Characterization of the Antibacterial Activity and Probiotic Properties of Lactic Acid Bacteria Isolated from Raw Fish and Nham-Plaa

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ABSTRACT

Isolates of lactic acid bacteria from fish fillets and nham-plaa were screened to select the most suitable strains for the formulation of starter cultures with probiotic properties. Using an agar spot test, 30 of the 180 isolates that were tested inhibited at least one of four indicator strains; *Staphylococcus aureus* TISTR 118, *Pediococcus acidilactici* TISTR 051, *Lactobacillus bulgaricus* TISTR 415 and *Listeria monocytogenes* DMST 11256. Two bacterial isolates,13IS3 and 13IS4, from fish fillets were resistant to a low pH in lactic acid (pH 2.9-3.2) and in HCl (pH 2.2) and a high concentration of NaCl (6.0%) and bile salts (5.25%). These isolates were identified by morphological, biochemical and molecular analysis as *Lactococcus lactis* 13IS3 and *Lactobacillus sakei* 13IS4. The fermentation of nham-plaa model broth (NMB) by *L. lactis* 13IS3 seemed to be stimulated by 1.0% garlic extract and 0-1.5% NaCl at all temperatures of incubation. *L. sakei* 13IS4 fermented at a slower rate than *L. lactis* 13IS3, but fermented more rapidly in a 3.0%-garlic extract NMB with 0-1.5% NaCl at 30°C and in the same medium containing 1.0-1.5% NaCl at 35°C.

Key words: lactic acid bacteria, probiotic, nham-plaa, fermented fish, starter culture

INTRODUCTION

Lactic acid bacteria (LAB) have been reported to inhibit pathogenic bacteria such as *Listeria monocytogenes*, *L. innocua* and *Staphylococcus aureus* in processed fish products (∅stergaard *et al*., 1998; Thapa *et al*., 2006). Several researchers isolated LAB from fish and meat products and screened them for their antibacterial activity (∅stergaard *et al*., 1998; Pennacchia *et al*., 2004). These bacteria produce a variety of antimicrobial substances including organic acids, diacetyl, hydrogen peroxide, reuterin and bacteriocins. Since starter cultures are essential for successful fish fermentation, they are commonly used in protective starter cultures for fish fermentation. To develop multifunctional starter cultures, newly LAB-isolated strains may be selected either from traditionally-fermented food or from natural raw material using their antagonistic activity and additional selective criteria such as health-promoting ("probiotic") properties (Holzapfel *et al*., 2003).

Bacteria showing probiotic properties are

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well known to have beneficial effects on human health. They play a major role in the establishment of balanced microflora in the intestinal tract. Their presence has been reported to inhibit growth of undesirable bacteria in the intestinal tract, stimulate the immune system, improve lactose digestion, provide anticarcinogenic actions and reduce serum cholesterol in humans (Gilliland, 2001). In order to gain these health effects, these bacteria need to survive their passage through the gastrointestinal tract. Acid and bile tolerance are basic properties indicating the ability of probiotic microorganisms to survive in the gastrointestinal tract. Besides these properties, antagonistic activity of probiotic bacteria against pathogenic and spoilage bacteria is an important criterion for selecting a preferable probiotic strain (Saarela *et al.*, 2000).

As a result of the increased interest in adding LAB to probiotic starter cultures in the production of fermented meat and fish products, bacteriocin-producing LAB from these products have been screened, identified and characterized (∅stergaard *et al.*, 1998; Pennacchia *et al.*, 2004; Thapa *et al.*, 2006). Therefore, new probiotic strains of LAB isolated from fish and Thai fermented-fish product may be suitable for use in starter cultures. The purpose of this study was to isolate LAB from raw-fish fillets and nham-plaa, a Thai fermented-fish sausage and screen them for their antagonistic activity, as well as their acid, NaCl and bile salt tolerance. These bacteria were also identified and characterized for their growth and fermentation capabilities in a fermented-fish model broth.

MATERIALS AND METHODS

Raw fish and nham-plaa samples

Five samples of raw-fish fillets of two species: *Chitala ornata* (spotted featherback fish) and *Channa micropeltes* (giant snakehead fish) and 10 samples of nham-plaa (a Thai fermented-fish sausage) made from these two fish species were purchased at local markets around the Ladkrabang district in Bangkok, Thailand.

