

## Comparative Probiotic Properties of *Lactobacillus fermentum* Isolated from Thai Traditional Fermented Foods: Miang and Nham

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**Abstract:** The aim of this study, was to compare the probiotic properties of *Lactobacillus fermentum* isolated from Thai traditional fermented foods, miang and nham. The crucial probiotic properties such as acid and bile tolerance, antimicrobial activity, cell surface hydrophobicity and antimicrobial susceptibility of *L. fermentum* were investigated. *L. fermentum* from miang showed higher acid tolerance and antimicrobial activity but much lower cell surface hydrophobicity than that from nham. Drug susceptibility test indicated interesting different characteristics. *L. fermentum* from miang exhibited resistant to gentamycin and trimethoprim but sensitive to ciprofloxacin whereas, that from nham was resistant to ciprofloxacin but sensitive to both gentamycin and trimethoprim. This study revealed the different probiotic properties of *L. fermentum* from different food origin. The promising high acid and bile tolerance as well as strong inhibition of some pathogens suggested that both strains could be applied as potential probiotic bacteria. The difference on drug susceptibility of *L. fermentum* found in this study provided the beneficial knowledge on strain selection for further product development application.

**Key words:** *Lactobacillus fermentum*, fermented food, probiotics, miang, nham

### INTRODUCTION

Traditional fermented food is unique for historical countries in different parts of the world, e.g. fermented milk, yoghurt in Bulgaria; fermented vegetables; kimchi in Korea; gundruk, sinki and khalpi in India; dochi and suantsai in Taiwan; fermented soybean, natto and mizo in Japan; and fermented seafood, jeotgal in Korea. In Thailand, there are two famous traditional fermented foods called miang and nham. Miang is nonsalt-fermented tea leaves. The freshly boiled leaves of tea (*Camellia sinensis*) are mixed with suitable amount of water. Nham is a salt-fermented ground meat; mainly made of fresh pork mixed with small amount of garlic, pepper salt and cooked rice. The fresh mixtures of both miang and nham are pressed and fermented naturally (without starter) for certain period of time. These products demonstrate a typical pickled flavor and sour. Traditional fermented food has been reported to be one of the major sources of several lactic acid bacteria (Okada *et al.*, 1986; Tanasupawat and Komagata, 1995; Chen *et al.*, 2006). Lee *et al.* (2006) reported the highly acid resistant

*Lactobacillus plantarum* NK181 isolated from jeotgal. Albano *et al.* (2007) isolated potent antilisterial activity of lactic acid bacteria from alheiras, traditional Portuguese fermented sausages. Potential probiotic strains are expected to have several desirable properties to exert their beneficial effects. They should meet the requirement properties particularly to be tolerant to acid and bile, have a property related to an adherence to the intestinal epithelium of the host and possess antimicrobial activity against the pathogenic microorganisms. Even *Lactobacillus* sp. are classified as GRAS (Generally Recognised as Safe) microorganisms, it is important to assess the safety of those microorganisms because of the serious drug resistance strains were occasionally reported. Such probiotic strains may transfer the resistant gene to pathogenic bacteria (Teuber *et al.*, 1999). Therefore, the susceptibility properties to drugs particularly the antimicrobial agents of the probiotic strains intended for the market should be intensely examined. Conway *et al.* (1987) reported that *L. acidophilus* strains isolated from human digestive tract were significantly less stable in acid than those isolated

from chicken. *L. fermentum* strains isolated from swine intestine were reported to be much more bile tolerant but less adhesive than those isolated from poultry digestive tract (Lin *et al.*, 2007). During our ongoing project on searching for some potential lactic acid bacteria, two strains of *L. fermentum* were isolated from different food origins, miang and nham.

The aim of this study, thus is to evaluate some crucial properties of both strains in comparatively. The results of this study was expected to provide the beneficial knowledge of probiotic properties of these 2 strains in order to be applied in further study or in commercial product development application.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions:** The strains of *L. fermentum* were isolated from two different traditional fermented foods, miang and nham. The method of isolation was according to Schillinger (1999) and Rodriguez *et al.* (2003) using de Man Rogosa Sharpe (MRS) agar or broth (Merck, Darmstadt, Germany) as a medium. The isolated strains were identified by biochemical characterization based on the ability of the isolates to utilize or oxidize different carbon sources, which determined by API CH 50 system (BioMérieux, Lyon, France). The identified isolates were confirmed by mean of species specific PCR (Song *et al.*, 2000). All isolated strains were kept at -20°C in MRS broth supplement with 30% sterile glycerol for further experiments.

