Studies on *Selroti*, a Traditional Fermented Rice Product of the Sikkim Himalaya: Microbiological and Biochemical Aspects

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May 30, 2007

This is to certify that the work presented in the thesis entitled: **Studies on *Selroti*, a Traditional Fermented Rice Product of the Sikkim Himalaya: Microbiological and Biochemical Aspects** has been carried out by **Ms. Hannah Yonzan** 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 Ms. Yonzan 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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CONTENTS

INTRODUCTION

A fermented food is defined as an edible product prepared from raw or cooked materials of plant or animal origins by microorganisms either spontaneously or by adding mixed or pure culture (Hansen, 2002; Hulse, 2004). The essential objective of food fermentation is to carry over supplies from the time of plenty to those of deficit (Kwon, 1994; Tamang, 2000). Bacteria, mostly lactic acid bacteria, yeasts and filamentous moulds constitute the microbiota of fermented foods and beverages, which are present in or on the ingredients, utensils, environment, and are selected through adaptation to the substrates (Hesseltine, 1983, Steinkraus, 1997). Functional microorganisms play important roles in bringing some remarkable physico-chemical changes in the substrates during fermentation such as enrichment of the human dietary with acceptable flavour, texture and aroma, biopreservation of food, bioenrichment of substrates with vitamins, protein and essential amino acids, improved digestibility, detoxification of undesirable and anti-nutritive compounds, enhancement of bioactive compounds and stimulation of probiotic functions (Campbell-Platt, 1994; Holzapfel and Schillinger, 2001; Shah, 2001; Ammor *et al.*, 2007).

In India, mostly due to wide variation in agro-climatic conditions and diverse dietary cultures of the multi-ethnic groups of people, microorganisms associated with more familiar and less familiar traditional fermented foods and beverages may contribute significant microbial resources (Batra, 1986; Soni and Sandhu, 1990; Tamang, 1998). Eleven genera of lactic acid bacteria with several species have been reported from fermented foods and beverages which include *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997; Axelsson, 1998; Carr *et al.*, 2002). Lactic acid bacteria have been traditionally used as starter cultures in the production of fermented foods which contribute to the organoleptic, rheological, nutritional properties of the products, resist spoilage and food toxins by their antimicrobial properties (Nout and Ngoddy, 1997; Salminen and Wright, 1998; Leroy and de Vuyst, 2004). Functional yeasts genera associated with fermented foods and beverages are mostly *Brettanomyces* (its perfect stage, *Dekkera*), *Candida, Cryptococcus, Debaryomyces, Galactomyces, Geotrichum, Hansenula, Hanseniaspora* (its

asexual counterpart *Kloeckera*), *Hyphopichia, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Saccharomyces, Saccharomycodes, Saccharomycopsis, Schizosaccharomyces, Torulopsis, Trichosporon, Yarrowia* and *Zygosaccharomyces* (Kurtzman and Fell, 1998; Pretorius, 2000; Nout and Aidoo, 2002; Tsuyoshi *et al.*, 2005). Yeasts play vital roles in production of many traditional fermented foods and beverages across the world (Aidoo *et al.*, 2006) mostly enhancing sensory quality of the foods (Boekhout and Robert, 2003).

In developing countries, a global interest in rice and its fermented product is increasing due to their calorigenic value, unique quality characteristics and high acceptability (Steinkraus, 1994; Efiuvwevwere and Ezeama, 1996). Traditional cereal-based fermented foods are frequently used as complementary foods for infants and young children in Africa (Tou *et al.*, 2006). In most of the countries, rice is fermented either by using mixed-culture(s) into alcoholic beverages, or by natural fermentation into leavened batter formed dough breads which are usually baked or steamed (Sugihara 1985; Yokotsuka, 1991). Some of the common cereal-based fermented foods across the world have been

extensively studied. The well-documented Indian cereal-based non-alcoholic fermented foods are *idli* (Mukherjee *et al.* 1965; Steinkraus *et al.* 1967; Soni and Sandhu, 1991), *dosa* (Soni *et al.*, 1985, 1986), *jalebies* (Batra, 1986), *rabadi* (Gupta *et al.*, 1992a); and Indian cereal-based alcoholic beverages are *bhaati jaanr* (Tamang and Thapa, 2006) and *kodo ko jaanr* (Thapa and Tamang, 2004, 2006). Several cereal-based fermented foods of Africa and other parts of the countries have been well investigated which include *masa* of South Africa (Efiuvwevwere and Ezeama, 1996), *puto* of South-East Asia (Kelly *et al.*, 1995), *mawé* or *ogi* of Benin (Onyekwere *et al.*, 1989; Hounhouigan *et al.*, 1993a), *kisra* of Sudan (Mohammed *et al.*, 1991), *sourdough* of America and Europe (Brandt, 2007), *ben-saalga* of Burkino Faso (Tou *et al.*, 2007), *kenkey* of Ghana (Nche *et al.*, 1994; Nout *et al.*, 1996), *togwa* of Tanzania (Mugula *et al.*, 2003a), *tarhana* of Turkey (Erbas *et al.*, 2006), etc.

Sikkim is a mountainous state of India with an area of 7096 sq. km and has four administrative districts - North, East, South and West. Darjeeling is a picturesque hill district of West Bengal state with an area of 3075 sq. km. Three sub-divisions of Darjeeling district viz. Darjeeling, Kalimpong and

Kurseong are hilly, commonly known as the Darjeeling hills or officially as Darjeeling Gorkha Hill Council. Topographically, culturally and ethnically the Darjeeling hills and Sikkim have similarities. The Darjeeling hills and Sikkim are located in the Eastern Himalayan regions and are ecologically grouped as the Sikkim Himalaya. Three major ethnic communities of the Sikkim Himalaya are the Nepalis, the Bhutias and the Lepchas.

Traditional fermented foods and beverages constitute an important part of the local diet in the Sikkim Himalaya (Tamang, 2005a). Traditional foods in the Darjeeling hills and Sikkim are closely associated with socio-economical development, ethnic importance, religious and cultural practices, and has been evolved as the result of tradition and empirical experiences of generations over a period of time (Tamang *et al.*, 1988). Daily per capita consumption of fermented foods and beverages in the Sikkim Himalaya is 87.6 g representing 10 % of the total daily food consumed in the local diet (Yonzan and Tamang, 1998). Depending on the altitudinal variation, various types of cereals crops such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), finger millet (*Eleusine coracana* Gaertn.), wheat (*Triticum aestivum* L.),

barley (*Hordeum vulgare* L.) and buckwheat (*Fagopyrum esculentum* Moench.) are cultivated and eaten as staple food items in the Sikkim Himalaya. Annual production of cereals in Sikkim during 2004-05 was 94.73 tonnes with annual yield as 1439.68 kg/hectare (Annual Progress Report, 2005).

Varieties of fermented foods and beverages are traditionally prepared and consumed, and even marketed locally in North East India (Tamang, 2001). Some of the ethnic fermented foods and beverages of North East India were extensively studied (Tamang, 2005b), however, several less familiar fermented foods are yet to be investigated scientifically. *Selroti* is a popular fermented rice-based food which is ring-shaped, spongy, pretzel-like, deep fried and commonly consumed in Sikkim and the Darjeeling hills in India, Nepal and Bhutan. To the best of our knowledge, no microbiological and biochemical aspects of *Selroti* of the Sikkim Himalaya have been studied. The present dissertation will focus on the microbiological and biochemical aspects of *Selroti* batter fermentation, and also on optimization of the traditional processing method using starter cultures in order to minimize the production time, maximize the substrate utilization and improve the quality of the product.

Objectives

The present thesis was aimed to document the indigenous knowledge of traditional processing of *Selroti* batter preparation in the Sikkim Himalaya; and to isolate, characterise and identify the predominant microorganisms in fermented batters collected from different sources. The objective of the thesis was also to study the effect of seasonal variation on microbial load, technological properties of the identified strains of functional lactic acid bacteria and yeasts such as acidifying capacity, enzymatic profiles, and their antimicrobial activities against pathogenic bacteria. Microbial population dynamics of lactic acid bacteria and yeasts during *in situ* fermentation were also examined. The strains of lactic acid bacteria and yeasts were selected and tested for production of fermented batters under laboratory conditions, and subjected to sensory evaluation. Proximate composition of raw materials and *Selroti* batters were also calculated.

REVIEW OF LITERATURE

There are two major types of cereal-based fermented products: (1) Alcoholic food beverages and (2) Non-alcoholic foods. Majority of alcoholic food beverages are prepared using mixed cultures with co-existence of filamentous moulds, yeast and lactic acid bacteria in the form of flattened ball, dry-cake like starter, mostly in Asia (Nout and Aidoo, 2002; Dung *et al.*, 2006). Consumption of rice as a staple food in Asia has resulted in a traditional cereal fermentation with moulds and yeasts (Haard *et al.*, 1999). Varieties of traditional non-alcoholic cereal-based fermented foods are mostly prepared and consumed in Africa as staple foods (Oyewole, 1997; Nout, 2001; Blandino *et al.*, 2003). Cereal-based fermented gruels are generally used as naturally fortified weaning foods for young children in Africa (Nout, 1991; Efiuvwevwere and Akona, 1995; Tou *et al.*, 2007).

Table A shows a comprehensive list of more familiar cereal-based fermented products of the world. Review of available literature on some common cereal-based fermented foods across the world has been mentioned below.

Table A. More familiar cereal-based fermented products of the world

Fermented Product	Substrate	Nature and use	Country	Reference
Ben-saalga	Pearl millet	Fermented gruel; complementary weaning food	Burkina Faso	Tou *et al.* (2007)
Bhaati jaanr	Rice	Alcoholic, sweet-acidic paste; staple food	India, Nepal, Bhutan	Tamang and Thapa (2006)
Dosa	Rice and black gram	Shallow-fried, thin crisp pancake; staple food	India, Sri Lanka	Soni and Sandhu (1990)
Enjera	Tef flour, wheat	Sour, leavened, pancake-like bread; staple food	Ethiopia	Gashe (1985)
Idli	Rice and black gram	Soft, moist and spongy, sour flavour cake; breakfast food	India, Sri Lanka	Reddy *et al.* (1981)
Jalebi	Wheat flour	Crispy, deep fried pretzels; confection snack	India, Nepal	Batra (1986)
Kenkey	Maize	Acidic, maize dumpling, steamed; staple food	Ghana	Halm *et al.* (1993)
Kisra	Sorghum	Thin pancake bread; staple food	Sudan	Elkhalifa (2000)
Kodo ko jaanr	Finger millet	Mild-alcoholic, acidic beverage	India, Nepal, Bhutan	Thapa and Tamang (2004)
Lao-Chao	Rice	Sweet taste with fruity aroma; dessert	China	Wang and Hesseltine (1970)

Fermented Product	Substrate	Nature and use	Country	Reference
Mahewu (Magou)	Maize	Sour, non-alcoholic beverage; drink	South Africa	Gadaga *et al.* (1999)
Masa	Rice/millet or sorghum	Shallow fried millet or sorghum flour cake; snack food	Nigeria	Efiuvwevwere and Ezeama (1996)
Mawè	Maize	Sour-dough, porridge or steamed bread; staple food	Togo and Benin	Hounhouigan *et al.* (1991)
Ogi	Maize, sorghum, millet	Starchy-cake, porridge; staple food	Nigeria	Onyekwere *et al.* (1989)
Puto	Rice	Rice cake; breakfast and snack food	Philippine, China	Kelly, *et al.* (1995)
Rabadi	Barley, maize, wheat, pearl millet	Cereal flour with buttermilk; staple food	India	Gupta *et al.* (1992a)
Sourdough bread	Rye, wheat or mixed flour	Acidic-tasting aerated bread, staple food	Europe and South America	Brandt (2007)
Tapé	Rice or cassava	Sweet-sour paste with an alcoholic flavour; staple food	Indonesia	Ko (1982)
Tarhana	Wheat, milk products, vegetables	Crushed wheat boiled in milk, stored as dry biscuit; consumed as soup	Turkey	Erbas *et al.* (2006)
Togwa	Sorghum, millet, maize, cassava	Food beverage; drink	Tanzania	Mugula *et al.* (2003a)

Ben-saalga

Ben-saalga is a popular fermented gruel produced from pearl millet in Burkina Faso, which is frequently used as complementary foods for infants and young children in Africa (Tou *et al.*, 2006). The processing steps for this food include: washing (optional), soaking of grains (first fermentation step), grinding and sieving of the wet floor, settling (second fermentation step), and cooking (Blandino *et al.*, 2003). These processes were chosen according to their potential ability to favour partial starch hydrolysis (pre-cooking and addition of malt) and also to promote rapid acidification by lactic acid fermentation (back-slopping) to improve hygienic conditions (Nout *et al.*, 1989). The soaking step was mainly characterized by alcoholic fermentation whereas lactic acid fermentation occurred during the settling step (Tou *et al.*, 2006). Glucose and fructose were the main substrates observed for lactic acid fermentation during the settling step; however unbalanced fermentation led to the hypothesis that starch hydrolysis products may also serve as substrates for lactic acid fermentation (Tou *et al.*, 2006).

In the combining cooking and addition of malt process combination, the start of fermentation was considerably

delayed due to the marked reduction in natural microflora during cooking (Tou *et al.*, 2007). Some species of LAB isolated from *ben-saalga* produced bacteriocin (Omar *et al.*, 2006). The dry matter content of *ben-saalga* is very low (Guyot *et al.*, 2003).

Bhaati jaanr

Bhaati jaanr is an inexpensive high calorie mild-alcoholic beverage prepared from the steamed glutinous rice, consumed as a staple food beverage in the Eastern Himalayan regions of Nepal, India and Bhutan (Thapa, 2001). It was revealed that *Saccharomycopsis fibuligera* and *Rhizopus* spp. play the important roles in saccharification process of rice in *bhaati jaanr* fermentation (Tamang and Thapa, 2006).

Dosa

Dosa is a light, shallow-fried, thin crisp pancake, made from finely grounded rice and dehulled black gram in South India (Steinkraus, 1983). *Dosa* batter is very similar to *idli* batter, except that both the rice and black gram are finely grounded. Bacteria alone or in combination with yeasts were

found to be responsible for the fermentation of *dosa* and ordinarily the microorganisms developed during the initial soak and fermentation are sufficient to bring about the fermentation (Soni *et al.*, 1986). Overall increase in batter volume, microbial load, total nitrogen, soluble proteins, reducing sugar and decrease of pH has been noted after 30 h fermentation of *dosa* (Soni *et al*, 1985). The combination of *Saccharomyces cerevisae* and natural bacterial flora was found to be the best microbial factor for standardizing the dosa fermentation (Soni and Sandhu, 1989a).

Enjera

Enjera (Injera) is thin soft bread, with numerous eyes, or gas holes, baked in Ethiopia from the cereal tef (*Eragrotis tef*) and eaten at nearly every meal with meat, vegetable or legume stew, with each person eating two or three per day (Gashe, 1985). Stewart and Getachew (1962) isolated fungi including *Pullaria, Aspergillus, Penicillium, Rhodotorula, Hermodendrum, Candida guilliermondii* and a number of unidentified bacteria from samples of *enjera* batter. Gashe *et al.* (1982) reported the initial fermentation was carried out by gram-negative aerogenic rods such as *Enterobacter, Hafnia,*

Citrobacter, Klebsiella, Escherichia and *Proteus. Streptococcus, Leuconostoc* and *Lactobacillus* develop during fermentation lowering the pH below 4.0 (Gashe *et al.*, 1982). *Leuconostoc mesenteroides* has been reported from *enjera* (Oyewole, 1997). Yeasts appeared in all stages of *enjera* fermentation but disappeared later due to decrease in pH of the product (Gashe, 1985).

Idli

Idli is an acid-leavened and steamed cake made by bacterial fermentation of a thick batter made from coarsely ground rice and dehulled black gram (Veer *et al,*, 1967). *Idli* cakes are soft, moist and spongy, sour flavour, and are eaten as breakfast in South India. *Idli* makes an important contribution to the diet as a source of protein, calories and vitamins B-complex, compared to the raw unfermented ingredients (Reddy *et al.*, 1981). Though, lactic acid bacteria mostly pediococci, lactobacilli and enterococci, are predominant microflora in *idli* (Thyagaraja *et al.*, 1992), yeasts have also been reported in *idli* mostly *Saccharomyces cerevisiae, Debaryomyces hansenii, Hansenula anomala, Torulopsis candida* and *Trichosporon beigelii.*from (Soni and

Sandhu, 1989b, 1990, 1991). Addition of yeasts in *idli* fermentation contributes to leavening and flavour development and results in enhanced contents of thiamine and riboflavin (Soni and Sandhu, 1989a). However, the presence of yeasts can interfere with acidification of the batter since the yeasts utilize a portion of the fermentable sugars that otherwise would be used for production of lactic acid supplementation of the batter ingredients (Venkatasubbaiah *et al.*, 1985; Steinkraus, 1996). The riboflavin content decreased due to the presence of *Streptococcus faecalis* (Veer *et al.*, 1967). The predominant microorganism responsible for souring as well as for gas production was found to be *Leuconostoc mesenteroides* (Mukherjee *et al.*, 1965). *Idli* batter obtained from the hotels and restaurants showed yeast participation in leavening process in addition to *Leuconostoc mesenteroides* (Venkatasubbaiah *et al.*, 1984). During *idli* fermentation the volume, total acid and soluble solids increased whereas the pH, total nitrogen and soluble nitrogen decreased (Steinkraus *et al*, 1967).

Jalebi

Jalebi is a traditional Indian crispy sweet, deep fried pretzels made from wheat flour and eaten as confection snack food (Chitale, 2000). The batter is prepared by mixing wheat flour with curd and then fried in oil and the fried *jalebi* are taken out from the pan and soak in sugar syrup immediately for 4-5 hours (Batra, 1986). LAB *Lb. fermentum, Lb. buchneri, Lb. bulgaricus, Streptococcus lactis, S. faecalis, S. thermophilus,* and yeasts *Saccharomyces bayanus, S. cerevisiae* and *Hansenula anomala* have been reported from *jalebi* (Batra and Millner, 1974; Soni and Sandhu, 1990). The pH decreases from 4.4 to 3.3 and there is a 9 % volume increase in the batter while both amino nitrogen and free sugar decreases during fermentation (Ramakrishnan, 1979).

Kenkey

Kenkey is a popular fermented maize product of Ghana which is acidic, dumpling like, wrapped in leaves or maize cob sheaths, and usually steamed, eaten as a staple food with soup (Amoa and Muller, 1976; Halm *et al.*, 1993). *Leuconostoc mesenteroides, Pediococcus acidilactici* and *Lactobacillus fermentum,* and yeast *Geotrichum candidum*

were reported from *kenkey* (Christian, 1970; Halm *et al.*, 1993). *Kenkey* dough containing LAB was used successfully to accelerate the fermentation time (Nche *et al.*, 1994). Decrease in endogenous activity of protease and carbohydrates were recorded when soaking temperature was raised to 60° C (Nche *et al.*, 1996). The lysine availability in *kenkey* increased during soaking, cooking and fermentation of maize and maize-cowpea doughs (Nche *et al.*, 1995). The incorporation of aflata into the dumpling prior to fermentation using starter dough, shortened the fermentation time in *kenkey* (Nout *et al.*, 1996). LAB are mainly responsible for acidification in *kenkey* (Nche *et al.*, 1995).

Kisra

Kisra is a thin pancake like fermented bread made from whole sorghum flour and it is the staple food of Sudan served regularly for at least in one of the three meals of the day (Elkhalifa, 2000). The general procedures practiced by most of the housewives are as follows: a thick paste (*ajeen*) is prepared by mixing approximately 60 parts of flour and 40 parts of water. The *ajeen* is left to stand fermenting for approximately 12-24 hour in an earthenware container, by

which time it develops a sour taste. The hot plate on the fire is rubbed with a damp oily cloth before spreading the batter. Now the women swiftly spread the batter into a very thin layer using a 4-5 inch strip of a dry piece of palm leaf in very quick and smooth sideways movements. In less than 1 minute, the *kisra* is done and ready to peel off (Dirar, 1992).

Kisra made from sorghum has 11.3 %, 14.1 % and 12.6 % of protein content respectively (Elkhalifa, 2000). The predominant microorganisms in *kisra* are *Lactobacillus* sp., *Acetobacter* sp. and *Saccharomyces cerevisiae* (Steinkraus, 1996). Mohammed *et al.* (1991) reported *Pediococcus pentosaceus, Lb. confusus, Lb. brevis, Erwinia ananas, Klebsiella pneumoniae, Enterobacter cloacae, Candida intermedia, Debaryomyces hansenii, Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Rhizopus* sp.

Kodo ko jaanr

Kodo ko jaanr is the most common fermented mild-alcoholic beverage prepared from dry seeds of finger millet (*Eleusine coracana*), by using a mixed cultures locally called *marcha* in Sikkim, the Darjeeling hills and North East hills in India, Nepal, Bhutan and Tibet in China (Tamang, 2005a).

Kodo ko jaanr contributes to the mineral intake in daily diet of the local people (Thapa, 2001). Because of high calorie, ailing persons and post-natal women consume the extract of *kodo ko jaanr* to regain the strength. Population of yeasts and LAB was detected at the level of 10^7 cfu/g and 10^5 cfu/g, respectively. Yeasts consisted of *P. anomala, S. cerevisiae, C. glabrata, Saccharomycopsis fibuligera,* and LAB consisted of *P. pentosaceus* and *Lb. bifermentans* in *kodo ko jaanr* samples. Microorganisms necessary for fermentation of finger millets into *kodo ko jaanr* are supplemented by *marcha,* a traditional mixed starter culture (Thapa and Tamang, 2004).

Thapa and Tamang (2006) studied the microbiological and physico-chemical changes during fermentation of *kodo ko jaanr* and found that *Saccharomycopsis fibuligera* and *Rhizopus* spp. play the dominant role in saccharification process of *kodo ko jaanr* fermentation breaking starch of substrates into glucose for ethanol production.

Lao-chao

Lao-chao is a popular Chinese fermented food with sweet taste and fruity aroma, made from rice by using *chiu-yueh* or *peh-yueh* as starters (Wang and Hesseltine, 1970). It

is served as a dessert and is also a traditional diet for new mothers who believe that it helps them regain their strength. Wei and Jong (1983) reported the presence of *Rhizopus*, *Amylomyces*, *Torulopsis*, and *Hansenula* in *lao-chao*. Pure culture fermentation method of *lao-chao* was developed by Wang and Hesseltine (1970) and showed that good fermented rice was made when a mold, *Rhizopus chinensis* NRRL 3671, and yeast, *Endomycopsis* (*Saccharomycopsis*) sp. NRRL Y7067, used as inocula instead of a commercial starter.

Mahewu (Magou)

Mahewu (Magou) is a traditional, sour, non-alcoholic maize beverage popular among the Bantu people of South Africa and Zimbabwe (Steinkraus, 1996; Gadaga *et al.*, 1999). *Mahewu* contains about 8 to 10 % solids and has a pH of about 3.5 with a titratable acidity of 0.4 to 0.5 % (lactic acid) (Schweigart and de Wit, 1960; Schweigart *et al.*, 1960). LAB associated with *mahewu* are *Lb. bulgaricus Lb. delbrueckii, Lb. acidophilus* and *S. lactis* (van der Merwe *et al.*, 1964, 1965).

Masa

Masa is a popular shallow fried fermented product which is obtained through fermentation of rice, millet or sorghum and is widely produced in Northern Nigeria (Efiuvwevwere, and Ezeama, 1996). The product or a comparable product is known as *kisra* in Sudan (Dirar, 1993). Whereas these products generally have a sour-sweet quality due to a wide range of microorganisms, some variations exist in the fermentation parameters such as fermentation time, formation paste or slurry (ratio of water to flour or grain), addition or exclusion of *Kanwa* and baking or frying temperatures (Steinkraus, 1983; Dirar, 1993). A wide range of microorganisms was isolated during the early stage of *masa* fermentation between 0-6 hours with *Bacillus* spp., *Lactobacillus* spp., *Saccharomyces* spp., *Enterobacter* spp., *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp. being the dominant organisms (Efiuvwevwere and Ezeama, 1996). As the fermentation progresses beyond 8 hours, *Enterobacter* spp., *Aspergillus* spp. and *Penicillium* spp. disappear while *Lactobacillus* spp., *Saccharomyces* spp. became dominant with few *Bacillus* spp. occurring (Efiuvwevwere and Ezeama, 1996).

Mawè

Mawè is a sour-dough fermented food prepared from dehulled maize of Togo and Benin (Hounhouigan *et al.*, 1991). *Lactobacillus* spp. constitute the majority of the strains of LAB involved in *mawè* fermentation and others were *P. pentosaceus, P. acidilactici* and *Leuc. mesenteroides* (Hounhouigan *et al.*, 1993b). The titratable acidity of home made and commercially produced *mawè* ranged from 1.2-1.4 % and pH (4.2), protein, fat, fibre, and ash of home produced *mawè* were found to be little higher than commercially produced *mawè* (Hounhouigan *et al.*, 1993c). Fermentation significantly increased the swelling and thickening capabilities of *mawè* (Hounhouigan *et al.*, 1993a). Aflatoxins and fumonisins that occur in maize, was studied through the traditional processing of naturally contaminated maize in *mawè*, where overall reduction of mycotoxin level was more significant during the fermentation (Fandohan *et al.*, 2005).

Ogi

Ogi is a traditional African fermented starch cake processed from maize, sorghum, and millet grains by soaking in water, followed by wet milling, sieving, further

fermentation, and decantation (Onyekwere, 1989). *Ogi* is also known as *akamu* by the Hausas of Nigeria (Akingbala *et al.*, 1987). The predominant microorganism involved in the fermentation of maize during *ogi* processing is *Lb. plantarum* (Banigo and Muller, 1972). Moulds associated with the surface microflora of the fermenting maize in *ogi* are *Cephalosporium, Rhizopus, Oospora, Cercospora, Fusarium, Aspergillus* and *Penicillium,* but these moulds are eliminated within 6 hour of steeping (Akinrele, 1970). Olukoya *et al.* (1994) developed an improved 'ogi' named 'Dogik' by using a starter culture of *Lactobacillus* with antimicrobial activities against some diarrhoeagenic bacteria, including *Salmonella, Shigella, Campylobacter, Aeromonas, Pleisiomonas, Escherichia coli, Yersinia enterolytica* and *Vibrio cholera.*

Puto

Puto is a fermented rice cake consumed as a breakfast and snack food in the Philippines and is generally served with grated coconut; it is closely related to Indian *idli* except that it contains no legume (Sanchez, 1996). *Leuconostoc mesenteroides* has been reported to be the predominant organisms in *puto* (Cooke *et al.*, 1987). However, Kelly *et al.*

(1995) reported other three species *Leuc. citreum, Leuc. pseudomesenteroides, Leuc. fallax* along with *Leuc. mesenteroides* subsp. *mesenteroides.* During *puto* fermentation, the yeasts and microaerophilic bacteria increased in number with time where the predominant organism was always *Leuc. mesenteroides,* followed by *S. faecalis* and then *S. cerevisiae* (Rosario, 1987). The prevalence of *Leuc. mesenteroides* in *puto* was 45 % to 89 % of the total microbial population and was responsible for most of the acid and gas production (Sanchez, 1996). It was found out that the yeast along with *Leuc. mesenteroides* played an important role in leavening the batter for *puto* (Tongananta and Orillo, 1996).

Rabadi

Rabadi is a fermented cereal-based food of North-West India (Gupta *et al.*, 1992a). It is prepared by mixing flour of wheat, barley, pearl millet or maize with buttermilk in an earthen or metallic vessel and then allowing the mixture to ferment in the open sun for 5-6 hour in the hot summer, and after fermentation, it is boiled, salted to taste and consumed (Gupta *et al.*, 1992a). *P. acidilactici* (3.6×10^5/g), *Bacillus* sp.

(1.1 x 10^6/g), and *Micrococcus* sp. (7.9 x 10^5/g) were reported from *rabadi* (Ramakrishnan, 1979). Phytic acid content decreased during *rabadi* fermentation (Mahajan and Chauhan, 1987; Gupta *et al.*, 1992a). *Rabadi* fermentation of barley flour-buttermilk at 30° C, 35° C and 40° C for 6 hour, 12 hour, 24 hour and 48 hour lowered pH, enhanced titratable acidity but did not change fat and total mineral (Gupta *et al*, 1992b).

