Studies on Microbial Diversity Associated with Some Fish Products of the Eastern Himalayas

Thesis Submitted for the Degree of Doctor of Philosophy (Science) of the

UNIVERSITY OF NORTH BENGAL

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Dedicated to my father-in-law

Late (Capt.) S.M. Tamang

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December 20, 2002

This is to certify that Mrs Namrata Thapa has worked under my supervision in the Department of Zoology, University of North Bengal for Ph.D. thesis entitled: **"STUDIES ON MICROBIAL DIVERSITY ASSOCIATED WITH SOME FISH PRODUCTS OF THE EASTERN HIMALAYAS".** I am forwarding her thesis for the Ph.D. degree (Science) of the University of North Bengal.

I recommend that she has fulfilled all requirements according to the rules of the University of North Bengal regarding the works embodied in her thesis.

Joydeb Pal

(Joydeb Pal)

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During the preparation of this thesis, period of changes in my life occurred. Someway it was a gift, a baby-boy Ashwaath, my second baby was born and otherway, it was a loss to my family, and my father-in-law expired. I wished to show the thesis to him, who always used to inspire me to complete the work. This thesis is dedicated to him.

Namrata Thapa

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CONTENTS

INTRODUCTION

Fish is extremely perishable proteinaceous food that spoils rapidly (Barile *et al.*, 1985). Traditional processing of fish such as fermentation, salting, drying and smoking are the principal methods of fish preservation without refrigeration in South-East Asia (Cooke *et al.*, 1993). Traditional fermented fish products are of two major types: fish-salt formulation, e.g. fish sauce/paste, and fish-salt-carbohydrate mixture eg. pla-ra in Thailand and burong-isda in the Philippines (Adams, 1998; Adams *et al.*, 1985). Fermented fish products contribute significantly to the diet by increasing protein intake to a larger population of the world (Beddows, 1984).

In Asia, traditionally processed fish products which are prepared by fermentation, drying, salting in vast quantities for human consumption for long centuries (Hesseltine, 1979; Steinkraus, 1983; Campbell-Platt, 1987). Fish fermentation technology is home-based traditional technique where varieties of fermented fish products mostly fish-sauce are prepared and used as staple foods, side-dishes and condiments in Asia (Lee *et al.*, 1993). Some of these traditional fish products include patis of the Philippines (Arroyo *et al.*, 1978; Sakai *et al.*, 1983a,b), nam-pla and plaa-raa of Thailand (Phithakpol, 1993), shottsuru and shiokara of Japan (Itoh *et al.*, 1993), jeot of Korea (Lee, 1993; Mheen, 1993), pindang of Indonesia (Putro, 1993), budu of Malaysia (Merican, 1977) and nga-pi of Myanmar (Tyn, 1993).

Microorganisms bring about some biochemical changes in the substrates during fermentation such as enrichment of human diet with acceptable flavour, texture and aroma, biopreservation of perishable foods, bioenrichment of substrates with vitamins, protein and essential amino acids, and detoxification of undesirable components (Campbell-Platt, 1994;

Steinkraus, 1994; Stiles and Holzapfel, 1997). Bacteria, mostly lactic acid bacteria, yeasts and filamentous fungi constitute the microflora associated with the traditional fermented foods which are present in or on the ingredients, utensils, environment, and are selected through adaptation to the substrate (Hesseltine, 1983; Tamang, 1998). Interest in using biological system for preserving foods is increasing and has been mainly directed at lactic acid bacteria (Jeppesen and Huss, 1993). Variety of indigenous fermented foods including fermented leafy vegetable, fermented soybeans, fermented milk products, as well as alcoholic beverages are prepared and consumed by different ethnic groups of people living in the Eastern Himalayan regions and its adjoining North-Eastern hills (Tamang *et al.*, 1988; Tamang, 2001).

The Teesta and Rangit river system alongwith their tributaries in Sikkim and the Darjeeling hills exhibit wide rage of gradients from sub-tropical to alpine zone. Altogether 44 species of fish have been reported from the Sikkim river system (Tamang, 1992). The river system in Assam and Arunachal Pradesh consists of the River Brahmaputra and its tributaries where 126 species of fish belonging to 26 families are reported (Motwani *et al.*, 1962; Jhingran, 1977). Logtake lake in Manipur which provides the main fishery resources in the state has varieties of ichthyofauna mostly dominated by species of *Puntius, Channa, Anabas*, etc. (Chaudhuri and Banerjea, 1965).

Seafood is uncommon to the Eastern Himalayan regions. The people of these regions catch the available fishes from the various sources mainly from hill rivers, streams and lakes, and majority of fish is consumed as

fresh. Some of these fishes are preserved or processed using indigenous knowledge of fermentation/drying/smoking for consumption. Preparation of traditional foods has been mainly influenced by the agro-climatic conditions, ethnic preference, socio-economy and cultural ethos in the Eastern Himalayas. Modern methods of preservation are still uncommon to majority of people, traditional way of preservation of locally available fish by fermentation, smoking, drying is the common practice.

Traditionally processed lesser-known fish products are consumed by different ethnic groups of people living in the Eastern Himalayan regions including Eastern Nepal; the Darjeeling hills, Sikkim, Assam, Meghalaya and Manipur in India, and Bhutan. Literature on traditionally processed fish products for human consumption in the Eastern Himalayas is lacking. Documentation on production statistics of common and lesser-known traditional fish products of the Eastern Himalayas, and information on microbiology and safety are not available. The proposed dissertation is aimed to study in depth the microbial diversity associated with some traditionally processed fish products, their identity and characteristic properties of few selected strains such as antimicrobial activity, enzymatic activity, ability to produce biogenic amines and hydrophobicity. Study of microbial diversity in the lesser-known fish products of the Eastern Himalayas, may contribute a significant unknown microbial gene pool, which should be preserved.

OBJECTIVES

- Documentation of types of fish products (fermented, smoked, dried), indigenous knowledge of preparation and preservation of locally available fish, and consumption pattern.
- Collection of fish products from different regions of the Eastern Himalayas.
- Isolation, enrichment and purification of microorganisms.
- Characterization and identification of isolates using conventional and modern biochemical techniques.
- Study of microbial population of major microbial groups.
- Study of enumeration of pathogenic contaminants in the products.
- Screening of predominant fermentative bacteria to produce biogenic amines.
- Enzymatic and antimicrobial properties of some of the isolated strains to know their role in processing.
- Analysis of food value of products.

Traditionally preserved fish products are largely confined to east and south-east Asia which are still produced principally on a cottage industry or domestic scale (Adams, 1998). As commonly applied, the term 'fermented fish' covers two categories of product (Adams *et al.*, 1985): (i) fish-salt formulations, e.g. fish sauce products such as the fish pastes and sauces tend to contain relatively high levels of salt, typically in the range 15-25% and are used mainly as a condiment; and (ii) fish-salt-carbohydrate mixtures, e.g. pla-ra in Thailand and burong-isda in the Philippines. In hot countries, particularly in rural areas, fermented fish products continue to play a vital role in adding protein, flavour and variety to rice-based diets (Campbell-Platt, 1987). Table (A) summarises some of the common traditionally processed fish products of Asia.

Among the fermented fish products, the more widely used are fish sauces and pastes (van Veen, 1965; Orejana, 1983). In the fish-salt-carbohydrate product, lactic fermentation occurs and contributes to the extended shelf life (Adams *et al.*, 1985; Owens and Mendoza, 1985). Lactic acid fermented products can be prepared in a shorter time than the fish-salt products, which depend primarily on autolytic processes and offer greater scope for low-cost fish preservation in South East Asia than the simply low water activity products (Adams *et al.*, 1985). The principal carbohydrate source used in these traditional lactic fermented products is cooked rice, although in some products partially saccharified rice (mouldy rice: ang-kak, or pre-fermented rice) is used or, on occasion, small amounts of cassava flour, or cooked millet, e.g. sikhae in Korea (Lee, 1984).

Table A. Some common traditionally processed fish products of Asia

Product	Substrate	Type and use	Country	Reference
Pindang	Fish	Dried/salted; side-dish	Indonesia	Putro (1993)
Pedah	Mackerel	Partly-dried and salted; side-dish	Indonesia	Westenberg (1951)
Trassi	Shrimps/fish	Fermented paste; side-dish	Indonesia	Van Veen (1965)
Shottsuru	Marine fish	Fish sauce; condiment	Japan	Itoh *et al.* (1993)
Shiokara	Squid	Fermented; side-dish	Japan	Fujii *et al.* (1999)
Jeot-kal	Fish	High-salt fermented; staple	Korea	Lee (1993)
Sikhae	Fish-cereals	Low-salt fermented; sauce	Korea	Lee (1993)
Gulbi	Shell-fish	Salted and dried; side-dish	Korea	Kim *et al.* (1993)
Budu	Anchovies	Fish sauce; condiment	Malaysia	Merican (1977)
Pekasam	Freshwater fish-roasted rice	Fermented; side-dish	Malaysia	Karim (1993)
Belacan	Shrimp	Shrimp paste; condiment	Malaysia	Wong and Jackson (1977)
Mehiawah	Marine fish	Fermented paste; side-dish	Middle–East	Al-Jedah *et al.* (1999)

Product	Substrate	Type and use	Country	Reference
Nga-pi	Fish	Fermented fish paste; condiment	Myanmar	Tyn (1993)
Ngan-pyaye	Fish	Fish sauce; condiment	Myanmar	Tyn (1993)
Patis	Marine fish	Fish sauce; condiment	The Philippines	Baens-Arcega (1977)
Bagoong	Fish	Fish paste; condiment	The Philippines	Mabesa and Babaan (1993)
Bagoong alamang	Shrimp	Shrimp paste; condiment	The Philippines	Mabesa and Babaan (1993)
Balao-balao	Shrimp	Fermented shrimp; condiment	The Philippines	Arroyo *et al.* (1978)
Burong isda	Rice-fish	Fermented rice-fish mixture; sauce or main dish	The Philippines	Sakai *et al.* (1983b)
Nam-pla	Anchovies	Fish sauce; condiment	Thailand	Saisithi *et al.* (1966)
Kapi	Shrimp	Shrimp paste; condiment	Thailand	Phithakpol (1993)
Plaa-raa	Fish, rice	Fermented paste; condiment and main dish	Thailand	Phithakpol (1987)
Nuoc-mam	Marne fish	Fish sauce; condiment	Vietnam	van Veen (1965)

Few food anthropologists have mentioned the antiquity and cultural aspects of fermented fish products in Asia. It may be hypothesized that the deliberate preparation of fermented fish originated by accident when a batch of old or improperly prepared salted fish fermented, and the umami taste was first observed and found acceptable (Ishige, 1986). Fermented fish production may have originated independently in many different locations, but it would have only developed and been deliberately elaborated where the taste was culturally acceptable and where the products were found complementary to the established cuisine (Ishige and Ruddle, 1987). Ishige (1993) advocated that the Mekong and associated basins of south-west China, Laos and northern and north-west Thailand were the most probable place of origin of fermented fish products.

Fermented fish products are prepared from freshwater and marine finfish, shellfish, and crustaceans that are processed with salt to cause fermentation and thereby to prevent putrefaction (Ishige, 1993). If cooked carbohydrates are added to the fish and salt mixture, the product is called narezushi and if no vegetables are added, the salt-fish mixture yields fish sauce, which is commonly used as a condiment, and if the product of fish and salt that preserves the whole or partial shape of the original fish, it is called 'shiokara', which when comminuted by either pounding or grating yields shiokara paste (Ishige and Ruddle, 1987). Shiokara paste has synonyms in South-East Asia, as in Myanmar it is known as nga-pi, in Kampuchea as pra-hoc (Ishige, 1993). Shiokara is used mostly as a side dish, and is important in the food life of Kampuchea, Laos, north and north east Thailand; lower Myanmar, Luzon and the Visayas in the Philippines, and Korea (Ishige, 1993).

Squid skiokara is the most popular fermented sea food in Japan (Fujii *et al.*, 1999).

Fermented fish products are of very minor importance outside Asia. In Europe, fish sauces known as liquamen or garum were ubiquitous condiments for the Romans, who had adopted them from the Greeks (Adams, 1998). Norwegian gravlaks, or buried salmon, is a traditional, relatively mild tasting product; more heavily fermented products, rakefisk or surfisk, the most popular varieties of which are rakörret, fermented trout, in Norway and surströmming, made from herring in Sweden (Riddervold, 1990; Kobayashi *et al.*, 2000).

The diversity of fish used in fermented products and in the way in which they are handled prior to processing means that the initial microbial levels are far from uniform, and counts ranging from 10^4/g to $>10^7$/g have been reported (Adams, 1998). Lactic fermented fish products are often associated with inland areas such as the Central Luzon region of the Philippines and the north-east of Thailand where the freshwater fish are the usual raw material, and their microflora tends to reflect their local environment more than that of marine species (Adams, 1986). In a survey of fermented fish products in north-east Thailand, it was found that several different sources were used for the fish - the flooded rice fields, paddy ponds beside rice fields used for collecting fish when the field has dried up, and a large local freshwater reservoir (Dhanamitta *et al.*, 1989).

A key factor limiting fish utilization is its perishability, since fish flesh offers microorganisms conditions of good nutrient availability coupled with a high water activity (a_w) and moderate pH (Shewan, 1962). In tropical countries the problem posed by the intrinsic suitability of fish flesh as a medium for microbial growth is further compounded

by a high ambient temperature (Adams *et al.,* 1987). Fish stored under these conditions is considered spoiled within 12 hours (FAO, 1981). In developing countries the necessity for low-cost methods of fish preservation has reduced the applicability of technologies such as chilling, freezing and canning (Cutting, 1999). Traditional curing processes, which depend upon the reduction of a_w as the principal preservative factor, are important, e.g. salting, drying and smoking (Sperber, 1983).

In several products, the rice may be added in a partially saccharified form such as kao-mark (Thailand), or, alternatively, a saccharifying agent such as koji (Japan) or ang-kak (the Philippines) may be added separately, which increase the amount of soluble sugars produced and thus the range of lactic acid bacteria that can grow lowering pH (Adams, 1998). In burong-isda where ang-kak is used pH decreases to 3.0-3.9 compared with the product without ang-kak addition (pH 4.1-4.5) despite other factors, such as salt content, being similar (Sakai *et al.,* 1983b). Sikhae, the Korean fermented fish product, is something of an exception since millet is used as the carbohydrate source (Lee, 1984). Saisithi (1987) reported that the function of the roasted rice added during pla-ra production is to improve texture and slow the fermentation rather than to serve as a substrate for lactic fermentation. Carbohydrate mix prevented yeasts growth in fermented fish products (Avurthi and Owens, 1990).

Most fermented fish products use salt as ingredient; therefore, both fermented fish and salted fish are alike in that both require the use of salt as a preservative (Ishige, 1986). A survey of fish products in Thailand noted that there were two empirical rules governing formulations: (i) the use of higher salt levels results in a longer

production phase but a better keeping quality product, and (ii) inclusion of more carbohydrate gives a faster fermentation and a stronger acid taste (Adams *et al.,* 1985). The salt levels in some high-salt fermented fish sauces and pastes are sufficient to check microbiological spoilage (Saonos and Dhamcharee, 1986).

Although the ability to ferment starch is not widespread in the lactic acid bacteria, a number of amylolytic species have been described and an amylolytic strain of *Lactobacillus plantarum* has been isolated from burong bangus in the Philippines (Sakai *et al.,* 1983a). Solidum (1977) reported Gram-positive coccii in the initial stage of fermentation of balao-balao and in the later stage, high acid-producing rods predominated the product.

Species of *Lactobacillus, Pediococcus, Micrococcus, Bacillus* and few types of yeast including species of *Candida* and *Saccharomyces* were reported from nam-plaa and kapi, fermented fish products of Thailand (Watanaputi *et al.*, 1983; Phithakpol, 1987). *Micrococcus* and *Staphylococcus* are dominant microorganisms during ripening of shiokara (Nishimura and Shinano, 1991; Tanasupawat *et al.*, 1991; Fujii *et al.*, 1994; Wu *et al.*, 2000).

Tetragenococcus muriaticus and *Tetragenococcus halophilus* were isolated from the Japanese fermented puffer fish ovaries (Satomi *et al.*, 1997; Kobayashi *et al.*, 2000c). Kobayashi *et al.* (2000a,b) isolated and identified *Haloanaerobium praevalens* in surströmming, fermented herrings of Sweden and *Haloanaerobium fermentans* from the Japanese puffer fish ovaries.

Crisan and Sands (1975) reported that *Bacillus* spp. were found to be the predominant isolates, probably reflecting their ability as spore formers to survive rather than any capacity to multiply under the

prevailing conditions in nam-pla and patis. Species of *Bacillus* mostly *Bacillus stearothermophilus, B. shaercus, B. circulans*, etc. are predominant microflora in nga-pi, fermented fish paste of Myanmar (Tyn, 1993).

Halobacterium and *Halococcus* sp were isolated from nam-pla with microbial load reaching upto 10^8/ml after 3 weeks of fermentation and also demonstrated their significant proteolytic activity during the first month of fermentation, suggesting that halophilic bacteria do play an important role in the production of fish sauce (Thongthai and Suntinanalert, 1991).

Bacillus licheniformis, Staphylococcus sp., *Aspergillus* sp. and *Candida* sp. were recovered from gulbi, salted and dried fish product of Korea (Kim *et al.*, 1993).

Avurhi and Owens (1990) found that any effective measure to exclude air from a fermenting fish/carbohydrate mix prevented spoilage yeasts. Traditional packing techniques used with lactic fermented fish products, such as wrapping in banana leaves, will not reliably exclude air, and surface mould growth has been shown to be a cause of rejection in the Thai product som-fak which suggests that the use of oxygen-impermeable wrapping films could extend the shelf-life of such products (Adams, 1999).

Fish is more susceptible to spoilage than certain other animal protein foods (Cutting, 1999). Studies have shown that the number of bacteria declines rapidly during the production of the Thai fish sauce nam-pla and that, after one month the product contained about 500 cfu/g comprising mainly *Micrococcus* and *Bacillus* spp. (Velankar, 1957; Saisithi *et al.*, 1966). Similar results were seen with patis, the

Philippines fish sauce, where the total counts dropped from 10^7 cfu/g to $<10^3$ cfu/g after 14 days and $<10^2$ cfu/g after 40 days (Velankar, 1957).

Fermentation promotes chemical change and the development of umami from amino acids where the fish flesh, itself a tasteless protein, is converted to amino acids, a simpler component with a characteristic taste (Kawamura and Kara, 1987). Narezushi, which is fish fermented together with rice or another starch, has a characteristic taste that develops from the auto-digestion of meat (Ishige, 1986). Free amino acids which increased during processing of gulbi were reported as important flavour components (Lee and Kim, 1975). Fujii *et al.* (1999) reported that the dominant microorganisms which included *Micrococcus* and *Staphylococcus* in shiokara during ripening produced organic acids imparting flavour to the product.

Lactic and anaerobic bacteria proliferate, and yeast is also an important ingredient, which act on the sugary content of the rice to produce many organic acids and alcohol (Mizutani *et al.*, 1988). The effect of the organic acids is to lower the pH, controlling the growth of putrefying bacteria, thus, narezushi can be preserved for long periods (Mizutani *et al.*, 1988). A novel fibrinolytic enzyme was purified from *Bacillus* sp, isolated from jeot-gal of Korea (Kim *et al.*, 1997).

Dried fish is produced with a moisture content of 17% to 45% (Clucas and Sutcliffe, 1981). Fermented fish products are generally high in protein and amino compounds (Beddows, 1985). Beddows *et al.* (1979) observed that budu, fermented fish product of Malaysia contains the amino acid profile of the original fish, and thus nutritive value is high. Teshima *et al.* (1967a,b) investigated the volatile compounds in shiokara and identified formic, ethanoic, propanoic, iso-butanoic, n-

pentanoic acids as well as ammonia, amino-butane and 2-methyl propylamine due to microbial action.

Biogenic amines which are organic basic compounds are found to occur in fish products, cheese, wine, beer, dry sausages and other fermented foods (Ten Brink *et al.*, 1990; Halász *et al.*, 1994). Biogenic amines are formed by decarboxylation of their precursor amino acids, as a result of the action of either endogenous amino acid decarboxylase activity (Halász *et al.*, 1994) or by the growth of decarboxylase positive microorganisms (Silla-Santos, 2001). Histamine is generally found in spoiled scombroid fish and other marine fish that contain high levels of histidine in their muscle tissues (Lukton and Olcott, 1958), and is associated with the chemical intoxication called scombroid fish poisoning (Fujii *et al.*, 1997). Many workers isolated various histamine-producing bacteria from the fish which had been involved in food poisoning incidents (Stratton and Taylor, 1991).

Lactic acid bacteria frequently produce histamine and tyramine in a processed fish (Stratton *et al.*, 1991; Leisner *et al.*, 1994). Trace quantities of putrescine, tyramine, agmatine and tryptamine have been detected in Ghanaian fermented fish (Yankah *et al.*, 1993). Ornithine and citrulline were detected as decomposition products of arginine in fish sauce (Mizutani *et al.*, 1992). In anchovies, high level of biogenic amines was detected during manufacturing process (Ridriguez-Jerez *et al.*, 1994).

MATERIALS AND METHODS

CULTURE MEDIA

Anaerobic Agar (HiMedia M288)

Arginine Hydrolysis Medium (Thornley, 1960)

Peptone	10.0 g
Yeast extract	5.0 g
D (+) glucose	0.5 g
$K_2HPO_4.3H_2O$	2.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Sodium acetate	5.0 g
Tri-sodium citrate	20.0 g
Tween 80	1.0 ml
Arginine	0.3 %
Phenol red	0.01 g
Distilled water	1000 ml
pH	5.0

Ascospore Agar (HiMedia M804)

***Bacillus cereus* Agar Base** (HiMedia M833)

Baird Parker Agar Base (HiMedia M043)

Basal Medium (Gordon *et al.*, 1973)

Diammonium hydrogen phosphate	1.0 g
Potassium chloride	0.2 g
$MgSO_4.7H_2O$	0.2 g
Yeast extract	0.2 g
Bromocresol purple	0.4 g
Distilled water	1000 ml
pH	7.0

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Biogenic Amine Sub-culturing Medium
(Bover-Cid and Holzapfel, 1999)

	g/l
MRS Broth (HiMedia M369)	52.2
D-Tyrosine (HiMedia RM 1520)	1.0
L-Histidine monohydrochloride (Merck)	1.0
L-Lysine monohydrochloride (Merck)	1.0
L-Ornithine monohydrochloride (Merck)	1.0
Pyridoxal-5-Phosphate (HiMedia RM 1554)	0.001
pH	6.00

Biogenic Amine Screening Medium
(Joosten and Northold, 1989; modified by Bover-Cid and Holzapfel, 1999)

	g/l
Tryptone	5.0
Yeast extract	5.0
Meat extract	5.0
Sodium chloride	2.5
Glucose	0.5
Tween 80	1.0
K_2HPO_4	2.0
Ammonium citrate	2.0
Calcium carbonate	0.1
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
$FeSO_4.7H_2O$	0.04
Thiamine	0.001
Pyridoxal-5-phosphate	0.005
Bromocresol purple	0.05
Agar	22.0
Amino acid	5.0

Amino acids are D-Tyrosine (pH 5.3) (HiMedia RM 1520); L-Histidine monohydrochloride (pH 5.0) (Merck); L-Lysine monohydrochloride (pH 5.15) (Merck); L-Ornithine monohydrochloride (pH 5.0) (Merck); No amino acid (pH 5.15).

