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Microbial and analytical characterization of Chhu—A traditional fermented milk product of the Sikkim Himalayas

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Chhu, a traditional fermented milk product prepared from cow milk or yak milk in Sikkim, India, contains: lactic acid bacteria (LAB), 8.1-8.8; yeasts, 6.0-6.9; and total viable counts, 8.9-9.2 Log cfu/g. No mould was detected. LAB were identified as *Lactobacillus alimentarius, Lb. farciminis, Lb. salivarius, Lb. bifermentans, Lb. brevis* and *Lactococcus lactis* subsp. *cremoris*, all of which showed a high degree of hydrophobicity, suggesting a possible probiotic character. Yeasts were identified as *Saccharomycopsis crataegensis* and *Candida castellii*. LAB produced a wide spectrum of enzymes. None of the strains produced bacteriocin and biogenic amines. Most strains of LAB coagulated skim milk with a moderate drop in pH. A proximate composition of Chhu was similar to a typical cheese.

Keywords: Chhu, Fermented milk, Lactic acid bacteria
IPC Code: C12P

Introduction

Dairy products constitute basic diet of ethnic people of Sikkim Himalayas in India[1]. Chhu (Sheden) is a strong-flavoured traditional cheese-like product prepared from cow milk or yak milk in Sikkim and the Darjeeling Hills, parts of Arunachal Pradesh, and Ladakh in India, besides Nepal, Bhutan and China (Tibet). During its preparation, curd is churned in a bamboo or wooden vessel, butter-milk is collected and cooked for 10-15 min to get a soft mass, which is sieved out and put inside a muslin cloth to drain out the remaining whey. Soft mass is placed in a closed vessel and is left for natural fermentation for 5-7 days at room temperature to prepare Chhu (Fig. 1). It is consumed as curry by cooking in butter along with onions, tomatoes and chilies (Fig. 2), and is mixed with beef or yak meat. Ema dachi, hot-curry of Chhu, is most delicious food in Bhutan.

This study analyzes microbial profile and technological properties of predominant functional microorganisms and a proximate composition of Chhu.

Materials and Methods

Collection of Samples

A total of 35 samples of Chhu {cow Chhu (CC), 21; yak Chhu (YC), 14} were collected aseptically from different places of Sikkim.

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Microbiological Analysis

Samples (10 g) were homogenized with sterile physiological saline (90 ml) in a stomacher lab-blender (400, Seward, London, UK) for 1 min and serially diluted in the same diluent. Lactic acid bacteria (LAB) were isolated on MRS agar (M641, HiMedia, Mumbai) plates, after incubation under anaerobic conditions in an anaerobic gas-pack system (LE002, HiMedia, Mumbai) at 30°C for 48-72 h. Total viable counts were determined using plate count agar (M091A, HiMedia, Mumbai) incubated aerobically at 30°C for 48-72 h. Aerobic spore-forming bacteria were isolated on nutrient agar, after inactivation of vegetable cells by heating at 100°C for 2 min[2], and were incubated at 37°C for 24 h. Moulds and yeasts were isolated on potato dextrose agar (M096, HiMedia, Mumbai) and YM agar (M424, HiMedia, Mumbai), supplemented with 10 IU/ml benzylpenicillin and 12 μg/ml streptomycin sulphate, respectively and incubated aerobically at 28°C for 72 h. Samples were tested[3] for enumeration of *Bacillus cereus* using selective *B. cereus* agar base (M833, HiMedia, Mumbai), *Staphylococcus aureus* using Baird Parker agar base (M043, HiMedia, Mumbai) and enterobacteriaceae using violet red bile glucose agar (M581, HiMedia, Mumbai).