The pathogenic bacterial strains used as indicator for antimicrobial activity study were *Escherichia coli* TISTR 780, *Staphylococcus aureus* TISTR 029 and *Salmonella* Typhi DMST 5784. The first two indicators were obtained from Microbiological Resources Center for Southeast Asia (MIRCEN) at Thailand Institute of Scientific and Technology Research. *Salmonella* Typhi DMST 5784 was supplied by the Culture Collection for Medical Microorganism Department of Medical Sciences Thailand. All three indicator strains were stored at -20°C in Trypticase soy broth supplement with 30% sterile glycerol.

**Test of acid tolerance:** This experiment was carried out according to the method described by Brashears *et al.* (2003) with some modification. A suspension of overnight culture of *L. fermentum* in MRS broth was centrifuged at 6,000 rpm for 10 min. The cell pellets were mixed with 0.05 M sodium phosphate buffer pH 2.0 or 3.0 to yield  $10^7$ - $10^8$  cfu mL<sup>-1</sup>. Each mixture was incubated at 37°C. After 2 h incubation, viable cell count was determined by plating serial dilutions (in phosphate buffer pH 7.0) on

MRS agar. These plates were then incubated at 37°C in an anaerobic atmosphere for 48 h. The property of acid tolerance was expressed as the percentage cell survival calculated by comparison of the bacterial cell count at initial with those at final after 2 h incubation as the following Eq:

$$\text{Survival(\%)} = \frac{\log \text{cfu after 2 h of incubation}}{\log \text{cfu at initial}} \times 100$$

**Test of bile tolerance:** This test was done by the methods of Yu and Tsen (1993) and Tsai *et al.* (2005) with some modification. The overnight culture with  $10^7$ - $10^8$  cfu mL<sup>-1</sup> was inoculated to the broth containing 0, 0.3 and 1.0% w/v Oxgall (Difco, Detroit, USA). The mixtures were incubated at 37°C under anaerobic condition for 24 h. The procedure of cell count determination was similar to those described for the acid tolerance test. The property of bile tolerance was expressed as the percentage cell survival calculated by comparison of the bacterial cell count in MRS broth with Oxgall to those without Oxgall after 24 h incubation as the following equation;

$$\text{Survival(\%)} = \frac{\log \text{cfu in MRS containing Oxgall}}{\log \text{cfu in MRS without Oxgall}} \times 100$$

**Detection of antibacterial activity:** The antibacterial activity of the isolated strains was determined by the method introduced by Schillinger and Lücke (1989) with some modification. *Escherichia coli* TISTR 780 and *Salmonella* Typhi DMST 5784 were used as gram negative pathogenic indicators whereas *Staphylococcus aureus* TISTR 029 was of gram positive. A portion of 1 µL of each *L. fermentum* culture in stationary growth phase was spotted on the surface of an MRS agar plate (containing 0.2% glucose) and incubated under anaerobic condition for 48 h to develop the colony. A volume of 250 µL of an exponential culture of the indicator bacterial strains was mixed with 9 mL of Brain Heart Infusion (Merck, Darmstadt, Germany) soft agar (0.7% agar). This mixture was immediately poured over the MRS plate on which the tested *L. fermentum* was grown. The plates were incubated aerobically at 37°C for 24 h. The antibacterial activity was determined by measuring the diameter of inhibition clear zone and growth spot with calipers. The inhibition activity of the test strains was expressed as the difference of these two diameters.

**Determination of cell surface hydrophobicity:** This study was done by the bacterial adherence to hydrocarbon assay modified from method of Vinderola and Reinheimer (2003). The test *L. fermentum* were grown in MRS broth at

37°C under anaerobic condition. The 18-24 h (stationary phase) test culture was harvested by centrifugation at 6,000 rpm for 10 min, washed twice and resuspended in 50 mM K<sub>2</sub>HPO<sub>4</sub> (pH 6.5) buffer to an optical density (OD<sub>560</sub>) of 0.8-1.0 (A<sub>0</sub>). A portion of 0.6 mL of n-hexadecane (Sigma Chemicals, St.Louis, USA) was added into 3 mL of bacterial suspension. The mixtures were blended by using vortex mixer for 120 sec. The tubes were allowed to stand at 37°C for 15 min to separate the two phases. The aqueous phase was carefully removed and the OD<sub>560</sub> of the aqueous phase (A) was measured. Percentage of cell surface hydrophobicity (%H) was calculated as the decrease in the optical density of the initial bacterial suspension due to cells partitioning into a hydrocarbon layer as the following the equation;