Egg Yolk Emulsion (HiMedia FD045)

Egg Yolk Tellurite Emulsion (HiMedia FD046)

Fermentation Basal Medium (Wickerham, 1951)

Yeast extract	4.5 g
Peptone	7.5 g
Distilled water	1000 ml
Bromothymol blue (Till sufficiently dense green colour appears)	

Lactate Configuration Medium (Holzapfel, unpublished)

Peptone from casein	10.0 g
Yeast extract	4.0 g
Glucose	20.0 g
Di-potassium hydrogen phosphate	2.0 g
Tween 80	1.0 g
Di-ammonium hydrogen phosphate	2.0 g
Magnesium sulphate	0.2 g
Manganese sulphate	0.04 g
Distilled water	1000 ml

Malt Extract Agar (HiMedia M137)

Malt Extract Agar (Kreger-van Rij, 1984)

Malt extract	100.0 g
Agar	20.0 g
Distilled water	1000 ml
pH	5.4

Milk Agar (Gordon *et al.*, 1973)

Skim milk powder (HiMedia RM1254)	5.0 g in 50 ml distilled water
Agar	1.0 g in 50 ml distilled water

Autoclaved separately at 121° C for 20 min, cooled to 45° C, mixed together and poured into Petri-dishes. The plates were allowed to stand at 37° C for 24 h to dry the surface of the agar.

MRS Agar (HiMedia M641)

MRS Broth (de Man *et al.*, 1960)

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
K_2HPO_4	2.0 g
Diammonium citrate	2.0 g
Glucose	20.0 g
Tween 80	1.0 g
Sodium acetate	5.0 g
$MgSO_4.7H_2O$	0.58 g
$MnSO_4.4H_2O$	0.28 g
Distilled water	1000 ml
pH	6.2-6.4

Agar (2% w/v) was added to prepare MRS agar.

MRS Broth (HiMedia M369)

Nitrate Broth (Gordon *et al.*, 1973)

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	1.0 g
Distilled water	1000 ml
pH	7.0

Nutrient Agar (HiMedia MM012)

Nutrient Broth (HiMedia M002)

Plate Count Agar (HiMedia M091)

Polymyxin B Selective Supplement (HiMedia FD003)

Potato Dextrose Agar (HiMedia M096)

Phytone broth (Nagai *et al.,* 1994)

Phytone peptone	15.0 g
Mono-Sodium glutamate	15.0 g
Sucrose	15.0 g
Potassium dihydrogen phosphate	2.5 g
Disodium hydrogen phosphate	1.7 g
Sodium chloride	0.05 g
Magnesium chloride	0.05 g
Biotin	0.1 µg/ml
Distilled water	1000 ml
pH 7.0	

Starch Agar (Gordon *et al.*, 1973)

Starch (HiMedia RM089)	1.0 g in cold distilled water
Tryptone	5.0 g
Yeast extract	15.0 g
Potassium dihydrogen phosphate	3.0 g
Agar	20.0 g
Distilled water	1000 ml

Tributyrin Agar (Stolp and Gadkari, 1981)

Peptone	5.0 g
Yeast extract	3.0 g
Tributyrin (HiMedia FD081)	10.0 g
Agar	12.0 g
Distilled water	1000 ml
pH	7.4-7.6

Tryptone Soya Agar (HiMedia 290)

Violet Red Bile Glucose Agar w/o Lactose (HiMedia M581)

Voges-Proskauer (VP) Broth (Gordon *et al.*, 1973)

Peptone	7.0 g
Glucose	5.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml
pH	6.5

Yeast Malt Agar (HiMedia M424)

Yeast Malt Broth (HiMedia M425)

Yeast Extract-Malt Extract Agar (Wickerham, 1951)

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	20.0 g
Distilled water	1000 ml
pH	5-6

Yeast Morphology Agar (HiMedia M138)

Yeast Nitrogen Base (HiMedia M139)

REAGENTS

Acidic Ninhydrin

1-Butanol/water saturated	465 ml
Acetic acid	35 ml
Ninhydrin	2.5 ml

Burke's Iodine Solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Gram's Crystal Violet (HiMedia S012)

Malachite Green (HiMedia S020)

Nitrate Reduction Test Reagent

Solution A

Sulphanilic acid	0.8 g
5 N acetic acid	100 ml

(Glacial acetic acid: water, 1: 2.5)

Solution B

∝-Napthylamine	0.5 g
5 N acetic acid	100 ml

The solutions A and B were mixed in equal quantities just before use.

Phenolphthalein (HiMedia I009)

Reagents for α-amylase assay

100 mM Tris - HCl buffer, pH 7.0

1.5% soluble starch was dissolved in 100 mM Tris (hydroxymethyl) aminomethane – HCl buffer, pH was adjusted to 7.0.

Stop solution

0.5 N Acetic acid – 0.5 N HCl (5:1)

Iodine solution

I_2 = 0.01%
KI = 0.1%

Reagents for Protease activity assay

100 mM phosphate buffer pH 6.8

0:1 M/L $Na_2HPO_4.12H_2O$
0.1 M/L KH_2PO_4
1% Azocasein was dissolved in 0.1M phosphate buffer (Sigma Chemicals, St. Louis, USA), pH 6.8

10% Trichloroacetic acid

1N NaOH

Safranin (HiMedia S027)

Voges-Proskauer test reagent

Potassium hydroxide (40%)
Ethanolic α-napthol (5%)

REFERENCE STRAINS

Reference Strains	Origin	Purpose in this experiment
Listeria monocytogenes 20600	DSM	Indicator strain (Antimicrobial activity)
Enterococcus faecium 20477	DSM	Indicator strain (Antimicrobial activity)
Streptococcus mutans 6178	DSM	Indicator strain (Antimicrobial activity)
Bacillus cereus 2010	CCM	Indicator strain (Antimicrobial activity)
Lactobacillus plantarum 20174	DSM	Standard (+) strain for meso-diaminopimelic acid determination

Originally these reference strains were obtained from DSM (Deutsche Sammlung von Mikroorganismen, Göttingen, Germany) and CCM (Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia). *Listeria monocytogenes* DSM 20600 and *Bacillus cereus* CCM 2010 were propagated in standard nutrient agar (HiMedia M002), *Enterococcus faecium* DSM 20477, *Streptococcus mutans* DSM 6178 and *Lactobacillus plantarum* DSM 20174 were cultivated in MRS broth (HiMedia M369). The cultures were maintained as frozen stocks at –20° C in 15% glycerol.

EXPERIMENTAL

Survey

A survey was conducted in different places of the Eastern Nepal, the Darjeeling hills, Sikkim, Assam, Meghalaya and Manipur to get detailed information on the types of fish products, traditional methods of preparation and mode of consumption of various fermented, smoked, dried/salted fish products used by the ethnic people. A questionnaire was prepared to collect information on the various types of fish products.

Collection of samples

Variety of fermented, smoked, dried/salted fish products samples used by the ethnic people were collected from different regions and markets of the Eastern Nepal in Maglung, Therathum and Aitabare; Gidhang village in Kalimpong of the Darjeeling hills, local "Lal Bazar" of Gangtok in Sikkim; local market of Shillong in Meghalaya, Ima market of Imphal in Manipur and local "Palten Bazar" of Guwahati. Samples were collected aseptically in pre-sterile poly-bags as well as sterile bottles, sealed, lebelled and stored at –20° C for analyses.

Microbial analysis

Ten g of sample was suspended in 90 ml of 0.85 % (w/v) sterile physiological saline and homogenized in a stomacher lab-blender 400 (Seward, UK) for 1 min. For aerobic spore-counts, 1 ml dilution was mixed with 9 ml sterile physiological saline, and heated for 2 min in continuously boiling water (Tamang and Nikkuni, 1996). Decimal dilution series were prepared in sterile diluent and diluted suspension of

sample was mixed with the molten media and poured into plates. Lactic acid bacteria (LAB) were selectively isolated on MRS agar (HiMedia M641) plates supplemented with 1 % $CaCO_3$ and incubated under anaerobic condition in an Anaerobic Gas-Pack system (HiMedia LE002) at 30° C for 3 days. Spore-forming bacteria were isolated on nutrient agar (HiMedia MM012) and incubated at 37° C for 1 day. Total viable counts were determined using plate count agar (HiMedia M091A) and incubated aerobically at 30° C for 2 days. Moulds and yeasts were isolated on potato dextrose agar (HiMedia M096) and yeast extract-malt extract agar (HiMedia M424), respectively supplemented with 10 IU/ml benzylpenicillin and 12 µg/ml streptomycin sulphate and incubated aerobically at 28° C for 3 days. Colonies are either selected randomly or all sampled if the plate contained less than 10 colonies, according to Leisner *et al.* (1997).

Purity of the isolates was checked by streaking again on fresh agar plates of the isolation media, followed by microscopic examinations. Colonies appeared were counted as colony forming units (cfu) per g of sample. The isolated strains were picked up on slants of their respective media and kept at 4° C. Cultures were sub-cultured after every two months. Identified representative strains of the different groups of the isolates were deposited and preserved in the respective broth media with 15 % glycerol in cryotubes at –20° C.

Characterization of bacterial isolates

Gram staining

The method of Bartholomew (1962) was followed. A suspension of a 24 h-old bacterial culture on slant was prepared in distilled water. A drop of that suspension was taken on grease-free slide and a smear was made. It was then heated-fixed, flooded by crystal violet stain for 1 min, and washed for 5 sec with water. The smear was flooded with Burke's iodine solution, allowed to react for 1 min, and washed again for 5 sec with water. Holding the slide against a white surface, 95% ethanol was poured drop-wise from the top edge of the slide until no more colour came out from the lower edge of the slide. After washing with water, the smear was stained with safranin for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

Cell morphology

An air-dried (not heated-fixed) smear of a 24 h-old bacterial culture was stained for 30 sec with safranine, washed in water, air-dried (Norris *et al.*, 1981) and observed under oil-immersion objective. Cell dimensions were measured with a standardized ocular micrometer.

Motility

A drop of a 24 h-old culture in MRS broth for LAB; nutrient broth for *Bacillus* and *Micrococcus*, was used to prepare a hanging drop in a cavity slide following the method of Harrigan (1998). The prepared culture was observed in a phase contrast microscope (Olympus CH3-BH-PC, Japan) for motility test of the strains

Production of catalase

The 0.5 ml of 10 % hydrogen peroxide solution was added to a 24 h-old culture and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.*, 1981).

Hydrolysis of arginine

Tubes of 5 ml arginine hydrolysis medium (Thornley, 1960) were inoculated with 24 h-old culture. The tubes were incubated at 30° C for 3 days and observed for the formation of ammonia from arginine (Schillinger and Lücke, 1987).

Gas ($C0_2$) production from glucose

For lactic acid bacteria, tubes of 10 ml MRS broth without citrate and containing inverted Durham tubes was inoculated with 24 h-old cultures and incubated at 30° C (Schillinger and Lücke, 1987). Accumulation of gas in the inverts indicated positive result.

Acid and gas production from glucose

For spore forming bacteria, tubes of 10 ml basal medium containing 0.5% w/v sugars and inverted Durham tubes were inoculated with the isolates and incubated at 37° C for 3 days (Norris *et al.*, 1981).

Growth at different pH

The pH of MRS broth was adjusted to different levels using 1 N HCl or 10% w/v NaOH. The medium was distributed into tubes containing 5 ml in each. They were autoclaved, cooled to room temperature and inoculated with 24 h-old MRS broth culture. The tubes were incubated at 30° C for 24 h and observed for growth (Dykes *et al.*,

1994). For spore forming bacteria the pH of nutrient broth was adjusted to 6.8, inoculated with 24 h-old cultures and incubated at 37° C for 3 days.

Growth at different temperatures

MRS broth and nutrient broth were inoculated with 24 h-old cultures and incubated at 10° C and 15° C for 7 days, 37° C and 45° C for 3 days, respectively and observed for growth (Dykes *et al.*, 1994).

Salt Tolerance

Salt tolerance was tested by inoculating a loop-full of culture in MRS broth supplemented with 6.5%, 10.0% and 18.0% NaCl, respectively, and incubated for 3 days at 30° C in a slanting position to improve aeration (Schillinger and Lücke, 1987). For spore forming bacteria, nutrient broth was supplemented with 7.0% w/v NaCl, inoculated with 24 h-old culture and incubated at 37° C for 7 days in a slanting position to improve aeration. Cultures were observed for growth after incubation.

Acid from carbohydrates

The method was based on Schillinger and Lücke (1987). Tubes of 5 ml MRS broth without beef extract and glucose containing 0.5% w/v of different carbohydrates and 0.004% phenol red indicator were inoculated and incubated at 30° C for 2-5 days. Colour change from red to yellow indicated acid production.

Voges-Proskauer reaction

Tubes of 10 ml Voges-Proskauer broth were inoculated with the isolates and incubated at 37° C for 7 days. To the culture, 0.6 ml 5% w/v ethanolic α- naphthol and 0.2 ml 40% w/v aqueous potassium hydroxide were added and kept for 1 h at room temperature for the production of a pink colour, indicating positive reaction. Initial and final pH of the broth was measured using pH meter (Gordon *et al.*, 1973).

Reduction of nitrate

Cultures were grown in 5 ml nitrate broth incubated at 37° C. After 3, 7 and 14 days, 1 ml of the culture was mixed with 3 drops of the reagent for nitrate reduction test and observed for the development of a red of yellow colour, indicating the presence of nitrate. A small amount of zinc dust was added to the tube that was negative even after 14 days and observed for the development of red colour, indicating the presence of nitrate i.e. absence of reduction (Norris *et al.*, 1981).

An alternative method was also followed. A strip of filter paper moistened with 10% w/v aqueous potassium iodide and then with a few drops of 1 N hydrochloric acid was touched with a drop of the culture. It was observed for the production of purple colour, indicating the presence of nitrite (Claus and Berkeley, 1986).

Anaerobic growth

Anaerobic agar (HiMedia M228) was distributed into culture tubes in amount sufficient to give 7.5 cm depth of the medium and sterilized by autoclaving at 121° C for 20 min. The tubes were inoculated with a small (outside diameter 1.5 m) loop-full of 24 h-old nutrient broth culture by stabbing up to the bottom of the column. They

were incubated at 37° C for 3 and 7 days, and observed for growth along the length of the stab (anaerobic) and on the surface of the agar (aerobic) (Claus and Berkeley, 1986).

Lactic acid configuration

The configuration of lactic acid produced was determined enzymatically using D-lactate and L-lactate dehydrogenase kits (Boehringer-Mannheim GmbH, Cat. No. 1112821, Germany) based on the method of Boehringer-Mannheim (1989). Lactic acid bacteria strains were grown in lactate configuration medium (Holzapfel, unpublished) at 37^0 C overnight. One ml culture was centrifuged in a microcentrifuge (Heraeus, Germany) at 10,000 rpm for 5 min. The 20 µl of the supernatant was mixed with 980 µl of redistilled water to obtain 1:50 sample dilution. The 1 ml of Solution (1), 0.2 ml of Solution (2), 0.02 ml of Suspension (3), 0.1 ml sample solution and 0.9 ml of redistilled water was pipetted into a cuvette, followed by gentle swirling to mix the contents of the cuvette after closing it with parafilm. Similarly, a blank was prepared by adding all the reagents except the sample solution being replaced with 1.0 ml of redistilled water. After 5 minutes the absorbance of the solutions (A_1) was measured in UV-VIS Spectrophotometer (Analytik Jena, Germany) at 340 nm. The absorbance differences (A_2-A_1) for both, blank and sample was determined and the difference of the absorbance difference of the blank from that of the sample ($\Delta A_{D\text{-lactic acid}}$) was calculated. The reaction was started by adding 0.02 ml of Solution (4) to the sample as well as to the blank. The cuvettes were swirled gently to mix the contents by closing it with parafilm. After 30 minutes the absorbance (A_2) of the sample and

the blank were measured immediately one after another at 340 nm. The 0.02 ml of Solution (5) was added to both the sample and the blank followed by mixing. These were allowed to stand for 30 minutes. The absorbance (A_3) was measured immediately one after another for the sample as well as for the blank at 340 nm. The absorbance differences (A_3-A_2) for both, blank and sample was determined and the difference of the absorbance difference of the blank from that of the sample ($\Delta A_{L\text{-lactic acid}}$) was calculated. The lactic acid isomer concentration was calculated as:

$$c = \frac{V \times MW \times \Delta A}{\varepsilon \times d \times v \times 1000} \text{ (g/l)}$$

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of lactic acid = 90.1 (g/mol)

d = light path = 1 cm

ε = extinction coefficient of NADH at 340 nm = 6.3 (l/mmol × cm)

The result was multiplied by the dilution factor.

meso-Diaminopimelic acid (meso-DAP)

The presence of meso-diaminopimelic acid in the cell walls of lactic acid bacteria was determined using thin-chromatography on cellulose plate (Schillinger and Lücke, 1987). Cells were grown in 5 ml MRS broth for 48 hours and were harvested by centrifuging at 13,000 rpm for 5 min, and washed with 3.0 ml of distilled water. The sediment was resuspended in 1.0 ml 6 N HCl and transferred to screw-capped tubes. The cells were hydrolysed overnight at 100° C in a water-bath. The contents of the tubes were blow-dried while immersed in boiling

water. The sediment was resuspended in 1.0 ml of distilled water and blow dried again and oven dried for 1 h. Finally, the sediment was suspended in 0.1 ml of distilled water and each sample (5 µl) was spotted on thin-layer chromatography plates on cellulose plates (Merck, Germany). Descending one-dimensional chromatography was done by keeping the plates in a TLC chamber in a solvent solution containing methanol: pyridine: 10 N HCl: water (32:4:1:7). The solvent solution was prepared 1 hour before use. After keeping for 4-5 h the plates were dried with a hair drier and the chromatograms were developed by spraying acidic ninhydrin and when almost dried, placed for 5 minutes in 100° C oven. Spots representing meso-diaminopimelic acid appeared dark green to grey and turned yellow within 24 hour. *Lactobacillus plantarum* DSM 20174 was used as standard (meso-DAP positive).

API Tests

The ability to ferment various carbon sources by lactic acid bacteria was determined using API 50 CHL system (bioMérieux, France) according to manufacturer's instructions and also based on the method described by Tamang and Holzapfel (1999). Cultures were grown on MRS agar at 30° C for 48 hour. The growth was harvested in 2 ml sterile normal NaCl solution which was used to prepare suspensions, corresponding to 10^7 cells/ml. The incubation box was prepared by distributing about 10 ml of sterile water into the honeycombed base of the 50 CHL trays. The strips were unpacked, placed them in the trays and the tubes were filled with the bacterial suspensions. The inoculated strips were kept slightly tilted and incubated at 30° C for 48 h. The results were read by referring to the manufacturer's interpretation table

at 24 hour and 48 hour, respectively. All spontaneous reactions were recorded.

Characterisation of yeast isolates

Cell morphology

Sterile yeast morphology agar (HiMedia M138) slants were inoculated with an actively growing (24 h-old) yeast culture and incubated at 28° C for 3 days and observed for cell morphology and mode of vegetative reproduction (Yarrow, 1998). Dimensions of cells were measured with a standardized ocular micrometer.

Pseudo- and True-mycelium

For observation of pseudo-mycelium and true-mycelium of yeast isolates, the slide culture method described by Kreger-van Rij (1984) was followed. A petri-dish, containing U-shaped glass rod supporting two glass slides, was autoclaved at 121° C for 20 min. Molten potato dextrose agar (HiMedia M096) was poured onto the slides. The solidified agar on the slides was inoculated very lightly with yeast isolates in two lines along each slide. Four sterile coverslips were placed over part of the lines. Some sterile water was poured into the petri-dish to prevent the agar from drying out. The culture was then incubated at 28° C for 4 days. The slides were taken out of the petri-dish and the agar was wiped off from the back of the slide. The edges of the streak under and around the coverslips were examined microscopically for the formation of pseudo-mycelium or true-mycelium.

Characteristics of asci and ascospore

Sterile ascospore agar (HiMedia M804) slants were streaked with a 24 h-old yeast isolates, incubated at 28° C for 3 days and examined at weekly intervals up to 4 weeks for observation of asci and ascospores. A heat fixed smear was flooded with 5 % w/v aqueous malachite green (HiMedia S020) for 30 to 60 sec, heated to steaming 3 to 4 times over the flame of a spirit lamp and counterstained with safranin (HiMedia S027) for 30 sec and observed under the microscope (Yarrow, 1998).

Reduction of nitrate

Cultures were grown in 5 ml nitrate broth incubated at 28° C. After 3, 7 and 14 days, 1 ml of the culture was mixed with 3 drops of the reagent for nitrate reduction test and observed for the development of a red or yellow colour, indicating the presence of nitrate. A small amount of zinc dust was added to the tube that was negative even after 14 days and observed for the development of red colour, indicating the presence of nitrate, i.e. absence of reduction (Yarrow, 1998).

Growth at 37° C

Slants of malt-extract agar (HiMedia M137) were inoculated with cells of young yeast isolates and incubated at 37° C for 4 days and observed for growth (Yarrow, 1998).

Sugar fermentation

The method was based on Kreger-van Rij (1984) and Yarrow (1998). Cells were grown at 28° C on yeast extract-malt extract agar (HiMedia M424) slants for 3 days. Tubes of 10 ml of fermentation basal medium (Wickerham, 1951) supplemented with 2 % w/v sterile sugars

containing inverted Durham tubes, were inoculated with the above yeast culture and incubated at 28° C and were shaken regularly to observe gas accumulation in the inverts.

Sugar assimilation

The method was based on Kreger-van Rij (1984) and Yarrow (1998). Yeast isolates were grown at 28° C on yeast extract-malt extract agar (HiMedia M424) slants for 3 days. Tubes containing 5 ml mixture of yeast nitrogen base (HiMedia M139) and carbon source were inoculated with cultures and incubated at 28° C for 3 to 7 days. Control test tube was made by adding 0.5 ml of yeast nitrogen base (HiMedia M139) in 4.5 ml of sterilized distilled water (devoid of any carbon source). Assimilation of carbon sources was observed by comparing with the control.