Single as well as mixed culture fermentation of pearl millet with yeast (*S. cerevisiae* or *S. diastaticus*) and LAB (*Lb. brevis* or *Lb. fermentum*) was developed for utilization of pearl millet by fermentation (Khetarpaul and Chauhan, 1990a,b). Fermentation of pearl millet with pure cultures of yeast and lactobacilli has been found effective method for improving its nutritive value: increased bioavailability of minerals (Khetarpaul and Chauhan, 1989); improved starch and protein digestibility (Khetarpaul and Chauhan, 1990a); increased total soluble sugar, reducing and non-reducing sugar content with decrease in starch (Khetarpaul and Chauhan, 1990b); elimination of anti-nutrients (Khetarpaul and Chauhan, 1991a); and brought an improvement in biological utilization (Khetarpaul and Chauhan, 1991b).

***Sourdough* bread**

Sourdough bread is acidic-tasting aerated bread, made from rye, wheat or mixed flours of Europe and South America (Campbell-Platt, 1987). Sourdough fermentation is a mixture of flour, water and salt and if kept at room temperature, it will undergo a natural fermentation that involves the growth of indigenous yeasts and LAB (Brandt, 2007). The microbial ecology of these fermentations has been well studied, leading to the recognition that *Saccharomyces cerevisiae* is the principal yeast of most bread fermentations (Jenson, 1998). Gas (CO_2) production causes expansion and leavening of the dough, ultimately affecting bread texture, density and volume (Hammes *et al.*, 2005). Some of the CO_2 dissolved to form carbonic acid. Ethanol produced by the yeast, yeast enzymes that affect cereal proteins and carbonic acid, influence the rheological properties of the dough again impacting on final bread texture and structure (Hammes and Ganzle, 1998). The principle requirements of the strains are rapid production of CO_2 from maltose and glucose, and generation of good bread flavours (Decock and Cappelle, 2005). There is significant physiological, biochemical and genetic diversity in strains of *S. cerevisiae* used for bread production (Jenson, 1998). In

some cases, species other than *S. cerevisiae* could offer better functionality in some criteria such as *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* are used to prepare frozen dough breads (Alves-Araújo *et al.*, 2004).

Many breads, especially in European countries, are still produced by traditional processes where no commercial strains of baker's yeast (*S. cerevisiae*) are added (Hammes and Ganzle, 1998). Dough fermentation is conducted by indigenous yeasts and LAB, and the resultant products are generally called sourdough breads because they have higher contents of lactic acid and acetic acid due to the bacterial growth (Hammes *et al.*, 2005; de Vuyst and Neysens, 2005; Rehman *et al.*, 2006). San Francisco sourdough bread falls into this category (Romano *et al.*, 2006). Various studies have been conducted in recent years to understand the microbial ecology of this fermentation (Viljoen, 2006). While indigenous *S. cerevisiae* is still prominent in many of these fermentations, the presence of other yeasts are significant and these include *S. exiguus, Candida milleri, C. humilis, C. krusei* (*Issatchenkia orientalis*), *P. anomola, P. membranifaciens* and *Yarrowia lipolytica* (Paramithiotis *et al.*, 2000; Gullo *et al.*, 2002; Foschino *et al.*, 2004; Veinocchi *et*

al., 2004). These yeasts have evolved to grow in temperature with LAB of these doughs, including *Lb. sanfranciscensis* (unique to these eco-systems), *Lb. plantarum*, and various other species *of Lactobacillus, Pediococcus* and *Leuconostoc* (Hammes *et al.*, 2005). Commercial starter cultures of these yeast-bacterial combinations are now available (Decock and Cappelle, 2005). Growth of *Pichia burtonii* produces visible, white or chalky discoloration (Legan and Voysey, 1991).

Tapé

Tapé is a sweet-sour paste with an alcoholic flavour, prepared from glutinous rice or cassava or other cereals by using starter *ragi* in Indonesia (Ko, 1982). A combination of *Aspergillus rouxii* and *Saccharomycopsis burtonii* reduced total solids by 50 % in 192 hour at 30° C, which raised the crude protein in tapé ketan by 16.5 % (Cronk *et al.*, 1977). Suprianto *et al.* (1989) reported that *Saccharomycopsis fibuligera* produced mainly α-amylase, and *Rhizopus* sp. produced glucoamylase in tapé fermentation and found that liquefaction was not caused by amylases of *Saccharomycopsis* even though it produced high activity of ∝-amylase.

Tarhana

Tarhana is a traditional Turkish fermented food made with wheat, milk products, various vegetables and spices using yoghurt bacteria and baker's yeast as culture and is commonly consumed as soup (Erbas *et al.*, 2006). It is prepared in biscuit form for storage after boiling the crushed cereal in fermented milk and dried (Daglioglu, 2000). During fermentation, count of *Lactobacillus* spp. decreased from 6.4 to 5.4 Log cfu/g and count of total mesophilic aerobic bacteria decreased from 6.4 to 5.9 Log cfu/g with increase in acid content of *tarhana* dough (Erbas *et al.*, 2005a). Changes in water-soluble vitamins were investigated in *tarhana* dough during fermentation, and the contents of riboflavin, thiamine, niacin, vitamin B_6 and folic acid increased significantly (Cartel *et al.*, 2007). The free amino acids in *tarhana* increased significantly during fermentation and storage (Erbas *et al.*, 2005b).

Togwa

Togwa is a Tanzanian fermented food prepared from sorghum, millet, maize or cassava which is consumed as liquid drink by adults and children (Mugula *et al.*, 2003a). During the natural fermentation of *togwa*, the process was predominated by *Lb. plantarum, Lb. brevis, Lb. fermentum, Lb. cellobiosus, P. pentosaceous, Weissella confuse, Issatchenkia orientalis, S. cerevisiae, C. pelliculosa* and *C. tropicalis* (Mugula *et al.*, 2003a). Starter cultures of LAB and yeasts isolated from native *togwa* were tested singly or in combination for their ability to ferment maize-sorghum gruel to produce *togwa* (Mugula *et al.*, 2003b). All species of bacteria showed an ability to ferment the gruel by lowering the pH from 5.87 to 3.24 and increasing the titratable acidity from 0.08 % to 0.30 % in 24 hour, and the yeasts used singly, showed little activity within 12 hour, but lowered the pH to 3.57-4.81 and increased the acidity to 0.11-0.21 % in 24 hour (Mugula *et al.*, 2003b).

MATERIALS AND METHODS

MEDIA USED

(1). 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

(2). Ascospore Agar (M804, HiMedia)

(3). *Bacillus cereus* Agar Base (M833, HiMedia)

(4). Baird Parker Agar Base (M043, HiMedia)

(5). Egg Yolk Emulsion (FD045, HiMedia)

(6). Egg Yolk Tellurite Emulsion (FD046, HiMedia)

(7). Fermentation Basal Medium for yeasts (Wickerham, 1951)

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

(8). Lactate Configuration Medium (Tamang *et al.*, 2005)

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

(9). *Listeria* Identification Agar Base (M 1064, HiMedia)

(10). *Listeria* Selective Supplement (FD 061, HiMedia)

(11). Malt Extract Agar (M137, HiMedia)

(12). MRS Agar (M641, HiMedia)

(13). MRS Broth (M369, HiMedia)

(14). 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

(15). Nutrient Agar (MM012, HiMedia)

(16). Nutrient Broth (M002, HiMedia)

(17). Plate Count Agar (M091, HiMedia)

(18). Potato Dextrose Agar (M096, HiMedia)

(19). *Salmonella-Shigella* Agar (M108, HiMedia)

(20). Skim Milk Powder (RM1254, HiMedia)

(21). Sucrose Agar (Garvie, 1984)

Tryptone	10 g
Yeast extract	5.0 g
K_2HPO_4	5.0 g
Trisodium citrate	5.0 g
Sucrose	50 g
Agar	15 g
Distilled water	1000 ml

Ingredients were dissolved in distilled water by heating in a steamer and sterialised by autoclaving.

(22). Tryptone Soya Agar (M290, HiMedia)

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

(24). Yeast-Malt Extract (YM) Agar (M424, HiMedia)

(25). Yeast Malt Extract (YM) Broth (M425, HiMedia)

(26). Yeast Morphology Agar (M138, HiMedia)

(27). Yeast Nitrogen Base (M139, HiMedia)

REAGENTS

(1). Burke's Iodine Solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

(2). Gram's Crystal Violet (S012, HiMedia)

(3). Malachite Green (S020, HiMedia)

(4). Nessler's Reagent

Potassium iodide	50.0 g
Mercuric chloride (saturated)	35.0 ml
Distilled water (ammonia free)	25.0 ml
Potassium hydroxide (50 %)	400.0 ml

Potassium iodide was dissolved in 35 ml of distilled water followed by addition of saturated aqueous solution of mercuric chloride till the appearance of precipitate. Then, 400 ml of potassium hydroxide was added and made the final volume to 1000 ml by adding distilled water. The solution was left for a week; the supernatant was decanted and stored in capped amber bottle at 4° C.

(5). 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.

(6). Phenolphthalein (I009, HiMedia)

(7). Safranin (S027, HiMedia)

Reference Strains

Reference Strains	Origin	Purpose in this experiment
Bacillus cereus CCM 2010	CCM	Indicator strain for antimicrobial activity
Klebsiella pneumoniae subsp. *pneumoniae* BFE 147	BFE	Indicator strain for antimicrobial activity
Listeria monocytogenes DSM 20600	DSM	Indicator strain for antimicrobial activity
Staphylococcus aureus S1	FMR	Indicator strain for antimicrobial activity

Originally, these reference strains were obtained from DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), CCM (Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia), BFE (Institute of Hygiene and Toxicology, Karlsruhe, Germany), and FMR (Food Microbiology Laboratory, Sikkim Government College, Gangtok, India).

Listeria monocytogenes DSM 20600, *Staphylococcus aureus* S1, *Bacillus cereus* CCM 2010, and *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147 were propagated in standard nutrient agar. The cultures were maintained as frozen stocks at –20° C in 15 % glycerol.

METHODS

Survey

Survey was conducted in randomly selected 214 households in villages located in all four districts of Sikkim – North (54 households), West (48 households), South (42 households) and East (70 households), representing the major ethnic communities, namely the Nepalis, Bhutias and Lepchas. Information was collected on consumption of *Selroti* using questionnaire (Table B) during 2001-2005. Amount of *Selroti* consumed in every meal by each person was weighed directly by a portable weighing balance (Ishida, Germany) and daily per capita consumption was estimated as g/capita/day. Feeding frequency of *Selroti* by each family was also recorded in percentage. A data base was developed from the field data collection and analysed using statistical software (SPSS) at the Indian Statistical Institute, Kolkata. Indigenous knowledge on traditional method of *Selroti* batters practised by the ethnic people of the Sikkim Himalaya, their mode of consumption, socio-economy and ethnical importance of the product was also documented based on the information sought from the local people of the respective places.

Table B. Questionnaire on survey on *Selroti* consumption of Sikkim

- Name of the Informant:
- Ethnic Group:
- Name of (i) Village/Revenue Block:; (ii) Sub-division; (iii) District:
- Approximate number of population of the Village:
- Nearest Market:
- Kindly provide information on fermented product:

Fermented product	Local name	Raw material	Consume (daily/times per week/occasionally)	Whether prepare at home or market purchase/both
Selroti (deep-fried)				

- Traditional method of preparation:
- Mode of consumption:
- Socio-economy of the product:
- Ethnical importance:

Collection of samples

A total of 78 samples of home-made *Selroti* batters were collected directly from different villages located in the Darjeeling hills and Sikkim. Similarly, 36 market-samples of *Selroti* batters were collected from different restaurants, local food stalls and canteens located in Gangtok. Samples of *Selroti* batters prepared at our laboratory following the traditional method were also included. All samples were collected aseptically in sterile bottles and poly-bags, which

were kept in an ice-box container, and transported to the laboratory for analyses.

Microbiological analysis

Ten g of sample were homogenised with 90 ml of 0.85 % (w/v) sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min. A serial dilution (10^{-1} to 10^{-8}) in the same diluent was made. Lactic acid bacteria (LAB) were isolated on MRS agar (M641, HiMedia) plates supplemented with 1 % $CaCO_3$, and were incubated at 30° C under anaerobic condition kept in an Anaerobic Gas-Pack container (LE002, HiMedia) for 48-72 hour. Aerobic mesophilic counts (AMC) were determined in plate count agar (M091A, HiMedia) plates and incubated at 30° C for 48-72 hour. Moulds and yeasts were isolated on potato dextrose agar (M096, HiMedia) and yeast-malt extract (YM) agar (M424, HiMedia), supplemented with 10 IU/ml benzylpenicillin and 12 µg/ml streptomycin sulphate, respectively, and incubated aerobically at 28° C for 72 hour. Colonies were 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 and sub-culturing on corresponding broths/agar, followed by microscopic examinations. Microbiological data obtained were transformed into logarithms of the numbers of colony forming unit (cfu) per g of sample. Identified strains of LAB were preserved in MRS broth using 15 % (v/v) glycerol at –20° C.

Characterisation of Bacterial Isolates

Cell morphology

The smear of a 24 hour-old bacterial culture was made in a grease free slide, air-dried (not heated-fixed), stained for 30 seconds with safranin (S027, HiMedia), washed in water, air-dried (Harrigan, 1998) and observed under oil-immersion objective. Cell dimensions were measured with a standardized ocular micrometer.

Gram staining

Bacterial isolates were Gram-stained following the method of Bartholomew (1962). A suspension of a 24 hour-old bacterial culture on slant was prepared, and smear was made in a grease-free slide. Then heated-fixed, flooded by crystal violet stain for 1 minute, and washed for 5 second

with water. The smear was flooded with Burke's iodine solution, allowed to react for 1 minute, and washed again for 5 second 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 minute and washed again with water. The slide was air-dried and observed under oil-immersion objective.

Motility

A hanging drop of a 24 hour-old culture in MRS broth was prepared 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 of the strains

Production of catalase

The production of gas bubbles by the isolates were observed by adding 0.5 ml of 10 % hydrogen peroxide solution (Merck) to the cultures indicating the presence of catalase (Schillinger and Lücke, 1987).

Arginine hydrolysis

Tubes of 5 ml arginine hydrolysis medium (Thornley, 1960) were inoculated with 24 hour-old culture. The tubes were incubated at 30° C for 3 days and formation of ammonia from arginine was detected by spotting 100 µl cultures onto a white porcelain tile and adding equal volume of Nessler's reagent. Appearance of dark orange colour indicated presence of ammonia (Schillinger and Lücke, 1987).

Gas production from glucose

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

Growth at different pH

The pH of MRS broth was adjusted to 3.9 and 9.6 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 hour-old MRS

broth culture. The tubes were incubated at 30° C for 24-72 hour and observed for growth (Dykes *et al.*, 1994).

Growth at different temperatures

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

Growth in different NaCl concentrations

Salt tolerance was tested by inoculating a loop-full of culture in MRS broth supplemented with 6.5 %, 10 % and 18 % NaCl, respectively, and incubated for 3 days at 30° C in a slanting position to improve aeration (Schillinger and Lücke, 1987). 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, containing 0.5 % w/v of different carbohydrates instead of glucose 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.

Production of dextran from sucrose

Dextran production of *Leuconostoc* isolates were tested by growth on the 5 % (w/v) sucrose agar (Garvie, 1984) and observed for mucoid appearance on the agar plates (Kelly *et al.*, 1995).

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). The LAB strains were grown in lactate configuration medium (Tamang *et al.*, 2005) at 30° C overnight. One ml culture was centrifuged in a microcentrifuge (Heraeus, Germany) at 8,000 *g* for 5 minutes. 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: $c = (V \times MW \times \Delta A)/(\varepsilon \times d \times v \times 1000)$ (g/l), where, 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/m mol × cm). The result was multiplied by the dilution factor.

API Tests

Carbohydrate fermentation patterns of LAB were determined using API 50 CHL and API 20 STREP test strips (bioMérieux, France) according to manufacturer's instructions as well as 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 hour. The results were read by referring to the manufacturer's

interpretation table at 24 hour and 48 hour, respectively. All spontaneous reactions were recorded. The APILAB PLUS database identification software (bioMérieux, France) was used to interpret the results.

Identification of Bacteria

Bacterial species were identified following the taxonomic keys of Bergey's Manual (Sneath *et al.*, 1986), Simpson and Taguchi (1995), Wood and Holzapfel (1995), and by APILAB PLUS Database software of API tests (bioMérieux, France).

Characterisation of yeast Isolates

Cell morphology

Cell morphology and mode of vegetative reproduction of yeast was observed following the method of Yarrow (1998). Sterile yeast morphology agar (M138, HiMedia) slants were inoculated with an actively growing (24 hour-old) yeast culture and incubated at 28° C for 3 days. 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 minutes. The glass slides were quickly removed from the glass rod with a flame sterialized pair of tweezers, and were dipped into the molten potato dextrose agar (M096, HiMedia) after which they were replaced on the glass rod support. 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 (M804, HiMedia) slants were streaked with actively grown yeast cultures, 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 (S020, HiMedia) for 30 to 60 second, heated to steaming 3 to 4 times over the flame of a spirit lamp and counterstained with safranin (S027, HiMedia) for 30 second and observed under the microscope (Yarrow, 1998).

Reduction of nitrate

Yeast 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 (M137, HiMedia) were inoculated with actively grown yeast isolates and incubated at 37° C for 4 days and observed for growth (Yarrow, 1998).

Sugar fermentation

Yeasts isolates were grown at 28° C on yeast-malt extract (YM) agar (M242, HiMedia) 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 (Yarrow,1998).

Sugar assimilation

Yeast isolates were grown at 28° C on yeast-malt extract (YM) agar (M242, HiMedia) slants for 3 days. Tubes containing 5 ml mixture of yeast nitrogen base (M139, HiMedia) 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 in 4.5 ml of sterilized distilled water (devoid of any carbon source).

Assimilation of carbon sources was observed by comparing with the control (Yarrow, 1998).

Identification of Yeast

Yeast isolates were identified to the genus level according to the criteria laid down by Kreger-van Rij (1984), Kurtzman and Fell (1998) and Yarrow (1998).

Pathogenic contaminants

Enumeration of pathogenic contaminants from the samples were done in selective media such as *Bacillus cereus* agar base (M833, HiMedia) for *Bacillus cereus*, Baird Parker agar base (M043, HiMedia) for *Staphylococcus aureus* and Violet Red Bile Glucose agar w/o lactose (M581, HiMedia) for enterobacteriaceae (Han *et al.*, 2001). *Salmonella-Shigella* Agar (M108, HiMedia) was used for the detection of *Salmonella* and *Shigella* and *Listeria* identification agar base (M1064, HiMedia) with *Listeria* selective supplement (FD 061, HiMedia) for *Listeria* in the samples following the standard method of Metaxopolous *et al.* (2001). Ten g of sample were blended with 90 ml of peptone-physiological saline (0.1 % neutral peptone, 0.85 % NaCl) and homogenized in a

stomacher lab-blender 400 (Seward, UK) for 1 minute. 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 (M833, HiMedia) with appropriate additions of Polymyxin B Selective Supplement (FD003, HiMedia) and Egg yolk emulsion (FD045, HiMedia). The inoculated plates were incubated at 30° C for 24-48 hour. Characteristic turquoise to peacock blue colonies surrounded by zone of precipitate of the same colour was regarded as presumptive *Bacillus cereus*.

***Staphylococcus aureus*:** Spread plates of Baird Parker agar base (M043, HiMedia) with appropriate additions of Egg yolk tellurite emulsion (FD046, HiMedia) was used for selective enumeration of *Staphylococcus aureus*. After serial dilution plates were overlaid with the medium and incubated at 30° C for 48 hours. The black colonies surrounded by clear zone extending 2-5 mm into the opaque medium appeared were regarded as presumptive *Staphylococcus aureus*.

Enterobacteriaceae: Sample dilutions in tryptone soya broth (M011, HiMedia) were allowed to resuscitate on thinly plated tryptone soya agar (M290, HiMedia) plates for 2 hour at 27° C, followed by a thick overlay of selective Violet Red Bile Glucose agar (without lactose) (M581, Himedia, Mumbai) medium and incubated at 30° C for 20 hour. Pink colonies appeared were regarded as presumptive enterobacteriaceae.

***Listeria* sp:** *Listeria* identification agar base (M1064, HiMedia) with *Listeria* selective supplement (FD061, HiMedia) media were used for detection of *Listeria* in the samples following the standard method of Metaxopolous *et al.* (2001). Inoculated plates were incubated at 30° C for 48 hour and observed in dark background for smooth glistening colonies indicating presence of *Listeria.*

***Salmonella* and *Shigella*:** *Salmonella-Shigella* agar (M108, HiMedia) was used for the detection of *Salmonella* and *Shigella* in samples following the method of Metaxopolous *et al.* (2001). After serial dilution plates were inoculated, followed by an overlay of the SS agar and incubated at 37° C for 48 hour and observed in dark background for

presumptive colonies. *Salmonella* colonies appear dark-centred while colourless colonies are regarded as presumptive *Shigella.*

Technological Properties of Isolates

Acidification and coagulation

Effect of acidification and coagulation of the LAB and yeasts isolates were assayed by inoculating 10 % skim milk with 24 hour old cultures (RM1254, HiMedia) (centrifuged at 8,000 *g* for 20 minutes and sterialized at 110° C for 10 minutes) at 1 % level, and incubated at 30° C. Observation was made for commencement of clotting, and pH was measured after 72 hour of incubation (Olasupo *et al.*, 2001).

Enzymatic profile by API-zym

The enzymatic profile of LAB and yeast isolates were assayed following the method of Arora *et al.* (1990) using API-zym (bioMérieux, France) galleries by testing for the activity of the following 19 enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine-, valine- and cystine-arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-

galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. LAB cultures were grown on MRS broth, and yeast cultures were grown on YM broth, respectively and cells were harvested in 2 ml sterile distilled water which was used to prepare suspension of 10^7 cells/ml. The 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 hour. 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 and bacteriocin activity

Agar Spot Test: The LAB isolates were screened for antimicrobial activity against some of the pathogenic bacteria by agar spot method of Schillinger and Lücke (1989). Cultures were grown on the respective broth media for 24 hour. 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 hour. The indicator

strains *Bacillus cereus* CCM 2010, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Listeria monocytogenes* DSM 20600 and *Staphylococcus aureus* S1 were propagated in standard nutrient agar (M002, HiMedia). 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 hour. After incubation, the plates were checked for inhibition zones (clearing of the medium) around the producer colony.

Bacteriocin Activity: Bacteriocin activity of the LAB isolates was estimated using an agar spot assay as described by Uhlman *et al.* (1992) and Schillinger *et al.* (1993). The antimicrobial-positive strains were grown in MRS broth at 30° C for 24 hour 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 minutes in block-thermostat (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 hour, 0.01 ml of the culture supernatant was spotted onto the agar surface. The plates were incubated at 30° C for 24 hour and subsequently examined for zones of inhibition.

Successional studies during *Selroti* batter fermentation

Preparation of Selroti batters at Laboratory

Selroti batter was prepared in the laboratory following the traditional method based on the combination of three popular methods being practiced at different places in Sikkim. Rice (*Oryza sativa* L.) local variety 'attey' was purchased from Gangtok market. One kg of rice was sorted, washed and soaked overnight at ambient temperature (20-22° C). Water was decanted from the soaked rice, spread over a tray for 1 hour, pounded and sieved to get rice flour. The rice flour was thoroughly mixed with 250 g of wheat flour (refined), 250 g sugar, 100 g butter and 25 g powdered spices including large cardamom (*Amomum subulatum* Roxb.), cloves (*Syzygium aromaticum* Merr.), coconut (*Cocos nucifera* L.), fennel (*Foeniculum vulgare* Mill), nutmeg (*Myristica fragrans* Houtt.), cinnamon (*Cinnamomum zeylanicum* Breyn.), and

small cardamom (*Elletaria cardamomum* Maton.). Milk was added to the mixture and kneaded into soft dough. More milk was added to make the batter with easy flow during preparation. Equal volumes of batter were distributed in 11 sterile 500 ml Duran bottles and were loosely capped, labeled and incubated at 28° C. Successional studies were carried at every 1 hour interval within a range of 0 to10 hours.

Microbial changes

Samplings were made at one hour interval till 10th hour for microbial changes during *in situ* fermentation. LAB, yeasts, *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae isolated from fermenting batters in every hour (0-10 hour) were analysed for their microbial load.

Physico-chemical changes

Samplings were made at every one hour interval till 10th hour for analysis of physico-chemical (pH, titratable acidity, temperature of fermenting batters, batter volume and batter weight) changes during *in situ* fermentation.

Sensory evaluation

Sensory properties of product was evaluated in terms of aroma, taste, texture, colour and general acceptability as method described by Meilgaard *et al.* (1990). *Selroti* batters were prepared at laboratory following the traditional method as described above. Fermenting batters were taken out in every hour and deep-fried in hot edible oil to make a *Selroti.* Deep-fried *Selroti* prepared from every hour fermenting batters were served to 10 judges for sensory evaluation with score rate of 1 as bad (hard texture) and 5 as excellent (soft texture); the market deep-fried *Selroti* was considered as control with scoring rate of 3, moderate (Table C).

Table C. Format for sensory evaluation of *Selroti*

Please use market Selroti as a control with scoring rate of 3 (moderate)

Sample code:

Name:

Attribute	Score					Comment
Aroma	Bad				Excellent	
	1	2	3	4	5	
Taste	Bad				Excellent	
	1	2	3	4	5	
Texture:	Hard				Soft	
	1	2	3	4	5	
Colour:	Bad				Excellent	
	1	2	3	4	5	
General Acceptability:	Bad				Excellent	
	1	2	3	4	5	

Date: Signature of the judge

Requirements for high grade *Selroti*

Aroma: Typical *Selroti* flavour
Taste: Sweet
Texture: Soft
Colour: Golden brown

Testing of strain(s) for *Selroti* Preparation

Starter cultures preparation

A loopful of LAB culture was inoculated in 5 ml MRS broth (M369, HiMedia) and incubated overnight at 30° C. The 1 ml of each culture was centrifuged (Biofuge pico, Heraeus, Germany) at 8,000 *g* for 5 minutes, the supernatant was discarded, 1 ml of sterile distilled water was added to the pellet, cells were resuspended and again centrifuged at 8,000 *g* for 5 minutes. Cells were again suspended in 1 ml sterile distilled water. This procedure achieved an inoculum size containing 10^8 cfu/ml, and was checked as viable count in MRS agar (M641, HiMedia) plates. A loopful of different yeast cultures were inoculated in 5 ml YM broth (M425, HiMedia) separately and incubated overnight at 28° C. These cultures were centrifuged and washed as described previously and an inoculum containing 10^7 cfu/ml, was made.

Sensory evaluation of Selroti prepared by starter culture

Pounded rice along with all ingredients are mixed thoroughly and soft dough was made as described earlier. Equal volumes (nearly 350 ml) of batter were distributed in 14 sterile 500 ml Duran bottles with screw-caps. *Selroti*

batter samples were inoculated with 1 ml of LAB and yeast inocula per 100 g of batter. Different starter cultures were used. In mixed fermentation, 1 ml of each inoculum was used for the same batter sample. Inoculated samples were mixed thoroughly by sterile spatula, loosely capped, labeled and incubated at 28° C. The pH and titratable acidity of the fermenting batters were determined at 0 hour, 4 hour and 6 hour. Fermenting batters were taken out at 4 hour and 6 hour and deep-fried in hot edible oil to make a *Selroti.* Deep-fried *Selroti* prepared from fermenting batters were served to 10 judges for sensory evaluation with score rate of 1 as bad (hard texture) and 5 as excellent (soft texture); the market deep-fried *Selroti* was considered as control with scoring rate of 3, moderate (Table C).

Consumers' Preference Trial

Freshly fried *Selroti* purchased from Gangtok market as well as fried *Selroti* prepared from batters made in the laboratory by using a mixture of selected isolates were served to 50 consumers who were familiar with *Selroti.* The 9-point scale used in consumers' preference trial ranged from score 1 (dislike extremely) to score 9 (like extremely) (IS, 1971).

Physical and Biochemical Analysis

pH

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

Determination batter volume

The volume in the batter during fermentation was determined following the method described by Thyagaraja *et al.* (1992). The batter (50 ml) was transferred to a sterile measuring cylinder of 100 ml capacity, covered with aluminium foil and incubated at 30° C. The increase in volume at different stages of fermentation was recorded and expressed as the percent volume increase over the initial volume.

Determination of batter weight

Batter weight (~25 g) was taken at every hour during fermentation using a digital balance (Lutron GM-500,

Taiwan) to observe an increase/decrease on weight of fermenting batter.

Batter temperature

The temperature (°C) change of the fermenting substrates during *Selroti* batter fermentation was recorded directly by a thermometer (Thapa, 2001).

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).

