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Lactic acid bacteria isolated from ethnic preserved meat products of the Western Himalayas

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ABSTRACT

We used culture- and molecular-biology-based methods to investigate the diversity of lactic acid bacteria (LAB) in the ethnic chevon (goat) meat products *chartayshya*, *jamma* and *arjia* of the Western Himalayas. In six *chartayshya*, six *jamma* and four *arjia* samples, LAB were the predominant microbial component involved in the fermentation of these samples, and the total LAB population in *arjia* (7.8 ± 0.1 log cfu g^{-1}; mean ± SD) was significantly higher ($P < 0.05$) than in *chartayshya* (6.9 ± 0.1 log cfu g^{-1}) and *jamma* (7.5 ± 0.1 log cfu g^{-1}). We identified 53 LAB samples by 16S rRNA and phenylalanyl-tRNA synthase (*pheS*) genes sequencing. The LAB isolates were identified as *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, and *Weissella cibaria*. These results revealed that there is a high level of diversity of LAB in the Himalayan ethnic preserved meat products.

1. Introduction

Meat is highly susceptible to microbial spoilage (Adams, 2010). Drying, smoking or fermentation of meat is critical steps in the traditional processing of meat in the Himalayas (Tamang, 2010). Ethnic people of the Western Himalayan regions of India and Nepal prepare and consume ethnic meat products such as *chartayshya, arjia* and *jamma. Chartayshya* is a chevon (goat) meat product consumed by the ethnic people of Uttarakhand state of India and West Nepal. During the preparation of *chartayshya*, red meat is cut into pieces of about 3–4 cm^3 and mixed with 1% salt. The pieces are strung together on a long thread which is hung from bamboo stripes or wooden sticks, then exposed in the corridors of the houses for 15–20 days (Rai et al., 2009). The prepared *chartaysha* is usually kept at room temperature for several weeks before it is eaten as a component of curry dishes.

Jamma or *geema* is an ethnic fermented sausage of the Western Himalayas prepared from chevon meat. Red meat is chopped into fine pieces; and mixed with 1% ground finger millet (*Eleusine coracana*), 0.5% wild pepper locally called 'timbur' (*Zanthoxylum* sp.), 0.5% chili powder and salt (Rai et al., 2009). A little fresh animal blood is also added. The meat mixture is made semi-liquid by addition of water and is poured into portions of goat small intestine 2–3 cm in diameter and 100–120 cm long with the help of funnel. Both ends of the filled intestine are tied, then it is pricked randomly to prevent bursting while boiling. After boiling for 15–20 min, the stuffed intestine is smoked above the kitchen oven for 15–20 days if they are not eaten immediately after boiling. The method of preparation of *jamma* is similar to *kargyong* of the Eastern Himalayas (Rai et al., 2009, 2010a). It is eaten as cooked sausage or as a curry component.

The preparation of *arjia* is similar to that for *jamma*. However, a mixture of chopped lungs of goat, salt, chili powder, *Zanthoxylum* sp. and fresh animal blood are stuffed into goat large instead of small intestine. *Arjia* is boiled and smoked as is *jamma*, and is consumed in curry dishes or as deep fried sausage.

The diversity of lactic acid bacteria (LAB) associated with these ethnic preserved meat products of the Western Himalayas was investigated using culture- and molecular-biology-based methods. Limited functional activities such as antimicrobial, enzymatic activities, and production of biogenic amines were also studied to ascertain the role of microorganisms in the products.

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Table 1
Microbiological populations of ethnic meat products of the Western Himalayas.

Product	pH	Log cfu g^{-1} sample					
		Bacteria			Yeast	Moulds	TVC
		LAB	Bacilli	Micrococcaceae			
Chartayshya ($n = 6$)	6.5 ± 0.1	6.9 ± 0.1	2.8 ± 0.1	6.6 ± 0.1	4.9 ± 0.1	1.6 ± 0.1	7.9 ± 0.1
Jamma ($n = 6$)	5.5 ± 0.2	7.5 ± 0.2	3.5 ± 0.1	5.5 ± 0.2	5.7 ± 0.1	2.7 ± 0.1	8.0 ± 0.2
Arjia ($n = 4$)	6.3 ± 0.1	7.8 ± 0.1	3.8 ± 0.1	6.4 ± 0.1	4.7 ± 0.1	DL	9.0 ± 0.2

cfu, colony forming unit; *n*, number of samples; DL, detection limit less than 10 cfu; TVC, total viable count
Data represent the means (±SD) of triplicate sets of experiments.

2. Materials and methods

2.1. Meat samples

Six samples of *chartayshya*, six samples of *jamma*, and four samples of *arjia* were each collected from different manufacturing places in Uttarakhand in India and western districts of Nepal. About 50 g of each sample) were collected aseptically in sterile bottles and microbiological analyses were performed within 2 days of sample collection. For determination of pH, samples were stored at −20 °C. The pH of the samples (10 g) was determined directly using a digital pH metre (Type 361, Systronics, Mumbai, India) calibrated with standard buffer solutions (Merck), after homogenisation in 20 ml of carbon-dioxide free distilled water.

