The Journal of Microbiology (2012) Vol. 50, No. 3, pp. 535–539
DOI 10.1007/s12275-012-1409-x

NOTE

Antibiotic Resistance and Probiotic Properties of Dominant Lactic Microflora from *Tungrymbai*, an Ethnic Fermented Soybean Food of India

Sharmila Thokchom and Santa Ram Joshi*

Microbiology Laboratory, Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong-793022, India

(Received August 18, 2011 / Accepted January 10, 2012)

The present investigation was conducted to assess lactic acid bacteria present in traditionally fermented food of ethnic tribes in India for probiotic properties, antibacterial activity, and antibiotic tolerance behavior. *Enterococcus* sp., *Lactobacillus* sp., and *Lactococcus* sp. showed antibacterial activity against *Bacillus cereus* MTCC 430, *Staphylococcus aureus* subsp. *aureus* MTCC 740, and *Salmonella enterica* ser. *paratyphi* A MTCC 735. *Lactococcus* sp. and *Lactobacillus* sp. could tolerate acidic conditions (pH 2) and high bile salt concentration (4000 ppm). The lactic microflora were found to be sensitive to most common antibiotics, except for cloxacillin (5 μg), cephalexin (30 μg), and cephalothin (30 μg).

Keywords: traditionally fermented food, lactic acid bacteria, probiotic, antibiotic, antibacterial activity

Tungrymbai, a fermented soybean food, is consumed by the ethnic Khasi tribes of India and is prepared using indigenous technology. It forms an intricate part of the food culture and serves as a cheap source of high-protein food in the local diet. The preparation and consumption of this food reflects the deep-rooted food culture of these ethnic communities (Sohliya *et al.*, 2009). Lactic acid bacteria (LAB) are a group of gram-positive bacteria that excrete lactic acid as a main fermentation product into the medium. The acidification and enzymatic processes accompanying the growth of LAB impart the unique flavor, texture, and preservative qualities to a variety of fermented foods (Klaenhammer *et al.*, 2002).

In terms of functional food, there is an increasing interest in probiotic products that contain LAB of intestinal origin. Probiotic LAB strains must be chosen according to accurate selection criteria in order to survive the transition through the gastrointestinal tract and colonize the intestinal tract for a sufficiently long period to achieve the desired health effect. One of the most important properties of probiotics is protection against pathogens in the intestinal tract of the host. Therefore, in the present study, to determine the potential uses of lactic microflora as probiotic strains, LAB isolated from the traditionally fermented soybean food *Tungrymbai* were analyzed for acid resistance, bile tolerance, antibiotic resistance, and antagonism against food-borne pathogens.

Samples of marketed *Tungrymbai* were collected aseptically in sterile containers and analyzed within 24 h of sampling. Isolation of LAB was done on M17 (HiMedia Laboratories Pvt. Ltd., India) agar. They were characterized by physiological and biochemical tests according to the criteria of Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 2000). Carbohydrate fermentation patterns were determined using the HiCarbohydrate kit (HiMedia Laboratories Pvt. Ltd.), according to the manufacturer's instructions.

For molecular characterization, the following primers were used for the amplification of the 16S rRNA gene: 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACTT-3′). PCR amplification was performed as described by Zamudio-Maya *et al.* (2008).

The acid tolerance of LAB was determined according to Lim and Im (2009) with slight modification. The M17 broth was acidified to pH 2, 2.5, and 3 using HCl containing 1,000 units of pepsin (HiMedia Laboratories Pvt. Ltd.). The viable cells were counted after exposure to acidic conditions for 0, 1, 2, 3, and 4 h.

The bile tolerance of LAB was screened according to Arihara *et al.* (1998), with a slight modification. Each LAB strain was grown in M17 broth at 37°C for 24 h without bile. Ten-fold serial dilutions were then prepared, and 0.1 ml aliquots were spread evenly on M17 agar containing bile salt (HiMedia Laboratories Pvt. Ltd.) concentrations of 2000, 3000, and 4000 ppm. Bacterial growth was determined after incubation at 37°C for 24 h.