Reducing sugar

Reducing sugar content of the 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 minutes. This was allowed to cool for 5 minutes 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 minutes, 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

Total Sugar

Total sugar of the sample was estimated by determining reducing sugar in hydrolysed sample with HCl (AOAC, 1990). In a 300 ml conical flask fitted with condenser, 2 g of sample was blended in 20 ml of distilled water to which 160 ml of distilled water and 20 ml of HCl (25 %) were added. It was heated in vigorously boiling water bath for 3 hour, cooled in a running tap water, neutralized with 10 % NaOH using pH

meter (Type 361, Systronics, India) and diluted to 500 ml with distilled water. It was filtered and the filtrate was taken for determining reducing sugar as described above. Total sugar was calculated in percentage.

Total sugar (%) = $(As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 500/V_2 \times 100/S$

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)

500 = total volume of extract (ml)

100 = %

S = sample size for preparation of extract

Moisture

Moisture content of the batters was calculated by drying 2.5–3.0 g of well-mixed sample at 135 ± 1° C for 2 hour 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 hour. 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 minutes, cooling and weighing was repeated until the difference between two successive weighing was ≤ 1 mg.

Fat

Fat content of the sample 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 hour. The flat bottomed flask was dried for 1 hour at 100° C to evaporate ether and moisture, cooled in desiccator and weighed (W_2). Fat was calculated: Fat (%) = $W_2 - W_1$/Sample weight × 100

Protein

Total nitrogen of the 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 hour. 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 (I007, HiMedia). 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 – C.F.) × N of standard NaOH] ×1.4007/weight of sample (g). Correction factor (C.F.)

= (titre of standard NaOH against blank – ml of standard acid).

Protein content was determined by multiplying total nitrogen value with 6.25 (AOAC, 1990). Protein (%) = Total Nitrogen (%) × 6.25

Water-soluble nitrogen

For estimation of water-soluble nitrogen, homogenised samples was centrifuged at 7,000 *g* for 10 minutes and 10 ml of accurately weighed supernatant was digested and distilled as described for total nitrogen (Tamang and Nikkuni, 1996).

TCA-soluble nitrogen

For determination of TCA-soluble nitrogen, homogenised sample was added with equal volume of 8 % trichloroacetic acid (TCA) (w/v) and kept at room temperature for 60 minutes. Mixture was centrifuged at 7,000 *g* for 10 minutes and 10 ml of accurately weighed samples was digested and distilled as described above (Tamang and Nikkuni, 1996).

Carbohydrate

The carbohydrate content of the samples was calculated by difference: 100 – (% protein + % fat + % ash) (Standal, 1963).

Food Value

Food value of each batter sample 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 (Indrayan *et al.*, 2005).

Minerals

The method of AOAC (1990) for determination of mineral was followed. The ash after heating the sample at 550° C for 3 hour 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 hour. 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,

sodium and potassium were estimated in flame-photometer (Model CL 361, Elico, India) at 623, 589 and 766 nm, respectively following the method of Ranganna (1986).

Statistical analysis

The data were analysed by determining standard deviation (SD), standard error of measurement (SEM) and analysis of variance (ANOVA) (Snedecor and Cochran, 1989).

RESULTS

SURVEY ON *SELROTI*

Survey was conducted in randomly selected 214 households in villages located in all four districts of Sikkim – North (54 households), West (48), South (42) and East (70), representing the Nepalis, the Bhutias and the Lepchas. Data on acquiring of *Selroti* from home-made or market purchase, annual production and per capita consumption of *Selroti* by ethnic groups in the four districts of Sikkim were calculated (Table 1-2). *Selroti* is prepared at home (75.6 %) comparable to market purchase (14.3 %) (Table 1). Among the ethnic groups, 89 % of Nepali prepared *Selroti* followed by the Lepcha 6.6% and the Bhutia 4.4% respectively. Per capita consumption of *Selroti* in Sikkim was calculated as 8 g/day (Table 2). Average annual production of *Selroti* per household in Sikkim was 18.5 kg (data not shown). Feeding frequency of *Selroti* in rural Sikkim has been summarized in Table 3. Average feeding frequency of *Selroti* in Sikkim was 95.7 % occasionally (means once in 3 months), 4.5 % monthly (once in a month) and 0.3 % weekly (once in a week). The daily consumption of *Selroti* in rural Sikkim was not found throughout the survey.

Table 1: Distribution of households indicating source of acquiring *Selroti* by ethnic groups in Sikkim

Ethnic groups	Source of acquiring *Selroti* (%)			Total consumption (%)
	Home prepared	Market purchase	Both (Home + Market)	
Nepali	93.4	4.1	2.5	89
Bhutia	66.7	16.7	16.7	4.4
Lepcha	66.7	22.2	11.1	6.6

Total households surveyed in Sikkim was 214.

Table 2. Per capita consumption of *Selroti* (gram) per day by ethnic groups as well as by districts in Sikkim

Statistics	Per capita consumption (g/day)				
	Ethnic groups				
	Nepali	Bhutia	Lepcha		Total
Mean ± SD	8.4 ± 13.5	3.6 ± 3.1	5.9 ± 2.1		8.0 ± 12.8
	District				Sikkim
	North	South	East	West	
Mean ± SD	0.6 ± 0	5.0 ± 6.3	10.8 ± 19.2	7.3 ± 3.8	8.0 ± 12.8

Total households surveyed was 214.

Table 3. Feeding frequency of *Selroti* in the Sikkim Himalaya

Region	Feeding frequency (%)			
	Daily	Weekly	Monthly	Occasionally
East district	0	1.1	15.6	83.3
West district	0	0	0	100
North district	0	0	0	100
South district	0	0	2.3	97.7
Average (Sikkim)	0	0.3	4.5	95.3

Weekly means once in a week. Monthly means once in a month. Occasionally means once in three months.

Values are the means of 214 households surveyed in rural areas of Sikkim.

PRODUCT CHARACTERISATION

Traditional method of preparation, mode of consumption, socio-economy and ethnic importance of *Selroti* was documented.

Selroti is a popular fermented rice-based ring shaped, spongy, pretzel-like, deep-fried food item commonly consumed in Sikkim and the Darjeeling hills in India, Nepal and Bhutan. It is prepared during religious festivals and

special occasions. *Selroti* is an important and special food item of the Nepalis.

Synonym of Selroti

Selroti is a Nepali word for ring-shaped rice-based bread. Different ethnic groups in the Sikkim Himalaya call it by their different names. Such as "*Selsoplay*" by Mukhia, "*Selgaeng*" by Tamang, "*Selpempak*" by Rai, etc.

Traditional Method of Preparation

Selroti is the traditional food item prepared specially by the Nepalis. During *Selroti* preparation, rice (*Oryza sativa* L.) local variety 'attey' is sorted, washed, and soaked in cold water for overnight or 4 hour to 8 hour at ambient temperature. The use of milled rice in *Selroti* preparation is also commonly practiced in the Sikkim Himalaya. Water is then decanted from the rice by using bamboo made sieve called 'chalni' and spread over a woven tray made up of bamboo, locally called 'naanglo' and dried for 1 hour. Soaked rice (Photo 1) is pounded into coarse powder in a wooden mortar and pestle known as 'okhali' and 'mushli', respectively

(Photo 2). Larger particles of pounded rice flour are separated from the rest by winnowing in a bamboo tray. Then, the rice flour is mixed with nearly 25 % refined wheat (*Triticum aestivum* L.) flour, 25 % sugar, 10 % butter or fresh cream and 2.5 % spices/condiments containing large cardamom (*Amomum subulatum* Roxb.), cloves (*Syzygium aromaticum* Merr.), coconut (*Cocos nucifera* L.), fennel (*Foeniculum vulgare* Mill.), nutmeg (*Myristica fragrans* Houtt.), cinnamom (*Cinnamomum zeylanicum* Bl.), and small cardamom (*Elletaria cardamomum* Maton.), are added to the rice flour and mixed thoroughly. Some people add tablespoon full of honey or unripe banana or baking powder (sodium bicarbonate) to the mixture, depending on quantity of the mixture (Photo 3). Milk (boiled/unboiled) or water is added, kneaded into a soft dough (Photo 4) and finally into batter with easy flow. Batter is left to ferment (Photo 5) naturally at ambient temperature (20-28° C) for 2 to 4 hours during summer and at 10-18° C for 6 to 8 hours during winter (Fig 1-3). The oil is heated in a cast-iron frying pan locally called 'tawa'.

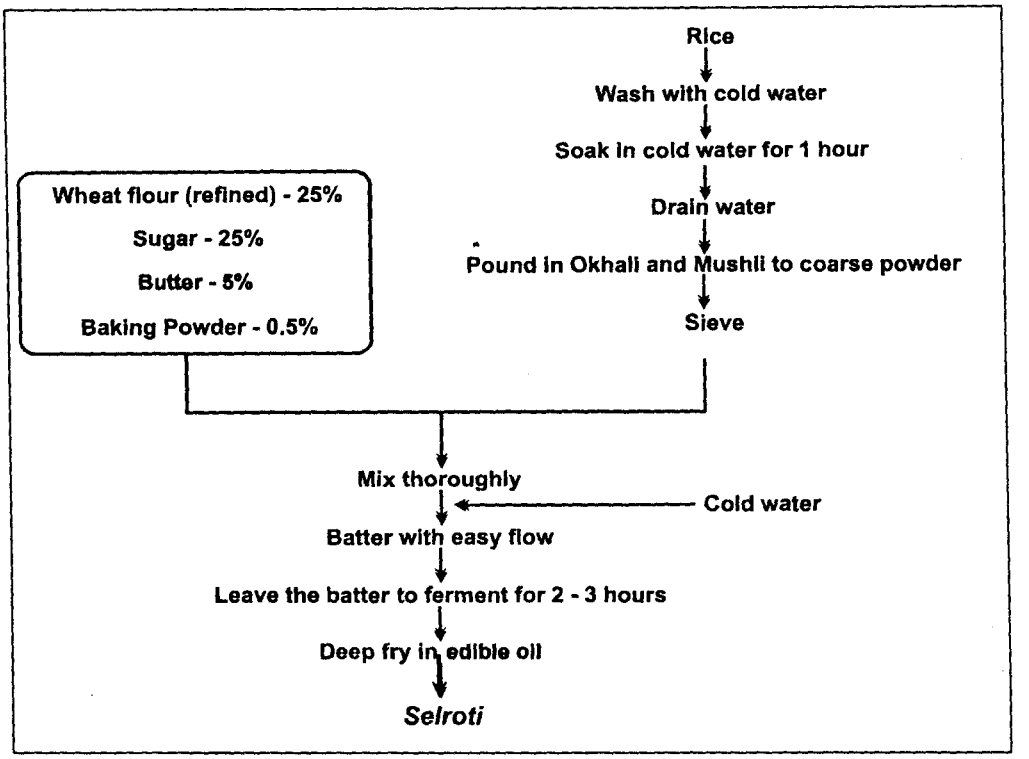


Fig 1. Flow sheet of traditional method of *Selroti* preparation practiced at Adampool village in East Sikkim

The fermented batter is squeezed by hand or 'daaru' (metallic serving spoon), deposited as continuous ring onto hot edible oil and fried until golden brown and is drained out from hot oil by poker locally called 'jheer' or 'suiro' or also by a spatula locally called 'jharna'. Deep-fried *Selroti* is served as confectionery (Photo 12).

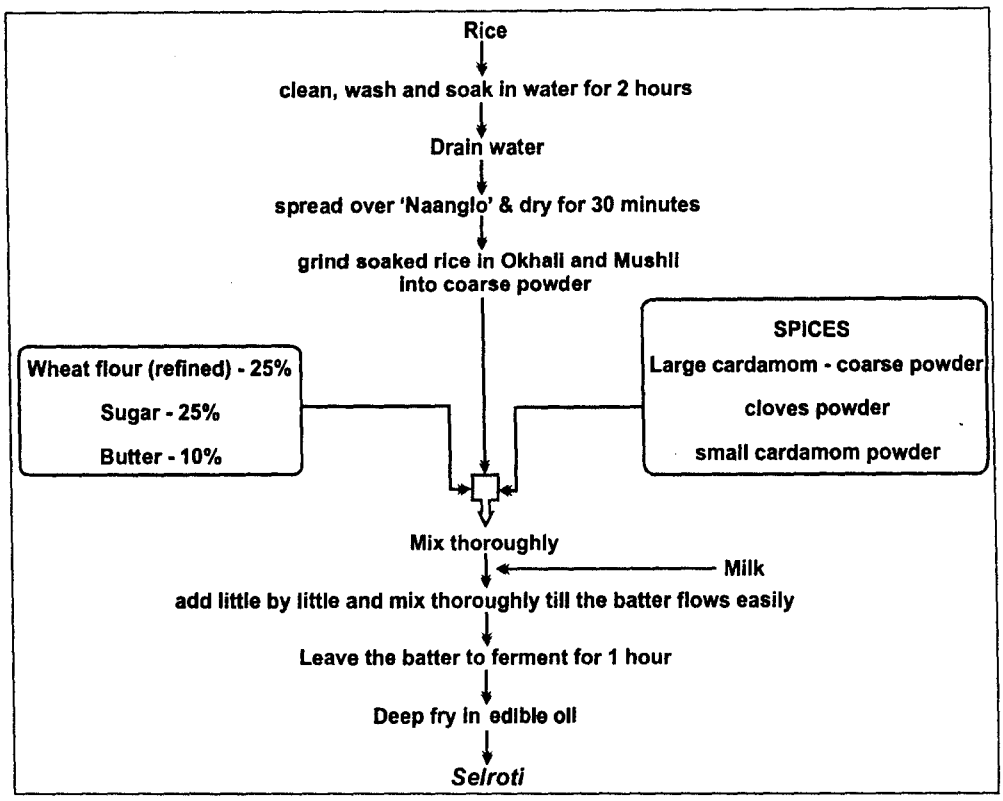


Fig 2. Flow sheet of traditional method of *Selroti* preparation practiced at Rangpo in East Sikkim

Equipment used

The following traditional equipments were used during traditional method of *Selroti* preparation as documented during survey.

'Okhali' and **'mushli'** – It is a pair of wooden mortar and pestle respectively used to pound soaked rice (Photo 7).

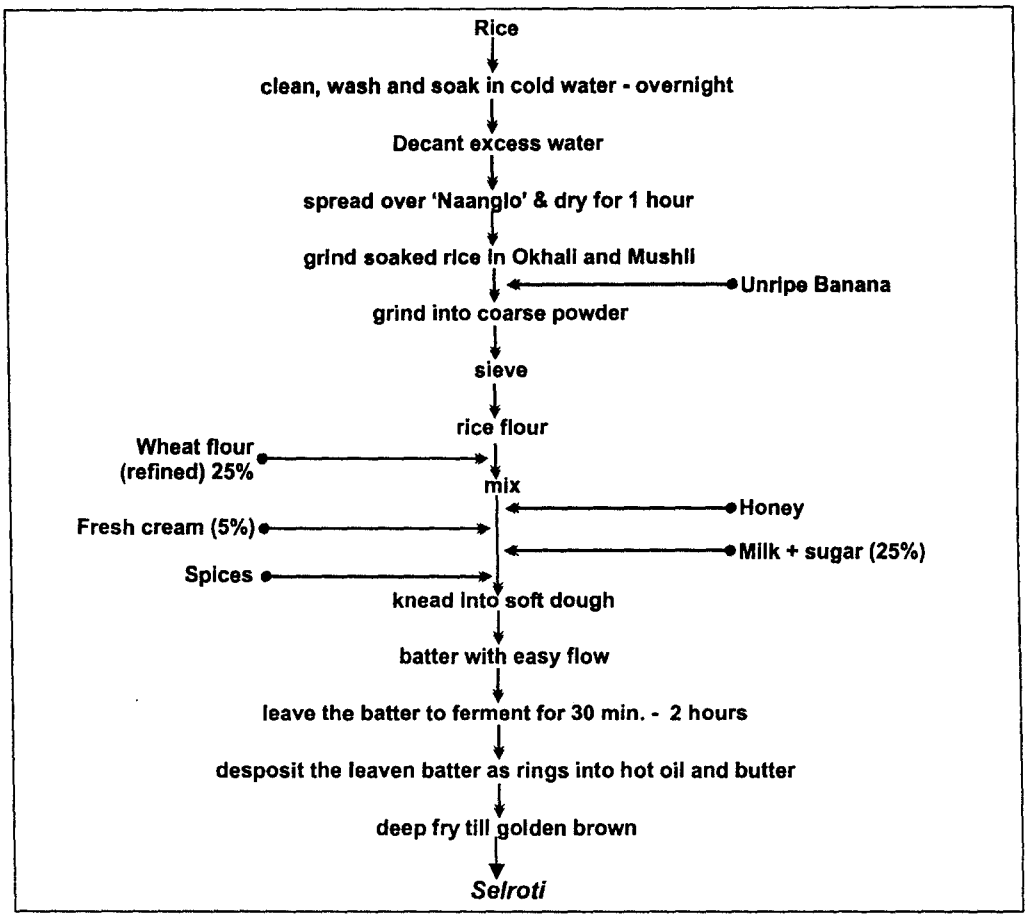


Fig 3. Flow sheet of traditional method of *Selroti* preparation practiced at Algarah in Kalimpong

'Naanglo' – It is a bamboo stripes woven tray used to dry soaked rice (Photo 8a).

'Chalni' – It is a sieve either made up of metal wire (Photo 8b) or bamboo stripes (Photo 9a). Sieve made up of bamboo has bigger holes to drain water from soaked rice. Sieve made up of metal wire is finely woven and is used to sieve pounded rice.

'Suiro' – It is a pointed bamboo stick used to turn *Selroti* upside down, lift, drain oil and to take out the fried *Selroti* (Photo 9b).

'Jheer' – It is a poker made up of metal wire to drain out the deep-fried *Selroti* from hot oil (Photo 9c).

'Daaru' – It is a metallic serving spoon used to pour batter onto the hot edible oil (Photo 9d).

'Tawa' – It is a cast-iron frying pan used to fry *Selroti* (Photo 10).

'Jharna' – it is a metal spatula and has a wide flat blade with holes. It is used to drain oil from the fried *Selroti.*

'Thumsey' – It is a bamboo-made basket use to store freshly fried *Selroti.*

Mode of Consumption

Selroti is served as confectionery bread with *aalu dam* (boiled potato curry) (Photo 13), *simi ko acchar* (pickle prepared from string beans) and meat. It can be served hot or cold. *Selroti* can be stored at room temperature for two weeks.

Socio-economy

The preparation of *Selroti* is an art of technology and is a family secret passed from mother to daughter. Women prepare it and men help them in pounding the soaked rice. Survey result indicated that *Selroti* is mostly prepared at home. It is also sold in canteens, local food stalls and restaurants (Photo 11). Some people are economically dependent upon this product. In local food stalls, it is sold at the average rate of Rs. 10/- per plate, each plate containing four pieces.

Ethnical Importance

Since the time immemorial *Selroti* is known as a ceremonial food in Sikkim, the Darjeeling hills, Nepal and Bhutan. It marks a special occasion of the Nepalis such as marriage, religious and cultural festivals. Ethnical importance of *Selroti* was documented during survey and noted in discussion chapter.

Photo 1. Soaked rice

Photo 2. Rice being pounded in *okhali* by *mushli*

Photo 3. Pounded rice and spices

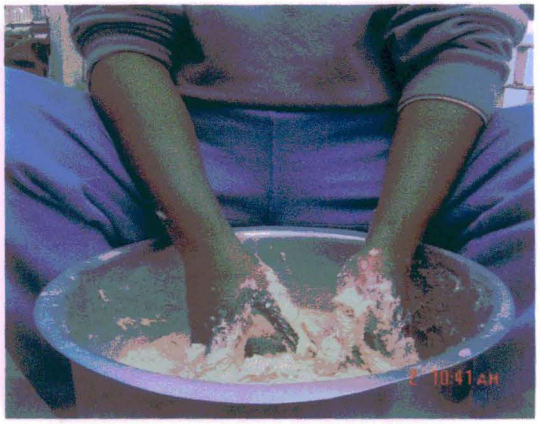


Photo 4. Mixing and kneading to make batter

Photo 5. Batter fermentation

Photo 6. *Selroti* being deep-fried in hot oil

Photo 7. a: *okhali* and **b:** *mushli*

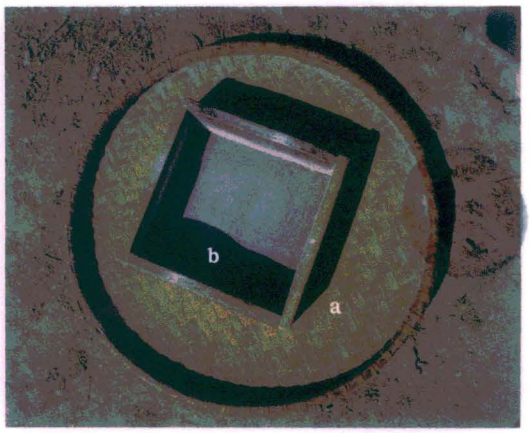


Photo 8. a: *Nanglo* and **b:** metal wired sieve

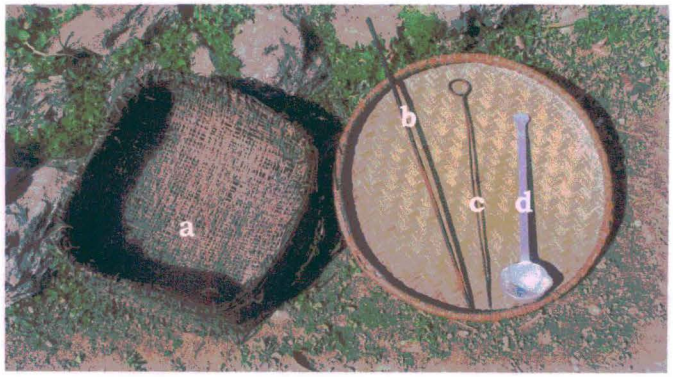
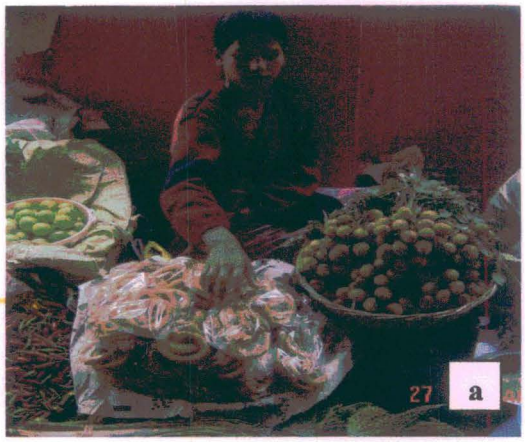


Photo 9. a: *Chalni*, **b:** *suiro*, **c:** *jheer*, **d:** *daaru*

Photo 10. Frying pan *tawa*



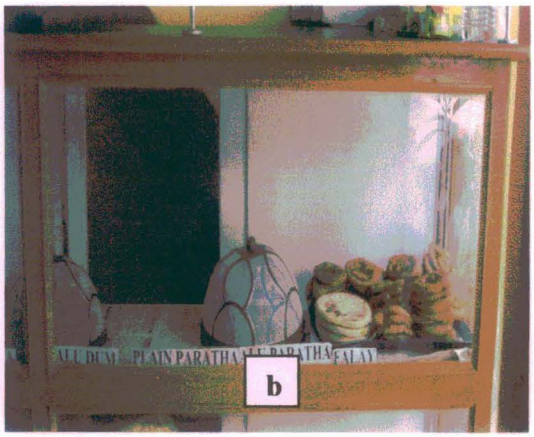


Photo 11. *Selroti* sold at market (**a :** Lal Market, and **b:** Tadong Bazar)

Photo 12. Different varieties of *Selroti*

Photo 13. *Selroti* being served with *alu dam*

MICROBIOLOGICAL ANALYSIS

Microbial population

A total of 78 samples of home-made *Selroti* batters were collected from different places of the Sikkim Himalaya- Gangtok (15), Adampool (10), Rangpo (12), Tadong (10), Namchi (13) in Sikkim, and Algarah (8) and Takdha (10) in the Darjeeling hills (Fig a). Similarly, 36 market-samples of *Selroti* batters were collected from different restaurants, local food stalls and canteens located in Lal Market of Gangtok (15), Tadong bazaar (10) and Tibet Road of Gangtok (11) (Fig b). A total of 11 samples of *Selroti* batters prepared at our laboratory following the traditional method were also included. All samples were analysed for microbiological populations (Table 4 and 5). In all home-made samples of *Selroti* batters, the numbers of lactic acid bacteria (LAB) as well as yeasts were in the range of 10^4 to 10^8 cfu/g and 10^4 to 10^5 cfu/g, respectively (Table 4). Average count of LAB and yeasts in market samples of fermented batters was 10^8 cfu/g and 10^5 cfu/g, respectively, whereas microbial population of LAB and yeasts in lab-made samples was 10^7 cfu/g and 10^5 cfu/g, respectively (Table 5). Total aerobic mesophilic counts

in all samples of fermented batters collected from households, market and lab-made sources was ranging in between 10^4 cfu/g and 10^8 cfu/g. Filamentous moulds were detected in a few samples of *Selroti* batters collected from Namchi, however, the detection limit was less than 10 cfu/g.

Table 4. Microbial load of *Selroti* batters collected from different households of the Sikkim Himalaya

Household		Log cfu/g sample		
Region	Place of collection	LAB	Yeast	AMC
East Sikkim	Gangtok (n = 15)	5.6 ± 0.1	4.5 ± 0.2	6.1 ± 0.1
	Adampool (n = 10)	7.6± 0.2	4.6 ± 0.1	7.6 ± 0.1
	Rangpo (n = 12)	6.6 ± 0.3	5.8 ± 0.2	6.8 ± 0.1
	Tadong (n = 10)	5.3 ± 0.1	4.5 ± 0.1	5.3 ± 0.4
South Sikkim	Namchi (n = 13)	8.0 ± 0.2	5.4 ± 0.2	8.0 ± 0.2
Kalimpong	Algarah (n = 8)	8.0 ± 0.2	5.6 ± 0.2	8.1 ± 0.1
Darjeeling	Takdha (n = 10)	5.4 ± 0.1	4.8 ± 0.1	5.8 ± 0.1

n, number of samples collected.

Data represents the means (± SD) of number of samples.

LAB, lactic acid bacteria; AMC, aerobic mesophilic count.

Moulds were detected only in few samples of Namchi at the level (<10 cfu/g).

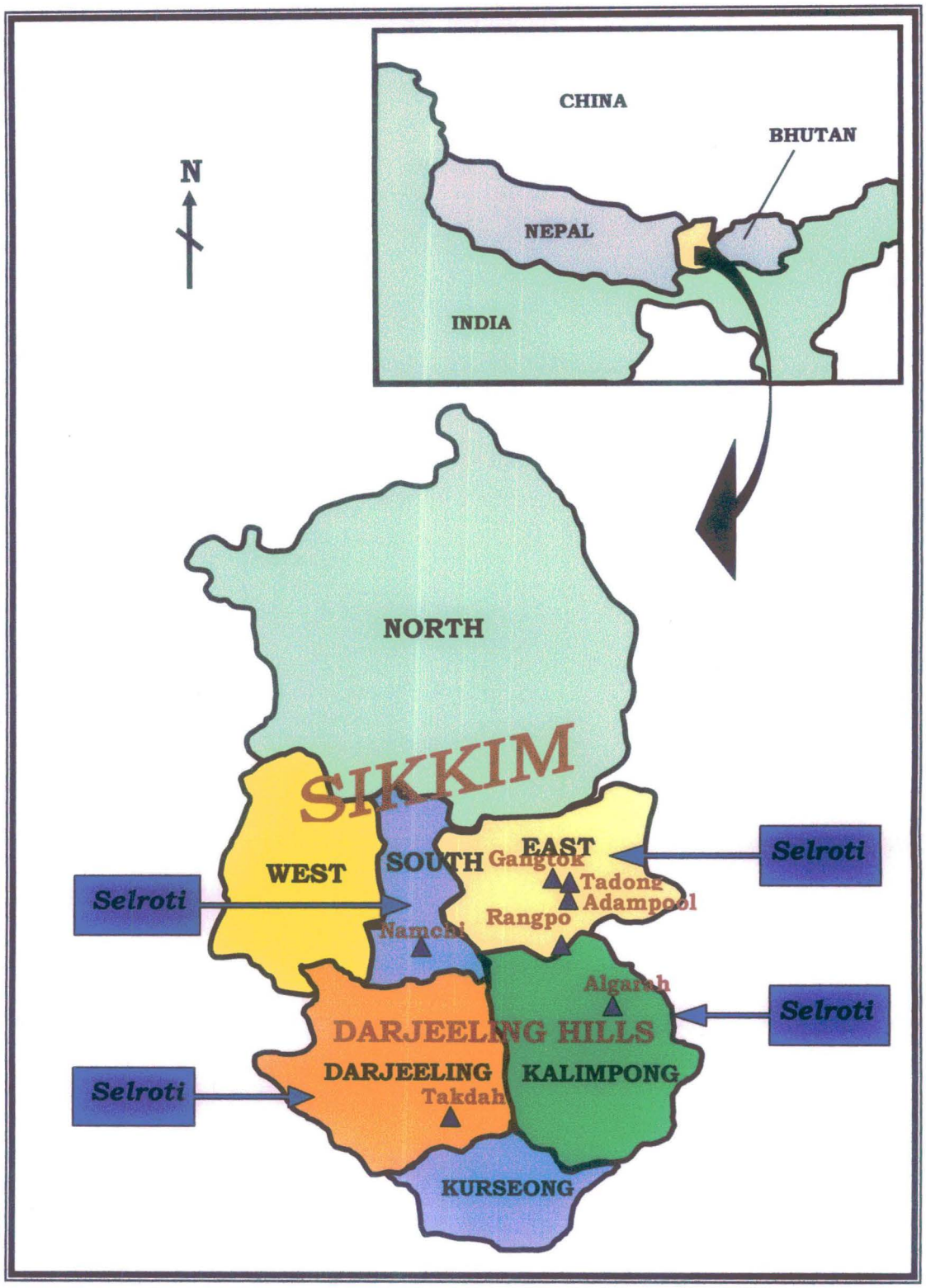


Fig a. Location map of the Sikkim Himalaya from where samples of *Selroti* batters were collected.

