Microbiological Evaluation of Indigenous Fermented Milk Products of the Sikkim Himalayas

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This is to certify that the work presented in the thesis entitled: **Microbiological Evaluation of Indigenous Fermented Milk Products of the Sikkim Himalayas**, has been carried out by ***Shri Sailendra Dewan*** under my guidance and supervision at Food Microbiology Laboratory of Department of Botany, Sikkim Government College, Gangtok. The results incorporated in this thesis have not been submitted for any degree elsewhere.

I also certify that Shri Dewan has followed the rules and regulation of this college in carrying out the work, and also fulfilled the conditions for submission of thesis to North Bengal University.

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In this age of rapid modernization, age-old cultures are being lost each day. Traditional food culture too suffers from such onslaught. Perhaps the only way to save our indigenous food culture from being lost for eternity is to make proper study on the usefulness and benefits of these indigenous food products so that they can be used in new ways. The traditional foods of the Eastern Himalayas have received worldwide attention and recognition due to the relentless hardwork and dedication of Dr Jyoti Prakash Tamang, my research supervisor. His efforts have brought out the traditional fermented foods of the Himalayas from the narrow confines of domestic consumption to the scientific curiosity and study worldwide. I am eternally thankful to him for initiating me into this novel field, for all the valuable guidance, support and help in my research work and for all the learning experience under him. Words will not be sufficient to express my gratitude to him fully.

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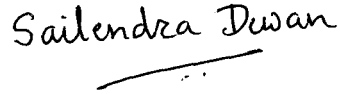

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CONTENTS

INTRODUCTION

Milk is a universal food and is polyphasic emulsion having a range of physical, chemical and biological properties (Huria, 2002). Fermented milk products are prepared from whole milk, or partially or fully skimmed milk, or concentrated milk by microbial fermentation mainly by lactic acid bacteria (Oberman, 1985). Fermented milk products account for about 20% of all fermented food production (Campbell-Platt, 1987). Popularity of fermented milk is attributed to its taste as well as its extended shelf-life, played by pure or mixed cultures of lactic acid bacteria which are used to produce the proper amount of diacetyl and other desirable flavour (Steinkraus, 1994). In fermented milk products, lactic acid bacteria largely convert the lactose into more digestible lactate and proteins into free amino acids imparting digestibility to the product (Tamang and Holzapfel, 1999).

Studies on microbiology and biochemistry of some common indigenous fermented milk products of India such as misti dahi (Ray and Srinivasan 1972, Gupta *et al.*, 2000), dahi (Ramakrishnan, 1979; Misra 1992), shrikhand (Patel and Chakraborty, 1985; Sarkar and Misra, 1997) have been carried. Production statistics, microbial biodiversity, nutritive value, development of starter culture(s), antimicrobial activities of cultures, of some common fermented milk products such as cheese (Galloway and Crawford, 1985), yoghurt (Pazakova *et al.*, 1997; Robinson, 1999), kefir and koumiss (Kosikowski, 1977), and probiotics properties of some lactic acid bacteria isolated from fermented milk products (Holzapfel *et al.*, 1997) have been well documented and investigated.

Food fermentation is one of the oldest 'biotechnological processes' from which development of fermented foods and beverages,

based on trial and error, has been rooted in the cultural history of human being (Geisen and Holzapfel, 1996). Fermented foods are prepared by the action of microorganism(s), either spontaneously or by adding starter culture(s), which modify the substrates biochemically and organoleptically into edible products, and are thus generally palatable, safe and nutritious (Kwon, 1994; Campbell-Platt, 1994). 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).

Sikkim, a tiny Himalayan state of India, with an area of 7096 sq. km and altitudes ranging from 300 m to 8500 m is bounded in the north by the Tibetan Plateau, in the east by the Chumbi Valley of Tibet and Bhutan, in the west by Nepal and in the south by the Darjeeling hills of West Bengal. The state comprises four districts viz.North, East, South and West. Three major ethnic groups of people the Nepali, the Bhutia and the Lepcha comprise the population of Sikkim. Agriculture forms a major component of the mixed farming system and is the main economy of the state. Livestock mostly plays a subsidiary role in this mixed farming set up. Cattle rearing are common practice in the mixed agricultural farming system in Sikkim. The important domestic livestock

of Sikkim are cattle, buffaloes, yaks, sheep, goats, pigs, etc. (Balaraman and Golay, 1991). Cow milk is consumed and is particularly fermented into a number of indigenous milk products. In the alpine and sub-alpine regions between 2100 m to 4500 m altitudes, yak (*Bos grunniens*) is also reared mostly for milk and its fermented products, meats, skin and hairs. Inter-specific crossing between yak and cattle called 'Joe' gives better yield in milk production and work capacity, and can tolerate warmer weather conditions.

The *Bhat-dal-tharkari-achar* (rice-legume soup-curry-pickle) followed by milk products and meat constitute the daily basic diet of the people in Sikkim (Tamang *et al.*, 1988; Tamang, 2001). Milk and its fermented products form an important part of food consumption. Some of the common fermented milk products of these regions are chhurpi, dahi, mohi, philu, chhu, etc., and their documentation may be worthwhile after thorough survey. Information in the literature related to few milk products of Sikkim has been limited to its traditional preparation and consumption pattern (Tamang *et al.*, 1988; Yonzan and Tamang, 1998), compositional and sensory characteristics of chhurpi (Katiyar *et al.*, 1991) and optimisation of process parameters of chhurpi (Pal *et al.*, 1993, 1994, 1995, 1996). Production of indigenous fermented milk products is mainly confined to the unorganised sector as well as individual household levels in the Sikkim Himalayas. Preliminary data on production statistics of common and lesser-known traditional milk products, information on microbiology and on economical aspects are not available. The proposed dissertation is aimed to study in depth the microbiological evaluation of common as well as lesser-known indigenous fermented milk 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 indigenous fermented milk products of the Sikkim Himalayas, may contribute a significant unknown microbial gene pool, which should be preserved.

Objectives

The proposed research is aimed at the achievement of the following objectives →

- Documentation of indigenous knowledge of traditional processing of milk: common as well as lesser-known fermented milk products of the Sikkim Himalayas.
- Analysis of food value of the products such as protein, fat, carbohydrate, caloric content, minerals.
- Isolation, characterization and identification of predominant microorganisms associated with the indigenous fermented milk products.
- Study of microbial population, enzymatic activities of major microbial groups.
- Study of pathogenic contaminants, and the potential of fermentative bacteria to produce biogenic amines.
- Study of antimicrobial activities of dominating microorganisms.
- Determination of degree of hydrophobicity, as presumptive probiotic properties of dominant microorganisms.

REVIEW OF LITERATURE

According to FIL-IDF (1981), fermented milk is defined as product prepared from milk–whole, partially or fully skimmed, concentrated milk or milk substituted from partially or fully skimmed dried milk homogenized or not, pasteurized or sterilized and fermented by means of specific microorganisms.

Fermented milk products are generally classified into four types: (1) acid/alcohol-type such as kefir and koumiss, (2) high acid-type such as Bulgarian sour milk, (3) medium acid-type such as acidophilus milk and yoghurt and (4) low acid-type such as cultured buttermilk and cultured cream (Kosikowski, 1977). Today, there are more than 70 bifidus- and acidophilus containing milk products produced worldwide (Shah, 2001). Some of the widely consumed fermented milk products are listed in Table A.

Acidophilus milk

Acidophilus milk is sour milk where starter culture *Lactobacillus acidophilus* is inoculated to sterilized milk (Robinson and Tamime, 1981). Acidophilus milk contains from 1.5 to 2.0 % acid (as lactic) and no alcohol and is used therapeutically but its high acidity makes it poor table beverage (Kosikowski, 1977). Acidic conditions produced by *Lactobacillus acidophilus* in the intestinal tract discourage the growth and proliferation of gas-forming putrefactive bacteria in the gut (Oberman, 1985). *Lactobacillus acidophilus* has probiotic properties (Shah, 2001). Acidophilus milk fermented by *Lb. acidophilus* reduce serum cholesterol level through several mechanisms (Ashar and Prajapati, 2001a,b).

Table A. Traditional fermented milk products of the world (Campbell-Platt, 1987)

Product	Milk type	Nature	Regions of production
Acidophilus milk	Cow milk	Sour milk	Russia, East Europe, Greece, Turkey, North America, Scandinavia
Bulgarian buttermilk	Cow milk	Sour milk	Yugoslavia, Bulgaria, Greece, Turkey, Albania, Romania
Butter	Milk	Soft paste	All parts of the world
Buttermilk	Cow milk	Sour milk	USA, Canada, Russia, Scandinavia, Middle East, Egypt, Ethiopia, India, Australia, New Zealand
Cheese	Milk	Soft or hard solid	All parts of the world
Dahi	Cow or buffalo milk	Yoghurt-like fermented milk	Indian subcontinent
Ghee	Milk	Soft paste	Indian subcontinent, Middle East, Africa, South East Asia
Kefir	Goat, sheep, or cow milk	Acidic, mildly alcoholic, effervescent milk	Russia, Europe, Middle East, North Africa
Kishk	Sheep milk-wheat	Milk-wheat mixture; dried balls	Greece, Turkey, Egypt, Libya, Middle East, Iran
Koumiss/ Kumiss	Horse, donkey or camel milk	Acid/alcoholic milk	Scandinavia, Russia, Mongolia, China
Lassi	Milk	Buttermilk or dahi, sometimes sweetened	Indian subcontinent, Mongolia, Middle East, North Africa, West Africa, Europe
Laban	Milk	Yoghurt-like fermented milk	Egypt, Turkey, Middle East
Misti dahi	Milk	Sweet yoghurt-like	Eastern India
Paneer	Buffalo, cow, milk	Cheese-like solid	Indian subcontinent, Middle East
Rabri	Buttermilk, cereals, pulses	Thick slurry-like product	India
Shrikhand	Cow or buffalo milk	Sweetened dewatered dahi	Western and southern India
Trahanas	Sheep milk, wheat	Wheat- fermented sheep milk; consumed as sweet-sour soup or biscuit	Cyprus, Greece, Turkey
Yoghurt	Cow, goat, sheep, buffalo or camel milk	Fermented milk	All parts of the world

Bulgarian buttermilk

Bulgarian or bulgaricus buttermilk is extremely sour milk prepared from boiled milk of goat or cow, inoculated with a portion of previous fermented milk (Oberman, 1985). Bulgarian buttermilk might have originated from Trak's tradition i.e. from the tradition of the sheep breeders who came to Asia from Bulgaria in the 15th century (Oberman, 1985). *Lactobacillus bulgaricus* is the primary fermenting organism in Bulgarian milk and is high acid milk in which total acidity (as lactic acid) may reach from 2.0 to 4.0 % (Kosikowski, 1977). Incubation temperature for bulgaricus buttermilk is from 38° C to 47° C (Steinkraus, 1983). *Lactobacillus bulgaricus* convert the milk lactose to lactic acid, and produce the flavour compounds acetaldehyde (Marshall, 1982).

Buttermilk

True buttermilk is the fluid remaining after the cream is churned into butter (Oberman, 1985). However, cultured buttermilk is made from fresh skim milk or from partially skimmed pasteurised milk fermented commonly with one or more selected strains of *Lactococcus lactis*, *Lactococcus cremoris* and one or more species of citric acid fermenting streptococci, *Leuconostoc cremoris*, sometimes *and Lactococcus lactis* subsp. *diacetylactis* (Robinson and Tamime, 1981). Increase in bacterial population was very rapid, the fungal and yeast population increased gradually in buttermilk (Viajayalakshmi and Murugesan, 2001).

Cheese

Cheese and cheese products derived from the fermentation of milk are of major nutritional and commercial importance throughout the world (Galloway and Crawford, 1985). According to USDA (1978), cheese can be classified into four major groups: very hard (grating) type, hard, semi-soft and soft. Some of the common varieties of cheese are: asiago old, parmesan, romano, sapsago, spalen, cheddar, caciocavallo, swiss, emmentaler, gruyère, brick, munster, limburger, port du salut, trappist, roquefort, gorgonzola, blue stilton, blue wensleydale, brie, camembert, neufchâtel, cottage, etc. (Androuet, 1976). Conversion of lactose to lactic acid in cheese is achieved by LAB, particularly *Lactococcus* spp. (Carr, 1981).

Biogenic amines which are organic basic compounds are found to occur in cheese, fish products, wine, beer, dry sausages and other fermented foods (Ten Brink *et al.*, 1990; Halász *et al.*, 1994). Cheese is the most commonly implicated food associated with histamine poisoning (Silla-Santos, 2001). The first reported case of histamine poisoning occurred in 1969 in the Netherlands and involved gouda cheese (Stratton *et al.*, 1991). Many studies have been undertaken to determine the amine contents of cheese products, and a variety of amines, such as histamine, tyramine, cadaverine, putrescine, tryptamine and phenylethylamine, have been found in different cheeses (Besancon *et al.*, 1992; Abd-Alla *et al.*, 1996; Schneller *et al.*, 1997; Vale and Gloria, 1997). Lactic acid bacteria frequently produce histamine and tyramine in fermented foods including dairy products (Stratton *et al.*, 1991; Leisner *et al.*, 1994). *Enterococcus faecalis* has been associated with tyramine in cheese and other fermented milk products (Holt *et al.*, 1994; Celano *et al.*, 1996).

Dahi

Dahi is the most popular fermented milk product in India and is obtained by lactic acid fermentation of cow or buffalo milk (Ramakrishnan, 1979). Dahi is well known for its palatability and nutritive value (Rathi *et al.*, 1990). It resembles plain yoghurt in appearance and consistency and differs in having less acidity (Batra and Millner, 1976; Mital, 1977; Shuaib and Azmey, 1977). Preparation and consumption of dahi has been recorded since 2000 B.C. (Prakash, 1961). *Lactobacillus bulgaricus, Lb. acidophilus, Lb. helviticus, Lb. casei, Lb. brevis, Streptococcus thermophilus, Lactococcus lactis, Lactococcus cremoris, Enterococcus faecalis* were isolated from dahi (Laxminarayana *et al.*, 1952; Ranganathan *et al.*, 1964; Ramakrishnan, 1979; Mohanan *et al.*, 1984).

According to BIS (1980a) specification, dahi is either plain or flavoured and should have 0.6 to 0.8 % acidity, not more than 18 coliforms/g, 100 yeast and moulds/g and a negative phosphatase test. A commercial production of dahi using starter culture combination of *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *diacetylactis* or with *Leuconostoc* spp. has been described by Misra (1992).

Kefir

Kefir is acidic, mildly alcoholic, distinctly effervescent milk in Russia (Hartles *et al.*, 1977). It can be made from the milk of goat, sheep or cow. Kefir is served in a glass and can be either drunk or eaten with a spoon, or it may be sweetened with sugar like yogurt or combined with fruits or biscuits (Mogilevsky, 1977). The essential material, along with the milk substrate, is the kefir grains (Hartles *et al.*, 1977).

Predominant yeasts in kefir include *Torulopsis holmii* and *Saccharomyces delbrueckii* in a ratio of about 10:1, both of which are lactose-negative (la Rieviere, 1963; Hartles *et al.*, 1977). The total number of viable lactose negative yeasts per g wet weight of grains amount to 1.4 to 3.3 $\times 10^8$ (la Rieviere, 1969). Predominant bacterium is *Lactobacillus brevis* (la Rieviere *et al.*, 1967). Both lactose and non-lactose fermenting species of yeasts *Klyuveromyces marxianus, Candida kefir, Candida pseudotropicalis, Saccharomyces cerevisiae, Saccharomyces exiguous* and *Torula holmii* were isolated from kefir (Chin Wen *et al.*, 1999).

During kefir fermentation, pH may drop below 3; total acid increases from 0.85 to 1.0% (as lactic), carbon dioxide is produced, making the product effervescent (la Rieviere *et al.*, 1967). Generally, less than 1% ethanol is produced. All these changes produce desirable organoleptic qualities (la Rieviere *et al.*, 1967). Kefir is a low-cost method of preserving milk (Steinkraus, 1983).

Kishk

Kishk is a fermented milk-wheat mixture stored in the form of dried balls of Egypt (Abd-el-Malek and Demerdash, 1977). Kishk is a popular food among the rural populations and the Bedouins of Egypt, Syria, Lebanon, Jordan, Iraq and North Africa (Basson, 1981). Kishk is a balanced food with excellent keeping quality, richer in B vitamins than either wheat or milk and well adapted to hot climates by its content of lactic acid and has a therapeutic value (Morcos *et al.*, 1973). The principal lactobacilli involved in kishk fermentation are *Lactobacillus casei, Lb. plantarum, Lb. brevis* (Abd-el-Malek and Demerdash, 1977). *Bacillus* spp. were also reported in kishk (Mahmoud, 1977).

Koumiss

Koumiss is an effervescent acid/alcoholic fermented milky white/greyish liquid made primarily from mare milk (Kosikowski, 1977). It has been known since ancient times and is the principal food of wandering tribes in European Russia and the plains of south, western and central Asia (Auclair and Accolas, 1974).

The description of traditional method is illustrated below (Kosikowski, 1977; Steinkraus, 1983; Campbell-Platt, 1987). In early times mare milk was stored in smoked horse skins, but now fresh mare or goat milk is placed in a wooden vessel. Boiling water is added to the warm mare milk in the proportion of 1:6 (v/v). An eighth part (v/v) of old koumiss is added and the mixture is covered and held for 15 to 24 h. Additional heat and agitation is applied if necessary to stimulate the fermentation. The fermentation is complete when the milk is thoroughly sour and sends up a thick mass to its surface. It is then beaten and stirred until the curd is thoroughly broken and forms a thick liquid. It is again covered and fermented for an additional 24 h or longer, and blended until perfectly smooth. Koumiss is then ready to drink.

The primary fermenting microorganisms in koumiss are *Lactobacillus bulgaricus*, yeasts *Candida kefyr, Torulopsis* spp. (Kosikowski, 1977; Tamime, 1981). The primary fermentation products are lactic acid (0.7-1.8 %), ethanol (1-2.5 %), carbon dioxide and these products account for the effervescence and sour, alcoholic flavor (Kosikowski, 1977). Koumiss is not only regarded as a food high in nutritional quality, it is also considered to be therapeutic, particularly in the treatment of pulmonary tuberculosis (Auclair and Accolas, 1974).

Kosikowski (1977) reported that more than 50 Russian sanatoria offer koumiss treatment for tuberculosis.

Lassi

Lassi is a by-product obtained in the preparation of country butter (ghee) from dahi by indigenous methods (Mital, 1977). Dahi is churned with frequent addition of water until butter granules are formed. The product obtained by manual removal of butter granules is called lassi (Laxminarayan and Shankar, 1980). The composition of lassi is water, 96.20 %; fat, 0.80 %; protein, 1.29 %; lactose, 1.20 %; lactic acid, 0.44 %; ash, 0.40 %; calcium, 0.60 % and phosphorus, 0.04 % (Rangappa and Achaya, 1974). Patidar and Prajapati (1998) reported that lassi prepared by combination of *Lactobacillus acidophilus* and *Streptococcus thermophilus* was organoleptically acceptable and stable.

Laban rayeb

Laban rayeb (laben) has a slightly acid taste with aroma resembling that of buttermilk (Morcos, 1977; Oberman, 1985). Laban is used as dairy spread and yoghurt cheese (El-Samargy, 1997). The pH of laban varies between 4.1 and 4.8 with acidity ranging from 0.8-1.3 % (Morcos, 1977). The predominating organisms are *Lactococcus lactis* subsp. *lactis, Streptococcus thermophilus, Lactobacillus bulgaricus* and lactose fermenting yeasts (Vedamuthu, 1982).

Misti dahi

Misti dahi (sweetened dahi, mishti doi, lal dahi or payodhi) is a sweetened fermented milk product from the eastern part of India (Ray and Srinivasan, 1972). Traditionally, milk with cane sugar is heated in

for 6-7 hours to evaporate part of the moisture. After cooling, the mix is inoculated with commercial starter culture kept from the previous day and transferred to earthenware pots. Curdling takes place at room temperature overnight (Ghosh and Rajorhia, 1990). Mixture of starters *Lactococcus lactis, Lactococcus diaceytylactis, Lactococcus cremoris* and *Leuconostoc* is most appropriate for commercial production of misti dahi from buffalo milk (Ghosh and Rajorhia, 1990). Wide variations in total solids (27-43 %), non fat milk solids (11-16 %) and sucrose (13-19 %) in the market samples of misti dahi (Ghosh and Rajorhia, 1987). Gupta *et al.*, (2000) optimized the production of misti dahi from buffalo milk using starter combinations comprising (i) *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*; and (ii) *Lactobacillus acidophilus*, *Lactococcus lactis* subsp. *lactis* and *Saccharomyces cerevisiae.*

Rabri

Rabri, an indigenous fermented milk-based, thick slurry-like product of India, is prepared by fermenting cereals and pulses including wheat, barley, maize, pearlmillet, chickpea, etc. with country buttermilk (Gupta *et al.,* 1991; Chatterjee *et al.*, 1994). Cooked maize flour is cooled and combined with buttermilk to make rabdi. The mixture is fermented overnight and consumed. *Pediococcus acidilactici* (3.6×10^5/g), *Bacillus* sp. (1.1×10^6/g), and *Micrococcus* sp. (7.9×10^5/g) have been isolated from fermented rabid (Ramakrishnan, 1977). The pH changes from 6.7 to 6.4 and a slight volume increase of 5% occurs. There is no change in amino nitrogen or free sugar (Ramakrishnan, 1977).

Shrikhand

Shrikhand is an indigenous, concentrated sweetened lactic fermented milk product, widely consumed in western part of India (Sarkar and Misra, 1997). It has a distinctive rich flavour and fairly long shelf-life due to higher acidity, reduced water content (Garg *et al.*, 1983) and addition of sugar (Patel and Chakraborty, 1988). Shrikhand possesses antibacterial properties against pathogenic as well as spoilage organisms (Sarkar *et al.*, 1996). Shrikhand preparation involves intentional fermentation and coagulation and generally buffalo milk is used for the manufacture of chakka because of higher yield and consumer preference (BIS, 1980b). Chakka, the basic raw material, is obtained by drainage of whey from the acid curd (Boghra and Mathur, 2000).

Trahanas

Trahanas, known as kapestoes or zamplaricos in Greek and tarhanocirv in Turkish, are fermented food made from crushed wheat and fermented sheep milk, which are boiled together, dried and stored in the form of biscuits (Economidou, 1975). They are made into a thick, sweet-sour soup for consumption, the fermentation and the products are closely related to Egyptian kishk.

Trahanas is found in every home and consumed mainly during the winter and are widely used for feeding weaned infants and young children (Economidou and Steinkraus, 1977). Economidou (1975) reported the presence of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* as the major fermenting organisms in trahanas.

Yoghurt

According to some sources yoghurt originated in Asia (Oberman, 1985). Yoghurt is prepared from milk of cow, goat sheep, buffalo or camel using 2-5 % lactic starters (Campbell-Platt, 1987). The predominant role in production of yoghurt lie with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Robinson, 1999). Widely distributed yeasts *(Candida mycoderma, Candida krusei, Candida tropicalis)* were regarded as spoilage microorganisms whereas bacterial strains, *Lactococcus lactis, Lactococcus lactis* subsp. *diacetylactis, Leuconostoc* spp., *Lactococcus lactis* var. *taette* (slime producer), were regarded as supplementary microflora (Robinson, 1990). In original Bulgarian and Yugoslavian yoghurts, *Geotrichum candidum* was also found (Robinson, 1990). The metabolic activity of yoghurt bacteria results in a considerable increase in cell numbers, the total count of viable yoghurt bacteria ranges between 200 and 1000 million per ml of fresh yoghurt but decreases during subsequent storage (Chandan, 1989). Finished yoghurt with pH 4.2-4.3 is thus the end product of a symbiotic culture of *Streptococcus thermophilus* and of *Lactobacillus bulgaricus* growing at temperatures in the range 40-45° C (Gilliland, 1985). A proportion of 1:1 of the 'rods' and 'cocci' forms is considered to be optimum for flavour and texture production but 1:5 or 1:10 or 2.1:1.2 are also favourable (Rasic and Kurmann, 1978; Vedamuthu, 1982). *Lactobacillus bulgaricus* demonstrates a much stronger proteolytic activity than does *Streptococcus thermophilus* (Tamine and Robinson, 1985).

Yoghurts exhibit an antagonistic effect against a number of pathogenic and saprophytic organisms but this effect shows many variations depending on the bacterial strains used, and on their particular

antagonistic properties (Shah, 2001). Yoghurt bacteria produce higher β-galactosidase activity than probiotic bacteria (Shah and Jelen, 1990; Shah, 1994). Yoghurt to be considered as a probiotic product, *Lactobacillus acidophilus* and *Bifidobacterium* spp. are incorporated as a dietary adjunct (Shah, 2000). Yoghurt containing these two probiotic bacteria is referred as "AR" yoghurt (Shah, 2001).

MATERIALS & 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

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 (Prof. W.H. 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 reducing sugar estimation

Reagent A

Anhydrous sodium carbonate	25.0 g
Sodium potassium tartarate	25.0 g
Sodium hydrogen carbonate	20.0 g
Anhydrous sodium sulphate	200.0 g

These are dissolved in 800 ml distilled water and diluted to 1000 ml.

Reagent B

$CuSO_4.5H_2O$	30.0 g
Distilled water with 4 drops of conc. H_2SO_4	200 ml

Reagent C

(a) Ammonium molybdate	25.0 g
Distilled water with 21 ml of conc. H_2SO_4	450 ml
(b) Disodium hydrogen arsenate heptahydrate	3.0 g
Distilled water	25 ml

Solution (b) was added to solution (a) slowly with stirring, then diluted to 500 ml, kept at 37° C to 40° C overnight and stored in a brown bottle.

Reagent D (Somogyi copper solution)

25 ml of Reagent A was mixed with 1 ml of Reagent B. Freshly prepared Reagent D was used.

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

Collection of samples

For sampling of milk products, the method of Harrigan (1998) was followed. Samples were collected directly from their place of preparation in different villages located in the Sikkim Himalayas. For liquid samples, the liquid mass was thoroughly mixed up and down with a sterile ladle before the sample was taken. For soft milk products a sterile knife was used to make a wedge by two cuts radiating from the center of the product mass. For hard milk products a sterile cheese trier was inserted obliquely towards the center of the product on one of the flat surface not less than 10-20 cm from the wedge. The weight of the sample taken was not less than 250 g and the sampling equipments were sterilized before use. Samples were collected aseptically in pre-sterile poly-bags as well as sterile bottles, sealed, labelled and stored at −20° C for analyses.

Survey

A survey was conducted on the fermented milk products of the Sikkim Himalayas based on the method of the Indian Statistical Institute, Kolkata (Dr B. Mukhopadhay, unpublished). Three villages from each of the nine sub-divisions of the state of Sikkim were randomly selected. The method employed for selection of villages was unbiased and incorporated a high degree of randomness. The names of all villages/revenue blocks listed in the District Census Handbook of Sikkim (Census of India, 1991) were written in small, equal-sized pieces of paper, folded similarly and mixed well. Three slips corresponding to three villages were picked from each group for the sub-division and these villages were selected for the survey. Questionnaire was prepared

to collect information on the various types of fermented milk products produced and consumed by the people, the traditional methods of preparation, the ingredients used, the equipments used, mode of consumption and the socio-economic importance of these milk products.

A total of 228 households were surveyed in Sikkim. Based on the information collected, average annual production per household and per capita consumption per day of the various fermented milk products were statistically calculated using SPSS (Statistical Package for Social Sciences) 7.5 for Windows.

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. Aerobic mesophilic 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 were 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 at Food Microbiology Laboratory of Department of Botany, Sikkim Government College, Gangtok, Sikkim, India.