Isolation of lactic acid bacteria

Total viable LAB counts in all samples of raw fish and nham-plaa were determined on deMan Rogosa Sharpe agar (MRS, pH 7.1 ± 0.2, containing 0.5 % calcium carbonate) under microaerophilic condition at 37°C for 48 h. Colonies with a clear zone were counted and selected for morphological characterization. The pure cultures were stored in MRS agar containing 1.0% w/v calcium carbonate.

Screening of inhibitory substance-producing lactic acid bacteria

The antibacterial activity of the LAB isolates was assessed by an agar spot test (Fleming *et al.*, 1985). A loopful of each LAB isolate on an MRS agar slope was transferred into 10 ml of MRS broth and incubated at 37°C for 48 h. The cultures were inoculated on the surface of a bacteriocin screening medium (BSM: 2.0 g/l meat extract, 4.0 g/l yeast extract, 10.0 g/l tryptone, 2.0 g/l glucose, 2.0 g/l citric acid diammonium salt, 1.0 g/l tween 80, 0.2 g/l $MgSO_4.7H_2O$, 0.05 g/l $MgSO_4.4H_2O$, 8.7 g/l $K_2HPO_4.3H_2O$, 8.0 g/l KH_2PO_4 and 15.0 g/l agar; Tichaczek *et al.*, 1992). The plates were incubated under microaerophilic condition at 37° C for 48 h and then covered with 5.0 ml of semisoft (1.0% agar) TSA (Tryptic Soy Agar, Difco) or MRS agar containing 2.0% each of the four indicator strains (i.e. *Staphylococcus aureus* TISTR 118, *Pediococcus acidilactici* TISTR 051, *Lactobacillus bulgaricus* TISTR 415 and *Listeria monocytogenes* DMST 11256). The isolates inhibiting at least one strain of those bacteria by the indication of a clear zone were selected to study their acid, bile and salt tolerance.

Selection of acid, bile and salt tolerant isolates

Inoculum of each LAB isolate on the

MRS slope was prepared by adding a loopful of 24 h growth into 10 ml of MRS broth and then incubated at 37°C for 24 h. Cells were then collected by centrifugation at 3,000 ×g for 20 min, washed twice and resuspended in 10 ml of 0.1% peptone water. The turbidity of each suspension was adjusted to match the turbidity of "3 McFarland standard" (10^6-10^7cfu/ml).

To study lactic acid tolerance, a cell suspension (50 µl) of each isolate was inoculated into 150 µl of MRS broth acidified with 85% (w/v) lactic acid at 8 different pH levels (pH 6.3, 5.3, 4.0, 3.7, 3.35, 3.2, 2.9 and 2.7) on a microliter plate. After incubation at 37°C for 24 h, the turbidity was measured using a microplate reader (iEMS Reader MF, Labsystems company) at 620 nm. For the hydrochloric acid tolerant study, the same procedure was followed, except that the MRS broth was adjusted to a pH of 6.3, 5.0, 3.7, 3.0, 2.6, 2.2, 2.0 and 1.9 with 5M hydrochloric acid.

To determine the bile salt tolerance, 50 ml of cell suspension was inoculated into 150 µl of the MRS broth containing bile salts (Mast diagnostics company) at 8 concentrations (0.00, 0.75, 1.50, 2.25, 3.00, 3.75, 4.50 and 5.25% w/v) using a microliter plate and incubated at 37°C for 24 h. To determine sodium chloride resistance, the same procedure was followed, except that the MRS broth contained sodium chloride at 8 concentrations (0.00, 1.50, 3.00, 4.50, 6.00, 7.50, 9.00 and 10.50% w/v).

Isolates that showed growth at the lowest pH level of lactic acid or hydrochloric acid and at the highest concentration of bile salts or sodium chloride in the MRS broth were considered as high potential candidates for acid, bile and salt tolerance.

Identification of the potential tolerant isolates

Morphological and biochemical characterization

The isolates were initially tested for the characteristics of: gram staining, cell morphology, colony morphology, motility, carbon dioxide production from glucose, growth at 10°C and 45° C, growth in 6.5% NaCl and 18% NaCl, growth at a pH of 4.4 and 9.6 and catalase reaction by 3% hydrogen peroxide (Axelsson, 2004). The isolates were then characterized by their carbohydrate fermentation patterns using the API-50 CH system (bioMerieux, France).

