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Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas

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Abstract

Sukako maacha, gnuchi, sidra and sukuti are traditional smoked and sun-dried fish products of the Eastern Himalayan regions of Nepal and India. A total of 40 samples of sukako maacha (14), gnuchi (6), sidra (10) and sukuti (10) were collected and were analysed for microbial load. Population of lactic acid bacteria (LAB) as well as aerobic mesophilic counts ranged from 4.7–8.3 to 5.1–8.5 log cfu g^{-1}, respectively. A total of 189 strains of LAB were isolated from sukako maacha, gnuchi, sidra and sukuti samples, out of which 171 strains were cocci and 15 strains, were heterofermentative lactobacilli. LAB were identified on the basis of phenotypic characters including API system as *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus plantarum*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Enterococcus faecalis*, *Pediococcus pentosaceus* and *Weissella confusa*. LAB strains produced a wide spectrum of enzymes. Some strains of LAB showed antagonistic properties against pathogenic strains. None of the strains produced biogenic amines in the method applied. This paper is the first report on the microbial composition, mostly lactic acid bacteria, of traditionally processed fish products of Eastern Himalayas.

Keywords: Traditional fish products; Sukako maacha; Gnuchi; Sidra; Sukuti; Lactic acid bacteria

1. Introduction

Fresh fish is an extremely perishable proteinaceous food that spoils due to the metabolism of spoilage microbiota which ends up in foods because of cross contamination (Barile et al., 1985; Gram and Huss, 1996). Dehydration, smoking, salting and fermentation are the best methods for preservation of available perishable fish (Beddows, 1985). People of the Eastern Himalayan regions of Nepal, India and Bhutan consume varieties of traditionally processed smoked, sun-dried, fermented and salted fish products (Tamang, 2001). Sukako maacha and gnuchi are typical smoked and dried fish products prepared and consumed in eastern Nepal, the Darjeeling hills and Sikkim in India by the Nepalis and the Lepcha, respectively. Some villagers sell them in the markets. As the products are manufactured by the rural people during the appropriate season, they are regarded as a special dish for them (Thapa, 2002).

Two types of fishes are preferred for the preparation of sukako maacha by the people residing near streams or river. These hill river fish are mostly 'dothay asala' (*Schizothorax richardsoni*) and 'chuchay asala' (*Schizothorax progastus*) (Tamang, 1992). The fish are collected in a bamboo basket from the river, are degutted, washed, and mixed with salt and turmeric powder. Degutted fish are hooked in a bamboo-made string and are hung above the earthen-oven in a kitchen for 7–10 days. Sukako maacha is kept inside the bamboo-made basket, locally called 'perungo', and can be stored for 3–4 months at room temperature.

Gnuchi is a typical smoked and dried fish product common to the Lepcha. Fish are caught early morning and are collected in a bamboo basket tied around the waist of the fisherman while fishing. Fish captured include *S. richardsonii*, *Labeo dero*, *Acrossocheilus* spp., *Channa* sp., etc. Fish are degutted, mixed with salt and turmeric powder and hung one after the other in a bamboo stripe above the earthen-oven

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and smoked for 7–10 days. Gnuchi can be kept at room temperature for 2–3 months.

Sidra is a sun-dried fish product commonly consumed by the Nepalis living in the Eastern Himalayan regions of eastern Nepal, the Darjeeling hills and Sikkim in India and Bhutan. During its preparation, the whole fish (*Puntius sarana*) is collected, washed and dried in the sun for 4–7 days. Sidra can be stored at room temperature for 3–4 months for consumption. It is usually consumed as a pickle. Sukuti is also a very popular sun-dried fish product among the Nepalis. During preparation of sukuti, fish (*Harpodon nehereus*) is collected, washed, rubbed with salt and dried in the sun for 4–7 days, and can be stored at room temperature for 3–4 months. Traditionally processed fish products are cooked or boiled before consumption which are usually eaten as pickle, soup and curry.

There is no information on the microbiology of these traditionally processed fish products of the Eastern Himalayas. The aim of this study was to characterize and identify the predominant lactic acid bacteria present in sukako maacha, gnuchi, sidra and sukuti. Some technological properties such as enzymatic activities, antimicrobial properties and ability to produce biogenic amines of isolates were also examined. To the best of our knowledge, this is the first report on the microbial composition of traditionally processed fish products of the Eastern Himalayas.