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 safranin, 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* 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 (Prof. W.H. Holzapfel, Institute of Molecular Biology and Biotechnology, Karlsruhe, Germany, 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).

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

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 anacrobic 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) Sarkar *et al.* (2002)

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 Petri-plates 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 (HiMedia 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.

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.

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

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 (for milk products) (AOAC, 1990).

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

Carbohydrate

The carbohydrate content was calculated by difference (Standal, 1963).

Carbohydrate (%) = 100 - [protein (%) + fat (%) + ash (%)]

Energy Value

The energy value was determined by multiplying the protein, fat and carbohydrate contents by the factors 4, 9 and 4, respectively, and adding all the multiplication values to get kcal per 100 g. (Gopalan *et al.*, 1995).

Reducing sugar

Reducing sugar content of sample was determined by modified colorimetric method of Somogyi (1945) using glucose as standard solution. To 1 ml of sample extract in a 20 ml capped glass tube, 1 ml of Reagent D was added and heated in a vigorously boiling water-bath for 20 min. This was allowed to cool for 5 min in running tap water and 1 ml of Reagent C was added and the test tube shaken until no bubbles evolved. After standing for 20 min, this was diluted to 25 ml with

distilled water and absorbance was measured at 520 nm in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). Reducing sugar was calculated in percentage.

Glucose (%) = $(As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 250/V_2 \times 100/10$

As = absorbance of sample

Ab = absorbance of blank

Ag = absorbance of glucose

[G] = concentration of glucose solution (µg/ml)

10^{-3} = mg to g

V_1 = total dilution volume for reaction (ml)

1= 1 ml for reaction

V_2 = pipetting volume of extract for dilution (ml)

250 = total volume of extract (ml)

100 = %

10 = sample size for preparation of extract

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).

Microbial and physico-chemical changes during fermentation

Soft chhurpi was prepared in the laboratory following the traditional method. Fresh cow milk was collected from a local milk producer. It was boiled, cooled and allowed to stand for 2 days at room temperature after which dahi was produced. The dahi was churned for 1 h to produce mohi and gheu. The mohi was boiled for 15 min to produce soft chhurpi, which in turn was hung in a muslin cloth for 1 day to drain out excess whey. The soft chhurpi was then put into a bottle and covered with a lid. This was allowed to ferment for 6 days at room temperature. Samples were taken every one day and the microbial and physico-chemical (pH, titratable acidity, reducing sugar) analysis done.

RESULTS

SURVEY ON FERMENTED MILK PRODUCTS

Survey was conducted in randomly selected 228 households within entire state of Sikkim (Fig 1). The number of households surveyed in North district was 54, South district was 48, East district was 78 and West district was 48. The various types of indigenous fermented milk products, their traditional methods of preparation, equipment used, mode of consumption, socio-economy and ethnical importance were documented (Table 1). Based on the survey, per capita consumption of different indigenous fermented milk products in the four districts of Sikkim was calculated (Table 2). The annual production of the various indigenous fermented milk products in Sikkim was also calculated (Table 3).

Some of the common indigenous fermented milk products are **dahi, mohi, gheu, soft chhurpi, dudh chhurpi,** and lesser-known milk products are **chhu, somar** and **philu**. These milk products are prepared from cow milk as well as kno (female yak) milk in Sikkim. Traditional method of preparation with flow-sheet, mode of consumption, socio-economy of some of the indigenous fermented milk products were documented.

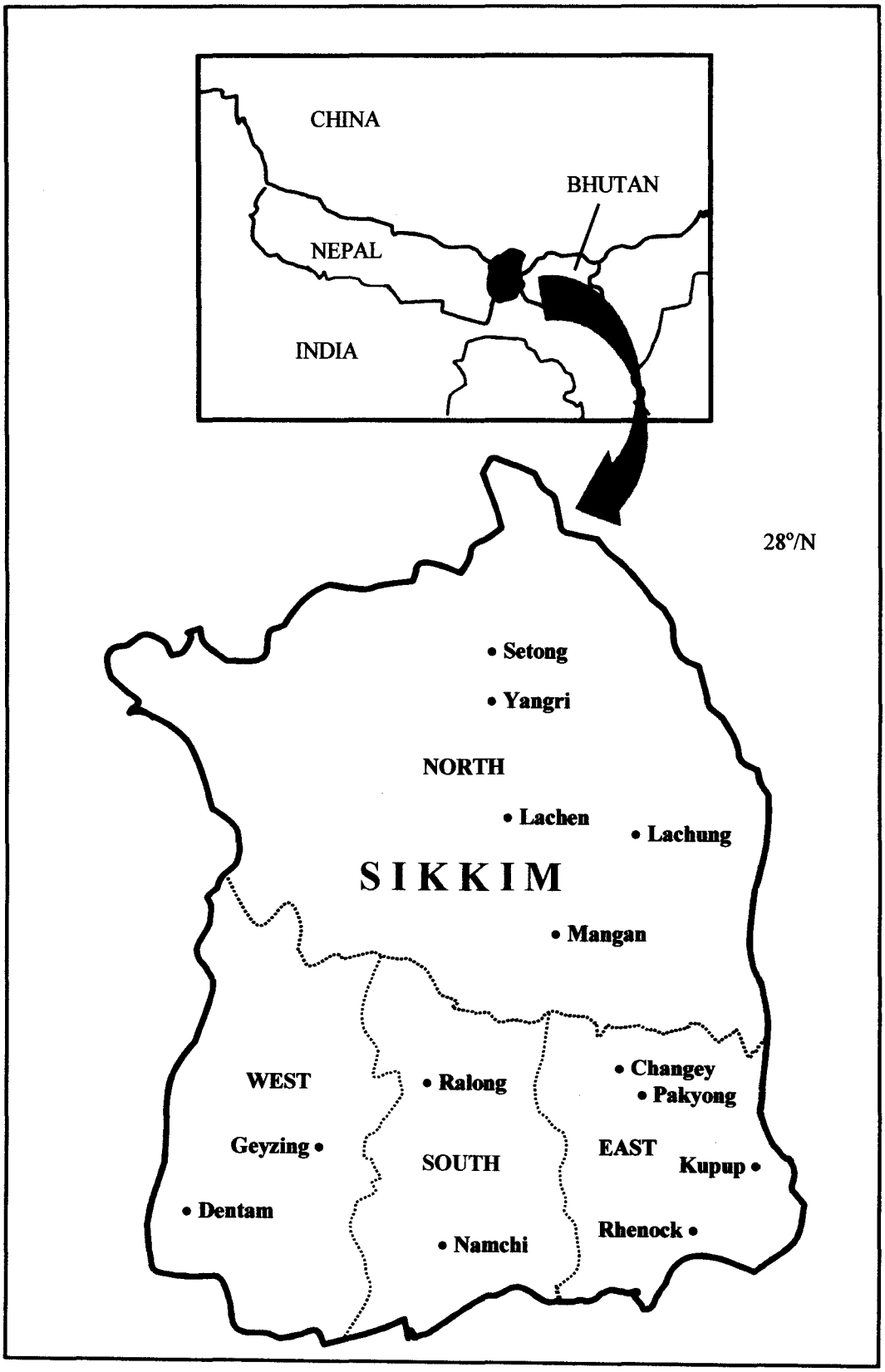

Fig 1. Location map of indigenous fermented milk products making villages in the Sikkim Himalayas

Table1. Indigenous fermented milk products of the Sikkim Himalayas

Product	Substrate	Nature and use	Dominant consumer
Dahi	Cow/kno[a] milk	Thick-gel; refreshing beverage, savory	Nepali, Bhutia, Lepcha
Mohi	Cow/kno milk	Buttermilk; refreshing beverage	Nepali, Bhutia, Lepcha
Gheu	Cow/kno milk	Butter; important ingredient in many other local foods	Nepali, Bhutia, Lepcha
Soft Chhurpi	Cow milk	Soft, rubbery, crumby-mass; curry, pickle, soup, side-dish	Nepali, Bhutia, Lepcha
Dudh chhurpi	Cow milk	Hard-mass; masticatory	Nepali, Bhutia, Lepcha
Chhu	Cow/kno milk	Semi-solid, strong flavoured; soup, curry, side-dish	Bhutia
Somar	Cow milk	Soft paste with strong flavour; condiment, soup	Sherpa
Philu	Cow/kno milk	Soft solid cream; cooked with butter and salt to produce a syrup delicacy, side-dish	Bhutia

[a] kno, female yak

Table 2. Per capita consumption of indigenous fermented milk products of the Sikkim Himalayas

Product	Per capita consumption (g per day)				
	District				
	North[a]	South[b]	East[c]	West[d]	Sikkim[e]
Dahi	29.9 ± 54.4	28.8 ± 35.5	46.2 ± 48.1	19.2 ± 19.2	33.0 ± 38.8
Ghee	10.3 ± 12.7	4.4 ± 4.8	9.8 ± 21.3	1.7 ± 2.0	7.1 ± 14.5
Soft chhurpi	5.0 ± 7.5	4.5 ± 9.9	1.6 ± 5.0	0.7 ± 1.7	2.8 ± 6.7
Chhu	3.4 ± 5.9	3.5 ± 9.9	0.3 ± 0.9	0.4 ± 1.8	1.7 ± 5.6
Somar	0	0.003 ± 0.017	0.1 ± 1.0	0	0.04 ± 0.56
Philu	0.4 ± 0.8	0.08 ± 0.31	0.006 ± 0.040	0.03 ± 0.13	0.1 ± 0.4
Dudh chhurpi	0.0005 ± 0.0009	0.001 ± 0.007	0.005 ± 0.026	0	0.002 ± 0.016
	Per capita consumption (ml per day)				
Mohi	22.7 ± 32.9	52.5 ± 56.7	68.6 ± 89.9	53.8 ± 57.5	51.2 ± 68.1

Products included both cow and kno fermented milk products.

Data represent the means of households[a] SD ± 54; households[b] SD ± 48; households[c] SD ± 78; households[d] SD ± 48; households[e] SD ± 228.

Table 3. Annual production of indigenous fermented milk products in each household of Sikkim

(kg per household)						
Product						
Dahi	Ghee	Soft chhurpi	Chhu	Somar	Philu	Mohi[a]
76.2 ± 97.7	14.2 ± 28.5	5.0 ± 13.3	4.0 ± 12.3	0.1 ± 1.2	0.2 ± 1.0	125.4 ± 171.0

[a] Litre per household

Data represent the means SD± 228 of households.

DAHI

Dahi is a traditional curd of the Sikkim Himalayas. It is a whitish semi-solid with a typical aroma and sour taste. Dahi is produced in most of the households for direct consumption as well as for the preparation of a number of other milk products like gheu, mohi, soft chhurpi, chhu, etc. Dahi is the Nepali word. The Bhutias and Lepchas call it shyow.

Traditional method of preparation

Fresh milk obtained from cow or kno (female yak) is boiled in a vessel. After boiling, the milk is cooled to room temperature. Sometimes a little amount of previous dahi (called 'mau' in Nepali) is added to the milk to speed the fermentation process. This is left for 1-2 days in summer or for 2-4 days in winter at room temperature for natural fermentation (Fig 2). The duration of fermentation depends on the season as well as on the geographical location of the place.

Milk
↓
Boiled
↓
Cooled to room temperature
↓
Previous dahi ('mau') added
↓
Fermentation (1-2 days)
↓
Dahi

Fig 2. Flow sheet of dahi preparation in Sikkim

Mode of consumption

Dahi is consumed directly as a refreshing non-alcoholic beverage. Sugar may or may not be added before consumption. It is also consumed after mixing it with rice or 'chewra' (beaten-rice). Dahi is used to prepare a number of other milk products like gheu, mohi, soft chhurpi, chhu, etc. Dahi is one of the important items in local diet including boiled rice, vegetable, pickle in meal (Plate 1). Per capita consumption of dahi is 33 g/day in Sikkim (Table 2).

Socio-economy

Many people in Sikkim produce dahi not only for self-consumption but also for selling the product in the local markets. Dahi, being very popular, finds a good market in all places of Sikkim. It costs about Rs. 16 per kg in Gangtok market.

Ethnical importance

Besides consumption, dahi is an essence in many religious occasions. Details of ethnical importance are discussed in discussion chapter of this thesis.

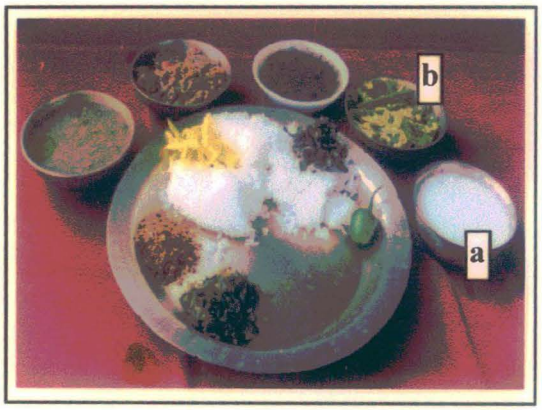

Plate 1. Typical Nepali meal including (a) dahi and (b) chhurpi curry

Plate 2. Churning of dahi in a (a) theki with (b) madani to produce gheu and mohi

Plate 3. Gheu being taken out of the theki

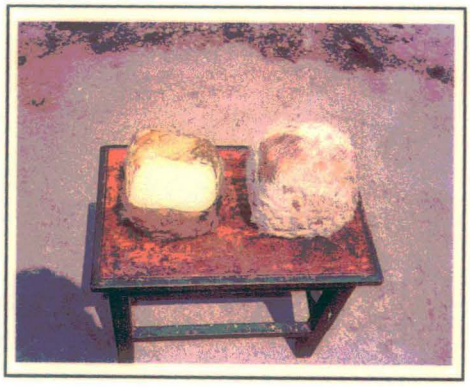

Plate 4. Maa stored inside the sheep skin as maata

MOHI

Mohi is buttermilk, liquid fermented milk product. It is whitish-coloured with a sour taste. Mohi is the by-product during the production of gheu (butter) from dahi. However it serves as an important item in the diet of the local people. Mohi is the Nepali name. The Bhutias and Lepchas call it kachhu.

Traditional method of preparation

Fresh milk obtained from cow or kno is boiled in a vessel. After boiling, the milk is cooled to room temperature. In most cases a little amount of previous dahi (called 'mau' in Nepali) is added to the milk and left for fermentation for 1-2 days in summer or 2-4 days in winter at room temperature. Fermentation leads to the formation of dahi. The dahi is churned to produce gheu and mohi. The churning can be done in a two ways. The most common and easy-to-use method is by using a long bamboo vessel, variously called as 'Tolung', 'Somg', or 'Padung' and a 'Madani', a long stick (Shar) with a circular or star-shaped flat wooden disc (Pangra) at one end. The dahi is poured into the bamboo vessel and the dahi is churned by lifting and lowering of the madani inside the bamboo vessel (Plate 2). The churning is done for 30-45 minutes with the addition of either cold or warm water as the weather demands to facilitate better separation of the gheu (butter) from the liquid mohi (buttermilk). After a big lump of gheu is formed and seen floating on the mohi, it is carefully lifted out with the hand and transferred to another vessel. Another method of churning is by using a 'Theki', a hollow wooden vessel and a 'Madani' consisting of the 'Ghurra', the churning part and the pulling string called 'Neti'. The dahi is kept inside the Theki and churned by pulling the Neti with either hand so that the Madani rotates in alternating clockwise and anticlockwise direction in the Theki. Cold or warm water is added as the

weather demands to facilitate better separation of the gheu (butter) from the liquid mohi (buttermilk). The gheu is collected as in the first method. The liquid that remains behind is mohi (Fig 3).

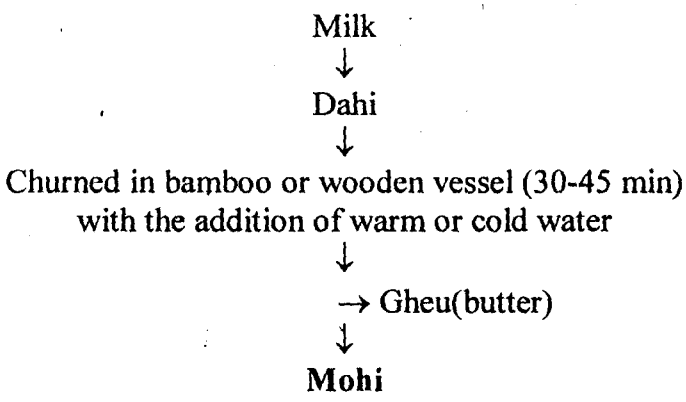

Fig 3. Flow sheet of mohi and gheu preparation in Sikkim

Mode of consumption

Mohi is consumed as a cooling beverage during hot days and also to overcome tiredness. It is also processed further to produce other fermented milk products like soft chhurpi, chhu, dudh chhurpi, etc. Per capita consumption of mohi is 51.2 ml/day in Sikkim (Table 2).

Socio-economy

Mohi is consumed at home as buttermilk drink.

GHEU

Gheu (butter) is a white-coloured, soft paste fermented milk product with a typical pleasant flavour and aroma. It is important commercially as well as for various other functions. Gheu is the Nepali name. The Bhutias and the Lepcha call maa and mor, respectively.

Traditional method of preparation

The dahi is churned to produce gheu (Plate 3) and mohi as described in mohi preparation (Fig 3). In North Sikkim, the Bhutias store gheu (maa) by wrapping it in a cubical shaped dried skin of 'bhenglung' (high altitude sheep) by stitching on all the edges from the inside called as 'maata' (Plate 4). Maata can be stored for several months or years for later consumption of the maa inside.

Mode of consumption

Freshly prepared gheu (called 'nuhaune gheu') is rarely consumed as it is. It is clarified further by boiling till the oily liquid separates from the unwanted dark-brown precipitate ('bilauni' or 'khar'). The clarified gheu (butter) is then consumed in a variety of ways. Gheu is mixed with boiled rice and eaten. It provides excellent aroma to the rice. It is also spread over bread and consumed. Gheu is used to prepare a large number of dishes like 'parathas', 'selroti', 'khabjay', 'pulao', various sweets and dishes, etc. Per capita consumption of gheu is 7.1 g/day in Sikkim (Table 2).

Socio-economy

Gheu is a highly priced milk product and serves as a major source of income for farmers and cattle-rearers in the Sikkim Himalayas. It is sold in the local markets all the year round. Cow milk gheu costs about Rs.160 per kg in Gangtok market.

SOFT CHHURPI

Soft chhurpi (Plate 5) is a cheese-like fermented milk product. It has a rubbery texture with slightly sour taste and excellent aroma when it is fresh. Soft chhurpi is used to prepare various dishes and its popularity is increasing as it provides a different taste to food.

Traditional method of preparation

Dahi is churned in a bamboo or wooden vessel, with the addition of warm or cold water, to produce gheu and mohi. The mohi is cooked for about 15 minutes till a soft, whitish mass is formed. This mass is seived out and put inside a muslin cloth, which is hung by a string to drain out the remaining whey. The product is called soft-variety of chhurpi (Fig 4).

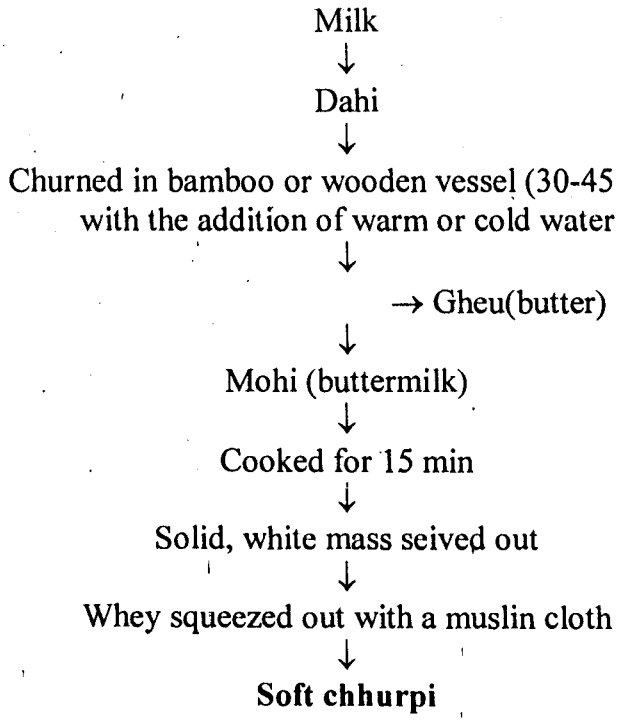

Fig 4. Flow sheet of soft chhurpi preparation in Sikkim

Mode of consumption

Soft chhurpi is prepared into a curry by cooking it in oil along with onions, tomato and chilies. Curry is also be prepared with wild edible ferns (Plate 6), locally called 'sauney ningro' (*Diplazium polypodiodes*) and 'kali ningro' (*Diplazium* sp.). This curry is consumed with rice or chapattis. It is also used to prepare 'achar' or pickle by mixing it with chopped cucumber, radish, chilies, etc. Soup prepared from soft chhurpi can be consumed as a substitute for dal along with rice. Per capita consumption of soft-variety of chhurpi is 2.8 g/day in Sikkim (Table 2).

Socio-economy

Soft chhurpi is sold in the local markets (Plate 8). It is consumed as an excellent source of protein and as a substitute for vegetables. Soft chhurpi costs about Rs. 60 per kilogram in Gangtok market.

DUDH CHHURPI

Dudh chhurpi (Plate 7) is a common fermented milk product consumed as a masticatory in the Sikkim Himalayas. It is usually prepared from cow milk and is available as small cubical shaped white solid mass in the local markets. A sweet, white powdery surface covers the hard-textured mass inside. In the market, dudh chhurpi pieces are sold after sewing in long strings. The Bhutias and Lepchas call it khamu.

Traditional method of preparation

Cow milk is boiled, cooled and kept for 1-2 days at room temperature to produce dahi. Dahi is churned in a bamboo vessel to produce gheu (butter) which is separated from the liquid mohi (buttermilk). The mohi is cooked in a vessel over fire for about 15 minutes to produce a solid white mass. This mass is taken out using a bamboo sieve (called 'chucha') and after the

Plate 5. **Soft chhurpi**

Plate 6. **Soft chhurpi curry with edible ferns and red chilli**

Plate 7. **Dudh chhurpi**

Plate 8. **Gheu and soft chhurpi being sold in the local market**

Plate 9. **Dudh chhurpi being sold in the local market**

liquid whey is drained off, it is placed inside a jute-sack. It is then pressed with a heavy stone for 2-4 hours to drain out the liquid. The solid is taken out of the sack, cut to cubical pieces (about 6.0 g weight). 'Thake', a thick paste prepared from the cooked mohi and milk, is applied on the surface of theses pieces, which are then, are woven in a thread and hung in the open air for sun drying for 3-4 days (Fig 5). After drying, this unique dairy product is consumed as masticatory.

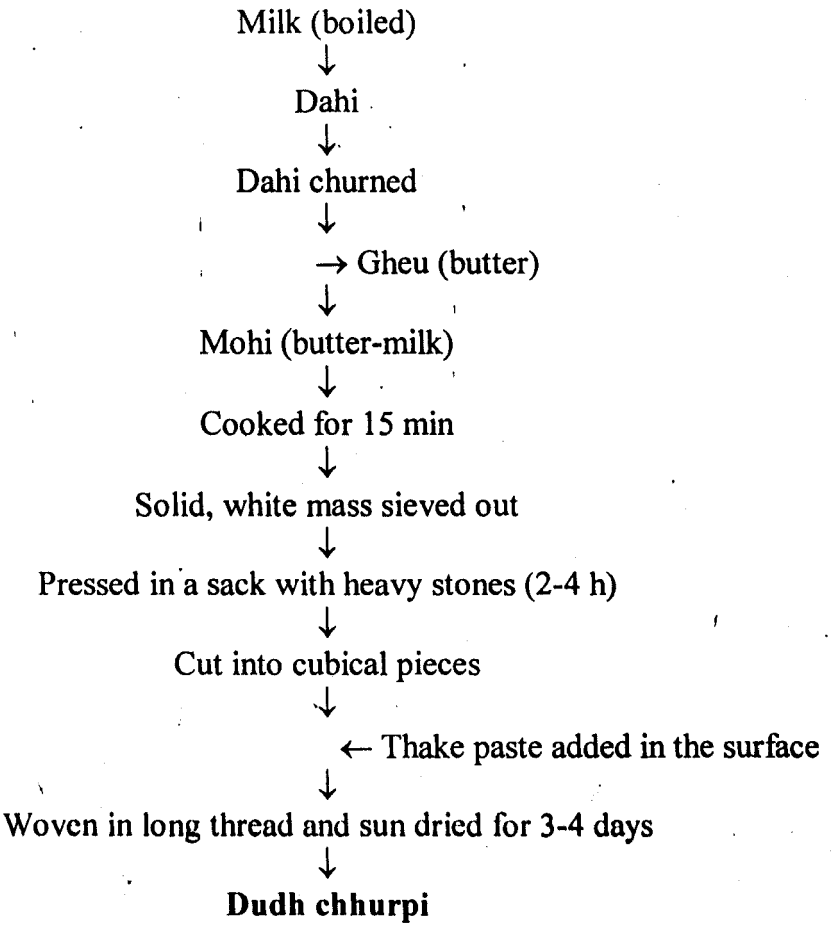

Fig 5. Flow sheet of dudh chhurpi preparation in Sikkim

Mode of consumption

Dudh chhurpi is consumed as a nutritious masticatory (like a chewing gum) by the people of Sikkim.

Socio-economy

Dudh chhurpi is sold in all local markets of Sikkim (Plate 9). It is available in chains woven in strings or as individual pieces. Dudh chhurpi costs about Rs.1 to 2 per piece in Gangtok market.

CHHU

Chhu (Plate 10) is a traditional fermented milk product consumed mostly by the Bhutia community in Sikkim. It is prepared from boiled or unboiled milk. Like chhurpi, it initially has a rubbery texture with slightly sour taste when it is fresh. But after further fermentation it becomes more liquid, creamish to pale yellow coloured and develops a strong flavour.

Traditional method of preparation

Dahi ('shyow') is prepared from boiled or unboiled milk (which helps in the formation of a highly sour and rapidly fermenting chhu later on). The dahi is churned in a bamboo or wooden vessel, with the addition of warm or cold water to produce gheu ('maa') and mohi ('kachhu'). The mohi is cooked for 15 minutes till a soft, whitish mass is formed. This mass is seived out and put inside a muslin cloth, which is hung by a string to drain out the remaining whey. The product is called chhu (Fig 6). The chhu is placed in a closed vessel and kept for several days to months to ferment the product further after which it is consumed.

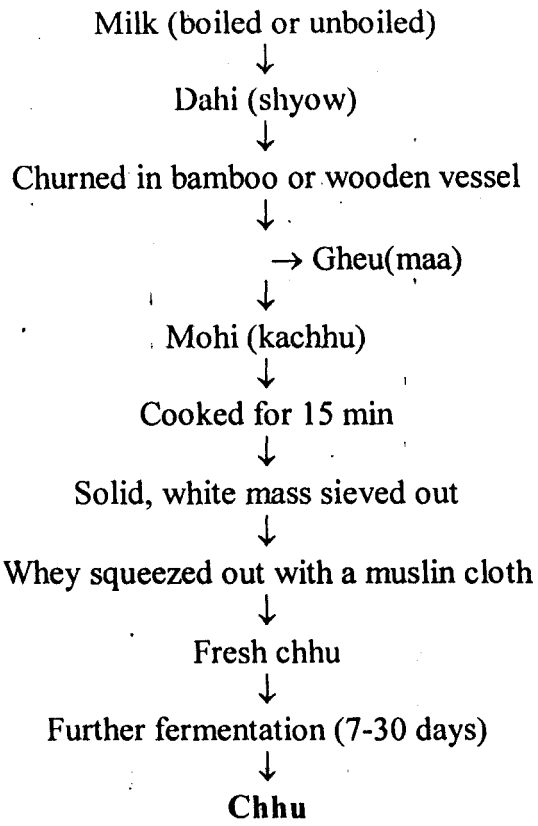

Fig 6. Flow sheet of chhu preparation in Sikkim

Mode of consumption

Fresh chhu is placed inside a vessel with a tight lid and left for fermentation at room temperature for a few days to several months. Chhu is prepared into a curry by cooking it in oil along with onions, tomato and chilies. This curry is consumed with cooked rice. Per capita consumption of chhu is 1.7g/day in Sikkim (Table 2).