$$H(\%) = \frac{A_0 - A}{A_0} \times 100$$

**Antimicrobial susceptibility test:** The susceptibility test to antimicrobial agents was carried out by using standard agar dilution method recommended by the Scientific Committee on Animal Nutrition (SCAN) (European Commission, 2001) in Mueller-Hinton agar (Merck, Darmstadt, Germany). The antimicrobial agents used were ampicillin, chloramphenicol, ciprofloxacin, quinupristin, erythromycin, gentamycin, kanamycin, linezolid, rifampicin, streptomycin, tetracycline and vancomycin, all were from Sigma Chemicals (St. Louis, USA). An exact concentration of each antimicrobial drug was prepared in the appropriate diluent and two-fold dilutions were carried out in distilled water before incorporating into the molten agar. All plates were allowed to set at room temperature. An inoculum was prepared for each test organism by suspending cells from a plate in normal saline in order to achieve a turbidity equivalent to that of a 0.5 McFarland standard. Then 1 mL of this suspension was spotted on the agar surface. The plates were incubated in the anaerobic chamber at 37°C for 48 h. MIC was determined as the lowest concentration of antimicrobial agent that resulted in either no growth or a few colonies as a significant drop-off in the amount of growth. The microbiological breakpoints for *Lactobacillus* sp. recommended by SCAN (European Commission, 2001) were used to evaluate the resistant rate of the isolated strains.

## RESULTS AND DISCUSSION

**Acid tolerance and bile tolerance:** Microbial strains suitable for probiotic should be able to tolerate in acid

Table 1: Effect of acid and bile condition on the viability of the isolated *L. fermentum*

Source of <i>L. fermentum</i>	Survival in acid (%)		Survival in bile salt (%)	
	pH 2.0	pH 3.0		
miang	62.1±1.2	99.9±0.2	97.9±0.5	95.5±1.1
nham	57.7±1.4	98.8±0.5	93.9±3.2	93.2±1.2

Table 2: Antimicrobial activity of the isolated *L. fermentum*

Source of <i>L. fermentum</i>	Inhibition zone (mm)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>S. Typhi</i>
miang	5.4±2.2	7.3±1.6	6.7±1.6
nham	4.7±1.4	5.0±1.3	5.9±0.7

media for at least 90 min since it is the food transit time through the human stomach (Havenaar *et al.*, 1992). In this study, the media of pH 2.0 was used to represent the extreme acid condition of human stomach as in the case of fasting period. When the stomach is non-fasting, e.g. after meal, the gastric pH is usually raised up to 3.0 or more. The media of pH 3.0 used in this study represented the pH of non-fasting gastric condition. Results as shown in Table 1 indicated that at pH 3.0, *L. fermentum* from both sources showed significantly better stability than at pH 2.0. This was similar to *L. casei* reported by Mishra and Prasad (2005). Moreover, it was found that at the extremely low pH of 2.0, *L. fermentum* from miang showed higher acid tolerance than the strain isolated from nham. This result indicated the different survival property in acid between these two strains.

The wide variation of bile sensitivity was observed among various species of *Lactobacillus* (Du Toit *et al.*, 1998; Ibrahim and Bezkorovainy, 1993). Results of this study as shown in Table 1 demonstrated that *L. fermentum* from both food sources were bile resistant. However, *L. fermentum* from miang showed comparatively better bile tolerance. The viability of *L. fermentum* from nham in bile condition was constantly low of about 93% even the bile salt was decrease from 1.0-0.3% whereas this range of bile concentration affected the survival of the strain from miang which showed approximately of 95-98%, respectively.

**Antimicrobial activity:** The rapid emergence of drug resistant strains and chronic toxicity (Cetinkaya *et al.*, 2000; Mody *et al.*, 2003) of chemical antimicrobial agents following the widespread use of antibiotics encourage scientists to seek for the safety tools in treatment of bacterial infection. The use of lactic acid bacteria with potential antimicrobial activity is the promising alternative treatment for such problems. The antimicrobial activity of the isolated *L. fermentum* against 3 pathogenic bacteria was shown in Table 2. It was found that *L. fermentum* strain from miang had more inhibitory potential than that from nham. Among 3 test