2. Materials and methods

2.1. Samples

Fourteen samples of sukako maacha, six samples of gnuchi, ten samples each of sidra and sukuti were collected from eastern Nepal in Maglung, Therathum and Aitabare; Gidhang village in Kalimpong of the Darjeeling hills, and the local market of Gangtok in Sikkim in India. Samples were collected aseptically in sterile poly-bags kept in an ice-box, and transported to the laboratory for analyses.

2.2. Microbiological analysis

2.2.1. Isolations

Ten g of sample were homogenised with 90 ml of 0.85% (w/v) sterile physiological saline in a Stomacher lab-blender (400, Seward, London, UK) for 1 min and serially diluted (10^{-1} to 10^{-8}) in the same diluent. One milliliter of these dilutions was pour-plated in the respective media for LAB, aerobic mesophilic bacteria, yeasts and moulds. LAB were isolated on MRS agar (M641, HiMedia, Mumbai, India) plates supplemented with 1% $CaCO_3$, after incubation under anaerobic conditions in an Anaerobic Gas-Pack system (LE002, HiMedia, Mumbai, India) at 30 °C for 48–72 h. Representative strains of LAB were obtained from MRS plates of the highest sample dilutions. Aerobic mesophilic counts were determined using plate count agar (M091A, HiMedia, Mumbai, India) incubated at 30 °C for 48–72 h. Aerobic spore-forming bacteria were isolated on nutrient agar, after inactivation of vegetable cells by heating at 100 °C for 2 min (Tamang and Nikkuni, 1996), and were incubated at 37 °C for 24 h. Moulds and yeasts were isolated on potato dextrose agar (M096, HiMedia, Mumbai, India) and yeast extract–malt extract agar (M424, HiMedia, Mumbai, India), supplemented with 10 IU ml^{-1} benzylpenicillin and 12 μg ml^{-1} streptomycin sulphate, respectively and incubated aerobically at 28 °C for 72 h. Colonies were either selected randomly or all sampled if the plate contained less than 10 colonies (Leisner et al., 1997). Purity of the isolates was checked by streaking again to fresh agar plates of the isolation media, followed by microscopic examinations. Microbiological data were transformed into logarithms of the numbers of colony forming unit (cfu) g^{-1}. Identified strains of LAB were preserved in MRS broth with 15% (v/v) glycerol at −20 °C.

Samples were examined for *Bacillus cereus*, *Staphylococcus aureus* and enterobacteriaceae following the method of Han et al. (2001). For *B. cereus* selective enumeration was carried out on spread plates of *B. cereus* agar (M833, HiMedia, Mumbai, India) added polymyxin B (FD003, HiMedia, Mumbai, India) and egg yolk emulsion (FD045, HiMedia, Mumbai, India), incubated at 30 °C for 24–48 h. Characteristic peacock blue colonies surrounded by a zone of precipitate of the same colour were regarded as presumptive *B. cereus*. For *S. aureus* selective enumeration was carried out on spread plates of Baird Parker agar (M043, HiMedia, Mumbai, India) added egg yolk tellurite emulsion (FD046, HiMedia, Mumbai, India), incubated at 30 °C for 24–48 h. The black colonies were recorded as presumptive *S. aureus*. For enterobacteriaceae diluted samples in tryptone soya broth (M011, HiMedia, Mumbai, India) were allowed to resuscitate on tryptone soya agar (290, HiMedia, Mumbai, India) plates for 1–2 h at 27 °C, followed by a thick overlay of selective violet red bile glucose (without lactose) agar medium, and incubated at 30 °C for 20 h. Typical colonies were recorded as presumptive enterobacteriaceae.

2.2.2. Characterisation of isolates of lactic acid bacteria

Cell morphology and motility of all isolates of LAB were observed using a phase contrast microscope (CH3-BH-PC, Olympus, Japan) following the method of Harrigan (1998). Isolates were Gram-stained and tested for catalase production. Preliminary identification and grouping was on the basis of cell morphology and phenotypic properties using CO_2 production from glucose, hydrolysis of arginine, growth at different temperatures (10, 15 and 45 °C), and at different pH (3.9 and 9.6) as well as the ability to grow in different concentrations of NaCl {6.5% (w/v), 10% (w/v) and 18% (w/v)}in MRS broth as described by 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 DAP in the cell walls of LAB was determined using thin-chromatography on cellulose plates (Tamang et al., 2000). Carbohydrate fermentation patterns of LAB were determined using API 50 CHL and API 20 STREP test strips (bioMérieux, France). The APILAB PLUS database identification software (bioMérieux, France) was used to interpret the results.