Molecular identification

A loopful of each isolate grown on Luria-Bertani (LB) agar was suspended in 400 µl of 50 mM Tris-HCl buffer, containing 5 mM EDTA (TE, pH 8.0) and 2 mg/ml of lysozyme. The mixture was incubated at 37°C for 10 min and subsequently 1/20 volume of 10% SDS was added and mixed well, before extracting with 1 volume of TE-saturated phenol. The aqueous phase was supplemented with 1/10 volume of 3M sodium acetate and 2.5 volume of absolute ethanol. The mixture was kept at -70°C for 30 min before centrifugation at 13,000 ×g for 10 min. The DNA pellet was washed with 70% ethanol, dried and dissolved in 50 ml TE buffer. A 600 bp segment of the 16S rDNA gene was amplified by PCR with a pair of primers, FDNA, 5′-TCCTACGGG AGGCAGCAGT-3′ and RDNA, 5′-TTGTGC GGGCCCCCGTCAAT-3′ (Phunpruch and Baebprasert, 2003). For DNA amplification, 100 ng of genomic DNA was added to a 100 µl of PCR mixture containing 2.5 units of *Taq* DNA polymerase (Promega, USA), 20 pmol of each deoxynucleoside triphosphate, 1.5 µM $MgCl_2$, and 25 pmol of each primer. The PCR protocol consisted of a denaturing step of 95°C for 5 min, followed by 30 cycles of denaturing for 30 sec at 95°C, annealing for 30 sec at 55°C and an extension at 72°C for 40 sec. A final extension at 72°C for 10 min was then performed. The PCR product was cloned and sequenced in both strands with a Big-Dye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) by using an ABI PRISMR 3700 DNA analyzer. Sequence comparisons were performed using the program

BLAST (Altschul *et al*., 1990) available on the internet (www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were performed using CLUSTALW (Higgins *et al*., 1994).

Detection of inhibitory activity of selected LAB isolates

Ten ml of the MRS broth were inoculated with each of the selected LAB isolates and incubated at 37°C for 24 h. Cell-free supernatant of each isolate was obtained by centrifugation at 3000 rpm 4°C for 20 min. The supernatant was then adjusted to a pH of 6.5 with 1M NaOH followed by filter sterilization through a 0.2-mm cellulose acetate filter (Whatman). The inhibitory effect of hydrogen peroxide was eliminated by the addition of 5 mg/ml of catalase. The inhibitory activity of the bacteriocin produced by each selected LAB was assessed by agar well diffusion assay (Schillinger and Lücke, 1989). The MRS agar, containing 0.7% agar was inoculated with each of the four indicator bacteria (10^7 cfu/ml) and poured into separate petridishes. A well of 5 mm diameter was cut into each dish and 30 μl of the culture supernatant was placed into each well. All plates were incubated at 37°C for 24 h and examined for the formation of inhibition zones.

Growth and fermentation capability of selected LAB isolates

The selected LAB isolates were tested for their optimum growth temperature and also their tolerance to sodium chloride and garlic extract concentrations in nham-plaa model broth (NMB). A water extract of fresh garlic was prepared as described by Swetwiwathana, 1998. The cell suspenion (10^7 cfu/ml) of each selected LAB was inoculated into NMB (10 g/l meat extract, 10 g/l tryptone, 0.5 g/l sodium ascorbate, 3 g/l sodium tripolyphosphate, 10 g/l glucose and 0.1 g/l sodium nitrite) with varying concentrations of sodium chloride (0.0, 1.0, 1.5 and 2.5% w/v) and garlic extract (0.0, 1.0, 3.0 and 5.0% w/v). They were incubated at three temperature levels (25, 30 and 35°C) for 5 days. The turbidity (absorbance at 620 nm) and pH of the fermentation medium were measured at day 0, 1, 2, 3, 4 and 5 of incubation to assess the growth and fermentation capability of the selected isolates. The experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Isolation and initial screening of lactic acid bacteria

The number of total LAB counts in all raw-fish fillet samples ranged from 3.0×10^5 – 2.5×10^6 cfu/g. In nham-plaa made from spotted featherback fish, the total LAB counts ranged from 4.2×10^8 – 2.1×10^9 cfu/g, whereas in nham-plaa made from giant snakehead fish the counts were $\geq 6.8 \times 10^8$ cfu/g. Thirty isolates (21.7%) of the 138 LAB counts from the fish fillets and nham-plaa samples inhibited at least one of the four tested organisms.

