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Phenotypic and genotypic identification of lactic acid bacteria isolated from ethnic fermented bamboo tender shoots of North East India

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Abstract

Mesu, soidon, soibum and soijim are ethnic fermented bamboo tender shoot products prepared by the people in North East India. Microbiological analysis of mesu, soidon, soibum and soijim showed the population dominated by lactic acid bacteria (LAB) ranging up to 10^8 cfu g^{-1}. The phenotypic characterisation of predominant LAB isolated from the fermented bamboo shoot products was based on general morphology, physiological tests, API and Biolog systems. The genotypic characterisation of LAB was based on RAPD-PCR, rep PCR, species-specific PCR techniques, 16S rRNA gene sequencing and DNA–DNA hybridisation. Predominant functional LAB strains associated with the fermented bamboo shoot products were identified as *Lactobacillus brevis*, *Lb. plantarum, Lb. curvatus*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuc. fallax*, *Leuc. lactis, Leuc. citreum* and *Enterococcus durans*.
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1. Introduction

Ethnic people living in the sub-Himalayan regions of North East India, Nepal and Bhutan prepare and consume a variety of domesticated and wild bamboo tender shoots (Sharma, 1989) and their fermented products for centuries (Tamang, 2001). About 26.2 tons, 435 tons and 426.8 tons of bamboo shoots are harvested annually in Sikkim, Meghalaya and Mizoram states, respectively, located in North East region of India (Bhatt et al., 2003). Some popular fermented bamboo tender shoots of North East India are mesu of Sikkim, soidon, soibum and soijim of Manipur, ekung, eup and hiring of Arunachal Pradesh, lung-siej or syrwa of Meghalaya (Tamang, 2005).

Mesu is an ethnic fermented bamboo tender shoot consumed by the Nepalis, Bhutias and Lepchas of Sikkim and the Darjeeling hills in India, eastern hills of Nepal and Bhutan. In the Limboo (one of the castes of Nepali) dialect, 'me' means young bamboo shoot and 'su' means sour (Tamang, 2000). The Bhutia calls it 'rakyu' and the Lepcha calls it 'sitit'. During preparation of mesu, tender shoots of bamboo (*Dendrocalamus sikkimensis*, *D. hamiltonii* and *Bambusa tulda*) are collected, their outer hard casings are removed and the inner portion is then chopped into small pieces with a knife. The chopped pieces are washed thoroughly with clean water, drained off and pressed tightly into a bamboo-made cylindrical vessel. This vessel is made air tight with a lid, placed in an upside-down position to drain out any liquid, and allowed to ferment under natural anaerobic conditions for 7–15 days. Mesu is eaten as a curry, pickle or soup. It is sold in the local markets during the months of July and September, when young bamboo tender shoots are plenty by some rural women who are dependent upon this product for their livelihood.

Soibum is an ethnic fermented young bamboo shoot product of Meities of Manipur. It is prepared from succulent bamboo

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shoots of *B. balcooa, D. strictus* and *Melocana baccifera* (Sarangthem and Singh, 2003). It is whitish in colour and with faint aroma and sour taste. During preparation, the outer casings are removed from young bamboo shoots, the inner part is chopped into pieces, and these are washed and left in a covered earthen pot to ferment for 20 days. It can be prepared from the single species of bamboo or from intermixed material of more than one species. Soibum is commonly sold in local vegetable markets in Manipur. Soibum is a special delicacy of the Meities of Manipur, eaten as pickle and curry mixed with fermented fish.

Soidon is a fermented product of Manipur prepared from the tip of matured bamboo shoots. The tips are collected; outer casings are removed and cut into small pieces. Chopped pieces of bamboo shoot tips are merged in water in an earthen pot, covered and fermented for 3–7 days at room temperature. After fermentation, chopped pieces of bamboo shoots called soidon are taken out, and the acidic liquid portion called soijim is kept in a bottle, and stored at room temperature for a year or more. Soidon is consumed both as a curry and as pickle. Soijim is used as condiment to supplement the sour-taste in curry.

