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 pre-dominant 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 (Hölzapfel *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