In this study, a large number of LAB was found in the fish fillets. These bacteria have been found in varying proportions on the outer and inner surfaces of live fish (Liston, 1980). A relatively large number of lactobacilli have been found in the intestines of fresh water fish (Bucio *et al*., 2006). Gonzálaz *et al*. (2000) reported that 22 strains of 249 LAB strains were isolated from freshwater fish, (mainly wild brown trout) and their surrounding environments and were identified as from the genus *Lactobacillus*, *Enterococcus*, *Lactococcus* or *Vagococcus*. The presence of a high number of LAB in the fish may have been due to some important intrinsic factors which influenced the microbiology and spoilage of fish. In general, fish flesh has a very high post-mortem pH (> 6.0) as it contains small amounts of carbohydrate (<0.5%) in the muscle tissue and has a high (positive) redox potential (Eh) because of the presence of trimethylamine oxide (TMAO). The TMAO usually causes positive Eh in fish flesh. Moreover, fish flesh consists of low-molecular-weight water-soluble nitrogen containing compounds such as free amino acids and nucleotides, which are a readily available substrate for bacterial growth (Gram and Huss, 1996).

A high number of lactic acid bacteria was found in nham-plaa. These LAB have commonly been isolated from seafoods and fish products as they are widely used in lactic starter cultures. ∅stergaard *et al*. (1998) found that the number of LAB in Thai low-salt fermented-fish products (som-fak and plaa-som) was between 8.3 to 9.2 log cfu/g. Similarly, Paludan-Müller *et al*. (2002a) found that plaa-som made from striped snake-head fish contained a high number of lactic acid bacteria (10^8-10^9 cfu/g).

Selection of acid, bile and salt tolerant isolates

All 30 isolates (100%) were able to grow in the MRS broth acidified with lactic acid to a pH of 4.0, whereas 36.7, 23.3, 6.7 and 6.7% of the 30 isolates were able to grow in lactic acid at a pH of 3.7, 3.35, 3.2 and 2.9, respectively (Table 1). Isolates 13IS4 and 14IS9 could grow in lactic acid at the lowest pH (pH 2.9). Similarly, the highest percentage (40%) of the 30 isolates was able to tolerate hydrochloric acid at a pH of 2.6, while 26.7, 20 and 10% of the isolates were resistant to hydrochloric acid at a pH of 3.7, 3.0 and 2.2, respectively. Isolates 5IS9, 13IS3, 13IS4 and 13IS5 grew at a pH of 2.2, but no isolate grew in hydrochloric acid at a pH of 2.0 or 1.9. In the test of bile salt tolerance, the majority of the isolates (90%) grew in the MRS broth that contained 5.25% (w/v) bile salts, whereas only a small proportion (3.33%) of the isolates (3IS3, 3IS6 and 14IS7) were tolerant to bile salts at concentrations of 1.5, 2.25 and 3.0% (w/v), respectively. All 30 isolates were able to grow in the MRS broth containing 4.5% sodium chloride, but 17 isolates (56.7%) were able to grow in the presence of 6.0% (w/v) sodium chloride. In addition, 33.3, 3.3 and 6.6% of the 30 isolates could grow in the MRS broth containing 7.5, 4.5 and 9.0% (w/v) sodium chloride, respectively. Consequently, isolates 13IS3 and 13IS4 that had a high ability to tolerate lactic acid, hydrochloric acid, bile salts and sodium chloride, were selected for identification.

The experimental design was intended to select the LAB isolates that had an ability to produce inhibitory substances and an ability to tolerate acid, bile and salt for use in probiotic starter cultures for fermented meat and fish products. It was important to consider the ability of the organisms to tolerate acid, bile and salt to ensure their ability to survive through the harsh conditions of fermentation and in the human intestinal tract, in order to gain the health promoting effects (Saarela *et al*., 2000). In this study, the isolates 13IS3 and 13IS4, identified as *L. lactis* and *L. sakei,* were selected as they showed a high ability to tolerate acid, bile and salt. The lowest pH of hydrochloric acid that they were able to tolerate (pH 2.2) was lower than the lowest pH of lactic acid (2.9 or 3.2). This was probably because lactic acid (a weak acid) may have been more toxic to the cells, compared to hydrochloric acid (a strong acid). Weak acid usually existed in an undissociated form and could penetrate the lipid bilayer of cell membrane more easily and finally dissociate in the cell (Davidson, 1997). Cook and Russel (1994) reported that decreasing the internal pH of *L. lactis* and *Streptococcus bovis* was more effective when the external pH was adjusted with lactic acid than with hydrochloric acid. In general, LAB are inherently more acid resistant than many other bacteria (Yousef and Courtney, 2003). Therefore, they may survive when exposed to gastric juice in the gut. Although the pH of excreted hydrochloric acid in the stomach is 0.9, the pH rises to 3.0 in the presence of food (Erkkila and Petaja, 2000). In addition, some components of the gastric juice may protect bacterial cells for survival at a low acidic pH (Conway *et al*., 1987).