2.3. Technological properties

2.3.1. Proteolytic activity

Surface-dried plates of milk agar (Gordon et al., 1973) were streaked with 24-h-old cultures, after incubation at 30 °C for 4 days, and examined for any clearing of casein around and underneath the growth for assessment of proteolytic activity.

2.3.2. Amylolytic activity

Surface-dried plates of starch agar (Gordon et al., 1973) were streaked with 24-h-old cultures, after incubation at 30 °C for 4 days. The plates were flooded with iodine solution for 15–30 min and examined the clear zone underneath (after the growth was scrapped off) for amylolytic activity.

2.3.3. Lipolytic activity

Surface-dried plates of tributyrin agar (Leuschner et al., 1997) were streaked with 24-h-old culture, after incubation at 30 °C for 4 days. Lipolytic activity was detected by a clear zone surrounding the culture in the turbid tributyrin agar.

2.3.4. Protease activity assay

Protease activity was measured by a modification of the method of Maeda et al. (1993). Cultures were grown in phytone broth (Nagai et al., 1994) on a rotary shaking incubator at 30 °C at 180 rpm for 72 h. Cultures were immediately centrifuged at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The enzyme solution and the substrate solution containing 1% Azocasein (Sigma Chemical Co., USA) was dissolved in 100 mM phosphate buffer, (pH 6.8) were pre-incubated separately at 37 °C for 5 min in a water-bath incubator (RSB-12, Remi, India). The enzyme reaction was started by adding 2 ml of 1% Azocasein to 1 ml of enzyme solution and incubated at 37 °C for 20 min. The reaction was quenched by the addition of 2.5 ml of 10% (*w*/*v*) trichloroacetic acid. After centrifugation at 15,000 rpm for 10 min, 2 ml of supernatant was neutralized with equal amount of 1 N NaOH and the absorbance was measured at 450 nm in UV–VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). One unit of protease activity was defined as the quantity required to increase the absorbance by 0.1 under the above conditions.

2.3.5. α-Amylase activity assay

The blue value method of Fuwa (1954) as modified by Kawaguchi et al. (1992) was followed for determination of α-amylase activity. Cultures were grown on broth medium (1.0% soluble starch, 1.0% beef extract, 1.0% peptone, and 0.3% NaCl, pH 7.0) on a rotary shaking incubator at 30 °C at 180 rpm for 48 h. The cultures were immediately centrifuged at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The enzyme solution and 1.5% soluble starch dissolved in 100 mM Tris–HCl buffer (pH 7.0) were pre-incubated separately at 37 °C for 5 min in water-bath shaker (RSB-12, Remi, India). Then, the reaction mixture was started by adding 1 ml of 1.5% soluble starch (RM089, HiMedia, Mumbai, India) to 0.5 ml enzyme solution and incubated at 37 °C for 10 min. Reaction was stopped by addition of 2.5 ml of stop solution (0.5 N acetic acid–0.5 N HCl, 5:1). The 100 ml of the reaction mixture was added to potassium iodide solution, left at room temperature for 20 min and the absorbance at 660 nm of the resulting solution was measured in UV–VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). One unit of α-amylase activity was defined as the amount of α-amylase which produced 10% reduction in the intensity of blue colour at the above condition.

2.3.6. Enzymatic profile by API-zym system

The enzymatic profile of lactic acid bacteria were assayed following the method of Arora et al. (1990) using API-zym (bioMérieux, France) galleries by testing for the activity of the following 19 enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine-, valine- and cystine-arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and fucosidase.

2.3.7. Antagonistic activity

Isolates of LAB were screened for antagonistic activity by the agar spot method of Schillinger and Lücke (1989). The indicator strains used for antagonisms included: *Listeria innocua* DSM 20649, *Listeria monocytogenes* DSM 20600, *B. cereus* CCM 2010, *S. aureus* S1, *Enterococcus faecium* DSM 20477, *Streptococcus mutans* DSM 6178, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Enterobacter cloacae* BFE 282, *Enterobacter agglomerans* BFE 154 and *Pseudomonas aeruginosa* BFE 162.