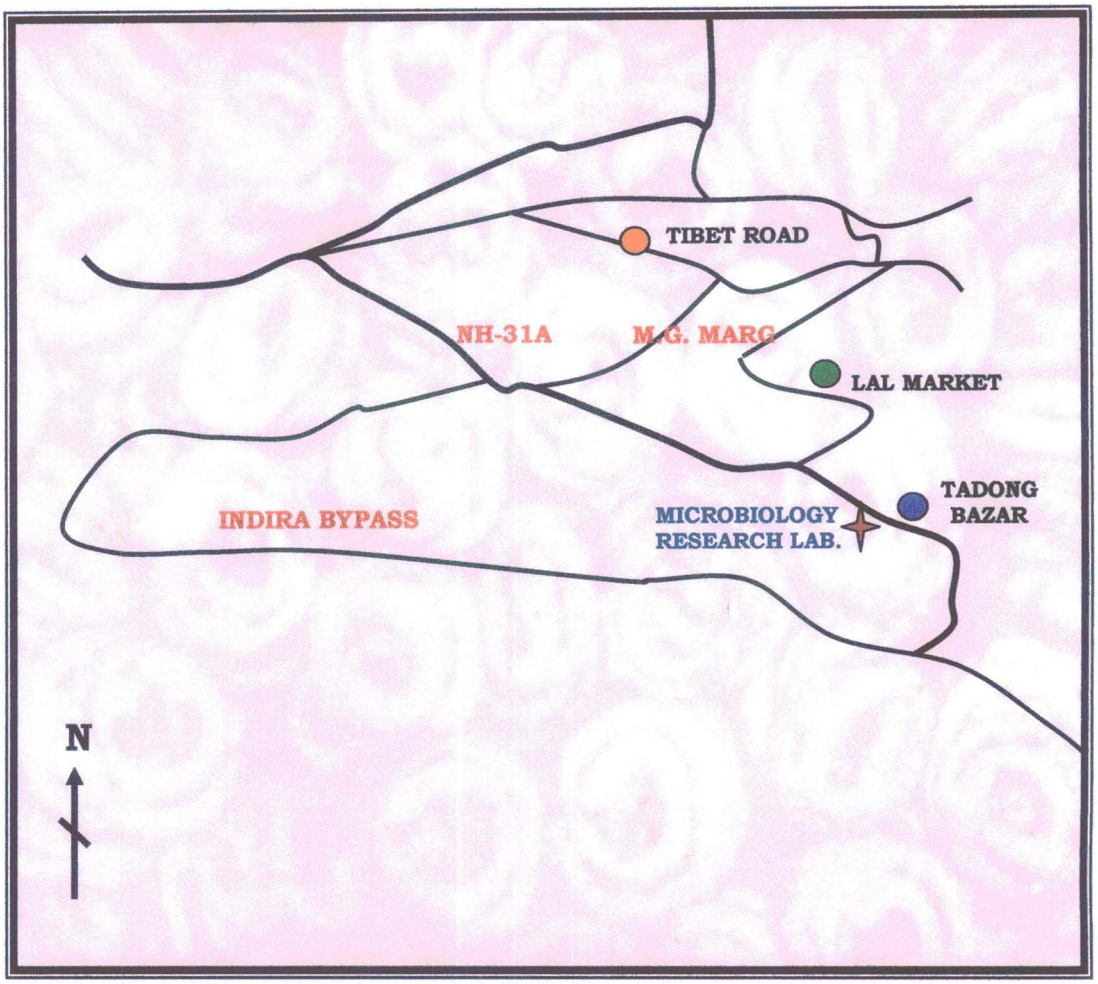


Fig b. Location map of Gangtok showing collection sites of market samples of *Selroti* batters and products.

Table 5. Microbial load of *Selroti* batters collected from different markets of the Sikkim Himalaya, and *Selroti* batters prepared in the laboratory

Source		Log cfu/g sample		
Preparation	Place of collection	LAB	Yeast	AMC
Market prepared *Selroti* batter	Lal Market of Gangtok (n = 15)	8.3 ± 0.1	5.7 ± 0.2	8.4 ± 0.1
	Tadong Bazaar of Gangtok (n = 10)	8.0 ± 0.1	5.6 ± 0.2	8.2 ± 0.1
	Tibet Road of Gangtok (n = 11)	8.6 ± 0.1	6.5 ± 0.2	8.7 ± 0.1
Lab - prepared *Selroti* batter[a]	Laboratory (n = 11)	7.6 ± 0.7	5.3 ± 0.3	7.9 ± 0.4

n, number of samples collected.

Data represents the means (± SD) of number of samples.

LAB, lactic acid bacteria; AMC, aerobic mesophilic count.

Moulds were not detected in any sample.

[a] *Selroti* prepared at Laboratory following the traditional method.

Grouping of representative LAB strains

A total of 167 bacterial isolates were isolated from *Selroti* batters collected from different sources: home-made batters (63), market batters (59) and lab-made batters (45). All isolates were purified in MRS broth, and their cell morphology and preliminary taxonomical tests were

performed. All 167 bacterial isolates were presumptive considered lactic acid bacteria (LAB) due to their growth in anaerobic agar and formation of clear zones of halo in MRS agar plates supplemented with calcium carbonate, were Gram-positive, catalase-negative, non-motile and did not form spores.

A grouping of all LAB isolates was based on cell morphology, gas production from glucose and arginine hydrolysis (Table 6). The representative strains of LAB were selected randomly from each grouped strains having similar morphology, ability to produce gas from glucose and hydrolyse arginine, isolated from the respective sample. The representative strains of LAB were grouped for detailed identification. Representative strains were assigned the strain code number indicating the sample names and source.

Table 6. Grouping of representative strains of the LAB isolated from *Selroti* batters of the Sikkim Himalaya

Source[a]	Cell shape	Gas from glucose	Arginine hydrolysis	Grouped strains	Representative strains	
					Total number	Strain code
Home prepared *Selroti* batter (63)	Rod	–	–	8	2	BP:B1, BR1:B1
	Coccoid rod	+	–	27	3	BG1:B1, BT1:B2, BS1:B1
	Coccus	–	+	10	3	BG3:B3, BP:B2, BS1:B2
	Coccus tetrad	–	+	18	6	BG2:B2, BG3:B2, BP:B3, BA1:B3, BN1:B3, BN2:B3
Market prepared *Selroti* batter (59)	Rod	–	–	9	1	S5:B1
	Coccoid rod	+	–	23	1	S6:B1
	Coccus	–	+	11	4	S1:B3, S5:B6, S1:B4, S4:B4
	Coccus tetrad	–	+	16	3	S2:B3, S4:B2, S5:B2
Lab-prepared *Selroti* batter (45)	Rod	–	–	5	1	L2:B1
	Coccoid rod	+	–	21	2	L0:B1, L1:B4
	Coccus	–	+	12	4	L1:B2, L3:B2, L6:B4, L10:B3
	Coccus tetrad	–	+	7	3	L9:B2, L1:B3, L3:B3

[a]Total number of isolates from each source is given in parenthesis.

All strains of LAB were Gram-positive, catalase-negative, non-motile and non-sporing.

Characteristics and identity of LAB

All representative strains of LAB were phenotypically characterised including growth at different temperatures, pH, tolerance in different concentrations of salts, determination of lactic acid configuration following the taxonomic keys described by Sneath *et al.* (1986), Schillinger and Lücke (1987), Dykes et al. (1994), Wood and Holzapfel (1995), and the sugar fermentation profiles data interpreted in APILAB PLUS Database software of API tests (bioMérieux, France). Out of 33 representative strains of LAB isolated from *Selroti* batters, 4 were homofermentative rods, 6 were coccoid rods, 11 were homofermentative cocci and 12 were tetrad-forming cocci (Table 6). All cocci forming tetrads were presumptively grouped as pediococci. Further differentiation of all tetrad forming representative strains was performed by using the key proposed by Simpson and Taguchi (1995) based on the ability to grow at pH 8.5, pH 4.2, at 50° C and in the presence of 10 % NaCl. On the basis of these tests, tetrad strains L9:B2, S4:B2, S5:B2, BG2:B2, L1:B3, L3:B3, S2:B3, BP:B3, BG3:B2, BN1:B3, BN2:B3, BA1:B3 were identified as *Pediococcus pentosaceus* (Table 7). The sugar fermentation

profiles using API identification profile also confirmed the identity of tetrad strains as *Pediococcus pentosaceus* (Plate a).

All homofermentative rod strains isolated from *Selroti* batters were curved, bean-shaped rods with rounded ends, in pairs and short chains and closed rings of usually four cells or horse-shoe forms were frequently observed. These strains grew well at 15° C, and produced DL lactate from glucose (Table 7). They were able to ferment ribose, trahalose, mannose, esculin, salicin, cellobiose and maltose (Table 7) and were identified as *Lactobacillus curvatus* (Plate b).

All coccoid strains isolated from *Selroti* batters produced D (-) lactate from glucose, were arginine-negative and showed the typical leuconostoc-like ovoid cells, produced dextran when grown on 5 % sucrose agar. However, they fermented sucrose, galactose, maltose, mannose and xylose. Sugar fermentation profiles using API confirmed their identity as *Leuconostoc mesenteroides* (Table 7) (Plate c).

Cocci strains (L1:B2, L3:B2, S1:B3, L6:B4, S5:B6, BS1:B2, BP:B2, L10:B3, BG3:B3, S1:B4, S4:B4) were non-gas producer, arginine-positive, grew well in 6.5 % NaCl, and at 45° C (Table 7). On the basis of sugar fermentation profiles

Table 7. Phenotypic characteristics of LAB from *Selroti* batters

Isolate code	Cell Morphology	Cell size (µm)	Arginine hydrolysis	CO_2 from glucose	Growth at/in								Lactate 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	
BP:B1	Rod	l = 2.8 (2.4 - 3.0) b = 0.7 (0.5 - 1.0)	–	–	+	+	–	+	+	+	–	–	DL	–	–	–	–	+	+	–	+	–	+	+	+	+	–	–	–	+	*Lactobacillus curvatus*
BR1:B1	Rod	l = 2.5 (2.4 - 2.8) b = 0.6 (0.4 - 1.0)	–	–	+	+	–	+	+	+	w	–	DL	–	–	–	–	+	+	–	+	–	+	+	+	+	–	–	–	+	*Lactobacillus curvatus*
S5:B1	Rod	l = 1.4 (0.8 - 2.2) b = 0.6 (0.5 - 0.8)	–	–	+	+	–	+	+	+	–	–	DL	–	–	–	–	+	+	–	+	–	+	+	+	+	–	–	–	+	*Lactobacillus curvatus*
L2:B1	Rod	l = 2.9 (2.4 - 3.4) b = 0.7 (0.6 - 0.8)	–	–	+	+	–	+	+	+	–	–	DL	–	–	–	–	+	+	–	+	–	+	+	+	+	–	–	–	+	*Lactobacillus curvatus*
L0:B1	Coccoid rod	l = 1.2 (0.8 - 1.6) b = 0.7 (0.3 - 1.0)	–	+	+	+	–	+	+	+	–	–	D	–	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	–	*Leuconostoc mesenteroides*
S6:B1	Coccoid rod	l = 1.1 (0.8 - 1.3) b = 0.6 (0.5 - 0.8)	–	+	+	+	–	+	+	+	–	–	D	–	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	–	*Leuconostoc mesenteroides*
BG1:B1	Coccoid rod	l = 1.4 (1.1 - 1.6) b = 0.6 (0.5 - 0.7)	–	+	w	+	–	+	–	+	–	–	D	–	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	+	*Leuconostoc mesenteroides*
BS1:B1	Coccoid rod	l = 1.1 (0.8 - 1.3) b = 0.6 (0.5 - 0.8)	–	+	+	+	–	+	–	+	–	–	D	–	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	–	*Leuconostoc mesenteroides*
BT1:B2	Coccoid rod	l = 1.1 (0.8 - 1.3) b = 0.6 (0.5 - 0.7)	–	+	+	+	–	+	–	+	–	–	D	–	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	–	*Leuconostoc mesenteroides*
L1:B4	Coccoid rod	l = 1.2 (0.8 - 1.6) b = 0.7 (0.3 - 1.0)	–	+	+	+	–	+	+	+	–	–	D	–	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	–	*Leuconostoc mesenteroides*
L1:B2	Coccus	d = 1.1 (0.8 - 1.3)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	+	*Enterococcus faecium*

l, length; b, breadth; d, diameter; w, weak; +, positive; -, negative.

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

Continued (Table 7)

Isolate code	Cell Morphology	Cell size (um)	Arginine hydrolysis	CO_2 from glucose	Growth at/in								Lactate 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	Celloblose	Maltose	Lactose	Mellbiose	Raffinose	Melezitose	
L3:B2	Coccus	d = 1.1 (0.8 - 1.3)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
S1:B3	Coccus	d = 1.1 (0.7 - 1.4)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
L6:B4	Coccus	d = 1.0 (0.7 - 1.4)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	–	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
S5:B6	Coccus	d = 1.1 (0.8 - 1.3)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
BS1:B2	Coccus	d = 1.3 (1.0 - 1.8)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
BP:B2	Coccus	d = 1.3 (1.0 - 1.8)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
L10:B3	Coccus	d = 1.3 (1.0 - 1.8)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
BG3:B3	Coccus	d = 1.4 (1.0 - 1.9)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
S1:B4	Coccus	d = 1.4 (0.8 - 2.1)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
S4:B4	Coccus	d = 1.4 (0.8 - 2.1)	+	–	+	+	+	+	+	+	–	–	L	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	*Enterococcus faecium*
L9:B2	Coccus/tetrad	d = 1.1 (0.8 - 1.3)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	+	–	–	–	*Pediococcus pentosaceus*

l, length; b, breadth; d, diameter; +, positive; -, negative.

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

Continued (Table 7)

Isolate code	Cell Morphology	Cell size (um)	Arginine hydrolysis	CO_2 from glucose	Growth at/in								Lactate 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	
S4:B2	Coccus/tetrad	d = 0.9 (0.8 - 1.1)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	–	–	–	–	*Pediococcus pentosaceus*
S5:B2	Coccus/tetrad	d = 0.9 (0.8 - 1.2)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	+	–	–	–	*Pediococcus pentosaceus*
BG2:B2	Coccus/tetrad	d = 0.8 (0.6 - 1.1)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	–	–	–	–	*Pediococcus pentosaceus*
L1:B3	Coccus/tetrad	d = 1.0 (0.6 - 1.4)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	–	–	–	–	*Pediococcus pentosaceus*
L3:B3	Coccus/tetrad	d = 1.1 (0.8 - 1.3)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	+	–	–	–	*Pediococcus pentosaceus*
S2:B3	Coccus/tetrad	d = 1.1 (0.6 - 1.4)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	–	–	–	–	*Pediococcus pentosaceus*
BP:B3	Coccus/tetrad	d = 1.0 (0.6 - 1.1)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	–	–	–	–	*Pediococcus pentosaceus*
BG3:B2	Coccus/tetrad	d = 1.3 (1.0 - 1.8)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	–	–	–	–	*Pediococcus pentosaceus*
BN1:B3	Coccus/tetrad	d = 1.4 (0.8 - 2.1)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	–	–	–	–	*Pediococcus pentosaceus*
BN2:B3	Coccus/tetrad	d = 1.4 (0.8 - 2.1)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	+	–	–	–	*Pediococcus pentosaceus*
BA1:B3	Coccus/tetrad	d = 1.4 (1.0 - 1.9)	+	–	+	+	–	+	–	+	–	–	DL	–	+	–	–	–	+	–	+	–	+	+	+	+	+	–	–	–	*Pediococcus pentosaceus*

l, length; b, breadth; d, diameter; +, positive; -, negative.

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

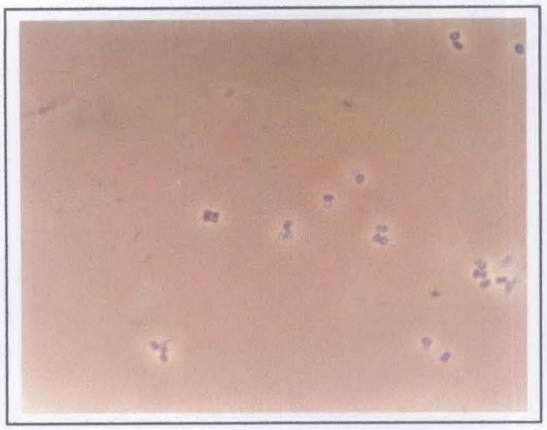


Plate (a). *Pediococcus pentosaceus* BG2:B2 (MRS broth, 3 days, 30° C), isolated from *Selroti* batter, showing tetrads in a phase contrast micrograph (× 825).

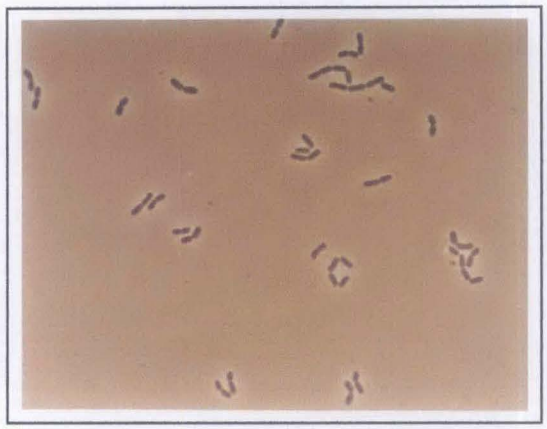


Plate (b). *Lactobacillus curvatus* BP:B1 (MRS broth, 3 days, 30° C), isolated from *Selroti* batter, showing non-sporing rods in a phase contrast micrograph (× 825).

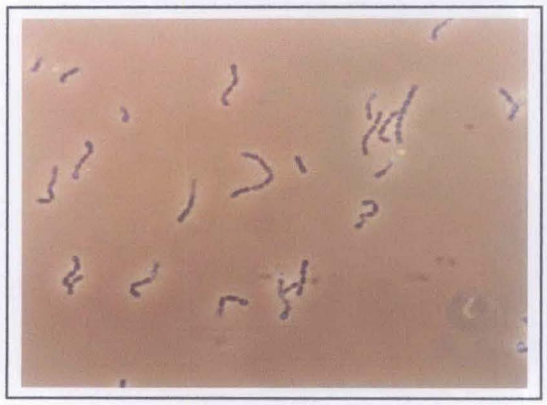


Plate (c). *Leuconostoc mesenteroides* BS1:B1 (MRS broth, 3 days, 30° C), isolated from *Selroti* batter, showing coccoid rods in a phase contrast micrograph (× 825).

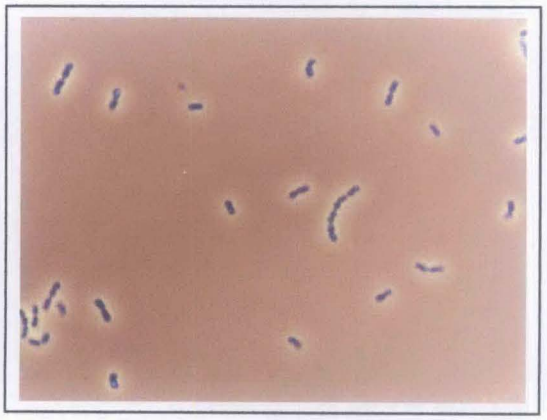


Plate (d). *Enterococcus faecium* BS1:B2 (MRS broth, 3 days, 30° C), isolated from *Selroti* batter, showing cocci in a phase contrast micrograph (× 825).

using API kits, they were identified as *Enterococcus faecium* (Plate d).

Based on the detailed characterisations and identification profiles, the following genera and species of functional lactic acid bacteria isolated from *Selroti* batters were identified as *Lactobacillus curvatus, Leuconostoc mesenteroides, Enterococcus faecium* and *Pediococcus pentosaceus.*

Grouping of representative yeast strains

Though the dominant microflora in all samples of *Selroti* batters was LAB, a sizable number of yeasts were also recovered in all samples. A total of 141 yeast isolates were isolated from *Selroti* batters collected from different sources: home-made batters (45), market batters (57) and lab-made batters (39).

The representative strains of yeast were selected randomly from each grouped strains having similar colony appearance, cell shape, type of mycelia and ascopsore for detailed identification (Table 8). Representative strains were

assigned the strain code number indicating the sample names and source.

Table 8. Grouping of representative strains of yeasts from *Selroti* batters of the Sikkim Himalaya

Source[a]	Colony	Cell shape	Mycelium	Ascospore	Grouped strains	Representative strains	
						Total number	Strain code
Home prepared *Selroti* batter (45)	Ds	O - Cy	True and Pseudo	Hat-shaped	89	3	BG1:Y1, BG3:Y1, BA1:Y1
	Ss	O - E	Pseudo	Globose	23	3	BG1:Y2, BA1:Y2, BA2:Y5
	Ss	O - E	Pseudo	Spheroidal	9	2	BG1:Y4, BR1:Y4
	Fs	O - E	Pseudo	Globose	4	1	BG1:Y3
Market prepared *Selroti* batter (57)	Ds	O - Cy	True and Pseudo	Hat - shaped	9	1	S1:Y1
	Ss	O - E	Pseudo	Globose	29	3	S2:Y2, S1:Y5, S1:Y6
	Ss	O - E	Pseudo	Spheroidal	10	2	S4:Y4, S5:Y4,
	Fs	O - E	Pseudo	Globose	9	2	S1:Y3, S3:Y3
Lab-prepared *Selroti* batter (39)	Ds	O - Cy	True and Pseudo	Hat-shaped	6	1	L1:Y1
	Ss	O - E	Pseudo	Globose	21	2	L9:Y6, L1:Y7
	Ss	O - E	Pseudo	Spheroidal	6	1	L1:Y4
	Fs	O - E	Pseudo	Globose	6	1	L3:Y3

[a]Total number of isolates from each source is given in parenthesis. All isolates reproduced by multilateral budding.

Ds, dusty surface; Ss, smooth surface; Fs, fringed surface; O-Cy, oval to cylindrical; O – E, oval to ellipsoidal.

Characteristics and identity of yeasts

Sugar fermentation and assimilation tests of randomly selected representative strains of yeasts were carried out (Table 9). Following the taxonomical keys of Kreger-van Rij (1984), and Kurtzman and Fell (1998), representative strains BG1:Y1, BA1:Y1, BG3:Y1, S1:Y1, L1:Y1 had dusty, dry and powdery surfaced colonies fringed with many strands of mycelia when grown on agar plates. They formed expanding septate hyphae with conidia borne on denticles. There were 1 to 4 hat-shaped ascospores per ascus. All of them fermented glucose, galactose, maltose, raffinose and sucrose. They were able to grow in 10 % NaCl and 5 % glucose in yeast nitrogen base. They were identified as *Pichia burtonii* (Plate e). Representative strains BG1:Y2, BA1:Y2, S2:Y2, BA2:Y5, S1:Y5 and L1:Y7 had smooth surfaced colonies, showing globose ascospores and fermented vigorously. They were identified as *Saccharomyces cerevisiae* (Plate f). Representative strains BG1:Y3, S1:Y3, S3:Y3 and L3:Y3 had smooth surfaced colonies fringed with pseudohyphae, showing globose ascospores and fermented sucrose. They were identified as *Saccharomyces kluyveri.* Representative

Table 9. Phenotypic characteristics of yeast isolated from *Selroti* batters

Isolate code	Cell Morphology	Cell size (µm)	Mycelium	Ascospore	Nitrate Reduction	Growth at 37°C	Sugars Fermented								Sugars Assimilated															Identity
							Glucose	Galactose	Lactose	Maltose	Raffinose	Sucrose	Starch	Trehalose	Arabinose	Cellobiose	Galactose	Glycerol	Inositol	Lactose	Maltose	Melibiose	Mannitol	Raffinose	Rhamnose	Sucrose	Starch	Trehalose	Xylose	
BG1:Y1	O - Cy	l = 8.5 (4.8 -11.2) b = 2.9 (2.4 - 3.2)	True, Pseudo	Hat-shaped	–	w	+	+	–	+	+	+	–	+	–	+	+	+	–	+	+	–	+	w	–	+	+	+	+	*Pichia burtonii*
BA1:Y1	O - Cy	l = 10.7 (6.4 - 14.4) b = 4.8 (3.2 - 6.4)	True, Pseudo	Hat-shaped	–	w	+	+	–	+	+	+	–	–	–	w	+	+	–	+	+	–	+	+	–	+	+	+	+	*Pichia burtonii*
BG3:Y1	O - Cy	l = 13.5 (5.1 - 19.6) b = 5.1 (3.7 - 6.6)	True, Pseudo	Hat-shaped	–	+	+	+	–	+	+	+	–	–	–	+	+	+	–	+	+	–	+	+	–	+	+	+	+	*Pichia burtonii*
S1:Y1	O - Cy	l = 14.4 (4.8 - 24.0) b = 6.1 (1.6 - 10.4)	True, Pseudo	Hat-shaped	–	+	+	+	–	+	+	+	–	+	–	+	+	+	–	+	+	–	+	+	–	+	+	+	+	*Pichia burtonii*
1:Y1	O - Cy	l = 13.9 (5.6 - 24.7) b = 5.7 (2.3 - 6.4)	True, Pseudo	Hat-shaped	–	w	+	+	–	+	+	+	–	–	–	w	+	+	–	+	+	–	+	+	–	+	+	+	+	*Pichia burtonii*
BG1:Y2	O - E	l = 3.8 (2.4 - 4.8) b = 2.0 (1.3 - 2.9)	Pseudo	Globose	–	+	+	+	–	+	+	+	+	–	–	–	+	+	–	w	+	+	–	+	–	+	+	+	–	*Saccharomyces cerevisiae*
BA1:Y2	O - E	l = 3.2 (1.6 - 4.8) b = 2.1 (1.2 - 3.2)	Pseudo	Globose	–	+	+	+	–	+	+	+	+	–	–	–	+	+	–	–	+	+	–	+	–	+	+	+	–	*Saccharomyces cerevisiae*
S2:Y2	O - E	l = 4.8 (3.2 - 5.6) b = 2.9 (1.6 - 4.8)	Pseudo	Globose	–	w	+	+	–	+	+	+	+	–	–	–	+	+	–	–	+	+	–	+	–	+	w	+	–	*Saccharomyces cerevisiae*
BG1:Y3	O - E	l = 4.4 (2.4 - 8.0) b = 1.6 (1.3 - 2.5)	Pseudo	Globose	–	w	+	+	–	–	–	+	–	–	–	+	+	+	–	–	+	+	+	+	–	+	–	+	+	*Saccharomyces kluyveri*
S1:Y3	O - E	l = 3.5 (3.2 - 4.0) b = 2.1 (1.6 - 2.4)	Pseudo	Globose	–	+	+	+	–	–	–	+	–	–	–	+	+	+	–	–	+	+	+	+	–	+	+	+	+	*Saccharomyces kluyveri*
S3:Y3	O - E	l = 3.6 (1.6 - 5.6) b = 2.0 (1.6 - 2.4)	Pseudo	Globose	–	+	+	+	–	–	–	+	–	–	–	+	+	+	–	–	+	+	+	+	–	+	+	+	+	*Saccharomyces kluyveri*