Pathogenic contaminants

Samples were tested for enumeration of pathogenic contaminants such as *Bacillus cereus* using selective *Bacillus cereus* agar base (HiMedia M833), *Staphylococcus aureus* using Baird Parker agar base (HiMedia M043) and enterobacteriaceae using Violet Red Bile Glucose agar w/o lactose (HiMedia M581) (Nout *et al.*, 1998). Ten g of sample was blended with 90 ml of peptone-physiological saline (0.1% neutral peptone, 0.85% NaCl) in a stomacher lab-blender 400 (Seward, UK) for 1 min. Serial decimal dilution series was prepared in the same diluent in duplicates.

***Bacillus cereus*:** Selective enumeration was carried out on spread plates of *Bacillus cereus* agar base (HiMedia M833) with appropriate additions of Polymyxin B Selective Supplement (HiMedia FD003) and Egg yolk emulsion (HiMedia FD045). The inoculated plates were incubated at 30° C for 24 h to 48 h. Characteristic turquoise to peacock blue colonies surrounded by zone of precipitate of the same colour were regarded as presumptive *Bacillus cereus*. A representative number (usually five per plate counted) were isolated and purified on *Bacillus cereus* agar base, followed by nutrient agar. Confirmation was on the basis of endospore formation, fermentation of glucose, xylose and arabinose and ability to grow at 50° C.

***Staphylococcus aureus*:** Selective enumeration was carried out on spread plates of Baird Parker agar base (HiMedia M043) with appropriate additions of Egg yolk tellurite emulsion (HiMedia FD046) and incubated at 30° C for 24 h to 48 h. The black colonies appeared which were regarded as presumptive *Staphylococcus aureus*.

Enterobacteriaceae: Sample dilutions in Tryptone soya broth (HiMedia M011) were allowed to resuscitate on thin Tryptone soya agar (HiMedia 290) plates for 1-2 h at 27° C, followed by a thick overlay of selective Violet Red Bile Glucose agar (without lactose) medium and incubated at 30° C for 20 h. Colonies appeared were regarded as presumptive enterobacteriaceae.

Identification

For identification of bacterial species, taxonomic keys laid down in Bergey's Manual of Systematic Bacteriology, volume 2 (Sneath *et al.*, 1986) and keys described by Wood and Holzapfel (1995) were followed. Endospore-forming rod-shaped bacteria were identified according to the keys based on Claus and Berkeley (1986), Slepecky and Hemphill (1992) (Table B). Yeast strains were identified according to the criteria laid down by Kreger-van Rij (1984), and Kurtzman and Fell (1998).

Table B: Phenotypic key used for tentative identification of Gram-positive endospore forming rod-shaped bacteria[a]

1	Allantoin or urate required	Positive	*Bacillus fastidiosus*
		Negative	2
2	Catalase	Positive	3
		Negative	20
3	Voges-Proskauer	Positive	4
		Negative	11
4	Growth in anaerobic agar	Positive	5
		Negative	10
5	Growth at 50° C	Positive	6
		Negative	7
6	Growth in 7% NaCl	Positive	*Bacillus licheniformis*
		Negative	*Bacillus coagulans*
7	Acid and gas from glucose	Positive	*Paenibacillus polymyxa*
		Negative	8
8	Reduction of NO_3^- to NO_2^-	Positive	9
		Negative	*Paenibacillus alvei*
9	Parasporal body in sporangium	Positive	*Bacillus thuringiensis*
		Negative	37
10	Hydrolysis of starch	Positive	*Bacillus subtilis*
		Negative	*Bacillus pumilus*
11	Growth at 65° C	Positive	32
		Negative	12
12	Hydrolysis of starch	Positive	13
		Negative	17
13	Acid and gas from glucose	Positive	*Paenibacillus macerans*
		Negative	14
14	Width of rod ≥ 1.0 mm	Positive	34
		Negative	15

15	Growth at pH 6.8	Positive	16
		Negative	*Bacillus alcalophilus*
16	pH in VP broth < 6.0	Positive	28
		Negative	26
17	Growth in 10 % NaCl	Positive	*Bacillus pasteurii*
		Negative	18
18	Growth in anaerobic agar	Positive	*Brevibacillus laterosporus*
		Negative	19
19	Acid from glucose	Positive	30
		Negative	24
20	Growth at 65° C	Positive	33
		Negative	21
21	Growth in anaerobic agar	Positive	22
		Negative	*Bacillus azotoformans*
22	Decomposition of casein	Positive	35
		Negative	23
23	Parasporal body in sporangium	Positive	*Paenibacillus popilliae*
		Negative	35
24	Growth at 50° C	Positive	*Bacillus badius*
		Negative	25
25	Growth at 5° C	Positive	*Bacillus insolitus*
		Negative	*Bacillus sphaericus*
26	Acid from arabinose	Positive	*Bacillus lentus*
		Negative	27
27	Growth at 5° C	Positive	30
		Negative	31
28	Growth at 5° C	Positive	*Paenibacillus macquariensis*
		Negative	29
29	Growth in 10% NaCl	Positive	*Virgibacillus pantothenticus*
		Negative	*Bacillus circulans*
30	Hydrolysis of urea	Positive	*Bacillus globisporus*
		Negative	*Bacillus marinus*
31	pH in VP broth > 7	Positive	*Brevibacillus brevis*
		Negative	*Bacillus firmus*
32	Hydrolysis of starch	Positive	33
		Negative	*Bacillus schlegelii*
33	Growth at pH 6.8	Positive	*Bacillus stearothermophilus*
		Negative	*Alicyclobacillus acidocaldarius*
34	Growth in anaerobic agar	Positive	*Bacillus thuringiensis*
		Negative	*Bacillus megaterium*
35	Growth in 10% NaCl	Positive	*Bacillus pasteurii*
		Negative	36
36	Growth at 40° C	Positive	*Paenibacillus larvae*
		Negative	*Paenibacillus lentimorbus*
37	Colony rhizoidal	Positive	*Bacillus mycoides*
		Negative	38
38	Cells motile	Positive	*Bacillus cereus*
		Negative	*Bacillus anthracis*

[a] Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by a species name (based on Claus and Berkeley, 1986; Slepecky and Hemphill, 1992)

Enzymatic activity

Proteolytic activity

Surface-dried plates of milk agar (Gordon *et al.*, 1973) were streaked with 24 h-old cultures, incubated at 30° C for 4 days (lactic acid bacteria) and 37° C for 2 days (spore forming bacteria), and examined for any clearing of casein around and underneath the growth for assessment of proteolytic activity.

Amylolytic activity

Surface-dried plates of starch agar (Gordon *et al.*, 1973) were streaked with 24 h-old cultures, incubated at 30° C for 4 days (lactic acid bacteria) and 37° C for 2 days (spore forming bacteria). After incubation 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.

Lipolytic activity

Surface-dried plates of tributyrin agar (Stolp and Gadkari, 1981) were streaked with 24 h-old culture and incubated at 30° C for 4 days (lactic acid bacteria) and 37° C for 2 days (spore forming bacteria). Lipolytic activity was detected by a clear zone surrounding the culture in the turbid tributyrin agar (Leuschner *et al.*, 1997).

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 rev/min for 72 h. Cultures were immediately centrifuged at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. Then, the enzyme solution and the substrate solution containing 1% Azocasein (Sigma Chemical Co., USA) was dissolved in 0.1 M phosphate buffer, (pH 6.8) were pre-incubated separately at 37° C for 5 min in a water-bath incubator (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 1N NaOH and the absorbance was measured at 450 nm in UV-VIS Spectrophotometer (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.

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 rev/min 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 0.1M Tris-HCl buffer (pH 7.0) were

pre-incubated separately at 37° C for 5 min in water-bath incubator. Then, the reaction mixture was started by adding 1 ml of 1.5% soluble starch (HiMedia RM089) to 0.5 ml enzyme solution and incubated at 37° C for 10 min. The reaction was stopped by the 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 5 ml of 0.01% I_2 – 0.1% KI solution, left at room temperature for 20 min and the absorbance of the resulting solution was measured at 660 nm in UV-VIS Spectrophotometer (Analytik Jena, Germany). One unit of α-amylase activity (dextrinizing power) was defined as the amount of α-amylase which produced 10% reduction in the intensity of blue colour at the above conditions.

Enzymatic profiles by API-zym system

The enzymatic profile of selected strains of lactic acid bacteria were assayed following the method of Arora *et al.* (1990) uisng API zym (bioMérieux, France) galleries by testing for the activity of the following 19 enzymes: phosphatase alkaline, esterase (C4), esterase lipase (C8), lipase (C14), leucine, valine and cystine arylamidase, trypsin, chymotrypsin, phosphatase acid, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase. Cultures were grown on MRS broth and growth was harvested in 2 ml sterile distilled water which was used to prepare suspension of 10^7 cells/ml. The API zym strip was unpacked and 2 drops of cell suspensions was inoculated in each cupule of the strip containing ready-made enzyme substrates and incubated at 30° C for 6 h. After incubation, 1 drop of ready-made zym-A and zym-B

reagents was added and observed for colour development based on the manufacturer's colour chart.

Antimicrobial Activity

Agar Spot Test

The method was based on Schillinger and Lücke (1989) and Uhlman *et al.* (1992). Cultures were grown on the respective broth media for 24 h. Sterilized petriplates were plated with MRS agar (containing 0.2% glucose) and allowed to dry. These were spotted with a drop of the broth culture of the producer strain and incubated at 30° C for 24 h. The indicator strains *Listeria monocytogenes* DSM 20600 and *Bacillus cereus* CCM 2010 were propagated in standard nutrient agar (HiMedia M002), *Enterococcus faecium* DSM 20477 and *Streptococcus mutans* DSM 6178 were cultivated in MRS broth (HiMedia M369). The 0.1 ml of an overnight culture (~10^7 cells) of each indicator strain was inoculated into 7 ml of soft MRS agar (containing 0.7% agar) and poured over the plate on which the producer was grown, respectively. These were incubated at 30 °C for 24 h. After incubation the plates were checked for inhibition zones (clearing of the medium) around the producer colony. Inhibition was scored positive if the width of the clear zone around the colonies of the producer strain was 1 mm or larger.

Bacteriocin Activity

Bacteriocin activity was estimated using an agar spot assay as described by Schillinger *et al.* (1993). The antimicrobial-positive strains were grown in MRS broth at 30° C for 24 h and a cell-free extract was obtained by centrifuging the culture in a microcentrifuge (Heraeus,

Germany). The supernatant was heated at 100° C for 5 min in blockthermostat (Staurt Scientific, UK). The cell-free supernatant was adjusted to pH 6.5 by addition of 1 N NaOH. Agar plates overlaid with 7 ml soft MRS agar (containing 0.7% agar) were inoculated with 0.1 ml of an overnight culture of the indicator strains (as mentioned above), respectively. After incubation at 25° C for 24 h, 0.01 ml of the culture supernatant was spotted onto the agar surface. The plates were incubated at 30° C for 24 h and subsequently examined for zones of inhibition.

Biogenic Amine

The ability 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). Freshly grown cultures were sub-cultured in 5 ml of biogenic amine Sub-culturing medium (Bover-Cid and Holzapfel, 1999) twice and incubated at 30° C for lactic acid bacteria and 37° C for *Bacillus* for 24 h to promote decarboxylase activity. The modified biogenic amine screening medium of Joosten and Northold (1989) (Bover-Cid and Holzapfel, 1999) was prepared, poured on to sterilized Petri-plates and allowed to dry. Bromocresol purple was used as pH indicator. These plates were streaked with the broth cultures in duplicates and incubated at 30° C for lactic acid bacteria and 37° C for *Bacillus* species for 4 days under aerobic and anaerobic conditions. Control plate lacked the amino acid. After incubation observation for positive reaction was made by the purple coloration of the colony and in case of tyramine production a clear halo due to tyrosine precipitate disappearance.

Hydrophobicity

The degree of hydrophobicity of the strains was determined by employing the methods described by Rosenberg (1984) and Ding and Lämmler (1992). These methods were based on adhesion of cells to hexadecane droplets. Cultures were grown in 5 ml of MRS broth (HeMedia M369). The 4 ml of this broth culture was centrifuged at 7,500 rpm for 5 min and the supernatant was discarded. The cell pellet was washed with 9 ml of Ringer solution (Merck), resuspended in a cylomixer and again centrifuged at 7,500 rpm for 5 min. The supernatant was again discarded; the cell pellet was washed with 9 ml of Ringer solution (Merck) and resuspended in a cylomixer. The 1 ml of this suspension was taken and the absorbance at 580 nm was measured in UV-VIS Spectrophotometer (Analytik Jena, Germany). The 1.5 ml of the suspension was mixed with 1.5 ml of n-Hexadecane (HiMedia RM 2238) in duplicates and mixed thoroughly in a cylomixer for 2 min. The two phases were allowed to separate for 30 min. The 1 ml of the lower phase was taken and the absorbance was measured at 580 nm in UV-VIS Spectrophotometer. The percentage hydrophobicity of strain adhering to hexadecane was calculated using the equation:

$$\text{Hydrophobicity (\%)} = \frac{OD_{580}\,(\text{initial}) - OD_{580}\,(\text{with hexadecane})}{OD_{580}\,(\text{initial})} \times 100$$

Adherence value greater than 75% were considered hydrophobic, less than 25% as hydrophilic and those between 25% and 75% as intermediate.

Proximate composition

pH

Ten g of sample was mixed with 20 ml carbon dioxide-free distilled water in a blender for 1 min and the pH of the slurry was determined directly (AOAC, 1990) using a μ pH meter (Systronics, Type.361) calibrated with standard buffer solutions (Merck).

Titratable Acidity

Titratable acidity of sample was calculated by titrating the filtrates of a well blended 10 g sample in 90 ml carbon-dioxide free distilled water with 0.1 N sodium hydroxide to end point of phenolphthalein (0.1 % w/v in 95 % ethanol) (AOAC, 1990).

Moisture

Moisture content of sample was calculated by drying 2.5–3.0 g of well-mixed sample at 135 ± 1° C for 2 h to constant weight (AOAC, 1990).

Ash

A sample (~ 2 g) was accurately weighed into a previously dried and weighed porcelain crucible and placed in a muffle furnace preheated to 550° C for 3 h. The crucible was transferred directly to a desiccator, allowed to cool to room temperature and weighed immediately (AOAC, 1990). The process of heating for 30 min, cooling and weighing was repeated until the difference between two successive weighing was ≤ 1 mg.

Protein

Total nitrogen of sample was determined following the method described in AOAC (1990). Approximately 1 g of sample was taken in a digestion flask, 0.7 g catalyst ($CuSO_4$: K_2SO_4, 1:9) and 25 ml of concentrated H_2SO_4 were added to it. The flask was heated gently until frothing ceased, boiled briskly until the solution became clear and then continued the boiling for about 1 h. The solution was transferred quantitatively to a round-bottomed flask, and mixed with approximately 100 ml of distilled water and 25 ml 4 % w/v aqueous Na_2S to precipitate mercury. A pinch of zinc granules to prevent bumping and a layer of 40 % w/v NaOH were added carefully. The flask was immediately connected to a distillation apparatus and the tip of the condenser was immersed in standard 0.1 N H_2SO_4 containing about 5 drops of methyl red indicator (HiMedia I007). The flask was rotated to mix the contents thoroughly and heated until all the ammonia had distilled. The receiver was removed and the tip of the condenser was washed with distilled water. The remaining acid in the receiver was titrated with standard 0.1 N NaOH solution. The blank determination on reagents was considered for correction. Nitrogen was calculated in percentage.

Total nitrogen (%) =

(ml of standard acid × N of standard acid) – (ml of standard NaOH × N of standard NaOH) ×1.4007/weight of sample (g).

Protein content was determined by multiplying total nitrogen value with 6.38 (AOAC, 1990).

Protein (%) = Total Nitrogen (%) × 6.38

Fat

Fat content was determined by ether extraction using glass soxhlet (AOAC, 1990). Flat-bottomed flask was oven dried and kept in a desiccator for cooling. The weight (W_1) of the round-bottomed flask was taken. A cellulose thimble (dry and fat free) was taken and in which ~ 2 g of sample was placed and put in the soxhlet. Fat was extracted by using petroleum ether with boiling range 40-60° C, on a heating mantle at 60° C for 5 h. The flat bottomed flask was dried for 1 h at 100° C to evaporate ether and moisture, cooled in desiccator and weighed (W_2). Fat was calculated in percentage.

$$\text{Fat (\%)} = W_2 - W_1/\text{Sample weight} \times 100$$

Minerals

The method was based on AOAC (1990). The ash after heating the sample at 550° C for 3 h was dissolved in 5 ml of 20 % HCl. The solution was evaporated to dryness on a hot plate at a temperature of 100-110° C and in an oven at 110° C for 1 h. The minerals in the dried residue were dissolved in about 10 ml of 100 % HCl and the solution was heated on a hot plate at a temperature of 100-110° C for 3-4 times. The solution was made up to 100 ml with 1 % HCl. Calcium, iron, magnesium, manganese and zinc was estimated in an atomic absorption spectrophotometer (Model 3110, Perkin-Elmer).

RESULTS

INDIGENOUS FISH PRODUCTS

The field survey was conducted to document the varieties of traditionally processed fish products, their methods of preparation, mode of consumption from different parts of the Eastern Himalayan regions of Nepal; the Darjeeling hills, Sikkim, and some North Eastern states in India. The various ethnic groups of people living in these regions prepare and consume a variety of traditionally processed smoked/sun-dried/fermented/salted fish products using available fish in the region for long centuries (Fig 1). The ethnic people use their indigenous knowledge of fish preservation and processing without using any extra chemical. Such fish processing technique has been still present in those regions or villages, which are located near water bodies with plenty of freshwater fishes. Some villagers sell them in the market area and economically dependent. About ten major types of traditionally processed fish products has been listed out, which are consumed in local diet by the people of the Eastern Himalayan regions (Table 1). These traditionally processed fish products are **sukako maacha** and **gnuchi** (smoked and sun-dried fish products), **sidra** and **sukuti** (sun-dried/salted fish products) of eastern Nepal, the Darjeeling hills and Sikkim; **ngari** and **hentak** (fermented fish products) of Manipur; **tungtap** (fermented fish product) of Meghalaya; **karati, bordia** and **lashim** (sun-dried and salted fish products) of Assam.

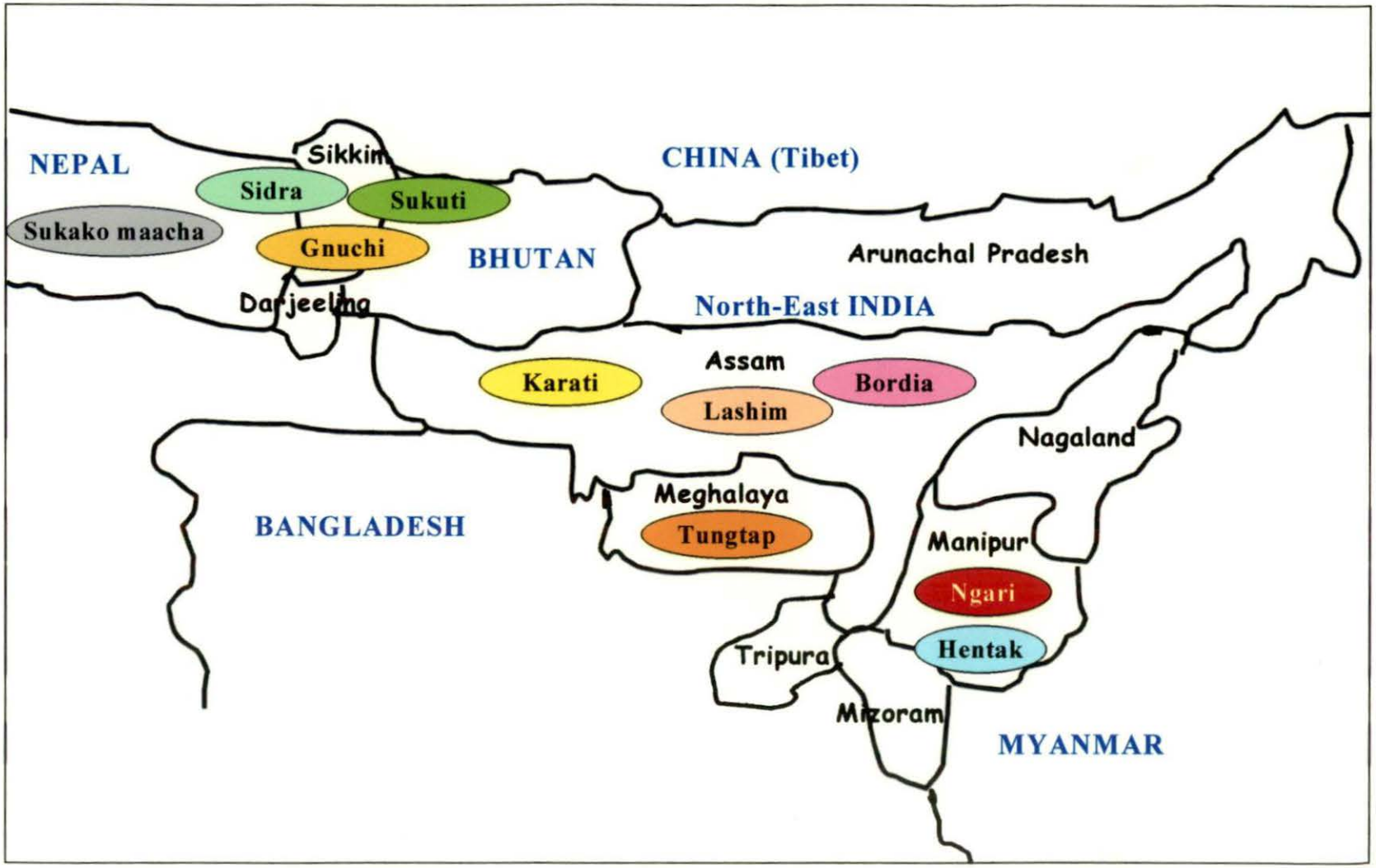


Fig 1. Map showing the traditionally processed fish production sites in the Eastern Himalayas

Table 1. Indigenous fish products of the Eastern Himalayan regions

Products	Substrate	Nature	Region
Sukako maacha	*Schizothorax* spp.	Dried/Smoked	Eastern Nepal, the Darjeeling hills, Sikkim, Bhutan
Gnuchi	*Schizothorax* spp., *Labeo* sp.	Smoked	The Darjeeling hills, Sikkim
Sidra	*Puntius sarana*	Dried	Eastern Nepal, the Darjeeling hills, Sikkim, Bhutan
Sukuti	*Harpodon nehereus*	Dried, salted	Eastern Nepal, the Darjeeling hills, Sikkim, Bhutan
Ngari	*Puntius sophore*	Fermented	Manipur
Hentak	*Esomus danricus*, petioles of *Alocasia macrorhiza*	Fermented paste	Manipur
Tungtap	*Danio* sp.	Fermented	Meghalaya
Karati	*Gudusua chapra*	Dried, salted	Assam, Meghalaya
Bordia	*Pseudeutropius atherinoides*	Dried, salted	Assam, Manipur, Mizoram, Arunachal Pradesh
Lashim	*Cirrhinus reba*	Dried, salted	Assam, Meghalaya

SUKAKO MAACHA

Sukako maacha is an indigenous smoked fish product commonly prepared in the river-site villages of Maglung, Therathum and Aitabare in eastern Nepal; Teesta and Rangit River basins in the Darjeeling hills and Sikkim in India.