2.2. Microbiological analysis

Microbiological analysis was performed as previously described (Tamang et al., 2005). Ten gram portion of each sample was put into a sterile plastic bag containing with 90 ml of 0.85% (w/v) sterile physiological saline and homogenised in a lab-blender (Stomacher 400, Seward, London, UK) for 1 min. Ten fold serial dilutions in the same diluent were prepared. Portions of dilution (0.1 ml) were spreaded on plates of MRS agar (de Man et al. 1960) (M641, HiMedia, Mumbai, India) plates supplemented with 1% $CaCO_3$. The plates were incubated at 30 °C in an Anaerobic Gas-Pack container (LE002, HiMedia) for 48 h. Mannitol-salt Phenol-red Agar (Merck, Darmstadt, Germany) was used for recovery of Micrococcaceae from the samples following the method of Papamanoli et al. (2002). Spread plates of Baird Parker agar base (M043, HiMedia) with appropriate additions of egg yolk tellurite emulsion (FD046, HiMedia) incubated at 35 °C for 48 h (Papamanoli et al., 2002) was used for recovery of staphylococci. Spore-forming bacilli were isolated on nutrient agar (MM012, HiMedia) plates incubated at 37 °C for 24 h, after inactivation of vegetable cells by heating dilutions at 100 °C for 2 min (Tamang and Nikkuni, 1996). Moulds and yeasts were isolated on potato dextrose agar (M096, HiMedia) and yeast-malt extract (YM) agar (M424, HiMedia), supplemented with 10 IU/ml benzylpenicillin and 12 μg/ml streptomycin sulphate, respectively. The plates were incubated aerobically at 28 °C for 72 h. Total viable count (TVC) was determined on the plate count agar (M091A, HiMedia) plates which were incubated at 30 °C for 48–72 h. Colonies of all microorganisms were selected randomly and purity of the isolates was checked by streaking again on fresh agar plates of the isolation media and sub-culturing in the corresponding broths, followed by microscopic examinations. Isolates were preserved in their respective isolation media supplemented with 15% (v/v) glycerol, at −20 °C. Samples were also tested for the presence of pathogenic bacteria using *Bacillus cereus* agar base (M833, HiMedia,) for *B. cereus*; Violet Red Bile Glucose agar without lactose (M581, HiMedia) for Enterobacteriaceae (Han et al., 2001); Salmonella-Shigella Agar (M108, HiMedia) for the detection of *Salmonella*; and *Shigella*, and *Listeria* identification agar base (M1064, HiMedia) with *Listeria* selective supplement (FD 061, HiMedia) for *Listeria* following the standard method of Metaxopoulos et al. (2001).

2.3. Phenotypic characterisation

Phenotypic characterisation was performed as previously described by Tamang et al. (2005). Cell morphology and the motility of isolates were determined by smearing wet materials under phase contrast illumination. LAB isolates were Gram-stained and tested for catalase production, gas production from glucose, hydrolysis of arginine, and production of dextran from sucrose; and growth at various temperatures (8 °C, 10 °C, 15 °C and 45 °C), pH values (3.9 and 9.6) and concentrations of sodium chloride (6.5%, 10% and 18%), in MRS broth, following the methods of Schillinger

Table 2
Phenotypic characteristics of isolates of LAB isolated the ethnic meat products of the Western Himalayan.

Product	No of isolates	Cell shape	Gas from glucose	NH_3 from arginine	Lactate isomer	Growth at 8 °C	Growth at 45 °C	Sugars fermented			
								Arabinose	Ribose	Xylose	Galactose
Chartayshya (18)	3	Rod	+	+	L	+	−	−	+	+	+
	9	Coccus/Tetrad	−	+	DL	+	+	−	+	+	+
	6	Coccus	−	+	L	−	+	−	+	+	+
Jamma (21)	3	Rod	+	+	L	+	−	−	+	2/1	+
	2	Rod	+	−	DL	−	−	−	1/1	+	+
	5	Coccus/Tetrad	−	+	DL	−	+	−	+	+	+
	8	Coccoid	+	1/7	D	−	−	−	+	+	+
	2	Coccus	−	+	L	−	+	−	+	−	+
	1	Coccus	−	−	L	−	+	−	+	−	+
Arjia (14)	7	Coccus/Tetrad	−	−	DL	−	+	−	+	+	+
	7	Coccus	−	+	L	−	+	−	+	+	+

All isolates were Gram-positive, catalase-negative, non-motile and non-sporeformers. The 53 representative strains were chosen by grouping of the 182 LAB strains which were isolated from MRS agar plates, on the basis of their phenotypic characteristics described in Table. Number of isolates is given in parenthesis; +, all strains positive; −, all strains negative; (../..), number of positive/negative strains.