Socio-economy

Fresh chhu is sold in the local markets. It costs about Rs. 60 per kilogram of fresh chhu in Gangtok market.

SOMAR

Somar (Plate 11) is a traditional fermented milk product prepared from cow milk consumed mostly by the Sherpa community belonging to Nepalis ethnic group resides at high altitudes in the Sikkim Himalayas. It is a soft paste with slightly bland and bitter taste and has a highly fermented smell. Somar is kept for a long period of time by cooking it with gheu (butter) and 'hardi' (tumeric) to produce a soft-brown paste to form a new type of somar. This is consumed as soup along with rice or 'dhero', a dish prepared from fingermillet.

Traditional method of preparation

Dahi ('shyow') is prepared from boiled and cooled cow milk. The dahi is churned in a bamboo vessel ('tolung') with a 'madani' for 30 minutes, with the addition of warm or cold water, to separate butter ('mor') from buttermilk ('thara'). The mohi is cooked for 15 minutes till a soft, whitish mass is formed. This mass is seived out with a cloth or plastic sieve. The product is called 'shergem' (same as soft chhurpi). The shergem is placed in a closed glass or plastic vessel and kept for several days (about 15 days) to ferment the product further. The fermented product is then cooked with milk, 'mor' (butter) and 'hardi' (tumeric) to prepare a thick brown paste, a new form of somar (Fig 7). This type of somar is stored several months for later consumption.

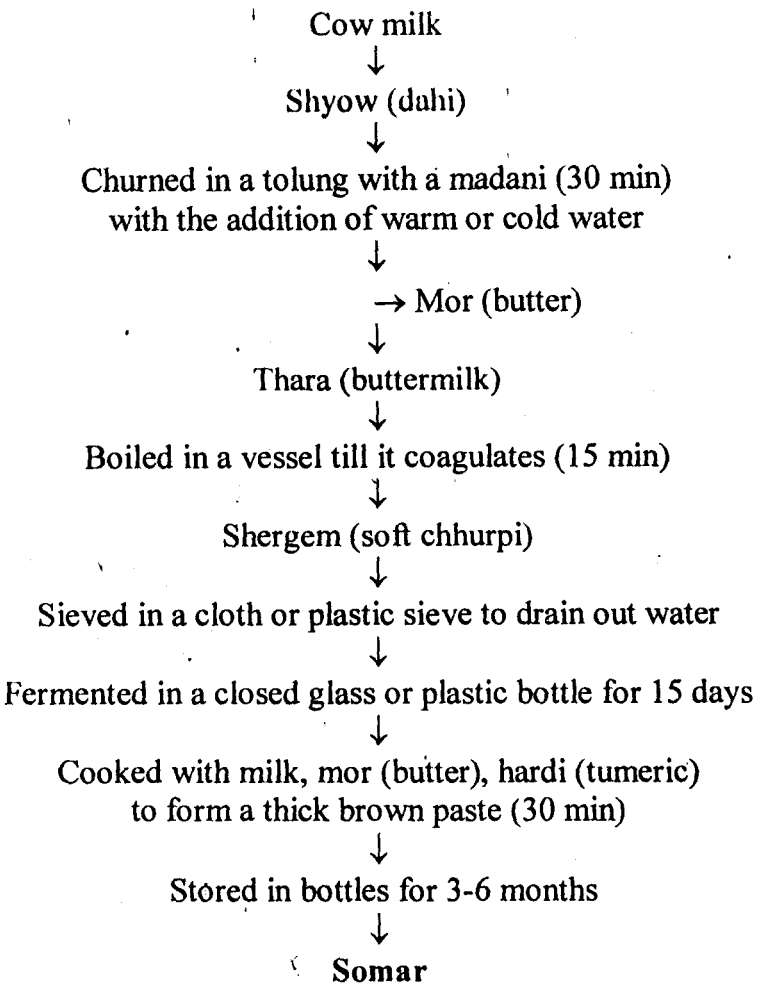

Fig 7. Flow sheet of somar preparation in Sikkim

Mode of consumption

A thick soup of somar is made by mixing with oil, garlic and salt. Somar soup is consumed with rice or 'dhero', a fingermillet dish.

Socio-economy

Somar is not sold in the market due to limited number of consumers. It is occasionally prepared in the homes of the Sherpas for self-consumption.

PHILU

Philu (Plate 12) is a typical indigenous fermented butter-like milk product obtained from cow or kno (female yak) milk. It is commonly consumed as cooked-paste delicacy with boiled rice by the Bhutias of Sikkim. Philu produced from cow milk is white coloured with a butter-like texture. It has a faint butter-like aroma and slightly bland taste. The philu obtained from kno milk has a creamish-white colour with an inconsistent semi-solid texture. It is more flavoured and sour in taste.

Traditional method of preparation

During the traditional method of philu preparation, fresh milk collected in cylindrical bamboo vessels (locally called 'dhungtuk' and 'dzydung' by the Bhutias) or in wooden vessels (called 'yadung' or 'thongba' in North Sikkim) is slowly swirled around the walls of these vessels by rotating these vessels for a few minutes (Plate 13). Sometimes a thick mesh of dried creeper is kept inside the vessel to increase the surface area of the vessel. A creamy mass sticks to the walls of the vessels and around the creeper. The milk is then poured off and utilized elsewhere. The vessel is then kept in an upside down position to drain out the remaining liquid. This process is repeated daily for about 6 to 7 days until a thick, white cream-layer is formed on the vessel walls and the creeper surface (Fig 8). This soft mass, known as philu, is scraped off and sold or consumed.

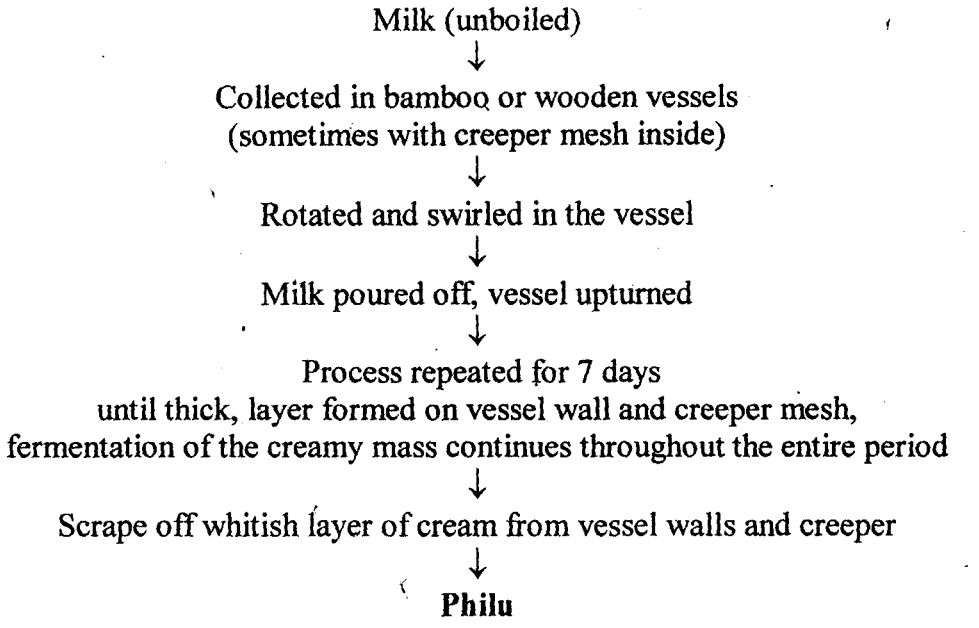

Fig 8. Flow sheet of philu preparation in Sikkim

Mode of consumption

Philu is consumed as a side dish with rice. Gheu ('maa') is cooked for sometime in a vessel. To this philu is added and cooked further after adding a little salt. Rich gravy is prepared and consumed along with boiled rice.

Socio-economy

Philu is a high-priced traditional milk product of the Sikkim Himalayas. Cow milk philu costs Rs. 200 per kg in Gangtok market. Many people are dependent on this product for their livelihood. In North Sikkim, philu is produced mostly from kno milk and is produced and consumed at the household level by the Bhutias.

Plate 10. Chhu

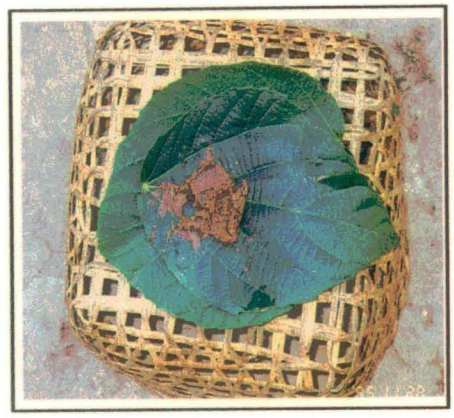

Plate 11. Somar

Plate 12. Philu and philu wrapped in a leaf for selling purpose

Plate 13. Preparation of philu in a bamboo vessel

MICROORGANISMS

Microbial load

One hundred and eighty-five samples of cow milk-fermented milk products and eighty-one samples of kno (female yak) milk-fermented milk products were collected from different places of Sikkim as shown in Table 4 and 5, respectively. Samples were analysed for microbial load (Table 6 and 7). The lactic acid bacteria (LAB) numbers were generally higher ranging from 10^4 to 10^8 cfu/g in all the fermented milk products analysed. The population of spore-formers was recovered in unboiled milk of cow and kno, soft chhurpi, cow chhu and somar at the level of 10 to 10^3 cfu/g. The yeast population ranged at the level of 10^3 to 10^7 cfu/g. However, yeasts were not detected in samples of kno mohi, kno maa and kno philu. No filamentous mould was detected in any of the samples of fermented milk products analysed. Aerobic mesophilic counts were 10^8 cfu/g in unboiled milk (cow and kno), 10^8 to 10^9 cfu/g in dahi, chhu, philu, 10^8 cfu/g in mohi and soft chhurpi, 10^5 cfu/g in somar, 10^4 cfu/g in dudh chhurpi and 10^6 cfu/g in maa.

Out of 1050 isolates of microorganisms obtained from 266 samples of fermented milk products, 772 isolates were lactic acid bacteria, 74 were spore-formers and 204 were yeasts.

Table 4. Collection of cow milk and its fermented products from different places of Sikkim

Product	Sample code	Place of collection	District	No. of samples collected
Milk (unboiled)	CMN	Namchi	South	3
	CMR	Ranka	East	4
	CMS	Syari	East	3
	CMD	Dentam	West	3
Dahi	CDM	Mangan	North	3
	CDR1	Ralong	South	5
	CDN	Namchi	South	5
	CDC	Changey	East	4
	CDR	Ranka	East	5
	CDS	Samdong	East	4
	CDP	Pakyong	East	4
	CDR2	Rhenock	East	5
	CDB	Bhusuck	East	6
	CDD	Dentam	West	5
Mohi	COM	Mangan	North	4
	CON	Namchi	South	5
	COS	Samdong	East	4
	COR	Ranka	East	6
	COD	Dentam	West	4
Soft chhurpi	CCM	Mangan	North	5
	CCN	Namchi	South	6
	CCS	Samdong	East	5
	CCR1	Ranipool	East	5
	CCR2	Ranka	East	4
	CCB	Bhusuck	East	6
	CCP	Pakyong	East	6
	CCG	Geyzing	West	5
	CCD	Dentam	West	4
Chhu	CUR	Ralong	South	6
	CUN	Namchi	South	5
	CUG1	Gangtok	East	5
	CUG2	Geyzing	West	5
Somar	CSR1	Rongli	East	5
	CSR2	Rongli	East	5
	CSN1	Begha	West	6
	CSN2	Begha	West	4
Philu	PC1	Changey	East	5
	PC2	Changey	East	5
Dudh chhurpi	DC1	Changey	East	3
	DC2	Changey	East	3

Table 5. Collection of kno (female yak) milk and its fermented products from different places of Sikkim

Product	Sample code	Place of collection	District	No. of samples collected
Milk (unboiled)	KML	Lachen	North	3
	KMLg	Lachung	North	3
	KMY	Yangri	North	3
	KMS	Setong	North	3
	KMK	Kupup	East	3
Dahi	KSY	Yangri	North	5
	KSS	Setong	North	6
	KSK	Kupup	East	5
Mohi	KOY	Yangri	North	5
	KOS	Setong	North	5
Chhu	KCY	Yangri	North	5
	KCS	Setong	North	4
	KCK	Kupup	East	5
Philu	KPY	Yangri	North	6
	KPS	Setong	North	5
Maa	KAL	Lachen	North	3
	KAY	Yangri	North	4
	KAS	Setong	North	5
	KAK	Kupup	East	3

Table 6. Microbial load of fermented cow milk products of the Sikkim Himalayas

Product	Log cfu/g fresh weight			
	LAB	Yeast	Sporeformer	Aerobic mesophilic count
Milk (unboiled)	8.2 (8.0-8.3)	7.3 (7.2-7.4)	2.0 (1.8-2.3)	8.5 (8.4-8.7)
Dahi	8.6 (8.5-8.7)	6.7 (6.6-6.8)	<DL	8.9 (8.7-9.0)
Mohi	8.5 (8.5-8.6)	6.3 (6.2-6.4)	<DL	8.8 (8.5-8.9)
Soft chhurpi	8.4 (8.4-8.6)	6.6 (6.3-6.9)	2.5 (2.4-2.7)	8.6 (8.5-8.7)
Chhu	8.2 (7.9-8.3)	6.8 (6.6-6.9)	3.1 (2.7-3.2)	9.0 (8.9-9.1)
Somar	4.4 (4.2-4.6)	3.0 (2.7-3.3)	3.2 (3.0-3.3)	5.3 (5.1-5.4)
Philu	8.1 (7.9-8.2)	6.5 (6.4-6.7)	<DL	9.5 (9.2-9.6)
Dudh chhurpi	4.0 (3.8-4.1)	3.3 (3.0-3.7)	<DL	4.5 (4.3-4.7)

cfu, colony forming units; LAB, lactic acid bacteria; DL, detection limit was 10 cfu/g

Data represent the means of 13 samples of unboiled milk; 46 samples of dahi; 23 samples of mohi; 46 samples of soft chhurpi; 21 samples of chhu; 20 samples of somar; 10 samples of philu; 6 samples of dudh chhurpi.

Ranges are given in parentheses. Mould was not detected.

Table 7. Microbial load of fermented kno (female yak) milk products of the Sikkim Himalayas

Product	Log cfu/g fresh weight			
	LAB	Yeast	Spore-former	Aerobic mesophilic count
Milk (unboiled)	7.1 (7.0-7.3)	5.0 (4.9-5.2)	1.2 (0.8-1.4)	8.2 (8.1-8.3)
Dahi	8.5 (8.3-8.6)	6.7 (6.7-6.8)	<DL	9.1 (9.0-9.2)
Mohi	8.7 (8.5-8.8)	<DL	<DL	8.9 (8.8-9.0)
Maa	6.2 (6.1-6.6)	<DL	<DL	6.9 (6.8-7.0)
Chhu	8.7 (8.5-8.8)	7.2 (7.0-7.3)	<DL	9.3 (9.2-9.4)
Philu	8.0 (7.9-8.1)	<DL	<DL	8.5 (8.4-8.6)

cfu, colony forming units; LAB, lactic acid bacteria; DL, detection limit was 10 cfu/g

Data represent the means of 15 samples of unboiled milk; 16 samples of dahi; 10 samples of mohi; 15 samples of maa; 14 samples of chhu; 11 samples of philu.

Ranges are given in parentheses. Mould was not detected.

Lactic acid bacteria

Out of 772 lactic acid bacteria strains isolated from 266 samples of fermented milk (both cow and kno) products, 663 isolates were non-sporeforming rods and 109 isolates were coccii (Table 8).

Table 8. Selection of representative strains of LAB isolated from fermented milk products of the Sikkim Himalayas

Product[a]	Number of strains isolated	Cell Shape	Gas from glucose	NH_3 from arginine	Grouped strains	Representative strains
Cow milk (unboiled)	45	Coccus	–	–	2	CMS2:C1
		Coccus	–	+	2	CMR2:C1
		Rod	–	–	34	CMN1:R1
		Rod	+	–	7	CMD1:R1
Cow dahi	92	Coccus	–	–	27	CDM1:C1
		Coccus	–	+	4	CDN1:C4
		Rod	–	–	58	CDC1:R1
		Rod	+	–	3	CDP1:R1
Cow mohi	46	Coccus	–	–	17	COM1:C3
		Coccus	–	+	14	CON1:C4, COS1:C1
		Rod	–	–	15	COD1:R2
Cow soft chhurpi	93	Coccus	–	–	4	CCB1:C1
		Rod	–	–	23	CCD1:R1
		Rod	–	+	18	CCD2:R1
		Rod	+	+	28	CCN1:R1
		Rod	+	–	20	CCR2:R1
Cow chhu	70	Coccus	–	–	10	CUR1:C1
		Rod	–	–	46	CUG3:R1
		Rod	–	+	8	CUG1:R2
		Rod	+	+	6	CUG2:R2

Product[a]	Number of strains isolated	Cell Shape	Gas from glucose	NH_3 from arginine	Grouped strains	Representative strains
Cow somar	25	Coccus	–	–	10	CSR2:C1
		Rod	–	–	15	CSR1:R1
Cow philu	80	Coccus	–	+	19	PC1:C4
		Rod	–	–	17	PC2:R6
		Rod	+	+	18	PC1:R11
		Rod	+	–	26	PC1:R1
Cow dudh chhurpi	49	Rod	–	–	5	DC2:R1
		Rod	–	+	4	DC1:R2
		Rod	+	+	20	DC4:R1
		Rod	+	–	20	DC4:R2
Kno milk	58	Rod	–	–	25	KMK1:R1
		Rod	–	+	15	KML1:R2
		Rod	+	+	9	KMK3:R1
		Rod	+	–	9	KMY1:R1
Kno dahi	53	Rod	–	–	23	KSY1:R2, KSY2:R2
		Rod	+	–	30	KSK1:R1
Kno chhu	81	Rod	–	–	41	KCK1:R1
		Rod	+	+	12	KCY1:R1
		Rod	+	–	28	KCK2:R4
Kno philu	80	Rod	–	–	80	KPY1:R1

[a]Number of samples is given in Table 6 and 7.

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

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. Representative strains CMN1:R1 (cow milk), CCD2:R1 (cow soft chhurpi), KMK1:R1 (kno milk), CMD1:R1 (cow milk), CDP1:R1 (cow dahi), PC1:R11 (cow philu), PC1:R1 (cow philu), DC4:R2 (cow dudh chhurpi), KMY1:R1 (kno milk), KSK1:R1 (kno dahi), KCK2:R4 (kno chhu), CDC1:R1 (cow dahi), COD1:R2 (cow mohi), CCD1:R1 (cow soft chhurpi), CUG3:R1 (cow chhu), CCN1:R1 (cow chhu), CCR2:R1 (cow soft chhurpi), CUG2:R2 (cow chhu), KCY1:R1 (kno chhu), PC2:R6 (cow philu), DC2:R1 (cow dudh chhurpi), KPY1:R1 (kno philu), CSR1:R1 (cow somar), KSY1:R2 (kno dahi), KSY2:R2 (kno dahi), DC4:R1 (cow dudh chhurpi), KMK3:R1 (kno milk), CUG1:R2 (cow chhu), DC1:R2 (cow dudh chhurpi), KML1:R2 (kno milk), KCK1:R1 (kno chhu) were non-sporeforming rods.

On the basis of sugar fermentation using the API system, lactic acid configuration and meso-diaminopimelic determination (Table 9) and also the taxonomical keys described by Sneath *et al.* (1986) and Wood and Holzapfel (1995), homo-fermentative lactic CMN1:R1, CCD2:R1, KMK1:R1 were identified as *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.* (Plate a), strains CDC1:R1, COD1:R2, CCD1:R1, CUG3:R1 as *Lactobacillus alimentarius* Reuter, strains CSR1:R1, KSY1:R2, KSY2:R2 as *Lactobacillus casei* subsp. *pseudoplantarum* Abo-Elnaga and Kandler, strains PC2:R6, DC2:R1, KPY1:R1 as *Lactobacillus casei* subsp. *casei* (Orla-Jensen) Hansen and Lessel, strains CUG1:R2, DC1:R2, KML1:R2 as *Lactobacillus farciminis* Reuter, strain KCK1:R1 as *Lactobacillus salivarius* Rogosa, Wiseman, Mitchell and Disraely; whereas hetero-fermentative lactics CMD1:R1, CDP1:R1, PC1:R11, PC1:R1, DC4:R2, KMY1:R1, KSK1:R1, KCK2:R4 were identified as *Lactobacillus bifermentans* Kandler, Schillinger and Weiss, strain CCN1:R1 as

Lactobacillus hilgardii Douglas and Cruess, CCR2:R1 as *Lactobacillus kefir* Kandler and Kunath, strains CUG2:R2, KCY1:R1 as *Lactobacillus brevis* (Orla-Jensen) Bergey *et al.* and strains DC4:R1, KMK3:R1 as *Lactobacillus confusus* (Holzapfel and Kandler) Sharpe, Garvie and Tilbury

Representative strains CMR2:C1 (cow milk), CDN1:C4 (cow dahi), CON1:C4 (cow mohi), COS1:C1 (cow mohi), CMS2:C1 (cow milk), CDM1:C1 (cow dahi), COM1:C3 (cow mohi), CUR1:C1(cow chhu), CSR2:C1 (cow somar) were coccii in shape, grew well at 10° C and 15° 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 and the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), strains CMR2:C1, CDN1:C4, CON1:C4, COS1:C1 were identified as *Lactococcus lactis* subsp. *lactis* Schleifer *et al.* and CMS2:C1, CDM1:C1, COM1:C3, CUR1:C1, CSR2:C1 as *Lactococcus lactis* subsp. *cremoris* Schleifer *et al.*

Representative strains CCB1:C1 (cow soft chhurpi), PC1:C4 (cow philu) were coccus-shaped, grew well at 10° C, 15° C and 45° C, pH 3.9 and pH 9.6, grew in NaCl 6.5 %, produced no gas from glucose. Following sugar fermentation pattern of isolates using API 50 CHL system and the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), strains CCB1:C1, PC1:C4 were identified as *Enterococcus faecium* (Orla-Jensen) Schleifer and Kilpper-Bälz (Plate b).

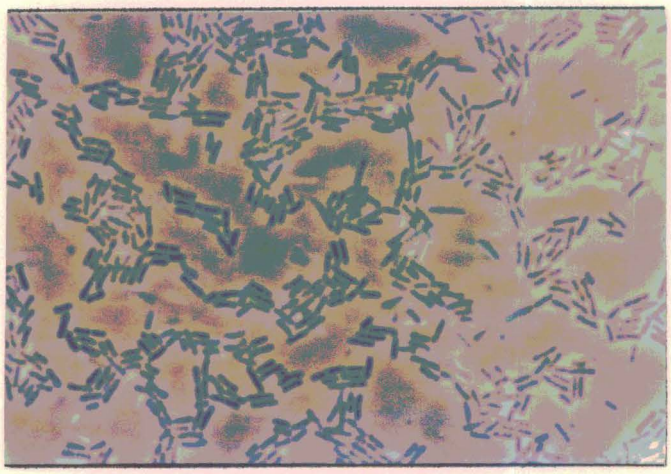

Plate (a). *Lactobacillus plantarum* CCD2:R1 (MRS agar, 3d, 30° C), isolated from soft chhurpi, showing non-sporeforming rod cells in phase contrast micrograph (× 825).

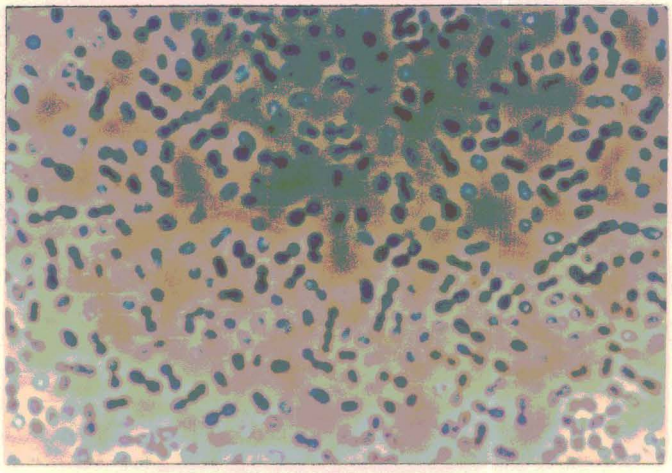

Plate (b). *Enterococcus faecium* PC1:C4 (MRS agar, 3 d, 30° C), isolated from philu, showing coccus cells in phase contrast micrograph (× 825).

Plate (c). *Bacillus subtilis* CSR2:S1 (NA, 2 d, 30° C), isolated from somar, showing endospore-forming rods in phase contrast micrograph (× 825).