2.3.8. Biogenic amine formation

The ability of LAB isolates to produce biogenic amines was determined qualitatively on an improved screening medium as described by Bover-Cid and Holzapfel (1999) using a 'cocktail' of four precursor amino acids (histidine, lysine, ornithine and tyrosine). Change of the bromocresol purple indicator to purple was considered as index of significant amino acid decarboxylase activity, corresponding to >350 mg of a particular amino acid L^{-1} (Olasupo et al., 2001).

3. Results

3.1. Microorganisms

A total of 40 samples of sukako maacha (14), gnuchi (6), sidra (10) and sukuti (10) were analysed for microbial counts (Table 1). In all traditionally processed fish products, the population of LAB as well as aerobic mesophilic counts ranged from 10^4 to 10^8 cfu g^{-1}. Bacterial endospores were detected at the range of level of <10–10^4 cfu g^{-1}. The load of *B. cereus*, *S. aureus* and enterobacteriaceae was less than 10^2 and 10^3 cfu g^{-1}, respectively. Yeasts were detected only in samples of sukako maacha. Moulds were not recovered in any sample analysed.

Table 1
Microbial characteristics of traditional fish products of the Eastern Himalayas

	Log cfu g^{-1} sample			
	Sukako maacha ($n=14$)	Gnuchi ($n=6$)	Sidra ($n=10$)	Sukuti ($n=10$)
Lactic acid bacteria	7.3 (5.4–8.3)	6.6 (5.8–6.9)	5.3 (4.8–6.5)	5.4 (4.7–6.2)
Bacterial endospores	2.7 (<DL–4.6)	3.2 (3.1–3.5)	3.1 (2.8–3.3)	<DL
Bacillus cereus	0.7 (<DL–2.2)	1.2 (<DL–1.3)	2.0 (1.2–2.4)	1.5 (1.2–1.8)
Staphylococcus aureus	2.3 (<DL–2.5)	3.1 (<DL–3.3)	3.0 (1.6–3.2)	3.1 (2.3–3.5)
Enterobacteriaceae	2.3 (<DL–2.5)	3.3 (<DL–3.6)	2.7 (1.9–3.0)	2.3 (1.8–2.7)
Yeast	3.2 (2.5–4.0)	<DL	<DL	<DL
Aerobic mesophilic count	7.6 (6.1–8.5)	6.8 (6.3–7.0)	5.8 (5.2–6.8)	5.7 (5.1–6.8)

Data represent the means of number of samples (n). Ranges are given in parentheses.
DL, detection limit is 10 cfu g^{-1}. Moulds were not detected.

All 189 strains isolated from sukako maacha (76), gnuchi (41), sidra (39) and sukuti (33) were considered to be lactic acid bacteria based on their positive Gram reactions, absence of motility, absence of spore formation and absence of catalase activity, out of which 171 strains were cocci and only 15 strains were heterofermentative lactobacilli (Table 2). A total of 14 representative strains were selected randomly from each group on the basis of cell morphology, gas production from glucose and arginine hydrolysis (Table 2) for further phenotypic characterisation including determination of the sugar fermentation pattern, lactate configuration and DAP (Table 3) following the taxonomical keys of Wood and Holzapfel (1995). Based on phenotypic characterisation and interpretation of APILAB PLUS database, representative strain SM:T1 (isolated from sukako maacha) was identified as *L. lactis* subsp. *cremoris*, strains SM:M1 and SM:M2 (sukako maacha) and strain SG1:B1 (sukuti) showing ovoid cells were identified as *Leuconostoc mesenteroides*. Strain SM:A1 (sukako maacha) and strain GG6 (gnuchi) were tentatively identified as *E. faecium*, whereas strain CG1:B2 (sidra) was *Enterococcus faecalis*. Cocci strains CG1:B1 (sidra), and SG1:B3 and SG2:B1 (sukuti) were identified as *Lactococcus plantarum*, whereas SG1:B2 (sukuti) was *L. lactis* subsp. *lactis*. Strain SM:A2, isolated from sukako maacha, and strain GG2, isolated from gnuchi, showing cocci-tetrads were identified as *Pediococcus pentosaceus*. Heterofermentative rod CG1:B3, isolated from sidra, was identified as *Weissella confusa*.