O - Cy, oval to cylindrical, O - E, oval to ellipsoidal
w, weak positive, +, positive, –, negative
l, length; b, breadth

continued (Table 9)

Isolate code	Cell Morphology	Cell size (µm)	Mycelium	Ascospore	Nitrate Reduction	Growth at 37°C	Sugars Fermented								Sugars Assimilated															Identity
							Glucose	Galactose	Lactose	Maltose	Raffinose	Sucrose	Starch	Trehalose	Arabinose	Cellobiose	Galactose	Glycerol	Inositol	Lactose	Maltose	Melibiose	Mannitol	Raffinose	Rhamnose	Sucrose	Starch	Trehalose	Xylose	
L3:Y3	O - E	l = 3.5 (3.2 - 4.0) b = 2.1 (1.6 - 2.4)	Pseudo	Globose	–	+	+	+	–	–	–	+	–	–	–	+	+	+	–	–	+	+	+	+	–	+	+	+	+	*Saccharomyces kluyveri*
BG1:Y4	O - E	l = 1.8 (1.2 - 2.4) b = 1.6 (1.2 - 2.4)	Pseudo	Spheroidal	–	–	w	–	–	–	–	–	–	–	–	+	+	+	–	–	+	–	+	+	+	+	+	+	+	*Debaryomyces hansenii*
BR1:Y4	O - E	l = 4.2 (1.6 - 5.6) b = 2.4 (1.6 - 4.0)	Pseudo	Spheroidal	–	–	w	–	–	–	–	–	–	–	–	+	+	+	–	–	+	–	+	+	+	+	+	+	w	*Debaryomyces hansenii*
S4:Y4	O - E	l = 3.7 (1.6 - 6.4) b = 3.0 (1.6 - 5.6)	Pseudo	Spheroidal	–	–	w	–	–	–	–	–	–	–	–	+	+	+	–	w	+	–	+	+	+	+	+	+	w	*Debaryomyces hansenii*
S5:Y4	O - E	l = 3.4 (1.6 - 5.6) b = 2.9 (1.6 - 4.0)	Pseudo	Spheroidal	–	–	w	–	–	–	–	–	–	–	–	+	+	+	–	–	+	w	+	+	+	+	+	+	w	*Debaryomyces hansenii*
L1:Y4	O - E	l = 3.3 (1.6 - 5.5) b = 2.5 (1.6 - 4.0)	Pseudo	Spheroidal	–	–	w	–	–	–	–	–	–	–	–	+	+	+	–	w	+	w	+	+	+	+	+	+	w	*Debaryomyces hansenii*
BA2:Y5	O - E	l = 3.5 (1.6 -4.0) b = 2.7 (1.6 - 3.2)	Pseudo	Globose	–	+	+	+	–	+	+	+	+	–	–	–	+	+	–	–	+	+	–	+	–	+	+	+	–	*Saccharomyces cerevisiae*
S1:Y5	O - E	l = 4.8 (3.2 - 5.6) b = (2.9 (1.6 - 4.8)	Pseudo	Globose	–	w	+	+	–	+	+	+	+	–	–	–	+	+	–	–	+	+	–	+	–	+	+	+	–	*Saccharomyces cerevisiae*
S1:Y6	O - E	l = 3.4 (1.6 - 5.2) b = 2.7 (1.6 - 5.2)	Pseudo	Globose	–	–	+	–	–	–	–	+	–	–	–	–	+	+	–	–	+	–	w	–	–	w	–	+	–	*Zygosaccharomyces rouxii*
L9:Y6	O - E	l = 3.3 (1.6 - 4.8) b = 2.9 (1.6 - 3.6)	Pseudo	Globose	–	–	+	–	–	–	–	+	–	+	–	–	+	+	–	–	+	–	w	–	–	+	–	+	–	*Zygosaccharomyces rouxii*
L1:Y7	O - E	l = 3.5 (1.6 -4.0) b = 2.7 (1.6 - 3.2)	Pseudo	Globose	–	+	+	+	–	+	+	+	+	–	–	–	+	+	–	–	+	+	–	+	–	+	+	+	–	*Saccharomyces cerevisiae*

O - Cy, oval to cylindrical; O - E, oval to ellipsoidal
w, weak; +, positive; –, negative
l, length; b, breadth

strains BG1:Y4, BR1:Y4, S4:Y4, S5:Y4 and L1:Y4 showed smooth surfaced colonies with spheroidal ascospores and fermented glucose weakly. They were identified as *Debaryomyces hansenii* (Plate g). Representative strains S1:Y6 and L9:Y6 showed smooth surfaced colonies with 1-4 globose ascospores and fermented glucose and sucrose. They were unable to grow on a medium containing 10 % glucose, 1 % tryptone, 1 % yeast extract, 2 % agar and 1 % acetic acid within 3 days. They were identified as *Zygosaccharomyces rouxii* (Plate h).

Compiling the identification profiles of the yeasts isolated from *Selroti* batters, four genera with five species comprising *Saccharomyces cerevisiae, Saccharomyces kluyveri, Debaryomyces hansenii, Pichia burtonii* and *Zygosaccharomyces rouxii* were reported in this thesis.

Prevalence of LAB

The most dominant lactic acid bacteria in all samples of *Selroti* batters collected from home-made, market and lab-made were *Leuconostoc mesenteroides* represented by 42.9 %, followed by *Pediococcus pentosaceus* (23.8 %), *Enterococcus*

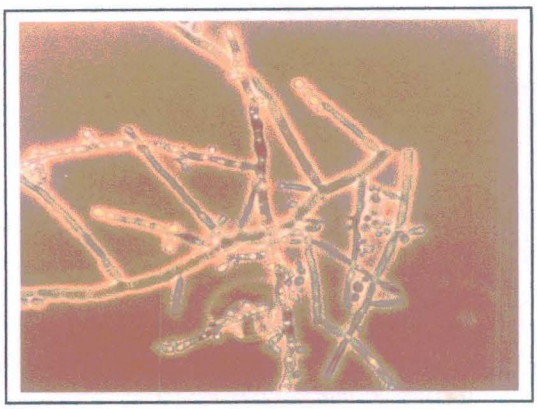


Plate (e). *Pichia burtonii* BG1:Y1 (YM broth, 3 days, 28° C), isolated from *Selroti* batter, showing denticles in a phase contrast micrograph (× 330).

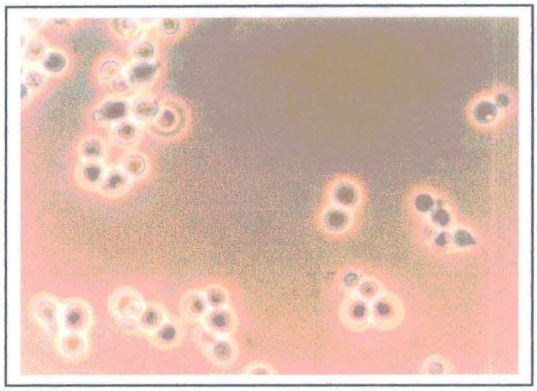


Plate (f). *Saccharomyces cerevisiae* BA1:Y2 (YM broth, 3 days, 28° C), isolated from *Selroti* batter, showing budding cells in a phase contrast micrograph (× 825).

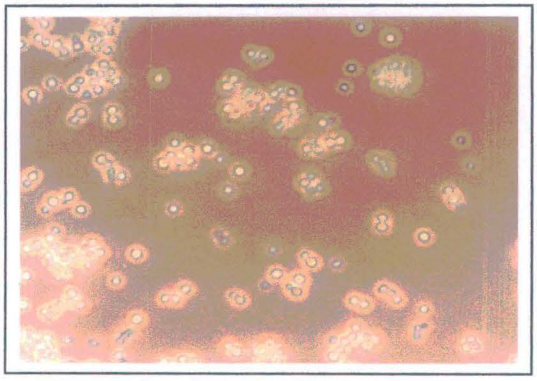


Plate (g). *Debaryomyces hansenii* BR1:Y4 (YM broth, 3 days, 28° C), isolated from *Selroti* batter, showing cells in a phase contrast micrograph (× 330).

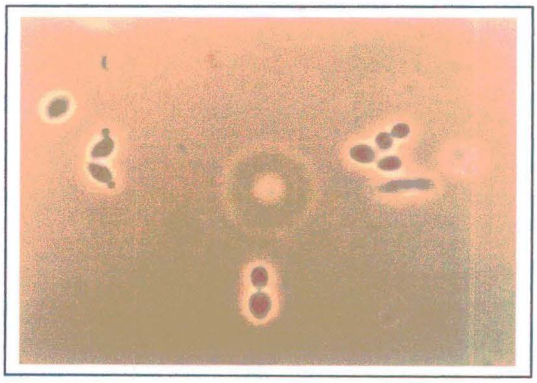


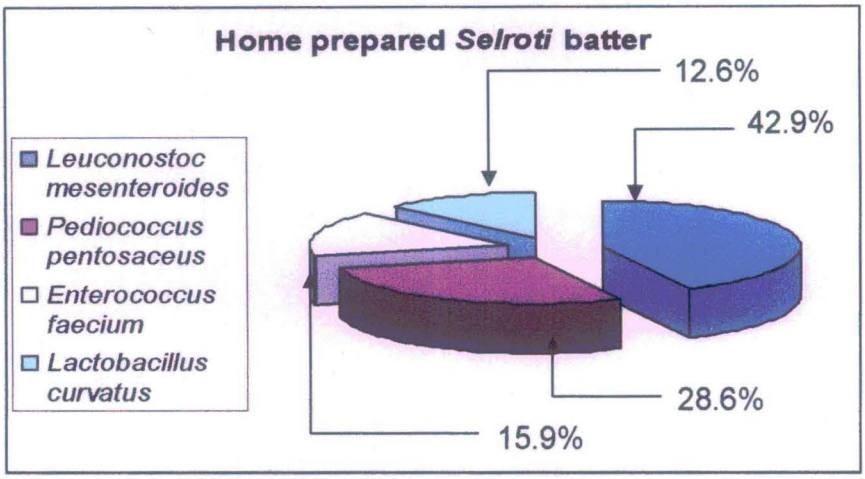
Plate (h). *Zygosaccharomyces rouxii* S1:Y6 (YM broth, 3 days, 28° C), isolated from *Selroti* batter, showing cells in a phase contrast micrograph (× 825).

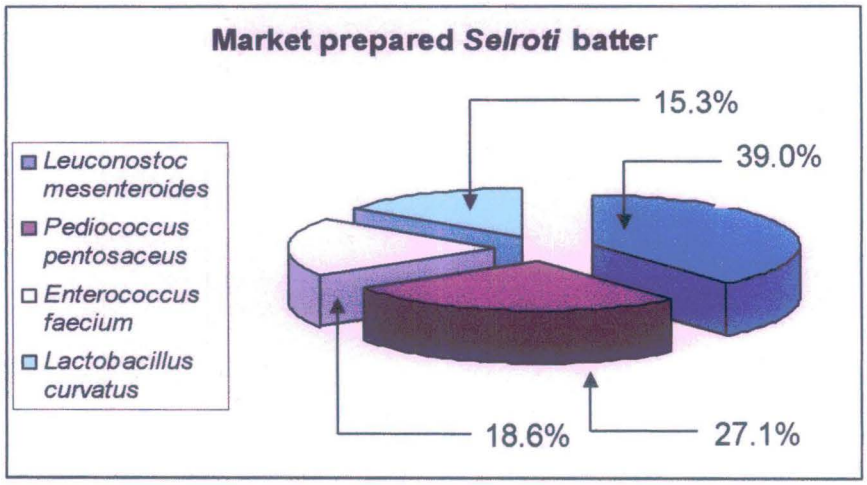
faecium (20.4 %) and *Lactobacillus curvatus* (13.0 %) out of 167 strains of LAB (Table 10, Fig 4 and Fig 5).

Table 10. Prevalence of functional LAB in *Selroti* batters collected from different sources

Source[a]	% of Prevalence			
	Leuconostoc mesenteroides	*Pediococcus pentosaceus*	*Enterococcus faecium*	*Lactobacillus curvatus*
Home prepared *Selroti* batter (63)	42.9	28.6	15.9	12.6
Market prepared *Selroti* batter (59)	39.0	27.1	18.6	15.3
Lab-prepared *Selroti* batter (45)	46.7	15.6	26.7	11.1
Average	42.9	23.8	20.4	13.0

[a]Total number of isolates from each source is given in parenthesis.





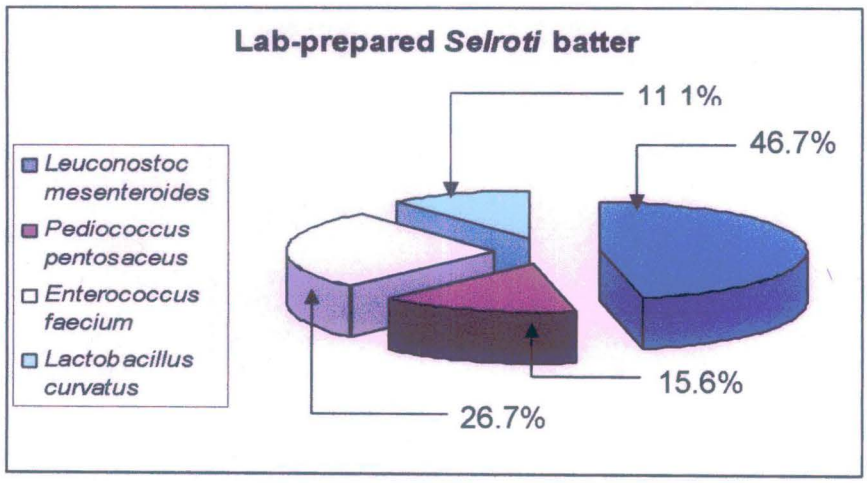


Fig 4. Graphic representation of prevalence of functional LAB in *Selroti* batters of the Sikkim Himalaya.

Prevalence of yeasts

The most dominant yeast recovered in all samples of *Selroti* batters collected from home-made, market and lab-made were *Saccharomyces cerevisiae* represented by 35.6 %, followed by *Debaryomyces hansenii* (17.6 %), *Pichia burtonii* (17.1 %), *Zygosaccharomyces rouxii* (16.3 %) and *Saccharomyces kluyveri* (13.4 %) out of 141 isolates of yeasts (Table 11, Fig 6 and Fig 7).

Table 11. Prevalence of functional yeasts in *Selroti* batters collected from different sources

Source[a]	% of Prevalence				
	Saccharomyces cerevisiae	*Debaryomyces hansenii*	*Pichia burtonii*	*Zygosaccharomyces rouxii*	*Saccharomyces kluyveri*
Home prepared *Selroti* batter (45)	33.3	20.0	20.0	17.8	8.9
Market prepared *Selroti* batter (57)	35.5	17.5	15.8	15.8	15.8
Lab-prepared *Selroti* batter (39)	38.4	15.4	15.4	15.4	15.4
Average	35.6	17.6	17.1	16.3	13.4

[a]Total number of isolates from each source is given in parenthesis.

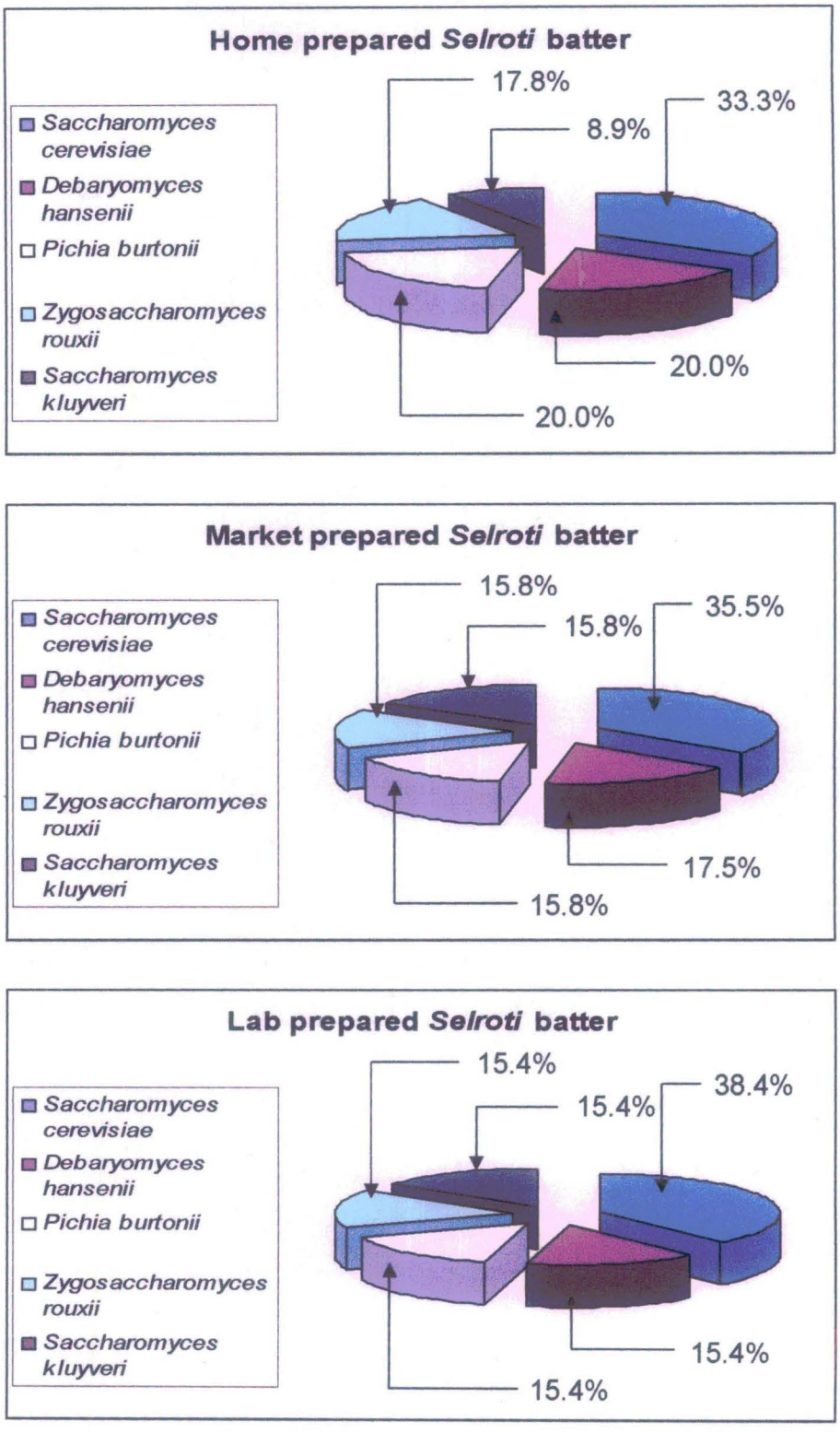


Fig 6. Graphic representation of prevalence of functional yeast in *Selroti* batters of the Sikkim Himalaya.

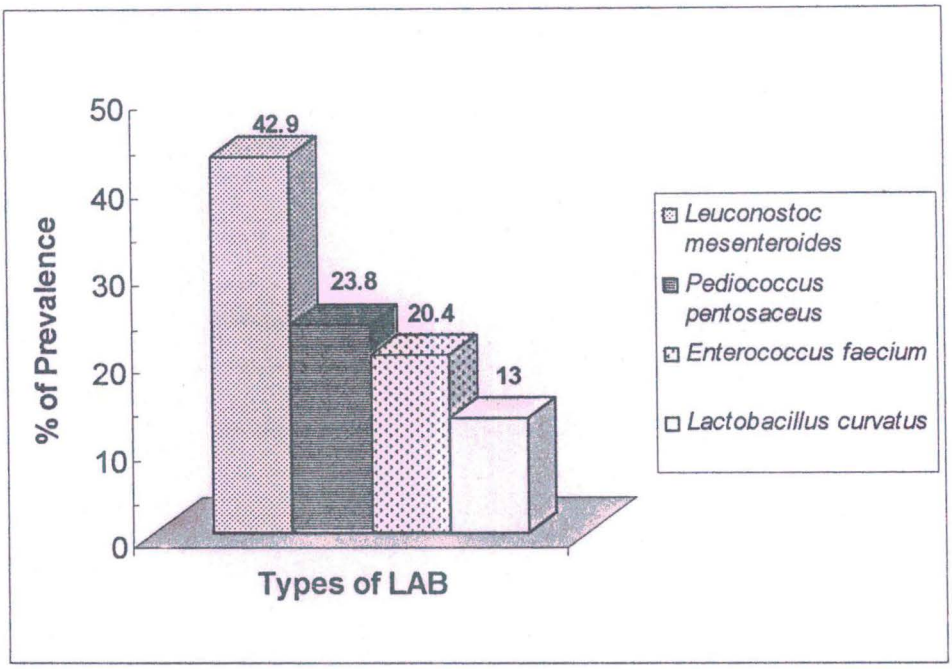


Fig 5. Graphic representation of average prevalence of functional LAB in *Selroti* batters of the Sikkim Himalaya.

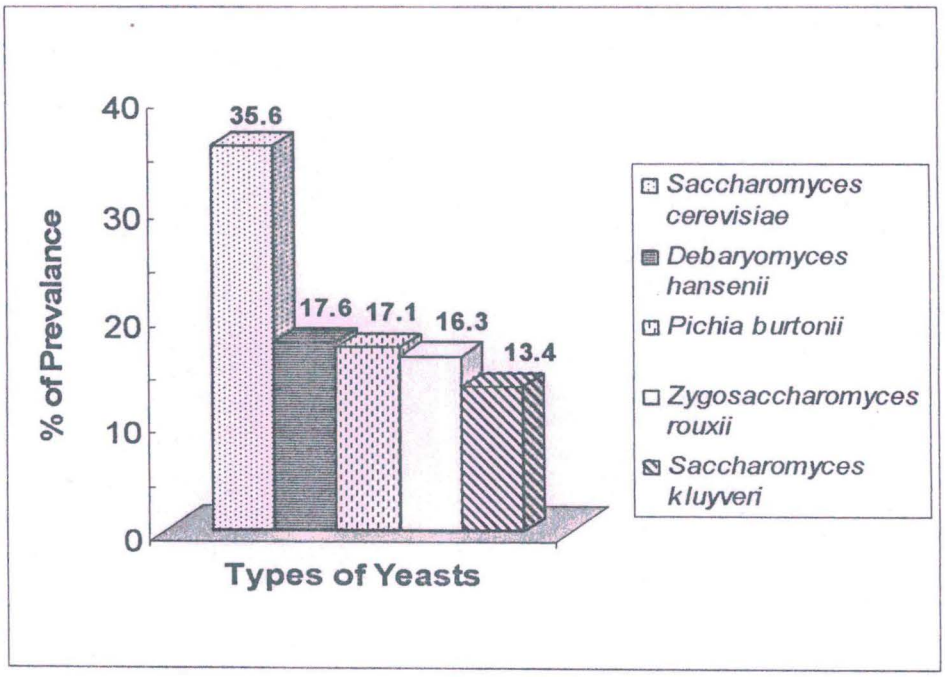


Fig 7. Graphic representation of average prevalence of functional yeast in *Selroti* batters of the Sikkim Himalaya.

Occurrence of bacterial contaminants

A total of 125 samples of *Selroti* batters collected from home-made batters (78), market- batters (36) and lab-made batters (11) were tested for presence of bacterial contaminants such as *Bacillus cereus,* enterobacteriaceae, *Listeria* sp., *Salmonella* sp., *Shigella* sp. and *Staphylococcus aureus* using selective media (Table 12). Food borne pathogens *Bacillus cereus, Listeria* sp., *Salmonella* sp. and *Shigella* sp. were not detected in any sample of fermented batters of the Sikkim Himalaya. In few samples, counts of enterobacteriaceae and *Staphylococcus aureus* were detected below 10^2 cfu/g. No bacterial contaminants were detected in any sample of lab-made *Selroti* batters except enterobacteriaceae around 10 cfu/g.

Table 12. Occurrence of bacterial contaminants in *Selroti* batters collected from different sources

Source	Log cfu/g sample					
	Bacillus cereus	Enterobacteriaceae	*Listeria* sp.	*Salmonella* sp.	*Shigella* sp.	*Staphylococcus aureus*
Home prepared *Selroti* batter (n = 78)	0	2.3 ± 0.4	0	0	0	1.1 ± 0.1
Market prepared *Selroti* batter (n = 36)	0	2.0 ± 0.1	0	0	0	1.3 ± 0.3
Lab-prepared *Selroti* batter[a] (n = 11)	0	1.1 ± 0.3	0	0	0	0

n, Number of samples collected from each source is given in parenthesis.

Data represent the means (± SD) of number of samples.

[a]*Selroti* prepared at Laboratory following the traditional method.

Effect of seasonal variation on microbial load

The effect of seasonal variation on microbial load of *Selroti* batters prepared during summer and winter seasons in Sikkim was studied (Table 13 and Fig 8). A total of 36 samples each were collected during summer and winter from different restaurants, food-stalls, canteens and street-vendors located at Gangtok and Tadong towns in East Sikkim. During summer, it was observed that the microbial load of LAB and yeast was found 10^8 cfu/g and 10^4-10^5 cfu/g, respectively. During winter, the microbial populations were around 10^7 cfu/g, slightly lower than that observed during summer. However, yeasts population increased up to 10^6 cfu/g during winter (Table 13). The average maximum temperature at Gangtok during summer is 22.2° C, whereas temperature in winter 13.8° C (Meteorological Surrey of India, Gangtok). The average pH of the samples during summer and winter was 4.7 and 5.0, respectively. The titratable acidity was 0.11 and 0.09 during summer and winter, respectively (Table 13).

Table 13. The effect of seasonal variation on microbial load of *Selroti* batters prepared during different seasons

Source	pH		Titratable acidity %		Yeasts		LAB	
	Summer[a]	Winter[b]	Summer	Winter	Summer	Winter	Summer	Winter
Lal market of Gangtok (n = 15)	5.4 ± 0.2	5.7 ± 0.3	0.07 ± 0.01	0.05 ± 0.01	5.1 ± 0.2	6.1 ± 0.2	8.5 ± 0.2	7.3 ± 0.2
Tadong bazaar of Gangtok (n = 10)	4.1 ± 0.1	4.5 ± 0.2	0.11 ± 0.02	0.15 ± 0.01	4.8 ± 0.2	6.4 ± 0.1	8.8 ± 0.1	7.1 ± 0.2
Tibet road of Gangtok (n = 11)	4.5 ± 1.0	4.7 ± 1.6	0.14 ± 0.01	0.09 ± 0.01	4.9 ± 0.4	6.4 ± 0.2	8.8 ± 0.2	7.8 ± 0.3
Average	4.7 ± 0.7	5.0 ± 0.6	0.11 ± 0.04	0.09 ± 0.1	5.0 ± 0.2	6.3 ± 0.2	8.7 ± 0.1	7.4 ± 0.3

n, number of samples collected.

Data represent the means (± SD) of number of samples.

[a]Maximum and minimum temperature of Gangtok during summer (May – August) is 22.2°C and 17.0°C, respectively.

[b]Maximum and minimum temperature of Gangtok during winter (Dec – March) is 13.8° C and 5.6° C, respectively.

