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In vitro screening of potential probiotic lactic acid bacteria isolated from intestinal contents and gills of Nile tilapia

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Abstract The potential probiotic lactic acid bacteria (LAB) were isolated from intestinal contents and gills of Nile tilapia (*Oreochromis niloticus*). Those properties included antagonistic activity against pathogenic bacteria, tolerance to pH, NaCl, fresh bile from Nile tilapia. Moreover, drug resistance profiles were examined. A total of forty isolates were collected. LAB isolated from the intestinal contents showed inhibitory activity against *Bacillus coagulans* TISTR1447, *Pseudomonas fluorescens* TISTR358 and *Salmonella* Typhimurium TISTR292, whereas those from the gills revealed only antagonistic activity against *Bacillus coagulans* TISTR1447, *S.* Typhimurium TISTR292 and *Escherichia coli* TISTR780. Isolates LI10 and LG5 were selected for further evaluation of probiotic properties. Both isolates could grow in pH 4–10, NaCl concentration up to 10% and fresh bile concentration up to 10%. These isolates were resistant to cefovecin, marbofloxacin, clindamycin and trimethoprim/sulfamethoxazole, meanwhile they were susceptible to benzylpenicillin, amoxicillin/clavulanic acid, vancomycin, tetracycline, nitrofurantoin and chloramphenicol. In addition, both isolates were identified by Matrix-assisted lazer desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) as *Enterococcus feacalis*. Therefore, these two isolates could potentially be used as probiotics.

Keywords: Probiotics, Nile tilapia, Gills, Intestine, Lactic acid bacteria

Introduction

Nowadays, the consumption of fish proteins, which are important for peoples, has been increasing. As a result, the aquaculture industry is growing

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rapidly. However, disease outbreak is considered major problem of commercial aquaculture since it affects economical and consumer health; this results in the use of various antibiotics. However, a number of antibiotics contribute to drug-resistant microorganisms which are disadvantageous to intestinal microflora of fish. An application of probiotics is one of the alternatives for disease prevention in aquaculture (Verschuere *et al.*, 2000; Pan *et al.*, 2008; Nayak, 2010; Ige, 2013; Mouriño *et al.*, 2016). Probiotics are defined as living microorganisms, which are beneficial to their host by improving microbial balance, activating immunity, and reducing diseases and stress. In addition, an adequate amount of probiotic can improve fish appetite, growth performance, feed utilization, and carcass quality (Fuller, 1989; Balcázar *et al.*, 2008). An investigation of the potential probiotic criteria is necessary; it includes antagonistic activity against pathogenic microorganisms, gastric acidity and bile salt tolerance (Allameh *et al.*, 2012; Rumjuankiat *et al.*, 2017). Several probiotic strains have been identified, including lactic acid bacteria (LAB), and characterized for application in aquaculture, such as those in the genera of *Lactobacillus, Pediococcus, Lactococcus, Weissella, Bifidobacterium, Pseudomonas, Fusobacterium, Vibrio, Enterococcus, Streptococcus, Saccharomyces, Debaryomyces, Aspergillus* and *Altermonas* (Verschuere *et al.*, 2000; Zorriehzahra *et al.*, 2016).

Nile tilapia (*Oreochromis niloticus* L.) is a tropical fish which well-adapt to warm water environment, but it is sensitive to cold stress (Abdel-Ghany *et al*., 2019). In addition, it is a favored fish species in global aquaculture due to its high performance and high market value (El-Naby *et al.,* 2019). Nowadays, the global production of tilapia has increased since 1990 (Delphino *et al.,* 2019).

The purpose of this research was to evaluate the preliminary criteria for the probiotic properties *in vitro* of LAB isolated from intestines and gills of Nile tilapia.

Materials and methods

Process of sample collection

Ten Nile tilapia were acquired from a private farm in Nakhon Pathom province, Thailand, after 3 months of rearing from the same batch with an average weight of 865 g. All fish were fed with KMITL diet in this study. Each of intestine and gill samples from individual fish was removed. Thereafter, all of the intestinal contents and gills were sampled for further microbial isolation. The samples were stored in an ice box and transferred to the Excellence Center

for Meat and Protein Innovation Technology, KMITL laboratory.