Table 2
Grouping of representative strains of LAB isolated from fish products of the Eastern Himalayas

Product[a]	Cell shape	CO_2 from glucose	Arginine hydrolysis	Grouped strains	Representative strains[b]
Sukako maacha (76)	Cocci	–	–	22	SM:T1
	Cocci/ovoid	+	–	30	SM:M1, SM:M2
	Cocci	–	+	10	SM:A1
	Cocci/tetrads	–	+	14	SM:A2
Gnuchi (41)	Cocci/tetrads	–	+	21	GG2
	Cocci	–	+	20	GG6
Sidra (39)	Cocci	–	–	13	CG1:B1
	Cocci	–	–	11	CG1:B2
	Rods	+	+	15	CG1:B3
Sukuti (33)	Cocci/ovoid	+	–	14	SG1:B1
	Cocci	–	–	19	SG1:B2, SG1:B3, SG2:B1

All strains of LAB were Gram-positive, catalase-negative and non-motile.
[a] Total number of isolates in each product are given in brackets.
[b] Randomly selected from each grouped strains.

3.2. Technological properties

Isolates of LAB were tested for protease, α-amylase and lipolytic activities, respectively (Table 4). All strains of LAB showed proteolytic activities (showing >2 mm hydrolysis zone in milk agar plate), though the estimated protease activity of these strains was 0.5–1.3 U ml^{-1}. The α-amylase activity of all 14 strains of LAB was found in between 1.5 and 5.8 U ml^{-1} (Table 4). Only five strains of LAB showed lipolytic activities (Table 4).

Enzymatic profiles of LAB strains were assayed using the API zym (bioMérieux, France) galleries. These strains showed relatively weak esterase and no lipase (C14) activities. *Lactoctoccus lactis* subsp. *cremoris* SM:T1, *E. faecium* SM:A1 and *Lactoctoccus lactis* subsp. *lactis* SG1:B2 showed strong phosphatase activities, while other strains showed moderate activities (result not shown). However, they showed no detectable proteinase activity with the applied method (result not shown).

Antagonistic properties of LAB isolates were tested against the indicator strains (*L. innocua* DSM 20649, *L. monocytogenes* DSM 20600, *B. cereus* CCM 2010, *S. aureus* S1, *E. faecium* DSM 20477, *S. mutans* DSM 6178, *K. pneumoniae* subsp. *pneumoniae* BFE 147, *E. cloacae* BFE 282, *E. agglomerans* BFE 154 and *P. aeruginosa* BFE 162). Some strains of LAB showed inhibition zones against few indicator strains. *Lactococcus lactis* subsp. *cremoris* SM:T1, isolated from sukako maacha showed strong inhibition zone against *L. innocua* and *S. aureus* (result not shown). Isolates of LAB were screened for their ability to produce biogenic amines. None of them produced biogenic amines in the method applied.

4. Discussion

Sukako maacha and gnuchi are typical smoked and dried fish products traditionally prepared and consumed in Nepal, and the Darjeeling hills and Sikkim in India, as side-dish by the

Table 3
Characteristics of LAB strains isolated from fish products of the Eastern Himalayas