Susceptibility of the LAB isolates to 15 types of antibiotics was determined by the disc diffusion method as described by Bauer *et al.* (1966). The commercially available antibiotic discs (HiMedia Laboratories Pvt. Ltd.) used were amoxicillin (10 μg), cloxacillin (5 μg), erythromycin (15 μg), tetracycline (10 μg), penicillin (2 units), co-trimoxazole (25 μg), penicillin–V (3 μg), cephalexin (30 μg), ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), clindamycin

*For correspondence. E-mail: srjoshi2006@yahoo.co.in

Table 1. Biochemical tests and carbohydrate fermentation profile of the LAB isolates

Isolates	Gram staining	Catalase test	Carbohydrate fermentation															
			Lac	Cel	Xyl	Mal	Raf	Treh	Meli	Inu	Sod. Glu	Dul	Glucs	Adon	α-Met	Rham	Esc. hyd	Rib
Enterococcus sp.	+ ve cocci	-	+	+	-	w	w	-	+	+	w	-	+	-	-	-	+	-
Vagococcus sp.	+ ve cocci	-	-	-	-	-	-	-	-	w	-	-	+	-	-	-	+	-
Lactococcus sp.	+ ve cocci	-	w	-	+	+	-	+	-	+	-	-	+	-	+	-	+	w
Weissella sp.	+ ve cocci	-	-	-	-	-	-	-	-	w	-	-	+	-	-	-	+	-
Lactobacillus sp.	+ ve rod	-	-	+	-	+	-	w	-	+	-	-	+	-	w	-	+	-

w, weakly positive; -, negative; +, positive
Lac, Lactose; Cel, Cellobiose; Xyl, Xylose; Mal, Maltose; Raf, Raffinose; Treh, Trehalose; Meli, Melibiose; Inu, Inulin; Sod. Glu, Sodium gluconate; Dul, Dulcitol; Glucs, Glucosamine; Adon, Adonitol; α-Met, α-Methyl-D-glucoside; Rham, Rhamnose; Esc. hyd, Esculin hydrolysis; Rib, Ribose.

(2 μg), gentamycin (10 μg), oxacillin (1 μg), and vancomycin (30 μg). Inhibition zone diameters were measured inclusive of the diameter of the discs. Results were expressed as sensitive, S (≥21 mm); intermediate, I (16–20 mm) and resistant, R (≤15 mm) according to Vlková *et al*. (2006).

Cell-free culture supernatants were prepared for the antibacterial assays by growing the isolates in M17 broth at 37°C for 24 h and centrifuging at 12,000×*g* for 10 min at 4°C. The pH was adjusted to 6.5–7.0 using 1 M NaOH, and two-step ammonium sulfate precipitation was performed: 30% (w/v) saturation for 2 h and then 50% (w/v) saturation overnight with continuous stirring at 4°C. The protein precipitates were obtained by centrifugation at 10,000×*g* for 30 min at 4°C. The pellet was resuspended in a minimal volume of sodium phosphate buffer (10 mM, pH 6.5) and dialyzed in the same buffer for 24 h.

Two different assays, the cross-streak method and the disc-diffusion method, were used to detect antibacterial activity. The cross-streak method was performed using Brain Heart Infusion (BHI) agar plates on which the LAB isolates were inoculated as lines 7.5 cm long and 0.6 cm wide, and incubated at 37°C for 48 h. The plates were then cross-streaked with indicator strains, incubated aerobically at 37°C for 24 h, and examined for growth inhibition of indicator strains around the streak line of LAB isolates.