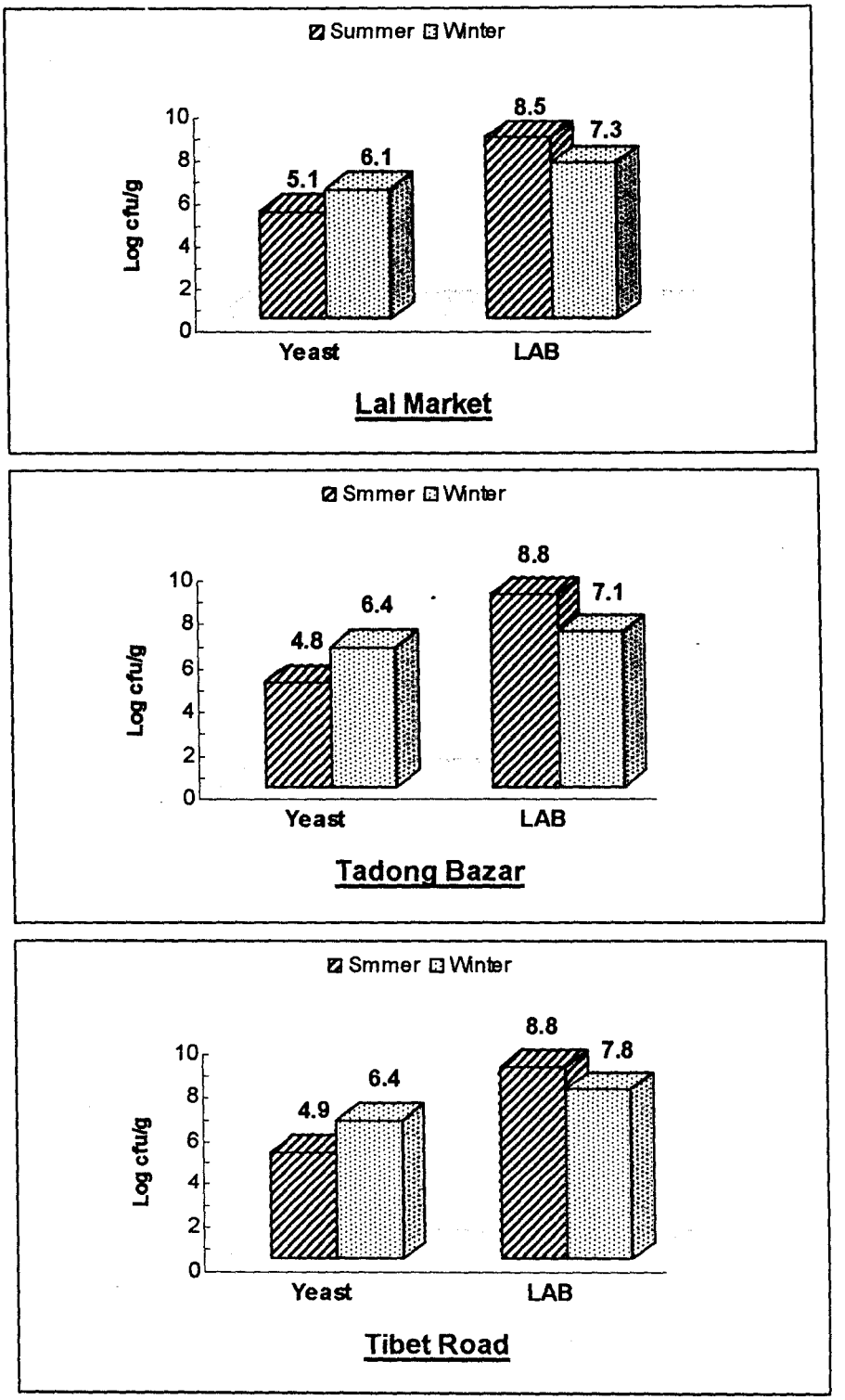


Fig 8. Graphic representation of the effect of different seasons on the yeast and LAB population in *Selroti* batter of market samples of the Sikkim Himalaya.

TECHNOLOGICAL PROPERTIES

Effect of acidification and coagulation

Effect of acidification and coagulation by the LAB and yeast strains isolated from *Selroti* batters from different sources were tested (Table 14 and 15). *Enterococcus faecium* strains BP:B2 and S4:B4 showed the lowest acidification value of pH 4.3 among all the tested strains of LAB, followed by *Enterococcus faecium* strains S5:B6, BS1:B2, L10:B3 and S1:B4 dropping the pH to 4.4. About 63.6 % of LAB strains caused coagulation of milk at 30° C with a significant drop in pH, as shown in Table 14. All strains of *Lactobacillus curvatus, Leuconostoc mesenteroides* and *Enterococcus faecium* coagulated skim milk. The coagulation time ranged from 24 to 30 hour at 30° C. The fastest coagulation time of 24 to 28 hour was observed in several strains of *Lb. Leuconostoc mesenteroides.* However, strains of *Pediococcus pentosaceus* did not coagulate skim milk.

Among yeasts, *Saccharomyces cerevisiae* strain S2:Y2 showed the lowest acidification value of pH 5.6, followed by *S. cerevisiae* strains BA1:Y2, BA2:Y5 and S1:Y5 and *D. hansenii* BR1:Y4 dropping the pH to 5.7 (Table 15). All strains of *S.*

cerevisiae and *D. hansenii* coagulated skim milk. The coagulation time ranged from 34 to 40 hour at 28° C. The fastest coagulation time of 34 to 36 hour was observed in many strains of *S. cerevisiae.* However, none of the strains of *Pichia burtonii, Saccharomyces kluyveri* and *Zygosaccharomyces rouxii* showed coagulating abilities in the applied method.

Table 14. Effect of LAB strains from *Selroti* on acidification and coagulation

Strains	30° C	
	Acidification (pH)	Coagulation
Control	6.4	–
BP:B1	4.8	+
BR1:B1	4.8	+
S5:B1	5.0	+
L2:B1	5.2	+
L0:B1	4.7	+
S6:B1	4.6	+
BG1:B1	4.5	+
BS1:B1	4.6	+
BT1:B2	4.6	+
L1:B4	4.5	+
L1:B2	4.6	+
L3:B2	4.6	+
S1:B3	4.5	+
L6:B4	4.5	+
S5:B6	4.4	+
BS1:B2	4.4	+
BP:B2	4.3	+
L10: B3	4.4	+
BG3:B3	4.5	+
S1:B4	4.4	+
S4:B4	4.3	+
L9:B2	6.0	–
S4:B2	6.1	–
S5:B2	6.0	–
BG2:B2	6.2	–
L1:B3	6.1	–
L3:B3	6.1	–
S2:B3	6.0	–
BP:B3	6.1	–
BG3:B2	6.1	–
BN1:B3	6.0	–
BN2:B3	6.1	–
BA1:B3	6.0	–

Data represents the mean value of 3 sets of experiment.
Coagulation occurred between 24 - 30 hour.
+, indicates coagulation; -, indicates absence of coagulation.

Table 15. Effect of yeast strains from *Selroti* on acidification and coagulation		
Strains	30° C	
	Acidification (pH)	Coagulation
Control	6.4	-
BG1:Y1	6.4	-
BA1:Y1	6.4	-
BG3:Y1	6.3	-
S1:Y1	6.3	-
L1:Y1	6.2	-
BG1:Y2	5.8	+
BA1:Y2	5.7	+
S2:Y2	5.6	+
BG1:Y3	6.4	-
S1:Y3	6.4	-
S3:Y3	6.3	-
L3:Y3	6.4	-
BG1:Y4	5.8	+
BR1:Y4	5.7	+
S4:Y4	5.8	+
S5:Y4	5.9	+
L1:Y4	5.8	+
BA2:Y5	5.7	+
S1:Y5	5.7	+
S1:Y6	6.4	-
L9:Y6	6.3	-
L1:Y7	5.8	+

Data represents the mean value of 3 sets of experiment.

Coagulation occurred between 34 - 40 hour.

+, indicates coagulation; -, indicates absence of coagulation.

Enzymatic profiles

Enzymatic profiles of LAB strains were assayed using the API-zym (bioMérieux, France) galleries (Tables 16). Each of the predominant LAB strain produced a wide spectrum of enzymes. These strains showed relatively weak esterase (C4), moderate phosphatase, and strong arylamidase activities. However, *E. faecium* strains BS1:B2 and S1:B3 showed moderate proteinase activity, whereas, phosphohydrolase activity was shown by all strains tested. *Lb. curvatus* strains BP:B1 and BS1:B1 showed the highest activity (>40 nanomoles) of α-glucosidase in API-zym test. *Leuc. mesenteroides* strains had high α-galactosidase and β-galactosidase activities. *P. pentosaceus* strains showed the highest β- glucosaminidase activities among the LAB strains. None of the strains tested showed alkaline phosphatase, trypsin, β- glucuronidase, α-mannosidase and α-fucosidase activities.

Enzymatic profiles of yeast strains were also assayed using the API-zym (bioMérieux, France) galleries (Tables 17). Yeast strains showed relatively weak esterase (C4) and weak to strong arylamidase and strong phosphatase activities.

However, they showed no detectable proteinase activity in the applied method. Acid phosphatase activity was shown by all strains tested, among which >40 nanomole activities was shown by *D. hansenii*, *P. burtonii* and *Z. rouxii* strains. Phosphohydrolase activity was also shown by all strains tested. None of the yeast strains from *Selroti* batters showed galactosidase activities except weak activities by *S. kluyveri.* Weak glucosaminidase activity was shown by yeast strains, isolated from *Selroti* batters.

Table 16. Enzymatic profiles of LAB strains from *Selroti* batters using API-zym

Enzyme	Strain (Activity in nanomoles)							
	BP:B1	S5:B1	BS1:B1	S6:B1	BS1:B2	S1:B3	BG2:B2	S4:B2
Control (without enzyme)	0	0	0	0	0	0	0	0
Alkaline phosphatase	0	0	0	0	0	0	0	0
Esterase (C4)	0	0	0	0	5	5	0	0
Esterase lipase (C8)	5	5	0	0	10	5	5	5
Lipase (C14)	5	5	0	0	0	0	5	5
Leucine arylamidase	≥40	30	20	10	5	10	≥40	≥40
Valine arylamidase	≥40	30	0	0	0	0	30	30
Cystine arylamidase	30	30	5	10	0	0	20	20
Trypsin	0	0	0	0	0	0	0	0
α-chymotrypsin	0	0	0	0	30	20	0	0
Acid phosphatase	10	10	10	10	5	5	10	10
Napthol-AS-BI-phosphohydrolase	10	10	10	20	10	20	30	30
α-galactosidase	5	5	≥40	≥40	0	0	0	0
β-galactosidase	0	0	≥40	≥40	5	5	0	0
β-glucuronidase	0	0	0	0	0	0	0	0
α-glucosidase	≥40	≥40	0	0	0	0	0	0
β-glucosidase	≥40	≥40	5	5	≥40	30	20	30
N-acetyl-β-glucosaminidase	30	20	0	0	10	10	≥40	≥40
α-mannosidase	0	0	0	0	0	0	0	0
α-fucosidase	0	0	0	0	0	0	0	0

Data represents the means of 3 sets of experiment.

BP:B1, *Lb. curvatus*; S5:B1, *Lb. curvatus*; BS1:B1, *Leuc. mesenteroids*; S6:B1, *Leuc. mesenteroids*; BS1:B2, *E. faecium*; S1:B3, *E. faecium*; BG2:B2, *P. pentosaceus*; S4:B2, *P. pentosaceus*.

Table 17. Enzymatic profiles of yeast strains from *Selroti* batters using API-zym

Enzyme	Strain (Activity in nanomoles)									
	BR1:Y4	S4:Y4	BG1:Y1	S1:Y1	BA1:Y2	S2:Y2	S3:Y3	BG1:Y3	S1:Y6	L9:Y6
Control (without enzyme)	0	0	0	0	0	0	0	0	0	0
Alkaline phosphatase	30	20	20	20	5	5	5	10	0	0
Esterase (C4)	5	5	5	5	5	5	5	5	5	5
Esterase lipase (C8)	5	5	10	10	10	10	5	5	10	20
Lipase (C14)	0	0	0	0	5	5	0	0	0	0
Leucine arylamidase	5	5	30	30	30	≥40	20	20	20	20
Valine arylamidase	0	0	0	0	20	20	5	5	10	5
Cystine arylamidase	0	0	0	0	5	5	0	0	0	0
Trypsin	0	0	0	0	0	0	0	0	0	0
α-chymotrypsin	0	0	0	0	0	0	0	0	0	0
Acid phosphatase	≥40	≥40	≥40	≥40	30	30	20	30	≥40	≥40
Napthol-AS-BI-phosphohydrolase	20	20	30	30	20	30	30	30	30	20
α-galactosidase	0	0	0	0	0	0	5	5	0	0
β-galactosidase	0	0	0	0	0	0	0	0	0	0
β-glucuronidase	0	0	0	0	0	0	0	0	0	0
α-glucosidase	5	5	20	20	30	≥40	≥40	≥40	0	0
β-glucosidase	0	0	20	20	10	30	30	20	0	0
N-acetyl-β-glucosaminidase	5	5	5	5	0	0	0	0	0	0
α-mannosidase	0	0	0	0	0	0	0	0	0	0
α-fucosidase	5	10	0	0	5	5	0	0	0	0

Data represents the means of 3 sets of experiment.

BR1:Y4, *Debaryomyces hansenii*; S4:Y1, *D. hansenii*; BG1:Y1, *Pichia burtonii;* S1:Y1, *P. burtonii*; BA1:Y2, *Saccharomyces cerevisiae*; S2:Y2, *S. cerevisiae*; S3:Y3, *S. kluyveri*; BG1:Y3, *S. kluyveri*; S1:Y6, *Zygosaccharomyces rouxii*; L9:Y6, *Z. rouxii.*

Screening of Bacteriocin activities

Strains of LAB isolated from fermented batters against different pathogenic bacteria such as *Bacillus cereus* CCM 2010, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Listeria monocytogenes* DSM 20600 and *Staphylococcus aureus* S1 were tested for their antimicrobial activities. Most of the LAB strains showed the clear inhibition zones in agar-spot plates against the used pathogenic bacteria, showing antagonisms (data not shown). All strains of LAB were tested for bacteriocin assay against the respective pathogenic bacteria. Cell-free supernatant extract of 24 hour old culture of LAB strains were spotted onto the plates containing the indicator pathogenic organisms. However, none of the tested strains of LAB from *Selroti* batters produced bacteriocin against used pathogenic bacteria under the applied conditions (Table 18).

Table 18. Screening of bacteriocin-producing LAB strains isolated from *Selroti* batters by cell-free culture supernatants

Strains	Indicator strains			
	B. cereus CCM 2010	*K. pneumoniae* subsp. *pneumoniae* BFE 147	*L. monocytogenes* DSM 20600	*S. aureus* S1
BP:B1	-	-	-	-
BR1:B1	-	-	-	-
S5:B1	-	-	-	-
L2:B1	-	-	-	-
L0:B1	-	-	-	-
S6:B1	-	-	-	-
BG1:B1	-	-	-	-
BS1:B1	-	-	-	-
BT1:B2	-	-	-	-
L1:B4	-	-	-	-
L1:B2	-	-	-	-
L3:B2	-	-	-	-
S1:B3	-	-	-	-
L6:B4	-	-	-	-
S5:B6	-	-	-	-
BS1:B2	-	-	-	-
BP:B2	-	-	-	-
L10:B3	-	-	-	-
BG3:B3	-	-	-	-
S1:B4	-	-	-	-

Continued (Table 18)

S4:B4	–	–	–	–
L9:B2	–	–	–	–
S4:B2	–	–	–	–
S5:B2	–	–	–	–
BG2:B2	–	–	–	–
L1:B3	–	–	–	–
L3:B3	–	–	–	–
S2:B3	–	–	–	–
BP:B3	–	–	–	–
BG3:B2	–	–	–	–
BN1:B3	–	–	–	–
BN2:B3	–	–	–	–
BA1:B3	–	–	–	–

Data shows 3 sets of experiments.

–, no zone of inhibition

PROXIMATE COMPOSITION

The proximate composition of unfermented rice and wheat flour obtained from local markets at Gangtok, and samples of *Selroti* batters collected from different sources were analysed (Table 19 and 20). Food value and limited mineral contents (calcium, sodium and potassium) were also determined. The content of moisture, reducing sugar, total sugar, fat and carbohydrate in *Selroti* batters increased over the raw materials. There was a marked increase in water-soluble nitrogen and TCA-soluble nitrogen in products over the raw materials. The food value of fermented batters was found to be increased slightly than the unfermented raw materials (Table 19). Contents of sodium and calcium increased in fermented products (Table 20).

Table 19. Proximate composition of raw materials and fermented product (*Selroti* batter)

Parameter	Raw materials[a]		*Selroti* batter			
	Rice (n = 6)	Wheat flour (n = 6)	Gangtok (n = 15)	Namchi (n =13)	Kalimpong (n = 8)	Darjeeling (n = 10)
pH	5.5 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	5.6 ± 0.5	5.4 ± 0.4	6.1 ± 0.5
Titratable acidity % (as lactic acid)	0.09 ± 0.01	0.1 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.08 ± 0.01
Moisture %	16.3 ± 0.4	18.4 ± 0.7	40.5 ± 7.8	44.5 ± 1.8	44.6 ± 1.9	38.2 ± 6.9
Reducing sugar %	0.01 ± 0.01	0.02 ± 0.01	1.6 ± 0.5	2.5 ± 0.4	2.1 ± 0.9	1.8 ± 0.1
Total sugar %	63.8 ± 0.9	58.4 ± 1.2	67.5 ± 5.8	71.9 ± 6.2	73.2 ± 4.7	63.4 ± 0.9
Ash (% DM)	0.7 ± 0.06	0.5 ± 0.07	0.7 ± 0.02	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Fat (% DM)	1.0 ± 0.01	0.9 ± 0.01	2.9 ± 0.2	2.6 ± 0.7	2.5 ± 0.1	2.6 ± 0.2
Water-soluble nitrogen (% DM)	0.016 ± 0.01	0.056 ± 0.01	0.07 ± 0.02	0.09 ± 0.01	0.036 ± 0.006	0.059 ± 0.03
TCA-soluble nitrogen (% DM)	0.0016 ± 0.001	0.0017 ± 0.003	0.006 ± 0.004	0.003 ± 0.001	0.002 ± 0.0007	0.003 ± 0.001
Protein (% DM)	8.3 ± 0.01	11.0 ± 0.5	5.7 ± 1.0	5.8 ± 0.3	5.9 ± 0.3	5.2 ± 0.3
Carbohydrate (% DM)	90.0 ± 1.0	87.6 ± 0.9	90.7 ± 1.2	90.8 ± 0.5	90.8 ± 0.3	91.5 ± 0.5
Food value (Kcal / 100g DM)	42.2 ± 0.4	402.5 ± 0.5	411.8 ± 0.8	409.8 ± 0.3	409.3 ± 0.4	410.2 ± 0.3

n, total number of samples (n) collected from each source is given in parenthesis.

Data represents the means (± SD) of triplicate of each sample.

DM, dry matter. TCA, trichloro-acetic acid.

[a]Raw materials purchased from Lal Market, Gangtok.

Table 20. Mineral contents of raw materials and fermented *Selroti* batter

Minerals (mg/100g)	Raw materials[a]		*Selroti* batter			
	Rice (n = 6)	Wheat flour (n = 6)	Gangtok (n = 15)	Namchi (n =13)	Kalimpong (n = 8)	Darjeeling (n = 10)
Sodium	5.9 ± 0.7	5.9 ± 0.5	8.5 ± 1.1	9.1 ± 0.5	8.9 ± 0.3	8.9. ± 0.3
Potassium	47.4 ± 1.1	117.5 ± 2.5	35.3 ± 1.9	36.9 ± 0.4	22.5 ± 1.1	24.2 ± 0.5
Calcium	9.4 ± 0.5	20.8 ± 0.2	21.6 ± 1.0	28.8 ± 1.0	24.2 ± 4.4	20.7 ± 0.1

n, total number of samples.

Data represents the means (± SD) of triplicate of each sample.

[a]Raw materials purchased from Lal Market, Gangtok.

SUCCESSIONAL STUDIES DURING FERMENTATION

Selroti batter was prepared in the laboratory following the traditional method as mentioned earlier. Successional studies were carried at every one-hour interval within a range of 0-10 hours during *in situ* fermentation of *Selroti* batter.

Microbiological changes during natural fermentation

Changes in microbial population in fermenting rice during *in situ Selroti* batter fermentation was carried out (Table 21 and Fig 10). Diverse microflora has been recovered in raw rice used as substrates before fermentation (Fig 9). Load of lactic acid bacteria increased significantly ($P<0.05$) from 10^4 cfu/g to 10^8 cfu/g within 0 to 8 hours, and beyond 8 hour the increase in microbial load was insignificant. Subsequently, population of yeasts increased significantly ($P<0.05$) from 10^3 cfu/g to 10^5 cfu/g within 5 hours, and remained relatively constant at the same level till the end. Mould was not detected during natural fermentation of *Selroti* batter. Significant ($P<0.05$) decrease in the beginning and finally disappearance of the contaminant bacteria *Bacillus cereus* and enterobacteriaceae which were present in raw rice grains was observed during fermentation. *Staphylococcus*

aureus was not detected in raw materials as well as in fermented products (Table 21).

Table 21. Microbial changes during *in situ* fermentation of *Selroti* batter

Fermentation time (Hour)	Microbial load (log cfu/g)				
	LAB	Yeast	*Bacillus cereus*	Enterobacteriaceae	*Staphylococcus aureus*
UR	3.1 ± 0.1^{g}	2.8 ± 0.3^{e}	1.1 ± 0.1^{a}	1.5 ± 0.1^{bc}	0
WR	1.8 ± 0.1^{h}	1.8 ± 0.2^{f}	0^{b}	1.9 ± 0.4^{a}	0
0	4.3 ± 0.1^{fj}	3.5 ± 0.1^{cd}	0^{b}	2.1 ± 0.2^{a}	0
1	4.7 ± 0.2^{ej}	3.6 ± 0.1^{c}	0^{b}	1.8 ± 0.1^{ac}	0
2	5.1 ± 0.3^{e}	3.8 ± 0.4^{bc}	0^{b}	1.4 ± 0.2^{bc}	0
3	6.5 ± 0.3^{d}	4.2 ± 0.2^{b}	0^{b}	1.1 ± 0.1^{bd}	0
4	7.0 ± 0.3^{c}	4.7 ± 0.2^{a}	0^{b}	0.8 ± 0.1^{d}	0
5	7.6 ± 0.2^{b}	5.4 ± 0.3^{a}	0^{b}	0	0
6	7.8 ± 0.2^{b}	5.4 ± 0.2^{a}	0^{b}	0	0
7	8.0 ± 0.2^{bi}	5.4 ± 0.2^{a}	0^{b}	0	0
8	8.3 ± 0.1^{ai}	5.6 ± 0.5^{a}	0^{b}	0	0
9	8.7 ± 0.2^{a}	5.7 ± 0.3^{a}	0^{b}	0	0
10	8.8 ± 0.2^{a}	5.4 ± 0.2^{a}	0^{b}	0	0

Data represents the means (±SD) of 3 sets of experiment.
Values bearing different superscripts in each column differ significantly ($P<0.05$).
UR, unwashed rice; WR, washed rice.
LAB, lactic acid bacteria. Mould was not detected.

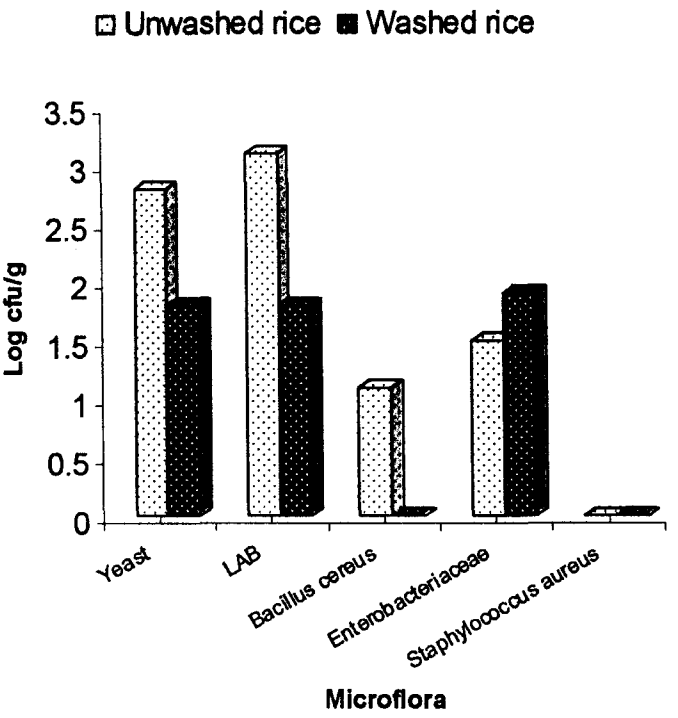


Fig 9. Graphic representation of microorganisms in unwashed and washed rice used for *Selroti* batter fermentation.

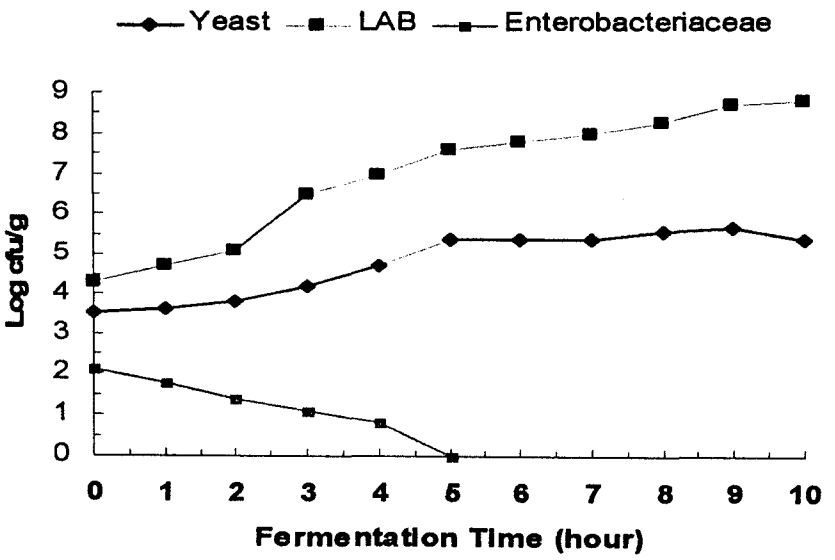


Fig 10. Graphic representation of microbial changes during *in situ Selroti* batter fermentation.

Physico-chemical changes

The mean pH value decreased significantly ($P<0.05$) from 6.5 to 4.1 during *in situ* fermentation (Table 22 and Fig 11). Titratable acidity increased significantly ($P<0.05$) from 0.07 % to 0.15 % throughout the fermentation. The temperature of fermenting cereal batters remained around 29° C at the end of the fermentation. Batter volume and batter weight remained moderately same during natural fermentation.

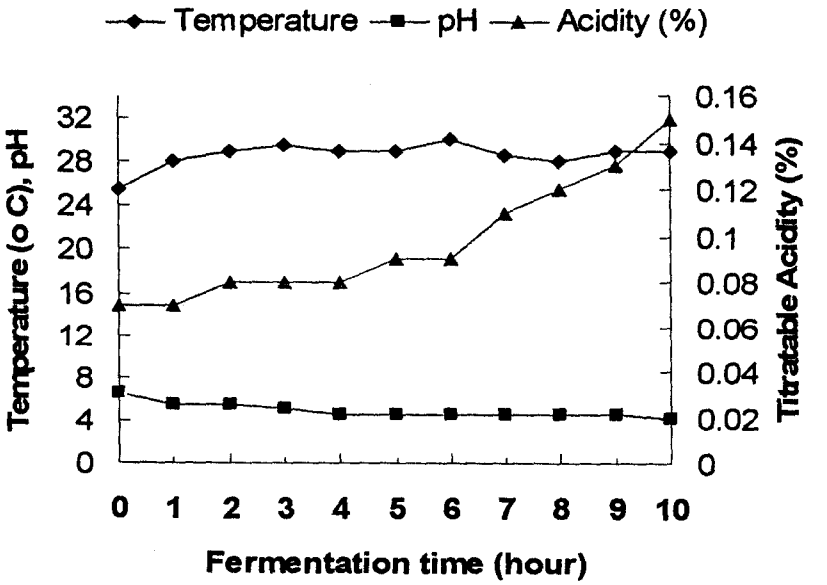


Fig 11. Changes in temperature, pH and acidity during *Selroti* batter fermentation. Values are means of three batches of fermentation.

Table 22. Physico-chemical changes during *Selroti* batter fermentation

Fermentation time (Hour)	pH	Titratable acidity (%)	Batter temperature (°C)	Batter volume (ml)	Batter weight (g)
UR	6.4 ± 0.1^{a}	0.07 ± 0.01^{g}	NA	NA	NA
WR	6.5 ± 0.1^{a}	0.07 ± 0.01^{g}	NA	NA	NA
0	6.5 ± 0.1^{b}	0.07 ± 0.01^{g}	25.5 ± 0.1^{f}	52.0 ± 0.1^{c}	23.95 ± 0.01^{a}
1	5.5 ± 0.1^{b}	0.07 ± 0.01^{g}	28.0 ± 0.1^{e}	52.0 ± 0.1^{c}	23.95 ± 0.01^{a}
2	5.5 ± 0.1^{c}	0.08 ± 0.01^{fg}	29.0 ± 0.1^{c}	52.0 ± 0.2^{c}	23.92 ± 0.01^{b}
3	5.0 ± 0.1^{d}	0.08 ± 0.01^{fg}	29.5 ± 0.2^{b}	52.0 ± 0.1^{c}	23.91 ± 0.02^{bc}
4	4.5 ± 0.1^{d}	0.08 ± 0.01^{fg}	29.0 ± 0.1^{c}	52.0 ± 0.01^{c}	23.89 ± 0.02 ^{bc}
5	4.5 ± 0.2^{d}	0.09 ± 0.01^{ef}	29.0 ± 0.1^{c}	52.0 ± 0.1^{c}	23.88 ± 0.02^{cd}
6	4.5 ± 0.1^{d}	0.09 ± 0.01^{ef}	30.0 ± 0.2^{a}	52.0 ± 0.2^{c}	23.86 ± 0.01^{de}
7	4.5 ± 0.1^{d}	0.11 ± 0.01^{d}	28.5 ± 0.3^{d}	52.0 ± 0.1^{c}	23.86 ± 0.01^{e}
8	4.5 ± 0.2^{d}	0.12 ± 0.01^{cd}	28.0 ± 0.1^{e}	52.1 ± 0.1^{bc}	23.85 ± 0.01^{e}
9	4.5 ± 0.2^{d}	0.13 ± 0.01^{bc}	29.0 ± 0.1^{c}	52.3 ± 0.1^{ab}	23.82 ± 0.02^{f}
10	4.1 ± 0.1^{e}	0.15 ± 0.02^{a}	29.0 ± 0.2^{c}	52.5 ± 0.2 ^{a}	23.82 ± 0.02^{f}

Data represents the means (±SD) of 3 sets of experiment.