Table 9: Characteristics of LAB strains isolated from fermented milk products of the Sikkim Himalayas

Product	Strain code	Cell Morphology	Cell Size (µm)	Gram-Stain	Catalase	Arginine Hydrolysis	CO_2 from Glucose	Growth at/in										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%	m-Dpm		Arabinose	Ribose	Xylose	Galactose	Sucrose	Trehalose	Rhamnose	Mannose	Sorbitol	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Raffinose	Melezitose	
Cow milk	CMN1:R1	Rod	1.1 (0.8-1.2) x 0.5 (0.4-0.6)	+	–	–	–	+	+	–	+	–	+	–	–	+	DL	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	+	*Lactobacillus plantarum*
	CMD1:R1	Rod	1.0 (0.8-1.2) x 0.5 (0.4-0.8)	+	–	–	+	+	+	–	+	–	–	–	–	–	DL	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	+	*Lactobacillus bifermentans*
	CMR2:C1	Coccus	1.3 (0.8-1.6)	+	–	+	–	+	–	–	+	–	–	–	–	nd	L	–	+	–	+	–	+	–	+	–	+	+	+	+	+	+	–	–	*Lactococcus lactis* subsp. *lactis*
	CMS2:C1	Coccus	1.2 (0.8-1.6)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	+	–	+	–	+	–	+	–	+	+	+	+	+	+	–	–	*Lactococcus lactis* subsp. *cremoris*
Cow dahi	CDC1:R1	Rod	1.0 (0.8-1.2) x 0.5 (0.4-0.8)	+	–	–	–	+	+	–	+	–	+	–	–	–	L(D)	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	+	*Lactobacillus alimentarius*
	CDM1:C1	Coccus	1.1 (0.8-1.6)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	–	+	–	+	–	+	–	+	–	+	+	+	+	+	+	–	–	*Lactococcus lactis* subsp. *cremoris*
	CDN1:C4	Coccus	1.2 (0.8-1.6)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	+	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	+	*Lactococcus lactis* subsp. *lactis*
	CDP1:R1	Rod	1.0 (0.8-1.2) x 0.5 (0.4-0.8)	+	–	–	+	+	+	–	+	–	+	–	–	–	DL	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Lactobacillus bifermentans*
Cow mohi	COD1:R2	Rod	0.9 (0.8-1.2) x 0.4 (0.3-0.5)	+	–	–	–	+	+	–	+	–	+	–	–	–	L(D)	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Lactobacillus alimentarius*
	COM1:C3	Coccus	1.1 (0.8-1.6)	+	–	–	–	+	+	–	+	–	+	–	–	nd	L	+	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Lactococcus lactis* subsp. *cremoris*
	CON1:C4	Coccus	1.3 (0.8-1.6)	+	–	+	–	+	+	–	+	–	+	–	–	nd	L	+	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Lactococcus lactis* subsp. *lactis*
	COS1:C1	Coccus	1.1 (0.8-1.6)	+	–	+	–	+	+	–	+	–	+	–	–	nd	L	+	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Lactococcus lactis* subsp. *lactis*
Cow soft chhurpi	CCD1:R1	Rod	0.9 (0.6-1.3) x 0.5 (0.3-0.6)	+	–	–	–	+	+	–	+	–	+	–	–	–	L(D)	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	+	*Lactobacillus alimentarius*
	CCD2:R1	Rod	1.2 (1.0-1.6) x 0.6 (0.5-0.6)	+	–	+	–	+	+	–	+	–	+	–	–	+	DL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	*Lactobacillus plantarum*
	CCN1:R1	Rod	1.5 (1.0-1.9) x 0.7 (0.5-0.8)	+	–	+	+	+	+	–	+	–	+	–	–	–	DL	+	+	+	+	–	–	–	–	–	–	–	–	+	–	+	–	–	*Lactobacillus hilgardii*
	CCR2:R1	Rod	0.7 (0.5-0.8) x 0.4 (0.3-0.5)	+	–	–	+	+	+	–	+	–	–	–	–	–	DL	+	+	+	+	+	–	–	–	–	–	–	–	+	–	+	–	–	*Lactobacillus kefir*
	CCB1:C1	Coccus	1.3 (1.0-1.6)	+	–	–	–	+	+	+	+	+	+	–	–	nd	L	+	+	–	+	+	+	+	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*

nd, not determined

contd. Table 9: Characteristics of LAB strains isolated from fermented milk products of the Sikkim 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		
Cow chhu	CUG2:R2	Rod	1.2 (0.8-1.6) x 0.6 (0.4-0.8)	+	–	+	+	+	+	–	+	–	+	–	–	–	DL	+	+	+	+	–	–	–	–	–	–	–	–	+	–	–	–	–	***Lactobacillus brevis***
	CUG3:R1	Rod	0.7 (0.6-0.8) x 0.5 (0.3-0.6)	+	–	–	–	+	+	–	+	–	+	–	–	–	L(D)	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	+	***Lactobacillus alimentarius***
	CUG1:R2	Rod	1.5 (1.0-1.9) x 0.4 (0.3-0.5)	+	–	+	–	+	+	–	+	–	w	–	–	–	L(D)	+	+	+	+	–	–	–	–	–	–	–	–	–	+	–	–	***Lactobacillus farciminis***
	CUR1:C1	Coccus	1.1 (0.8-1.5)	+	–	–	–	+	+	–	+	+	+	–	–	nd	L	–	+	–	+	–	+	–	+	–	+	+	+	+	+	–	–	–	***Lactococcus lactis* subsp. *cremoris***
Cow phllu	PC1:R11	Rod	0.8 (0.5-1.0) x 0.4 (0.3-0.5)	+	–	+	+	+	+	–	+	–	+	–	–	–	DL	–	+	+	–	–	–	–	–	–	–	–	+	–	–	–	–	***Lactobacillus bifermentans***
	PC1:C4	Coccus	1.0 (0.8-1.3)	+	–	+	–	+	+	+	+	+	+	–	–	nd	L	+	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	–	***Enterococcus faecium***
	PC2:R6	Rod	0.9 (0.8-1.2) x 0.5 (0.4-0.8)	+	–	–	–	+	+	–	+	–	+	–	–	–	L	–	+	–	+	+	+	–	+	+	+	+	+	+	–	+	–	–	***Lactobacillus casei* subsp. *casei***
	PC1:R1	Rod	1.3 (1.2-1.6) x 0.5 (0.3-0.6)	+	–	–	+	+	+	–	+	–	+	–	–	–	DL	+	+	+	–	+	–	–	–	–	–	–	–	+	–	–	–	–	***Lactobacillus bifermentans***
Cow somar	CSR1:R1	Rod	1.6 (0.8-2.4) x 0.9 (0.6-1.3)	+	–	+	+	+	+	–	+	–	+	–	–	–	DL	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	+	+	***Lactobacillus casei* subsp. *pseudoplantarum***
	CSR2:C1	Coccus	1.3 (1.0-1.6)	+	–	–	–	+	+	–	+	+	+	–	–	nd	L	–	+	–	+	–	+	–	+	–	+	+	+	+	+	+	–	–	***Lactococcus lactis* subsp. *cremoris***
Cow dudh chhurpi	DC1:R2	Rod	0.9 (0.8-1.2) x 0.5 (0.4-0.6)	+	–	+	–	+	+	–	+	–	+	–	–	–	L(D)	+	+	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	***Lactobacillus farciminis***
	DC2:R1	Rod	0.9 (0.6-0.9) x 0.4 (0.2-0.4)	+	–	–	–	+	+	–	+	–	+	–	–	–	L	–	+	–	+	+	+	–	+	+	+	+	+	+	+	–	–	–	***Lactobacillus casei* subsp. *casei***
	DC4:R1	Rod	1.1 (0.8-1.2) x 0.5 (0.4-0.6)	+	–	+	+	+	+	+	+	–	+	–	–	–	DL	+	+	+	+	–	+	+	+	–	+	+	+	+	–	–	–	–	***Lactobacillus confusus***
	DC4:R2	Rod	1.1 (0.8-1.2) x 0.6 (0.4-0.8)	+	–	–	+	+	+	+	+	–	+	–	–	–	DL	+	+	+	+	–	+	+	+	–	+	+	+	+	–	–	–	–	***Lactobacillus bifermentans***

nd, not determined

contd. Table 9: Characteristics of LAB strains isolated from fermented milk products of the Sikkim 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		
Kno milk	KML1:R2	Rod	0.9 (0.8-1.6) x 0.4 (0.2-0.4)	+	–	+	–	–	+	+	+	–	+	–	–	–	L(D)	–	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	*Lactobacillus farciminis*
	KMK1:R1	Rod	0.9 (0.6-1.2) x 0.5 (0.4-0.6)	+	–	–	–	–	+	–	+	–	+	–	–	+	DL	–	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+	–	*Lactobacillus plantarum*
	KMK3:R1	Rod	1.0 (0.8-1.2) x 0.5 (0.4-0.6)	+	–	+	+	–	–	–	+	–	–	–	–	–	DL	–	–	–	+	+	+	–	+	–	–	–	–	+	+	+	+	–	*Lactobacillus confusus*
	KMY1:R1	Rod	1.0 (0.8-1.6) x 0.5 (0.4-0.8)	+	–	–	+	+	+	–	+	–	+	–	–	–	DL	–	–	–	+	+	+	–	+	–	–	–	–	+	+	+	+	–	*Lactobacillus bifermentans*
Kno dahi	KSK1:R1	Rod	1.1 (0.8-1.6) x 0.6 (0.4-0.8)	+	–	–	+	–	–	–	+	–	–	–	–	–	DL	–	+	–	+	+	+	–	+	–	+	+	+	+	+	+	–	+	*Lactobacillus bifermentans*
	KSY1:R2	Rod	1.0 (0.8-1.2) x 0.4 (0.2-0.4)	+	–	–	–	–	+	–	+	–	–	–	–	–	DL	–	+	–	+	+	+	–	+	–	+	+	+	+	+	–	+	*Lactobacillus casei* subsp. *pseudoplantarum*
	KSY2:R2	Rod	0.8 (0.6-0.8) x 0.4 (0.2-0.4)	+	–	–	–	–	+	–	+	–	–	–	–	–	DL	–	+	–	+	+	+	–	+	–	+	+	+	+	+	–	+	*Lactobacillus casei* subsp. *pseudoplantarum*
Kno phiu	KPY1:R1	Rod	1.1 (0.8-1.4) x 0.5 (0.4-0.8)	+	–	–	–	+	+	–	+	–	+	–	–	–	L	–	–	–	+	+	+	–	–	–	–	–	–	+	+	+	+	–	*Lactobacillus casei* subsp. *casei*
Kno chhu	KCK1:R1	Rod	1.6 (0.8-2.4) x 0.3 (0.2-0.6)	+	–	–	–	+	+	–	+	–	+	–	–	–	L	+	–	+	+	+	+	–	+	–	–	+	–	+	+	+	+	–	*Lactobacillus salivarius*
	KCK2:R4	Rod	1.9 (0.8-2.9) x 0.5 (0.4-0.8)	+	–	–	+	+	+	–	+	–	+	–	–	–	DL	+	–	–	+	+	+	–	+	–	–	–	–	+	+	+	–	–	*Lactobacillus bifermentans*
	KCY1:R1	Rod	0.9 (0.5-1.2) x 0.6 (0.5-0.6)	+	–	+	+	+	+	–	+	–	–	–	–	–	DL	–	+	–	+	+	+	–	+	+	+	+	+	+	+	–	+	–	*Lactobacillus brevis*

nd, not determined

Spore-former rods

Seventy-four strains of endospore-forming rods were isolated from two hundred and sixty-six samples of fermented milk (both cow and kno) products. All 74 spore-forming strains were Gram-positive, catalase-positive, aerobic and motile (Table 10). Based on dichotomous key of Slepecky and Hemphill (1992) embodying all 34 species of *Bacillus* described by Claus and Berkeley (1986), representative strains CCD1:S1 (cow soft chhurpi), CCG1:S2 (cow soft chhurpi), CUN1:S1 (cow chhu), CUN2:S2 (cow chhu), CSR2:S1 (cow somar) (Plate c), CSN1:S2 (cow somar), were identified as *Bacillus subtilis* (Ehrenberg) Cohn (Table 11).

Table 10. Selection of representative strains of spore-formers isolated from fermented cow milk products of the Sikkim Himalayas

Product[a]	Number of strains isolated	Cell Shape	Catalase	Starch hydrolysis	Representative strains
Soft chhurpi	19	Rod	+	+	CCDI:S1, CCG1:S2
Chhu	25	Rod	+	+	CUN1:S1, CUN2:S2
Somar	30	Rod	+	+	CSR2:S1, CSN1:S2

[a] Number of samples is given in Table 6.

All isolates were Gram-positive, aerobic, motile and endo-sporeformers.

Table 11: Characteristics of *Bacillus* species isolated from fermented milk products of the Sikkim Himalayas, based on taxonomical keys of Claus and Berkeley (1986); Slepecky and Hemphill (1992)

Product	Strain code	Colony Morphology	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-Proskauer Reaction	pH in VP Broth	Identity
Cow soft chhurpi	CCD1:S1	White, irregular, on surface	Rod	L= 3.3 (2.8-4.0) B= 0.9 (0.8-1.2)	+	+	–	+	+	+	+	–	+	+	5.5	***Bacillus subtilis***
	CCG1:S2	White, irregular, on surface	Rod	L= 3.3 (3.2-3.6) B= 0.9 (0.8-1.2)	+	+	–	+	+	+	+	–	+	+	5.5	***Bacillus subtilis***
Cow chhu	CUN1:S1	White, irregular, on surface	Rod	L= 3.2 (3.2-3.2) B= 1.3 (1.2-1.6)	+	+	–	+	+	+	–	–	+	+	5.5	***Bacillus subtilis***
	CUN2:S2	White, irregular, on surface	Rod	L= 3.5 (3.2-3.6) B= 0.9 (0.8-1.2)	+	+	–	+	+	+	+	–	+	+	5.5	***Bacillus subtilis***
Cow somar	CSR2:S1	White, irregular, on surface	Rod	L= 3.5 (3.2-3.6) B= 0.8 (0.7-0.9)	+	+	–	+	+	+	–	–	+	+	5.5	***Bacillus subtilis***
	CSN1:S2	White, irregular, on surface	Rod	L= 3.5 (3.2-3.6) B= 0.8 (0.4-1.2)	+	+	–	+	+	+	+	–	+	+	5.5	***Bacillus subtilis***

L, length; B, breadth

Yeasts

Representative strains of yeasts were selected on the basis of colony, cell morphology and type of mycelium among 204 yeasts isolates (Table 12). Sugar fermentation and assimilation tests of randomly selected representative strains of yeasts were carried out (Table 13). Two types of genera of yeast were grouped. All cylindrical-shaped yeasts showed true type of mycelia and had globose-shaped ascospores. Following the taxonomical keys described by Kreger-van Rij (1984) and Kurtzman and Fell (1998), these cylindrical-shaped strains CMR1:Y1 (cow milk), CDC1:Y1 (cow dahi), COM1:Y1 (cow mohi), CCD1:Y1 (cow soft chhurpi), CUG1:Y1 (cow chhu), PC1:YC1 (cow philu), CSN1:Y1 (cow somar), KMK1:Y1 (kno milk), KSK1:Y1 (cow dahi), KCK1:Y1 (cow chhu) were identified as *Saccharomycopsis crataegensis* Kurtzman *et* Wickerham (Plate d).

No ascus and ascospore were present in the oval to elliptical-shaped yeast strains. All of them showed the multilateral budding. On the basis of the taxonomical keys laid down by Kreger-van Rij (1984) and Kurtzman and Fell (1998), oval-shaped strains of yeasts CUR1:Y1 (cow chhu), and DC1:Y1 (cow dudh chhurpi) were identified as *Candida castellii* (Capriotti) Meyer *et* Yarrow (Plate e). However, strains CMR1:Y6 (cow milk), CDR2:Y1 (cow dahi), COS1:Y1 (cow mohi), CCG1:Y1 (cow soft chhurpi), PC1:YO1 (cow philu) could be grouped as *Candida*. Species identification could not be confirmed.

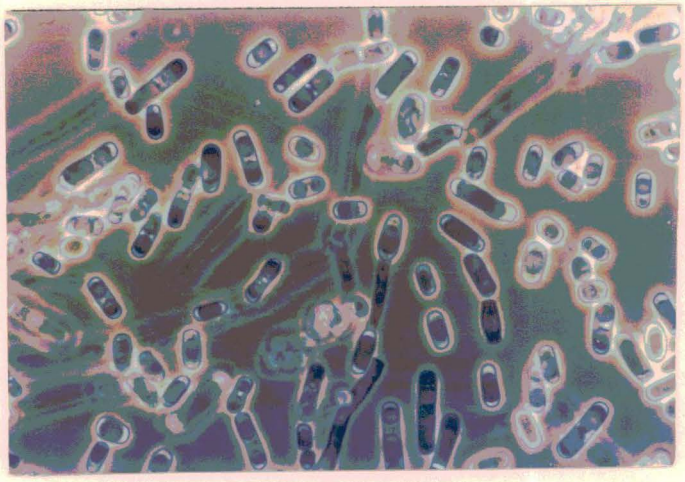

Plate (d). *Saccharomycopsis crataegensis* KCK1:Y1 (YM agar, 3 d, 28° C), isolated from chhu, showing cylindrical cells with true mycelia in phase contrast micrograph (× 330).

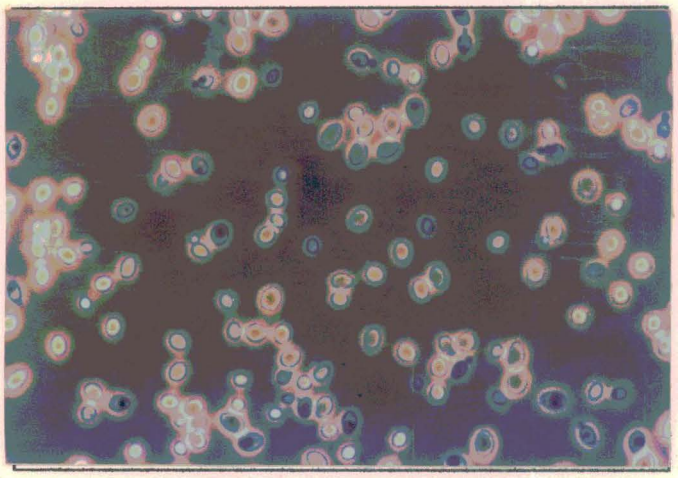

Plate (e). *Candida castellii* DC1:Y1 (YM agar, 3 d, 28° C), isolated from dudh chhurpi, showing elliptical cells with budding in phase contrast micrograph (× 330).

Table 12. Selection of representative strains of yeasts isolated from fermented milk products of the Sikkim Himalayas

Product[a]	Number of strains isolated	Colony	Cell Shape	Mycelium	Ascospore	Grouped strains	Representative strains
Cow milk	20	Ds	Cy	True	Globose	10	CMR1:Y1
		Ss	O-El	Absent	Absent	10	CMR1:Y6
Cow dahi	18	Ds	Cy	True	Globose	10	CDC1:Y1
		Ss	O-El	Absent	Absent	8	CDR2:Y1
Cow mohi	9	Ds	Cy	True	Globose	3	COS1:Y1
		Ss	O-El	Absent	Absent	6	COM1:Y1
Cow soft chhurpi	21	Ds	Cy	True	Globose	18	CCD1:Y1
		Ss	O-El	Absent	Absent	3	CCG1:Y1
Cow chhu	25	Ds	Cy	True	Globose	12	CUR1:Y1
		Ss	O-El	Absent	Absent	13	CUG1:Y1
Cow somar	5	Ds	Cy	True	Globose	5	CSN1:Y1
Cow philu	70	Ds	Cy	True	Globose	20	PC1:YC1
		Ss	O-El	Absent	Absent	50	PC1:YO1
Cow dudh chhurpi	5	Ds	Cy	True	Globose	5	DC1:Y1
Kno milk	9	Ds	Cy	True	Globose	9.	KMK1:Y1
Kno dahi	10	Ds	Cy	True	Globose	10	KSK1:Y1
Kno chhu	12	Ds	Cy	True	Globose	12	KCK1:Y1

[a]Number of samples is given in Table 6 and 7.

Ds, dusty surface; Ss, smooth surface; Cy, cylindrical; O-El, elliptical; All oval to elliptical yeasts showed multilateral budding.

Table 13: Characteristics of yeasts isolated from fermented milk products of the Sikkim Himalayas

Product	Strain code	Colony Morphology	Cell Morphology	Cell Size (µm)	Mycelium	Ascus and Ascospore	Nitrate Reduction	Growth at 37oC	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		
Cow milk	CMR1:Y1	Ds	Cylindrical	L= 10.0 (6.0-14.0) B= 6.0 (4.0-8.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	w	–	+	–	–	+	+	–	+	***Saccharomycopsis crataegensis***
	CMR1:Y6	Ss	Elliptical	L= 6.0 (2.0-8.0) B= 4.0 (2.0-6.0)	Absent	Absent	–	+	+	+	–	–	–	+	–	–	+	–	+	–	–	+	+	–	–	–	–	+	+	–	+	***Candida* sp.**
Cow dahi	CDC1:Y1	Ds	Cylindrical	L= 20.0 (12.0-28.0) B= 5.0 (4.0-6.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	+	–	+	–	–	+	+	–	+	***Saccharomycopsis crataegensis***
	CDR2:Y1	Ss	Elliptical	L= 6.3 (4.0-8.0) B= 4.7 (4.0-6.0)	Absent	Absent	–	+	+	+	–	–	–	+	–	–	+	w	+	–	–	+	w	–	+	+	–	+	+	–	+	***Candida* sp.**
Cow mohi	COS1:Y1	Ss	Elliptical	L= 7.0 (6.0-8.0) B= 4.7 (4.0-6.0)	Absent	Absent	–	+	+	+	+	–	–	+	–	–	+	+	+	w	–	+	+	–	+	–	+	+	+	–	+	***Candida* sp.**
	COM1:Y1	Ds	Cylindrical	L= 18.7 (12.0-24.0) B= 6.0 (4.0-8.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	w	–	+	–	–	+	+	–	+	***Saccharomycopsis crataegensis***
Cow soft chhurpi	CCD1:Y1	Ds	Cylindrical	L= 17.3 (12.0-24.0) B= 4.3 (4.0-5.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	w	–	+	–	–	+	+	–	+	***Saccharomycopsis crataegensis***
	CCG1:Y1	Ss	Elliptical	L= 5.0 (4.0-6.0) B= 3.7 (3.0-4.0)	Absent	Absent	–	+	+	+	–	–	–	+	–	–	+	w	+	–	–	–	–	–	–	–	+	+	+	–	w	***Candida* sp.**
Cow chhu	CUR1:Y1	Ss	Elliptical	L= 8.7 (6.0-11.0) B= 4.3 (4.0-5.0)	Absent	Absent	–	+	+	–	–	–	–	–	–	–	w	–	w	+	–	–	w	–	–	–	–	+	+	–	+	***Candida castellii***
	CUG1:Y1	Ds	Cylindrical	L= 24.0 (15.0-31.0) B= 4.7 (4.0-5.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	w	+	+	–	–	+	–	+	–	–	w	+	w	+	***Saccharomycopsis crataegensis***
Cow philu	PC1:YC1	Ds	Cylindrical	L= 20.0 (12.0-28.0) B= 4.3 (4.0-5.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	w	w	–	+	–	–	+	+	–	+	***Saccharomycopsis crataegensis***
	PC1:YO1	Ss	Elliptical	L= 8.0 (7.0-9.0) B= 4.0 (3.0-5.0)	Absent	Absent	–	+	+	+	+	–	–	+	–	–	+	w	+	w	–	+	w	–	w	–	+	+	+	–	+	***Candida* sp.**
Cow somar	CSN1:Y1	Ds	Cylindrical	L= 22.0 (12.0-29.0) B= 5.0 (4.0-6.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	w	+	+	–	–	w	–	+	–	–	+	w	–	+	***Saccharomycopsis crataegensis***
Cow dudh chhurpi	DC1:Y1	Ss	Elliptical	L= 8.0 (7.0-9.0) B= 6.0 (5.0-7.0)	Absent	Absent	–	+	+	–	–	–	–	–	–	–	w	–	+	+	–	–	+	–	–	–	–	+	w	–	+	***Candida castellii***
Kno milk	KMK1:Y1	Ds	Cylindrical	L= 22.7 (8.0-40.0) B= 6.0 (4.0-8.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	w	–	–	+	–	–	+	+	–	+	***Saccharomycopsis crataegensis***
Kno dahi	KSK1:Y1	Ds	Cylindrical	L= 22.7 (12.0-32.0) B= 5.7 (4.0-7.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	+	–	–	+	+	–	+	***Saccharomycopsis crataegensis***
Kno chhu	KCK1:Y1	Ds	Cylindrical	L= 19.0 (11.0-26.0) B= 6.3 (5.0-8.0)	True	Globose	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	+	+	–	+	–	–	+	***Saccharomycopsis crataegensis***

Ss, smooth surface; Ds, dusty surface; w, weak; L, length; B, breadth.

Prevalence of microorganisms

Prevalence of lactic acid bacteria was 100 % in both cow and kno milk as well as their products, that of yeasts was 60 % and 45.5 % in cow milk fermented product and kno milk fermented products, respectively, whereas that of *Bacillus* species was 50.6 % in cow milk fermented products (Fig 9). *Bacillus* was not detected in kno milk fermented product tested. However, prevalence of LAB, yeasts and *Bacillus* spp. was 100 % in both unboiled cow milk and kno milk.

Fig 10 shows the distribution of microflora in fermented milk products. Lactic acid bacterial strains were dominant microflora representing 74 % in fermented milk products followed by yeasts 19 % and *Bacillus* 7 %, respectively. Among LAB, rod-shaped lactics were represented by 86 % whereas coccus-shaped lactics were represented only by 14 % in the analysed samples of fermented milk products of the Sikkim Himalayas (Fig 11).

Occurrence of pathogenic contaminants

Table 14 summarises the distribution of the pathogenic contaminants mainly *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae in fermented milk (both cow and kno) products collected from different places of the Sikkim Himalayas. *Bacillus cereus* was detected in few samples of chhu, somar, philu and dudh chhurpi. *Staphylococcus aureus* was detected in soft chhurpi, chhu, somar, philu and dudh chhurpi (Table 14). Enterobacteriaceae was present in all samples except dahi, mohi and soft chhurpi. However, the load of these pathogenic contaminants was $<10^3$ cfu/g in all samples tested except somar which showed slightly higher numbers of $\sim10^4$ cfu/g (Table 14). Unboiled milk contained all pathogenic contaminants (Table 14).

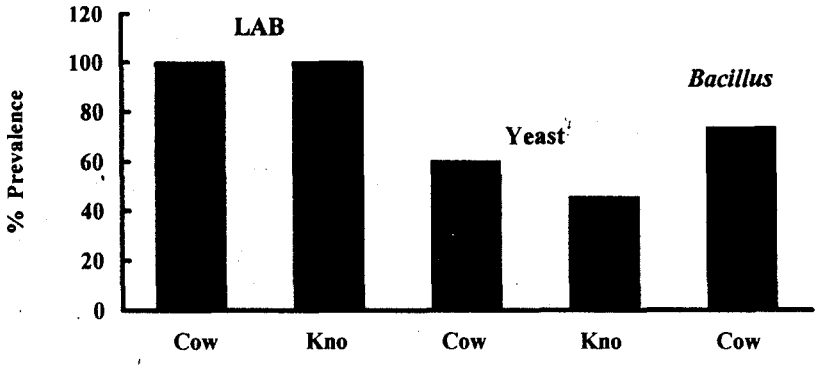

Fig 9. Prevalence of microorganisms in fermented milk products of the Sikkim Himalayas (SH)

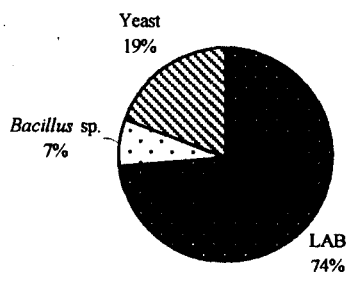

Fig 10. Distribution of microflora in fermented milk products of SH

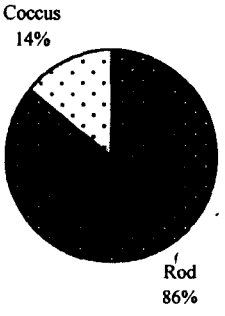

Fig 11. Distribution of LAB in fermented milk products of SH

Table 14. Occurrence of pathogenic bacteria in fermented milk products of the Sikkim Himalayas

Product	Log cfu/g fresh weight		
	Bacillus cereus	*Staphylococcus aureus*	*Enterobacteriaceae*
Cow milk	2.3 (1.8-2.7)	2.5 (2.0-2.8)	3.5 (2.5-3.8)
Cow dahi	0	0	0
Cow mohi	0	0	0
Cow soft chhurpi	0	2.0 (0-2.3)	0
Cow chhu	3.1 (2.8-3.2)	3.0 (2.8-3.3)	3.4 (3.2-3.5)
Cow somar	4.0 (3.5-4.5)	4.0 (3.8-4.2)	4.4 (4.2-4.5)
Cow philu	2.5 (2.0-2.7)	2.5 (2.4-2.6)	4.3 (4.0-4.5)
Cow dudh chhurpi	4.5 (4.2-4.7)	2.7 (2.6-2.8)	3.0 (2.8-3.3)
Kno milk	1.6 (0-1.8)	2.0 (1.7-2.3)	2.7 (2.4-2.8)
Kno dahi	0	0	0
Kno chhu	2.2 (0-2.4)	2.1 (1.5-2.5)	2.7 (2.3-2.9)

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

Enzymatic activity

Forty-eight selected representative strains of species of lactic acid bacteria and *Bacillus*, isolated from samples of fermented milk (both cow and kno) products were tested for proteolytic (Table 15), amylolytic (Table 16) and lipolytic activities (Table 17). Out of 42 LAB strains tested, only seven strains, viz. *Lactococcus lactis* subsp. *cremoris* COM1:C3, *Lactobacillus plantarum* CCD2:R1, *Lactobacillus plantarum* CCD2:R1, *Lactobacillus alimentarius* CUG3:R1, *Lactobacillus casei* subsp. *casei* DC2:R1, *Lactobacillus confusus* DC4:R1, *Lactobacillus casei* subsp. *casei* KPY1:R1 showed proteolytic activity (showing >2 mm hydrolysis zone in milk agar plate), though the estimated protease activity of these strains was <1.5 U/ml. All six strains of *Bacillus subtilis* showed proteolytic activity. However, the protease activity of these *Bacillus* strains, estimated was not more than 1.5 U/ml (Table 15). Thirteen strains of LAB were screened for amylolytic activity (showing >2 mm hydrolysis zone in starch agar plate) and α-amylase activity was estimated. The highest α-amylase activity was that of *Lactobacillus alimentarius* CDC1:R1 and *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 (>5 U/ml) (Table 16). All strains of *Bacillus subtilis* showed amylolytic activity. Five strains of LAB *Lactococcus lactis* subsp. *lactis* CMR2:C1, *Lactococcus lactis* subsp. *cremoris* CMS2:C1, *Enterococcus faecium* PC1:C4, *Lactobacillus plantarum* KMK1:R1, *Lactobacillus brevis* KCY1:R1 showed lipolytic activity on tributyrin agar plates and three strains of *Bacillus* viz. *Bacillus subtilis* CCG1:S2, *Bacillus subtilis* CSR2:S1 and *Bacillus subtilis* CSN1:S2 showed lipolytic activity (Table 17).