For disc diffusion, the method described by Gonzalez and Kunka (1987) was followed. A volume of 50 μl of the cell-free culture supernatant was used to test the antibacterial activity. The indicator strains used were *Bacillus cereus* MTCC 430, *Staphylococcus aureus* subsp. *aureus* MTCC 740, *Escherichia coli* MTCC 730, and *Salmonella enterica* ser. *paratyphi* A MTCC 735.

The LAB isolates identified from the marketed *Tungrymbai* belonged to the genera *Enterococcus*, *Vagococcus*, *Lactobacillus*, *Lactococcus*, *Aerococcus*, and *Weissella* (Table 1).

The amplified 16S rRNA gene sequences were analyzed for homologous sequences using NCBI BLAST, and the obtained sequences were used to construct a phylogenetic tree. The phylogenetic tree was constructed using the Neighbor-Joining method in Mega 4.1 (Beta 3) software (Fig. 1). The taxonomic position and the similarity percentage of the isolates are shown in Table 2. The sequences

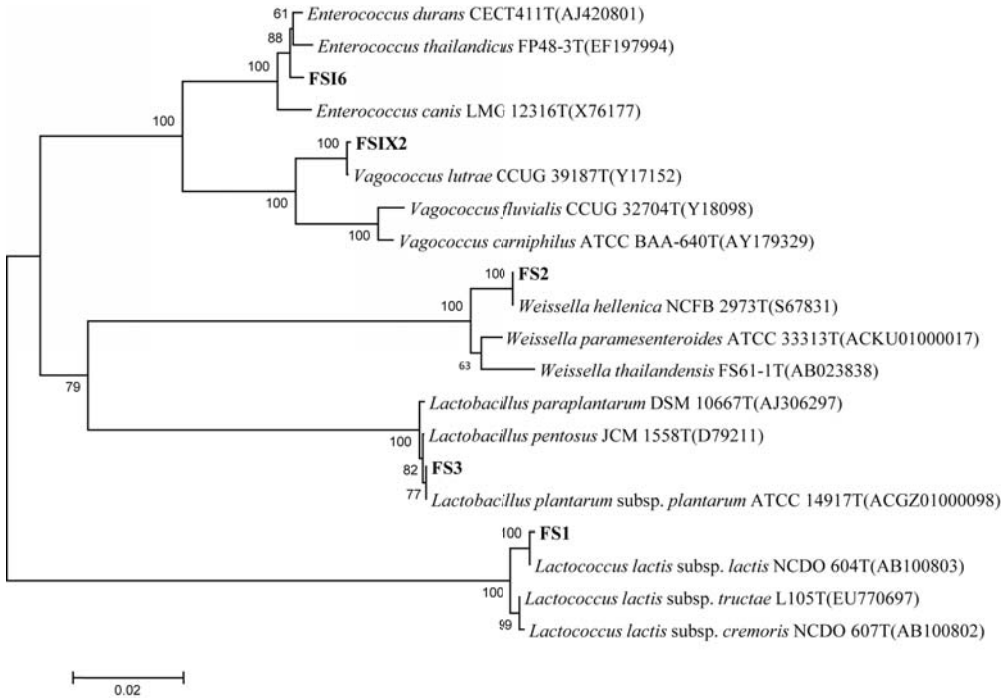

Fig. 1. Phylogenetic tree of the characterized LAB isolates constructed using the Neighbor-Joining method.

Table 2. Taxonomic position, and acid and bile tolerance of the characterized LAB isolates

Taxonomic position	rDNA type (isolates)	Representative isolate rDNA type (Accession No.[a])	Highest similarity[b]	Similarity (%)	Acid tolerance (pH 2 after 4 h)	Bile tolerance (4000 ppm bile salt)
Bacteria Firmicutes Lactobacillales	FSI6	JN029831	*Enterococcus canis* LMG 12316(T) [X76177]	98.474	-	-
	FSIX2	HQ728325	*Vagococcus lutrae* CCUG 39187(T) [Y17152]	98.864	+	-
	FS1	HQ728330	*Lactococcus lactis* subsp. *lactis* NCDO 604(T) [AB100803]	99.169	+	+
	FS2	HQ728331	*Weissella hellenica* NCFB 2973(T) [S67831]	99.796	-	-
	FS3	HQ728333	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917(T) [ACGZ01000098]	99.521	+	+