Values bearing different superscripts in each column differ significantly ($P<0.05$).

UR, unwashed rice; WR, washed rice.

NA, not applicable.

Sensory properties of Selroti

Batters prepared during natural fermentation of *Selroti* from each hour (0-10) were collected aseptically, and deep-fried in edible oil to make *Selroti* at laboratory, and were subjected to sensory evaluation by 10 judges (Table 23). There was no significant ($P<0.05$) difference in aroma attribute of *Selroti* prepared until 3 hours. They had flat and starchy flavour. There was significant ($P<0.05$) difference in aroma score of *Selroti* prepared at 8 to 9 hours. No significant ($P<0.05$) difference in texture of *Selroti* prepared during 0-10 hours was observed. Similarly, no significant ($P<0.05$) difference in colour attribute of *Selroti* prepared until 5 hours fermentation period was observed, however, the significant ($P<0.05$) increase was seen after 5 hours till 9 hours. In general acceptability, *Selroti* batter prepared for 8 hour period of fermentation showed significantly ($P<0.05$) highest score. Deep-fried *Selroti* prepared for 8 hour following the traditional method had soft texture, sweet taste and aroma, significantly ($P<0.05$) acceptable to judges (Table 23).

Table 23. Sensory evaluation of *Selroti* batter prepared during natural fermentation

Fermentation time (Hour)	Attribute				
	Aroma	Taste	Texture	Colour	General acceptability
0	1.2 ± 0.5^{e}	1.2 ± 0.1^{de}	2.6 ± 0.6^{a}	2.0 ± 0.1^{b}	1.2 ± 0.5^{gd}
1	1.2 ± 0.5^{e}	1.2 ± 0.1^{de}	2.6 ± 0.6^{a}	2.0 ± 0.1^{b}	1.2 ± 0.5^{gd}
2	1.2 ± 0.5^{e}	1.4 ± 0.5^{ce}	2.6 ± 0.6^{a}	2.0 ± 0.1^{b}	1.4 ± 0.6^{fd}
3	1.8 ± 0.5^{de}	1.4 ± 0.6^{ce}	2.8 ± 0.5^{a}	2.0 ± 0.1^{b}	1.6 ± 0.6^{ed}
4	2.0 ± 0.7^{cfe}	2.4 ± 0.6^{ae}	2.8 ± 0.5^{a}	2.0 ± 0.1^{b}	2.2 ± 0.5^{cdefg}
5	2.2 ± 0.5^{bdf}	2.6 ± 0.8^{abcd}	3.0 ± 0.6^{a}	2.0 ± 0.1^{b}	2.6 ± 0.6^{bcdef}
6	2.8 ± 0.5^{af}	2.6 ± 0.8^{abcd}	3.0 ± 0.4^{a}	2.4 ± 0.6^{ab}	3.0 ± 0.1^{abcd}
7	3.0 ± 0.7^{ab}	2.6 ± 1.1^{abcd}	3.8 ± 0.5^{a}	2.8 ± 0.5^{ab}	3.0 ± 0.1^{ab}
8	3.6 ± 0.6^{a}	4.0 ± 0.6^{a}	4.0 ± 0.4^{a}	3.8 ± 0.5^{a}	4.0 ± 0.7^{a}
9	3.6 ± 0.6^{a}	3.2 ± 1.3^{ab}	4.0 ± 0.6^{a}	3.8 ± 0.8^{a}	3.6 ± 0.9^{ab}
10	3.0 ± 1.0^{ab}	1.8 ± 1.1^{be}	3.8 ± 0.4^{a}	2.8 ± 0.8^{ab}	1.8 ± 0.7^{d}

Data represents the mean scores (±SD) of ten judges.

Values bearing different superscripts in each column differ significantly ($P<0.05$).

Market fried *Selroti* was used as control (score 3), score 1, bad/hard; score 5, excellent/soft.

STARTER CULTURE PREPARATION

Starter cultures of LAB and yeasts isolated from native *Selroti* batters were tested singly or in combination for their ability to ferment rice flour to produce *Selroti.* Different starter cultures and their combinations used were Starter A- cells of *Leuconostoc mesenteroides* BS1:B1; Starter B- mixture of cells of all LAB strains (*Enterococcus faecium* BS1:B2; *Lactobacillus curvatus* BP:B1; *Leuconostoc mesenteroides* BS1:B1; *Pediococcus pentosaceus* BG:B2); Starter C- cells of *Saccharomyces cerevisiae* BA1:Y2; Starter D- mixture of cells of all yeasts strains (*Debaryomyces hansenii* BR1:Y4; *Pichia burtonii* BG1:Y1; *Saccharomyces cerevisiae* BA1:Y2; *S. kluyveri* S3:Y3; *Zygosaccharomyces rouxii* S1:Y6); Starter E- mixture of B and D (LAB and yeasts) mentioned above; Starter F- mixture of cells of *Leuconostoc mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2. *Leuconostoc mesenteroides* BS1:B1 was selected as a starter culture based on its heterofermentative property, superior technological properties such as acidifying ability, antagonistic properties and high enzymatic profiles than most of the other genera. *Saccharomyces cerevisiae* BA1:Y2

was selected based on vigorous fermentative property and a wide spectrum of enzymes.

Sensory evaluation of Selroti prepared by starter culture

The batter was prepared following the traditional method as described earlier. About 350 g of batter was equally distributed in sterile 500-ml Duran bottles with screw caps. Each batter was inoculated with 1ml of the starter culture(s) per 100 g of batter either singly or in combinations as described before; mixed thoroughly by a sterile spatula and incubated at 28° C. The pH and titratable acidity were determined at 0 hour, 4 hour and 6 hour intervals. *Selroti*, prepared from different batter samples incubated for 4 hours and 6 hours were deep-fried in hot edible oil and served to 10 judges for sensory evaluation using the format (Table C), as described in Materials and Methods section.

There was a significant ($P<0.05$) decrease and increase in pH and titratable acidity of the fermenting batters, inoculated by starter culture(s), respectively from 0 hour to 6 hour (Table 24 and Fig 12 a,b). There was no significant ($P<0.05$) difference in all sensory attributes of *Selroti* prepared

by different combinations of pure cultures starters except Starter F, a mixture of *Leuc. mesenteroides* and *S. cerevisiae* (Table 25). Organoleptically, deep-fried *Selroti* prepared from 4-hour fermented batter inoculated with Starter F scored significantly ($P<0.05$) highest in taste, aroma, texture and general acceptability (Fig 13). *Selroti* prepared from the batter supplemented with Starter B, Starter D and Starter E strains had undesirable sweet sour taste and unpleasant acidic flovour due to high acid content, which were unacceptable to consumers. Fried *Selroti* prepared from batters fermented by a mixed starter culture of *Leuconostoc mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2 for 4 hour, had desirable sweet taste, typical *Selroti* flavour, soft texture, thus significantly ($P<0.05$) acceptable to judges.

Table 24. Changes in pH and titratable acidity during *Selroti* batter fermentation using selected starter cultures

Starter culture	pH			Titratable acidity %		
	0 hour	4 hour	6 hour	0 hour	4 hour	6 hour
A	6.1 ± 0.1^{a}	4.6 ± 0.1^{b}	4.0 ± 0.1^{de}	0.06 ± 0.01^{e}	0.15 ± 0.01^{dh}	0.21 ± 0.01^{bf}
B	6.1 ± 0.1^{a}	4.2 ± 0.1^{b}	3.8 ± 0.1^{e}	0.06 ± 0.01^{e}	0.16 ± 0.01^{dg}	0.22 ± 0.02^{b}
C	6.1 ± 0.1^{a}	4.6 ± 0.1^{b}	4.1 ± 0.1^{d}	0.05 ± 0.01^{e}	0.14 ± 0.02^{d}	0.17 ± 0.02^{cgh}
D	6.2 ± 0.1^{a}	4.2 ± 0.1^{b}	4.0 ± 0.1^{de}	0.05 ± 0.01^{e}	0.17 ± 0.01^{cgh}	0.21 ± 0.01^{bf}
E	6.1 ± 0.1^{a}	4.0 ± 0.1^{de}	3.8 ± 0.1^{e}	0.06 ± 0.01^{e}	0.19 ± 0.01^{cf}	0.25 ± 0.01^{a}
F	6.1 ± 0.1^{a}	4.4 ± 0.1^{c}	3.9 ± 0.1^{e}	0.06 ± 0.1^{e}	0.16 ± 0.01^{dg}	0.22 ± 0.02^{b}
G	6.2 ± 0.1^{a}	4.7 ± 0.1^{b}	4.1 ± 0.1^{d}	0.06 ± 0.01^{e}	0.14 ± 0.02^{d}	0.17 ± 0.02^{cgh}

Data represents the means scores ±SD of three sets of experiments. Values bearing different superscripts in each column differ significantly ($P<0.05$).

A, *Leuconostoc mesenteroides* BS1:B1.

B, all strains of LAB (*Enterococcus faecium* BS1:B2; *Lb. curvatus* BP:B1; *Leuconostoc mesenteroides* BS1:B1; *Pediococcus pentosaceus* BG2:B2).

C, *Saccharomyces cerevisiae* BA1:Y2.

D, all strains of yeasts (*Debaryomyces hansenii* BR1:Y4; *Pichia burtonii* BG1:Y1; *Saccharomyces cerevisiae* BA1:Y2; *S. kluyveri* S3:Y3; *Zygosaccharomyces rouxii* S1:Y6).

E, mixture of B and D (LAB + Yeasts) mentioned above.

F, mixture of *Leuc. mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2.

G, without inoculum.

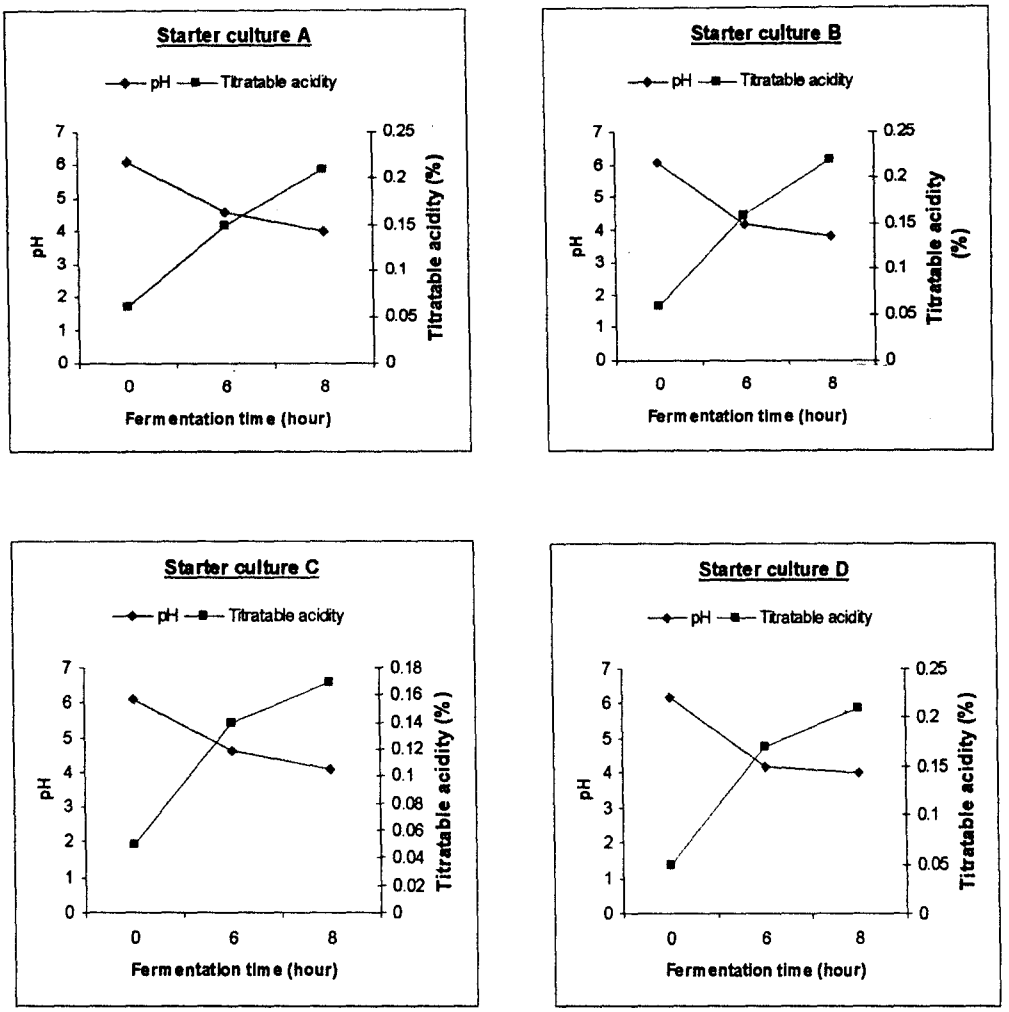


Fig 12 a. Changes in pH and titratable acidity during *Selroti* batter fermentation using selected starter culture. Values are means of three batches of fermentation.

A, *Leuconostoc mesenteroides* BS1:B1;

B, all lactic acid bacteria isolated from *Selroti* batter (*Enterococcus faecium* BS1:B2, *Lb. curvatus* BP:B1, *Leuconostoc mesenteroides* BS1:B1, *Pediococcus pentosaceus* BG2:B2);

C, *Saccharomyces cerevisiae* BA1:Y2;

D, all yeasts isolated from *Selroti* batter (*Debaryomyces hansenii* BR1:Y4, *Pichia burtonii* BG1:Y1, *Saccharomyces cerevisiae* BA1:Y2, *S. kluyveri* S3:Y3, *Zygosaccharomyces rouxii* S1:Y6);

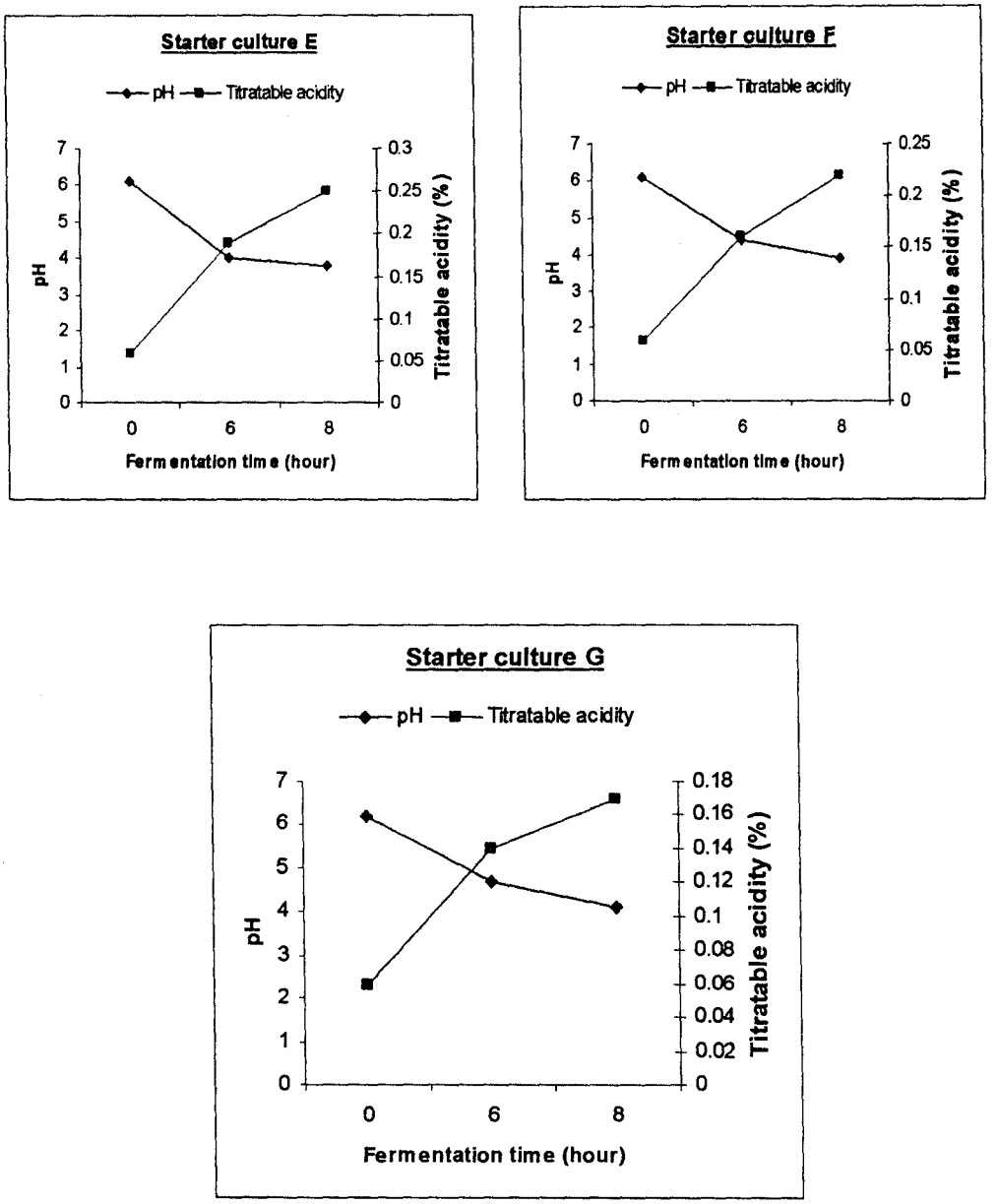


Fig 12 b. Changes in pH and titratable acidity during *Selroti* batter fermentation using selected starter culture. Values are means of three batches of fermentation.

E, mixture of B and D mentioned above;

F, mixture of *Leuc. mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2;

G, without inoculum.

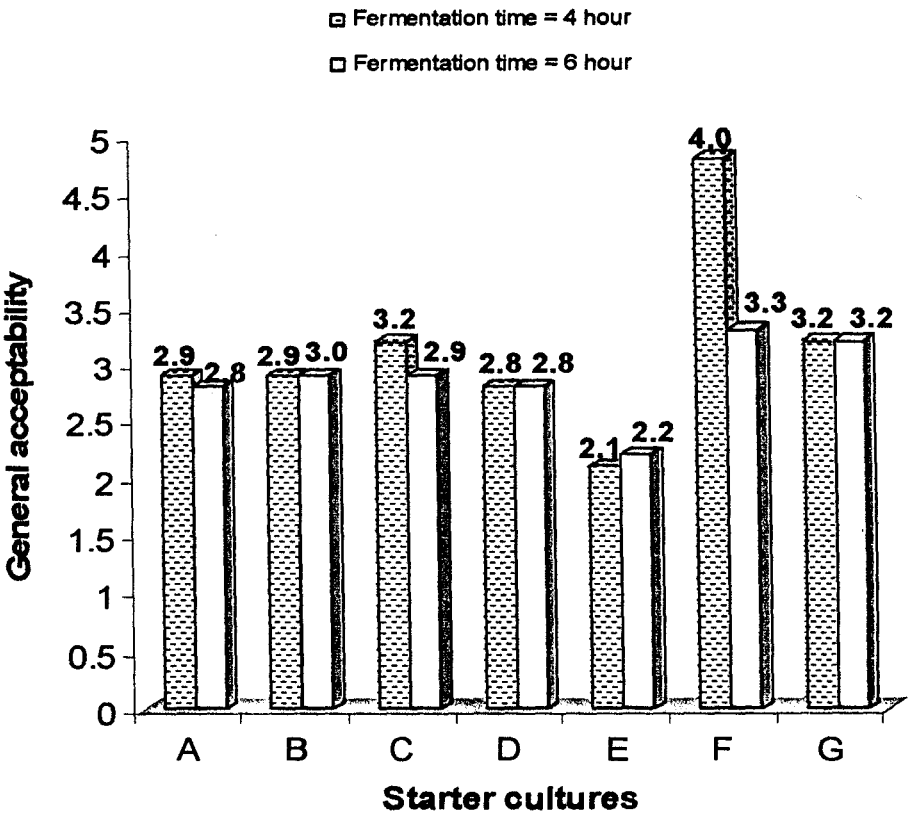


Fig 13. Graphic representation of sensory general acceptability of *Selroti* prepared using starter cultures different combinations of starter cultures as mentioned below.

A, *Leuconostoc mesenteroides* BS1:B1;

B, all lactic acid bacteria isolated from *Selroti* batter (*Enterococcus faecium* BS1:B2, *Lb. curvatus* BP:B1, *Leuconostoc mesenteroides* BS1:B1, *Pediococcus pentosaceus* BG2:B2);

C, *Saccharomyces cerevisiae* BA1:Y2;

D, all yeasts isolated from *Selroti* batter (*Debaryomyces hansenii* BR1:Y4, *Pichia burtonii* BG1:Y1, *Saccharomyces cerevisiae* BA1:Y2, *S. kluyveri* S3:Y3, *Zygosaccharomyces rouxii* S1:Y6);

E, mixture of B and D mentioned above;

F, mixture of *Leuc. mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2;

G, without inoculum.

Table 25. Sensory evaluation of *Selroti* batter prepared using selected starter cultures

Starter culture	Fermentation time (Hour)	Attribute				
		Aroma	Taste	Texture	Colour	General acceptability
A	4	3.0 ± 0.6[a]	2.8 ± 0.7[b]	2.7 ± 0.7[a]	3.2 ± 0.7[a]	2.9 ± 0.6[b]
	6	2.7 ± 1.3[a]	2.8 ± 1.1[b]	3.7 ± 1.0[a]	3.3 ± 0.7[a]	2.8 ± 1.1[b]
B	4	2.9 ± 0.6[a]	2.8 ± 0.7[b]	3.3 ± 0.8[a]	2.2 ± 0.8[b]	2.9 ± 0.6[b]
	6	3.1 ± 0.6[a]	2.9 ± 0.6[b]	2.6 ± 1.0[a]	3.1 ± 0.6[a]	2.9 ± 0.6[b]
C	4	3.0 ± 0.7[a]	3.1 ± 0.7[a]	3.8 ± 0.8[a]	3.2 ± 0.3[a]	3.2 ± 0.6[b]
	6	2.7 ± 0.6[a]	2.7 ± 0.7[b]	2.6 ± 0.9[a]	3.3 ± 0.6[a]	2.9 ± 0.8[b]
D	4	2.9 ± 0.6[a]	2.9 ± 1.0[b]	2.7 ± 0.8[a]	2.2 ± 0.8[b]	2.8 ± 0.8[b]
	6	2.4 ± 0.6[a]	2.9 ± 0.6[b]	3.3 ± 0.8[a]	3.2 ± 0.5[a]	2.8 ± 0.8[b]
E	4	2.5 ± 0.9[a]	2.1 ± 1.0[b]	1.9 ± 1.2[b]	2.3 ± 0.9[b]	2.1 ± 1.0[b]
	6	2.7 ± 0.5[a]	2.1 ± 1.2[b]	2.0 ± 1.1[b]	2.6 ± 0.7[a]	2.2 ± 1.1[b]
F	4	4.0 ± 1.0[a]	4.6 ± 1.1[a]	4.0 ± 1.1[a]	3.9 ± 0.7[a]	4.8 ± 1.0[a]
	6	3.1 ± 1.0[a]	3.4 ± 1.1[a]	3.8 ± 1.1[a]	3.7 ± 0.8[a]	3.3 ± 1.3[b]
G	4	2.8 ± 0.8[a]	3.3 ± 0.8[a]	3.3 ± 0.7[a]	3.3 ± 0.7[a]	3.2 ± 0.8[b]
	6	3.7 ± 1.0[a]	3.8 ± 1.2[a]	4.0 ± 0.9[a]	3.9 ± 0.9[a]	3.2 ± 1.0[b]

Market fried *Selroti* was used as control (score 3), score 1, bad/hard; score 5, excellent/soft.

Data represents the means scores ±SD of 10 judges. Values bearing different superscripts in each column differ significantly ($P<0.05$).

A, *Leuconostoc mesenteroides* BS1:B1.

B, all strains of LAB (*Enterococcus faecium* BS1:B2; *Lb. curvatus* BP:B1; *Leuconostoc mesenteroides* BS1:B1; *Pediococcus pentosaceus* BG2:B2).

C, *Saccharomyces cerevisiae* BA1:Y2.

D, all strains of yeasts (*Debaryomyces hansenii* BR1:Y4; *Pichia burtonii* BG1:Y1; *Saccharomyces cerevisiae* BA1:Y2; *S. kluyveri* S3:Y3; *Zygosaccharomyces rouxii* S1:Y6).

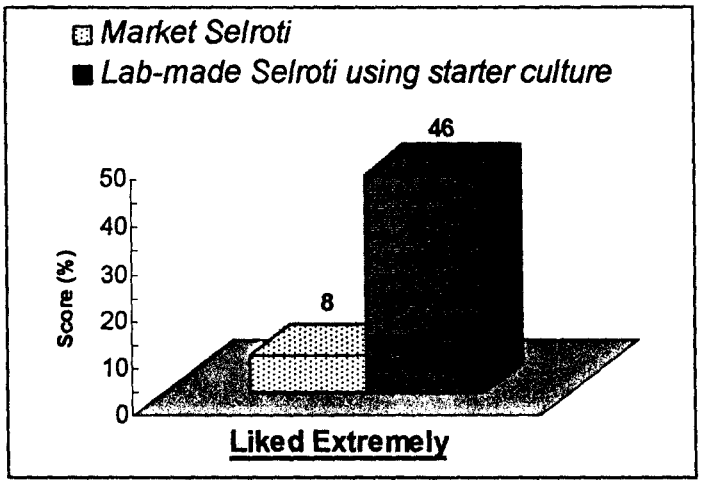
E, mixture of B and D (LAB + Yeasts) mentioned above.

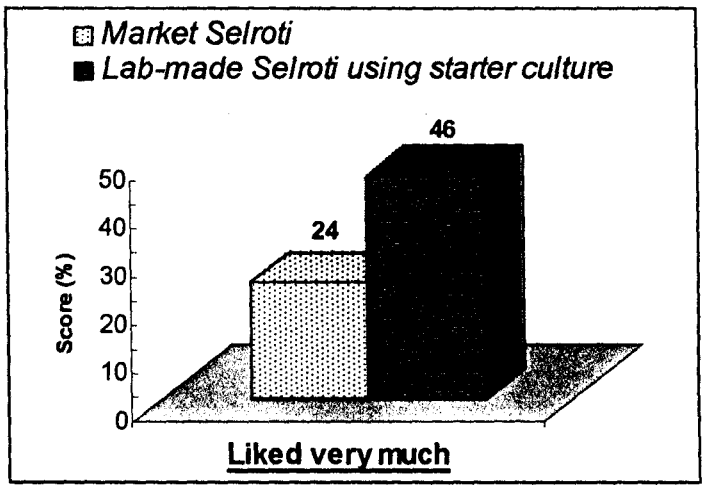
F, mixture of *Leuc. Mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2.

G, without inoculum.

Consumers' Preference Trial

Selroti batter was prepared by using starter culture of *Leuconostoc mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2 for 4 hours, deep-fried, and served freshly to 50 consumers from different places for consumers' preference trial as the method described in Materials and Metods. The consumers' preference trial showed that *Selroti* batter prepared in the laboratory by cell suspension mixture of *Leuc. mesenteroides* BS1:B1 and *S. cerevisiae* BA1:Y2 as starter was more acceptable than *Selroti* batters prepared by conventional method. Market *Selroti* was liked extremely (score, 9) by 8 %, very much (score, 8) by 24 % and moderately (score, 7) by 68 %, whereas deep-fried *Selroti* prepared from batters fermented by a pure cultures mixture of LAB + yeast (*Leuc. mesenteroides* + *S. cerevisiae*) was liked extremely by 46 %, very much by 48 % and moderately by 6 % of the consumers (Fig 14).