Table 15. Proteolytic activity of the selected strains isolated from fermented milk products of the Sikkim Himalayas

Product	Strain	Casein hydrolysis	Protease[a] (U/ml)
Cow milk	*Lactobacillus plantarum* CMN1:R1	–	
Cow milk	*Lactobacillus bifermentans* CMD1:R1	–	
Cow milk	*Lactococcus lactis* subsp. *lactis* CMR2:C1	–	
Cow milk	*Lactococcus lactis* subsp. *cremoris* CMS2:C1	–	
Cow dahi	*Lactobacillus alimentarius* CDC1:R1	–	
Cow dahi	*Lactococcus lactis* subsp. *cremoris* CDM1:C1	–	
Cow dahi	*Lactococcus lactis* subsp. *lactis* CDN1:C4	–	
Cow dahi	*Lactobacillus bifermentans* CDP1:R1	–	
Cow mohi	*Lactobacillus alimentarius* COD1:R2	–	
Cow mohi	*Lactococcus lactis* subsp. *cremoris* COM1:C3	+	1.0
Cow mohi	*Lactococcus lactis* subsp. *lactis* CON1:C4	–	
Cow mohi	*Lactococcus lactis* subsp. *lactis* COS1:C1	–	
Cow soft chhurpi	*Lactobacillus alimentarius* CCD1:R1	–	
Cow soft chhurpi	*Lactobacillus plantarum* CCD2:R1	+	1.0
Cow soft chhurpi	*Lactobacillus hilgardii* CCN1:R1	–	
Cow soft chhurpi	*Enterococcus faecium* CCB1:C1	–	
Cow soft chhurpi	*Lactobacillus kefir* CCR2:R1	–	
Cow philu	*Lactobacillus bifermentans* PC1:R11	–	
Cow philu	*Enterococcus faecium* PC1:C4	+	1.0
Cow philu	*Lactobacillus casei* subsp. *casei* PC2:R6	–	
Cow philu	*Lactobacillus bifermentans* PC1:R1	–	
Cow chhu	*Lactobacillus alimentarius* CUG3:R1	+	1.5
Cow chhu	*Lactococcus lactis* subsp. *cremoris* CUR1:C1	–	
Cow chhu	*Lactobacillus farciminis* CUGI:R2	–	

Product	Strain	Casein hydrolysis	Protease[a] (U/ml)
Cow chhu	*Lactobacillus brevis* CUG2:R2	–	
Cow somar	*Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1	–	
Cow somar	*Lactococcus lactis* subsp. *cremoris* CSR2:C1	–	
Cow dudh chhurpi	*Lactobacillus farciminis* DC1:R2	–	
Cow dudh chhurpi	*Lactobacillus casei* subsp. *casei* DC2:R1	+	1.0
Cow dudh chhurpi	*Lactobacillus confusus* DC4:R1	+	0.8
Cow dudh chhurpi	*Lactobacillus bifermentans* DC4:R2	–	
Kno milk	*Lactobacillus farciminis* KML1:R2	–	
Kno milk	*Lactobacillus plantarum* KMK1:R1	–	
Kno milk	*Lactobacillus confusus* KMK3:R1	–	
Kno milk	*Lactobacillus bifermentans* KMY1:R1	–	
Kno dahi	*Lactobacillus bifermentans* KSK1:R1	–	
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2	–	
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY2:R2	–	
Kno philu	*Lactobacillus casei* subsp. *casei* KPY1:R1	+	0.7
Kno chhu	*Lactobacillus salivarius* KCK1:R1	–	
Kno chhu	*Lactobacillus bifermentans* KCK2:R4	–	
Kno chhu	*Lactobacillus brevis* KCY1:R1	–	
Cow soft chhurpi	*Bacillus subtilis* CCD1:S1	+	1.0
Cow soft chhurpi	*Bacillus subtilis* CCG1:S2	+	1.2
Cow chhu	*Bacillus subtilis* CUN1:S1	+	0.9
Cow chhu	*Bacillus subtilis* CUN2:S2	+	0.9
Cow somar	*Bacillus subtilis* CSR2:S1	+	1.0
Cow somar	*Bacillus subtilis* CSN1:S2	+	1.1

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

Table 16. Amylolytic activity of the selected strains isolated from fermented milk products of the Sikkim Himalayas

Product	Strain	Starch hydrolysis	α-Amylase[a] (U/ml)
Cow milk	*Lactobacillus plantarum* CMN1:R1	+	3.0
Cow milk	*Lactobacillus bifermentans* CMD1:R1	+	4.6
Cow milk	*Lactococcus lactis* subsp. *lactis* CMR2:C1	–	
Cow milk	*Lactococcus lactis* subsp. *cremoris* CMS2:C1	–	
Cow dahi	*Lactobacillus alimentarius* CDC1:R1	+	5.6
Cow dahi	*Lactococcus lactis* subsp. *cremoris* CDM1:C1	–	
Cow dahi	*Lactococcus lactis* subsp. *lactis* CDN1:C4	–	
Cow dahi	*Lactobacillus bifermentans* CDP1:R1	+	3.0
Cow mohi	*Lactobacillus alimentarius* COD1:R2	+	2.3
Cow mohi	*Lactococcus lactis* subsp. *cremoris* COM1:C3	–	
Cow mohi	*Lactococcus lactis* subsp. *lactis* CON1:C4	–	
Cow mohi	*Lactococcus lactis* subsp. *lactis* COS1:C1	–	
Cow soft chhurpi	*Lactobacillus alimentarius* CCD1:R1	+	3.8
Cow soft chhurpi	*Lactobacillus plantarum* CCD2:R1	–	
Cow soft chhurpi	*Lactobacillus hilgardii* CCN1:R1	+	3.1
Cow soft chhurpi	*Enterococcus faecium* CCB1:C1	–	
Cow soft chhurpi	*Lactobacillus kefir* CCR2:R1	+	4.0
Cow philu	*Lactobacillus bifermentans* PC1:R11	–	
Cow philu	*Enterococcus faecium* PC1:C4	–	
Cow philu	*Lactobacillus casei* subsp. *casei* PC2:R6	–	
Cow philu	*Lactobacillus bifermentans* PC1:R1	–	
Cow chhu	*Lactobacillus alimentarius* CUG3:R1	+	3.3
Cow chhu	*Lactococcus lactis* subsp. *cremoris* CUR1:C1	–	
Cow chhu	*Lactobacillus farciminis* CUGI:R2	–	
Cow chhu	*Lactobacillus brevis* CUG2:R2	–	
Cow somar	*Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1	–	
Cow somar	*Lactococcus lactis* subsp. *cremoris* CSR2:C1	–	

Product	Strain	Starch hydrolysis	α-Amylase[a] (U/ml)
Cow dudh chhurpi	*Lactobacillus farciminis* DC1:R2	–	
Cow dudh chhurpi	*Lactobacillus casei* subsp. *casei* DC2:R1	–	
Cow dudh chhurpi	*Lactobacillus confusus* DC4:R1	–	
Cow dudh chhurpi	*Lactobacillus bifermentans* DC4:R2	+	4.2
Kno milk	*Lactobacillus farciminis* KML1:R2	–	
Kno milk	*Lactobacillus plantarum* KMK1:R1	–	
Kno milk	*Lactobacillus confusus* KMK3:R1	–	
Kno milk	*Lactobacillus bifermentans* KMY1:R1	–	
Kno dahi	*Lactobacillus bifermentans* KSK1:R1	+	4.4
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2	+	5.1
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY2:R2	+	3.7
Kno philu	*Lactobacillus casei* subsp. *casei* KPY1:R1	–	
Kno chhu	*Lactobacillus salivarius* KCK1:R1	–	
Kno chhu	*Lactobacillus bifermentans* KCK2:R4	–	
Kno chhu	*Lactobacillus brevis* KCY1:R1	–	
Cow soft chhurpi	*Bacillus subtilis* CCD1:S1	+	3.7
Cow soft chhurpi	*Bacillus subtilis* CCG1:S2	+	2.0
Cow chhu	*Bacillus subtilis* CUN1:S1	+	5.7
Cow chhu	*Bacillus subtilis* CUN2:S2	+	5.3
Cow somar	*Bacillus subtilis* CSR2:S1	+	2.0
Cow somar	*Bacillus subtilis* CSN1:S2	+	3.6

[a]Only the strains showing positive starch hydrolysis agar test (>2.0 mm) were assayed for α-amylase activity. The data represent the means of three set.

Table 17. Lipolytic activity of the selected strains isolated from fermented milk products of the Sikkim Himalayas

Product	Strain	Lipolytic activity
Cow milk	*Lactobacillus plantarum* CMN1:R1	–
Cow milk	*Lactobacillus bifermentans* CMD1:R1	–
Cow milk	*Lactococcus lactis* subsp. *lactis* CMR2:C1	+
Cow milk	*Lactococcus lactis* subsp. *cremoris* CMS2:C1	+
Cow dahi	*Lactobacillus alimentarius* CDC1:R1	–
Cow dahi	*Lactococcus lactis* subsp. *cremoris* CDM1:C1	–
Cow dahi	*Lactococcus lactis* subsp. *lactis* CDN1:C4	–
Cow dahi	*Lactobacillus bifermentans* CDP1:R1	–
Cow mohi	*Lactobacillus alimentarius* COD1:R2	–
Cow mohi	*Lactococcus lactis* subsp. *cremoris* COM1:C3	–
Cow mohi	*Lactococcus lactis* subsp. *lactis* CON1:C4	–
Cow mohi	*Lactococcus lactis* subsp. *lactis* COS1:C1	–
Cow soft chhurpi	*Lactobacillus alimentarius* CCD1:R1	–
Cow soft chhurpi	*Lactobacillus plantarum* CCD2:R1	–
Cow soft chhurpi	*Lactobacillus hilgardii* CCN1:R1	–
Cow soft chhurpi	*Enterococcus faecium* CCB1:C1	–
Cow soft chhurpi	*Lactobacillus kefir* CCR2:R1	–
Cow philu	*Lactobacillus bifermentans* PC1:R11	–
Cow philu	*Enterococcus faecium* PC1:C4	+
Cow philu	*Lactobacillus casei* subsp. *casei* PC2:R6	–
Cow philu	*Lactobacillus bifermentans* PC1:R1	–
Cow chhu	*Lactobacillus alimentarius* CUG3:R1	–
Cow chhu	*Lactococcus lactis* subsp. *cremoris* CUR1:C1	–
Cow chhu	*Lactobacillus farciminis* CUG1:R2	–
Cow chhu	*Lactobacillus brevis* CUG2:R2	–
Cow somar	*Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1	–
Cow somar	*Lactococcus lactis* subsp. *cremoris* CSR2:C1	–

Product	Strain	Lipolytic activity
Cow dudh chhurpi	*Lactobacillus farciminis* DC1:R2	–
Cow dudh chhurpi	*Lactobacillus casei* subsp. *casei* DC2:R1	–
Cow dudh chhurpi	*Lactobacillus confusus* DC4:R1	–
Cow dudh chhurpi	*Lactobacillus bifermentans* DC4:R2	–
Kno milk	*Lactobacillus farciminis* KML1:R2	–
Kno milk	*Lactobacillus plantarum* KMK1:R1	+
Kno milk	*Lactobacillus confusus* KMK3:R1	–
Kno milk	*Lactobacillus bifermentans* KMY1:R1	–
Kno dahi	*Lactobacillus bifermentans* KSK1:R1	–
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2	–
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY2:R2	–
Kno philu	*Lactobacillus casei* subsp. *casei* KPY1:R1	–
Kno chhu	*Lactobacillus salivarius* KCK1:R1	–
Kno chhu	*Lactobacillus bifermentans* KCK2:R4	–
Kno chhu	*Lactobacillus brevis* KCY1:R1	+
Cow soft chhurpi	*Bacillus subtilis* CCD1:S1	–
Cow soft chhurpi	*Bacillus subtilis* CCG1:S2	+
Cow chhu	*Bacillus subtilis* CUN1:S1	–
Cow chhu	*Bacillus subtilis* CUN2:S2	–
Cow somar	*Bacillus subtilis* CSR2:S1	+
Cow somar	*Bacillus subtilis* CSN1:S2	+

+ = the clearing zone was ≥ 3.0 mm

Enzymatic profiles of randomly selected lactic acid bacteria strains of milk products were assayed using the API zym (bioMérieux, France) galleries (Table 18). Each of the predominant LAB strains produced a wide spectrum of enzymes. These strains showed relatively weak esterase and no lipase (C14) activities. All seven strains of LAB showed strong phosphatase, β-galactosidase and β-glucosidase activities (Table 18). However, they showed no detectable proteinase activity with the methods applied.

Antimicrobial activity

Table 19 shows the antagonistic properties of the lactic acid bacterial strains and *Bacillus* strains, isolated from fermented milk (both cow and kno) 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 11 strains of LAB showed antimicrobial activities against the indicator strains used. *Lactococcus lactis* subsp. *cremoris* CDM1:C1, *Lactobacillus casei* subsp. *casei* DC2:R1 and *Lactobacillus bifermentans* KSK1:R1 inhibited the growth of *Listeria monocytogenes* DSM 20600. *Lactococcus lactis* subsp. *cremoris* CDM1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1, *Lactobacillus plantarum* KMK1:R1, *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 showed inhibition zone against *Bacillus cereus* CCM 2010, *Enterococcus faecium* CCB1:C1, *Lactococcus lactis* subsp. *cremoris* CUR1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1, *Lactobacillus brevis* KCY1:R1 against *Enterococcus faecium* DSM 20477 and *Lactobacillus bifermentans* CDP1:R1, *Lactococcus lactis* subsp. *lactis* COS1:C1, *Lactococcus lactis* subsp. *cremoris* CUR1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 against *Streptococcus mutans* DSM 6178. *Bacillus subtilis*

CUN1:S1, *Bacillus subtilis* CUN2:S2, *Bacillus subtilis* CSN1:S2 showed the antagonistic properties against the indicator strains.

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

Table 18. Enzymatic profiles using API zym system of representative strains of LAB isolated from fermented milk products of the Sikkim Himalayas

Enzyme	Activity (nanomoles)						
	A	B	C	D	E	F	G
Control (without enzyme)	0	0	0	0	0	0	0
Phosphatase alkaline	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Esterase (C4)	0	5	0	5	0	0	0
Esterase Lipase (C8)	5	5	5	5	0	5	5
Lipase (C14)	0	0	0	0	0	0	0
Leucine arylamidase	5	20	20	5	20	10	5
Valine arylamidase	0	0	0	0	0	0	0
Cystine arylamidase	0	5	20	0	5	10	0
Trypsin	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0
Phoshatase acid	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Napthol-AS-BI-phosphohydrolase	10	20	30	30	10	20	10
α-galactosidase	5	10	10	10	5	10	10
β-galactosidase	≥40	≥40	10	10	≥40	10	≥10
β-glucuronidase	0	0	0	0	0	0	0
α-glucosidase	20	30	5	5	5	10	10
β-glucosidase	≥40	≥40	10	10	≥40	10	≥10
N-acetyl-β-glucosaminidase	0	0	0	0	0	0	0
α-mannosidase	0	0	0	0	0	0	0
α-fucosidase	0	0	0	0	0	0	0

A = *Lactobacillus casei* subsp. *casei* PC2:R6 (philu); **B** = *Lb. bifermentans* KSK1:R1 (dahi); **C** = *Lactococcus lactis* subsp. *lactis* CDN1:C4 (dahi); **D** = *Enterococcus faecium* CCB1:C1 (soft chhurpi); **E** = *Lb. plantarum* CCD2:R1 (soft chhurpi); **F** = *Lb. brevis* CUG2:R2 (chhu); **G** = *Lactococcus lactis* subsp. *cremoris* CSR2:C1 (somar). Data represent the means of 2 replicate sets.

Table 19. Antimicrobial activity of bacterial strains isolated from fermented milk products of the Sikkim Himalayas

Product	Strain	Indicator Strains			
		Listeria monocytogenes DSM 20600	*Bacillus cereus* CCM 2010	*Enterococcus faecium* DSM 20477	*Streptococcus mutans* DSM 6178
Cow milk	*Lactobacillus plantarum* CMN1:R1	–	–	–	–
Cow milk	*Lactobacillus bifermentans* CMD1:R1	–	–	–	–
Cow milk	*Lactococcus lactis* subsp. *lactis* CMR2:C1	–	–	–	–
Cow milk	*Lactococcus lactis* subsp. *cremoris* CMS2:C1	–	–	–	–
Cow dahi	*Lactobacillus alimentarius* CDC1:R1	–	–	–	–
Cow dahi	*Lactococcus lactis* subsp. *cremoris* CDM1:C1	+	+	–	–
Cow dahi	*Lactococcus lactis* subsp. *lactis* CDN1:C4	–	–	–	–
Cow dahi	*Lactobacillus bifermentans* CDP1:R1	–	–	–	+
Cow mohi	*Lactobacillus alimentarius* COD1:R2	–	–	–	–
Cow mohi	*Lactococcus lactis* subsp. *cremoris* COM1:C3	–	–	–	–
Cow mohi	*Lactococcus lactis* subsp. *lactis* CON1:C4	–	–	–	–
Cow mohi	*Lactococcus lactis* subsp. *lactis* COS1:C1	–	–	–	+
Cow soft chhurpi	*Lactobacillus alimentarius* CCD1:R1	–	–	–	–
Cow soft chhurpi	*Lactobacillus plantarum* CCD2:R1	–	–	–	–
Cow soft chhurpi	*Lactobacillus hilgardji* CCN1:R1	–	–	–	–
Cow soft chhurpi	*Enterococcus faecium* CCB1:C1	–	–	+	–
Cow soft chhurpi	*Lactobacillus kefir* CCR2:R1	–	–	–	–
Cow philu	*Lactobacillus bifermentans* PC1:R11	–	–	–	–
Cow philu	*Enterococcus faecium* PC1:C4	–	–	–	–
Cow philu	*Lactobacillus casei* subsp. *casei* PC2:R6	–	–	–	–
Cow philu	*Lactobacillus bifermentans* PC1:R1	–	–	–	–
Cow chhu	*Lactobacillus alimentarius* CUG3:R1	–	–	–	–
Cow chhu	*Lactococcus lactis* subsp. *cremoris* CUR1:C1	–	–	+	+
Cow chhu	*Lactobacillus farciminis* CUGI:R2	–	–	–	–

Product	Strain	*Listeria monocytogenes* DSM 20600	*Bacillus cereus* CCM 2010	*Enterococcus faecium* DSM 20477	*Streptococcus mutans* DSM 6178
Cow chhu	*Lactobacillus brevis* CUG2:R2	–	–	–	–
Cow somar	*Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1	–	+	+	–
Cow somar	*Lactococcus lactis* subsp. *cremoris* CSR2:C1	–	–	–	–
Cow dudh chhurpi	*Lactobacillus farciminis* DC1:R2	–	–	–	–
Cow dudh chhurpi	*Lactobacillus casei* subsp. *casei* DC2:R1	+	–	–	–
Cow dudh chhurpi	*Lactobacillus confuses* DC4:R1	–	–	–	–
Cow dudh chhurpi	*Lactobacillus bifermentans* DC4:R2	–	–	–	–
Kno milk	*Lactobacillus farciminis* KML1:R2	–	–	–	–
Kno milk	*Lactobacillus plantarum* KMK1:R1	–	+	–	–
Kno milk	*Lactobacillus confuses* KMK3:R1	–	–	–	–
Kno milk	*Lactobacillus bifermentans* KMY1:R1	–	–	–	–
Kno dahi	*Lactobacillus bifermentans* KSK1:R1	+	–	–	–
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2	–	+	–	+
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY2:R2	–	–	–	–
Kno philu	*Lactobacillus casei* subsp. *casei* KPY1:R1	–	–	–	–
Kno chhu	*Lactobacillus salivarius* KCK1:R1	–	–	–	–
Kno chhu	*Lactobacillus bifermentans* KCK2:R4	–	–	–	–
Kno chhu	*Lactobacillus brevis* KCY1:R1	–	–	+	–
Cow soft chhurpi	*Bacillus subtilis* CCD1:S1	–	–	–	–
Cow soft chhurpi	*Bacillus subtilis* CCG1:S2	–	–	–	–
Cow chhu	*Bacillus subtilis* CUN1:S1	–	–	–	+
Cow chhu	*Bacillus subtilis* CUN2:S2	+	–	–	–
Cow somar	*Bacillus subtilis* CSR2:S1	–	–	–	–
Cow somar	*Bacillus subtilis* CSN1:S2	–	–	+	+

+, inhibition zone was > 1.0 mm; –, no inhibition zone

Biogenic amines

Forty eight strains of lactic acid bacterial and *Bacillus* isolated from fermented milk (both cow and kno) products were tested for biogenic amine production with the surface plate method applied (Table 20). None of the strains produced tyramine, cadaverine, histamine and putrescine in the applied method.

Hydrophobicity

Table 21 shows the percentage hydrophobicity of the LAB isolated from fermented milk (both cow and kno) products. Nineteen strains of LAB showed high degrees of hydrophobicity (>75%), among which *Lactobacillus casei* subsp. *casei* PC2:R6 (isolated from cow philu) and *Lactococcus lactis* subsp. *cremoris* CUR1:C1 (isolated from cow chhu) showed the highest percentage of hydrophobicity of 97.89 % and 97.15 %, respectively (Fig 12). All of the strains tested showed more than 36 % hydrophobicity.

Table 20. Screening of biogenic amines producing strains isolated from fermented milk products of the Sikkim Himalayas

Product	Strain	Tyr	Lys	Hist	Orn
Cow milk	*Lactobacillus plantarum* CMN1:R1	–	–	–	–
Cow milk	*Lactobacillus bifermentans* CMD1:R1	–	–	–	–
Cow milk	*Lactococcus lactis* subsp. *lactis* CMR2:C1	–	–	–	–
Cow milk	*Lactococcus lactis* subsp. *cremoris* CMS2:C1	–	–	–	–
Cow dahi	*Lactobacillus alimentarius* CDC1:R1	–	–	–	–
Cow dahi	*Lactococcus lactis* subsp. *cremoris* CDM1:C1	–	–	–	–
Cow dahi	*Lactococcus lactis* subsp. *lactis* CDN1:C4	–	–	–	–
Cow dahi	*Lactobacillus bifermentans* CDP1:R1	–	–	–	–
Cow mohi	*Lactobacillus alimentarius* COD1:R2	–	–	–	–
Cow mohi	*Lactococcus lactis* subsp. *cremoris* COM1:C3	–	–	–	–
Cow mohi	*Lactococcus lactis* subsp. *lactis* CON1:C4	–	–	–	–
Cow mohi	*Lactococcus lactis* subsp. *lactis* COS1:C1	–	–	–	–
Cow soft chhurpi	*Lactobacillus alimentarius* CCD1:R1	–	–	–	–
Cow soft chhurpi	*Lactobacillus plantarum* CCD2:R1	–	–	–	–
Cow soft chhurpi	*Lactobacillus hilgardii* CCN1:R1	–	–	–	–
Cow soft chhurpi	*Enterococcus faecium* CCB1:C1	–	–	–	–
Cow soft chhurpi	*Lactobacillus kefir* CCR2:R1	–	–	–	–
Cow philu	*Lactobacillus bifermentans* PC1:R11	–	–	–	–
Cow philu	*Enterococcus faecium* PC1:C4	–	–	–	–
Cow philu	*Lactobacillus casei* subsp. *casei* PC2:R6	–	–	–	–
Cow philu	*Lactobacillus bifermentans* PC1:R1	–	–	–	–
Cow chhu	*Lactobacillus alimentarius* CUG3:R1	–	–	–	–
Cow chhu	*Lactococcus lactis* subsp. *cremoris* CUR1:C1	–	–	–	–
Cow chhu	*Lactobacillus farciminis* CUG1:R2	–	–	–	–
Cow chhu	*Lactobacillus brevis* CUG2:R2	–	–	–	–
Cow somar	*Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1	–	–	–	–
Cow somar	*Lactococcus lactis* subsp. *cremoris* CSR2:C1	–	–	–	–

Product	Strain	Tyr	Lys	Hist	Orn
Cow dudh chhurpi	*Lactobacillus farciminis* DC1:R2	–	–	–	–
Cow dudh chhurpi	*Lactobacillus casei* subsp. *casei* DC2:R1	–	–	–	–
Cow dudh chhurpi	*Lactobacillus* □*onfuses* DC4:R1	–	–	–	–
Cow dudh chhurpi	*Lactobacillus bifermentans* DC4:R2	–	–	–	–
Kno milk	*Lactobacillus farciminis* KML1:R2	–	–	–	–
Kno milk	*Lactobacillus plantarum* KMK1:R1	–	–	–	–
Kno milk	*Lactobacillus confuses* KMK3:R1	–	–	–	–
Kno milk	*Lactobacillus bifermentans* KMY1:R1	–	–	–	–
Kno dahi	*Lactobacillus bifermentans* KSK1:R1	–	–	–	–
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2	–	–	–	–
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY2:R2	–	–	–	–
Kno philu	*Lactobacillus casei* subsp. *casei* KPY1:R1	–	–	–	–
Kno chhu	*Lactobacillus salivarius* KCK1:R1	–	–	–	–
Kno chhu	*Lactobacillus bifermentans* KCK2:R4	–	–	–	–
Kno chhu	*Lactobacillus brevis* KCY1:R1	–	–	–	–
Cow soft chhurpi	*Bacillus subtilis* CCD1:S1	–	–	–	–
Cow soft chhurpi	*Bacillus subtilis* CCG1:S2	–	–	–	–
Cow chhu	*Bacillus subtilis* CUN1:S1	–	–	–	–
Cow chhu	*Bacillus subtilis* CUN2:S2	–	–	–	–
Cow somar	*Bacillus subtilis* CSR2:S1	–	–	–	–
Cow somar	*Bacillus subtilis* CSN1:S2	–	–	–	–