Isolation and culture conditions of LAB

LAB culture was isolated from intestinal contents and gills of each fish. In brief, one gram of intestinal content or gills was diluted in 9 mL of 0.85% sterile sodium chloride (w/v), and were then homogenized by vortex. Ten-fold serial dilutions were carried out. Afterwards, the amount of 0.1 mL aliquots were cultured on De Man, Rogosa and Sharpe agar (MRS; Merck, Germany) containing 0.5% $CaCO_3$. MRS agar plates containing sample dilution were incubated at 37°C for 48 hr under anaerobic conditions (candle jar). Then, colonies with apparent clear zone were randomly selected. The strains were sub-cultured and steaked twice on new MRS agar plates to achieve purified isolates. Finally, 40 isolates were randomly sub-cultured in MRS broth added with 30% (v/v) glycerol and kept at -40°C for further probiotic characterization.

Microbial identification and species confirmation

Morphological identification

Initially, pure isolates were cultured in nutrient broth (Merck, Germany) and were further subjected to stain with Gram's staining kit (Merck, Germany). The identification of morphology was conducted using an optical microscope (Nikon Model Eclipse E200, Japan).

Microbial identification by MALDI-TOF MS

Species of the isolates were further confirmed by MALDI-TOF MS (VITEX MS^{TM}, BIOMÉRIEUX) at Kamphaeng Saen Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus. The isolates cultured from a pure colony were deposited on VITEX MS-DS target slides (BIOMÉRIEUX) followed by adding 1 µL matrix solution (VITEX MS-CHCA, BIOMÉRIEUX) on the sample smear. After air-drying, the slides were inserted into VITEX MS^{TM} (BIOMÉRIEUX, Marcy I' Etoile, France). Mass spectra were generated and compared by computing with an algorithm with databases. The results were displayed according to the confidence value for each isolate on MYLA® MS knowledge Base V3.2 used for spectrum analyses of the organisms. Values between 85 and 90% were considered an acceptable identification. A cut-off at 90% was chosen for the VITEX MS. *E. coli* ATCC 8739 was included as positive quality control in each run for both MALDI-TOF systems.

Probiotic characteristics

Antagonistic activity of the LAB isolate

Antagonistic activity of LAB strains against both pathogenic and spoilage microorganisms were examined by spot-on-lawn approach using the cell-free supernatant (CFS) of individual isolates. Species of microorganisms and their growth conditions used for antimicrobial test are listed in Table 1. LAB strains were cultured in MRS broth and incubated at 37 ℃ for 24 hr. CFS was achieved by centrifugation (WiseSpin(R)CF-10, Korea) at 12,000 rpm for 10 minutes and subsequently sterilized by filter (0.2 μm, Pall, U.S.A.). At the same time, the indicator strain was cultured in nutrient broth (Merck, Germany) and then incubated at 37 ℃ for 24 hr. Antimicrobial activity was performed by spotting 10 μL of CFS on the surface of agar plate, which was overlaid with 5 mL of soft agar (0.8-1% w/v) seeded with 10 μL of freshly-grown indicator strains. After incubation for the appropriate time and temperature, as shown in Table 1, the inhibition zone was observed and expressed as arbitrary units (AU/mL) (Ennahar *et al.*, 2001).

Table 1. List of indicator strains and their growth conditions

Indicator microorganisms	Medium*	Temperature (℃)
Bacillus coagulans TISTR1447	NB/aerobic	37
Pseudomonas fluorescens TISTR358	NB/aerobic	37
Salmonella Typhimurium TISTR292	NB/aerobic	37
Escherichia coli TISTR780	NB/aerobic	37
Listeria innocua ATCC33090^{T}	NB	37

*Growth under agitation at 200 rpm
NB = Nutrient broth (Merck, Germany)
TISTR = Thailand Institute of Scientific and Technological Research
ATCC = American Type Culture Collection