Strain code	Morphology	CO_2 from glucose	NH_3 from arginine	Growth at			DAP	Lactate isomer	Sugars fermented																Identification
				10 °C	15 °C	45 °C			Arabinose	Ribose	Xylose	Galactose	Sucrose	Trehalose	Rhamnose	Mannose	Sorbitol	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Raffinose	Melezitose	
SM:T1	Coccus	–	–	+	+	–	–	L	–	–	+	+	+	+	–	+	+	–	+	–	+	+	–	–	–	*Lactococcus lactis* subsp. cremoris
SM:M1	Coccus	+	–	+	+	–	–	D	+	–	+	–	+	+	–	–	+	–	–	+	+	+	–	–	–	*Leuconostoc mesenteroides*
SM:M2	Coccus	+	–	+	+	–	–	D	–	–	+	+	+	+	–	+	+	+	+	–	+	–	–	–	–	*Leuconostoc mesenteroides*
SM:A1	Coccus	–	+	+	+	+	–	L	+	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+	–	*Enterococcus faecium*
SM:A2	Coccus, tetrad	–	+	+	+	+	–	DL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	*Pediococcus pentosaceus*
GG2	Coccus, tetrad	–	+	+	+	+	–	DL	+	+	–	+	+	+	+	–	+	+	+	+	+	+	–	–	–	*Pediococcus pentosaceus*
GG6	Coccus	–	+	+	+	+	–	L	–	+	–	+	+	–	–	–	+	+	+	+	+	+	+	–	–	*Enterococcus faecium*
CG1:B1	Coccus	–	–	+	+	+	–	L	–	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	–	*Lactococcus plantarum*
CG1:B2	Coccus	–	–	+	+	+	–	L	–	–	–	+	+	–	–	+	+	+	+	+	+	–	–	+	–	*Enterococcus faecalis*
CG1:B3	Rod	+	+	+	+	–	–	DL	+	+	+	+	–	+	+	–	–	+	+	+	+	–	–	–	–	*Weissella confusa*
SG1:B1	Coccus	+	–	–	–	–	–	D	–	+	–	–	+	–	+	–	–	–	–	+	+	–	–	–	–	*Leuconostoc mesenteroides*
SG1:B2	Coccus	–	–	+	+	–	–	L	+	–	+	–	+	–	–	–	–	–	+	+	+	–	–	+	–	*Lactococcus lactis* subsp. *lactis*
SG1:B3	Coccus	–	–	+	+	–	–	L	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	*Lactococcus plantarum*
SG2:B1	Coccus	–	–	+	+	–	–	L	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	*Lactococcus plantarum*

All grew in pH 3.9, NaCl 6.5%. No growth in pH 9.6, NaCl 10% and 18%.

Table 4
Enzymatic activity of LAB strains, isolated from fish products of the Eastern Himalayas

Product	Strain	Protease[a] (U ml^{-1})	α-amylase[a] (U ml^{-1})	Lipolytic activity
Sukako maacha	SM:T1	0.8	3.5	–
	SM:M1	0.5	4.5	–
	SM:M2	0.6	3.6	+
	SM:A1	1.2	3.1	+
	SM:A2	1.3	3.2	–
Gnuchi	GG2	1.3	1.5	–
	GG6	0.8	4.8	–
Sidra	CG1:B1	1.1	2.5	–
	CG1:B2	1.0	5.8	–
	CG1:B3	0.7	3.2	+
Sukuti	SG1:B1	0.6	3.1	–
	SG1:B2	0.9	3.2	–
	SG1:B3	0.7	1.5	+
	SG2:B1	0.8	1.8	+

Data represent the means of three sets.
[a] Strains showing positive hydrolysis test (>2.0 mm) were assayed.

Nepalis and the Lepcha, respectively. Sidra and sukuti are sun-dried fish products common in the diet of the ethnic people of the region as a side dish or pickle. On the basis of phenotypic characteristics lactic acid bacteria isolated from sukako maacha, gnuchi, sidra and sukuti were identified as *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, *L. plantarum*, *L. mesenteroides*, *E. faecium*, *E. faecalis*, *P. pentosaceus* and *W. confusa.* However this identification is based on phenotypic characters and the API system, authentic identity of species, should be confirmed by molecular identification. The microbial load of traditionally processed fish products reveals that lactic cocci were predominant LAB. This may be due to gradations of concentration of salts used during processing, which control the bacterial flora (Tanasupawat et al., 1993). None of the LAB isolates obtained from the samples were halotolerant (i.e., 18% salt tolerance). The identity of LAB species seems to correspond with that of LAB typically reported from other Asian fish products (Mabesa et al., 1983; Tanasupawat et al., 1992; Phithakpol, 1993).

Isolates of LAB showed proteolytic activity with low protease activity. This indicates that lactic acid bacteria have very low proteolytic activities in the fish products. However, some isolates of LAB showed amylolytic activity which is essential in liquefaction during processing of fish products (Reddi et al., 1972). Proteolysis and liquefaction that occur during fish production has been reported to be largely the result of autolytic breakdown of the fish tissues, which is more rapid when whole fish are used since the head and viscera contain higher concentrations of proteolytic enzymes than other tissues (Backhoff, 1976). The use of the API-zym method is of relevance for selection of strains as potential starter cultures on the basis of superior enzyme profiles, especially peptidases and esterase, for accelerated maturation and flavour development of fish products. The absence of proteinases (trypsin and chymotrypsin) and presence of peptidase (leucine-, valine- and cystine-arylamidase) and esterase-lipase (C4 and C8) activities produced by the predominant LAB isolated from

fish products are possible traits of desirable flavour in the products (Thapa et al., 2004).