Method of preparation

Two types of fishes are preferred for the preparation of sukako maacha by the people of these regions residing near streams or river. These hill river fishes are mostly 'dothay asala' (*Schizothorax richardsoni* Gray) and 'chuchay asala' (*Schizothorax progastus* McClelland). The fishes are collected in a bamboo basket locally called 'bhukh' from the river or streams, and are degutted, washed, mixed with salt and turmeric powder. Degutted fishes are hooked in a bamboo-made string and are hung above the earthen-oven in kitchen for 7-10 days (Fig 2). The smoked fish is called sukako ko maacha. The shelf-life of sukako maacha is 3-4 months at room temperature.

Sukako ko maacha is sold in the local markets of Aitabare and Therathum in Nepal. The product is kept inside the bamboo-made closed basket locally called 'perungo' (Plate 1).

Mode of consumption

Sukako maacha is prepared as curry mixed with tomato, chilli and salt. It is also cooked with vegetable. It is eaten as side-dish in main meal with boiled rice by the Nepalis.

Plate 1. **Sukako ko maacha kept inside bamboo basket locally called 'perungo'**

Fish (*Schizothorax* spp.)
↓
Caught from river
↓
Degutted and washed
↓
Mixed with turmeric powder and salt
↓
Hooked in a bamboo string or spread on a bamboo tray
↓
Smoked above the earthen-oven for 7-10 days
↓
SUKAKO MAACHA

Fig 2. Method of preparation of sukako maacha in Therathum village in Nepal

GNUCHI

Gnuchi is a typical smoked and dried fish product common to the Lepcha tribes of the Darjeeling hills of Kalimpong sub-division living near the Ghish khola (river), and also near the Teesta river in Sikkim. The word 'gnuchi' means smoked fish in the Lepcha language.

Method of preparation

The Lepcha of Gidhang village catche fishes early in the morning in the Ghish river because they think that during this hour the fishes migrate near the banks which makes them easier to catch the fishes using fishing net locally called 'sangli'. Fishes are collected in a bamboo basket locally called 'tamfyok', which is woven by bamboo strips and is properly tied around the waist of the fisherman while fishing. Fishes captured inlcudes *Schizothorax richardsonii* Gray, *Labeo dero* Hamilton, *Acrossocheilus* spp., *Channa* sp., etc. According to the Lepcha fishermen, the best variety for gnuchi is *Schizothorax* sp.

Fishes are kept on a big bamboo tray called 'sarhang' to drain off water, degutted, mixed with salt and turmeric powder. Fish is separated according to their size. The bigger sized fish is selected and spread in an upside down manner on 'sarhang' and is kept above the earthen-oven in kitchen. The small sized fishes are hung one after the other in a bamboo stripe (Plate 2) above the earthen-oven (Plate 3) and keep for 10-14 days, gnuchi is ready to consume (Fig 3). Gnuchi can be kept at room temperature for 2-3 months.

Mode of consumption

Gnuchi is eaten as curry with boiled rice. It is also cooked with vegetable.

Plate 2. **Fish is hung in bamboo-string for gnuchi preparation**

Plate 3. **Smoking the fish for gnuchi preparation in Gidhang village**

Fish
↓
Collected, degutted
↓
Washed
↓
Mixed with turmeric powder and salt
↓
Sieved in a bamboo tray
↓
Spread thoroughly
↓
Smoked above the earthen-oven for 10-14 days
↓
GNUCHI

Fig 3. Traditional method of gnuchi preparation in Gidhang village of Kalimpong

SIDRA

Sidra (Plate 4) is 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.

Method of preparation

During its preparation, the whole fish (*Puntius sarana* Hamilton) is collected, washed and dry in the sun for 4-7 days (Fig. 4). Sidra can be stored at room temperature for 3-4 months for consumption. Sidra is sold at local market in these regions.

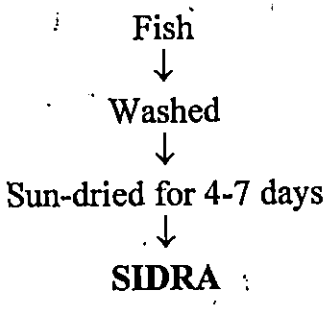


Fig 4. Traditional method of sidra preparation

Mode of consumption

Sidra is consumed as pickle. During pickle making, sidra is roasted and is mixed with dry chili, boiled tomato and salt to make a thick pickle paste. In the typical meal of the Nepalis, cooked rice and 'khalo dal' (black gram soup) is served with sidra pickle.

Plate 4. Sidra

Plate 5. Sukuti

SUKUTI

Sukuti (Plate 5) is also very popular sun-dried fish product among the Nepalis of the eastern Nepal, the Darjeeling hills and Sikkim in India and Bhutan.

Method of preparation

During preparation of sukuti, fish (*Harpodon nehereus* Hamilton) is collected, washed, and rubbed with salt and dry in the sun for 4-7 days (Fig 5). The sun-dried fish product, sukuti is stored at room temperature for 3-4 months for consumption. Sukuti is sold at local markets.

Fish
↓
Washed
↓
Rubbed with salt
↓
Sun-dried for 4-7 days
↓
SUKUTI

Fig 5. Traditional method of sukuti preparation

Mode of consumption

Sukuti is consumed as pickle, soup and curry. During curry preparation, sukuti is fried, mixed with dry chili, onion and salt to make a pickle. It is usually eaten with boiled rice and black gram soup.

NGARI

Ngari is a traditional fermented fish product with typical flavour of Manipur. Ngari preparation and consumption reflects the typical food culture of Manipur.

Method of preparation

During traditional preparation of ngari, whole fish (*Puntius sophore* Hamilton) is collected, rubbed with salt and dry in the sun for 3-4 days. Then, the sun-dried fish is washed briefly and spread on a bamboo mats, filled and pressed tightly in an earthen pot by feet (Plate 6). To the inner wall of the pot, a layer of mustard oil is applied before filling up the fishes. The pot is sealed airtight and then stored at room temperature for 4-6 months (Fig 6). After fermentation, lid is open and ngari is ready for consumption. It can be kept for more than a year at room temperature.

Ngari is sold at every local market in Manipur (Plate 7), and even in Assam.

Mode of consumption

Ngari is one of the most delicious food items in the diet of Manipuri people. It is eaten as side-dish curry. Sometimes, it is mixed with meat and vegetable and is eaten with boiled rice. Ngari is eaten almost daily.

Plate 6. Ngari preparation at Imphal

Plate 7. Ngari is sold at Imphal market

Fish (*Puntius sophore*)
↓
Sun-dried
↓
Washed
↓
Spread on a bamboo mats to dry
↓
Kept in a closed earthen pot
↓
Pressed tightly by feet, sealed the pot
↓
Fermented at room temperature for 4-6 months
↓
NGARI

Fig 6. Traditional method of ngari preparation in Manipur

HENTAK

Hentak (Plate 8) is a ball-like thick paste prepared by fermentation of a mixture of sun-dried fish (*Esomus danricus* Hamilton) powder and petioles of aroid plants (*Alocasia macrorhiza*) in Manipur. Hentek is one of the most delicious indigenous fish products in the local diet of Manipur.

Method of preparation

During the traditional method of preparation of hentak, finger sized fishes (*Esomus danricus* Hamilton) are collected and washed thoroughly, and sun-dried. The dried fishes are crushed to powder. Petioles of *Alocasia macrorhiza* are cut into pieces, washed with water and then exposed to sunlight for one day. An equal amount of the cut pieces of the petioles of *Alocasia macrorhiza* is mixed with powdered fish and ball-like thick paste is made. The ball-like mixture is kept in an earthen pot and the opening of the earthen pot is tightly sealed by a cloth or a lid to maintain the anaerobic condition of the container, and is fermented for 7-9 days preferably under sunshine (Fig 7). Hentak is prepared at every house in Manipur. It is not sold in the local market.

Mode of consumption

Hentak is consumed as curry as well as condiment with boiled rice. Sometime, hentak is given in small amounts to mothers in confinement and patients in convalesce.

Plate 8. Hentak

Fish (*Esomus danricus*)
↓
Sun-dried
↓
Crushed to powder
↓
← Added pieces of petioles of *Alocasia macrorhiza*
↓
Mixed
↓
Ball-like thick paste is made
↓
Kept in a closed earthen pot
↓
Fermented at room temperature for 7-9 days
↓
HENTAK

Fig 7. Traditional method of hentak preparation in Manipur

TUNGTAP

Tungtap (Plate 9) is a fermented fish paste, commonly consumed by the Khasia tribes of Meghalaya. Tungtap is one of the most delicious side-dishes in the diet of Khasia.

Method of preparation

During preparation of tungtap, sun-dried fish (*Danio* spp.) is collected, washed briefly, and is mixed with salt. The salted, sun-dried fish is kept in the earthen pot, made airtight and fermented for 4-7 days (Fig 8). Tungtap is ready for consumption.

Fish
↓
Sun-dried, washed and rubbed with salt
↓
Mixed
↓
Kept inside an airtight bottle/earthen pot
↓
Fermented at room temperature for 4-7 days
↓
TUNGTAP

Fig 8. Method of tungtap preparation in Meghalaya

Mode of consumption

Tungtap is consumed as pickle (Plate 10), mixed with onion, dry chili, garlic and seeds of *Zanthoxylum nitidum*. Sometime, it is cooked to make curry and is eaten with rice.

Plate 9. Tungtap

Plate 10. Tungtap pickle

Plate11. Karati

Plate 12. Dried fish products are sold at Guwahati market in Assam

KARATI, BORDIA and LASHIM

Karati (Plate 11), bordia and lashim are similar types of sun-dried and salted fish products sold at Guwahati markets (Plate 12) in Assam. Karati is prepared from *Gudusia chapra* Hamilton, bordia is prepared from *Pseudeutropius atherinoides* Bloch and lashim is prepared from *Cirrhinus reba* Hamilton. These fish products are eaten as side-dish along with boiled rice in Assam and other north-eastern parts of India.

Fish
↓
Collected
↓
Washed and mixed with salt
↓
Dry in the sun for 5-7 days
↓
KARATI/ BORDIA/LASHIM

Fig 9. Method of preparation of some sun-dried and salted fish products in Assam

MICROORGANISMS

Microbial load

Seventy-two samples of different fish products were collected from different places of Eastern Nepal (Maglung, Therathum and Aitabare), the Darjeeling hills (Gidhang in Kalimpong sub-division), Sikkim (Gangtok), Meghalaya (Shillong), Assam (Guwahati) and Manipur (Imphal) of the Eastern Himalayan regions as shown in Table 1. Samples were analysed for microbial load (Table 2-4). The load of lactic acid bacteria (LAB) was higher ranging from the level of 10^4 cfu/g to 10^8 cfu/g in all fish products analysed. Load of spore-formers was $<10^4$ cfu/g in all samples. No spore-formers were detected in samples of sukako ko maacha of Maglung area and sukuti. Whereas, yeasts population was found at the level of 10^3 cfu/g in sukako ko maacha, ngari, tungtap, karati and bordia only. Filamentous moulds were not recovered in any sample analysed. Total viable count was variable ranging from the level of 10^6 cfu/g to 10^8 cfu/g in sukako ko maacha, $\sim10^7$ cfu/g in ngari, $\sim10^6$ cfu/g in gnuchi, tungtap and lashim, $\sim10^5$ cfu/g in sidra, karati and bordia, $\sim10^4$ cfu/g in sidra and hentak.

Out of 527 isolates of microorganisms isolated from seventy-two samples of different fish products of the Eastern Himalayan regions, 369 were lactic acid bacteria, 77 were spore-formers, 27 were aerobic coccii and 54 were yeasts.

Table 2. Microbial load of fish products collected from Nepal, the Darjeeling hills and Sikkim

Product	Place of collection	Log cfu/g dry weight				
		LAB	Spore-former	Yeast	Mould	TVC
Sukako maacha	Maglung (Nepal)	6.2 (5.4-6.8)	<DL	3.0 (2.8-3.3)	0	6.6 (6.1-6.7)
Sukako maacha	Therathum (Nepal)	8.1 (7.6-8.3)	4.0 (3.6-4.5)	3.7 (3.3-4.0)	<DL	8.3 (7.0-8.5)
Sukako maacha	Aitabare (Nepal)	7.7 (7.2-7.9)	4.1 (3.5-4.6)	3.0 (2.5-3.2)	0	7.8 (7.0-8.4)
Gnuchi	Gidang (Kalimpong)	6.6 (5.8-6.9)	3.2 (3.1-3.5)	<DL	0	6.8 (6.3-7.0)
Sidra	Gangtok (Sikkim)	5.3 (4.8-5.6)	3.1 (2.8-3.3)	<DL	<DL	5.5 (5.2-5.7)
Sukuti	Gangtok (Sikkim)	4.1 (3.6-4.4)	<DL	<DL	<DL	4.6 (3.0-5.1)

LAB, lactic acid bacteria; TVC, total viable count; DL, detection limit (<10 cfu/g).

Data represent the means of 6 samples from each place. Ranges are given in parentheses.

Table 3. Microbial load of fish products collected from Manipur and Meghalaya

Product	Place of collection	Log cfu/g dry weight				
		LAB	Spore-former	Yeast	Mould	TVC
Ngari	Imphal (Manipur)	6.8 (5.8-7.2)	4.2 (3.3-4.6)	3.1 (2.8-3.3)	<DL	7.0 (6.3-7.2)
Hentak	Imphal (Manipur)	4.6 (4.0-4.8)	3.8 (3.3-4.2)	<DL	<DL	4.7 (4.3-4.9)
Tungtap	Shillong (Meghalaya)	5.9 (5.2-6.2)	3.2 (3.5-3.7)	3.0 (2.6-3.5)	<DL	6.2 (5.9-6.4)

LAB, lactic acid bacteria; TVC, total viable count; DL, detection limit (<10 cfu/g).

Data represent the means of 6 samples from each place. Ranges are given in parentheses.

Table 4. Microbial load of fish products collected from Assam

Product	Place of collection	Log cfu/g dry weight				
		LAB	Spore-former	Yeast	Mould	TVC
Karati	Guwahati	4.2 (4.0-4.4)	3.1 (2.8-3.3)	3.1 (2.7-3.3)	<DL	5.1 (4.2-5.5)
Bordia	Guwahati	5.3 (4.3-5.6)	3.5 (2.9-3.8)	2.2 (1.8-2.5)	<DL	5.6 (5.2-6.0)
Lashim	Guwahati	5.8 (4.2-6.2)	2.1 (0-2.6)	3.0 (2.2-3.3)	<DL	6.0 (5.6-6.3)

LAB, lactic acid bacteria; TVC, total viable count; DL, detection limit (<10 cfu/g).

Data represent the means of 6 samples from each place. Ranges are given in parentheses.

Lactic Acid Bacteria (LAB)

Out of 369 lactic acid bacteria strains isolated from seventy-two samples of fish products, 282 isolates were coccii and 87 isolates were non-sporeforming rods (Table 5). All isolates of lactic acid bacteria were Gram-positive, non-sporeforming, non-motile, catalase negative and facultative anaerobes; they did not hydrolyse casein, gelatin and starch (Table 6). Representative strains SM:T1 (Sukako maacha), CG1:B1 (Sidra), SG1:B2 (Sukuti) , SG1:B3 (Sukuti) , SG2:B1 (Sukuti), Ng2:L4 (Ngari), Ng2:L5 (Ngari), T2:L2 (Tungtap) and KA1 (Karati) were coccoid-rod in shape, grew well at 10^0 C and 15^0 C, pH 3.9 but not in pH 9.6, grew in NaCl 6.5 %, produced no gas from glucose. Following sugar fermentation pattern of isolates using API 50 CHL system (Table 6) and the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), strains SM:T1, T2:L2 and KA1 were identified as *Lactococcus lactis* subsp. *cremoris* Schleifer *et al.*, strains CG1:B1, SG1:B3, SG2:B1, Ng2:L4, Ng2:L5 were identified as *Lactococcus plantarum* Scheifer *et al.* and strain SG1:B2 was identified as *Lactococcus lactis* subsp. *lactis* Schleifer *et al.*

Representative strains SM:M1 (Sukako maacha), SM:M2 (Sukako maacha), BA4 (Bordia), BA5 (Bordia) and SG1:B1(Sukuti) were lenticular-coccii in shape, produced gas from glucose, arginine was not hydrolysed. Following the API 50 CHL system (Table 6) and the taxonomical keys described by Sneath *et al.* (1986) and Wood and Holzapfel (1995), strains SM:M1, SM:M2, BA4, BA5 and SG1:B were identified as *Leuconostoc mesenteroides* (Tsenkovskii) van Tieghem.

Representative strains SM:A2 (Sukako maacha) and GG2 (Gnuchi) were coccii in tetrads, grew well in 10 % NaCl but not in 18 % NaCl, produced no gas from glucose. Following the API 50 CHL system (Table

6) and the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), strains SM:A2 and GG2 were identified as *Pediococcus pentosaceus* Mees.

Representative strains SM:A1 (Sukako maacha), GG6 (Gnuchi) and CG1:B2 (Sidra) were coccii in pairs or chains, grew well at 45^0 C, produced no gas from glucose. Following the API 50 CHL system (Table 6) and the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), strains SM:A1and GG6 were identified as *Enterococcus faecium* (Oral- Jensen) Scheifer and Kilpper- Bälz (Plate b), and strain CG1:B2 as *Enterococcus faecalis* (Andrewes and Horder) Scheifer and Kilpper- Bälz.

Representative strains CG1:B3 (Sidra), HL1 (Hentak), H1:B1 (Hentak), T2:L1 (Tungtap), T2:L5 (Tungtap) and LG1 (Lashim) were non-sporeforming rods. On the basis of sugar fermentation using the API system, lactic acid isomer and meso-DAP determination, and also the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), hetero-fermentative lactic CG1:B3 was identified as *Lactobacillus confusus* (Holzapfel and Kandler) Sharpe, Garvie and Tilbury; strains HL1 and T2:L5 were identified as *Lactobacillus fructosus* Kodama (Plate a); whereas homo-fermentative lactic H1:B1 identified as *Lactobacillus amylophilus* Nakamura and Crowell, strain T2:L1 as *Lactobacillus corynifomis* subsp. *torquens* Abo-Elnaga and Kandler and strain LG1as *Lactobacillus plantarum* Orla-Jensen.

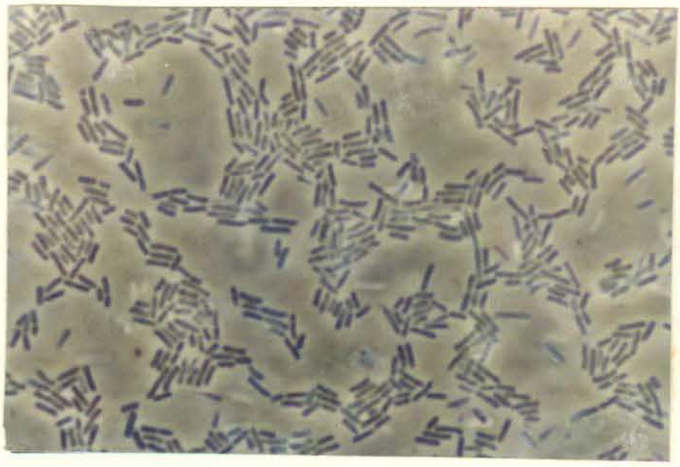


Plate (a). *Lactobacillus fructosu* T2:L5 (MRS agar, 3 d, 30° C) isolated from tungtap, showin non-sporeforming rod cells i phase contrast micrograp (× 825).

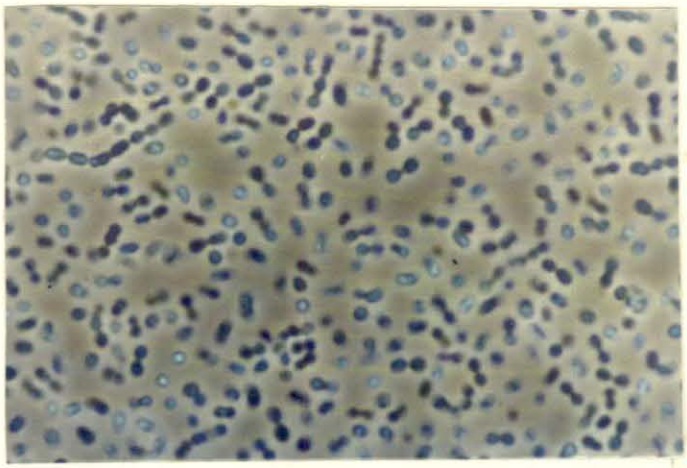


Plate (b). *Enterococcus faecium* SM:A1 (MRS agar, 3 d, 30° C), isolated from sukako maacha, showing coccus cells in phase contrast micrograph (× 825).

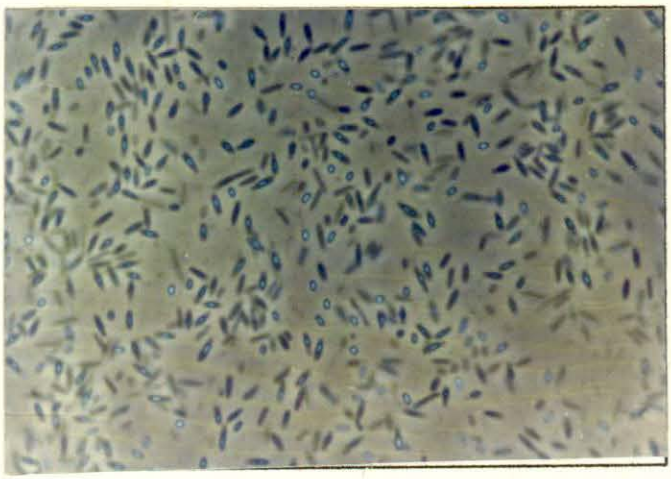


Plate (c). *Bacillus subtilis* MSG1:S1 (NA, 2 d, 30° C), isolated from gnuchi, showing endospore-forming rods in phase contrast micrograph (× 825).