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and Lücke (1987). The configuration of lactic acid produced from glucose was determined enzymatically using commercial D-lactate and L-lactate dehydrogenase test kits (Cat. No. 1112821, Boehringer-Mannheim GmbH, Germany). Carbohydrate fermentation patterns of LAB isolates were determined using the API 50 CHL test strips (bioMérieux, Lyon, France) and the result was obtained using the APILAB PLUS database identification software (bioMérieux).

2.4. Genotypic characterisation

Genomic DNA was prepared from 53 LAB isolates representing the groups resulting from preliminary phenotypic characterisation (Table 3).

2.4.1. DNA extraction

LAB isolates were cultivated and maintained in MRS broth at 30 °C for 1 day. The genomic DNA used for randomly amplified polymorphic DNA (RAPD)–PCR typing and 16S rRNA and *pheS* genes sequencing were prepared from the isolates according to the method of Watanabe et al. (2008). Briefly, 1-ml aliquots of stationary-phase bacterial culture were pelleted by centrifugation at 20,000×*g* for 3 min, and the cell pellet was suspended in 250 μl extraction buffer (100 mM Tris–HCl, 40 mM EDTA, pH 9.0). Seven hundred miligrams of glass beads (diameter, 0.1 mm) and 500 μl of benzyl chloride were added to the suspension, and the mixture was shaken vigorously for 30 s in a FastPrep FP120 homogenizer (Bio 101, Vista, CA, USA) at a speed setting of 6.5 m/s. Fifty microlitres of 10% sodium dodecyl sulphate was added to the suspension, and the mixture was vortexed vigorously at 50 °C for 20 min in a Shaking Incubator (SI300, AS ONE Corp., Osaka, Japan). Then, 150 μl of 3 M sodium acetate was added, and the mixture was cooled on ice for 3 min. After centrifugation at 20,000×*g* for 8 min, the supernatant was collected and DNA was precipitated with isopropanol. The DNA was diluted to 10 μg/ml in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.4.2. RAPD fingerprint

RAPD-PCR analysis was performed as previously described (Chao et al., 2008a). Primers A (5′-CCGCAGCCAA-3′), B (5′-AACGCGCAAC-3′) and C (5′-GCGGAAATAG-3′) were used in this study. PCR amplification was performed using 25 μl of a mixture containing 10 mM Tris–HCl (pH8.3), 50 mM KCl, 1 mM $MgCl_2$, 200 μM of each dNTP, 1.6 μM of primer, 2 U *Taq* polymerase (Takara Bio, Shiga, Japan) and 10 ng of template DNA. The PCR cycling program consisted of one cycle of 94 °C for 2 min; six cycles of 94 °C for 30 s, 36 °C for 1 min, 72 °C for 90 s; 30 cycles of 94 °C for 20 s, 36 °C for 30 s, 72 °C for 90 s; and a final incubation at 72 °C for 3 min. The PCR products were electrophoresed in 1.5% agarose gel. Gels were stained in ethidium bromide and photographed. Isolates with the same RAPD profile were placed in a group, and one representative strain from each group was chosen for 16S rRNA and *pheS* genes sequence analyses.

2.4.3. 16S rRNA and pheS genes sequencing and phylogenetic analyses

The conditions for PCR amplification of the partial 16S rRNA gene and subsequent DNA sequencing have been previously described (Chao et al., 2008b). Those for partial *pheS* gene were described by Naser et al. (2005). Primers pheS-21F (5′-CAYCCNGCHCGYGAYATGC-3′) and pheS-23R (5′-GGRTGTAC-CATVCCNGCHCC-3′) were used. PCR amplification was performed using 25 μl of a mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 200 μM of each dNTP, 0.4 μM of each primer, 1 U *Taq* polymerase (Takara Bio) and 10 ng template DNA. The amplification programme for *pheS* gene consisted of one cycle of 95 °C for 5 min, 3 cycles of 95 °C for 1 min, 46 °C for 2 min 15 s, 72 °C for 1 min 15 s; 30 cycles of 95 °C for 35 s, 46 °C for 1 min 15 s, 72 °C for 1 min 15 s; and a final incubation at 72 °C for 7 min. The PCR-amplified 16S rRNA and *pheS* genes were purified using AMPure Kit (Beckman Coulter Inc., Brea, CA, USA) and were subsequently sequenced using ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA, USA).

The closest known species of 15 representative LAB strains for each of 15 different RAPD-PCR types were determined by BLAST and sequences of closest species were extracted from DDBJ/GenBank/EMBL databases. Multiple alignments of the sequences were carried out with CLUSTAL_X program ver. 2.0.12 (Thompson et al., 1997), according to the Kimura two-parameter model (Kimura, 1980). Approximately 1450 bp of the 16S rRNA gene sequences (approx. 400 bp for the *pheS* gene) of the strains and related species were used for constructing with neighbour-joining method (Saitou and Nei, 1987). The statistical reliability of trees was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985).