Determination of acid and NaCl tolerance

To examine acid and NaCl tolerance, the methods of Hyronimus *et al.* (2000) and Rumjuankiat *et al.* (2017) were modified for probiotic selection from LAB. To examine acid tolerance, MRS broth was adjusted for different pH 2–10 with 0.5 mol l^{-1} HCl or 0.5 mol l^{-1} NaOH. To investigate NaCl tolerance, MRS broth was added with NaCl 0–10% concentration. Two percent of collection culture strain of probiotic was propagated in MRS broth at 37℃ for 10–12 hr. The turbidity of LAB culture was visually examined for growth

rate (++ meant maximum growth, + meant normal growth and – meant no growth).

Determination of fresh bile tolerance

The methods of Balcázar *et al.* (2008) and Rumjuankiat *et al.* (2017) were modified to study bile tolerance of the selected LAB strains. Fresh bile from Nile tilapia was collected and stored at -40 ℃ until used. The selected LAB strains were inoculated in MRS broth containing fresh bile at 0–10% concentrations. Determination of growth rate was carried out as mentioned above.

Antibiotic resistance assay

Antibiotic susceptibility and minimum inhibitory concentration (MIC) of the selected LAB strains were conducted using VITEX-2 Compact, an automated microbiology system for microbial identification and susceptibility test (BIOMÉRIEUX, France) at Kamphaeng Saen Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus. Antibiotics used for the assay included benzylpenicillin, amoxicillin/clavulanic acid, cefovecin, enrofloxacin, marbofloxacin, erythromycin, clindamycin, vancomycin, tetracycline, nitrofurantoin, chloramphenicol and trimethoprim/sulfamethoxazole.

Results

Antagonistic activity of LAB isolated from Nile tilapia intestines and gills

Based on spot-on-lawn method, it was found that CFS of 20 isolates showed inhibitory activity of 100 AU/mL against *B. coagulans* ITSTR 1447. Among those, one isolate from intestine, LI10, and four isolates from gills (LG-5, LG-10, LG16 and LG17) displayed antimicrobial activity of 100 AU/ml against *B. coagulans* TISTR 1147 and *S.* Typhimurium TISTR 292. In addition, LG5 and LI10 revealed inhibitory activity against three indicator strains including *B. coagulans* TISTR 1147, *S.* Typhimurium TISTR 292 and *E. coli* TISTR 780, as shown in Table 2. Therefore, two strains, LI10 and LG5, were selected for further study on their probiotic properties.

Table 2. Antimicrobial activity of LAB isolated from Nile tilapia intestines and gills against indicator bacteria

Isolates	Antimicrobial activity (AU/mL)				
	B. coagulans TISTR1447	*P. fluorescens* TISTR358	*S.* Typhimurium TISTR292	*E. coli* TISTR780	*L. innocua* ATCC33090^{T}
Intestines					
LI-1	100	-	-	-	-
LI-2	-	-	-	-	-
LI-3	-	-	-	-	-
LI-4	-	-	-	-	-
LI-5	-	-	-	-	-
LI-6	-	-	-	-	-
LI-7	-	-	-	-	-
LI-8	-	-	-	-	-
LI-9	100	-	-	-	-
LI-10	100	-	100	100	-
LI-11	100	-	-	-	-
LI-12	100	-	-	-	-
LI-13	100	-	-	-	-
LI-14	100	-	-	-	-
LI-15	100	-	-	-	-
LI-16	100	-	-	-	-
LI-17	100	-	-	-	-
LI-18	-	-	-	-	-
LI-19	-	100	-	-	-
LI-20	-	100	-	-	-
Gills					
LG-1	-	-	100	-	-
LG-2	-	-	100	-	-
LG-3	100	-	-	-	-
LG-4	100	-	-	-	-
LG-5	100	-	100	100	-
LG-6	100	-	-	-	-
LG-7	100	-	-	-	-
LG-8	100	-	-	-	-
LG-9	100	-	-	-	-
LG-10	100	-	100	-	-
LG-11	100	-	-	-	-
LG-12	100	-	-	-	-
LG-13	100	-	-	-	-
LG-14	100	-	-	-	-
LG-15	100	-	-	-	-
LG-16	100	-	100	-	-
LG-17	100	-	100	-	-
LG-18	100	-	-	-	-
LG-19	100	-	-	-	-
LG-20	100	-	-	-	-

Acid and NaCl tolerance

Two selected LAB isolates, LI10 and LG5, were able to survive in MRS broth at pH 4–10 and MRS containing up to 10% NaCl. Moreover, the growth of both isolates declined in lower pH and higher concentration of NaCl (Table 3).