Characterization and Identification

LAB isolates were Gram-stained and tested for catalase production. Cell morphology and motility test

Fig. 1—Cow-milk Chhu of Sikkim

Fig. 2—Chhu curry

were observed in a phase contrast microscope (CH3-BH-PC, Olympus, Japan). LAB isolates were identified[4] on the basis of phenotypic characters. The configuration of lactic acid produced was determined enzymatically using D-lactate and L-lactate dehydrogenase kits (Roche Diagnostic, France). The presence of meso-diaminopimelic acid (DAP) in the cell walls of LAB was determined using thin-chromatography[5]. Sugar fermentation of LAB was determined using API 50 CHL strips (bioMérieux, France). APILAB PLUS database identification software (bioMérieux, France) was used to interpret results. Method[6] was used to characterize and identify yeasts.

Enzymatic Profile by API-zym System

Enzymatic profiles of LAB were assayed using API-zym (bioMérieux, France) galleries by testing activity of 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- and β-galactosidases, β-glucuronidase, α-and β-glucosidases, N-acetyl-β-glucosaminidase, α-mannosidase and fucosidase.

Acidification and Coagulation

Acidification and coagulation of milk by LAB strains was assayed by inoculating 10 % skim milk (RM1254, HiMedia, Mumbai) at 1 % level and incubated at 30°C and 37°C. Observation was made for commencement of clotting, followed by pH measurement[7].

Antagonism and Bacteriocin Activity

LAB isolates were screened for antagonistic activity by the agar spot method[8] against *Listeria innocua* DSM 20649, *L. monocytogenes* DSM 20600, *B. cereus* CCM 2010, *Staphylococcus aureus* S1, *Enterococcus faecium* DSM 20477, *Streptococcus mutans* DSM 6178, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Enterobacter cloacae* BFE 282, *E. agglomerans* BFE 154 and *Pseudomonas aeruginosa* BFE 162. Cell-free neutralized supernatants fluids of LAB isolates were screened for bacteriocin production by the agar spot test method[9], using the bacteriocin screening medium[10].

Biogenic Amine

Ability of LAB isolates to produce biogenic amines was determined qualitatively on an improved screening medium[11] using a 'cocktail' of four precursor amino acids (histidine, lysine, ornithine, tyrosine). Change of bromocresol purple indicator to purple was index of significant amino acid decarboxylase activity, corresponding to >350 mg of a particular amino acid/l[7].

Hydrophobicity

To determine hydrophobicity of LAB strains[12], isolates were grown in MRS broth (M369, HiMedia, Mumbai) at 30°C for 24 h and centrifuged at 7,500

Table 1—Microbial characteristics of Chhu of Sikkim

Microorganisms	Range of counts (Log cfu/g sample)	
	Cow-Chhu (n = 21)	Yak-Chhu (n = 14)
Lactic acid bacteria	8.1-8.3	8.5-8.8
Bacterial endospores	<DL-2.7	<DL
Bacillus cereus	<DL-2.8	<DL-2.4
Staphylococcus aureus	1.7-2.4	<DL-1.5
Enterobacteriaceae	1.8-2.5	1.2-2.0
Yeast	6.6-6.9	6.0-6.7
Total viable count	8.9-9.1	8.8-9.2

Data represent the means of number of samples (n); Microbiological data were transformed into logarithms of the numbers of colony forming unit (cfu)/g; DL, detection limit is 10 cfu/g; Mould was not detected.

rpm for 5 min. The pellet was washed three times with 9 ml of Ringer solution (Merck, Germany) and thoroughly mixed in a vortex. Suspension (1 ml) was taken and the absorbance at 580 nm was measured. Then, 1.5 ml of suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia, Mumbai) in duplicates and mixed thoroughly. Phases were allowed to separate for 30 min at room temperature, after which aqueous phase was carefully removed and absorbance at 580 nm was measured. Percentage hydrophobicity was expressed as follows:

$$\text{Hydrophobicity (\%)} = \frac{\text{OD580 (initial)} - \text{OD580 (with hexadecane)}}{\text{OD580 (initial)}} \times 100$$

Analysis of Proximate Composition

The pH of sample was determined directly using a digital pH meter (Type 361, Systronics, India). Titratable acidity, moisture, ash, fat and protein contents of sample were determined using methods of AOAC[13]. Carbohydrate content was estimated by difference: 100–(% protein + % fat + % ash)[14]. Calcium, magnesium, manganese, iron and zinc were estimated in an atomic absorption spectrophotometer (model 2380, Perkin-Elmer, USA).