Table 5. Selection of representative strains of LAB, isolated from fish products

Product	Source[a]	Number of strains isolated	Cell shape	Gas from glucose	Arginine hydrolysis	Representative strains
Sukako maacha	Maglung	30	Coccus	–	–	SM:M1; SM:M2
Sukako maacha	Therathum	22	Coccus	+	–	SM:T1
Sukako maacha	Aitabare	24	Coccus	–	+	SM:A1; SM:A2
Gnuchi	Gidang	21	Coccus	–	+	GG2
		20	Coccus	–	–	GG6
Sidra	Gangtok	24	Coccus	–	–	CG1:B1; CG1:B2
		15	Rod	+	+	CG1:B3
Sukuti	Gangtok	23	Coccus	–	–	SG1:B2; SG2:B1
		10	Coccus	+	–	SG1:B1; SG1:B3
Ngari	Imphal	20	Coccus	+	–	Ng2:L4
		25	Coccus	–	–	Ng2:L5
Hentak	Imphal	13	Rod	+	–	HL1
		17	Rod	–	–	H1:B1
		8	Coccus	–	–	H2:B3
Tungtap	Shillong	10	Rod	–	–	T2:L1
		15	Rod	+	–	T2:L5
		13	Coccus	–	–	T2:L2
Karati	Guwahati	19	Coccus	–	–	KA1
Bordia	Guwahati	23	Coccus	+	–	BA4; BA5
Lashim	Guwahati	17	Rod	–	–	LG1

[a]Number of samples was 6 from each source. All isolates were Gram-positive, catalase-negative, non-sporeformers and non-motile.

Table 6: Characteristics of LAB strains isolated from fish products of the Eastern Himalayas

Product	Strain code	Cell Morphology	Cell Size (µm)	Gram-Stain	Catalase	Arginine Hydrolysis	CO_2 from Glucose	Growth at/in								m-Dpm	Lactic Acid Isomer	Sugars Fermented																		Identity
								10° C	15° C	45° C	pH 3.9	pH 9.8	NaCl 6.5%	NaCl 10%	NaCl 18%			Arabinose	Ribose	Xylose	Galactose	Sucrose	Trehalose	Rhamnose	Mannose	Sorbitol	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Raffinose	Melezitose		
Sukako maacha	SM:T1	Coccus	0.9 (0.8-1.2)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	–	+	+	+	+	–	+	+	–	+	–	+	+	–	–	–		*Lactococcus lactis* subsp. *cremoris*
	SM:M1	Coccus	0.8 (0.7-0.9)	+	–	–	+	+	+	–	+	–	+	–	–	nd	D	+	–	+	–	+	+	–	+	–	–	–	–	+	+	+	–	–		*Leuconostoc mesenteroides*
	SM:M2	Coccus	0.8 (0.7-0.9)	+	–	–	+	+	+	–	+	–	+	–	–	nd	D	–	–	+	–	+	+	–	+	–	+	+	–	–	–	–	–	–		*Leuconostoc mesenteroides*
	SM:A1	Coccus	0.9 (0.8-1.2)	+	–	+	–	+	+	+	+	–	+	–	–	nd	L	+	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–			*Enterococcus faecium*
	SM:A2	Coccus, Tetrad	0.8 (0.7-0.9)	+	–	+	–	+	+	+	+	–	+	+	–	nd	DL	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–			*Pediococcus pentosaceus*
Gnuchi	GG2	Coccus, Tetrad	0.8 (0.7-0.9)	+	–	+	–	+	+	+	+	–	+	+	–	nd	DL	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	–	–		*Pediococcus pentosaceus*
	GG6	Coccus	1.1 (0.8-1.2)	+	–	–	–	+	+	+	+	–	+	–	–	nd	L	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	–		*Enterococcus faecium*
Sidra	CG1:B1	Coccus	0.9 (0.8-1.2)	+	–	–	–	+	+	+	+	–	+	–	–	nd	L	–	–	–	–	+	+	–	–	–	–	+	–	+	–	–	–	–		*Lactococcus plantarum*
	CG1:B2	Coccus	0.9 (0.8-1.2)	+	–	–	–	+	+	+	+	–	+	–	–	nd	L	–	+	–	+	+	+	–	+	+	+	+	+	+	+	–	–	+		*Enterococcus faecalis*
	CG1:B3	Rod	1.3 (1.2-1.6) x 0.9 (0.8-1.2)	+	–	+	+	+	+	–	+	–	+	–	–	–	DL	+	+	+	+	–	+	+	+	–	+	+	+	+	–	–	–	–		*Lactobacillus confusus*
Sukuti	SG1:B1	Coccus	0.8 (0.6-0.9)	+	–	–	+	–	–	–	+	–	+	–	–	nd	D	–	+	–	–	+	–	–	+	–	–	+	–	+	–	–	–	–		*Leuconostoc mesenteroides*
	SG1:B2	Coccus	1.1 (0.8-1.2)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	+	–	+	+	+	–	–	–	–	+	–	+	+	–	–	+		*Lactococcus lactis* subsp. *lactis*
	SG1:B3	Coccus	0.9 (0.8-1.2)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–		*Lactococcus plantarum*
	SG2:B1	Coccus	0.8 (0.8-0.8)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–		*Lactococcus plantarum*

nd, not determined

contd. Table 6: Characteristics of LAB strains isolated from fish products of the Eastern Himalayas

Product	Strain code	Cell Morphology	Cell Size (µm)	Gram-stain	Catalase	Arginine Hydrolysis	CO_2 from Glucose	Growth at/in									m-Dpm	Lactic Acid Isomer	Sugars Fermented																		Identity
								10° C	15° C	45° C	pH 3.9	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%				Arabinose	Ribose	Xylose	Galactose	Sucrose	Trehalose	Rhamnose	Mannose	Sorbitol	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Raffinose	Melezitose	
Ngari	Ng2:L4	Coccus	0.9 (0.8-1.2)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	*Lactococcus plantarum*
	Ng2:L5	Coccus	1.0 (0.8-1.2)	+	–	–	–	–	–	–	+	–	+	–	–	nd	L	–	–	–	–	–	+	–	+	–	–	+	–	–	+	–	–	–	*Lactococcus plantarum*
Hentak	HL1	Rod	1.6 (1.2-2.0) x 0.9 (0.8-1.2)	+	–	–	+	+	+	–	+	–	+	–	–	–	D(L)	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	*Lactobacillus fructosus*
	H1:B1	Rod	1.8 (1.6-2.0) x 0.8 (0.4-1.2)	+	–	–	–	+	+	–	+	–	+	–	–	–	L	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	*Lactobacillus amylophilus*
Tungtap	T2:L1	Rod	1.7 (1.6-2.0) x 0.9 (0.8-1.2)	+	–	–	–	–	–	–	+	–	–	–	–	–	D	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	–	+	*Lactobacillus coryniformis* subsp. *torquens*
	T2:L2	Coccus	0.8 (0.4-1.2)	+	–	–	–	–	–	–	+	–	+	–	–	nd	L	–	–	–	+	+	+	–	+	–	–	+	+	+	+	–	–	–	*Lactococcus lactis* subsp. *cremoris*
	T2:L5	Rod	1.3 (1.2-1.6) x 0.8 (0.7-0.9)	+	–	–	+	–	–	–	+	–	–	–	–	–	D(L)	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	*Lactobacillus fructosus*
Karati	KA1	Coccus	0.9 (0.8-1.2)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	–	–	+	–	–	–	–	–	–	+	–	+	–	–	–	–	*Lactococcus lactis* subsp. *cremoris*
Bordia	BA4	Coccus	0.8 (0.8-0.8)	+	–	–	+	–	–	–	+	–	+	–	–	nd	D	+	+	+	+	+	+	–	+	–	–	+	–	+	–	–	–	–	*Leuconostoc mesenteroides*
	BA5	Coccus	0.9 (0.8-1.2)	+	–	–	+	–	+	–	+	–	+	–	–	nd	D	+	+	+	+	+	+	–	+	–	–	+	–	–	–	–	–	–	*Leuconostoc mesenteroides*
Lashim	LG1	Rod	1.6 (1.4-1.7) x 0.8 (0.7-0.9)	+	–	–	–	–	–	–	+	–	+	–	–	+	DL	–	+	–	–	–	–	–	–	–	–	+	–	+	–	+	–	–	*Lactobacillus plantarum*

nd, not determined

Spore-forming rods

Seventy-seven strains of endospore-forming rods were isolated from sixty samples of traditionally processed fish products. All 77 spore-forming strains were Gram-positive, catalase-positive, aerobic and motile (Table 7).

Based on dichomotous key of Slepecky and Hemphill (1992) embodying all 34 species of *Bacillus* described by Claus and Berkeley (1986), representative strains AFM1:S2 (Sukako maacha), MSG1:S1 (Gnuchi) (Plate c), CG2:S1 (Sidra), H1:S1 (Hentak), T1:S1 (Tungtap), K1:S1 (Karati) and BDG1:S1 (Bordia) were identified as *Bacillus subtilis* (Ehrenberg) Cohn; representative strains AFM2:S1 (Sukako maacha), Ng1:S1 (Ngari), LDG1:S1 (Lashim) were identified as *Bacillus pumilus* Meyer and Gottheil (Table 8).

Table 7. Selection of representative strains of spore-formers, isolated from fish products

Product	Source[a]	Number of strains isolated	Cell shape	Catalase	Starch hydrolysis	Representative strains
Sukako maacha	Maglung	10	Rod	+	–	AFM2:S1
	Maglung	8	Rod	+	+	AFM1:S2
Gnuchi	Gidang	7	Rod	+	+	MSG1:S1
Sidra	Gangtok	9	Rod	+	+	CG2:S1
Ngari	Imphal	10	Rod	+	–	Ng1:S1
Hentak	Imphal	8	Rod	+	+	H1:S1
Tungtap	Shillong	8	Rod	+	+	T1:S1
Karati	Guwahati	5	Rod	+	+	K1:S1
Bordia	Guwahati	5	Rod	+	+	BDG1:S1
Lashim	Guwahati	7	Rod	+	–	LDG1:S1

[a]Number of samples was 6 from each source.

All isolates were Gram-positive, aerobic, motile and spore-formers.

Table 8: Characteristics of *Bacillus* species isolated from fish products of the Eastern Himalayas, based on taxonomical keys of Claus and Berkeley (1986); Slepecky and Hemphill (1992)

Product	Strain code	Cell Morphology	Cell Size (µm)	Gram-Stain	Catalase	Gas from Glucose	Acid from Glucose	Nitrate Reduction	Growth at pH 6.8	Growth at NaCl 7.0%	Anaerobic Growth	Starch Hydrolysis	Voges-Proskaeur Reaction	pH in VP Broth	Identity
Sukako maacha	AFM2:S1	Rod	L= 3.5 (3.2-4.0) W= 0.8 (0.5-1.2)	+	+	–	–	–	+	+	–	–	+	5.5	*Bacillus pumilus*
	AFM1:S2	Rod	L= 2.9 (2.8-3.2) W= 0.5 (0.4-0.8)	+	+	–	–	–	+	+	–	+	+	5.5	*Bacillus subtilis*
Gnuchi	MSG1:S1	Rod	L= 3.5 (3.2-4.0) W= 0.7 (0.4-0.8)	+	+	–	–	–	+	+	–	+	+	5.5	*Bacillus subtilis*
Sidra	CG2:S1	Rod	L= 3.3 (2.8-3.2) W= 1.1 (0.8-1.2)	+	+	–	–	–	+	+	–	+	+	5.5	*Bacillus subtilis*
Ngari	Ng1:S1	Rod	L= 3.5 (3.2-4.0) W= 0.7 (0.4-0.8)	+	+	–	–	–	+	+	–	–	+	5.5	*Bacillus pumilus*
Hentak	H1:S1	Rod	L= 3.3 (2.0-4.0) W= 0.7 (0.4-0.8)	+	+	–	–	+	+	+	–	+	+	5.5	*Bacillus subtilis*
Tungtap	T1:S1	Rod	L= 3.7 (3.2-4.0) W= 0.8 (0.8-0.8)	+	+	–	–	–	+	+	–	+	+	5.5	*Bacillus subtilis*
Karati	K1:S1	Rod	L= 3.5 (3.2-4.0) W= 1.2 (0.8-1.6)	+	+	–	–	+	+	+	–	+	+	5.5	*Bacillus subtilis*
Bordia	BDG1:S1	Rod	L= 3.3 (2.0-4.0) W= 0.7 (0.4-0.8)	+	+	–	–	+	+	+	–	+	+	5.5	*Bacillus subtilis*
Lashim	LDG1:S1	Rod	L= 2.9 (2.8-3.2) W= 0.5 (0.4-0.8)	+	+	–	–	–	+	+	–	–	+	5.5	*Bacillus pumilus*

L, length; W, Width

Aerobic coccii

Twenty-seven strains of aerobic coccii were isolated from 18 samples of fish products (Table 9).

All isolates were Gram-positive, coccii in tetrads and also in clusters, non-spore-formers, non-motile, catalase-positive and arginine not hydrolysed (Table 10). Following the taxonomical key of Sneath *et al.* (1986), all representative strains SM:M1 (Sukako maacha), GM1 (Gnuchi), CG2:M1 (Sidra), SG1:M3 (Sukuti) and Ng1:M2 (Ngari) were identified as *Micrococcus*. However, species could not be identified due to limited tests.

Table 9. Selection of representative strains of aerobic coccii, isolated from fish products

Product	Source[a]	Number of strains isolated	Cell shape	Catalase	Arginine hydrolysis	Representative strains
Sukako maacha	Maglung	7	Coccus, Tetrad	+	–	SM:M1
Gnuchi	Gidang	5	Coccus, Tetrad	+	–	GM1
Sidra	Gangtok	6	Coccus, Tetrad	+	–	CG2:M1
Sukuti	Gangtok	4	Coccus, Tetrad	+	–	SG1:M3
Ngari	Imphal	5	Coccus, Tetrad	+	–	Ng1:M2

[a]Number of samples was 6 from each source.

All isolates were Gram-positive, non-motile and non-sporeformers.

Table 10: Characteristics of aerobic cocci isolated from fish products of the Eastern Himalayas

Product	Strain code	Cell Morphology	Cell Size Diameter (µm)	Gram-Stain	Catalase	Arginine Hydrolysis	CO_2 from Glucose	Motility	Anaerobic growth	Nitrate reduction	Growth In/at							Sugars Fermented					Identity
											37° C	45° C	pH 3.9	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%	Glucose	Galactose	Mannose	Esculin	Lactose	
Sukako maacha	SM:M1	Coccus, Tetrad	1.4 (0.6-2.0)	+	+	–	–	–	–	–	+	–	–	–	+	–	–	+	–	–	–	–	*Micrococcus* sp.
Gnuchi	GM1	Coccus, Tetrad	1.2 (0.5-1.8)	+	+	–	–	–	–	–	+	–	–	–	+	–	–	+	–	–	–	–	*Micrococcus* sp.
Sidra	CG2:M1	Coccus, Tetrad	0.9 (0.4-1.8)	+	+	–	–	–	–	–	+	–	–	–	+	–	–	+	–	–	–	–	*Micrococcus* sp.
Sukuti	SG1:M3	Coccus, Tetrad	1.3 (0.5-2.0)	+	+	–	–	–	–	–	+	–	–	–	+	–	–	+	–	–	–	–	*Micrococcus* sp.
Ngari	Ng1:M2	Coccus, Tetrad	1.0 (0.6-2.0)	+	+	–	–	–	–	–	+	–	–	–	+	–	–	+	–	–	–	–	*Micrococcus* sp.

Yeasts

Representative strains of yeasts were selected on the basis of colony, cell morphology and type of mycelium among 54 yeasts isolates, isolated from traditionally processed fish products of the Eastern Himalayan regions (Table 11). None of these strains produced ascus and ascospores.

Table 11. Selection of representative strains of yeasts

Product	Source[a]	Number of strains isolated	Colony shape	Cell shape	Mycelium	Representative strains
Sukako maacha	Maglung	11	Ss	Oval	Absent	AFM1:Y1
Sukako maacha	Therathum	10	Ss	Oval	Pseudo	AFM2:Y2
Ngari	Imphal	8	Ss	Oval	Absent	Ng:Y1
Tungtap	Shillong	6	Ss	Oval	Pseudo	T1:Y1
		6	Ds	Cylindrical	True	T1:Y2
Karati	Guwahati	8	Ss	Oval	Absent	K1:Y1
Bordia	Guwahati	5	Ss	Oval	Absent	BDG:Y1

[a]Number of samples was 6 from each source.

All isolates reproduced by multilateral budding except T1:Y2-group.

Ss, smooth surface; Ds, dusty surface.

Sugar fermentation and assimilation tests of randomly selected oval-shaped as well as cylindrical–shaped representative strains of yeasts were carried out (Table 12).

Table 12: Characteristics of yeasts isolated from fish products of the Eastern Himalayas

Product	Strain code	Colony Morphology	Cell Morphology	Cell Size (µm)	Mycelium	Nitrate Reduction	Growth at 37°C	Sugars Fermented								Sugars Assimilated														Identity
								Glucose	Galactose	Lactose	Maltose	Raffinose	Sucrose	Starch	Trehalose	Arabinose	Cellobiose	Galactose	Glycerol	Inositol	Lactose	Maltose	Melibiose	Mannitol	Rhamnose	Raffinose	Sucrose	Starch	Trehalose	Xylose	
Sukako maacha	AFM1:Y1	Ss	Oval	L= 5.6 (4.0-6.0) W= 3.0 (2.5-4.2)	Absent	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	+	–	+	–	+	+	–	+	–	***Candida* sp.**
	AFM2:Y2	Ss	Oval	L= 5.1 (5.0-5.3) W= 3.1 (3.0-3.2)	Pseudo	–	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	+	+	+	–	+	+	–	+	+	***Candida chiropterorum***
Ngari	Ng:Y1	Ss	Oval	L= 6.2 (6.1-6.4) W= 3.2 (2.2-4.5)	Absent	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	+	+	–	+	–	+	+	–	+	+	***Candida* sp.**
Tungtap	T1:Y1	Ss	Oval	L= 7.1 (5.7-7.7) W= 4.7 (3.5-4.9)	Pseudo	–	+	+	–	–	–	–	+	–	–	+	–	+	+	–	–	+	–	+	–	–	+	–	–	+	***Candida bombicola***
	T1:Y2	Ds	Cylindrical	L= 23.6 (20.1-25.0) W= 5.6 (4.1-7.1)	True	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	+	–	+	–	–	+	–	–	+	***Saccharomycopsis* sp.**
Karati	K1:Y1	Ss	Oval	L= 7.8 (7.6-7.9) W= 3.1 (2.1-3.3)	Absent	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	+	+	–	+	–	+	+	–	+	+	***Candida* sp.**
Bordia	BDG:Y1	Ss	Oval	L= 4.1 (4.1-4.2) W= 2.1 (2.0-2.2)	Absent	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	+	+	–	+	–	+	+	–	+	+	***Candida* sp.**

Ss, smooth surface; Ds, dusty surface; L, length; W, Width; Ascus and ascospores were absent in all strains.

Following the taxonomical keys described by Kreger-van Rij (1984) and Kurtzman and Fell (1998), the oval-shaped strain AFM2:Y2 (Sukako maacha) was identified as *Candida chiropterorum* Grose *et* Marinkelle (Plate e); and another oval-shaped strain T1:Y1 (Tungtap) as *Candida bombicola* (Spencer, Gorin *et* Tulloch) Meyer *et* Yarrow (Spencer *et al.*). Strains AFM1:Y1 (Sukako maacha), Ng:Y1 (Ngari), K1:Y1 (Karati) and BDG:Y1 (Bordia) were grouped as *Candida.* Species could not be identified.

Strain T1:Y2 (Tungtap) had dusty and dry surfaced colonies with horn-like projections made up of many strands of mycelia when grown on agar plates, and were cylindrical in shape. This strain was identified as *Saccharomycopsis* (Plate d). However, species identification could not be confirmed.

Prevalence of microorganisms

Prevalence of LAB was 100 %, whereas that of *Bacillus* species, *Micrococcus* species and yeasts was only 83 %, 41 % and 53 % in seventy-two samples analysed, respectively (Fig 10). Data shows (Fig 11) the dominant microflora in 72 samples of fish products was LAB (70%) followed by *Bacillus* spp. (15%), yeasts (10%) and *Micrococcus* (5%).

Out of 369 lactics isolated from the different fish products, 77% of coccii dominant the lactic flora, whereas only 27% rods were present in the fish products (Fig 12).

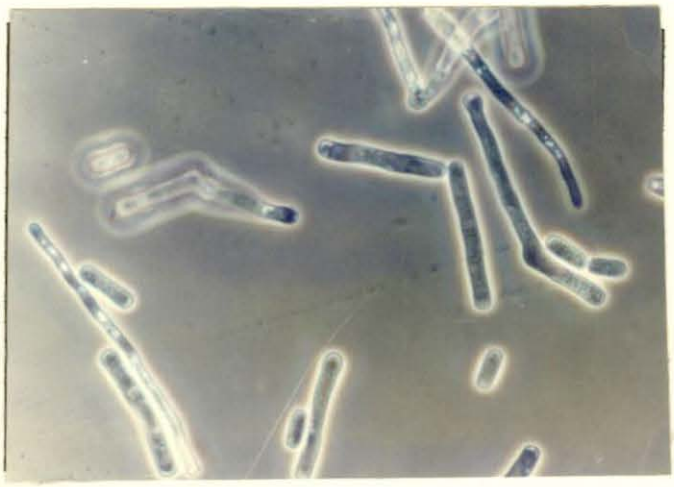
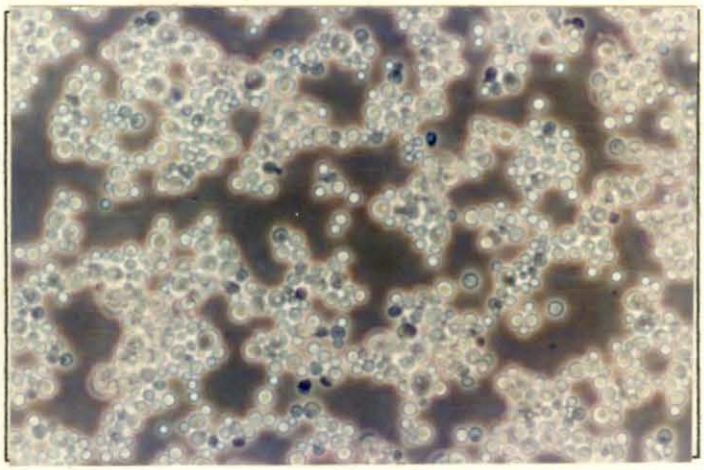


Plate (d). *Saccharomycopsis* sp. T1:Y2 (YM agar, 3 d, 28° C), isolated from tungtap, showing cylindrical cells with true mycelia in phase contrast micrograph (× 330).