Morphological, biochemical and molecular identification of selected lactic acid bacteria

Morphological and physiological tests were performed to identify the two selected isolates (13IS3 and 13IS4) at the genus level. Both isolates were gram positive bacteria. They had similar colony morphology and motility, but had different cell morphologies. Isolate 13IS3 was ovoid (cocci) in shape, but isolate 13IS4 was cylindrical. They were catalase negative and able

Table 1 Lactic acid, hydrochloric acid, bile salts and sodium chloride tolerance of lactic acid bacteria isolated from fish fillets and nham-plaa.

Source of isolates	LAB isolates	pH[a] of lactic acid	pH[a] of HCl	Bile salts (w/v) [b]	NaCl (%w/v) [b]
nham-plaa 1[c]	2IS1	4.00	3.70	5.25	6.00
	3IS3	3.70	3.70	1.50	6.00
	3IS4	4.00	3.70	5.25	6.00
	3IS6	3.70	3.00	2.25	6.00
	3IS7	4.00	3.70	5.25	7.50
	4IS1	4.00	3.70	5.25	7.50
	4IS3	3.70	3.00	5.25	6.00
	4IS5	3.70	3.00	5.25	7.50
	4IS6	4.00	2.60	5.25	7.50
	4IS8	3.70	3.00	5.25	6.00
	5IS2	4.00	2.60	5.25	6.00
	5IS4	4.00	3.00	5.25	9.00
	5IS8	3.70	2.60	5.25	6.00
	5IS9	3.70	2.20	5.25	6.00
nham-plaa 2[d]	9IS1	3.70	3.00	5.25	6.00
	9IS2	4.00	3.70	5.25	4.50
	9IS3	3.70	3.70	5.25	7.50
	9IS5	3.20	3.70	5.25	7.50
	9IS7	3.70	2.60	5.25	7.50
	9IS8	3.35	2.60	5.25	6.00
Fish fillet 1[c]	13IS2	3.35	2.60	5.25	6.00
	13IS3	3.20	2.20	5.25	6.00
	13IS4	2.9	2.20	5.25	6.00
	13IS5	3.35	2.20	5.25	6.00
Fish fillet 2[d]	14IS3	3.35	2.60	5.25	7.50
	14IS4	3.35	2.60	5.25	6.00
	14IS7	3.35	2.60	3.00	7.50
	14IS8	3.35	2.60	5.25	9.00
	14IS9	2.9	2.60	5.25	6.00
	14IS12	3.70	2.60	5.25	7.50

[a] The lowest pH of acid at which the isolates were able to grow.

[b] The highest concentration of bile salts or NaCl at which the isolates were able to grow.

[c] Sample from spotted featherback fish.

[d] Sample from giant snakehead fish.

to grow at 10°C, in the presence of 6.5% sodium chloride and at a pH of 4.4, but could not grow in the presence of 18% sodium chloride. However, isolate 13IS4 could grow at 45°C and produced carbon dioxide from glucose, but isolate 13IS3 could not. In contrast, isolate 13IS3 could grow at a pH of 9.6, but isolate 13IS4 could not. On the basis of the key characteristics, isolate 13IS3 could be presumptively identified as the genus *Lactococcus* and isolate 13IS4 was identified as *Lactobacillus* (Axelsson, 2004).

Identification of isolates 13IS3 and 13IS4 was confirmed by API 50CH and molecular techniques. The 16S rDNA gene sequences of isolates 13IS3 and 13IS4 were compared to the GenBank database which confirmed their identities as *L. lactis* and *L. sakei*, respectively.