2.5. Antimicrobial activity

LAB isolates were screened for antimicrobial activity by the agar spot method (Schillinger and Lücke, 1989). The indicator strains used for antagonisms were *B. cereus* CCM 2010^T, *Enterobacter agglomerans* BFE 154^T, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147^T, *Listeria innocua* DSM 20649^T, *Listeria monocytogenes* DSM 20600^T and *Pseudomonas aeruginosa* BFE 162^T. Cell-free neutralised supernatants fluids of LAB were screened for antimicrobial activity by the agar spot test (Uhlman et al., 1992), using the bacteriocin screening medium (MRS agar containing only 0.2% glucose) as described by Tichaczek et al. (1992).

Sugars fermented														
Sucrose	Trehalose	Rhamnose	Mannose	Sorbitol	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Raffinose	Melezitose	Mannitol	Identity
+	2/1	–	+	–	+	+	+	+	1/2	+	+	–	–	*L. divergens*
+	+	–	+	–	+	+	+	+	+	3/6	–	–	3/6	*Pediococcus pentosaceous*
+	+	–	+	–	+	+	+	+	+	+	+	–	3/3	*Enterococcus faecium*
+	2/1	–	+	2/1	+	+	+	+	+	+	+	–	–	*L. divergens*
+	+	–	+	–	+	+	+	+	+	+	+	–	–	*L. sanfransisco*
+	+	–	+	–	+	+	+	+	3/2	4/1	3/2	–	1/4	*P. pentosaceous*
+	+	–	+	–	+	+	+	+	+	6/2	+	–	5/3	*L. mesenteroides*
+	+	–	+	–	+	+	+	+	+	+	+	–	+	*E. faecium*
+	+	–	+	–	+	+	+	+	+	+	+	–	–	*E. cecorum*
5/2	+	–	+	–	6/1	+	+	+	6/1	2/5	–	–	–	*P. pentosaceous*
+	+	–	+	–	+	+	+	+	6/1	6/1	4/3	–	4/3	*E. faecium*

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Table 3
Genotypic characterisation of LAB isolates isolated form the Western Himalayan ethnic meat products.