Plate(e). *Candida chiropterorum* AFM2:Y2 (YM agar, 3 d, 28° C), isolated from sukako maacha, showing elliptical cells with budding in phase contrast micrograph (× 330).

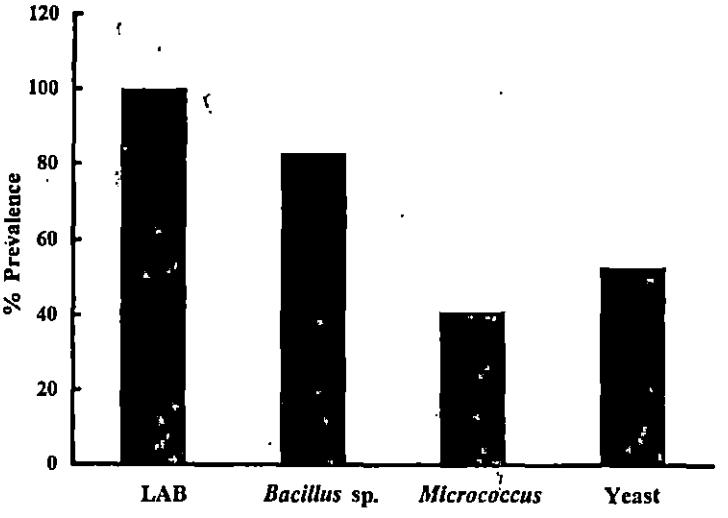


Fig 10. Prevalence of microorganisms in fish products of the Eastern Himalayas (EH)

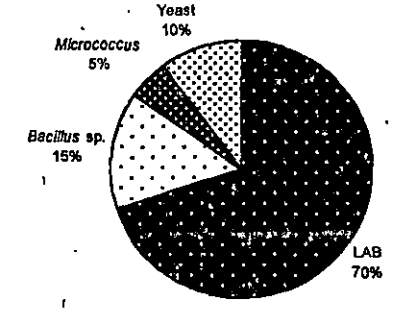


Fig 11. Distribution of microflora in fish products of EH

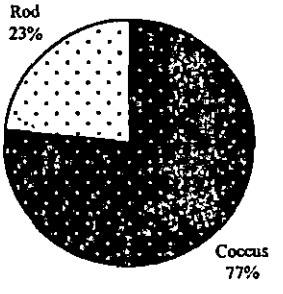


Fig 12. Distribution of LAB in fish products of EH

Occurrence of pathogenic contaminants

Pathogenic contaminants mainly *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae in the traditionally processed fish products were detected (Table 13-15). *Bacillus cereus* occurred in all products except sukako maacha collected from Therathum and Aitabare at the level of $<10^2$ cfu/g (Fig 13). Load of *Staphylococcus aureus* was $<10^3$ cfu/g in samples tested (Fig 13). Enterobacteriaceae occurred in all fish product samples, though the load was found not more than 10^3 cfu/g (Fig 13). Prevalence of *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae in the fish products analysed was 66%, 54.7% and 68.3%, respectively (Fig 14).

Table 13. Occurrence of *Bacillus cereus*, *Staphylococcus aureus* and Enterobacteriaceae in fish products collected from Nepal, the Darjeeling hills and Sikkim

Product	Place of collection	Log cfu/g dry weight		
		B. cereus	*S. aureus*	Enterobacteriaceae
Sukako maacha	Maglung (Nepal)	2.0 (0-2.2)	2.2 (0-2.5)	2.8 (0-3.0)
Sukako maacha	Therathum (Nepal)	0	2.0 (0-2.3)	2.0 (0-2.2)
Sukako maacha	Aitabare (Nepal)	0	2.7 (0-2.9)	2.1 (0-2.4)
Gnuchi	Gidang (Kalimpong)	1.2 (0-1.3)	3.1 (0-3.3)	3.3 (0-3.6)
Sidra	Gangtok (Sikkim)	2.0 (1.2-2.4)	3.0 (1.6-3.2)	2.7 (1.9-3.0)
Sukuti	Gangtok (Sikkim)	1.5 (1.2-1.8)	3.1 (2.3-3.5)	2.3 (1.8-2.7)

Data represent the means of 5 samples from each place. Ranges are given in parentheses.

Table 14. Occurrence of *Bacillus cereus*, *Staphylococcus aureus* and Enterobacteriaceae in fish products collected from Manipur and Meghalaya

Product	Place of collection	Log cfu/g dry weight		
		B. cereus	*S. aureus*	Enterobacteriaceae
Ngari	Imphal (Manipur)	2.3 (1.8-2.6)	3.0 (2.5-3.5)	3.3 (3.1-3.5)
Hentak	Imphal (Manipur)	2.2 (1.7-2.4)	2.8 (2.4-3.0)	3.0 (2.7-3.3)
Tungtap	Shillong (Meghalaya)	2.3 (1.7-2.5)	0	3.5 (2.9-3.9)

Data represent the means of 5 samples from each place. Ranges are given in parentheses.

Table 15. Occurrence of *Bacillus cereus*, *Staphylococcus aureus* and Enterobacteriaceae in fish products collected from Assam

Product	Place of collection	Log cfu/g dry weight		
		B. cereus	*S. aureus*	Enterobacteriaceae
Karati	Guwahati	2.6 (2.2-2.8)	1.8 (0-2.0)	2.8 (2.0-3.0)
Bordia	Guwahati	2.4 (1.8-2.8)	2.0 (0-2.2)	3.2 (2.8-3.5)
Lashim	Guwahati	2.2 (1.8-2.5)	2.0 (0-2.3)	3.1 (2.6-3.3)

Data represent the means of 5 samples from each place. Ranges are given in parentheses.

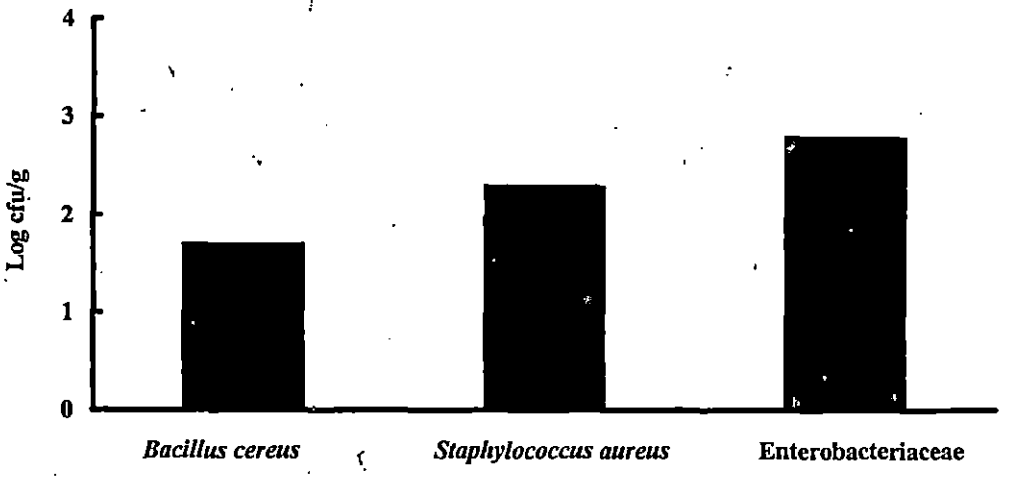


Fig 13. Average microbial load of *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae in fish products of EH

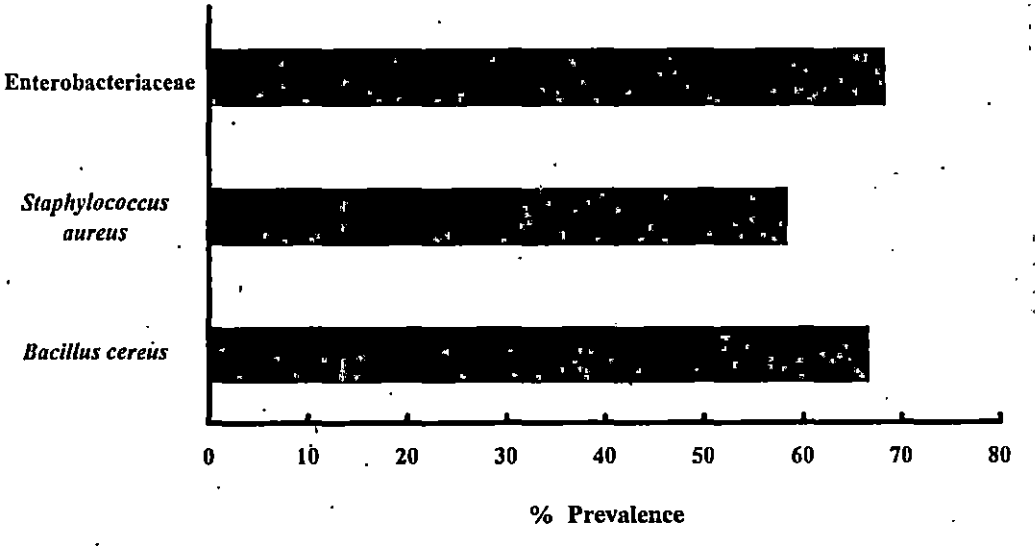


Fig 14. Prevalence of *Bacillus cereus*, *Staphylococcus aureus* and enterobacteriaceae in fish products of EH

Enzymatic activity

Thirty three selected representative strains of species of lactic acid bacteria and *Bacillus*, isolated from different fish products were tested for proteolytic activity (Table 16), for amylolytic activity (Table 17) and lipolytic activity (Table 18). Out of 25 LAB strains tested, only three strains, viz. *Enterococcus faecium* GG6, *Lactobacillus cornyformis* subsp. *torquens* T2:L1 and *Leuconostoc mesenteroides* BA4 showed proteolytic activity (showing >2 mm hydrolysis zone in milk agar plate), though the estimated protease activity of these strains was <1 U/ml. Whereas, all the strains of *Bacillus* showed proteolytic activity. However, the protease activity of these *Bacillus* strains, estimated was not more than 4 U/ml (Table 16). Seven strains of LAB were screened for amylolytic activity (showing >2 mm hydrolysis zone in starch agar plate) and α-amylase activity was estimated which was recorded 3.2 U/ml to 5.8 U/ml (Table 17). All *Bacillus subtilis* strains showed amylolytic activity, whereas *Bacillus pumilus* strains did not show any amylolytic activity. Six strains of LAB showed lipolytic activity on tributyrin agar plates and only four strains of *Bacillus* showed lipolytic activity (Table 18).

Enzymatic profiles of randomly selected lactic acid bacteria strains of fish products were assayed using the API zym (bioMérieux, France) galleries (Table 19). Each of the predominant LAB strains produced a wide spectrum of enzymes. These strains showed relatively weak esterase and no lipase (C14) activities. *Lactoctoccus lactis* subsp. *cremoris* SM:T1 and *Enterococcus faecium* SM:A1 showed strong phosphatase activities, while other strains showed moderate activities (Table 19). However, they showed no detectable proteinase activity with the methods applied.

Table16. Proteolytic activity of the selected strains, isolated from fish products

Product	Strain	Casein hydrolysis	Protease[a] (U/ml)
Sukako maacha	*Lactococcus lactis* subsp. *cremoris* SM:T1	–	
Sukako maacha	*Leuconostoc mesenteroides* SM:M1	–	
Sukako maacha	*Leuconostoc mesenteroides* SM:M2	–	
Sukako maacha	*Enterococcus faecium* SM:A1	–	
Sukako maacha	*Pediococcus pentosaceus* SM:A2	–	
Gnuchi	*Pediococcus pentosaceus* GG2	–	
Gnuchi	*Enterococcus faecium* GG6	+	0.8
Sidra	*Lactococcus plantarum* CG1:B1	–	
Sidra	*Enterococcus faecalis* CG1:B2	–	
Sidra	*Lb. confusus* CG1:B3	–	
Sukuti	*Leuconostoc mesenteroides* SG1:B1	–	
Sukuti	*Lactococcus lactis* subsp. *lactis* SG1:B2	–	
Sukuti	*Lactococcus plantarum* SG1:B3	–	
Sukuti	*Lactococcus plantarum* SG2:B1	–	
Ngari	*Lactococcus plantarum* Ng2:L4	–	
Ngari	*Lactococcus plantarum* Ng2:L5	–	
Hentak	*Lb. fructosus* HL1	–	
Hentak	*Lb. amylophilus* H1:B1	–	
Tungtap	*Lb. coryniformis* subsp. *torquens* T2:L1	+	1.0
Tungtap	*Lactococcus lactis* subsp. *cremoris* T2:L2	–	
Tungtap	*Lactobacillus fructosus* T2:L5	–	
Karati	*Lactococcus lactis* subsp. *cremoris* KA1	–	
Bordia	*Leuconostoc mesenteroides* BA4	+	0.8
Bordia	*Leuconostoc mesenteroides* BA5	–	
Lashim	*Lactobacillus plantarum* LG1	–	
Sukako maacha	*Bacillus pumilus* AFM2:S1	+	1.0
Sukako maacha	*Bacillus subtilis* AFM1:S2	+	2.4
Gnuchi	*Bacillus subtilis* MSG1:S1	+	3.7
Ngari	*Bacillus pumilus* Ng1:S2	+	1.0
Tungtap	*Bacillus subtilis* T1:S1	+	2.7
Karati	*Bacillus subtilis* K1:S1	+	4.5
Bordia	*Bacillus subtilis* BDG1:S1	+	4.3
Lashim	*Bacillus pumilus* LDG1:S1	+	1.0

[a]Only the strains showing positive casein hydrolysis agar test (>2.0 mm) were assayed for protease activity. Data represent the means of three set. Lb, *Lactobacillus*

Table 17. Amylolytic activity of the selected strains, isolated from fish products

Product	Strain	Starch hydrolysis	∝-amylase[a] (U/ml)
Sukako maacha	*Lactococcus lactis* subsp. *cremoris* SM:T1	–	
Sukako maacha	*Leuconostoc mesenteroides* SM:M1	+	4.5
Sukako maacha	*Leuconostoc mesenteroides* SM:M2	–	
Sukako maacha	*Enterococcus faecium* SM:A1	–	
Sukako maacha	*Pediococcus pentosaceus* SM:A2	+	3.2
Gnuchi	*Pediococcus pentosaceus* GG2	–	
Gnuchi	*Enterococcus faecium* GG6	–	
Sidra	*Lactococcus plantarum* CG1:B1	–	
Sidra	*Enterococcus faecalis* CG1:B2	+	5.8
Sidra	*Lb. confusus* CG1:B3	–	
Sukuti	*Leuconostoc mesenteroides* SG1:B1	+	4.0
Sukuti	*Lactococcus lactis* subsp. *lactis* SG1:B2	–	
Sukuti	*Lactococcus plantarum* SG1:B3	–	
Sukuti	*Lactococcus plantarum* SG2:B1	–	
Ngari	*Lactococcus plantarum* Ng2:L4	–	
Ngari	*Lactococcus plantarum* Ng2:L5	+	4.4
Hentak	*Lb. fructosus* HL1	–	
Hentak	*Lb. amylophilus* H1:B1	–	
Tungtap	*Lb. coryniformis* subsp. *torquens* T2:L1	–	
Tungtap	*Lactococcus lactis* subsp. *cremoris* T2:L2	+	3.1
Tungtap	*Lactobacillus fructosus* T2:L5	–	
Karati	*Lactococcus lactis* subsp. *cremoris* KA1	–	
Bordia	*Leuconostoc mesenteroides* BA4	+	4.2
Bordia	*Leuconostoc mesenteroides* BA5	–	
Lashim	*Lactobacillus plantarum* LG1	–	
Sukako maacha	*Bacillus pumilus* AFM2:S1	–	
Sukako maacha	*Bacillus subtilis* AFM1:S2	+	4.0
Gnuchi	*Bacillus subtilis* MSG1:S1	+	4.4
Ngari	*Bacillus pumilus* Ng1:S2	–	
Tungtap	*Bacillus subtilis* T1:S1	+	3.2
Karati	*Bacillus subtilis* K1:S1	+	3.0
Bordia	*Bacillus subtilis* BDG1:S1	+	3.0
Lashim	*Bacillus pumilus* LDG1:S1	–	

[a]Only the strains showing positive starch hydrolysis (>2 mm) agar test were selected for determination of ∝-amylase assay. Data represent the means of three set.

Table 18. Lipolytic activity of selected strains using tributyrin agar plates

Product	Strain	Lipolytic activity
Sukako maacha	*Lactococcus lactis* subsp. *cremoris* SM:T1	–
Sukako maacha	*Leuconostoc mesenteroides* SM:M1	–
Sukako maacha	*Leuconostoc mesenteroides* SM:M2	+
Sukako maacha	*Enterococcus faecium* SM:A1	+
Sukako maacha	*Pediococcus pentosaceus* SM:A2	–
Gnuchi	*Pediococcus pentosaceus* GG2	–
Gnuchi	*Enterococcus faecium* GG6	–
Sidra	*Lactococcus plantarum* CG1:B1	–
Sidra	*Enterococcus faecalis* CG1:B2	–
Sidra	*Lb. confusus* CG1:B3	–
Sukuti	*Leuconostoc mesenteroides* SG1:B1	–
Sukuti	*Lactococcus lactis* subsp. *lactis* SG1:B2	–
Sukuti	*Lactococcus plantarum* SG1:B3	+
Sukuti	*Lactococcus plantarum* SG2:B1	–
Ngari	*Lactococcus plantarum* Ng2:L4	–
Ngari	*Lactococcus plantarum* Ng2:L5	+
Hentak	*Lb. fructosus* HL1	–
Hentak	*Lb. amylophilus* H1:B1	–
Tungtap	*Lb. coryniformis* subsp. *torquens* T2:L1	–
Tungtap	*Lactococcus lactis* subsp. *cremoris* T2:L2	–
Tungtap	*Lactobacillus fructosus* T2:L5	+
Karati	*Lactococcus lactis* subsp. *cremoris* KA1	–
Bordia	*Leuconostoc mesenteroides* BA4	–
Bordia	*Leuconostoc mesenteroides* BA5	–
Lashim	*Lactobacillus plantarum* LG1	+
Sukako maacha	*Bacillus pumilus* AFM2:S1	–
Sukako maacha	*Bacillus subtilis* AFM1:S2	–
Gnuchi	*Bacillus subtilis* MSG1:S1	–
Ngari	*Bacillus pumilus* Ng1:S2	–
Tungtap	*Bacillus subtilis* T1:S1	+
Karati	*Bacillus subtilis* K1:S1	+
Bordia	*Bacillus subtilis* BDG1:S1	+
Lashim	*Bacillus pumilus* LDG1:S1	+

+ = the clear zone was >2 mm

Table 19. Enzymatic profiles of selected strains of LAB, isolated from fish products using API-zym system

Enzyme	Activity (nanomoles)					
	A	B	C	D	E	F
Control (without enzyme)	0	0	0	0	0	0
Phosphatase alkaline	≥40	≥40	20	10	10	20
Esterase (C4)	5	5	5	5	5	5
Esterase Lipase (C8)	5	10	5	10	5	5
Lipase (C14)	0	0	0	0	0	0
Leucine arylamidase	5	10	10	10	5	5
Valine arylamidase	0	5	5	5	5	0
Cystine arylamidase	5	5	5	5	5	5
Trypsin	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0
Phoshatase acid	≥40	≥40	20	10	10	10
Napthol-AS-BI-phosphohydrolase	5	10	5	10	5	10
α-galactosidase	0	0	0	0	0	0
β-galactosidase	0	0	20	0	0	0
β-glucuronidase	0	0	0	0	0	0
α-glucosidase	5	5	10	5	0	0
β-glucosidase	0	0	0	0	0	0
N-acetyl-β-glucosaminidase	0	0	0	0	0	0
α-mannosidase	0	0	0	0	0	0
α-fucosidase	0	0	0	0	0	0

A = *Lactococcus lactis* subsp. *cremoris* SM:T1; **B** = *Enterococcus faecium* SM:A1; **C** = *Pediococcus pentosaceus* GG2; **D** = *Leuconostoc mesenteroides* SG1: B1; **E** = *Lactococcus plantarum* Ng2:L4; **F** = *Lactobacillus plantarum* LG1.

Data represent the means of 2 replicate sets.

Antimicrobial activity

Table (20) shows the antagonistic properties of the lactic acid bacterial strains and *Bacillus* strains, isolated from fish products which were tested against the indicator strains (*Listeria monocytogenes* DSM 20600), *Bacillus cereus* CCM 2010, *Enterococcus faecium* DSM 20477 and *Streptococcus mutans* DSM 6178). Only 7 strains of LAB showed antimicrobial activities against the indicator strains used. *Enterococcus faecium* SM:A1, *Pediococcus pentasaceus* GG2, *Lactococcus plantarum* CG1:B1 and *Lactococcus plantarum* SG1:B3 inhibited the growth of *Listeria monocytogenes* DSM 20600. *Leuconoctoc mensenteroides* BA4 showed inhibition zone against *Bacillus cereus* CCM 2010, *Lactobacillus coryniformis* subsp. *torquens* T2:L1 against *Enterococcus faecium* DSM 20477 and *Lactococcus lactis* subsp. *cremoris* KA1 against *Streptococcus mutans* DSM 6178. Three strains of *Bacillus subtilis* showed the antagonistic properties against the indicator strains (Table 19), none of the strains of *Bacillus pumilus* showed the antimicrobial activity in the applied method.

None of the strains were found to produce any bcteriocin with the methods applied (data not shown).