In this study, the results of the biochemical identification of isolate 13IS3 were in agreement with those of the molecular identification which indicated that isolate 13IS3 was *L. lactis*. However, the results of the biochemical and molecular identification of isolate 13IS4 were different. Even though the API 50 CH biochemical profiles of isolate 13IS4 showed a 43.5% similarity to those of *L. curvatus*, the results of the 16S rDNA sequencing indicated that isolate 13IS4 should have been *L. sakei*. This was probably due to the fact that *L. curvatus* and *L. sakei* are very closely related (Berthier and Erhlich, 1999). These two bacterial species are not easily or rapidly differentiated by a biochemical test, since they differ only in the hydrolysis of arginine and the fermentation of melibiose. Moreover, some atypical strains of *L. sakei* may be found, such as melibiose-negative strains, which deviate from the standard melibiose pattern of regular *L. sakei* (Berthier and Erhlich, 1999; Lee *et al*., 2004). Therefore, the molecular identification results should be more reliable than the biochemical test results in the case of these two species.

Inhibitory activity of selected lactic acid bacteria against indicator microorganisms

The results of the agar well diffusion assay revealed that only culture supernatant of *L. lactis* 13IS3 caused the formation of an inhibition zone of up to 28 mm against *S. aureus* TISTR118, while the supernatant of *L. sakei* 13IS4 could not. This may have been due to the effect of inhibitory substances which could have been bacteriocins. Similarly, Imphol and Suriyawong (2003) found that *L. lactis* ssp. *lactis* C7 inhibited several LAB and food-borne pathogens. The inhibitory substance produced by this bacterium was bacteriocin C7. In addition, antibacterial activity of *L. lactis* ssp. *cremoris* isolated from sakako maacha, the traditional smoked and sun-dried fish product of the eastern Himalayas, was recently tested against some indicator organisms using an agar spot test. The results showed that this bacterium inhibited *Listeria innocua* and *Staphylococcus aureus* (Thapa *et al*., 2006).

Effect of sodium chloride and garlic extract on the fermentation and growth of selected lactic acid bacteria in nham-plaa model broth at 25, 30 and 35°C

Change of pH during fermentation

L. lactis 13IS3 isolated from fish fillets showed a slightly different fermentation pattern at 25, 30 and 35°C in NMB containing 0-2.5% sodium chloride and 0-5.0% garlic extract (Table 2). The pH decreased rapidly to <4.5 in NMB containing 0-2.0% sodium chloride and 1.0% garlic extract within one day at all three incubation temperatures. The fermentation of NMB by this bacterium seemed to be stimulated by the 1.0% garlic extract. The optimum concentrations of sodium chloride for fermentation were 1.5-2.0% at 25°C and 0-1.5% at the other two temperature levels. At a concentration of garlic extract (3.0-5.0%) and sodium chloride (2.5%), the pH of the fermentation medium slowly reduced to < 4.5 after two days or longer. However, *L. sakei* 13IS4

Table 2 Fermentation response of *Lactococcus lactis* 13IS3 and *Lactobacillus sakei* 13IS4 in nham-plaa model broth containing different concentrations of sodium chloride and garlic extract at 25, 30 and 35°C.

Sodium chloride (%)	Days to pH < 4.5											
	25°C				30°C				35°C			
	0 % GE[a]	1.0% GE	3.0 % GE	5.0 % GE	0 % GE	1.0 % GE	3.0 % GE	5.0 % GE	0 % GE	1.0 % GE	3.0 % GE	5.0 % GE
Lactococcus lactis 13IS3												
0.0	2	2	2	3	3	1	2	5	2	1	4	4
1.0	2	2	2	4	2	1	2	4	1	1	2	4
1.5	2	1	2	4	2	1	2	4	1	1	2	4
2.0	3	1	2	3	2	2	2	4	2	2	3	5
2.5	3	3	3	>5	3	2	3	5	2	2	4	>5
Lactobacillus sakei 13IS4												
0.0	>5	4	4	>5	>5	4	3	4	>5	>5	5	>5
1.0	>5	4	4	5	4	3	3	4	>5	4	3	3
1.5	>5	4	4	5	>5	4	3	4	>5	4	3	4
2.0	>5	>5	5	>5	>5	4	4	4	>5	4	4	4
2.5	>5	>5	>5	>5	4	5	4	4	>5	>5	5	4

[a]GE = Garlic extract

fermented at a slower rate than *L. lactis* 13IS3. At a 3.0% garlic extract, *L. sakei* 13IS4 fermented more rapidly (pH <4.5) within three days in NMB containing 0-1.5% sodium chloride at 30°C and in NMB containing 1.0-1.5% sodium chloride at 35°C. In most cases, the pH of the fermentation medium without the addition of sodium chloride and garlic extract slowly decreased to < 4.5 (after five or more days).