Tyr = tyramine precussor; Lys = Lysine, cadaverine precussor; His = histidine, histamine precussor; Orn = ornithine, putresine precussor

Table 21. Percentage hydrophobicity of LAB strains isolated from fermented milk products of the Sikkim Himalayas

Product	Strain	% Hydrophobicity
Cow milk	*Lactobacillus plantarum* CMN1:R1	58.74 (+)
Cow milk	*Lactobacillus bifermentans* CMD1:R1	73.57 (+)
Cow milk	*Lactococcus lactis* subsp. *lactis* CMR2:C1	82.05 (++)
Cow milk	*Lactococcus lactis* subsp. *cremoris* CMS2:C1	64.32 (+)
Cow dahi	*Lactobacillus alimentarius* CDC1:R1	48.61(+)
Cow dahi	*Lactococcus lactis* subsp. *cremoris* CDM1:C1	43.64 (+)
Cow dahi	*Lactococcus lactis* subsp. *lactis* CDN1:C4	37.64 (+)
Cow dahi	*Lactobacillus bifermentans* CDP1:R1	43.30 (+)
Cow mohi	*Lactobacillus alimentarius* COD1:R2	36.81 (+)
Cow mohi	*Lactococcus lactis* subsp. *cremoris* COM1:C3	81.48 (++)
Cow mohi	*Lactococcus lactis* subsp. *lactis* CON1:C4	70.85 (+)
Cow mohi	*Lactococcus lactis* subsp. *lactis* COS1:C1	80.31 (++)
Cow soft chhurpi	*Lactobacillus alimentarius* CCD1:R1	82.87 (++)
Cow soft chhurpi	*Lactobacillus plantarum* CCD2:R1	89.94 (++)
Cow soft chhurpi	*Lactobacillus hilgardii* CCN1:R1	65.32 (+)
Cow soft chhurpi	*Enterococcus faecium* CCB1:C1	68.25 (+)
Cow soft chhurpi	*Lactobacillus kefir* CCR2:R1	74.88 (+)
Cow philu	*Lactobacillus bifermentans* PC1:R11	69.64 (+)
Cow philu	*Enterococcus faecium* PC1:C4	71.29 (+)
Cow philu	*Lactobacillus casei* subsp. *casei* PC2:R6	97.89 (++)
Cow philu	*Lactobacillus bifermentans* PC1:R1	76.49 (++)
Cow chhu	*Lactobacillus alimentarius* CUG3:R1	74.33 (+)
Cow chhu	*Lactococcus lactis* subsp. *cremoris* CUR1:C1	97.15 (++)
Cow chhu	*Lactobacillus farciminis* CUGI:R2	96.28 (++)
Cow chhu	*Lactobacillus brevis* CUG2:R2	79.20 (++)
Cow somar	*Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1	53.94 (+)
Cow somar	*Lactococcus lactis* subsp. *cremoris* CSR2:C1	49.35 (+)
Cow dudh chhurpi	*Lactobacillus farciminis* DC1:R2	81.15 (++)

Product	Strain	% Hydrophobicity
Cow dudh chhurpi	*Lactobacillus casei* subsp. *casei* DC2:R1	84.39 (++)
Cow dudh chhurpi	*Lactobacillus confusus* DC4:R1	66.35 (+)
Cow dudh chhurpi	*Lactobacillus bifermentans* DC4:R2	42.46 (+)
Kno milk	*Lactobacillus farciminis* KML1:R2	85.22 (++)
Kno milk	*Lactobacillus plantarum* KMK1:R1	83.05 (++)
Kno milk	*Lactobacillus confusus* KMK3:R1	90.00 (++)
Kno milk	*Lactobacillus bifermentans* KMY1:R1	72.84 (+)
Kno dahi	*Lactobacillus bifermentans* KSK1:R1	86.19 (++)
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2	53.54 (+)
Kno dahi	*Lactobacillus casei* subsp. *pseudoplantarum* KSY2:R2	41.76 (+)
Kno philu	*Lactobacillus casei* subsp. *casei* KPY1:R1	53.98 (+)
Kno chhu	*Lactobacillus salivarius* KCK1:R1	85.54 (++)
Kno chhu	*Lactobacillus bifermentans* KCK2:R4	82.14 (++)
Kno chhu	*Lactobacillus brevis* KCY1:R1	85.95 (++)

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

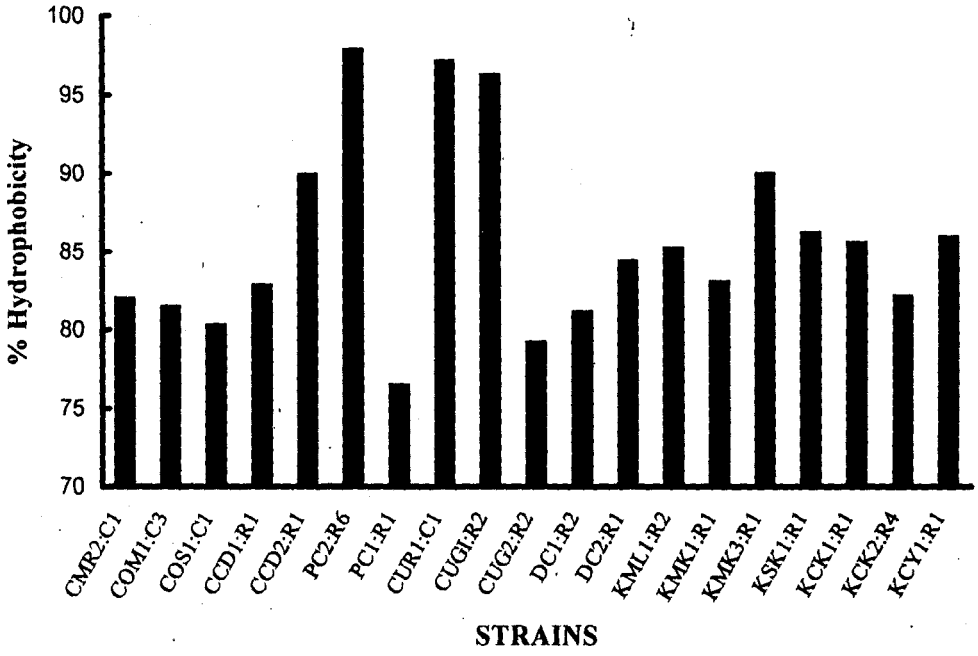

Fig 12. Percentage hydrophobicity of LAB strains isolated from fermented milk products of the Sikkim Himalayas

Proximate composition

Proximate composition of the indigenous fermented milk (both cow and kno) products is presented in Table 22 and 23. The pH of cow milk products was 3.9-4.3 except in chhu, somar and dudh chhurpi which had pH value of 6.0. The pH of kno milk products was 3.7-4.8 except maa showing pH 6.0. The titratable acidity ranged from 0.04 % (in somar) to 0.89 % in (kno mohi). Moisture content was low upto 12 % in kno maa, 16 % in dudh chhurpi but high in all other milk products at 36.5 % to 92.6 %. The ash content was also found to be around 1.1 to 7.7 % on dry matter basis. Fat content varied from product to product. Philu contained high fat whereas cow chhu had very low fat of 5.8 % dry matter basis. High content of protein was observed in all milk products except in kno maa. Carbohydrate content was also variable in the products. Energy value of cow philu (645.6 kcal/100g) and kno maa (876.3 kcal/100g) was the highest among the milk products analysed (Table 23). Among the minerals of the milk products, calcium content was higher than other minerals estimated (Table 24). Among the milk products analysed, cow chhu contained highest amount of calcium and magnesium.

Table 22. Proximate composition of fermented cow milk products of the Sikkim Himalayas

Product	Parameter							
		%		% on dry matter basis				Energy (kcal/ 100g DM)
	pH	Titratable Acidity	Moisture	Ash	Fat	Protein	Carbohydrate	
Milk	6.5 (6.4-6.6)	0.15 (0.13-0.16)	87.0 (84.4-89.6)	5.5 (4.2-6.0)	30.8 (29.1-39.1)	28.0) (27.4-28.4)	35.8 (32.4-39.2)	531.9
Dahi	4.2 (3.8-4.3)	0.73 (0.70-0.75)	84.8 (77.9-88.2)	4.7 (4.1-5.6)	24.5 (23.5-25.7)	22.5 (21.0-24.0)	48.2 (47.7-51.4)	503.6
Mohi	3.9 (3.7-4.0)	0.73 (0.71-0.75)	92.6 (90.6-95.0)	2.7 (2.4-3.0)	12.4 (12.0-12.8)	44.7 (39.6-51.8)	40.2 (32.5-46.0)	451.2
Soft chhurpi	4.3 (4.0-4.5)	0.61 (0.59-0.63)	73.8 (67.9-77.9)	6.6 (5.6-8.6)	11.8 (8.8-13.9)	65.3 (63.9-67.8)	16.3 (9.8-21.8)	432.4
Chhu	6.3 (5.1-7.3)	0.15 (0.14-0.18)	75.5 (71.0-79.6)	1.9 (1.6-2.3)	5.8 (4.6-7.8)	58.4 (55.7-58.7)	33.9 (31.2-38.1)	421.1
Somar	6.0 (5.9-6.0)	0.04 (0.04-0.04)	36.5 (32.2-39.2)	2.7 (1.9-4.0)	15.4 (14.8-15.8)	35.0 (33 9-35.9)	46.9 (44.3-49.5)	465.7
Philu	4.3 (4.2-4.4)	0.61 (0.59-0.62)	38.2 (37.4-38.8)	3.6 (2.6-4.1)	52.0 (47.5-57.0)	32.0 (31.2-32.6)	12.5 (6.4-18.7)	645.6
Dudh chhurpi	6.0 (5.8-6.2)	0.29 (0.27-0.32)	16.8 (8.0-24.6)	5.2 (4.0-6.1)	6.1 (5.3-6.7)	57.2 (55.3-58.6)	31.6 (28.5-35.4)	409.4

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

Table 23. Proximate composition of fermented kno (female yak) milk products of the Sikkim Himalayas

Product	Parameter							
		%		% on dry matter basis				Energy (kcal/ 100g DM)
	pH	Titratable Acidity	Moisture	Ash	Fat	Protein	Carbohydrate	
Milk	6.7 (6.4-6.8)	0.16 (0.14-0.19)	84.2 (79.8-87.4)	5.8 (5.5-6.1)	61.7 (60.0-63.2)	26.2 (25.4-27.0)	6.3 (3.7-9.1)	685.1
Dahi	4.0 (3.8-4.2)	0.82 (0.80-0.85)	86.3 (78.7-89.8)	7.5 (7.2-8.3)	69.4 (66.1-72.9)	28.2 (27.4-29.2)	5.1 (1.0-10.3)	758.0
Mohi	3.7 (3.5-3.8)	0.89 (0.86-0.92)	90.7 (89.8-91.8)	7.7 (7.1-8.2)	14.1 (12.9-16.0)	59.2 (56.9-62.4)	19.0 (13.4-23.1)	439.4
Maa	5.9 (5.4-6.2)	0.28 (0.26-0.30)	12.6 (12.5-12.8)	1.1 (1.1-1.1)	96.1 (94.8-97.0)	1.6 (1.3-1.8)	1.2 (0.1-2.8)	876.3
Chhu	4.8 (4.5-5.2)	0.44 (0.42-0.46)	70.1 (65.2-75.3)	6.1 (5.0-6.6)	11.2 (10.5-12.7)	62.5 (58.9-66.8)	20.2 (13.9-25.6)	431.6
Philu	4.8 (4.3-5.3)	0.79 (0.78-0.81)	47.9 (42.1-52.3)	1.4 (0.6-1.8)	48.0 (40.6-53.2)	38.7 (38.0-39.2)	12.0 (5.8-20.7)	634.5

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

Table 24. Mineral contents of fermented milk products of the Sikkim Himalayas

Product	mg/100 g				
	Calcium	Iron	Magnesium	Manganese	Zinc
Cow milk	103.8	1.7	39.7	1.2	57.7
Cow soft chhurpi	44.1	1.2	16.7	0.6	25.1
Cow chhu	111.0	4.5	64.3	3.1	87.6
Cow somar	31.2	0.4	13.7	0.5	17.2
Cow philu	34.9	0.8	16.9	0.9	27.1
Cow dudh chhurpi	19.8	0.5	6.3	0.4	10.0
Kno milk	76.9	1.0	34.7	1.0	49.1
Kno maa	81.2	1.0	32.4	1.8	43.9

Data represent the means of 2 samples.

Successional studies during chhurpi (soft-variety) fermentation

Chhurpi (soft-variety) was prepared in the laboratory following the traditional method as mentioned in Materials and Methods. Successional studies were carried at every 1 day interval within a range of 0-6 days.

Microbial changes

Table 25 shows the changes in microbial population in cow milk during soft-variety chhurpi fermentation. Load of lactic acid bacteria increased from 10^5 cfu/g in boiled milk to10^8 cfu/g at the end of fermentation (Fig 13). Population of yeasts increased from 10^3 cfu/g to 10^7 cfu/g during fermentation. Subsequently, load of *Bacillus* increased up to 10^3 cfu/g on the sixth day.

Physico-chemical changes

The mean pH value of boiled milk was decreased from 6.38 to 4.08 on the sixth day of fermentation (Table 26). Titratable acidity increased from 0 d till the end (Fig 14). Reducing sugar content was decreased remarkably during fermentation (Table 26).

Table 25. Microbial changes during soft chhurpi fermentation from cow milk

Fermentation time (days)	Log cfu/g		
	LAB	**Yeast**	***Bacillus***
0_{un}	7.8 (7.2-7.9)	7.0 (6.6-7.2)	2.0 (1.9-2.1)
0_b	5.2 (4.8-5.6)	3.9 (3.7-4.1)	1.8 (1.7-2.0)
1	8.0 (7.8-8.2)	4.3 (4.0-4.6)	2.1 (1.8-2.3)
2	8.5 (8.3-8.7)	5.0 (4.8-5.3)	2.2 (2.0-2.4)
3	8.9 (8.6-9.0)	5.6 (5.3-5.8)	2.2 (2.1-2.4)
4	8.9 (8.6-9.1)	6.9 (6.7-7.1)	2.3 (2.0-2.5)
5	8.7 (8.5-8.8)	7.0 (6.8-7.2)	2.8 (2.5-3.0)
6	8.6 (8.5-8.7)	7.6 (7.5-7.7)	3.4 (3.2-3.7)

0_{un} = Unboiled milk, 0 d; 0_b = Boiled milk, 0 d

Data represent the means of three batches of fermentation at 30° C. Ranges are given in parentheses.

Table 26. Physico-chemical changes during soft chhurpi fermentation from cow milk

Fermentation time (days)	pH	Acidity (%)	Reducing sugar (%)
0_{un}	6.45 (6.42-6.46)	0.16 (0.14-0.18)	6.8 (6.7-6.9)
0_b	6.38 (6.37-6.39)	0.18 (0.16-0.20)	6.6 (6.5-6.7)
1	4.28 (4.27-4.29)	0.59 (0.55-0.61)	5.4 (5.3-5.5)
2	4.22 (4.21-4.23)	0.61 (0.58-0.64)	1.7 (1.5-1.9)
3	4.20 (4.18-4.22)	0.62 (0.61-0.63)	0.7 (0.6-0.8)
4	4.17 (4.16-4.18)	0.66 (0.63-0.69)	0.6 (0.5-0.7)
5	4.13 (4.12-4.14)	0.75 (0.71-0.79)	0.5 (0.4-0.6)
6	4.08 (4.07-4.09)	0.82 (0.79-0.83)	0.4 (0.3-0.5)

0_{un} = Unboiled milk, 0 d; 0_b = Boiled milk, 0 d

Data represent the means of three batches of fermentation at 30° C. Ranges are given in parentheses.

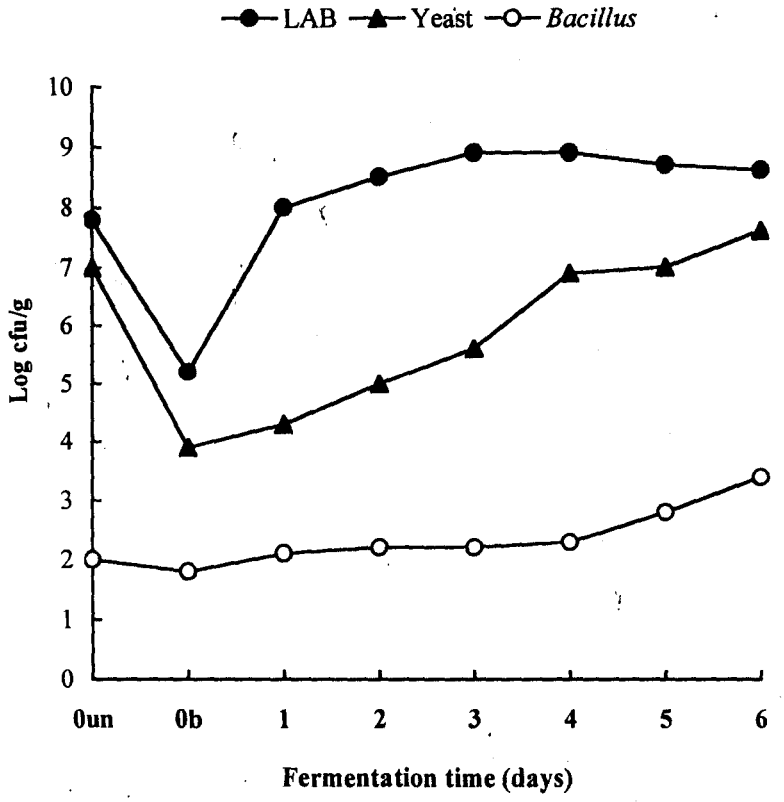

Fig 13. Changes in microbial load during soft chhurpi fermentation. Values are the means of three baches of fermentation.

0un = Unboiled milk, 0 day
0b = Boiled milk, 0 day

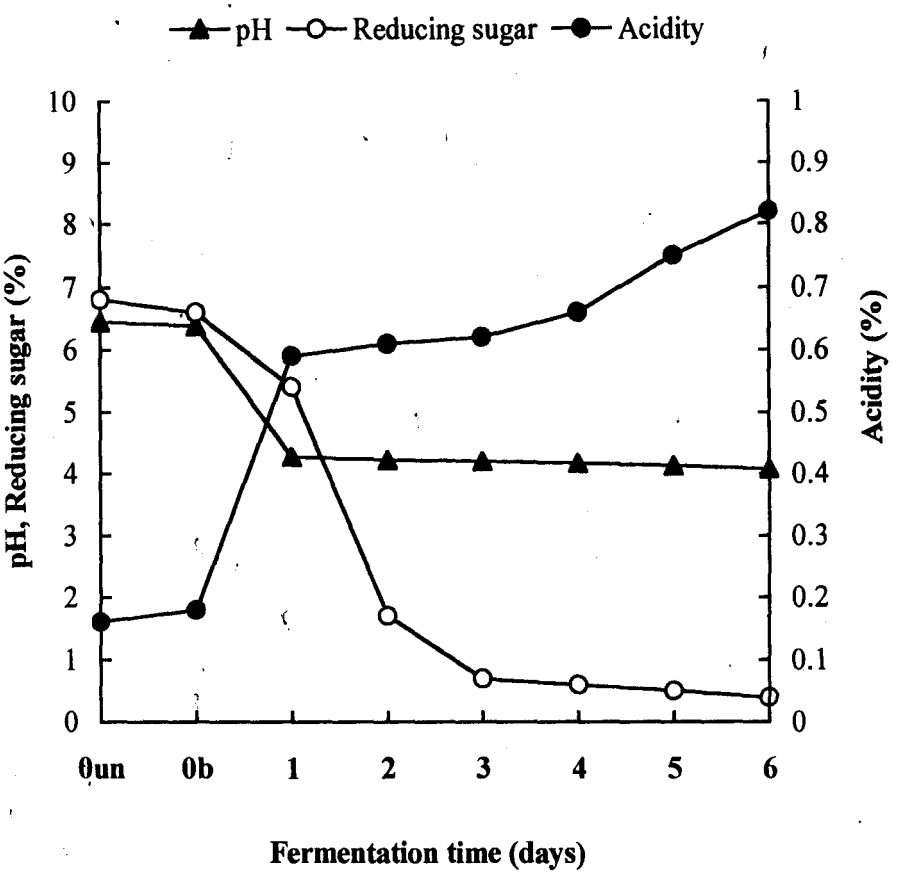

Fig 14. Changes in pH, acidity and reducing sugar during soft chhurpi fermentation. Values are the means of three batches of fermentation.

0un = Unboiled milk, 0 day
0b = Boiled milk, 0 day

DISCUSSION

Indigenous fermented milk products

Consumption of milk and milk products are the dietary culture of ethnic people living in the Sikkim Himalayas, where livestock and dairy play important role in mixed-farming system. Cow milk is popular. In the high altitude (>2100 m), yak rearing is common practice for milk, meat and skin and hair. Consumption of buffalo milk and goat milk is uncommon in this region. The people of the Sikkim Himalayas prepare a variety of indigenous fermented milk products for long centuries. Some of these products are common to all; few are confined to particular community and the region. Dahi, mohi, gheu, soft chhurpi, dudh chhurpi are common products and are prepared and consumed by all communities. However, chhu and philu are mostly consumed by the Bhutia and the Lepcha; somar is exclusively prepared and consumed by the Sherpa of the region living in high altitude. Similarly, kno (female yak) is common in sub-alpine and alpine region in East and North districts bordering with China (Tibet), and West Sikkim bordering with Nepal. Kno milk and its fermented products such as maa (butter), philu are produced only in these high altitude regions by the Bhutias.

Traditional foods have important bearing in the dietary habits of the Sikkimese people. Their indigenous knowledge of making various milk products is worth-documentation; probably the indigenous technology of food fermentation has evolved as a result of traditional wisdom and empirical experiences of generations over a period of time, based on agro-climatic condition, ethnical preference, socio-economic development status, religion and cultural practices. Milk and milk products play important role in dietary habit of the vegetarian Hindu belonging to majority Nepali communities. However, meat-eater Bhutia, Lepcha and other Nepali communities also enjoy eating the milk

products as part of their dietary culture. Rice is the staple food for all and is eaten with vegetable, meat or dairy products in every meal.

Fermented milk products are prepared at household levels using the indigenous knowledge. Use of standard starter culture is not a practice yet. They use the back-sloping technique i.e., use of previous fermented product as source of inocula into the freshly boiled milk. The Nepalis like mild-flavoured milk products, whereas the Bhutia, Lepcha and Lepcha, mostly living in high altitude prefer to have strong flavoured products such as chhu and somar. Dahi is still most popular milk product with annual production of 76.2 kg per individual household in Sikkim and per capita consumption is 33 g per day. Mohi is also most common by-product of dahi fermentation, consumed as buttermilk drink with cooked rice or as refreshing beverage.

Ethnic Importance

Dahi plays an important part in the socio-religious activities of the local community in Sikkim. For the Nepali community, dahi is a sacred item in many of their festivals and religious pujas. Dahi is used to prepare 'Tika' with rice and coloured-powder during 'Dashai', the main festival of the Nepali community. Dahi is mixed with beaten-rice (chiura) and makes essential food item during the festival such as 'Ashar ko pandra' signifying the beginning of work in the fields for the farmers. Dahi is offered to the bridegroom as a symbol of good luck during marriage in the Nepali. It is an essence to solemnize the marriage of Hindu. The Bhutias and the Lepchas too use dahi (shyow) in their religious and social events like marriages and funerals. Mohi is used as a beverage in meals during many social festivals and religious events. It is offered to guests and visitors in many of the homes in Sikkim with the intention of

relieving their tiredness. This social custom is still practiced today. For the Nepali community, gheu is a sacred item in all their religious customs. It is used in the birth, marriage, death as well as in other pujas as sacred offerings. The Nepalis, Bhutias and Lepchas use gheu for lighting the lamps for gods and goddesses in religious places.

Soft chhurpi is served as an important dish as curry and 'achar' in the various religious and social festivals of the people of the Sikkim Himalayas. Chhu is an important local food and is consumed by the people as soup along with rice when other foods are not easily available. As it is stored for a long time the problem of spoilage does not arise and can be consumed at any time. Presently, somar is consumed mostly by the older generation of the Sherpas. It is generally consumed to increase the appetite and to cure digestive problems.

MICROORGANISMS

The microbial load of indigenous fermented milk products analysed reveals that lactic acid bacteria (LAB) was the dominant microorganism with high population levels up to 10^8 cfu/g, followed by yeasts. The population of *Bacillus* was low. No filamentous mould was detected in any of the samples of fermented milk products analysed. The prevalence of lactic acid bacteria in milk (cow and kno) fermented products was 100 %, indicating their dominance in the milk products; that of yeasts was 60 % and 45.5 % in cow milk fermented and kno milk fermented products, respectively, whereas that of *Bacillus* species was 50.6 % in cow milk products.

Among LAB microflora, rods were represented by 86 % whereas coccii were represented only by 14 % in the analysed samples. 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. Gas production from glucose was used as a first step in the differentiation of lactic rods (Kandler, 1983). Two groups of non-sporeforming rods were identified: homo-fermentative and hetero-fermentative. On the basis of sugar fermentation using the API system, lactic acid configuration and meso-diaminopimelic determination and also the taxonomical keys described by Sneath *et al.* (1986) and Wood and Holzapfel (1995), homo-fermentative lactics were identified as *Lactobacillus plantarum*, *Lactobacillus alimentarius*, *Lactobacillus casei* subsp. *pseudoplantarum*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus farciminis* and *Lactobacillus salivarius*. Hetero-fermentative lactics were identified as *Lactobacillus bifermentans*, *Lactobacillus hilgardii*, *Lactobacillus kefir*, *Lactobacillus brevis* and *Lactobacillus confusus*

On the basis of API 50 CHL system and the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), coccus-shaped, non-motile and homofermentative strains of LAB were identified as *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium*.

Different species of LAB were found to be associated with the fermentation of different types of indigenous fermented milk products. Their relative importance in the fermentation process is indicated by their numbers exceeding 10^7/g, based on the dilution factors. Wide variety of microorganisms was present in raw milk of cow and kno. Microorganisms mostly LAB present in raw cow milk may contribute to the spontaneous fermentation of the milk (Gaya *et al.*, 1999; Gadaga *et al.*, 2001). It was observed that rods dominated the lactic acid microflora in the fermented milk products analysed. This finding appears to be

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novel and has not been reported in literature yet. The identity of the LAB seems to correspond with that of LAB typically reported for dairy products (Hammes and Vogel, 1995).

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. Species of *Bacillus* were also present in kishk (Mahmoud, 1977). It shows *Bacillus subtilis* also play some role in milk fermentation. There has been no record of outbreak of illness associated with *Bacillus subtilis* in fermented foods (Beumer, 2001).

Two types of yeasts were isolated: cylindrical-shaped with true mycelia and oval-shaped without ascoscope and ascus. Following the taxonomical keys described by Kreger-van Rij (1984) and Kurtzman and Fell (1998), the cylindrical-shaped strains were identified as *Saccharomycopsis crataegensis*. The oval to elliptical-shaped yeast strains CUR1:Y1 (cow chhu) and DC1:Y1 (cow dudh chhurpi) were identified as *Candida castellii*. Oval-shaped yeast strains CMR1:Y6 (cow milk), CDR2:Y1 (cow dahi), COS1:Y1 (cow mohi), CCG1:Y1 (cow soft chhurpi), PC1:YO1 (cow philu) were identified as *Candida*. Species identification could not be confirmed.