Table 20. Antimicrobial activities of strains, isolated from fish products against the indicator strains

Test Strain	Indicator Strains			
	Listeria monocytogenes DSM 20600	*Bacillus cereus* CCM 2010	*Enterococcus faecium* DSM 20477	*Streptococcus mutans* DSM 6178
Lactococcus lactis subsp. *cremoris* SM:T1	–	–	–	–
Leuconostoc mesenteroides SM:M1	–	–	–	–
Leuconostoc mesenteroides SM:M2	–	–	–	–
Enterococcus faecium SM:A1	+	–	–	–
Pediococcus pentosaceus SM:A2	–	–	–	–
Pediococcus pentosaceus GG2	+	–	–	–
Enterococcus faecium GG6	–	–	–	–
Lactococcus plantarum CG1:B1	+	–	–	–
Enterococcus faecalis CG1:B2	–	–	–	–
Lb. confusus CG1:B3	–	–	–	–
Leuconostoc mesenteroides SG1:B1	–	–	–	–
Lactococcus lactis subsp. *lactis* SG1:B2	–	–	–	–
Lactococcus plantarum SG1:B3	+	–	–	–
Lactococcus plantarum SG2:B1	–	–	–	–
Lactococcus plantarum Ng2:L4	–	–	–	–
Lactococcus plantarum Ng2:L5	–	–	–	–
Lactobacillus fructosus HL1	–	–	–	–
Lb. amylophilus H1:B1	–	–	–	–
Lb. coryniformis subsp. *torquens* T2:L1	–	–	+	–
Lactococcus lactis subsp. *cremoris* T2:L2	–	–	–	–
Lb. fructosus T2:L5	–	–	–	–
Lactococcus lactis subsp. *cremoris* KA1	–	–	–	+
Leuconostoc mesenteroides BA4	–	+	–	–
Leuconostoc mesenteroides BA5	–	–	–	–
Lb. plantarum LG1	–	–	–	–
Bacillus pumilus AFM2:S1	–	–	–	–
Bacillus subtilis AFM1:S2	–	–	–	–
Bacillus subtilis MSG1:S1	–	–	+	–
Bacillus pumilus Ng1:S2	–	–	–	–
Bacillus subtilis T1:S1	–	–	–	+
Bacillus subtilis K1:S1	–	–	–	–
Bacillus subtilis BDG1:S1	–	+	–	–
Bacillus pumilus LDG1:S1	–	–	–	–

+, inhibition zone was >1 mm; –, no inhibition zone

Biogenic amines

Twenty representative strains of lactic acid bacteria as well as eight representative strains of *Bacillus* spp. were screened for their ability to produce biogenic amines with the surface plate method as described by Bover-Cid and Holzapfel (1999). Interestingly, none of the strains produced tyramine, cadaverine, histidine and putrescine in the applied method (Table 21).

Hydrophobicity

Table 22 shows the percentage hydrophobicity of the lactic acid bacteria isolated from traditionally processed fish products. Nine strains of lactic acid bacteria showed high degrees of hydrophobicity (>75%), among which *Pediococcus pentosaceus* GG2 (isolated from gnuchi) showed the highest percentage of hydrophobicity of 94% (Fig 15). All tested strains had more than 30% hydrophobicity.

Proximate composition

Proximate composition of traditionally processed fish products namely, sukako maacha, gnuchi, sidra, sukuti, ngari, hentak, tungtap, karati, bordia and lashim as well as fresh river fish of Sikkim is presented in Table 23. The pH of all these products was 6.2-6.5 with titratable acidity ranging from 0.5 to 1.1%. Moisture content was low upto 10 % in sukako maacha, however, hentak, the simi-paste product contained 40% of moisture. High content of protein was observed in all fish products. Even the ash content was also found to be around 16%. Among the minerals of the fish products, calcium content was higher than other minerals estimated. Among the fish products analysed, gnuchi contained highest amount of calcium and magnesium (Table 24).

Table 21. Screening of biogenic amines producing strains isolated from fish products

Product	Strain	Tyr	Lys	His	Orn
Sukako maacha	*Lactococcus lactis* subsp. *cremoris* SM:T1	–	–	–	–
Sukako maacha	*Leuconostoc mesenteroides* SM:M1	–	–	–	–
Sukako maacha	*Leuconostoc mesenteroides* SM:M2	–	–	–	–
Sukako maacha	*Enterococcus faecium* SM:A1	–	–	–	–
Sukako maacha	*Pediococcus pentosaceus* SM:A2	–	–	–	–
Gnuchi	*Pediococcus pentosaceus* GG2	–	–	–	–
Gnuchi	*Enterococcus faecium* GG6	–	–	–	–
Sidra	*Lactococcus plantarum* CG1:B1	–	–	–	–
Sidra	*Enterococcus faecalis* CG1:B2	–	–	–	–
Sidra	*Lactobacillus confusus* CG1:B3	–	–	–	–
Sukuti	*Leuconostoc mesenteroides* SG1:B1	–	–	–	–
Sukuti	*Lactococcus lactis* subsp. *lactis* SG1:B2	–	–	–	–
Sukuti	*Lactococcus plantarum* SG1:B3	–	–	–	–
Sukuti	*Lactococcus plantarum* SG2:B1	–	–	–	–
Ngari	*Lactococcus plantarum* Ng2:L4	–	–	–	–
Ngari	*Lactococcus plantarum* Ng2:L5	–	–	–	–
Hentak	*Lactobacillus fructosus* HL1	–	–	–	–
Hentak	*Lactobacillus amylophilus* H1:B1	–	–	–	–
Tungtap	*Lactobacillus coryniformis* subsp. *torquens* T2:L1	–	–	–	–
Tungtap	*Lactococcus lactis* subsp. *cremoris* T2:L2	–	–	–	–
Tungtap	*Lactobacillus fructosus* T2:L5	–	–	–	–
Karati	*Lactococcus lactis* subsp. *cremoris* KA1	–	–	–	–
Bordia	*Leuconostoc mesenteroides* BA4	–	–	–	–
Bordia	*Leuconostoc mesenteroides* BA5	–	–	–	–
Lashim	*Lactobacillus plantarum* LG1	–	–	–	–
Sukako maacha	*Bacillus pumilus* AFM2:S1	–	–	–	–
Sukako maacha	*Bacillus subtilis* AFM1:S2	–	–	–	–
Gnuchi	*Bacillus subtilis* MSG1:S1	–	–	–	–
Ngari	*Bacillus pumilus* Ng1:S2	–	–	–	–
Tungtap	*Bacillus subtilis* T1:S1	–	–	–	–
Karati	*Bacillus subtilis* K1:S1	–	–	–	–
Bordia	*Bacillus subtilis* BDG1:S1	–	–	–	–
Lashim	*Bacillus pumilus* LDG1:S1	–	–	–	–

Ty r = Tyrosine, tyramine precursor; Lys = Lysine, cadaverine precursor; His = Histidine, histidine precursor; Ort = Ornithine, putrescine precursor.

Table 22. Percentage hydrophobicity of LAB strains isolated from fish products

Product	Strain	% Hydrophobicity
Sukako maacha	*Lactococcus lactis* subsp. *cremoris* SM:T1	78.71(++)
Sukako maacha	*Leuconostoc mesenteroides* SM:M1	81.01 (++)
Sukako maacha	*Leuconostoc mesenteroides* SM:M2	72.71 (+)
Sukako maacha	*Enterococcus faecium* SM:A1	41.36 (+)
Sukako maacha	*Pediococcus pentosaceus* SM:A2	35.94 (+)
Gnuchi	*Pediococcus pentosaceus* GG2	93.98 (++)
Gnuchi	*Enterococcus faecium* GG6	32.22 (+)
Sidra	*Lactococcus plantarum* CG1:B1	36.97 (+)
Sidra	*Enterococcus faecalis* CG1:B2	86.82 (++)
Sidra	*Lactobacillus confusus* CG1:B3	69.66 (+)
Sukuti	*Leuconostoc mesenteroides* SG1:B1	44.51 (+)
Sukuti	*Lactococcus lactis* subsp. *lactis* SG1:B2	81.62 (++)
Sukuti	*Lactococcus plantarum* SG1:B3	30.33 (+)
Sukuti	*Lactococcus plantarum* SG2:B1	33.21 (+)
Ngari	*Lactococcus plantarum* Ng2:L4	67.14 (+)
Ngari	*Lactococcus plantarum* Ng2:L5	52.97 (+)
Hentak	*Lactobacillus fructosus* HL1	84.27 (++)
Hentak	*Lactobacillus amylophilus* H1:B1	55.33 (+)
Tungtap	*Lactobacillus coryniformis* subsp. *torquens* T2:L1	63.70 (+)
Tungtap	*Lactococcus lactis* subsp. *cremoris* T2:L2	47.12 (+)
Tungtap	*Lactobacillus fructosus* T2:L5	80.90 (++)
Karati	*Lactococcus lactis* subsp. *cremoris* KA1	82.37 (++)
Bordia	*Leuconostoc mesenteroides* BA4	70.32 (+)
Bordia	*Leuconostoc mesenteroides* BA5	84.55 (++)
Lashim	*Lactobacillus plantarum* LG1	46.00 (+)

++ = hexadecane adherence ≥75% (hydrophobic); +, = hexadecane adherence 26-74% (intermediate)

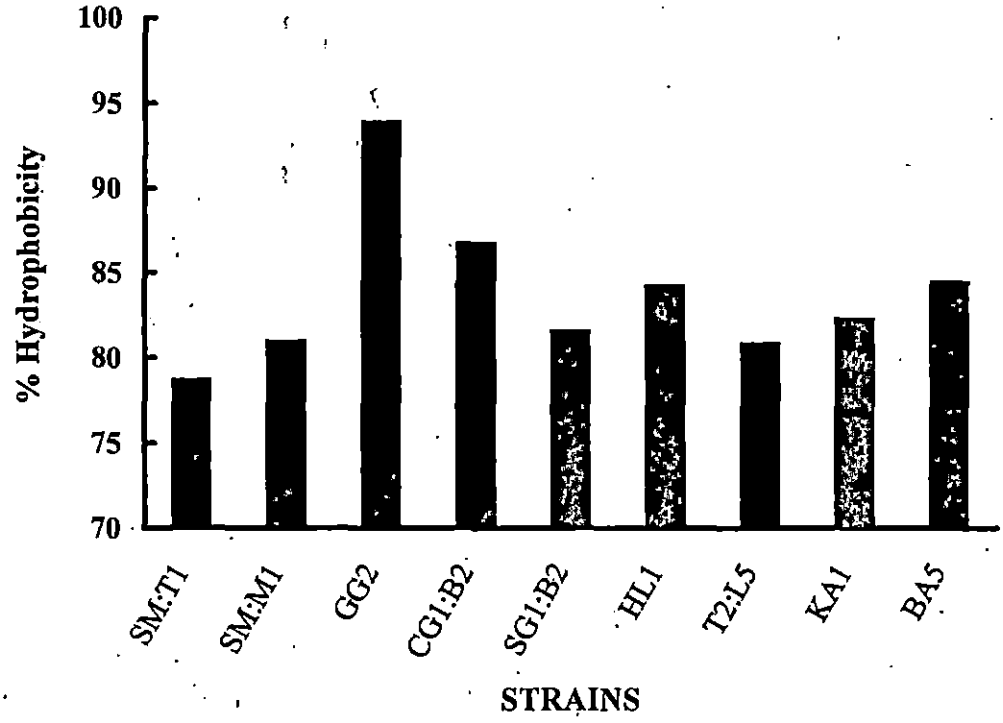


Fig 15. Percentage hydrophobicity of LAB strains isolated from fish products of the Eastern Himalayas

Table 23. Proximate composition of fish products of the Eastern Himalayas

Product	pH	%				
		Acidity	Moisture	Ash	Protein	Fat
Fresh fish[a]	7.1	0.4	62.4	3.2	32.2	5.0
Sukako maacha	6.4	1.0	10.4	16.2	35.0	12.0
Gnuchi	6.3	1.1	14.3	16.9	21.3	14.5
Sidra	6.5	0.9	15.3	16.6	25.5	12.2
Sukuti	6.4	0.6	12.7	13.6	36.8	11.4
Ngari	6.2	0.9	33.5	21.1	34.1	13.2
Hentak	6.5	0.5	40.0	15.0	32.7	13.6
Tungtap	6.2	1.1	35.4	18.9	32.0	12.0
Karati	6.3	1.0	11.8	14.5	35.0	12.4
Bordia	6.4	1.0	12.0	15.3	24.5	12.3
Lashim	6.4	0.9	9.6	12.8	28.3	11.8

[a]Asala fish (*Schizothorax* sp.), collected from Ranikhola near Gangtok.

Data represent the means of 5 samples.

Table 24. Mineral contents of some fish products of the Eastern Himalayas

Product[a]	mg/100 g				
	Ca	Fe	Mg	Mn	Zn
Fresh fish[a]	37.0	0.9	8.8	1.1	7.5
Sukako maacha	38.7	0.8	5.0	1.0	5.2
Gnuchi	37.0	1.1	8.8	1.1	7.5
Sidra	25.8	0.9	1.6	0.8	2.4
Sukuti	17.7	0.3	1.4	0.2	1.3
Ngari	41.7	0.9	0.8	0.6	1.7
Hentak	38.2	1.0	1.1	1.4	2.9
Tungtap	25.8	0.9	1.6	0.8	2.4

[a]Data represent the means of 2 samples.

DISCUSSION

Indigenous Fish Products

The people of the Eastern Himalayan regions of Nepal; the Darjeeling hills, Sikkim, Assam, Meghalaya, Manipur in India; and Bhutan, consume different types of traditionally processed smoked/sun-dried/fermented/salted fish products. **Sukako maacha** and **gnuchi** are typical smoked and dried fish products prepared and consumed in Nepal, the Darjeeling hills and Sikkim as curry or side-dish by the Nepalis and the Lepcha, respectively. **Sidra** and **sukuti** are sun-dried fish products common in the diet of the ethnic people of Nepal, the Darjeeling hills, Sikkim and Bhutan as side-dish or pickle. **Ngari** and **hentak** are unique fermented fish cuisine of Manipuri. **Tungtap** is a traditional fermented fish product consumed by the Khasia tribes in Meghalaya. **Karati, bordia** and **lashim** are sun-dried and salted fish products commonly sold in the local markets in Assam.

Some of these products are prepared using indigenous knowledge of the rural people for fish preservation. This process though practiced only by the villagers but seems to be very efficient one as there is no insect infestation occurs. Such fish processing technique has been still present in those regions or villages, which are located near water bodies with plenty of freshwater fishes. Some villagers sell them in the market area. As the products are manufactured by the rural people during appropriate season, they are regarded as a special dish for them. The fish products also seem to be an important source of protein in the local diet. According to some old people of the villages in these regions, interviewed during the survey, sukako ko maacha was produced in bulk in most of the places in eastern part of Nepal near the river-sites, even in the low-altitudes of rivers like Balasan, Teesta and Rangit in the

Darjeeling hills and Sikkim till seventies. Now-a-days, production of these traditional processed fish products is confined to limited areas and is hardly seen in the local markets due to decline in the fish population in hill rivers. The study reveals that decline in the fish population in the Teesta and Rangit River is mainly due to hydropower project activities leading soil erosion, siltation, water pollution by growing industries, sewage and pesticide (Tamang, 2002).

The traditional technique for fish preservation in the Eastern Himalayan regions involves dehydration (drying), smoking, fermentation and salting (low-salt) for preservation. Dehydration, smoking, salting and fermentation are the best methods for preservation of available perishable fish (Beddows, 1985). The products are whole fish, prepared from fresh water and lakes, and are eaten as side-dish or curry or pickle. No fish sauce and shrimp products are prepared and used as condiment in the local diet in the Eastern Himalayan regions. This may be due to use of spices to stimulate the appetite instead of using umami taste-producer such as fish sauce, soy-sauce (Kozaki, 1976; Kawamura and Kare, 1987).

Consumption of fish products in the local diet, though, is important diet, is comparatively less than other fermented products such as vegetable and dairy products in the Eastern Himalayan regions. This may be attributed by pastoral system of agriculture and the consumption of dairy products in these regions. Societies that are purely pastoral lack the custom of fish-eating (Ishige, 1993). Several reasons may be suggested to account for the decline in the importance of fermented fish products concomitant with the increase in that of products of plant origin. It is more efficient and cheaper to use materials of plant origin

than it is to use animal products. These give a much higher rate of return on the investment, the productive cycle is more reliable, products of plant origin are easier to transport, particularly when bulk manufacture continued, and fish and meat are generally preferred in the fresh forms.

Microorganisms

Microbial analysis reveals that lactic acid bacteria (LAB) were pre-dominant microflora in the traditionally processed fish products having the load upto 10^8 cfu/g, followed by *Bacillus* species with the population not more than 10^4 cfu/g. However, *Bacillus* species were not recovered from sukako ko maacha of Maglung area and sukuti samples. Yeasts were recovered only from few samples such as sukako ko maacha, ngari, tungtap, karati and bordia at the level of 10^3 cfu/g. Filamentous moulds were not recovered in any fish product analysed. Total viable count was variable ranging from the level of 10^4 cfu/g to 10^8 cfu/g in all samples analysed.

The total isolates from 72 samples of fish products comprised 70% LAB, 15% *Bacillus*, 5% *Micrococcus* and 10% Yeasts. This reveals that the LAB is predominant microflora in the traditionally processed fish products of the Eastern Himalayas. Bacterial fermentation is more dominant than yeast and mould fermentation in fish, significantly affected by proteolytic enzymes produced by bacteria (Shinano *et al.*, 1975).

All isolates of LAB were Gram-positive, non-sporeforming, non-motile, catalase negative and facultative anaerobes; they did not hydrolyse casein, gelatin and starch. Following the taxonomical keys described by Sneath *et al.* (1986) and Wood and Holzapfel (1995), and

also based on the API 50 CHL system, coccii lactics were identified as *Lactococcus lactis* subsp. *cremoris* Schleifer *et al.*, *Lactococcus plantarum* Scheifer *et al.*, *Lactococcus lactis* subsp. *lactis* Schleifer *et al.*, *Leuconostoc mesenteroides* (Tsenkovskii) van Tieghem, *Enterococcus faecium* (Oral- Jensen) Scheifer and Kilpper-Bälz, *Enterococcus faecalis* (Andrewes and Horder) Scheifer and Kilpper-Bälz and tetrads were identified as *Pediococcus pentosaceus* Mees.

Gas production from glucose was used as a first step in the differentiation of lactic rods (Kandler, 1983). Hetero-fermentative lactics were identified as *Lactobacillus confusus* (Holzapfel and Kandler) Sharpe, Garvie and Tilbury and *Lactobacillus fructosus* Kodama and homo-fermentative lactics were identified as *Lactobacillus amylophilus* Nakamura and Crowell, *Lactobacillus corynifomis* subsp. *torquens* Abo-Elnaga and Kandler and *Lactobacillus plantarum* Orla-Jensen on the basis of sugar fermentation using the API system, lactic acid isomer and meso-diaminopimelic acid determination, and also based on the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995).

LAB was prevalent in all samples indicating their pre-dominance in the product. It was observed that coccii dominated the lactic acid microflora in the fish products analysed. 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 strains obtained from theses samples were halotolerant (i.e., 18% salt tolerance). LAB species were also reported from other Asian fish products such as species of *Lactobacillus, Pediococcus* from nam-plaa and kapi, fermented fish products of Thailand (Watanaputi *et al.*, 1983;

Phithakpol, 1987; Tanasupawat *et al.*, 1992), *Pediococcus acidilactici* and *Leuçonostoc paramesenteroides* from burong isda, fermented rice-freshwater fish mixture of the Philippines (Mabesa *et al.*, 1983).

Spore-forming isolates were Gram-positive, catalase-positive, aerobic and motile. Following the dichotomous key of Slepecky and Hemphill (1992) embodying all 34 species of *Bacillus* described by Claus and Berkeley (1986), spore-forming rods were identified as *Bacillus subtilis* (Ehrenberg) Cohn and *Bacillus pumilus* Meyer and Gottheil. Prevalence of *Bacillus* species was 83 % in analysed samples of fish products. Though the load was around 10^4 cfu/g, their presence shows the dominance in fish products next to LAB. *Bacillus* species were found to be the predominant in the fish products due their ability as endospore formers to survive under the prevailing conditions (Crisan and Sands, 1975). Many workers have reported the presence of *Bacillus* species in several traditionally processed fish products such as in nam-pla and kapi (Watanaputi *et al.*, 1983; Phithakpol, 1987), gulbi (Kim *et al.*, 1993), anchovy sauce (Chaiyanan *et al.*, 1996), jeol-gat (Kim *et al.*, 1997). *Bacillus stearothermophilus, B. shaercus, B. circulans* are predominant microflora in nga-pi, fermented fish paste of Myanmar (Tyn, 1993). It shows *Bacillus subtilis and B. pumilus* also play some role in fish fermentation. There has been no record of outbreak of illness associated with *Bacillus subtilis* and *Bacillus pumilus* in fermented foods (Beumer, 2001).

All aerobic coccii isolates were Gram-positive, in tetrads and also in clusters, non-spore-formers, non-motile and catalase-positive. Following the taxonomical key of Sneath *et al.* (1986), aerobic coccii isolates were identified as *Micrococcus*. However, species could not be identified due to limited tests. Besides, LAB and *Bacillus* spp., species of *Micrococcus* have also been reported from some fermented fish products of Thailand (Watanaputi *et al.*, 1983; Phithakpol, 1987). *Micrococcus* and *Staphylococcus* are dominant microorganisms during ripening of the Japanese fermented fish, shiokara (Fujii *et al.*, 1994; Wu *et al.*, 2000).

Following the taxonomical keys described by Kreger-van Rij (1984), Yarrow (1998), and Kurtzman and Fell (1998), the oval-shaped strain AFM2:Y2 was identified as *Candida chiropterorum* Grose *et* Marinkelle; and another oval-shaped strain T1:Y1 as *Candida bombicola* (Spencer, Gorin *et* Tulloch) Meyer *et* Yarrow (Spencer *et al.*). However, strains AFM1:Y1, Ng:Y1, K1:Y1 and BDG:Y1 grouped as *Candida* could not be identified upto species level. Yeast strain T1:Y2 had dusty, dry surfaced colonies with horn-like projections made up of many strands of mycelia, cylindrical in shape and was identified as *Saccharomycopsis*. Species identification could not be confirmed. Prevalence of yeasts was 53 % in analysed samples of fish products. Species of *Candida* and *Saccharomyces* were also reported from nam-plaa and kapi (Watanaputi *et al.*, 1983; Phithakpol, 1987), *Candida* sp. were recovered from gulbi, salted and dried fish product of Korea (Kim *et al.*, 1993).

Pathogenic contaminants

Bacillus cereus occurred in 66 % of total samples analysed in fish products. However, none of the sample was found to contain more than 10^2 cfu/g of *Bacillus cereus* population in the different fish products of the Eastern Himalayan regions. Small number of *Bacillus cereus* in foods is not considered significant (Roberts *et al.*, 1996). Initial growth of *Bacillus cereus* was observed in fish sauce but was inhibited gradually due to growth of LAB (Aryanta *et al.*, 1991). *Bacillus cereus* was detected in some other fermented fish products of Asia such as patis and nam-pla (Crisan and Sands, 1975), mehiawah (Jehah *et al.*, 1999).

Load of *Staphylococcus aureus* was also found less than 10^3 cfu/g in all samples tested. *Staphylococcus aureus* is regarded as a poor competitor and its growth in fermented foods is generally associated with a failure of the normal microflora (Nychas and Arkoudelos, 1990). *Staphylococcus aureus* survives during shiokara fermentation but does not produce enterotoxin confirming the safety of traditional shiokara (Wu *et al.*, 1999). Enterobacteriaceae occurred widely in all fish product samples, however, the population was found not more than 10^3 cfu/g in samples analysed.