Phenotypic characterisation of *Pediococcus pentosaceus*, *Lactobacillus brevis* and *Lb. plantarum* of mesu samples collected from markets of Kalimpong, Darjeeling and Sikkim in India was reported by Tamang and Sarkar (1996). Chemical changes and sensory attributes of soibum during fermentation were studied by Pravabati and Singh (1986), Giri and Janmejay (1994, 2000). Sarangthem and Singh (2003) reported the presence of species of *Bacillus* and *Micrococcus* in soibum.

The aim of the present work was to identify the predominant lactic acid bacteria present in the ethnic fermented bamboo tender shoot products of Sikkim and Manipur of India on the basis of phenotypic characteristics including API and Biolog systems, and genotypic characteristics including repetitive element (rep)-PCR, randomly amplified polymorphic DNA (RAPD)-PCR and species-specific PCR techniques, 16S rRNA gene sequencing and DNA–DNA hybridisation.

2. Materials and methods

2.1. Samples

About 300 g each of 26 samples of mesu were collected from different bamboo-growing regions of Sikkim in India. Three samples of raw young tender shoots of about 1 ft height detached from bamboo, locally called ‘choya bans’ (*D. hamiltonii*) were collected from Lindogh village in East Sikkim. About 300 g each of 7 samples of soidon, 6 samples of soibum and 5 samples of soijim were obtained from Bishnupur and Imphal of Manipur in India. All samples were collected aseptically in sterile bottles kept in an ice-box, and transported to the laboratory for analyses.

2.2. Microbiological analysis

Samples (10 g) of each product were mixed with 90 ml of 0.85% (w/v) sterile physiological saline and homogenised in a Stomacher lab-blender (400, Seward, UK) for 1 min. A serial dilution in the same diluent was made. LAB were isolated on plates of MRS agar (M641, HiMedia, India) supplemented with 1% $CaCO_3$ and incubated at 30 °C in an anaerobic gas-jar (LE002, HiMedia, India) for 48–72 h. Total viable counts were determined on plate count agar (M091A, HiMedia, India) incubated at 30 °C for 48–72 h. Colonies of moulds and yeasts were examined on potato dextrose agar (M096, HiMedia, India) and yeast-malt (YM) agar (M424, HiMedia, India), supplemented with 10 $IUml^{-1}$ benzylpenicillin and 12 $\mu g\ ml^{-1}$ streptomycin sulphate, respectively, and which were incubated aerobically at 28 °C for 72 h. Isolated colonies based on colony morphology were selected randomly from the highest diluted plates. Purity of the isolates was checked by streaking again and sub-culturing on fresh agar plates of the isolation media, followed by microscopic examinations. Purified isolates of LAB were preserved at −20 °C in MRS broth (M369, HiMedia, India) with 15% (v/v) glycerol added.

2.3. Phenotypic characterisation

Cell morphology of all isolates and their motility were determined using a phase contrast microscope (Olympus CH3-BH-PC, Japan). Isolates were Gram-stained and tested for catalase production, and were preliminarily identified based on the phenotypic properties such as carbon dioxide production from glucose, ammonia production from arginine, growth at different temperatures and production of dextran from sucrose as well as the ability to grow in different concentrations of sodium chloride and pH in MRS broth, following the methods of Schillinger and Lücke (1987), and Dykes et al. (1994). The configuration of lactic acid produced from glucose was determined enzymatically using D-lactate and L-lactate dehydrogenase test kits (Roche Diagnostic, France). The presence of *meso*-diaminopimelic acid (DAP) in the cell walls of LAB was determined on cellulose plates using a thinlayer-chromatography (Tamang et al., 2000). Sugar fermentation patterns of LAB isolates were determined using the API 50 CHL test strips (bioMérieux, France). The Biolog microplate bacterial identification system (Biolog Inc., USA; Oxoid GmbH, Wesel, Germany), based on the utilisation of 95 single carbon sources was used for the identification of *Leuconostoc* species as described elsewhere (Tamang et al., 2005).