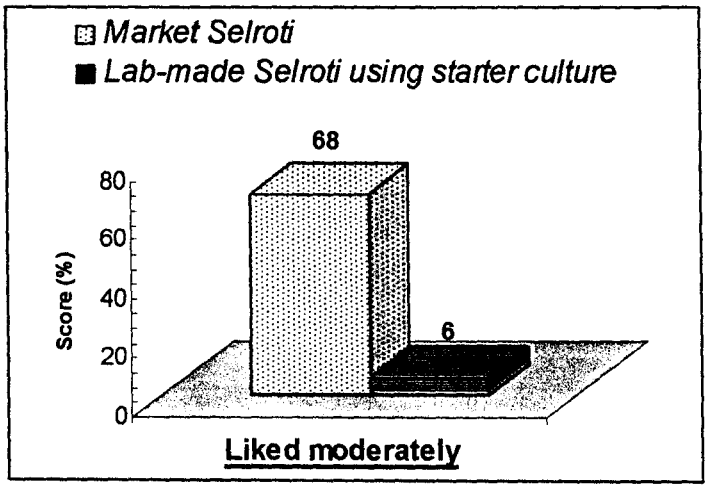


Fig 14. Consumers' preference trial of *Selroti*

DISCUSSION

Documentation of traditional knowledge

Traditional knowledge of the ethnic people living in the Sikkim Himalaya on production of fermented cereal-based food was worth documenting, both as functional foods, and for socio-cultural reasons. *Selroti* is an important fermented cereal-based food in the local diet of the Sikkim Himalaya, and are prepared at households. Its production practiced by the ethnic people of Sikkim is a unique type of food production by a natural fermentation.

Information on indigenous knowledge and antiquity of *Selroti* was sought from village elders and older women during survey, which has been well documented in this thesis. *Roti* is a Nepali word for bread. Out of many kinds of bread the Nepalis consume, two of them have a special place in the society. One is *Babari* and the other is *Selroti. Babari* is round, solid pancake whereas *Selroti* is ring shaped pretzel-like bread. Both are prepared from grounded rice flour. It was recorded during interview that in olden days only *Babari* was prepared and consumed by the people instead of *Selroti.* Since, the consumers found it difficult to fry in a pan, especially to turn it upside down; they started making rings with the batter. And to turn this ring-shaped bread they

started using a poker locally called 'suiro' (a pointed bamboo stick). Anything lifted with a 'suiro' is called 'saela' in the Nepali language. Probably, the word *Selroti* might have derived from the word 'saela'. That is how preparation and consumption of *Babari* among the Nepalis was slowly replaced by *Selroti* preparation, which has become a distinct food culture of the Nepalis.

There is a hypothesis on the nomenclature of *Selroti.* The word 'seli' is a name for local variety of rice cultivated in foot hills of Nepal. The product prepared from 'seli' variety of rice might have been called as *Selroti.* In Nepali, the word 'saal' means a year. Since *Selroti* is prepared during *Tiwar,* one of the main festivals of the Nepalis which is celebrated once in a year. Some people believe that the word *Selroti* has originated from the word 'saal' meaning confectionary bread prepare during festival once in a year. The antiquity of *Selroti* remains a myth, no historical documents or monographs were available on this product. Documentation of ethnical information on antiquity of *Selroti* during our survey will provide vital information on the history and food culture of the Nepalis in the Sikkim Himalaya.

In olden days, *Selroti* preparation did not include the use of spices or condiments but now-a-days, because of the development of diversified taste, people prefer to add spices during *Selroti* preparation.

Selroti has a deeply rooted ethnic importance among the Nepalis. *Selroti* is served during marriage ceremony of various castes of the Nepalis along with other traditional food items. It is a customary to hand over a basket full of freshly fried *Selroti* to bride's parents by the groom during marriage among the Nepalis. This is probably to supplement the sweet-dish, which is traditionally not common among the Nepalis, for greetings. Traditionally, newly married Nepali bride visits her parent's house once in a year. When she returns back to her husband's house she should carry a 'thumsey' (local name for bamboo basket) containing freshly fried-*Selroti.* This traditional is known as 'pani roti' in Nepali.

Selroti is traditionally served along with other traditional food items during *Bhai Tika,* a Hindu festival of the Nepalis, which is observed to honour the brothers by their sisters. Beside this, it is also served during other festivals of the Nepalis like *chaitay dasai, maghay sakranti, bara dasai,* etc.

Fried *Selroti* products can be preserved for about 10-15 days without refrigeration and consumed as it is or slightly warmed up. People might have invented such preservation technique to feed themselves while traveling. Carrying fried *Selroti* is a traditional practice in the Sikkim Himalaya while traveling for long distances (Tamang, 2005a).

It was understood from the feeding frequency summarized in Table 3 that *Selroti* is prepared and consumed occasionally may be because its preparation is labour-intensive, time consuming and costly. *Selroti* is mostly prepared at home (75.6 %) comparable to market purchase. Among the ethnic groups in Sikkim, 93.4 % of Nepali prepared *Selroti* at home, justifying that *Selroti* has an important bearing in the dietary habits of the Nepalis in the Sikkim Himalaya.

Microorganisms

The microbial population of *Selroti* batters collected from different sources revealed that lactic acid bacteria (LAB), comprising lactobacilli, pediococci, leuconostocs and enterococci were the predominant microorganisms present in viable numbers above 10^8 cfu/g, followed by yeasts around

10^5 cfu/g, respectively. The identity of the LAB strains seems to be similar with that of LAB typically present in many traditional cereal-based fermented foods of other regions (Soni and Sandhu, 1990; Oyewole, 1997; Brandt, 2007). Taxonomically diverse species of LAB have been identified from *Selroti* batters of the Sikkim Himalaya. They represented the four genera of LAB - *Lactobacillus, Leuconostoc, Pediococcus* and *Enterococcus.* Classification of LAB into different genera is largely based on morphology, gas production from glucose (Kandler, 1983), mode of glucose fermentation, and growth at different temperatures (Mundt, 1986; Dykes, 1994).

On the basis of a combination of phenotypic properties and the API sugar profile data, strains of LAB isolated from *Selroti* batters were identified as *Leuconostoc mesenteroides, Enterococcus faecium, Pediococcus pentosaceus* and *Lactobacillus curvatus. Leuc. mesenteroides* has been reported in several cereal-based fermented foods such as *idli* of India (Mukherjee *et al.,* 1965), *enjera* of Ethiopia (Oyewole, 1997), *puto* of the Philippines (Kelly *et al.,* 1995); *mawè* of Togo and Benin (Hounhouigan *et al.,* 1993b). Enteroccoci play beneficial role in production of many fermented foods

(Bouton *et al.*, 1998; Cintas *et al.*, 2000). *E. faecium* appears to pose a low risk for use in foods, because these strains generally harbour fewer recognised virulence determinants than *E. faecalis* (Franz *et al.*, 2003). *P. pentosaceous* along with several species of lactobacilli were reported as predominant LAB strains in Tanzanian *togwa* (Mugula *et al.*, 2003a); in *mawè* of Togo and Benin (Hounhouigan *et al.*, 1993b) and in *kodo ko jaanr* of India (Thapa and Tamang, 2004). There are not many reports on the occurrence of *Lb. curvatus* in fermented cereal-based foods. *Lb. curvatus* has been reported in *mawè*, a fermented maize food in lesser percentage of prevalence than other LAB (Hounhouigan *et al.*, 1993b).

Though the dominant microflora in fermented *Selroti* products was LAB, a sizable number of yeasts were also reported. Based on the detailed characterisations and identification profiles, the following yeasts were isolated and identified from *Selroti* batters: *Saccharomyces cerevisiae, Saccharomyces kluyveri, Debaryomyces hansenii, Pichia burtonii* and *Zygosaccharomyces rouxii. Saccharomyces cerevisiae* is the principal yeast of most bread fermentations (Jenson, 1998). Yeast fermentation serves several functions

in sourdough production (Hammes and Ganzle, 1998). Gas production causes expansion and leavening of the dough, ultimately affecting the texture, density and volume of the bread (Hammes *et al.*, 2005). *Saccharomyces kluyveri* has been reported in *nan*, a leaved bread of North India (Batra, 1986). *Debaryomyces hansenii* has been isolated from *idli* along with several other yeasts (Soni and Sandhu, 1991). *Pichia burtonii* has been reported in some Asian rice-based alcoholic starters such as *loog-pang* of Thailand (Limtong *et al.*, 2002) and *marcha* of Sikkim (Tsuyoshi *et al.*, 2005). However, *Pichia burtonii* produces visible, white or chalky discoloration in sourdough (Legan and Voysey, 1991). Acid-tolerant *Zygosaccharomyces rouxii* has not been reported in cereal-based fermented foods, though it has been reported in many fermented soybean foods of Asia which contribute aroma to the product (Aidoo *et al.*, 2006). Origin of *Z. rouxxii* is usually from sugar, honey and confectionery (Kreger-van Rij, 1984). Probably recovery of *Z. rouxii* in *Selroti* batters was their entry through sugars and honey which are added during *Selroti* batter preparation to make it sweet.

The most prevalent LAB and yeasts in all samples of *Selroti* batters were *Leuc. mesenteroids* and *S. cerevisiae*, respectively which were recovered in all samples analysed as predominant organisms. Predominance of *Leuc. mesenteroids* and *S. cerevisiae* have been common in other cereal-based fermented foods (Steinkraus, 1996; Brandt, 2007).

Occurrence of bacterial contaminants

Food borne pathogens *Bacillus cereus, Listeria* sp., *Salmonella* sp. and *Shigella* sp. were not detected in any sample of fermented batters of *Selroti*, due to slight acidic nature of the products. Fermentation of cereals reduces contamination of weaning foods in Ghana (Mensah *et al.*, 1990). These contaminants might have introduced during handling of raw materials for preparation when pH was not low enough to inhibit their growth. High population ($>10^8$ cfu) of LAB in *Selroti* batters (Table 4 and 5), could restrict the growth of other organisms simply by their physical occupation of available space and uptake of most readily assimilative nutrients (Adams and Nicolaides, 1997). It is also a fact that lactic acid produced by LAB may reduce pH to a

level where pathogenic bacteria may be inhibited (Holzapfel *et al.*, 1995; Tsai and Ingham, 1997; Adams and Nout, 2001).

Effect of seasonal variation on microbial load

It was observed that seasons affect the development and prevalence of microorganisms in the fermented batters. During summer, the microbial load of LAB increased due to rise in temperature which may accelerate fermentation rate. Whereas, winter was favourable for yeasts. Similar observation on effect of seasonal variation was made during *idli* fermentation favouring the bacterial load (Soni *et al.*, 1986).

Technological properties of isolates

Technological or technical properties of LAB strains isolated from fermented foods are important criteria for selection of starter cultures to be used in the manufacture of functional foods (Durlu-Ozkaya, 2001; Badis, 2004). Acidification is an important technological property in relevance of selection for starter culture among the LAB (de Vuyst, 2000). LAB and yeast strains isolated from *Selroti* batters were screened for their acidifying and coagulating

capacity, and found that most of the LAB strains acidified with lowering of pH up to 4.3 (Table 14). About 63.6 % of LAB strains caused coagulation of skim milk at 30° C (Table 14). Coagulation of milk by LAB strains shows their potential as starters or adjunct cultures in the production of fermented products. Among yeasts strains, only *S. cerevisiae* and *D. hansenii* showed acidification characters, though decrease in pH was limited to 5.6 (Table 15). These strains although originating from plant sources, appeared to be adapted to the milk ecology, since they coagulated and acidified the skim milk used in the applied method. The casein degradation initiated with milk clotting peptidases and proteinases, which produce peptides and amino acids (Mäyra-Mäkinen and Bigret, 1998).

The use of the API-zym technique has been reported (Arora *et al.*, 1990) as a rapid and simple means of evaluating and localising 19 different hydrolases of microorganisms associated with dairy fermentations. This method is also of relevance for selection of strains as potential starter cultures based on superior enzyme profiles, especially peptidases and esterases, for accelerated maturation and flavour development of other fermented products (Tamang *et al.*,

2000; Kostinek *et al.*, 2005). Absence of proteinases (trypsin) and presence of strong peptidase (leucine-, valine-, and cystine-arylamidase) activities produced by the predominant LAB strains isolated from *Selroti* batters are possible traits of desirable quality for their use in production of typical flavour and aroma. *Leuc. mesenteroides* isolated from Armada cheese showed the highest aminopeptidase activity (Herreros *et al.*, 2003). Yeasts strains from *Selroti* batters showed no detectable proteinase activity in the applied method. High activity of phosphatase by yeast strains (Table 17) showed their possible role in phytic acid degradation in cereal-based fermented foods. Anti-nutritive factors such as phytic acids and oligosaccharides are of particular significance in unbalanced cereal-based diets (Fredrikson *et al.*, 2002). Due to these nutritional consequences, the degradation of anti-nutritive factors in food products by fermentation is desirable as reported for a number of foods of plant origin (Chavan and Kadam, 1989; Mbugua *et al.*, 1992; Svanberg *et al.*, 1993). It was also shown that strain of *Leuc. mesenteroides* isolated from *Selroti* batters had high α-galactosidase and β-galactosidase activities. Presence of high activity of α-galactosidase (Table 16); probably indicated their ability to

hydrolyse oligosaccharides of raffinose family (Holzapfel, 2002). High activity of β-galactosidase exhibited by LAB strains are essential features in fermented milk products (Mathara *et al.*, 2004).

All strains of LAB showed antimicrobial activities against a number of pathogenic Gram-positive and Gram-negative bacteria in the applied method. However, the cell-free supernatant fluid extracts of LAB strains isolated from *Selroti* batters could not produce bacteriocin under the applied condition. Lactic acid bacteria compete with other microorganisms by screening antagonistic compounds and modifying the micro-environment by their metabolism (Lindgren and Dobrogosz, 1990; Tagg, 1992). Production of bacteriocin depends on a number of intrinsic and extrinsic factors including redox potential, water activity, pH and temperature (Yang and Ray, 1994; Delgado *et al.*, 2005). Species of LAB strains isolated from several cereal-based fermented foods have the antimicrobial activities including bacteriocins production (Olukoya *et al.*, 1993; Omar *et al.*, 2006).

Proximate composition

The proximate composition of unfermented rice and wheat flour and samples of *Selroti* batters collected from different sources were analysed (Table 19 and 20). Moisture content in *Selroti* batters was higher than that of raw materials due to soaking prior to fermentation and also due to addition of water and milk during its preparation. There was a remarkable increase in water-soluble and TCA-soluble nitrogen in *Selroti* batters due to solubilisation of proteins, indicating its protein digestibility. Increase in free amino acids in *tarhana* has been reported (Erbas *et al.*, 2005b). The food value of fermented batters was found to be increased slightly than the unfermented raw materials. Samples of fermented batters collected from Sikkim Himalaya had comparatively higher mineral content. Fermentation may have enhanced the nutritional and mineral contents of cereals (Amoa and Muller, 1973; Blandino *et al.*, 2003; Umeta *et al.*, 2005). Proximate and food value of *Selroti* batters are almost same as reported in other cereal-based fermented foods such as *idli* (Soni and Sandhu, 1989a), *rabadi* (Gupta *et al.*, 1992b) and *tarhana* (Erbas *et al.*, 2005a).

Fermentation dynamics *in situ*

During *in situ* fermentation of *Selroti* batter, indigenous lactic acid bacteria and yeasts changed spontaneously. Spontaneous change in LAB as well as yeast population during several cereal fermentations were reported (Soni and Sandhu, 1990; Mugula *et al.*, 2003a). As expected in a typical lactic fermentation (Vaughn, 1985; Lee, 1997), the pH of the fermenting substrates decreased and the titratable acidity increased as the batter fermentation progressed due to growth of LAB which, converts fermentable sugars into lactic acid (Buono *et al.*, 1990). Bacterial contaminants *Bacillus cereus* and enterobacteriaceae were associated with initial fermentation and finally disappeared during *Selroti* batter fermentation. Such similar observation was also made in *masa* fermentation (Efiuvwevwere and Ezeama, 1996). The LAB produced sufficient acid for inhibition of pathogenic microorganisms in foods (Adam and Nicolaides, 1997). By averting the invasion of these potential contaminants, lactic acid fermentation imparts attributes of robust stability and safety in the product like *Selroti.* Another safety aspect of *Selroti* is deep frying prior to consumption. There has been no report of any food poisoning or infectious disease infestation

by consuming *Selroti.* The results showed that various microbial genera occur at the early stages of *Selroti* batter fermentation. This may be partly attributed to the microbial diversity often associated with rice grains and plant materials (Sneath *et al.*, 1986; Efiuvwevwere and Ezeama, 1996). Rice grains contain a numerous microflora including yeasts *P. burtonii* (Kreger-van Riz, 1984), leuconostocs and pediococci (Wood and Holzapfel, 1995). There was no remarkable increase in physical properties of fermenting cereal such as batter temperature, volume and weight during *in situ* fermentation of *Selroti* batter. However, increase in batter volume during fermentation has been reported in *idli* and *dosa* (Soni *et al.*, 1986) and in *puto* (Tongananta and Orillo, 1996). Numerous chemical and physical factors influence the rate and growth of various microorganisms, as well as their sequence of appearance during cereal fermentation (Cartel *et al.*, 2007).

Batters prepared during *in situ* fermentation of *Selroti* from each hour ranging from 0 to 10 hour were deep-fried in edible oil to make *Selroti,* and served to consumers for sensory evaluation. *Selroti* batter prepared by *in situ* fermentation for 8 hour had significantly ($P<0.05$) high

sensory properties due to soft texture, sweet taste and aroma, acceptable to consumers. It is generally noted that a soft texture and sweet-taste, with golden brown colour fried *Selrori* is considered the best to the consumers. Yeasts play vital roles in production of many traditional fermented foods mostly enhancing sensory quality of the foods (Boekhout and Robert, 2003; Romano *et al.*, 2006).

Selroti batter preparation by selected starter cultures

Use of standard starter culture is not a practice in the Sikkim Himalaya except in alcoholic beverage production (Thapa and Tamang, 2004). Starter cultures of LAB and yeasts, previously isolated from native *Selroti* batters were tested singly or in combination for their ability to ferment rice flour to produce *Selroti.* The rationale behind is to use starter culture in order to supplement the natural microflora of *Selroti* batter. Sensory evaluations were carried out in order to choose the best culture combinations. It was found that *Selroti* batters produced using a mixture of pure culture strains of *Leuconostoc mesenteroides* BS1:B1 and *Saccharomyces cerevisiae* BA1:Y2, selected on the superior technological property as mentioned in the result section, at

28° C for 4 hour had organoleptically scored the highest acceptability among the consumers. This was also correlated by decrease and increase in pH and titratable acidity of the fermenting batters, respectively from 0 hour to 4 hour. Yeast contributes in flavour development to fermented maize product and LAB are responsible for acidification (Nche, 1995). None of the strains combinations of *E. faecium, Lb. curvatus, P. pentosaceus, D. hansenii, P. burtonii, S. kluyveri,* and *Z. rouxii,* used as starters could produce organoleptically acceptable *Selroti* product. The consumers' preference trial showed that *Selroti* batter prepared in the laboratory by cell suspension mixture of *Leuc. mesenteroides* BS1:B1 and *S. cerevisiae* BA1:Y2 was more acceptable than *Selroti* batters prepared by conventional method. This fried *Selroti* had desirable sweet taste, a typical *Selroti* flavour and soft texture with golden-brown colour. The principle requirements of the strains are rapid production of CO_2 from maltose and glucose, and generation of good bread flavours (Decock and Cappelle, 2005), which were performed by both isolates (*Leuc. mesenteroides* and *S. cerevisiae*) in *Selroti* batters (Table 7 and 9).

Application of starter cultures may appear appropriate in *Selroti* batter production at household level since it is cost-effective and may contribute to effective control and safeguarding of the fermentation process. *Selroti* prepared by using a starter culture had thus advantages over the traditional method, which resulted in a shorter fermentation time that eliminates the chance of growth of contaminants, hygienic conditions, maintaining consistency with better quality and flavour. The final product is not always consistent in natural fermentation; the use of a mixed starter culture could provide more consistent fermentations and products of higher quality (Gardner *et al.*, 2001; Zorba *et al.*, 2003). Modern starter cultures are selected, either as single or multiple strains, especially for their adaptation to a substrate or raw material, for example cereals, milk, meat, legumes, roots, and tubers (Buchenhüskes, 1993; Holzapfel *et al.*, 2003). Commercial starter cultures of the yeast-bacterial combinations are now available for sourdough production (Decock and Cappelle, 2005). The authentic identity of indigenous LAB isolated from traditional fermented cereal products and their detailed technological characters should be considered for development of starter culture.

Though, optimised process condition is always superior and advantageous than the conventional method, however, introduction and replacement of natural and easily operated traditional technology may be difficult to change for the producers or rural populace (Holzapfel, 1997). Authentic identity of functional microbes in fermented foods is necessary to develop the starter cultures isolated from conventionally prepared foods (Geisen and Holzapfel, 1996; Tamang and Holzapfel, 1999). Preservation and safeguarding of foods are still major objectives of fermentation (Holzapfel, 2002). Yet, other aspects such as wholesomeness, acceptability and overall quality have become increasingly important and valued features to account of the substrate, technical properties of the strain, food safety requirements and quality expectations (Holzapfel *et al.*, 2003).

Conclusion

Selroti is a cultural food of the Nepalis in the Sikkim Himalaya prepared from rice. The traditional knowledge of ethnic people for *Selroti* batter preparation was worth documenting. Scientific knowledge on *Selroti* of the Sikkim Himalaya is unknown outside this region. Species of

functional microorganisms associated with fermented batters of *Selroti* collected from different sources of the Sikkim Himalaya were LAB: *Leuconostoc mesenteroides, Enterococcus faecium, Pediococcus pentosaceus* and *Lactobacillus curvatus;* Yeasts: *Saccharomyces cerevisiae, Debaryomyces hansenii, Pichia burtonii, Zygosaccharomyces rouxii* and *Saccharomyces kluyveri.*

This study showed that strains of LAB and yeast play important and partly complex role in this traditional fermentation process by their functional properties related to a specific and partly a wide enzyme spectrum, their acidifying capacity and antimicrobial activities of LAB, though bactericion production was not observed. Microbial dynamics *in situ* fermentation of *Selroti* batter was studied to understand the microbial composition of the product. Some of these LAB strains possess interesting protective and functional properties, which render them interesting candidates for use as starter culture for controlled and optimised production of fermented cereal batters typical of Sikkim. *Selroti* batters prepared by a mixed pure culture strains of LAB had many advantages over the conventionally prepared products.

SUMMARY

This thesis has aimed to document the indigenous knowledge of traditional processing of *Selroti* batter preparation in the Sikkim Himalaya; and to isolate, characterise and identify the dominant microorganisms in fermented batters collected from different sources. The objective of the thesis was also to analyse microbial load, testing for occurrence of pathogenic contaminants in the product as food safety, to study the effect of seasonal variation, technological properties of functional lactic acid bacteria (LAB) and yeasts such as acidifying capacity, enzymatic profiles, and their antimicrobial activities against pathogenic bacteria. Microbial population dynamics during *in situ* fermentation of Selroti batter were also examined including some physico-chemical changes. Strains of LAB and yeasts were selected and tested for production of fermented batters, and subjected to sensory evaluation. Proximate composition of raw materials and *Selroti* batters were also calculated.

Selroti is an important fermented rice food of the Sikkim Himalaya. Information on indigenous knowledge of *Selroti* was sought during survey, which was documented in this thesis. Per capita consumption of *Selroti* in Sikkim is 8

g/day. *Selroti* is mostly prepared at home (75.6 %) comparable to market purchase.

Scientific knowledge on *Selroti* of the Sikkim Himalaya is unknown outside this region. A total of 125 samples of *Selroti* batters were collected from different villages and markets of the Sikkim Himalaya. A total of 167 isolates of LAB and 141 yeasts were isolated. The microbial population of *Selroti* batters revealed that LAB present in viable numbers above 10^8 cfu/g, followed by yeasts around 10^5 cfu/g, respectively. On the basis of a combination of phenotypic properties and the API sugar profile data, strains of LAB isolated from *Selroti* batters were identified as *Leuconostoc mesenteroides, Enterococcus faecium, Pediococcus pentosaceus* and *Lactobacillus curvatus.* Based on the detailed characterizations and identification profiles of yeasts, *Saccharomyces cerevisiae, Saccharomyces kluyveri, Debaryomyces hansenii, Pichia burtonii* and *Zygosaccharomyces rouxii* were identified. The most prevalent LAB and yeasts in *Selroti* batters were *Leuc. mesenteroids* (42.9 %) and *S. cerevisiae* (35.6 %), respectively. Food borne pathogens *Bacillus cereus, Listeria* sp., *Salmonella* sp. and *Shigella* sp. were not detected in any sample of fermented

batters, due to slight acidic nature of the products. It was observed that seasons affect the development and prevalence of microorganisms in the fermented batters. During summer, the microbial load of LAB increased, whereas, winter was favourable for yeasts.

LAB and yeast strains isolated from *Selroti* batters were screened for their acidifying and coagulating capacity, and found that most of the LAB strains acidified with lowering of pH up to 4.3. About 63.6 % of LAB strains caused coagulation of skim milk at 30° C. Among yeasts, only *S. cerevisiae* and *D. hansenii* showed acidification characters, though decrease in pH was limited to 5.6. The use of the API-zym technique has relevance for selection of strains as potential starter cultures. Absence of proteinases and presence of strong peptidase activities produced by the predominant LAB strains are possible traits of desirable quality for their use in production of typical flavour and aroma. Yeasts strains from *Selroti* batters showed no proteinase activity. High activity of phosphatase by yeast strains showed their possible role in phytic acid degradation in cereal-based fermented foods. It was also shown that strain of *Leuc. mesenteroides* isolated from *Selroti* batters had

high α-galactosidase and β-galactosidase activities, indicating their ability to hydrolyse oligosaccharides. Though, all strains of LAB showed antimicrobial activities against pathogenic bacteria, none of them produced bacteriocin under the applied condition.

The proximate composition of unfermented rice and samples of *Selroti* batters were analysed. Moisture content in *Selroti* batters was higher due to soaking prior to fermentation. There was a remarkable increase in water-soluble and TCA-soluble nitrogen in *Selroti* batters due to solubilisation of proteins, indicating its protein digestibility. The food value of fermented batters was found to be increased. Samples of fermented batters had comparatively higher mineral contents.

During *in situ* fermentation of *Selroti* batter, indigenous lactic acid bacteria and yeasts changed spontaneously. As expected in a typical lactic fermentation, the pH of the fermenting substrates decreased and the titratable acidity increased as the batter fermentation progressed due to growth of LAB. Bacterial contaminants were associated with initial fermentation and finally disappeared during *Selroti* batter fermentation. By averting the invasion of these

potential contaminants, lactic acid fermentation imparts attributes of robust stability and safety in the product like *Selroti.* Another safety aspect of *Selroti* is deep frying prior to consumption. There was no remarkable increase in physical properties of fermenting cereal such as batter temperature, volume and weight during *in situ* fermentation of *Selroti* batter.

Batters prepared during *in situ* fermentation of *Selroti* from each hour ranging from 0 to 10 hour were deep-fried in edible oil to make *Selroti,* and served to consumers for sensory evaluation. *Selroti* batter prepared by *in situ* fermentation for 8 hour had significantly ($P<0.05$) high sensory properties.

Starter cultures of LAB and yeasts, previously isolated from native *Selroti* batters were tested singly or in combination for their ability to ferment rice flour to produce *Selroti.* Sensory evaluations were carried out in order to choose the best culture combinations. It was found that *Selroti* batters produced using a mixture of pure culture strains of *Leuc. mesenteroides* BS1:B1 and *S. cerevisiae* BA1:Y2, selected on the superior technological property, at 28° C for 4 hour had organoleptically scored the highest

acceptability among the consumers. This was also correlated by decrease and increase in pH and titratable acidity of the fermenting batters, respectively from 0 hour to 4 hour. None of the other strains combinations, used as starters could produce organoleptically acceptable *Selroti.* The consumers' preference trial showed that *Selroti* batter prepared by cell suspension mixture of *Leuc. mesenteroides* BS1:B1 and *S. cerevisiae* BA1:Y2 was more acceptable than *Selroti* batters prepared by conventional method. This fried *Selroti* had desirable sweet taste, a typical *Selroti* flavour and soft texture with golden-brown colour. *Selroti* prepared by using a starter culture had thus advantages over the traditional method, which resulted in a shorter fermentation time that eliminates the chance of growth of contaminants, hygienic conditions, maintaining consistency with better quality and flavour.

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