Factors such as water activity (a_w), and pH can determine chances of survival or proliferation of microbial food contaminants (Hauschild, 1992; Sutherland *et al.*, 1996). The presence of *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae in fish products was due to contamination during processing either through smoking or drying. However, the population of these contaminants was not more than 10^3 cfu/g in the fish product sample tested, which would be the impact of competition and/or antagonistic reaction of pre-dominant lactic acid

bacteria that have prevented the proliferation (Adams and Nicolaides, 1997). Lactic acid, produced by LAB may reduce pH to a level where pathogenic bacteria (*Staphylococcus aureus, Bacillus cereus, Clostridium botulinum*) will be either inhibited or destroyed (Hólzapfel *et al.*, 1995).

There has been no reported case of toxicity or illness due to consumption of the traditionally processed fish products in the Eastern Himalayan regions. Salting in the initial stages of fish processing can inhibit the growth of pathogenic microorganisms (Steinkraus, 1983).

Enzymatic activities

Only three strains, viz. *Enterococcus faecium* GG6, *Lactobacillus cornyformis* subsp. *torquens* T2:L1 and *Leuconostoc mesenteroides* BA4, isolated from gnuchi, tungtap, bordia, respectively showed proteolytic activity with low protease activity (1.0 U/ml). This indicates that lactic acid bacteria have very low proteolytic activities in the fish products. Whereas, all strains of *Bacillus* strains showed proteolytic activity with protease activity of 4 U/ml. Seven strains of LAB showed amylolytic activity with 3.2 U/ml to 5.8 U/ml α-amylase activity. All *Bacillus subtilis* strains showed amylolytic activity. LAB as well as *Bacillus subtilis* with amylolytic activities are essential in liquefaction during processing of fish products. 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 (Reddi *et al.*, 1972; Backhoff, 1976). Proteolytic enzymes and lipase produced by microorganisms

might easily be accelerated through lipid decomposing processes in fatty fish species dark-fleshed fish (Cha and Lee, 1985). Six strains of LAB and four strains of *Bacillus* showed lipolytic activity on tributyrin agar plates. This result indicates that pre-dominant microorganism (both LAB and *Bacillus*) have enzymatic activities during processing of the fish products.

The use of the API-zym technique has been reported (Arora *et al.*, 1990) as a rapid and simple means of evaluating and localising 19 different hydrolases of microorganisms associated with fish products. This method is also 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 organisms isolated from fish products (Table 19) are possible traits of desirable flavour in the products.

Antimicrobial activity

Antagonism refers to the inhibition of other (undesired or pathogenic) microorganisms, caused by competition for nutrients, and by the production of antimicrobial metabolites (Holzapfel *et al.*, 1995). Lactic acid bacteria compete with other microbes by screening antagonistic compounds and modifying the micro-environment by their metabolism (Lindgren and Dobrogosz, 1990). The antagonistic properties of the strains, isolated from fish products of the Eastern Himalayan regions were tested against the indicator strains (*Listeria*

monocytogenes DSM 20600, *Bacillus cereus* CCM 2010, *Enterococcus faecium* DSM 20477 and *Streptococcus mutans* DSM 6178). Some of the strains such as *Enterococcus faecium* SM:A1, *Pediococcus pentasaceus* GG2, *Lactococcus plantarum* CG1:B1, *Lactococcus plantarum* SG1:B3, *Leuconoctoc mensenteroides* BA4, *Lactobacillus coryniformis* subsp. *torquens* T2:L1, *Lactococcus lactis* subsp. *cremoris* KA1 showed the antagonistic properties against the indicator strains. This reveals that some of these LAB strains have antimicrobial properties, which can reduce the number of other undesired microorganism in the fish products as well as help in the preservation of fish (Einarsson and Lauzon, 1995). The antimicrobial compounds produced by LAB are natural preservatives which could be used for safety of minimally processed foods (Niku-Paavola *et al.*, 1999).

None of the strains were found to produce any bacteriocin with the method applied. The antimicrobial activity of most bacteriocins is directed against species that are closely related to the producer and also against different strains of the same species as the producer (Schillinger *et al.*, 1996, 2001). Growth rate and competitiveness of a culture are determined by its adaptation to a substrate and by a number of intrinsic and extrinsic factors including redox potential (E_h), water activity (a_w), pH and temperature (Holzapfel *et al.*, 1995). Moreover, the inhibition zones were relatively small and not clear (among many strains, data not shown), which indicates that inhibition was probably caused by lactic acid production. According to Daeschel (1992), organic acids, such as lactic acid and acetic acid, cause a gradient of inhibition and therefore these somewhat diffuse inhibition zones, whereas substances such as bacteriocin and hydrogen peroxide give very sharp boundaries.

Biogenic amine screening

Biogenic amines have been reported in fish products (Ten Brink *et al.*, 1990; Halász *et al.*, 1994), which are formed by decarboxylation of their precursor amino acids, as a result of the action of either by decarboxylase activity (Halász *et al.*, 1994) or by the growth of decarboxylase positive microorganisms (Silla-Santos, 2001). Several toxicological problems resulting from the ingestion of food containing relatively high levels of biogenic amines have been reported (Ten Brink *et al.*, 1990). In susceptible human, biogenic amines can lead to a variety of cutaneous, gastrointestinal, haemodynamic and neurological symptoms (Taylor, 1986). Lactic acid bacteria frequently produce histamine and tyramine in a variety of foods such as processed fish, cheese, fermented vegetables and beverages (Stratton *et al.*, 1991; Leisner *et al.*, 1994).

None of the tested thirty three strains, isolated from different traditionally processed fish products were found to decarboxylase the used amino acids – tyrosine, lysine, histidine and ornithine. This result indicated that biogenic amine is not produced by the dominant microorganisms (LAB and *Bacillus* spp.) in fish products, which also correlated that these traditionally processed fish products are safe to eat. However, the lack of histamine, tyramine, cadaverine and putrescine producers isolated from traditionally processed fish products in our study, could possible be explained by the lack of free amino acids within the samples. The concentration of amino acids in food is important for biogenic amine formation (Joosten and Northolt, 1989). Another reason may be due to no or low proteolytic activity of the LAB strains, isolated from fish products. Strains with high proteolytic activity probably have

an increased potential for biogenic amine production in food system (Halász *et al.*, 1994).

Some authors have suggested that the main biological feature influencing biogenic amine formation is the extent of growth of microorganisms possessing decarboxylase activity (Yoshinaga and Frank, 1982; Gardini *et al.*, 2001). Enterobacteriaceae also play vital role in the metabolisms of biogenic amines, especially of putrescine and cadaverine (Simon-Sarkadi and Holzapfel, 1995). Before confirming the non-production of biogenic amine in the traditionally processed fish products, qualitative and quantitative analysis of biogenic amine is necessary.

Degree of Hydrophobicity

Bacterial adherence to hydrocarbons, such as hexadecane, proved to be a simple and rapid method to determine cell surface hydrophobicity (Rosenberg *et al.*, 1980; van Loosdrecht *et al.*, 1987). Adherence is one of the most important selection criteria for probiotic bacteria (Shah, 2001). Nine strains of LAB isolated from traditionally processed fish products of the Eastern Himalayas showed high degrees of hydrophobicity (>75%), among which *Pediococcus pentosaceus* GG2 (isolated from gnuchi) showed the highest degree of hydrophobicity of 94%, showing strong hydrophobic properties. All strains of LAB had more than 30% hydrophobicity, indicating that the strains isolated from fish products of the Eastern Himalayas were not hydrophilic in nature. The adherence of microorganisms to various surfaces seemed to be mediated by hydrophobic interactions (Rosenberg, 1984). Functional effects of probiotic bacteria include adherence to the intestinal cell wall

for colonization in the gastrointestinal tract with capacity to prevent pathogenic adherence or pathogen activation (Bernet *et al.*, 1993; Salminen *et al*, 1996).

High degree of hydrophobicity by the lactic acid bacteria isolated from lesser-known traditional fish products of the Eastern Himalayas indicates the potential of adhesion to gut epithelial cells of human intestine, advocating their 'probiotic' character (Holzapfel *et al.*, 1998). Lactic acid bacteria are normal residents of the complex ecosystem of the gastrointestinal tract (GIT) (Mitsuoka, 1992; Holzapfel *et al.*, 1997).

Proximate composition

Proximate composition of sukako maacha, gnuchi, sidra, sukuti, ngari, hentak, tungtap, karati, bordia and lashim was presented in Table 23. The pH of all these products was slightly acidic in nature, due to pre-dominance of LAB flora and also subsequent fermentation or processing of fish. Drying in the sun or smoking during preservation, as a result of dehydration, most of the fish products have low moisture content. Dried fish is produced with a moisture content of 17% to 45% (Clucas and Sutcliffe, 1981). Due to low moisture content and slightly acidic in nature, the shelf-like of the product can be prolonged and can be kept for longer period at room temperature. High content of protein was observed in all analysed fish products, indicating increasing protein intake in the local diet. Fermented fish products are generally high in protein and amino compounds (Beddows, 1985).

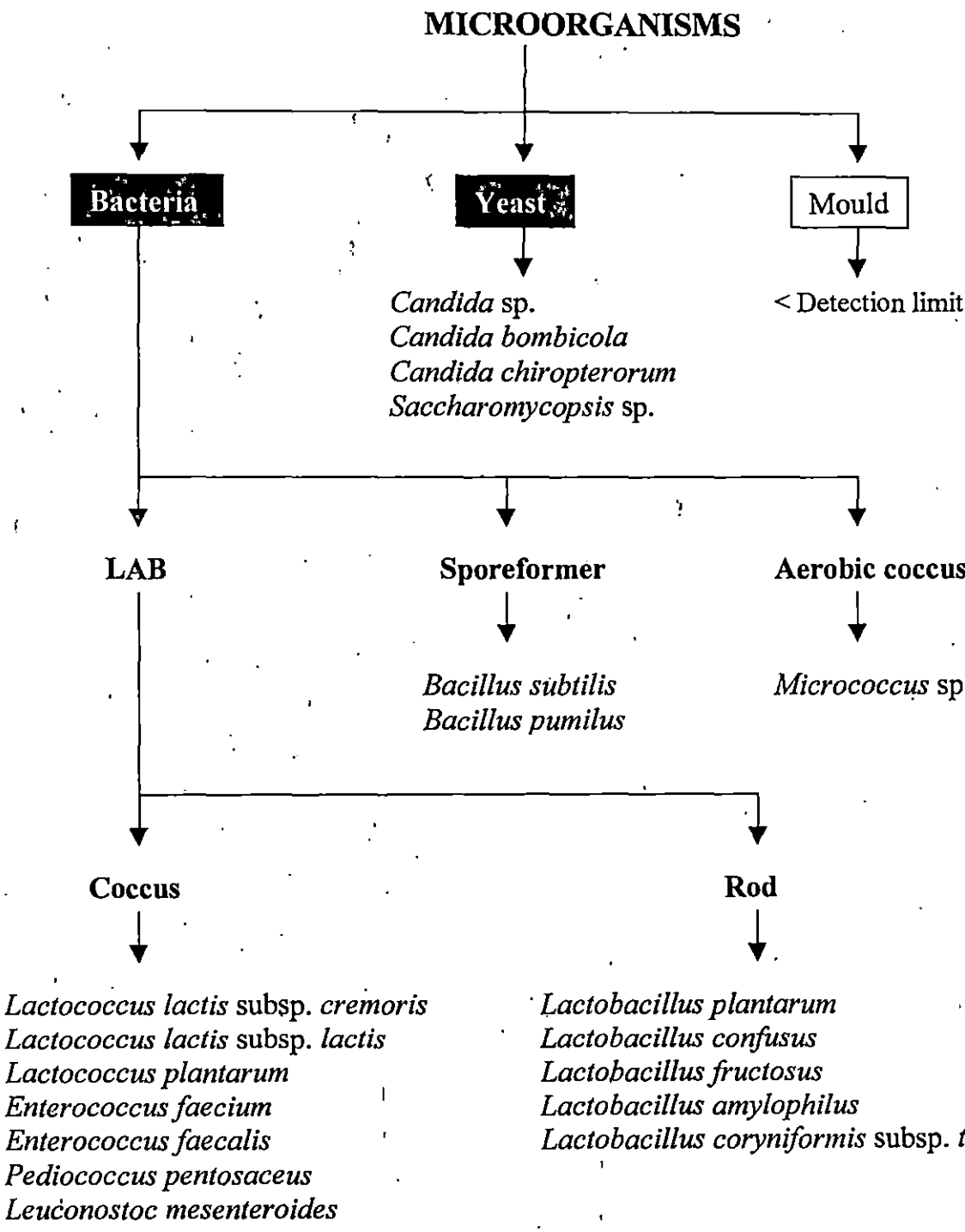
Conclusion

Traditional processing of perishable fish such as smoking, drying, salting and fermentation are principal methods of bio-preservation without refrigeration or addition of any synthetic preservative in the Eastern Himalayas. Traditional foods harness the dietary history of particular community. Indigenous knowledge of ethnic people for production of processed fish for consumption is worth-documentation. Though, the traditionally processed fish products are lesser-known, role of LAB in fermentation/process enhancing functional properties such as wide spectrum of enzymatic activities as well as enzymatic profiles, antimicrobial activities, probiotic (adherence character showing high degree of hydrophobcity), and even non-producer of biogenic amine is remarkable observation in this study. Some of these lactic acid bacteria strains possess the protective and functional properties which can be used as starter culture for controlled optimized production of fish preservation. The use of starter culture in the production of fermented foods increases the safety of processes and reduces losses caused by false fermentation (Geisen and Holzapfel, 1996).

This study has demonstrated that microbial diversity ranging from species of lactic acid bacteria belonging to coccii-lactics (*Lactococcus, Enterococcus, Pediococcus, Leuconostoc*) to species of homo- and hero-fermentative rods (*Lactobacillus*) belonging to lactic acid bacteria, *Bacillus subtilis, Bacillus pumilus., Micrococcus* spp. to species of yeasts (*Candida, Saccharomycopsis*) were present in the lesser-known traditionally processed fish products of the Eastern Himalayan regions. Biodiversity of strains within each analysed sample as well as the expression of strains specific characteristics was dependent on the

intrinsic and extrinsic parameters of food-related eco-system. Table C shows the schematic presentation of microbial diversity in the traditionally processed fish products of the Eastern Himalayan regions. The isolated, identified and preserved microorganisms from lesser-known fish products may contribute significant information on unknown microbial gene pool as genetic resources of the Himalayan regions.

Table C: Schematic presentation of microbial diversity in traditionally processed fish products (sukako maacha, gnuchi, sidra, sukuti, ngari, hentak, tungtap, karati, bordia and lashim) of the Eastern Himalayas



SUMMARY

The people of the Eastern Himalayan regions of Nepal; the Darjeeling hills, Sikkim, Assam, Meghalaya and Manipur in India consume different types of traditionally processed smoked/sun-dried/fermented/salted fish products. Sukako maacha and gnuchi are typical smoked and dried fish products prepared and consumed in Nepal, the Darjeeling hills and Sikkim as curry or side-dish by the Nepalis and the Lepcha, respectively. Sidra and sukuti are sun-dried fish products common in the diet of the ethnic people of Nepal, the Darjeeling hills, Sikkim and Bhutan as side-dish or pickle. Ngari and hentak are unique fermented fish cuisine of Manipuri. Tungtap is a traditional fermented fish product consumed by the Khasia tribes in Meghalaya. Karati, bordia and lashim are sun-dried and salted fish products commonly sold in the local markets in Assam.

Some of these products are prepared using indigenous knowledge of the rural people for fish preservation. The traditional technique for fish preservation in the Eastern Himalayan regions involves dehydration (drying), smoking, fermentation and salting (low-salt) for preservation. No fish sauce and shrimp products are prepared and used as condiment in the local diet in the Eastern Himalayan regions.

Seventy-two samples of different fish products were collected from different places of Eastern Nepal (Maglung, Therathum and Aitabare), the Darjeeling hills (Gidhang in Kalimpong sub-division), Sikkim (Gangtok), Meghalaya (Shillong), Assam (Guwahati) and Manipur (Imphal) of the Eastern Himalayan regions and were analysed for microbial load. Microbial load of lactic acid bacteria was 10^4 cfu/g to 10^8 cfu/g, spore-forming rods was $<10^4$ cfu/g, yeasts was 10^3 cfu/g and total viable count was 10^4 cfu/g to 10^8 cfu/g.

Though the load of spore-formers was around 10^4 cfu/g, their presence shows the dominance in fish products next to lactic acid bacteria. Filamentous moulds were not recovered. Out of 527 isolates of microorganisms isolated, 369 strains were lactic acid bacteria, 77 were spore-formers, 27 were aerobic coccii and 54 were yeasts.

Out of 369 lactic acid bacteria strains isolated from seventy-two samples of fish products, 282 isolates were coccii and 87 isolates were non-sporeforming rods. Lactic acid bacteria were identified as *Lactococcus lactis* subsp. *cremoris, Lactococcus plantarum, Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides, Enterococcus faecium, Enterococcus faecalis, Pediococcus pentosaceus, Lactobacillus confuses, Lactobacillus fructosus, Lactobacillus amylophilus, Lactobacillus corynifomis* subsp. *torquens* and *Lactobacillus plantarum.*

Endospore-forming rods were identified as *Bacillus subtilis* and *Bacillus pumilus*, aerobic coccii isolates were *Micrococcus*. Yeasts were identified as *Candida chiropterorum, Candida bombicola, Saccharomycopsis* spp.

Prevalence of lactic acid bacteria was 100 %, whereas that of *Bacillus* species, *Micrococcus* species and yeasts was only 83 %, 41 % and 53 % in seventy-two samples analysed, respectively. Lactic acid bacteria (70%) were the dominant microflora in 72 samples of fish products, followed by *Bacillus* spp. (15%), yeasts (10%) and *Micrococcus* (5%). Out of 369 lactics isolated from the different fish products, 77% of coccii dominant the lactic microflora, whereas only 27% rods were present in the fish products.

Pathogenic contaminant was detected in the selective media used. *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae were

detected in 66%, 54.7% and 68.3% of fish products, respectively. However, none of the sample was found to contain more than 10^2 cfu/g of *Bacillus cereus*, 10^3 cfu/g of *Staphylococcus aureus* and enterobacteriaceae population.

Only three strains, viz. *Enterococcus faecium* GG6, *Lactobacillus cornyformis* subsp. *torquens* T2:L1 and *Leuconostoc mesenteroides* BA4, isolated from gnuchi, tungtap, bordia, respectively showed proteolytic activity with low protease activity (1.0 U/ml). Seven strains of LAB showed amylolytic activity with 3.2 U/ml to 5.8 U/ml α-amylase activity. All *Bacillus subtilis* strains showed amylolytic activity. Six strains of LAB and four strains of *Bacillus* showed lipolytic activity on tributyrin agar plates.

Enzymatic profiles of randomly selected lactic acid bacteria strains of fish products were assayed using the API zym galleries. Each of the predominant LAB strains produced a wide spectrum of enzymes. These strains showed relatively weak esterase and no lipase (C14) activities. *Lactoctoccus lactis* subsp. *cremoris* SM:T1 and *Enterococcus faecium* SM:A1 showed strong phosphatase activities, while other strains showed moderate activities. This method is also 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 organisms isolated from fish products are possible traits of desirable flvaour in the products.

The antagonistic properties of the strains, isolated from fish products of the Eastern Himalayan regions were tested against the

indicator strains (*Listeria monocytogenes* DSM 20600, *Bacillus cereus* CCM 2010, *Enterococcus faecium* DSM 20477 and *Streptococcus mutans* DSM 6178). Some of the strains such as *Enterococcus faecium* SM:A1, *Pediococcus pentasaceus* GG2, *Lactococcus plantarum* CG1:B1, *Lactococcus plantarum* SG1:B3, *Leuconoctoc mensenteroides* BA4, *Lactobacillus coryniformis* subsp. *torquens* T2:L1, *Lactococcus lactis* subsp. *cremoris* KA1 showed the antagonistic properties against the indicator strains. This reveals that some of these LAB strains have antimicrobial activities, which can reduce the number of other undesired microorganism in the fish products as well as help in the preservation of fish. However, none of the strains were found to produce any bcteriocin with the methods applied.

Thirty three strains were tested for biogenic amine production with the surface plate method applied. None of the strains were produced tyramine, cadaverine, histidine and putrescine in the applied method. This result indicated that biogenic amine is not produced by the dominant microorganisms (LAB and *Bacillus* spp.) in fish products, which also correlated that these traditionally processed fish products are safe to eat.

Nine strains of LAB isolated from traditionally processed fish products showed high degrees of hydrophobicity (>75%), among which *Pediococcus pentosaceus* GG2 (isolated from gnuchi) showed the highest degree of hydrophobicity of 94%, showing strong hydrophobic properties. All strains of LAB had >30% hydrophobicity, indicating that the strains isolated from fish products of the Eastern Himalayas was not hydrophilic in nature. High degree of hydrophobicity by the lactic acid bacteria isolated from lesser-known traditional fish products of the

Eastern Himalayas indicates the potential of adhesion to gut epithelial cells of human intestine, advocating their 'probiotic' character.

Proximate composition of sukako maacha, gnuchi, sidra, sukuti, ngari, hentak, tungtap, karati, bordia and lashim was analysed. The pH of all these products was 6.2-6.5 with titratable acidity ranging from 0.5 to 1.1%. Moisture content was low upto 10 % in sukako maacha, however, hentak, the simi-paste product contained 40% of moisture. Drying in the sun or smoking during preservation, as a result of dehydration, most of the fish products have low moisture content. High content of protein was observed in all analysed fish products, indicating increasing protein intake in the local diet. Among the minerals of the fish products, calcium content was higher than other minerals estimated. Among the fish products analysed, gnuchi contained highest amount of calcium and magnesium. Due to low moisture content and slightly acidic in nature, the shelf-like of the product can be prolonged and can be kept for longer period at room temperature.

Traditional processing of perishable fish such as smoking, drying, salting and fermentation are principal methods of bio-preservation without refrigeration or addition of any synthetic preservative in the Eastern Himalayas. Though, the traditionally processed fish products are lesser-known, role of LAB in fermentation/process enhancing functional properties such as wide spectrum of enzymatic activities or profiles, antimicrobial activities, probiotic (adherence character showing high degree of hydrophobcity), and even non-producer of biogenic amine is remarkable observation in this study.

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Table C shows the schematic presentation of microbial diversity in the traditionally processed fish products of the Eastern Himalayan regions. The isolated, identified and preserved microorganisms from lesser-known fish products may contribute significant information on unknown microbial gene pool as genetic resources of the Himalayan regions.

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