Product	Isolates	Phenotypic identification	RAPD-PCR type				Genotypic identification
			Primer A	Primer B	Primer C	Type	
Chartayshya ($n = 18$)	CD:L2[a]	*Enterococcus faecium*	A-1	B-1	C-1	**1**	*Enterococcus hirae*
	CD:L5	*Enterococcus faecium*	A-1	B-1	C-1	**1**	*Enterococcus hirae*
	CD:L7-1	*Enterococcus faecium*	A-1	B-1	C-1	**1**	*Enterococcus hirae*
	CD:L7-2[a]	*Enterococcus faecium*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L12-1	*Enterococcus faecium*	A-1	B-1	C-1	**1**	*Enterococcus hirae*
	CD:L12-2	*Enterococcus faecium*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L14	*Enterococcus faecium*	A-1	B-1	C-1	**1**	*Enterococcus hirae*
	CD:L22[a]	*Enterococcus faecium*	A-1	B-3	C-1	**3**	*Enterococcus hirae*
	CD:L3[a]	*Lactobacillus divergens*	A-3	B-4	C-3	**4**	*Weissella cibaria*
	CD:L6	*Lactobacillus divergens*	A-3	B-4	C-3	**4**	*Weissella cibaria*
	CD:L21	*Lactobacillus divergens*	A-3	B-4	C-3	**4**	*Weissella cibaria*
	CD:L1	*Pediococcus pentosaceus*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L8	*Pediococcus pentosaceus*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L10	*Pediococcus pentosaceus*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L13	*Pediococcus pentosaceus*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L15	*Pediococcus pentosaceu*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L20	*Pediococcus pentosaceus*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
	CD:L26	*Pediococcus pentosaceus*	A-2	B-2	C-2	**2**	*Pediococcus pentosaceus*
Jamma ($n = 21$)	KJ:L4[a]	*Enterococcus faecium*	A-4	B-5	C-4	**5**	*Enterococcus durans*
	KJ:L5	*Enterococcus faecium*	A-4	B-5	C-4	**5**	*Enterococcus durans*
	KJ:L6[a]	*Lactobacillus divergens*	A-5	B-6	C-5	**6**	*Leuconostoc mesenteroides*
	KJ:L7	*Lactobacillus divergens*	A-5	B-6	C-5	**6**	*Leuconostoc mesenteroides*
	KJ:L8[a]	*Lactobacillus divergens*	A-6	B-7	C-6	**7**	*Pediococcus pentosaceus*
	KJ:L1[a]	*Lactobacillus sanfrancisco*	A-7	B-8	C-7	**8**	*Leuconostoc citreum*
	KJ:L15	*Lactobacillus sanfrancisco*	A-7	B-8	C-7	**8**	*Leuconostoc citreum*
	KJ:L2[a]	*Leuconostoc mesenteroides*	A-8	B-9	C-8	**9**	*Leuconostoc mesenteroides*
	KJ:L14-1[a]	*Leuconostoc mesenteroides*	A-9	B-10	C-9	**10**	*Enterococcus hirae*
	KJ:L14-2	*Leuconostoc mesenteroides*	A-6	B-7	C-6	**7**	*Pediococcus pentosaceus*
	KJ:L18	*Leuconostoc mesenteroides*	A-5	B-6	C-5	**6**	*Leuconostoc mesenteroides*
	KJ:L21	*Leuconostoc mesenteroides*	A-5	B-6	C-5	**6**	*Leuconostoc mesenteroides*
	KJ:L23	*Leuconostoc mesenteroides*	A-5	B-6	C-5	**6**	*Leuconostoc mesenteroides*
	KJ:L25	*Leuconostoc mesenteroides*	A-5	B-6	C-5	**6**	*Leuconostoc mesenteroides*
	KJ:L29	*Leuconostoc mesenteroides*	A-5	B-6	C-5	**6**	*Leuconostoc mesenteroides*
	KJ:L3-1[a]	*Pediococcus pentosaceus*	A-10	B-11	C-10	**11**	*Enterococcus faecium*
	KJ:L3-2[a]	*Pediococcus pentosaceus*	A-11	B-12	C-11	**12**	*Pediococcus pentosaceus*
	KJ:L10	*Pediococcus pentosaceus*	A-11	B-12	C-11	**12**	*Pediococcus pentosaceus*
	KJ:L13	*Pediococcus pentosaceus*	A-11	B-12	C-11	**12**	*Pediococcus pentosaceus*
	KJ:L16	*Pediococcus pentosaceus*	A-11	B-12	C-11	**12**	*Pediococcus pentosaceus*
	KJ:L31	*Pediococcus pentosaceus*	A-11	B-12	C-11	**12**	*Pediococcus pentosaceus*
Arjia ($n = 14$)	KA:L3[a]	*Enterococcus faecium*	A-13	B-14	C-13	**13**	*Enterococcus hirae*
	KA:L5	*Enterococcus faecium*	A-13	B-14	C-13	**13**	*Enterococcus hirae*
	KA:L6	*Enterococcus faecium*	A-13	B-14	C-13	**13**	*Enterococcus hirae*
	KA:L8	*Enterococcus faecium*	A-13	B-14	C-13	**13**	*Enterococcus hirae*
	KA:L13	*Enterococcus faecium*	A-13	B-14	C-13	**13**	*Enterococcus hirae*
	KA:L15	*Enterococcus faecium*	A-13	B-14	C-13	**13**	*Enterococcus hirae*
	KA:L20	*Enterococcus faecium*	A-13	B-14	C-13	**13**	*Enterococcus hirae*
	KA:L1[a]	*Pediococcus pentosaceus*	A-14	B-15	C-14	**14**	*Pediococcus pentosaceus*
	KA:L2	*Pediococcus pentosaceus*	A-14	B-15	C-14	**14**	*Pediococcus pentosaceus*
	KA:L4	*Pediococcus pentosaceus*	A-14	B-15	C-14	**14**	*Pediococcus pentosaceus*
	KA:L11-1[a]	*Pediococcus pentosaceus*	A-15	B-16	C-15	**15**	*Enterococcus faecalis*
	KA:L11-2	*Pediococcus pentosaceus*	A-14	B-15	C-14	**14**	*Pediococcus pentosaceus*
	KA:L16	*Pediococcus pentosaceus*	A-14	B-15	C-14	**14**	*Pediococcus pentosaceus*
	KA:L21	*Pediococcus pentosaceus*	A-14	B-15	C-14	**14**	*Pediococcus pentosaceus*

n, total number of representative strains.
[a] Representative strains of each RAPD-PCR typing group.
The numbers of RAPD types are the most important information in this table. So we use bold values to emphasize them.

2.6. Enzymatic activities by API ZYM

Enzymatic activities of LAB isolates were assayed using API ZYM strips (bioMérieux), based on the method described by Rai et al. (2010a).

2.7. Biogenic amines

The ability of LAB isolates to produce biogenic amines was determined qualitatively in screening medium (Bover-Cid and Holzapfel, 1999) using a mixture of four precursor amino acids (histidine, lysine, ornithine and tyrosine). Change of the bromocresol purple indicator to purple was considered to be indication of significant amino acid decarboxylase activity, corresponding to >350 mg of a particular amino acid/litre (Olasupo et al., 2001).