Growth response in Nham-Plaa Model Broth

L. lactis 13IS3 showed good growth in NMB containing 3.0% and 1.0% garlic extract at 25°C and 30°C, respectively. This bacterium also grew well in NMB containing 0-1.5% sodium chloride at all temperature levels of incubation (Table 3). However, the time to reach maximum cell density (OD_{620} 1.00-1.26) was one to two days longer than the time to reach a pH of <4.5 at all three temperatures of incubation.

The maximum cell density of *L. sakei* 13IS4 was higher at an incubation temperature of 30°C or 35°C than at 25°C. At 30°C, this bacterium grew well in NMB containing 2.0-2.5% sodium chloride with no garlic extract added (maximum OD_{620} 1.26-1.48), whereas optimum sodium chloride concentration was 1.0-1.5% in NMB with 0-1.0% garlic extract at 35°C (maximum OD_{620} 1.76-1.79). The time to reach the maximum cell density of *L. sakei* was 3-5 days.

These results indicated that garlic affected the growth of both *L. lactis* 13IS3 and *L. sakei* 13IS4 in NMB. The optimum concentration of garlic extract in NMB for the growth and fermentation of *L. lactis* 13IS3 was 1.0%, but this bacterium did not grow well in >1.0% garlic extract. Karaioannoglou *et al.* (1977) reported similar results which showed that >1.0% garlic extract inhibited *L. plantarum* in culture media. However, 3.0% garlic extract

Table 3 Growth response of *Lactococcus lactis* 13IS3 and *Lactobacillus sakei* 13IS4 in nham-plaa model broth containing different concentrations of sodium chloride and garlic extract during fermentation at 25, 30 and 35°C.

Sodium chloride (%)	25°C			30°C			35°C		
	Maximum growth[a]	Days to maximum OD_{620}	% Optimum[b] garlic extract	Maximum Growth	Days to maximum OD_{620}	% Optimum garlic extract	Maximum growth	Days to maximum OD_{620}	% Optimum garlic extract
Lactococcus lactis 13IS3									
0.0	1.17	4	1.0	1.26	4	1.0	1.18	3	1.0
1.0	1.00	4	3.0	1.19	4	1.0	1.13	3	1.0
1.5	1.14	3	3.0	1.15	4	1.0	1.11	3	1.0
2.0	0.96	4	3.0	1.06	4	1.0	0.98	4	0.0
2.5	0.84	4	3.0	0.86	4	3.0	0.77	4	1.0
Lactobacillus sakei 13IS4									
0.0	0.97	4	0.0	1.23	2	0.0	1.35	3	0.0
1.0	0.99	3	0.0	1.17	5	1.0	1.79	4	0.0
1.5	1.09	3	1.0	0.98	4	0.0	1.76	5	1.0
2.0	1.00	5	0.0	1.26	3	0.0	1.62	5	1.0
2.5	0.91	4	0.0	1.48	5	0.0	1.65	5	1.0

[a]Maximum OD_{620} observed at each sodium chloride concentration
[b]Concentration of garlic extract that provided maximum cell density

enhanced the fermentation of *L. sakei* 13IS4 in NMB. Some spices have been shown to stimulate the growth of LAB (Zaika and Kissinger, 1979). Paludan-Müller *et al.* (2002b) reported that no garlic resulted in a lack of acidification during fermentation of som-fak. This may have been due to the presence of manganese in the spices (Kröckel, 1995). LAB required manganese for several enzyme activities including pyruvate kinase and other enzymes in the Embden-Meyerhof pathway (Jessen, 1995). However, a high concentration of garlic extract resulted in the slow growth and fermentation of these bacteria. This may have been due to a high content of allicin and other sulfides in an essential oil of the garlic. Allicin or diallylthiosulfinic acid was identified as the major antimicrobial component in garlic bulbs (Conner, 1993).

CONCLUSION

The two LAB strains, *L. lactis* 13IS3 and *L. sakei* 13IS4, exhibited a high resistance to acid, sodium chloride and bile salts, but *L. lactis* 13IS3 showed better characteristics of antibacterial activity and fermentation capability that are desirable in a fish starter culture and could potentially be used as a probiotic starter culture for fermented-fish products. However, it was concluded that this bacterium required further investigation into: the production, purification and characterization of bacteriocins; starter culture production and cell viability during storage and fermentation; and its technical properties during fish fermentation.

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