[a] NCBI GenBank
[b] Validly published species in EzTaxon

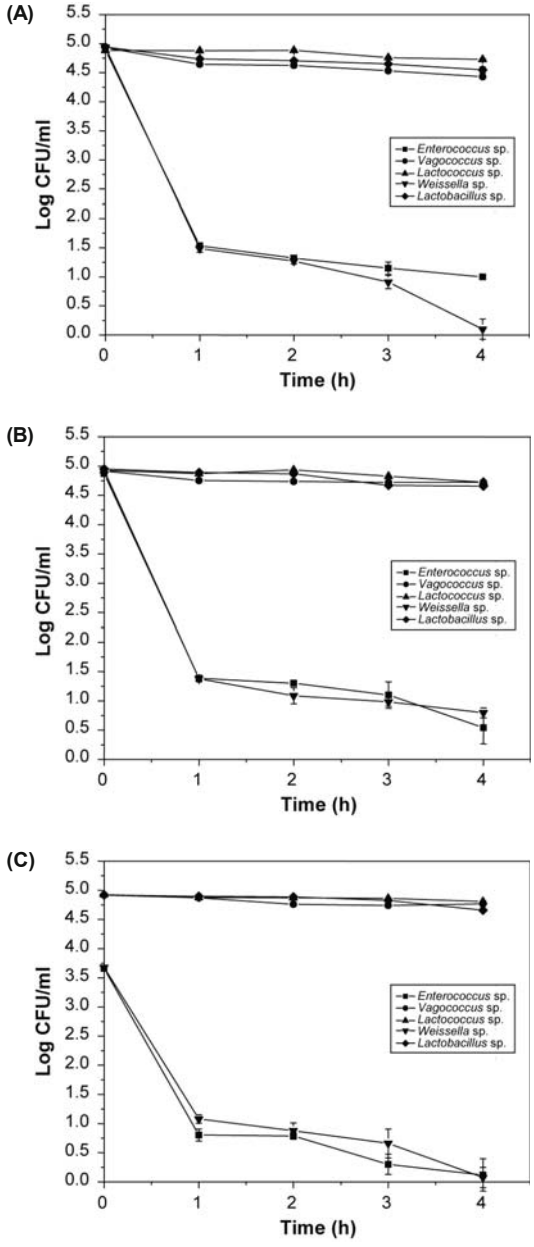

Fig. 2. Acid tolerance of LAB isolates. Viable count was determined after 0, 1, 2, 3, and 4 h incubation in M17 broth adjusted to pH 2.0 (A), pH 2.5 (B), pH 3.0 (C) (Error bars represent standard deviation of mean values).

were deposited in the GenBank database under the accession numbers HQ728325, HQ728330, HQ728331, HQ728333, and JN029831, which were identified as *Vagococcus* sp. (FSIX2), *Lactococcus* sp. (FS1), *Weissella* sp. (FS2), *Lactobacillus* sp. (FS3), and *Enterococcus* sp. (FSI6), respectively.

The identified isolates were analyzed for their acid and bile tolerance to evaluate their potential probiotic properties. It was found that *Lactococcus* sp., *Lactobacillus* sp., and *Vagococcus* sp. could tolerate acidic conditions (pH 2) even after incubation for 4 h while *Enterococcus* sp. and *Weissella* sp. were sensitive to acidic conditions (Fig. 2).

Lactococcus sp. and *Lactobacillus* sp. could tolerate a bile salt concentration of up to 4,000 ppm, while *Enterococcus* sp. and *Weissella* sp. could tolerate bile salt concentrations up to 3,000 ppm. *Vagococcus* sp., however, was highly sensitive to bile salt (Table 3).