3. Results and discussion

Microbiological population of 16 samples of ethnic preserved meat products of the Western Himalayas was analyzed (Table 1). The population of LAB was $>10^7$ cfu/g.

The bacterial strains were presumptive lactic acid bacteria due to their Gram-positive-staining, absence of catalase, non-spore-forming and non-motile characteristics. Of 182 LAB isolates 53 representative strains were selected on the basis of cell morphology, carbon-dioxide production from glucose and

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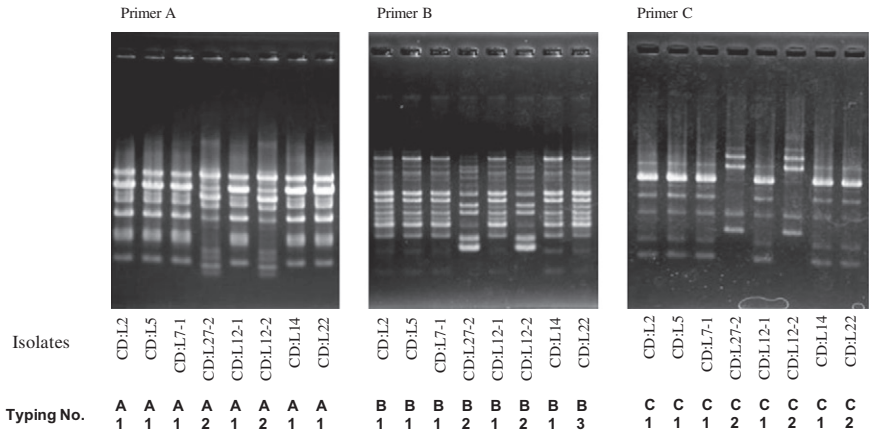

Fig. 1. RAPD-PCR profiles obtained from eight LAB isolates (CD:L2, CD:L5, CD:L7-1, CD:L7-2, CD:L2-1, CD:L2-2, CD:L14 and CD:L22) isolated from *chartayshya*. Three primers with random sequences (A, B and C) were used.

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of the 15 representative strains of each RAPD-PCR typing groups with closely related species. The tree was constructed by the neighbour-joining method on the basis of a comparison of approximately 1450 bp, and *L. delbrueckii* subsp. *delbrueckii* YIT 0080^T was used as an outgroup. Bootstrap values (%) based on 1000 replications are given at nodes. Bootstrap value (%) above 70% is shown. Bar, 3% sequence divergence.

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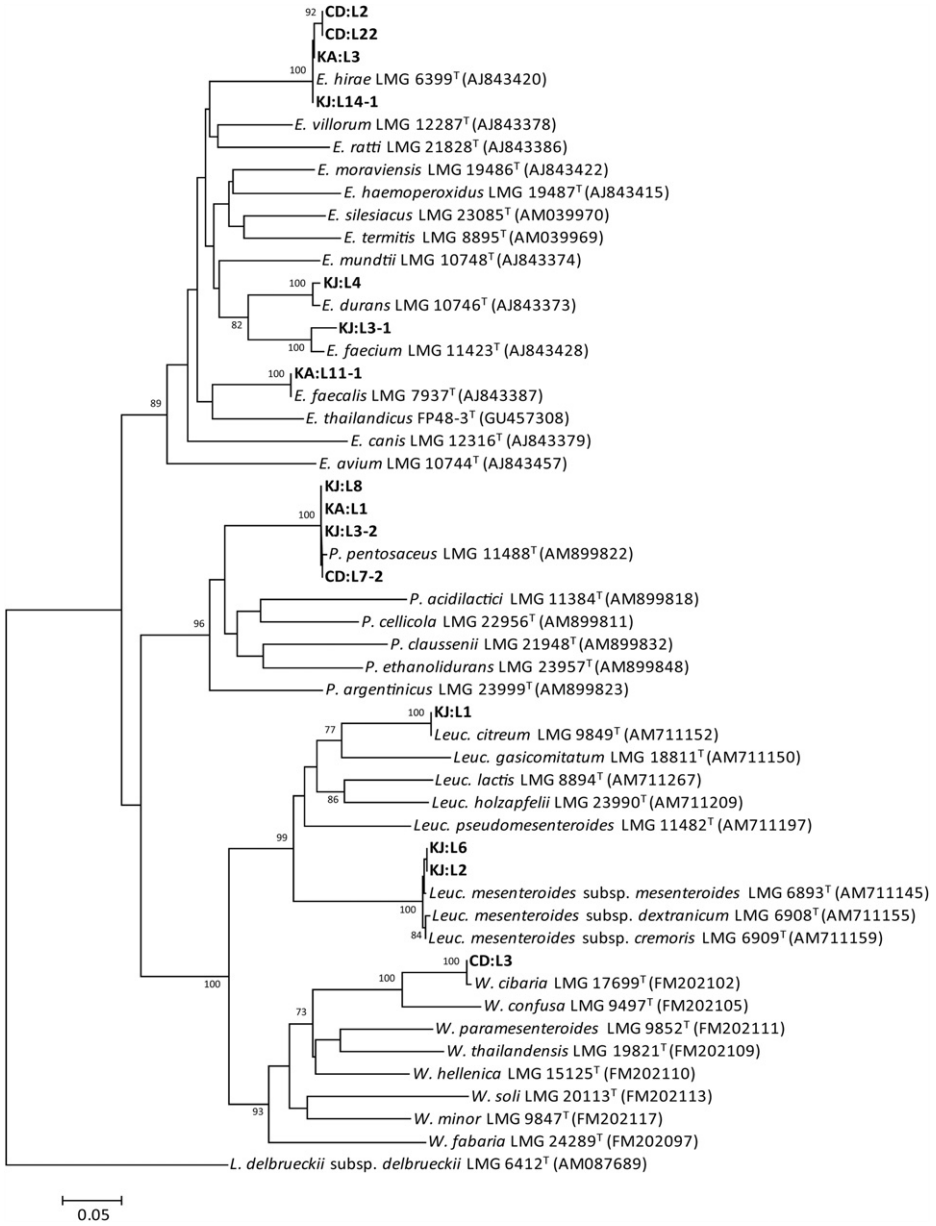