2.4. Genotypic characterisation

2.4.1. DNA extraction

Total genomic DNA of 60 strains of LAB was extracted from 2-ml samples of overnight cultures grown in MRS broth at 30 °C according to the methods of Pitcher et al., 1989). Reference strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), LTH (Universität Hohenheim, Germany) and ATCC (American Type Culture Collection).

2.4.2. RAPD-PCR analysis

The primer M13 (5’-GAG GGT GGC GGT TCT-3’) (Huey and Hall, 1989) was used for RAPD-PCR. Conditions of PCR

reactions and amplification were performed as described by Schillinger et al. (2003). The PCR amplification was conducted with a Primus 96 Plus thermal cycler (MWG Biotech, Ebensburg, Germany).

2.4.3. rep PCR

Repetitive element (rep)-PCR analysis of LAB strains was carried out using reaction and amplification conditions as previously described by Gevers et al. (2001). For rep-PCR, the primer GTG_5 (5'-GTG GTG GTG GTG GTG-3') recommended for LAB identification by Gevers et al. (2001) was used.

2.4.4. Species-specific PCR

For identification of *Lb. brevis*, a species-specific PCR was applied. The oligonucleotide primer 5'-CTTGCACTGATTT TAACA-3' and 5'-GGGCGGTGTGTACAAGGC-3' were used as forward and reverse primers, respectively (Guarneri et al., 2001). The PCR conditions were as described by Guarneri et al. (2001). To verify the identity of the PCR product, amplified fragments were digested with P*st*I (New England Biolabs, Frankfurt, Germany) in a 15 µl reaction mixture containing 11.5 µl of the PCR product, 1.5 µl incubation buffer and 10 U P*st*I (1 h, 37 °C).

2.4.5. 16S rRNA gene sequencing

The almost complete 16S rDNA nucleotide sequence was determined for one representative leuconostocs, a lactobacilli and an enterococci strain. The 16S rDNA gene was amplified by PCR using primers as described by Cibik et al. (2000). DNA was amplified in 50 µl volumes containing 100 ng template DNA, 200 µM dNTPs, 25 pM of the respective primers, 2.5 U *Pwo* DNA polymerase (Peqlabs, Konstanz, Germany) and 1×*Pwo* polymerase buffer. DNA was amplified in 32 cycles (denaturation, 94 °C for 1 min; annealing, 51 °C for 1 min; extension, 68 °C for 2 min). The PCR product was cleaned using Quantum-prep spin columns (Biorad, Munich, Germany) according to the manufacturer's instructions and subsequently commercially sequenced (GATC Biotech, Konstanz, Germany) using primers SP3, SP4 and SP5 as described by Cibik et al. (2000).

2.4.6. DNA–DNA hybridisation

Total genomic DNA for hybridisation experiments was extracted and purified according to the method of Marmur (1961) as modified by Stackebrandt and Kandler (1979). DNA–DNA reassociation analysis was performed using a Cary 100 spectrophotometer (Varian, Darmstadt, Germany). DNA homology values were calculated from renaturation rates according to standard methods (Huss et al., 1983).

2.4.7. Gel electrophoresis

Amplification products from RAPD-PCR and rep-PCR were subjected to electrophoresis in 1.8% agarose gels in 1×TBE buffer for 15–17 h at 48 and 55 V, respectively. The products resulting from the *Lb. brevis* specific PCR were separated on 1.2% agarose for 2 h at 100 V. A DNA molecular mass marker (100 and 500 bp molecular ladders from Biorad, München, Germany) was used as a standard. After electrophoresis, the gels were stained in ethidium bromide and after washing visualised with a UV transilluminator. Gels were photographed using the Fluorchem Imager 5500 system (Alpha Innotech, USA) and the digitised images were analysed and processed using the Bionumerics software (Applied Maths, Kortrijk, Belgium). Groupings of the fingerprints were performed by means of the Pearson product-moment correlation coefficient and the unweighted pair group method using arithmetic averages clustering algorithm (UPGMA).