Results and Discussion

Samples of Chhu prepared from cow milk (21) and from yak milk (14) were analyzed for microbial counts (Table 1). The population of LAB, yeasts and the total viable counts was found at the level of 10^8 cfu/g, 10^6 cfu/g and 10^9 cfu/g, respectively. Bacterial endospores were detected only in samples of CC. Counts of *B. cereus, S. aureus* and enterobacteriaceae were found less than 10^2 cfu/g in Chhu samples. Mould was not recovered in any sample.

A total of 120 strains of LAB isolated from Chhu were grouped randomly on the basis of gas production, arginine hydrolysis and cell morphology into representative strains. Based on phenotypic characterization including lactate configuration, DAP determination and interpretation of APILAB PLUS database, and also taxonomical key[15], homo-fermentative lactic strain CUG3: R1 (CC) was identified as *Lactobacillus alimentarius*, strain CUG1:R2 (CC) as *Lb. farciminis*, and strain KCK1:R1 (YC) as *Lb. salivarius*; hetero-fermentative strain KCK2:R4 (YC) was identified as *Lb. bifermentans*, and strains CUG2:R2 (CC) and KCY1:R1 (YC) as *Lb. brevis*. Coccus strain CUR1: C1 (CC) was identified as *Lactococcus lactis* subsp. *cremoris*. The lactobacilli were predominant lactic acid flora represented 93 % in Chhu samples. The identity of LAB seems to correspond with that of LAB typically reported for milk products[16]. LAB present in raw cow milk may contribute to the spontaneous fermentation[17].

Yeast strains (37) were grouped randomly on the basis of colony, cell morphology, vegetative characters, and sugar fermentation and assimilation tests. Following taxonomical keys[6], yeasts were identified as *Saccharomycopsis crataegensis* and *Candida castellii*. Presence of high number of yeasts (10^6 cfu/g) indicates some role during spontaneous fermentation of Chhu. Yeasts bring about desirable fermentation changes in fermented milk products[18].

Enzymatic profiles of LAB strains, assayed using the API zym (bioMérieux, France) galleries (Table 2), showed relatively weak esterase and strong leucine arylamidase, β-galactosidase and glucosidase activities, and no detectable proteinase activity. The use of API-zym technique has relevance for selection of strains as potential starter cultures for accelerated maturation and flavour development of milk products[5]. Absence of proteinases, and presence of high peptidase (leucine-arylamidase) and esterase-lipase (C4 and C8) activities produced by the LAB isolated from Chhu are traits of desirable quality for their use in production of typical flavour. High activity of β-galactosidase exhibited by LAB species is essential features in Chhu. All LAB strains except *Lb. brevis* caused coagulation of milk at both 30°C and 37°C with a significant drop in pH (Table 3). However, coagulation occurred faster (17-36 h) at 37°C than 30°C (20-38 h). Coagulation of