All the characterized isolates were highly resistant to cloxacillin (5 μg), cephalexin (30 μg), and cephalothin (30 μg), with the exception of *Lactococcus* sp., which was sensitive to cephalothin (30 μg). All these isolates were sensitive to amoxicillin (10 μg), penicillin – V (3 μg), ampicillin (10 μg), and clindamycin (2 μg). The sensitivity of these isolates to the other antibiotics analyzed varied with the organism (Table 4).

Enterococcus sp., *Lactobacillus* sp., and *Lactococcus* sp. inhibited the growth of *Bacillus cereus* MTCC 430, *E. coli* MTCC 730, and *Salmonella enterica* ser. *paratyphi* A MTCC 735. This growth inhibition was more pronounced in the disc diffusion method than in the cross-streak method. The other isolates, *Vagococcus* sp. and *Weissella* sp., did not show any antibacterial activity against the tested strains.

Although some studies have been conducted on the microbial diversity of the fermented soybean foods of Northeast India (Singh and Umabati, 1995; Tamang, 2003; Jeyaram *et al.*, 2008; Sohliya *et al.*, 2009), very few studies have evaluated the probiotic properties, antibiotic tolerance, and antibacterial activity of the microbes. In the present study, the predominant lactic microflora in the fermented soybean food *Tungrymbai* were identified as *Lactococcus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Aerococcus* sp., and *Weissella* sp. The presence of *Weissella* sp. in fermented foods such as fermented sausages, fish, and maize dough has been reported previously (Ampe *et al.*, 1999; Paludan-Muller *et al.*, 1999; Zamudio-Maya *et al.*, 2008); however, this is the first report of its presence in fermented soybean.

In the human GI tract, the mean bile salt concentration is believed to be 3,000 ppm, which is considered high enough

Table 3. **Bile salt tolerance of the characterized LAB isolates expressed as log CFU/ml**

Bile salt concentration (ppm)	Log CFU/ml				
	Enterococcus sp.	*Vagococcus* sp.	*Lactococcus* sp.	*Weissella* sp.	*Lactobacillus* sp.
Control	4.84±0.003	4.83±0.001	3.66±1.41	3.55±1.36	4.89±0.001
2000	4.79±0.004	NG	3.55±1.36	3.50±1.35	4.87±0.004
3000	3.53±0.035	NG	3.42±1.32	3.17±1.22	4.84±0.002
4000	NG	NG	3.51±1.35	NG	4.71±0.003

NG , no growth. Values are mean±SEM of three replicates.

to screen for resistant strains (Gilliland *et al*., 1984; Goldin and Gorbach, 1992). Acid tolerance is another fundamental property that indicates the ability of probiotic microorganisms to survive passage through the stomach. Thus, LAB isolates from *Tungrymbai* were screened for bile and acid tolerance. It was found that *Lactococcus* sp., *Enterococcus* sp., and *Lactobacillus* sp. could tolerate a bile salt concentration of 3000 ppm, which agrees with the results reported for *Lactobacillus* strains by Pennacchia *et al.* (2004). Erkkila and Petaja (2000) reported that strains of *Lactobacillus curvatus* (RM 10), and *Lactobacillus sake* (L2) were resistant to bile salt concentrations of 3,000 ppm at pH 6. The present investigation also showed that *Vagococcus* sp., *Lactococcus* sp., and *Lactobacillus* sp. could tolerate acidic conditions (pH 2) for 4 h of exposure. However, *Enterococcus* sp. and *Weissella* sp. were sensitive to acidic conditions. Lim and Im (2009) reported that *Lactobacillus plantarum* isolated from Korean fermented foods was resistant to acidic conditions during incubation for 2 h at pH 2.5, and was stable at 5% bile concentration. Pennacchia *et al.* (2004) reported a 60–80% survival rate for LAB incubated in PBS buffer at pH 2.5 for 3 h at 37°C.