The presence of high number of yeasts, indicate some role for this group, especially during the early stages of traditional and partly spontaneous fermentation processes. Depending on the substrate and environmental conditions, yeasts may show up to 10-fold higher metabolic activities as bacteria and may particularly influence the quality and acceptability of the final product. Yeasts bring about

desirable fermentation changes in some fermented milk products (Westall and Filtenborg, 1998). Yeasts are important in ripening of certain cheese (Olson, 1996). Two strains of lactose-fermenting *Candida* COS1:Y1 and PC1:YO1 were isolated from cow mohi and cow philu, respectively (Table 13). This result indicates that some of the strains of *Candida* have roles in fermentation of milk products particularly mohi and philu. Several lactose and non-lactose fermenting yeasts have been isolated from milk and fermented milk products (Ashenafi, 1989; Paula *et al.*, 1998). Presence of yeasts in fermented milk products particularly in chhu and somar may have roles in aging and flavour development. This observation may be justified by similar work on cheddar cheese, where yeasts have roles in aging and flavour development (Welthagen and Viljoen, 1998). Yeast growth in milk products is attributed to the ability of the yeasts to utilize milk constituents, such as protein, fat, lactose and citrate (Fleet, 1990). Though, many yeasts cause spoilage in milk products (Westall and Filtenborg, 1998), information on their significant role in lactose-fermentation in milk fermented milk is little.

Pathogenic contaminants

Bacillus cereus was detected in few samples of chhu, somar, philu and dudh chhurpi. However, none of these fermented milk samples was found to contain more than 10^3/g of *Bacillus cereus* population. Small number of *Bacillus cereus* in foods is not considered significant (Roberts *et al.*, 1996).

Staphylococcus aureus was detected in soft chhurpi, chhu, somar, philu and dudh chhurpi. Numbers of *Staphylococcus aureus* were $<10^3$/g in all milk product samples tested. Concentration of 10^5-10^6/g of *Staphylococcus aureus* in food samples is necessary to produce

enterotoxin (Bisping and Amtsberg, 1988). Moreover, *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). LAB can reduce the contents of *Staphylococcus aureus* during cold storage of yoghurt (Pazakova *et al.*, 1997). This observation shows that less numbers of *Staphylococcus aureus* in fermented milk products of Sikkim may not be harmful to consumers.

Enterobacteriaceae was present in all samples except dahi, mohi and soft chhurpi. However, the load of these pathogenic contaminant was $<10^3$ cfu/g in all samples tested except somar which showed slightly higher numbers of $\sim10^4$/g. Unboiled milk contained all pathogenic contaminants.

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 milk products was due to their presence in unsterilised milk. However, the population of these contaminants was not more than 10^3 cfu/g in the milk 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 (Holzapfel *et al.*, 1995). There has been no reported case of toxicity or illness due to consumption of the fermented milk products particularly aged and strong-flavoured chhu and somar in Sikkim.

Enzymatic activity

Seven strains of LAB viz. *Lactococcus lactis* subsp. *cremoris* COM1:C3, *Lactobacillus plantarum* CCD2:R1, *Lactobacillus plantarum* CCD2:R1, *Lactobacillus alimentarius* CUG3:R1, *Lactobacillus casei* subsp. *casei* DC2:R1, *Lactobacillus confusus* DC4:R1, *Lactobacillus casei* subsp. *casei* KPY1:R1 showed proteolytic activity. All strains of *Bacillus subtilis* showed proteolytic activity. Proteolysis induced an increase in free amino acids content (Rasic *et al.*, 1971), improves the digestibility of proteins (Breslaw and Kleyn, 1973). Thirteen strains of LAB showed amylolytic activity. The highest α-amylase activity was that of *Lactobacillus alimentarius* CDC1:R1 and *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 (>5 U/ml). All strains of *Bacillus subtilis* showed amylolytic activity. Five strains of LAB *Lactococcus lactis* subsp. *lactis* CMR2:C1, *Lactococcus lactis* subsp. *cremoris* CMS2:C1, *Enterococcus faecium* PC1:C4, *Lactobacillus plantarum* KMK1:R1, *Lactobacillus brevis* KCY1:R1 showed lipolytic activity on tributyrin agar plates and three strains of *Bacillus* viz. *Bacillus subtilis* CCG1:S2, *Bacillus subtilis* CSR2:S1 and *Bacillus subtilis* CSN1:S2 showed lipolytic activity.

The use of the API-Zym technique has been reported (Arora *et al.*, 1990) as a rapid and simple means of evaluating and localizing 19 different hydrolases of microorganisms associated with dairy fermentations. This method is also of relevance for selection of strains as potential starter cultures on the basis of superior enzyme profiles, especially peptidases, and esterases, for accelerated maturation and flavour development of milk products (Tamang *et al.*, 2000). The process of cheese maturation involves sequential breakdown of milk components, such as fat, protein and lactose by the starter bacteria

(Davies and Law, 1984). Therefore, a fundamental understanding of starter culture enzymes is of prime importance in evaluating their suitability and in predicting their influence on the final cheese quality.

The absence of proteinases (trypsin and chymotrypsin) and presence of high peptidase (leucine-, valine- and cystine-arylamidase) and esterase-lipase (C4 and C8) activities produced by the predominant LAB isolated from indigenous fermented milk products of Sikkim are traits of desirable quality for their use in production of typical flavours. Cheese starters with low proteinases and strong peptidases are also useful in reducing bitterness and improving body and textural defects, which are often caused by most of the microbial preparations when used as rennet substitutes (Davies and Law, 1984). In addition, soapiness defect caused by the accumulation of long-chained fatty acids in many ripened cheese varieties can be removed by using strains with high esterase and lipase activities (Davies and Law, 1984).

High activity of β-galactosidase exhibited by species of *Lactobacillus* are essential features in indigenous fermented milk products of Sikkim. The β-galactosidase activity shown by *Lactobacillus casei* subsp. *casei* PC2:R6, *Lactobacillus bifermentans* KSK1:R1 and *Lactobacillus plantarum* CCD2:R1, isolated from philu, dahi and soft-chhurpi, respectively indicates that these indigenous fermented milk products particularly philu, dahi and soft chhurpi are suitable for consumption by lactose-intolerant infants. β-galactosidase, responsible for hydrolysing the naturally occurring (1-6) linked galactosidase in milk has been demonstrated in *Lactobacillus acidophilus, Bifidobacterium bifidum* (Premi *et al.,* 1972). A decline in lactose content and an increase in lactase activity due to β-galactosidase

activity of the starter culture make fermented milk more suitable for lactose-intolerant infants (Shah, 2001). Similar observation was made in low-lactose dahi which was found suitable for infants suffering from lactose-intolerance due to presence of β-galactosidase (Chandrasekaran *et al.*, 1975; Rao *et al.*, 1985). Yoghurt bacteria produce higher β-galactosidase than probiotic bacteria (Shah and Jelen, 1990; Shah, 1994).

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 fermented milk products of the Sikkim Himalayas 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 strains *Lactococcus lactis* subsp. *cremoris* CDM1:C1, *Lactobacillus casei* subsp. *casei* DC2:R1 and *Lactobacillus bifermentans* KSK1:R1 inhibited the growth of *Listeria monocytogenes* DSM 20600. *Lactococcus lactis* subsp. *cremoris* CDM1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1, *Lactobacillus plantarum* KMK1:R1, *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 showed inhibition zone against *Bacillus cereus* CCM 2010, *Enterococcus faecium* CCB1:C1, *Lactococcus lactis* subsp. *cremoris* CUR1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1, *Lactobacillus*

brevis KCY1:R1 against *Enterococcus faecium* DSM 20477 and *Lactobacillus bifermèntans* CDP1:R1, *Lactococcus lactis* subsp. *lactis* COS1:C1, *Lactococcus lactis* subsp. *cremoris* CUR1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 against *Streptococcus mutans* DSM 6178. This reveals that some of these LAB strains have antimicrobial properties, which can reduce the number of other undesired microorganism in the milk products as well as help in the preservation of milk products (Einarsson and Lauzon, 1995). The inhibitory effect of LAB against *Bacillus cereus* was also observed in dahi samples collected from South Indian markets (Gandhi and Nambudripad, 1975; Balasubramanyam and Varadaraj, 1994). 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 amines

Fermentation may be important in the formation of biogenic amines through the action of added lactic acid cultures or the natural microflora (Silla-Santos, 2001). Biogenic amines have been reported in fermented milk 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 cheese, fish, fermented vegetables and beverages (Stratton *et al.*, 1991; Leisner *et al.*, 1994).

None of the strains of LAB produced tyramine, cadaverine, histamine and putrescine in the applied method. This result indicated that biogenic amine is not produced by the dominant microorganisms (LAB and *Bacillus* spp.) in fermented milk products of Sikkim, products. Amino acid decarboxylase activity has been shown to depend on the composition of the medium and the growth phase of the microorganisms (Halász *et al.*, 1994). The major biogenic amine producers in foods are enterobacteriaceae and enterococci (Nout, 1994). Most functional LAB do not produce significant levels of biogenic amines (Nout, 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 indigenous fermented milk 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). Nineteen strains of LAB showed high degrees of hydrophobicity (>75%), among which *Lactobacillus casei* subsp. *casei* PC2:R6 (isolated from cow philu) and *Lactococcus lactis* subsp. *cremoris* CUR1:C1 (isolated from cow chhu) showed the highest percentage of hydrophobicity of 97.89 % and 97.15 %, respectively, showing strong hydrophobic properties. All strains of LAB had more than 36 % hydrophobicity, indicating that the strains isolated from fermented milk products of the Sikkim 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 fermented milk products of the Sikkim 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 the indigenous fermented milk (both cow and kno) products is presented in Table 22 and 23. The pH of all these products was acidic in nature with higher acidity content, due to lactic acid fermentation. Moisture content of kno maa and dudh chhurpi was low due to drying after fermentation. Some of the products had high fat. High content of protein was observed in all milk products. Indigenous fermented milk products are high calorie-content foods of which maa (butter) made from female yak had high calorie value of 876.3 kcal/100g. Among the minerals of the milk products, calcium content was higher than other minerals estimated.

Microbial changes

During soft-variety chhurpi fermentation, LAB increased remarkable during fermentation. It shows that LAB are the main functional microorganisms in chhurpi fermentation. Due to spontaneous fermentation nature of chhurpi, yeasts population was just next to LAB and increased during fermentation. *Bacillus*, though, present in the initial stage, increased gradually. Due to lactic acid fermentation, pH went down and acidity increased during soft chhurpi fermentation. Reducing sugar decreased gradually during fermentation due to amylolytic activity of LAB, breaking glucose to lactic acid, thus increasing pH (Nout, 2001).

Conclusion

Traditional fermented milk products are typically produced at household levels in the Sikkim Himalayas. Traditional foods harness the dietary history of particular community. Indigenous knowledge of ethnic people for production of fermented milk products for consumption is worth-documentation. Though, the fermented milk products of Sikkim 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 fermented milk products. 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 coccus-lactics (*Lactococcus, Enterococcus*) to species of homo-and hero-fermentative rods (*Lactobacillus*), *Bacillus subtilis* to species of yeasts (*Candida, Saccharomycopsis*) were present in the indigenous fermented milk products. 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. Some of these strains particularly species of *Lactobacillus* showed high β-galactosidase activity in dahi, philu and soft chhurpi, indicating these products as low-lactose milk products, which can be suitable for consumption by lactose intolerance-infants. Isolated and identified microorganisms from common as well lesser-known fermented milk products may contribute significant information on unknown microbial gene pool as genetic resources of the Himalayan regions.

SUMMARY

The people of the Sikkim Himalayas prepare a variety of indigenous fermented milk products for long centuries. Some of these milk products are dahi, mohi, gheu, soft chhurpi, dudh chhurpi, chhu and philu. Their indigenous knowledge of food fermentation for production of various milk products has been documented, along with per capita consumption and annual production of each milk product in Sikkim. Dahi is the most popular milk product with annual production of 76.2 kg per individual household in Sikkim and per capita consumption is 33 g per day. Besides consumption, some of the indigenous fermented milk products have deep-rooted socio-ethnical importance. Dahi plays an important part in the socio-religious activities of the local community in Sikkim. It is an essence to solemnize the marriage of Hindu. The Bhutias and the Lepchas too use dahi (shyow) in the religious and social events. Gheu is a sacred item in all their religious customs. It is used in the birth, marriage, death as well as in other religious occasions

One hundred and eighty-five samples of cow milk-fermented products and eighty-one samples of kno (female yak) milk-fermented products were collected from different places of Sikkim and were analysed for microbial load. Lactic acid bacteria (LAB) was the dominant microorganism with high population levels up to 10^8 cfu/g, followed by yeasts with load of 10^7 cfu/g. The population of spore-formers was recovered in unboiled milk of cow and kno, soft chhurpi, cow chhu and somar at the level of $<10^3$ cfu/g. No filamentous mould was detected. Aerobic mesophilic counts were 10^8 cfu/g in unboiled milk (cow and kno), 10^8 to 10^9 cfu/g in dahi, chhu, philu, 10^8 cfu/g in mohi and soft chhurpi, 10^5 cfu/g in somar, 10^4 cfu/g in dudh chhurpi and 10^6 cfu/g in maa. Out of 1050 isolates of microorganisms obtained from

266 samples of fermented milk products, 772 isolates were lactic acid bacteria, 74 were spore-formers and 204 were yeasts.

Out of 722 LAB isolates, 663 strains were rod-shaped and 109 were coccus-shaped, Gram-positive, non-sporeforming, catalase-negative and non-motile. Homo-fermentative rods were identified as *Lactobacillus plantarum*, *Lactobacillus alimentarius*, *Lactobacillus casei* subsp. *pseudoplantarum*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus farciminis* and *Lactobacillus salivarius* and hetero-fermentative rods were identified as *Lactobacillus bifermentans*, *Lactobacillus hilgardii*, *Lactobacillus kefir*, *Lactobacillus brevis* and *Lactobacillus confuses*. Homofermentative coccus-latics were identified as *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium*. Endospore-forming isolates were identified as *Bacillus subtilis*. Yeasts were identified as *Saccharomycopsis crataegensis* and *Candida castellii*.

Prevalence of LAB in fermented milk (cow and kno) products was 100 %, that of yeasts was 60 % and 45.5 % in cow milk fermented and kno milk fermented products, respectively, whereas that of *Bacillus* species was 50.6 % in cow milk products. Lactic acid bacteria were dominant microflora representing 74 % in milk products followed by yeasts 19 % and *Bacillus* 7 %, respectively. Among LAB microflora, rods were represented by 86 % whereas coccii were represented only by 14 % in the analysed samples.

Bacillus cereus was detected in few samples of chhu, somar, philu and dudh chhurpi at the level $<10^3$/g. *Staphylococcus aureus* was detected in soft chhurpi, chhu, somar, philu and dudh chhurpi. Numbers of *Staphylococcus aureus* were $<10^3$/g in all milk product samples tested. Enterobacteriaceae was present in all samples except dahi, mohi

and soft chhurpi. However, the load of these pathogenic contaminant was $<10^3$ cfu/g in all samples tested except somar which showed slightly higher numbers of $\sim10^4$/g. Unboiled milk contained all pathogenic contaminants.

Lactococcus lactis subsp. *cremoris* COM1:C3, *Lactobacillus plantarum* CCD2:R1, *Lactobacillus plantarum* CCD2:R1, *Lactobacillus alimentarius* CUG3:R1, *Lactobacillus casei* subsp. *casei* DC2:R1, *Lactobacillus confusus* DC4:R1, *Lactobacillus casei* subsp. *casei* KPY1:R1 showed proteolytic activity. The highest α-amylase activity was that of *Lactobacillus alimentarius* CDC1:R1 and *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 (>5 U/ml). All strains of *Bacillus subtilis* showed proteolytic and amylolytic activity. *Lactococcus lactis* subsp. *lactis* CMR2:C1, *Lactococcus lactis* subsp. *cremoris* CMS2:C1, *Enterococcus faecium* PC1:C4, *Lactobacillus plantarum* KMK1:R1, *Lactobacillus brevis* KCY1:R1, *Bacillus subtilis* CCG1:S2, *Bacillus subtilis* CSR2:S1 and *Bacillus subtilis* CSN1:S2 showed lipolytic activity.

Enzymatic profiles of randomly selected lactic acid bacteria strains of fermented milk products were assayed using the API zym galleries. LAB strains produced a wide spectrum of enzymes. These strains showed relatively weak esterase and no lipase (C14) activities. All strains showed strong phosphatase, β-galactosidase and β-glucosidase activities. However, they showed no detectable proteinase activity with the methods applied. The absence of proteinases (trypsin and chymotrypsin) and presence of high peptidase (leucine-, valine- and cystine-arylamidase) and esterase-lipase (C4 and C8) activities produced by the predominant LAB isolated from indigenous fermented milk products of Sikkim are traits of desirable quality for their use in

production of typical flavours. High activity of β-galactosidase exhibited by species of *Lactobacillus* are essential features in indigenous fermented milk products of Sikkim.

The antagonistic properties of the strains, isolated from fermented milk products were tested against the indicator strains (*Listeria monocytogenes* DSM 20600, *Bacillus cereus* CCM 2010, *Enterococcus faecium* DSM 20477 and *Streptococcus mutans* DSM 6178). *Lactococcus lactis* subsp. *cremoris* CDM1:C1, *Lactobacillus casei* subsp. *casei* DC2:R1 and *Lactobacillus bifermentans* KSK1:R1 inhibited the growth of *Listeria monocytogenes* DSM 20600. *Lactococcus lactis* subsp. *cremoris* CDM1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1, *Lactobacillus plantarum* KMK1:R1, *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 showed inhibition zone against *Bacillus cereus* CCM 2010, *Enterococcus faecium* CCB1:C1, *Lactococcus lactis* subsp. *cremoris* CUR1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* CSR1:R1, *Lactobacillus brevis* KCY1:R1 against *Enterococcus faecium* DSM 20477 and *Lactobacillus bifermentans* CDP1:R1, *Lactococcus lactis* subsp. *lactis* COS1:C1, *Lactococcus lactis* subsp. *cremoris* CUR1:C1, *Lactobacillus casei* subsp. *pseudoplantarum* KSY1:R2 against *Streptococcus mutans* DSM 6178. This reveals that some of these LAB strains have antimicrobial properties, which can reduce the number of other undesired microorganism in the milk products as well as help in the preservation of milk products. None of the strains were found to produce any bacteriocin with the method applied.

Forty eight strains of lactic acid bacteria and *Bacillus* isolated from fermented milk (both cow and kno) products were tested for biogenic amine production with the surface plate method applied.

None of the strains produced tyramine, cadaverine, histamine and putrescine in the applied method. This result indicated that biogenic amine is not produced by the dominant microorganisms (LAB and *Bacillus* spp.) in fermented milk products of Sikkim, products.

Nineteen strains of LAB showed high degrees of hydrophobicity (>75%), among which *Lactobacillus casei* subsp. *casei* PC2:R6 (isolated from cow philu) and *Lactococcus lactis* subsp. *cremoris* CUR1:C1 (isolated from cow chhu) showed the highest percentage of hydrophobicity of 97.89 % and 97.15 %, respectively, showing strong hydrophobic properties. All strains of LAB had more than 36 % hydrophobicity, indicating that the strains isolated from fermented milk products of the Sikkim Himalayas were not hydrophilic in nature. High degree of hydrophobicity by the lactic acid bacteria isolated from lesser-known traditional fermented milk products of the Sikkim Himalayas indicates the potential of adhesion to gut epithelial cells of human intestine, advocating their 'probiotic' character.

Proximate composition of the indigenous fermented milk was analysed. The pH of cow milk products was 3.9-4.3 except in chhu, somar and dudh chhurpi which had pH value of 6.0. The pH of kno milk products was 3.7-4.8 except maa showing pH 6.0. The titratable acidity ranged from 0.04 % (in somar) to 0.89 % in (kno mohi). Moisture content was low upto 12 % in kno maa, 16 % in dudh chhurpi but high in all other milk products at 36.5 % to 92.6 %. The ash content was also found to be around 1.1 to 7.7 % on dry matter basis. Fat content varied from product to product. Philu contained high fat whereas cow chhu had very low fat of 5.8 % dry matter basis. High content of protein was observed in all milk products except in kno maa. Carbohydrate content was also variable in the products. Indigenous fermented milk products

are high calorie-content foods of which maa (butter) made from kno (female yak) is high calorie food having energy value of 876.3 kcal/100g. Among the minerals of the milk products, calcium content was higher than other minerals estimated.

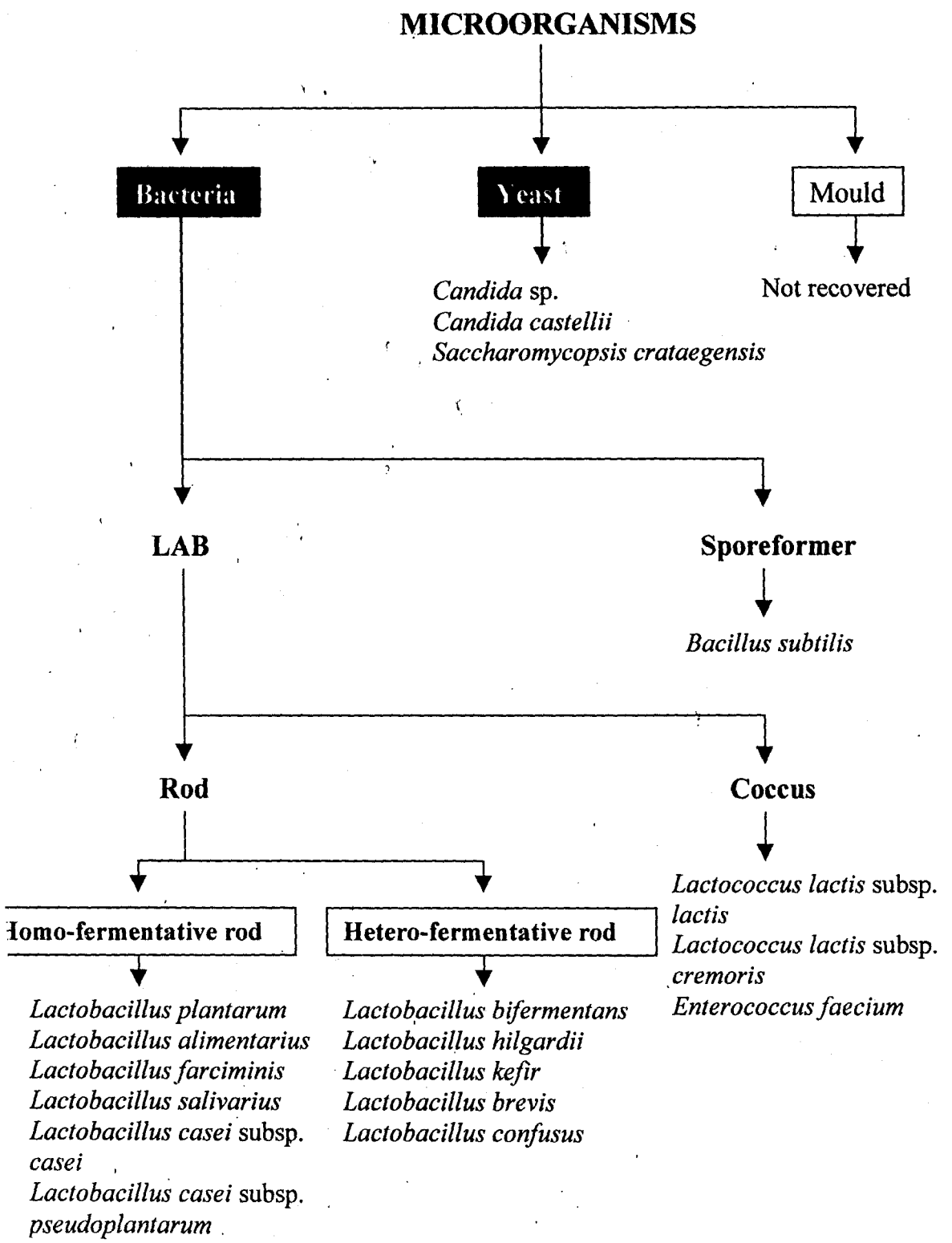
Chhurpi (soft-variety) was prepared in the laboratory following the traditional method. Succession studies were carried at every 1 day interval within a range of 0-6 days. Load of lactic acid bacteria increased from 10^5 cfu/g in boiled milk to10^8 cfu/g at the end of fermentation. Population of yeasts increased from 10^3 cfu/g to 10^7 cfu/g during spontaneous fermentation. Subsequently, load of *Bacillus* increased up to 10^3 cfu/g on the sixth day. The mean pH value of boiled milk was decreased from 6.38 to 4.08 and titratable acidity increased from 0 day till the end. Reducing sugar content was decreased remarkably during fermentation.

Traditional fermented milk products are typically produced at household levels in Sikkim Himalayas. Though, the fermented milk products of Sikkim 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 hydrophobicity), 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 fermented milk products.

This study has demonstrated that microbial diversity ranging from species of lactic acid bacteria belonging to coccus-lactics (*Lactococcus, Enterococcus*) to species of homo-& hetero-fermentative

rods (*Lactobacillus*), *Bacillus subtilis* to species of yeasts (*Candida, Saccharomycopsis*) were present in the indigenous fermented milk products. Table C shows the schematic presentation of microbial diversity in the indigenous fermented milk products of the Sikkim Himalayas. Isolated and identified microorganisms from common as well lesser-known fermented milk 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 indigenous fermented milk products of the Sikkim Himalayas



BIBLIOGRAPHY

Abd-Alla, E.A.M., El-Shafei, K., Ibrahim, G.A. and Sharaf, O.M. (1996) Changes in microflora and biogenic amines of some market processed cheeses during storage. *Egyptian Journal of Dairy Science* 24: 217-226.

Abd-ed-Malek, Y. and Demerdash, M. (1977) Microbiology of kishk. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Adams, M.R. and Nicolaides, L. (1997) Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control* 8 (5/6): 227-239.

Androuet, P. (1976) *Encyclopedia of Cheese*. Omnibus Press, New York.

AOAC (1990) *Official Methods of Analysis*, 15th Edition. Association of Official Analytical Chemists, Virginia.

Arora, G., Lee, B.H. and Lamoureux, M. (1990) Characteristics of enzyme profiles of *Lactobacillus casei* species by a rapid API-ZYM system. *Journal of Dairy Sciences* 73: 264-273.

Ashar, M.N. and Prajapati, J.B. (2001a) Role of probiotic cultures and fermented milks in combating blood cholesterol. *Indian Journal of Microbiology* 41: 75-86.

Ashar, M.N. and Prajapati, J.B. (2001b) Serum cholesterol level in human fed with acidophilus milk. *Indian Journal of Microbiology* 41: 257-263.

Ashenafi, M. (1989) Proteolytic, lipolytic and fermentative properties of yests isolsted from ayid, a traditional Ethiopian Cottage cheese Sinet. *Ethiopian Journal of Science* 12(2): 131-139.

Auclair, J. and Accolas, J.P. (1974) Koumiss (koumiss, coomys). In: *Encyclopèdia of Food Technology* (Eds: Johnson, A.H. and Peterson, M.S.), pp. 537-538. CT:AVI, Westport.

Balaraman, N. and Golay, M.M. (1991) *Livestock Production in Sikkim.* Sikkim Science Society, Gangtok.

Balasubramanyam, B.V. and Vardaraj, M.C. (1994). Dahi as a potential source of Lactic Acid Bacteria active against foodborne pathogenic and spoilage bacteria. *Journal of Food Science Technology* 31(3): 241-243.

Bartholomew, J.W. (1962) Variables influencing results and the precise definition of the steps in Gram staining as a means of standardizing the results obtained. *Stain Technology* 37:139-155.