Table 3. The growth of LI10 and LG5 isolates during exposure to different concentrations of pH and NaCl

Concentration	Growth level	
	Isolate LI10	Isolate LG5
Acid tolerance (pH)		
2	-	-
3	-	-
4	+	+
5	++	++
6	+++	+++
7	+++	+++
8	+++	+++
9	+++	+++
10	+++	+++
NaCl concentration		
0%	+++	+++
1%	+++	+++
2%	+++	+++
3%	+++	+++
4%	+++	++
5%	++	+
6%	+	+
7%	+	+
8%	+	+
9%	+	+
10%	+	+

Notation: Double positive sign (++), positive sign (+) and negative sign (-) were evaluated for maximum growth, normal growth, and no growth, respectively.

Fresh bile tolerance

It was found that both isolates could grow apparently at a concentration up to 8% of fresh bile, while lower growth was observed when utilizing 10% fresh bile, but still survived (Table 4).

Table 4. The growth level of selected isolates for fresh bile tolerance

Concentration	Growth level	
	Isolate LI10	Isolate LG5
0%	+++	+++
1%	+++	+++
2%	+++	+++
3%	+++	+++
4%	+++	+++
5%	+++	+++
6%	+++	+++
7%	+++	+++
8%	+++	+++
9%	++	++
10%	+	+

Notation: Double positive sign (++), positive sign (+) and negative sign (-) were evaluated for maximum growth, normal growth, and no growth, respectively.

Identification of selected strains for probiotic properties

After gram staining, it was found that both isolates, LI10 and LG5, were gram-positive with cocci shape. Species confirmation revealed that both isolates were *Enterococcus feacalis* with a confidence value of 99.9%. Therefore, these isolates were named as *E. faecalis* LI10 and *E. feacalis* LG5, respectively (Table 5).

Table 5. Morphological identification and species confirmation of LI10 and LG5 isolates

Isolate	Gram	Shape	Species confirmation by MALDI-TOF MS	Confidence value
LI10	+	Coccus	*E. faecalis*	99.9
LG5	+	Coccus	*E. faecalis*	99.9

Antibiotic resistance

Relied on the investigation with VITEK 2 instrument, both isolates, *E. faecalis* LI10 and *E. feacalis* LG5, were resistant to cefovecin, marbofloxacin, clindamycin and trimethoprim/sulfamethoxazole with MIC ≥8, ≥4, ≥4 and ≤10 µg/mL, respectively. However, they were susceptible to benzylpenicillin, amoxicillin/clavulanic acid, vancomycin, tetracycline, nitrofurantoin and chloramphenicol with MIC of 2, ≤2, 1, ≤1, ≤16 and ≤4, respectively (Table 6).

Table 6. Antibiotics susceptibility of *E. faecalis* LI10 and *E. faecalis* LG5

Antibiotics	***E. feacalis* LI10**		***E. feacalis* LG5**	
	MIC (µg/mL)	Interpretation	MIC (µg/mL)	Interpretation
Benzylpenicillin	1	S	2	S
Amoxicillin/ Clavulanic Acid	≤2	S	≤2	S
Cefovecin	≥8	R	≥8	R
Enrofloxacin	1	I	1	I
Marbofloxacin	≥4	R	≥4	R
Erythromycin	2	I	2	I
Clindamycin	≥4	R	≥4	R
Vancomycin	1	S	1	S
Tetracycline	≤1	S	≤1	S
Nitrofurantoin	≤16	S	≤16	S
Chloramphenicol	≤4	S	≤4	S
Trimethoprim/ Sulfamethoxazole	≤10	R	≤10	R

Notation: S = Susceptible: the infection may respond to treatment at the normal dosage;
I = Intermediate: the results are equivocal and the test should be repeated if the bacterium is not fully susceptible to an alternative drug;
R= Resistant: the bacterium is not inhibited by the usually achievable systemic concentrations of the antimicrobial agent and efficacy has not been reliable in clinical studies.