2.5. pH

The pH of the samples (10 g) was determined directly using a digital pH meter (Type 361, Systronics, India) calibrated with standard buffer solutions (Merck), after homogenisation in 20 ml of carbon-dioxide free distilled water.

3. Results and discussion

3.1. Microbial counts and pH

A total of 44 samples of ethnic fermented bamboo tender shoots comprising mesu (26), soidon (7), soibum (6) and soijum (5), and 3 samples of raw bamboo tender shoots (*Dendrocalamus hamiltonii*) were analysed for microbiological load (Table 1). In all fermented bamboo shoot products, the numbers of LAB as well as the total viable counts were in the range of 10^5 to 10^8 cfu g^{-1}. Yeasts were detected in a few samples of mesu. Moulds were not detected in any of the analysed samples. Numbers of LAB, yeasts and total viable counts of raw bamboo tender shoots were ca. 10^2 cfu g^{-1}, 10 cfu g^{-1} and 10^2 cfu g^{-1}, respectively. The mean pH of the samples ranged from 3.9 to 4.2, with mesu showing the lowest pH. The mean pH of raw bamboo shoot was 6.1 (Table 1).

3.2. Identification

A total of 60 bacterial strains isolated from raw bamboo shoot (4), mesu (35), soidon (7), soibum (7) and soijim (7) were considered as presumptive LAB because they were Gram positive, catalase-negative bacteria which did not form spores

Table 1
Microbiological populations of ethnic fermented bamboo tender shoot products of North East India

Product	pH	Log cfu g^{-1} sample		
		LAB	Yeasts	Total viable counts
Raw bamboo shoot (n=3)	6.1±0.1	2.2±0.2	1.3±0.4	2.5±0.2
Mesu (n=26)	3.9±0.2	7.4±1.1	3.9±3.0	7.6±1.0
Soidon (n=7)	4.2±0.1	6.0±0.2	<DL	6.1±0.2
Soibum (n=6)	4.2±0.1	5.8±0.3	<DL	5.8±0.3
Soijim (n=5)	4.1±0.2	7.2±0.4	<DL	7.3±0.1

Data represent the means (±SD) of number of samples (n). Ranges are given in parenthesis. Mould
was not detected. DL, detection limit less than 10 cfu g^{-1}.

Table 2
Phenotypic characteristics of the LAB from fermented bamboo shoot products of North East India

Product	Number of strains	Cell shape	Gas from glucose	NH_3 from arginine	Lactate isomer	Growth at 45 °C	Sugars fermented					
							Arabinose	Cellobiose	Esculin	Galactose	Lactose	Maltose
Row bamboo shoot	4	rod	+	+	DL	−	2/2	−	−	3/1	1/3	3/1
Mesu(35)	15	rod	−	−	DL	−	7/8	+	+	+	+	+
	1	rod	−	−	DL	−	−	−	−	+	−	+
	10	rod	+	7/3	DL	−	9/1	2/8	2/8	+	2/8	+
	4	coccoid	+	−	D	−	+	+	2/2	2/2	−	+
	5	Coccus (Tetrad)	−	+	DL	+	+	4/1	4/1	+	−	+
Soidon(7)	2	rod	+	−	DL	−	1/1	−	−	+	1/1	+
	4	rod	+	−	D	−	1/3	1/3	1/3	+	3/1	+
	1	coccoid	+	−	D	−	−	−	−	−	−	−
Soibum(7)	2	rod	−	−	DL	−	−	+	−	+	−	+
	1	rod	+	−	DL	−	+	−	−	+	−	+
	1	coccus	−	−	L	−	−	+	+	+	+	+
	1	rod	+	−	D	−	−	−	−	+	+	+
	1	coccoid	+	−	D	−	−	−	+	−	−	−
	1	coccoid	+	−	D	−	+	+	−	+	+	−
Soijim(7)	2	rod	+	−	DL	−	+	−	−	+	−	+
	3	rod	+	−	D	−	−	2/1	1/2	+	+	+
	2	coccoid	+	−	D	−	−	−	−	−	−	−

