agar spot test showed that all of the selected LAB could inhibit the growth of both *S. aureus* and *E. coli*. The inhibition zone was variable depended on LAB isolate and pathogenic bacteria strain. The results revealed that the LABs slightly inhibit Gram's positive bacteria better than Gram's negative bacteria.

Table 3 Antagonistic activity of cell free supernatant from lactic acid bacteria against *S. aureus* and *E. coli*

Lactic acid bacteria	Inhibition Zone (mm)	
	S. aureus	*E. coli*
UP 2	13.2	12.0
UP9	14.6	12.3
UP16	13.1	12.1
UP20	12.5	11.0

β-Galactosidase production

In qualitative β- galactosidase screening, isolates UP2, UP9, UP16, and UP20 possessed the presence of β-galactosidase activity after 24 h of incubation at 37° C (data not showed).

Cell surface hydrophobicity

Bacterial cell surface hydrophobicity was determined as the ability of bacteria to adhere to hydrocarbons (MATS: Microbial Adhesion to Solvents). Cell surface hydrophobicity of the LAB isolates tested was highly

variable (10%-81%) depending upon bacterial cell (Figure 3). In general, isolates UP20 and UP2 possessed a high percent hydrophobicity (81% and 68.8%, respectively) compared to other isolates investigated.

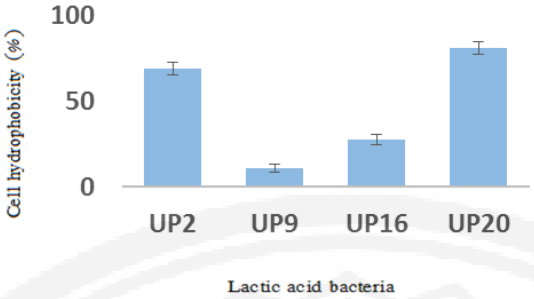

Figure 3 Cell hydrophobicity test as the ability of bacteria to adhere to hydrocarbons

Discussion

In the present work, LABs were isolated from fermented fish (Pla-som) based on critical technological criteria to obtained probiotic lactic acid bacteria. Isolation and identification of probiotic strain from fermented products make these worthwhile as they are safe and offer various health benefits. Lactic acid bacteria to be used as probiotic must overcome the unfriendly condition of the human gastrointestinal tract (GIT) and subsequently colonize the intestinal tract. In order to reach active and survive through GIT, they should be resistant to acid and bile.

The in vitro low pH tolerance test showed that 4 isolates at pH 2 exhibited a capability to survive in an acid condition. These results corresponded to the finding of Guo et al. (2010). They found that the viable counts of lactic acid bacteria were significantly affected by the low acidity, especially at pH 2. Prasad, Gill, Smart, and Gopal (1998) reported that various hydrogen ion concentrations affected the growth of bacteria and suppressed growth. Lactobacilli are considered intrinsically resistant to acid environments. They respond to acid stress condition through the stress response protein. The F_0F_1-ATPase is a known mechanism that gram-positive organisms use for protection against acidic conditions. The F_0F_1-ATPase consisting of a catalytic portion (F_1) and an integral membrane portion (F_0) which function as a membranous channel for proton translocation. The present of F_0F_1-ATPase can increase the intracellular pH at a low extracellular pH. Thus, the acid tolerance of lactobacilli is attributed to the presence of a constant gradient between extracellular and cytoplasmic pH (Corcoran, Stanton, Fitzgerald, & Ross, 2005). According to the previous report, while consumption of probiotic bacteria it will be mixed with food and carrier matrix molecules. The probiotic bacteria did not expose directly to the stomach HCl concentration. (Prasad et al., 1998)

Bile salt tolerance is considered one of the essential property required for probiotic bacteria to survive in the small intestine (Succi et al., 2005). In this study, most of the isolates showed resistance to 0.3% bile concentration. Bile plays a fundamental role in the specific and nonspecific defense mechanism of the gut. Therefore, bile tolerance is considered as an essential characteristic of LAB strains, which enables them to survive, grow, and exert their action on gastrointestinal transit.

Selected LAB isolates were tolerant of the NaCl concentration used in this study (Table 2). Especially, isolate UP20 could tolerate to the maximum concentration at 9% NaCl. For the application of LAB as starter cultures in fermented fish (Pra-ra and Pla-som), these LABs must be capable of tolerating stressful conditions

such as salt stress. To tolerate NaCl, various mechanisms developed by LABs were described, for example, the uptake or synthesis of a limited number of solutes (Bremer & Krämer, 2000).

For LAB to be a probiotic, it should resist to the toxic metabolites, primarily phenols, produced during the digestion process (Hoier, 1992). Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated by bacteria in the gut leading to the formation of phenols, which have bacteriostatic properties (Suskovic, Brkic, Matosic, & Maric, 1997).

Selected isolates were tested for antimicrobial activity against *S. aureus* and *E. coli*. All selected LABs showed higher inhibition zone against *S. aureus* than *E. coli*. Previous reports showed inhibition of similar pathogens by LAB at varying degrees (Klayraung, Viernstein, Sirithunyalug, & Okonogi, 2008). The antimicrobial mechanism involved is likely to be the production of classic bacteriocins, proteinaceous compounds produced by lactic acid bacteria with a bactericidal effect against taxonomically closely related bacteria (Lara-Villoslada et al., 2007).

Beta-galactosidase activity is also plentifully present in the colon of human beings. It catalyzes the first step of lactose fermentation in the colon and is often tested as an indication of the capacity of colonic microbiota to utilize lactose present in the intestine (Jain et al., 2007). The presence of this enzyme in bacteria intended for use as probiotic due to the reduction of lactose intolerance perspective. This enzyme also leads to the formation of galacto-oligosaccharides (GOS), the prebiotic oligosaccharide that can stimulate the growth and colonization of bifidobacteria in the human intestine and suppress potentially harmful bacteria such as *Clostridium* and *Bacteroides* species in the intestine (Sako, Matsumoto, & Tanaka, 1999).

Selected isolates were evaluated for cell surface properties, and LAB strains showed a variable degree of hydrophobicity (Mathara et al., 2008). LAB, after successful passage over the stomach and the small intestine, have to establish themselves through adhesion to epithelial cells of the large intestine where they can demonstrate their probiotic properties. Hydrophobicity was used to indicate adhesion properties in this study as the positive relationships between hydrophobicity and adhesion were reported by a different report (Klayraung et al., 2008).

Conclusion and Suggestions

Now a day, there is increasing research on traditional fermented food products. Fish fermentation, like many traditional fermenting processes, is spontaneous and uncontrolled could be a valuable source of LAB. In the present study, several LAB isolated from fermented fish products were characterized and shown to exhibited potential functional properties, including β-galactosidase, acidifying and hydrophobicity, and antimicrobial activity. The results obtained from this study concluded that all selected LAB isolates showed good probiotic potential; therefore, Its could be considered as potential candidate lactic acid bacteria. However, further studies may be performed to confirm their potential health benefits such as cholesterol removal activity, bile salts hydrolases activity, and immune modulation activity. The potential to be used of selected LAB as starter microorganism for fermented fish production will be further investigated as well.

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