Discussion

This study evaluated LAB strains for potential probiotic properties using intestines and gills from Nile tilapia. Forty isolates were selected for assessing antagonistic activity, including tolerance to acid, NaCl, and bile salt. In this study, LI10 from the intestine and LG5 from the gills, displayed antagonistic activity against pathogenic bacteria, including *S.* Typhimurium and *E. coli* and spoilage bacteria, including *B. coagulands.* Antagonistic activity is one of the important criteria for probiotic selection (Prabhurajeshwar and Chandrakanth, 2017). In general, LAB produce several antimicrobial substances including organic acid, bacteriocin, diacetyl and hydrogenperoxide, which inhibit the growth of other microorganisms (Holzapfel and Wood, 1995; Verschuere *et al.*, 2000; Narakaew *et al.*, 2010; Ige, 2013; Prabhurajeshwar and Chandrakanth, 2017). Another important criterion to select probiotic strains included the survival of strain during passing through the gastrointestinal tract (Ruiz-Moyano *et al.*, 2019). Therefore, two isolates, LI10 and LG5, were further characterized for acid, NaCl and bile salt tolerance. This study found that both isolates survived after exposure to pH ranged 4–10, NaCl at concentration up to 10% and fresh bile up to 10% concentration. As previously mentioned,

probiotic strain properties were resistant to pH, NaCl, and bile salt (Allameh *et al.*, 2012). Likewise, the selected probiotic isolates in this study could survive in high concentrations of bile salt. Several preceding studies reported that probiotics should be capable of resisting inhibitory ingredients in the gastrointestinal tract, such as bile salts (Ehrmann *et al.*, 2002; Narakaew *et al.*, 2010; Allameh *et al.*, 2012; Prabhurajeshwar and Chandrakanth, 2017). The antibiotic susceptibility test, in the present study, revealed that both LI10 and LG5 isolates were sensitive to benzylpenicillin, amoxicillin/clavulanic acid, vancomycin, tetracycline, nitrofurantoin and chloramphenicol. Both isolates were resistant to cefovecin, marbofloxacin, clindamycin, trimethoprim/ sulfamethoxazole. Recently, consumers have concerned the problem of antibiotic resistance seriously. Arumugam *et al.* (2017) isolated *E. feacalis* from tilapia and studied antibiotic resistance; they found that such strain was resistant to amoxiclav, ampicillin, erythromycin, gentamicin, kanamycin, nitrofurantoin, penicillin G, streptomycin, sulphafurazole, and vancomycin.

According to MALDI-TOF MS and Gram staining, the two selected isolates, LI10 and LG5, were *Enterococcus faecalis* and named as *E. faecalis* LI10 and *E. faecalis* LG5. *E. faecalis*, respectively. Moreover, they were gram-positive bacteria in the genera of LAB, which can be found in the intestinal tract of animals. It has been reported that strains used as probiotics and immune stimulant in fish usually belong to the species in genera *Enterococcus* and *Lactobacillus*. The genus *Enterococcus* harbors many different species, but two of them are of importance in terms of probiotics, including *E. faecium* and *E. faecalis* (Klein *et al.*, 1998; Nayak, 2010).

In conclusion, among forty LAB isolates, *E. faecalis* LI10 (from intestine) and *E. faecalis* LG5 (from gill) showed higher spectra for antagonistic activity against spoilage and pathogenic bacteria. Moreover, they tolerated low pH and high concentration of NaCl and bile salts up to 10%. Therefore, these strains should be further studied regarding their probiotic properties and potential benefits for future use.

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