+, all strains positive; −, all strains negative; (../..), number of positive/negative strains. All strains grew at 10 °C and 15 °C. *DAP positive.

and were non-motile. The strains of LAB were phenotypically characterised on the basis of cell morphology, gas production from glucose, arginine hydrolysis and lactate configuration (Table 2). In addition, growth at different temperatures, pH, the sugar fermentation patterns and in case of the homofermentative lactobacilli the presence of *meso*-diaminopimelic acid (DAP) were determined.

All 4 strains of LAB isolated from raw bamboo shoots were heterofermentative rods, unable to grow at 45 °C. They produced ammonia from arginine and both isomers of lactate from glucose. Of the 35 strains of LAB isolated from mesu, 16 were homofermentative rods, 10 strains were heterofermentative rods, 4 strains were gas-producing coccoid cells and 5 strains were tetrad-forming cocci presumptively classified as belonging to the genus *Pediococcus*. Seven of the heterofermentative rods were scored positive in arginine hydrolysis. All homofermentative rods, except one strain (BFE 2941) contained DAP in cell wall and produced both isomers of lactate from glucose. Eighteen of the 21 strains isolated from soidon, soibum, and soijim, were gas producing coccoid cells or rods, and among the remaining 3 strains, two were homofermentative, DAP-positive rods and another strain from soibum was a homofermentative coccus producing L (+)-lactate from glucose. Phenotypic characterisation including sugar fermentation pattern, arginine hydrolysis and growth behaviour at 50 °C justified the identification of the five *Pediococcus* strains as *P. pentosaceus*.

A total of 19 strains including the four strains from raw bamboo shoots, ten strains from mesu, two from soidon and soijim each and one strain from soibum were CO_2 forming DL lactate producers and therefore classified as heterofermentative lactobacilli. All of them were identified as *Lb. brevis* using species-specific PCR. Amplification of the 16S rRNA gene with *Lb. brevis*-specific oligonucleotide primers resulted in the 1340 bp fragment typical for *Lb. brevis* (Guarneri et al., 2001; Tamang et al., 2005).

The 15 DAP-positive homofermenters from mesu and the 2 homofermentative *Lactobacillus* strains (BFE 2953 and BFE 2954) from soibum were identified as *Lb. plantarum* using rep-PCR technique with GTG_5 primer (Fig. 1). The remaining homofermentative *Lactobacillus* (BFE 2941) from mesu differed from the *Lb. plantarum* strains and hence16S rDNA gene sequencing was performed. The sequence obtained was 100% identical to the 16S rRNA gene of *Lb. curvatus* DSM 20019.

A total of nine strains produced D (−) lactate from glucose, were arginine-negative and showed the typical leuconostoc-like ovoid cell shape. Four of them (BFE 925, BFE 929, BFE 931 and BFE 2958) isolated from soidon, soibum and soijim only fermented a small range of carbohydrates (Table 2). The fingerprints of these strains obtained with primer GTG_5 in the rep-PCR were highly similar to the type strain of *Leuconostoc fallax* (results not shown). The application of the Biolog system based on the utilisation patterns of 95 single carbon sources confirmed this result. The Biolog system also allowed identifying the fifth gas producing ovoid strain BFE 930 as *Leuc. mesenteroides* subsp. *mesenteroides*. The remaining four coccoid strains (BFE 2936, BFE 2937, BFE 2938 and BFE 2939) isolated from mesu, were identified as *Leuc. citreum* by using rep-PCR. The fingerprints obtained with GTG_5 primer showed a high similarity with the type strain of this species (data not shown).