The studied LAB isolates exhibited sensitivity or intermediate sensitivity to most of the common antibiotics tested. However, the isolates were resistant to cloxacillin (5 µg), cephalexin (30 µg), and cephalothin (30 µg). It was found that *Enterococcus* sp. was resistant to a few of the antibiotics analyzed. *Lactococcus* sp. and *Lactobacillus* sp., which showed acid and bile tolerance, were sensitive to most of the antibiotics tested. Enterococcal isolates both from raw meat (Klein *et al*., 1998; Davies and Roberts, 1999; Robrido *et al*., 2000) and fermented milk and meat products (Batish and Ranganathan, 1986; Teuber and Perreten, 2000; Franz *et al*., 2001; Giraffa, 2002) were analyzed for resistance to a broader range of different antibiotics using phenotypic susceptibility testing. Data from these studies suggest a high prevalence of (multiple) antibiotic-resistant enterococci in the food samples analyzed, which were mostly susceptible to the clinically relevant antibiotics ampicillin and vancomycin.

In the present study, the inhibitory effect of the cell-free culture supernatant of each isolate was evaluated. Antibacterial activity against pathogenic bacteria was observed in 3 isolates: *Enterococcus* sp., *Lactobacillus* sp., and *Lactococcus* sp. Earlier reports have shown that gram-positive bacteria are more sensitive to bacteriocins produced by LAB than Gram-negative bacteria. Nisin A produced by *Lactococcus lactis* subsp. *lactis* and pediocin PA-1 produced by *Pediococcus acidilactici* exert bactericidal activity against many Gram-positive bacteria and sublethally stressed Gram-negative bacteria associated with food spoilage and food-borne disease outbreaks (Yang *et al*., 1999).

These probiotic properties, together with the possibility of antibiotic resistance gene transfer shown in the present study, reveal that *Lactobacillus* sp. and *Lactococcus* sp. characterized from the marketed traditionally fermented soybean food *Tungrymbai* could be considered as potent probiotic candidates due to their acid and bile tolerance as well as their antibacterial activity against both Gram-positive and Gram-negative bacteria. These isolates were found to be sensitive to most of the antibiotics tested, reducing the likelihood of antibiotic resistance gene transfer to pathogenic microbes.

We are grateful to the Department of Biotechnology, Govt. of India, for providing financial assistance to carry out this research.

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Table 4. **Antibiotic sensitivity pattern of the characterized LAB isolates**

Isolates	Antibiotics														
	Am (10 µg)	Cx (5 µg)	E (15 µg)	T (10 µg)	P (2 units)	Co (25 µg)	Pv (3 µg)	Cp (30 µg)	A (10 µg)	Ch (30 µg)	C (30 µg)	Cd (2 µg)	G (10 µg)	Ox (1 µg)	Va (30 µg)
Enterococcus sp.	S	R	R	S	S	I	S	R	S	R	S	S	S	I	S
Vagococcus sp.	S	R	R	S	I	S	S	R	S	R	S	S	S	R	S
Lactococcus sp.	S	R	S	S	S	S	S	R	S	S	S	S	S	S	I
Lactobacillus sp.	S	R	I	S	I	S	S	R	S	R	S	S	S	S	S
Weissella sp.	S	R	I	S	S	S	S	R	S	R	S	S	S	I	S

[sensitive, S (≥ 21 mm); intermediate, I (16–20 mm); resistant, R (≤ 15 mm)]
Am, Amoxycillin; Cx, Cloxacillin; E, Erythromycin; T, Tetracycline; P, Penicillin; Co, Co-trimoxazole; Pv, Penicillin-V; Cp, Cephalexin; A, Ampicillin; Ch, Cephalothin; C, Chloramphenicol; Cd, Clindamycin; G, Gentamycin; Ox, Oxcillin; Va, Vancomycin.

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