Species of *Lactococcus* and *Enterococcus* isolated from sukako maacha and sidra showed antagonistic activities against *L. innocua* and *S. aureus*, and also against *P. aeruginosa*; which can reduce the number of other undesired microorganisms in the fish products as well as help in the preservation of fish (Einarsson and Lauzon, 1995).

Lactic acid bacteria frequently produce biogenic amines mostly histamine and tyramine in processed fish, cheese, fermented vegetables and beverages (Stratton et al., 1991; Leisner et al., 1994). However, the inability of LAB strains isolated from traditionally processed fish products of the Eastern Himalayas to produce biogenic amines is a good indication of their acceptability in the possible development of starter cultures. The production of biogenic amines by LAB to be selected as starter cultures is not a desirable property (Buchenhüskes, 1993). Before confirming the non-production of biogenic amine in the fish products, qualitative and quantitative analysis of biogenic amine is necessary.

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References

Arora, G., Lee, B.H., Lamoureux, M., 1990. Characteristics of enzyme profiles of *Lactobacillus casei* species by a rapid API–ZYM system. Journal of Dairy Science 73, 264–273.

Backhoff, P.H., 1976. Some chemical changes in fish silage. Journal of Food Technology 11, 353–363.

Barile, L.E., Milla, A.D., Reilly, A., Villadsen, A., 1985. Spoilage pathogens of mackerel (*Rastrelliger faughni*): 1. Delays in icing. ASEAN Food Journal 1, 70–77.

Beddows, C.G., 1985. Fermented fish and fish products. In: Wood, B.J.B. (Ed.), Microbiology of Fermented Foods, vol. 2. Elsevier Applied Science Publishers, London, pp. 1–39.

Bover-Cid, S., Holzapfel, W.H., 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. International Journal of Food Microbiology 53, 33–41.

Buchenhüskes, H.J., 1993. Selection criteria for lactic acid bacteria to be used as starter cultures in various food commodities. FEMS Microbiology Reviews 12, 253–272.

Dykes, G.A., Britz, T.J., von Holy, A., 1994. Numerical taxonomy and identification of lactic acid bacteria from spoiled, vacuum packaged Vienna sausages. Journal of Applied Bacteriology 76, 246–252.

Einarsson, H., Lauzon, H.L., 1995. Biopreservation of brined shrimp (*Pandalus borealis*) by bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 61, 669–676.

Fuwa, H., 1954. A new method for microdetermination of amylase activity by the use of amylose as the substrate. Journal of Biochemistry 41 (5), 583–603.

Gram, L., Huss, H., 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology 33, 589–595.

Gordon, R.E., Haynes, W.C., Pang, C.H.-N., 1973. The Genus *Bacillus,* Handbook no. 427. United States Department of Agriculture, Washington, DC.

Han, B.Z., Beumer, R.R., Rombouts, F.M., Nout, M.J.R., 2001. Microbiological safety and quality of commercial sufu—a Chinese fermented soybean food. Food Control 12, 541–547.

Harrigan, W.F., 1998. Laboratory Methods in Food Microbiology, 3rd edition. Academic Press, London.

Kawaguchi, T., Nagae, H., Murao, S., Arai, M., 1992. Purification and some properties of a haim-sensitive α-amylase from newly isolated *Bacillus* sp. No 195. Bioscience, Biotechnology, and Biochemistry 56, 1792–1796.

Leisner, J.J., Millan, J.C., Huss, H.H., Larsen, L.M., 1994. Production of histamine and tyramine by lactic acid bacteria isolated from vacuum-packed sugar-salted fish. Journal of Applied Bacteriology 76, 417–423.

Leisner, J.J., Rusul, G., Wee, B.W., Boo, H.C., Mohammad, K., 1997. Microbiology of Chili Bo, a popular Malaysian food ingredient. Journal of Food Protection 60, 1235–1240.