Batra, L.R. and Millner, P.D. (1976) *Asian fermented foods and beverages. Development in Industrial Microbiology* 17: 117-128.

Basson, P. (1981) Women and traditional food technologies. Changes in rural Jordan. *Ecology and Food Nutrition* 11:17-23.

Bernet, F.M., Brassart, D., Nesser, J.R. and Servin, A. (1993) Adhesion of human bifidobacterial starters to cultured human intestinal epithelial cells and inhibition of enteropathogen cell interaction. *Applied and Environmental Microbiology* 59: 4121-4128.

Besancon, X., Smet, C., Chabalier, C., Rinemale, M., Reverbel, J.P., Ratomehenina, R. and Galzy, P. (1992) Study of surface yeast

flora of Roquefort cheese. *International Journal of Food Microbiology* 17: 9-18.

Beumer, R.R. (2001) Microbiological hazards and their control: bacteria. In: *Fermentation and Food Safety* (Eds: Adams, M.R. and Nout, M.J.R.), pp. 141-157. Aspen Publishers, Inc. Gaithersburg, Md., USA.

BIS (1980a) IS: 9617. *Specification for dahi.* Bureau of Indian Standards, Manak Bhawan, New Delhi.

BIS (1980b) IS: 9532. *Indian Standard Specification for chakka and shrikhand.* Bureau of Indian Standards, Manak Bhawan, New Delhi.

Bisping, W. and Amtsberg, G. (1988) *Colour Atlas for The Diagnosis of Bacterial Pathogens In Animals,* 1st Edition, Paul Parey Scientific Publishers, Berlin and Hamburg.

Boehringer-Mannheim (1989) UV method for the determination of L-lactic acid and D-lactic acid in foodstuffs and other materials. In: *Methods of Biochemical Analysis and Food Analysis using Single Reagents* pp. 78-81. Boehringer-Mannheim GmbH, Biochemica.

Bogra, V.R. and Mathur, O.N. (2000) Physico-chemical status of major milk constituents and minerals at various stages of shrikhand preparation. *Journal of Food Science and Technology* 37(2): 111-115.

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

Brenslaw, E.S. and Kleyn, D.H. (1973) *In Vitro* digestibility of protein in yoghurt at various stages of processing. *Journal of Food Science* 38: 1061-1021.

Campbell-Platt, G. (1987) *Fermented Foods of the World: A Dictionary and Guide*. Butterworths, England.

Campbell-Platt, G. (1994) Fermented foods - a world perspective. *Food Research International* 27: 253-257.

Carr, S. (1981) *Cheese*. Mitchell Beazley, London.

Celano, G.V., Cafarchia, C. and Tiecco, G. (1996) Formation of biogenic amines in Pecorino Pugliese cheese. *Ind. Aliment.* 35: 249-252.

Census of India (1991) *District Census Handbook, Series 22, Sikkim,* Government of India.

Chandan, R.C. (1989) *Yogurt: Nutritional Health Properties*. National Yogurt Association, Virgnia.

Chandrasekharan, R., Kumar, V., Walia, B.N.S. and Moorthy, B. (1975) Carbohydrate intolerance in infants with acute diarrhoea and its implications *Acta Paediatrica Scandinavica* 64: 483.

Chatterjee, T.K., Sarkar, S. and Biswas, G. (1994) Quality characteristics of some marketed indigenous milk products: major constituents and mineral composition of rabri. *Journal of Food Science and Technology* 31 (5): 426-427.

Chin Wen, L., Hsiao Ling, C. and Jeruei, L (1999) Identification and characterization of lactic acid bacteria and yeasts isolated from

Kefir grains in Taiwan. *Australian Journal of Dairy Technology* 54(1): 14-18.

Claus, D. and Berkeley, R.C.W. (1986) Genus *Bacillus* Cohn 1872, 174. In: *Bergey's Manual of Systematic Bacteriology*, Vol. 2, (Eds: Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G.), pp. 1105-1139. Williams & Wilkins, Baltimore, MD.

Daeschel, M. (1992) Procedures for the detection of antimicrobial activity of microorganisms. In: *Food Biopreservation of Microbial Origin.* (Eds: Bibek, R. and Daeschel, M.), pp. 57-81. CRC Press, Mexico.

Davies, F.L. and Law, B.A. (1984) *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk.* Elsevier Applied Science Publishers, New York.

de Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* 23: 130-135.

Ding, H. and Lämmler, C. (1992). Cell surface hydrophobicity of *Actinomyces pyogenes* determined by hexadecane adherence- and salt aggregation studies. *Zentralblatt für Veterinärmedizin* (B) 39: 132-138.

Dykes G.A., Britz T. J. and von Holy A (1994). Numerical taxonomy and identification of lactic acid bacteria from spoiled, vacuum packaged Vienna sausages. *Journal of Applied Bacteriology* 76: 246-52.

Economidou, P.L. (1975) *Studies on Greek trahanas-a wheat fermented milk food.* M.S. Thesis, Cornwell University, USA.

Economidou, P.L. and Steinkraus, K.H. (1977) Greek trahanas. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok

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

El-Samaragy, Y.A. (1997) Labneh or Yoghurt cheese: A review. *Egyptian Journal of Dairy Science* 25 Chin(2): 165-178.

FIL-IDF (1981) *Consumption statistics for milk and milk products, 1979.* IDF Document 131.

Fleet, G. H. (1990) Yeasts in dairy products - a review. *Journal of Applied Bacteriology.* 68: 199-211.

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

Gadaga, T.H., Mutukumira, A.N. and Narhus, J.A. (2001) Growth characteristics of *Candida kefyr* and two strains of *Lactococcus lactis* subsp. *lactis* isolated from Zimbabwean naturally fermented milk. *International Journal of Food Microbiology* 70: 11-19.

Galloway, J.H. and Crawford, R.J.M. (1985). Cheese fermentations. In: *Microbiology of Fermented Foods* Vol. I, (Ed: Wood, B.J.B.) pp. 111-165. Elsevier Applied Science Publishers, London.

Gandhi, D.N. and Nambudripad, V.K.N. (1975) A note on antimicrobial properties of dahi (curd). *Indian Journal of Dairy Science* 28: 72-73.

Gardini, F., Martuscelli, M., Caruso, M.C., Galgano, F., Crudele, M.A., Favati, F., Guerzoni, M.E. and Suzzi, G. (2001) Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *International Journal of Food Microbiology* 64: 105-117.

Gaya, P., Babin, M., Medina, M. and Nunez, M. (1999). Diversity among lactococci isolated from ewes' raw milk and cheese. *Journal of Applied Microbiology* 87: 849-855.

Garg, S.K., Bhale, P. and Rawat, R.S. (1983) *Shrikhand* – An indigenous fermented milk product. *Indian Dairyman.* 35: 657-662.

Geisen, R. and Holzapfel W.H. (1996) Genetically modified starters and protective cultures. *International Journal of Food Microbiology* 30: 315-324.

Ghosh, J. and Rajorhia, G.S. (1987) Chemical, microbiological and sensory properties of Misti dahi sold in Calcutta. *Asian Journal of Dairy Research* 6: 11-16.

Ghosh, J. and Rajorhia, G.S. (1990) Selection of starter culture for production of indigenous fermented milk product (*Misti dahi*). *Lait* 70: 147-154.

Gilliland, S.E. (1985) *Bacterial starter Cultures for Foods*. CRC Press, Boca Raton, Florida.

Gordon, R.E., Haynes, W.C. and Pang, C.H.-N (1973) The genus *Bacillus,* Handbook No. 427. United States Department of Agriculture, Washington D.C.

Gopalan, C., Rama Sastri, B.V. and Balasubramanian, S.C. (1995) *Nutritive Value of Indian Foods*. National Institute of Nutrition, Hyderabad.

Gupta, M., Khetarpaul, N. and Chauhan, B.M. (1991) Effect of *rabadi* fermentation on HCl-extractability of minerals of wheat. *Food Chemistry* 42: 111-117.

Gupta, R.C., Mann, B., Joshi, V.K. and Prasad, D.N. (2000). Microbiological, chemical and ultrastructural characteristics of misti doi (sweetened dahi). *Journal of Food Science and Technology* 37(1): 54-57

Halász A., Baráth, A., Simon-Sarkadi, L. and Holzapfel, W.H. (1994) Biogenic amines and their production by microorganisms in food. *Trends in Food Science and Technology* 5: 42-49.

Hammes, W.P. and Vogel, R.H. (1995) the genus *Lactobacillus*. In: *The Lactic Acid Bacteria,* Vol. 2: *The Genera of Lactic Acid Bacteria.* (Eds: Wood, B.J.B. and Holzapfel. W.H.), pp. 19-54. Blackie Academic & Professional, An Imprint of Chapman and Hall, London.

Harrigan, W.F. (1998) *Laboratory Methods in Food Microbiology,* 3rd Edition. Academic Press, London.

Hartles, P., van Hooidonk, J. and la Riviere, J.W.M. (1977) Kefir. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Hauschild, A. H. W. (1992) Epidemilogy of human foodborne botulism. *Clostridium botulinum*: Ecology and Control in Foods. Marcel Dekker, New York.

Hesseltine, C.W. (1979) Some important fermented foods of Mid-Asia, the Middle East, and Africa. *Journal of American Oil Chemists' Society* 56: 367-374.

Hesseltine, C.W. (1983) Microbiology of oriental fermented foods. *Annual Review of Microbiology* 37: 575-601.

Holt, J.G., Krieg, N.R., Sfneath, T.H.A., Stanley, J.T. and William, S.T. (1994) *Bergey's Manual of Determinative Bacteriology*, 9th Edition. Baltimore, MD.

Holzapfel, W.H., Giesen, R. and Schillinger, U. (1995) Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology* 24: 343-362.

Holzapfel, W.H., Schillinger, U., Toit, M.D. and Dicks, L. (1997) Systematics of probiotic lactic acid bacteria with reference to modern phenotypic and genomic methods. *Microecology and Therapy* 26: 1-10

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J.H.J. (1998) Overview of gut flora and probiotics. *International Journal of Food Microbiology* 41: 85-101.

Huria, V.K. (2002) Milk: the classic food of modern civilization. *Indian Food Industry* 21 (4): 24-32.

Joosten, H.M.L.J. and Northolt, M.D. (1989) Detection, growth and amine-producing capacity of lactobacilli in cheese. *Applied Environmental Microbiology* 55: 2356-2359.

Kandler, O. (1983) Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 49: 209-224.

Katiyar, S.K., Bhasin, A.K. and Bhatia, A.K. (1991) Traditionally processed and preserved milk products of Sikkimese Tribes. *Science and Culture* 57(10,11): 256-258.

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

Kosikowski, F.V. (1977) *Cheese and Fermented Milk Products*, 2nd Edition. Edwards Bros. Inc., Ann Arbor, Michigan.

Kreger-van Rij, N.J.W. (1984) *The Yeasts - a Taxonomic Study*. Elsevier Science Publishers, Amsterdam.

Kurtzman, C.P. and Fell, J.W. (1998) *The Yeast, A Taxonomic Study*, 4th Edition, Elsevier, Amsterdam.

Kwon, T.W. (1994) The role of fermentation technology for the world food supply. In: *Lactic Acid Fermentation of Non-dairy Food and Beverages*, (Eds: Lee, C.H., Adler-Nissen, J. and Bärwald, G.), pp. 1-7. Ham Lim Won, Seoul.

La Riviere, J.W.M. (1963) Studies on the kefir grain. *Journal of General Microbiology* 31:V.

La Riviere, J.W.M. (1969) Ecology of yeasts in kefir grain. *Antonie van Leeuwenhoek* 35: Supplement:Yeasts Symposium.

La Riviere, J.W.M., Kooiman, P. and Schmidt, K. (1967) Kefiran, a novel polysaccharide produced in the kefir grain by *Lactobacillus brevis*. *Arch. fur Mikrobiol.* 59:269-278.

Laxminarayana, H., Nambudripad, V.K.N., Laxmi, N.V., Anantaramaiah, S.H., Sreenivasamurty, V. and Iya, K.K. (1952) Studies on dahi. III. Taxonomy of lactic acid bacteria of dahi. *Indian Journal of Vetinary Science and Animal Husbandry* 22: 13-25.

Laxminarayana, H. and Shankar, P.A. (1980) Fermented milk in human nutrition. *Indian Dairyman* 32: 121-129.

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

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

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

Lindgren, S.E. and Dobrogosz, W.J. (1990) Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews* 87: 149-164.

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

Mahmoud, S.A.Z. (1977) Chemical and microbiological composition of Egyptian kishk. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Marshall, V.M. (1982) Flavour compounds in fermented milks. *Perfumer and Flavorist* 7(2): 27-34.

Misra, A.K. (1992) Commercial production of dahi. *Indian Dairyman.* 44: 501-503.

Mital, B.K. (1977) Indian fermented milks. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Mitsuoka, T. (1992) The human gastrointestinal tract. In: *The Lactic Acid Bacteria, Vol. 1, The lactic acid bacteria in health and disease* (Ed: Wood, B.J.B.), pp. 69-114. Elsevier Applied Science, London.

Mogilevsky, S. (1977) Kefir. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Mohanan, K.R., Shankar, P.A. and Laxminarayana, H. (1984) Microflora of *dahi* prepared under household conditions of Bangalore. *Journal of Food Science and Technology* 21: 45-46.

Morcos, S.R., Hegazi, S.M. and El-Damhoughy, S.T. (1973) Fermented foods in common use in Egypt I The nutritive value of kishk. *Journal of Science of Food and Agriculture*. 24:1153-1156.

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

Nikku-Paavola, M.-L., Laitila, A., Mattila-Sandholm, T. and Haikara, A. (1999) New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* 86: 29-35.

Norris, J.R., Berkeley, R.C.W., Logan, N.A. and O'Donnell, A.G. (1981) The Genus *Bacillus* and *Sporolactobacillus*. In: *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria,* Vol. 2, (Eds: Starr, M.P., Stolp, H.G., Trüper, A., Balows and Schlegel, H.G.), pp. 1711-1742. Springer-Verlag, New York.

Nout, M.J.R. (1994) Fermented foods and food safety. *Food research International* 27: 291-298.

Nout, M.J.R. (2001) Fermented foods and their production. In: *Fermentation and Food Safety* (Eds: Adams, M.R. and Nout, M.J.R.), pp. 1-38. Aspen Publishers, Inc. Gaithersburg, Md., USA.

Nout, M.J.R., Bakshi, D. and Sarkar, P.K. (1998) Microbiology safety of kinema, a fermented soya bean food. *Food Control* 9(6): 357-362.

Nychas, G.J.E. and Arkoudelos, J.S. (1990) Staphylococci: their role in fermented sausages. *Journal of Applied Bacteriology Symposium Supplement* 19: 167S-188S.

Oberman, H. (1985) Fermented Milks. In: *Microbiology of Fermented Foods* Vol I (Ed. Wood B.J.B.), pp. 167-195. Elsevier Applied Science Publishers, London.

Olson, N.F. (1996) *Yeasts. Ripened Semi-Soft Cheeses*. Pfizer Cheese Monographs. Vol IV, *Pfizer,* New York.

Pal, P.K., Hossain, S.A. and Sarkar P.K. (1993). An assessment of manufacturing methods and sensory characteristics of market churpi. *Journal of Hill Research* 6: 73-76.

Pal, P.K., Hossain, S.A., Sarkar P.K. and Patil, G.R. (1994). Compositional and sensory characteristics of kachcha chhurpi. *Journal of Food Science and Technology* 31: 71-72

Pal, P.K., Hossain, S.A. and Sarkar P.K. (1995). The effect of different coagulants on quality of chhurpi. *Indian Journal of Dairy Science* 48: 562-565.

Pal, P.K., Hossain, S.A. and Sarkar P.K. (1996). Optimisation of process parameters in the manufacture of churpi. *Journal of Food Science and Technology*. 33: 219-223.

Paula, C.R., Gamble, W., Laria, S.T. and Reis Filho, S.A. (1998) Occurrence of fungi in butter, available for public consumption in Sao Paul City, *Revistade Microbiology* 19(3): 317-320.

Prakash, O. (1961) *Food and Drinks in Ancient India.* Munshi Ram Monoharlal Publication, Delhi.

Patel, R.S. and Chakraborty, B.K. (1985) Reduction of curd-forming period in shrikhand manufacturing process. *Lait* 65: 55-64.

Patel, R.S. and Chakraborty, B.K. (1988) *Shrikhand* – a Review. *Indian Journal of Dairy Sciences*. 41: 109-115.

Patidar, S.K. and Prajapati, J.B. (1998) Standardization and evaluation of lassi prepared using *Lactobacillus acidophilus* and *Streptococcus thermophilus*. *Journal of Food Science and Technology* 35 (5): 428-431.

Pazakova, J., Turek, P. and Laciakova, A. (1997) The survival of *Staphylococcus aureus* during the fermentation and storage of yoghurt. *Journal of Applied Microbiology*. 82: 659-662.

Premi, L., Sandine, W.E. and Elliker, P.R. (1972) Lactose- hydrolyzing enzymes of *Lactobacillus* species. *Applied Microbiology* 24: 51-57.

Rao, D.R., Pulusani, S.R. and Chawan, C.B. (1985) Role of fermented milk products in milk intolerance and other clinical conditions. *Advances in Nutrition Research* 7: 203.

Ramakrishnan, C.V. (1977) The use of fermented foods in India. . In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Ramakrishnan, C.V. (1979) Studies on Indian fermented foods. *Baroda Journal of Nutrition* 6: 1-57.

Ranganathan, B., Nambrudipad, V.K.N., Dunani, A.T. and Iya, K.K. (1964) *Bacterial flora of milk and milk products of India.* Indian Council of Agricultural Research, Series No. 37.

Rangappa, K.S. and Achaya, K.T. (1974) *Indian Dairy Products.* Asia Publishing House, New Delhi.

Rasic, J.L., Stojsavljevic, T. and Curcic, R. (1971) A study of amino acids of yoghurt II Amino Acids content and biological value of proteins of different kinds of yoghurt. *Milchwiss* 26: 219-224.

Rasic, J.L. and Kurman, J.A. (1978) *Yoghurt - Scientific Grounds, Technology, Manufacture and Preparations.* Technical Dairy Publishing House, Copenhagen.

Rathi, S.D., Deshmukh, D.K., Ingle, U.M., Syed, H.M. (1990) Studies on the physico-chemical properties of freeze-dried dahi. *Indian Journal of Dairy Science* 43: 249-251.

Ray, H.P. and Srinivasan, R.A. (1972) Use of microorganisms for production of indigenous fermented milk products (sweetened dahi). *Journal of Food Science and Technology* 9: 62-65.

Roberts, T.A., Baird-Parker, A.C. and Tompkin, R.B. (1996) In: *Microorganisms in Foods, 5, Microbiological Specifications of Food Pathogens*, Blackie Academic and Professional, London.

Robinson, R.K. (1990) *Dairy Microbiology*, 2nd Edition. Chapman and Hall, London.

Robinson, R.K. (1999) Fermented milks-yoghurt. In: *Encyclopedia of Food Microbiology*, vol. I (Eds: Robinson, R.K., Bhatt, C.A. and Patel, P.D.), pp. 1-7. Academic Press, London.

Robinson, R.K. and Tamme, A.Y. (1981) Microbiology of fermented milks. In: *Dairy Microbiology* vol 2 The Microbiology of Milk Products (Ed. Robinson, R.K.), pp. 245-278. Applied Science, London.

Rosenberg, M. (1984) Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. *FEMS Microbiology Letters* 22: 289-295.

Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters* 9: 29-33.

Salminen, S., Isolauri, E., Salminen, E., (1996). Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains for future challenges. *Antonie van Leeuwenhoek.* 70: 347-358.

Sarkar, S. and Misra, A.K.(1997) Commercial production of shrikhand. *Indian Food Industry* 16(1,2): 48-52.

Sarkar, S., Kuila, R.K. and Misra, A.K. (1996) Anti-bacterial properties of *shrikhand. Indian Journal of Dairy Sciences* 49: 270-276.

Schillinger, U. and Lücke, F.K. (1987) Identification of lactobacilli from meat and meat products. *Food Microbiology* 4: 199-208.

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

Schillinger, U., Stiles, M.E. and Holzapfel, W.H. (1993) Bacteriocin production by *Carnobacterium piscicola* LV 61. *International Journal of Food Microbiology* 20: 131-147.

Schillinger, U., Geisen, R. and Holzapfel, W.H. (1996) Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science and Technology* 7: 158-164.

Schillinger, U., Becker, B., Vignolo, G. and Holzapfel, W.H. (2001) Efficacy of nisin in combination with protective cultures against *Listeria monocytogenes* Scott A in tofu. *International Journal of Food Microbiology* 71: 159-168.

Schneller, R., Good, P. and Jenny, M. (1997) Influence of pasteurized milk, raw milk and different ripening cultures on biogenic amine concentrations in semi-soft cheeses during ripening. *Food Research Technology* 204: 265-272.

Shah, N.P. and Jelen, P. (1990) Survival of lactic acid bacteria and their lactases under acidic conditions. *Journal of Food Science* 55: 506-509.

Shah, N.P. (1994) *Lactobacillus acidophilus* and lactose intolerance: A review. *ASEAN Food Journal* 9: 47-54.

Shah, N.P. (2000) Probiotic bacteria: Selective enumeration and survival in dairy foods. *Journal of Dairy Science* 83: 894-907.

Shah, N.P. (2001) Functional foods from probiotics and prebiotics. *Food Technology* 55 (11): 46-53.

Shuaib, A.C.A. and Azmey, M.S.M. (1977) Fermented foods of Sri Lanka. In: *The Proceeding of the Symposium on Indigenous Fermented Foods,* November 21-27, 1977, GIAM-V, Bangkok.

Silla-Santos, M.H. (2001) Toxic nitrogen compounds produced during processing: biogenic amines, ethyl carbamides, nitrosamines. In: *Fermentation and Food Safety* (Eds: Adams, M.R. and Nout, M.J.R.), pp. 119-140. Aspen Publishers, Inc. Gaithersburg, Md., USA.

Simon-Sarkadi, L. and Holzapfel, W.H. (1995) Biogenic amines and microbial quality of sprouts. *Zeitschrift für Lebensmittel-Untersuchung und-Forschung* 200: 261-265.

Slepecky, R.A. and Hemphill, H.E. (1992) The genus *Bacillus*-non-medical. In: *The Prokaryotes, A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Application,* 2nd Edition., Vol 2., (Eds: Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H.), pp. 1663-1696. Springer-Verlag, New York.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (1986) *Bergey's Manual of Systematic Bacteriology*, Vol. 2. Williams & Wilkins, Baltimore, MD.

Somogyi, M. (1945) A new reagent for the determination of sugars. *Journal of Biological Chemistry* 160: 61-62.

Standal, B.R. (1963) Nutritional value of proteins of oriental soybean foods. *Journal of Nutrition* 81: 279-285.

Sperber, W.H. (1983) Influence of water activity on foodborne bacteria: a review. *Journal of Food Protection* 46: 142-150.

Steinkraus, K.H. (1983) *Handbook of Indigenous Fermented Food*, Vol. 9. Marcel Dekker, Inc., New York.

Steinkraus, K.H. (1994) Nutritional significance of fermented foods. *Food Research International* 27: 259-267.

Stiles, M.E. and Holzapfel, W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology* 36: 1-29.

Stolp, H. and Gadkari, D. (1981) Nonpathogenic members of the genus *Pseudomonas* In: *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria,* vol. 1, (Eds: Starr, M.P., Stolp, H.G., Trüper, A., Balows and Schlegel, H.G.), pp. 719-741. Springer-Verlag, New York.

Stratton, J.E., Hutkins, R.W. and Taylor, S.L. (1991) Biogenic amines in cheese and other fermented foods. A review. *Journal of Food Protection* 54: 460-470.

Sutherland, J.P., Aherne, A. and Beaumont, A.L. (1996) Preparation and validation of a growth model for *Bacillus cereus*: the effects of temperature, pH, sodium chloride and carbon dioxide. *International Journal of Food Microbiology* 30: 359-372.

Tamime, A.Y. (1981) Microbiology of starter cultures. In: *Dairy Microbiology* vol 2 The Microbiology of Milk Products, (Ed: Robinson, R.K.), pp. 113-156, Applied Science, London.

Tamime, A.Y. and Robinson, R.K. (1985) *Yogurt – Science and Technology*. Pergamon Press, Oxford.

Tamang, J.P. (1998) Role of microorganisms in traditional fermented foods. *Indian Food Industry* 17 (3): 162-167.

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

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

Tamang, J.P. and Holzapfel, W.H. (1999) Microfloras of fermented foods. In: *Encyclopedia of Food Microbiology*, Vol. 2 (Eds: Robinson, R.K., Bhatt, C.A. and Patel, P.D.) pp. 249-252. Academic Press, London.

Tamang, J.P., Sarkar. P.K. and Hesseltine, C.W. (1988) Traditional fermented foods and beverages of Darjeeling and Sikkim - a review. *Journal of Science of Food and Agriculture* 44: 375-385.

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

Taylor, S.L. (1986) Histamine food poisoning: toxicology and clinical aspects. *CRC Critical Review in Toxicology* 17:s91-128.

Ten Brink, B., Damink, C., Joosten, H.J. and Huis in't Veld, J. (1990) Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology* 11: 73-84.

Thornley, M.J. (1960) The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *Journal of Applied Bacteriology* 23: 37-52.

Uhlman, L., Schillinger, U., Rupnow, J.R. and Holzapfel, W.H. (1992) Identification and characterization of two bacteriocin-producing strains of *Lactococcus lactis* isolated from vegetables. *International Journal of Food Microbiology* 16:141-151.

United States Department of Agriculture (1978) *Cheese Varieties and Descriptions*. USDA, Washington, D.C.

Vale, S.R. and Gloria M.B.A. (1997) Determination of biogenic amine in cheese. *Journal of AOAC International* 80: 1006-1012.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G. and Zehnder, A.J.B. (1987). The role of bacterial cell wall hydrophobicity in adhesion. *Applied and Environmental Microbiology* 53 (8):1893-1897.

Vedamuthu, E.R. (1982) Fermented milks. In: *Economic Microbiology*, Vol 7. Fermented Foods, (Ed: Rose, A.H.), Academic Press, London.

Vijayalakshmi, R. and Murugesan, T. (2001) Shelf life and microbiological quality of selected dairy products. *Journal of Food Science and Technology*. 38(4): 385-386.

Welthagen, J.J. and Viljoen, B.C. (1998) Yeast profile in Gouda cheese during processing and ripening. *International Food Microbiology* 41(3): 184-359.

Westall, S. and Filtenborg, O. (1998) Yeast occurrence in Danish Feta cheese. *Food Microbiology* 15(2): 215-222.

Wickerham, L.J. (1951) Taxonomy of yeasts. *Technical Bulletin 1029*, United States Department of Agriculture, Washington, D.C.

Wood, B.J.B. and Holzapfel. W.H. (1995) *The Lactic Acid Bacteria,* Vol. 2: *The Genera of Lactic Acid Bacteria.* Blackie Academic & Professional, An Imprint of Chapman and Hall, London.

Yarrow, D. (1998) Methods for the isolation, maintenance and identification of yeasts. In: *The Yeast, a Taxonomic Study*, 4th Edition, (Eds: Kurtzman, C.P. and Fell, J.W.), pp. 77-105. Elsevier Science, Amsterdam.

Yonzan, H. and Tamang, J.P. (1998) Consumption pattern of traditional fermented foods in the Sikkim Himalaya. *Journal of Hill Research* 11(1): 112-115.

Yoshinaga, D.H. and Frank, H.A. (1982) Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwomus pelamis*). *Applied and Environmental Microbiology* 44: 447-452.