Another eight heterofermentative strains (four from soidon, one from soibum and three from soijim) were difficult to identify. They were rods and produced gas from glucose and therefore, they were presumptively classified as heterofermentative lactobacilli. However, they produced only D (−)-lactate

Mannose	Melezitose	Melibiose	Raffinose	Rhamnose	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose	Identity
−	−	2/2	−	−	+	−	−	−	−	+	*Lb. brevis*
+	+	13/2	12/3	−	9/6	+	6/9	+	+	1/14	**Lb. plantarum*
+	−	−	−	−	+	+	−	+	+	−	*Lb. curvatus*
2/8	2/8	7/3	−	−	+	2/8	2/8	2/8	2/8	+	*Lb. brevis*
+	1/3	−	−	−	−	3/1	−	+	+	1/3	*Leuc. citreum*
+	−	−	−	+	+	4/1	−	−	+	2/3	*P. pentosaceus*
1/1	−	+	1/1	−	1/1	−	−	1/1	1/1	+	*Lb. brevis*
3/1	−	+	3/1	−	1/3	2/2	−	3/1	3/1	+	*Leuc. lactis*
+	−	−	−	−	−	−	−	+	+	−	*Leuc. fallax*
+	−	−	−	−	+	+	1/1	+	+	−	**Lb. plantarum*
−	−	+	−	−	+	−	−	−	−	+	*Lb. brevis*
+	−	+	−	−	+	+	−	−	+	−	*Ent. durans*
+	−	+	+	−	−	−	−	+	+	+	*Leuc. lactis*
+	−	+	−	−	−	+	−	+	+	+	*Leuc. fallax*
+	−	+	−	−	−	+	−	+	+	−	*Leuc. mesenteroides*
−	−	+	−	−	+	−	−	−	−	+	*Lb. brevis*
+	−	+	+	−	−	1/2	−	+	+	+	*Leuc. lactis*
+	−	−	−	−	−	−	−	+	1/1	−	*Leuc..fallax*

from glucose and were unable to hydrolyse arginine as it is known for *Leuconostoc* and some *Weissella* species. They also formed dextran from sucrose. The commercial identification systems API 50 CHL and Biolog did not allow a clear identification of these strains. The patterns of the 8 strains obtained using RAPD-PCR with primer M13 showed a high similarity (results not shown). Therefore, 16S rRNA gene sequencing of two of the eight strains (BFE 926 and BFE 932) was performed and the comparison with the 16S rDNA gene sequence of *Leuc. lactis* DSM 20202 revealed identities of 99.8% and 99.7%, respectively. In addition, DNA relatedness of the same two strains with the type strain of *Leuc. lactis* DSM 20202 was