Leuschner, R.G., Kenneally, P.M., Arendt, E.K., 1997. Method for the rapid quantitative detection of lipolytic activity among food fermenting microorganisms. International Journal of Food Microbiology 37, 237–240.

Mabesa, R.C., Castillo, M.M., Revilla, S.V., Bandian, V.T., 1983. Safety evaluation of fermented fish and shellfish products: I. Microbiological contaminants. Philippines Journal of Sciences 112 (1-2), 91–102.

Maeda, T., Takagi, M., Imanaka, T., 1993. Purification and characterization of a new metal protease which hydrolyzes the cyclic decapeptide, gramicidin S. Journal of Fermentation and Bioengineering 75, 173–177.

Nagai, T., Nishimura, K., Suzuki, H., Banba, Y., Sasaki, H., Kiuchi, K., 1994. Isolation and characterization of *Bacillus subtilis* strain producing natto with strong Umami-taste and high viscosity. Nippon Shokuhin Kogyo Gakkaishi 41, 123–128.

Olasupo, N.A., Schillinger, U., Holzapfel, W.H., 2001. Studies on some technological properties of predominant lactic acid bacteria isolated from Nigerian fermented foods. Food Biotechnology 15 (3), 157–167.

Phithakpol, B., 1993. Fish fermentation technology in Thailand. In: Lee, C.H., Steinkraus, K.H., Alan Reilly, P.J. (Eds.), Fish Fermentation Technology. United Nations University Press, Tokyo, pp. 155–166.

Reddi, P.K., Constantinides, S.M., Dymsza, H.A., 1972. Catheptic activity of fish muscle. Journal of Food Science 37, 643–647.

Schillinger, U., Lücke, F.K., 1987. Identification of lactobacilli from meat and meat products. Food Microbiology 4, 199–208.

Schillinger, U., Lücke, F.K., 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Applied and Environmental Microbiology 55 (8), 1901–1906.

Stratton, J.E., Hutkins, R.W., Taylor, S.L., 1991. Biogenic amines in cheese and other fermented foods—a review. Journal of Food Protection 54, 460–470.

Tamang, J.P., 2001. Food culture in the Eastern Himalayas. Journal of Himalayan Research and Cultural Foundation 5 (3-4), 107–118.

Tamang, J.P., Nikkuni, S., 1996. Selection of starter culture for production of kinema, a fermented soybean food of the Himalaya. World Journal of Microbiology and Biotechnology 12, 629–635.

Tamang, J.P., Dewan, S., Thapa, S., Olasupo, N.A., Schillinger, U., Holzapfel, W.H., 2000. Identification and enzymatic profiles of predominant lactic acid bacteria isolated from soft-variety *chhurpi*, a traditional cheese typical of the Sikkim Himalayas. Food Biotechnology 14 (1&2), 99–112.

Tamang, P., 1992. Sytematics, Distribution and Ecology of the Ichthyospecies of Sikkim and Their Bearing on the Fish and Fisheries of the State. Ph. D. Thesis, Guwahati University, Guwahati, India.

Tanasupawat, S., Okada, S., Suzuki, K., Kozaki, M., Komagata, K., 1992. Identification of *Enterococcus hirae*, *E. faecalis*, *E. faecium*, and *E. casseliflavus* strains from fermented foods. Bulletin of the Japan Federation for Culture Collection 8 (2), 86–94.

Tanasupawat, S., Okada, S., Suzuki, K., Kozaki, M., Komagata, K., 1993. Lactic acid bacteria, particularly heterofermentative lactobacilli, found in fermented foods of Thailand. Bulletin of the Japan Federation for Culture Collection 9, 65–78.

Thapa, N., 2002. Studies on Microbial Diversity Associated With Some Fish Products of the Eastern Himalayas. Ph.D. Thesis, North Bengal University, Siliguri, India.

Thapa, N., Pal, J., Tamang, J.P., 2004. Microbial diversity in ngari, hentak and tungtap, fermented fish products of North-East India. World Journal of Microbiology and Biotechnology 20 (6), 599–607.

Wood, B.J.B., Holzapfel, W.H., 1995. The Lactic Acid Bacteria. The Genera of Lactic Acid Bacteria, vol. 2. Blackie Academic and Professional, London.