Fig. 3. Phylogenetic tree based on *pheS* gene sequences showing the relationship of 15 representative strains of each RAPD-PCR typing groups with closely related species. The tree was constructed by the neighbour–joining method on the basis of a comparison of approximately 390 bp, and *L. delbrueckii* subsp. *delbrueckii* LMG 6412^T was used as an outgroup. Bootstrap values (%) based on 1000 replications are given at nodes. Bootstrap value above 70% is shown. Bar, 5% sequence divergence.

hydrolysis of arginine, and were phenotypically characterised on the basis of growth temperatures, pH and salt tolerance, lactate configuration and fermentation of sugar (Table 2). Strains of LAB were identified as *Enterococcus cecorum*, *Enterococcus faecium*, *Lactobacillus divergens* (senior synonym of *Carnobacterium divergens*), *Lactobacillus sanfrancisco* (senior synonym of *Lactobacillus sanfranciscensis*), *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*. Phenotypic characterisation including sugar fermentation pattern, arginine hydrolysis and growth behaviour at 50 °C justified identification of all tetrad-forming cocci as *P. pentosaceus*. The sugar fermentation profiles confirmed the identity of *P. pentosaceus* isolates. Coccoid strains were identified as *E. cecorum* and *E. faecium*. Coccoid strains which produced dextran on MRS agar modified by the addition of 5% (w/v) sucrose, and fermented sucrose, galactose, maltose, mannose and xylose, were tentatively identified as *L. mesenteroides*.

A total of 53 LAB isolates that were selected by reference to their distinct phenotypic characteristics (18 from *chartayshya*, 21 from *jamma*, and 14 from *arjia*) were subjected to RAPD-PCR analysis. On the basis of the resulting RAPD profiles the 53 isolates were categorized into 15 groups; four from *chartayshya*, eight from *jamma* and three from *arjia* (Table 3 and Fig. 1).

One representative LAB strain from each group was chosen and used for 16S rRNA gene sequence analysis. The four representative strains from *chartayshya* belonged to the genera *Enterococcus*, *Pediococcus* and *Weissella*. The eight representative strains from *jamma* belonged to the genera *Enterococcus*, *Leuconostoc* and *Pediococcus*. The representative strains from *arjia* belonged to the genera *Enterococcus* and *Pediococcus*. Since all the representative strains had >97% similarities with their closest known neighbours (Fig. 2), no representative strains were clearly discriminated at the species level by the 16S rRNA gene-based method (Stackebrandt

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Table 4
Enzymatic profiles of LAB strains from the Western Himalayan ethnic meat products.

Enzyme	Strain (Activity in nanomoles)					
	CD:L3	CD:L26	KJ:L4	KJ:L18	KA:L4	KA:L6
Alkaline phosphatase	0	0	0	0	0	0
Esterase (C4)	5	0	0	0	0	0
Esterase lipase (C8)	0	5	0	0	0	0
Lipase (C14)	0	5	0	0	0	0
Leucinearylamidase	5	5	5	0	5	5
Valinearylamidase	>40	>40	>40	10	>40	>40
Cystinearylamidase	>40	>40	>40	0	>40	>40
Acid phosphatase	5	5	5	0	5	5
Naphthol-AS-BI-phosphohydrolase	0	0	0	0	0	0
α-galactosidase	0	0	0	0	0	0
β-galactosidase	30	5	10	5	10	10
α-glucosidase	20	20	10	10	10	20
β-glucosidase	0	0	0	30	0	0
N-acetyl-β- glucosaminidase	>40	>40	>40	0	>40	10