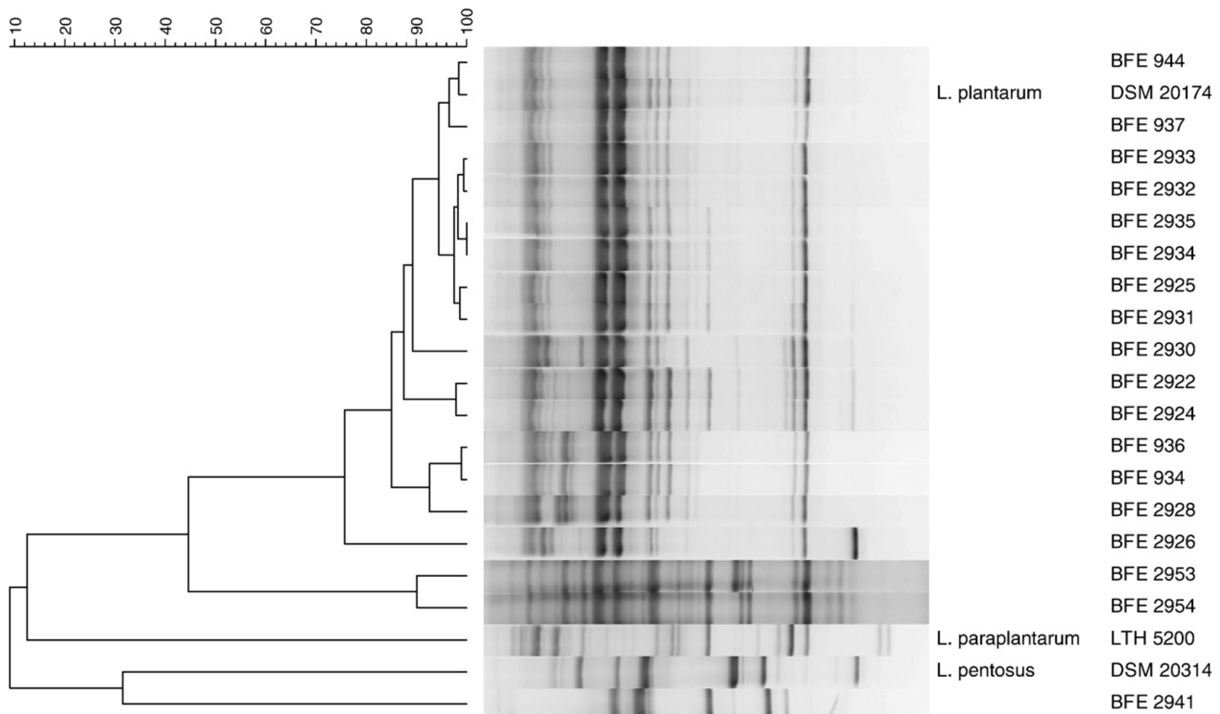

Fig. 1. Dendrogram obtained from $(GTG)_5$-PCR fingerprints of strains of the *Lb. plantarum* group. Patterns were grouped with the unweighted pair group algorithm with arithmetic averages (UPGMA).

determined. Strains BFE 926 and BFE 932 both showed a high DNA similarity (above 95%) with *Leuc. lactis* and a low level of relatedness with the control strain *Leuc. fallax* DSM 10615 (34%).

Strain BFE 2955 from soibum was the only homofermentative coccus and was identified as *Enterococcus durans* using 16S rRNA gene sequencing. The sequence of this strain shared 99.87% identity with *E. durans* ATCC 59607. *Lb. brevis* was detected in all fermented bamboo shoot products except soibum and is apparently the predominating heterofermentative *Lactobacillus* species associated with the lactic fermentation of bamboo shoots. *Lb. plantarum* was obviously the main species found in mesu (65% of the isolates). Leuconostocs were not recovered from mesu; representatives of several *Leuconostoc* species, however, were present in all other fermented bamboo products.

The microbial profile of mesu, soidon, soibum and soijim samples revealed that LAB comprising lactobacilli, pediococci, leuconostocs and enterococcus were the predominant microorganisms present in viable numbers above 10^7 $cfug^{-1}$. Similar fermented bamboo shoot product called naw-mai-dong or nor-mai-dorng of Thailand also contained lactobacilli, leuconostocs and pediococci (Dhavises, 1972; Phithakpol et al., 1995). On the basis of a combination of phenotypic properties and molecular techniques such as RAPD-PCR, rep-PCR, species-specific PCR, 16S rRNA gene sequencing and DNA–DNA hybridisation, strains of LAB isolated from mesu, soidon, soibum and soijim were identified as *Lb. brevis*, *Lb. plantarum, Lb. curvatus, P. pentosaceus, Leuc. mesenteroides* subsp. *mesenteroides, Leuc. fallax*, *Leuc. lactis, Leuc. citreum* and *E. durans*. Findings of *Leuc. mesenteroides* subsp. *mesenteroides, Leuc. fallax, Leuc. lactis*, *Leuc. citreum* and *E. durans* in ethnic fermented bamboo shoots of North East India is reported for the first time. The technological properties of these strains can now be investigated in order to determine their usefulness for development of a starter culture preparation.

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