Table 3: Antimicrobial susceptibility of isolated *L. fermentum*

Antimicrobial agent*	MIC ( $\mu\text{g mL}^{-1}$ ) of <i>L. fermentum</i>	
	From miang	From nham
Ampicillin (2)	<1	2
Chloramphenicol (16)	<8	<8
Ciprofloxacin (4)	4	8
Quinipristin (4)	<2	<2
Erythromycin (4)	<0.5	<0.5
Gentamycin (1)	1	<0.5
Kanamycin (32)	<16	<16
Linezolid (4)	4	4
Rifampicin (32)	1	1
Streptomycin (16)	<8	<8
Tetracycline (16)	<2	<2
Trimethoprim (32)	32	<16
Vancomycin (4)	>8	>8

\*Values in parenthesis are the breakpoints by SCAN category

microorganisms, the Inhibition activity of *L. fermentum* from miang against *E. coli* TISTR 780 was the highest followed by *S. Typhi* DMST 5784 and *S. aureus* TISTR 029, respectively. The inhibition of *L. fermentum* isolated from nham was different. It could inhibit *S. Typhi* DMST 5784 better than *E. coli* TISTR 780 and *S. aureus* TISTR 029, respectively. Interestingly, it was noticed that both strains showed higher potent against gram negative than gram positive bacteria. The inhibition activity of those strains was expected to be due to the organic acids, bacteriocin and hydrogen peroxide produced by lactic acid bacteria cells (Lorca *et al.*, 2001; Silvia and Nakaia, 2003). Normally, gram negative bacteria possess strongly defended structure of cell membrane (Scherrer and Gerhardt, 1971) which impermeable to several antimicrobial agents resulting less sensitive to many drugs. The high inhibition power against gram negative bacteria of *L. fermentum* obtained in this study suggested the higher potential of these probiotics than several drug substances.

**Cell surface hydrophobicity:** High cell surface hydrophobicity may favor the colonization of mucosal surfaces and play a role in the adhesion of bacteria to gastrointestinal host epithelial cells and extracellular matrix proteins (Zareba *et al.*, 1997). *L. fermentum* from miang exhibited extremely low cell surface hydrophobicity value of  $20.9 \pm 1.6\%$ , whereas that from nham was of  $68.7 \pm 6.2\%$ . This high different level usually occurs among bacteria of different species.

The results from our study revealed that different strains of same species isolated from different food sources possessed different hydrophobicity. This result was consistent with the report of Garriga *et al.* (1998) when two strains of *L. salivarius* exhibited the different colonizing capacity.

**Antimicrobial susceptibility:** Lactobacilli are increasing incorporated into foods and other nutraceutical products due to their established health benefits (Saarela *et al.*, 2000). In probiotic applications, viable bacterial cells are consumed in high daily dose and the safety of the applied strain is therefore of utmost importance. One of the safety assessments is that the probiotic should be inhibited by common antimicrobial agents.

In this study, the susceptibility to certain antimicrobial agents was compared between two strains of *L. fermentum*. Results as shown in Table 3 revealed that both strains were sensitive to chloramphenicol, quinipristin, erythromycin, kanamycin, rifampicin, streptomycin and tetracycline as the MIC values of these drugs to both strains were lower than drug-corresponding breakpoints. They were considered to have the same resistant property to ciprofloxacin, linezolid and vancomycin. However, interesting differences were observed. *L. fermentum* from miang revealed sensitive to ampicillin while the strain from nham showed resistant to this drug. Moreover, *L. fermentum* from miang showed resistant to gentamycin and trimethoprim, whereas that from nham was sensitive to these drugs. Klare *et al.* (2005) reported that antimicrobial activity of *B. bifidum* was partially influenced by growth medium. As the material source of isolated *L. fermentum* in this study was different, this was considered to be a major effect which influenced certain intrinsic factors of the obtained bacterial strains.

## CONCLUSION

Data from our study suggested that some bacterial properties are not species specific. The results demonstrated different probiotic properties between *L. fermentum* strains isolated from two distinct Thai traditional fermented food sources, miang and nham. *L. fermentum* from miang showed slightly higher acid tolerance and antimicrobial activity but much lower cell surface hydrophobicity than that from nham. Both strains showed bile resistant. Drug susceptibility test indicated interesting different characteristics. *L. fermentum* from miang exhibited resistant to both gentamycin and trimethoprim but sensitive to ciprofloxacin whereas that from nham was resistant to ciprofloxacin but sensitive to gentamycin and trimethoprim.

Results from this study revealed that the food origin might affect some intrinsic factors of *L. fermentum*. The promising high acid and bile tolerance as well as strong inhibition of some pathogens suggested that both strains could be applied as potential probiotic bacteria.

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