Data represents the means of 3 sets of experiment.
Trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase were not hydrolysed by any LAB strain.
CD:L3, *Weissella cibaria*; CD:L26, *Pediococcus pentosaceous* (*chartayshya*); KJ:L4, *Enterococcus durans*; KJ:L18, *Leuconostoc mesenteroides* (*jamma)*; KA:L4, *Pediococcus pentosaceous*; KA:L6, *Enterococcus hirae* (*arjia*).
0, no enzyme activity; 5, 10, 20, 30, >40 indicates nanomoles of hydrolysed substrate after 6 h of incubation at 30 °C.

and Goebel, 1994). No strain isolated from these ethnic meat products was confirmed as belonging to the genus *Lactobacillus* whereas some strains were originally identified as *Lactobacillus* species by phenotypic characterisation. This discordance in results probably cause because phenotypic characterisation is sensitivity to culture conditions and bacterial growth phase. Moreover the rod-shape of both *Lactobacillus* and *Weissella* gave rise to similar phenotypic characterisation of isolates of the two genera (Björkroth et al., 2002).

In recent studies, the sequences of alpha subunits of phenylalanyl-tRNA synthase (*pheS*) gene were used as alternative approach to discriminate closely related species of the genera *Enterococcus*, *Leuconostoc*, *Pediococcus* and *Weissella* (Naser et al., 2005; de Bruyne et al., 2007, 2008, 2010). In this study, the greater discriminative power of *pheS* gene sequencing compared to 16S rRNA gene sequencing was also confirmed for all the representative strains of LAB (Fig. 3). By *pheS* gene analysis, the four strains from *chartayshya* were identified as *Enterococcus hirae*, *P. pentosaceus* and *Weissella cibaria*. The eight strains from *jamma* were identified as *Enterococcus durans*, *E. faecium*, *E. hirae*, *Leuconostoc citreum*, *L. mesenteroides* and *P. pentosaceus*. The three strains from *arjia* were identified as *Enterococcus faecalis*, *E. hirae* and *P. pentosaceus*. Although all strains were clearly differentiated at the species level, the strains in the *L. mesenteroides* could not be identified at subspecies level by *pheS* gene sequencing (Fig. 3). Identification by genotypic method, especially by using housekeeping gene sequencing, was evidently an essential procedure for evaluation the diversity of LAB from ethnic meat products.

Enterobacteriaceae, *Listeria* sp., *Salmonella* sp., and *Shigella* sp. were not detected in samples, which indicates that the ethnic meat products of the Western Himalayas are generally safe to eat. LAB strains isolated from the ethnic meat products showed very weak lipolytic activity in the API ZYM test (Table 4). Weak lipolytic activity of LAB strains has been observed during fermentation of *Nham*, a fermented beef or pork sausage of Thailand (Montel et al., 1998; Visessanguan et al., 2006). The absence of proteinases (trypsin and chymotrypsin) and presence of strong peptidase (leucine-, valine-, and cystine-arylamidase) activities of the LAB strains possibly imparting flavours to the sausages (Henriksen and Stahnke, 1997). LAB strains showed antimicrobial activity against *E. agglomerans* BFE 154^T and *K. pneumoniae* subsp. *pneumoniae* BFE 147^T (data not shown). However, the cell free supernatant fluids of the LAB strains did not exhibit antimicrobial activity. None of the strains produced biogenic amines. The inability of LAB strains to produce biogenic amines may be a good indication of their potential for possible development as starter cultures.

The diversity of LAB in the ethnic preserved meat products of the Western Himalayas indicates that they provide nutrient-rich environments for LAB. Although sugar is not added during preparation of these ethnic meat products, the carbohydrate composition of *chartayshya*, *jamma* and *arjia* was 38.6 ± 4.3, 82.8 ± 2.0 and 82.8 ± 2.0, respectively (Rai et al., 2010b). Despite of having high population of LAB, the pH value of all samples had pH 5.5–6.3 which is not so acidic. This indicates that acidification by LAB which catabolise the carbohydrate was weakened by *Bacillus* and Micrococcaceae through alkaline-fermentation (Baruzzi et al., 2006). Further investigations are needed to study the microbial dynamics and mechanisms of fermentation which would contribute to a better understanding of the fermentation process of the Western Himalayan ethnic preserved meat products.

4. Conclusion

Diversity of microorganisms ranging from species of lactic acid bacteria, bacilli, micrococci and yeasts was observed in the Western Himalayan ethnic preserved meat products. No pathogenic bacteria were recovered from the ethnic meat products. Dominant microorganisms exhibited some functional properties such as possessing antimicrobial activities, diverse range of enzymatic activities, and non-production of biogenic amines. Like commercial sausages, the ethnic sausages and meat products are also important dietary components in the Himalayas.

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