Table 2—Enzymatic profiles using API zym system of LAB isolated from Chhu

Enzyme	Activity (nanomoles) of LAB strains						
	CUG3: R1	CUR1: C1	CUG1: R2	CUG2: R2	KCK1: R1	KCK2: R4	KCY1: R1
Control (without enzyme)	0	0	0	0	0	0	0
Phosphatase alkaline	0	0	0	≥40	0	0	≥40
Esterase (C4)	5	5	5	5	5	5	5
Esterase lipase (C8)	5	10	0	5	0	5	5
Lipase (C14)	0	0	0	0	0	0	0
Leucine arylamidase	≥40	≥40	≥40	30	≥40	≥40	30
Valine arylamidase	5	30	5	0	5	5	0
Cystine arylamidase	0	30	5	0	5	5	0
Trypsin	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0
Phoshatase acid	5	≥40	5	≥40	5	10	≥40
Napthol-AS-BI-phosphohydrolase	10	20	10	30	10	5	20
α-Galactosidase	10	0	30	10	30	30	10
β-Galactosidase	≥40	10	≥40	10	≥40	≥40	20
β-Glucuronidase	0	0	0	0	0	0	0
α-Glucosidase	≥40	5	≥40	10	≥40	≥40	10
β-Glucosidase	≥40	5	≥40	10	≥40	≥40	10
N-Acetyl-β-glucosaminidase	0	0	0	0	0	0	0
α-Mannosidase	0	0	0	0	0	0	0
α-Fucosidase	0	0	0	0	0	0	0

Data represent the means of duplicate set

Table 3—Effect of LAB strains isolated from Chhu on coagulation and acidification of milk, and the degree of hydrophobicity

Product	LAB strain	Coagulation, 30° C[a]	Coagulation, 37° C[b]	Hydrophobicity, %
Control	Sterile skim milk	– (6.8)	– (6.8)	
Cow- Chhu	CUG3: R1	+ (5.0)	+ (5.4)	75.3
	CUR1: C1	+ (4.5)	+ (4.5)	97.2
	CUG1: R2	+ (5.2)	+ (5.1)	96.3
	CUG2: R2	– (5.4)	– (5.7)	79.2
Yak-Chhu	KCK1: R1	+ (4.6)	+ (5.0)	85.5
	KCK2: R4	+ (4.5)	+ (5.1)	82.1
	KCY1: R1	– (6.0)	– (5.8)	86.0

Numbers in brackets represent the *p*H values; Data represent the means of triplicate sets.
[a]Coagulation occurred between 20-38 h; [b]coagulation occurred between 17-36 h.

milk by LAB strains reveals their potential as starters or adjunct cultures in the production of Chhu.

Some strains of LAB that showed antagonistic activities were not found to produce any bacteriocin against indicator strains in the applied method. None of the strains produced biogenic amines in the applied method. This is a good indication of their acceptability in the possible development of starter cultures. The production of biogenic amines by LAB to be selected as starter cultures is not a desirable property[19]. All strains of LAB showed a high degree of hydrophobicity (>75%), among which *Lactococcus lactis* subsp. *cremoris* CUR1:C1, isolated from CC, showed highest percentage of hydrophobicity (Table 3). Bacterial adherence to hydrocarbons, such as hexadecane, proved to be a simple method to determine cell surface hydrophobicity[20]. High degree of hydrophobicity by LAB isolated from Chhu probably indicates the potential of adhesion to gut epithelial cells of human intestine, suggesting a possible probiotic character[21], provided these strains are consumed in a viable state.

Table 4—Proximate composition of raw milk and fermented milk product (Chhu)

Parameter	Cow milk		Yak milk	
	Raw milk	Chhu	Raw milk	Chhu
pH	6.7 ± 0.09	4.3 ± 0.25	6.6 ± 0.16	4.8 ± 0.28
Titratable acidity (% as lactic acid)	0.15 ± 0.01	0.54 ± 0.02	0.16 ± 0.02	0.45 ± 0.02
Moisture, %	87.0 ± 2.12	75.4 ± 3.51	84.2 ± 3.12	70.2 ± 4.12
Ash, % DM	5.5 ± 0.76	5.6 ± 0.25	5.8 ± 0.25	5.9 ± 0.67
Fat, % DM	30.8 ± 4.37	6.1 ± 1.32	61.7 ± 1.31	11.2 ± 0.92
Protein, % DM	28.0 ± 0.41	58.4 ± 1.35	26.2 ± 0.65	61.8 ± 2.14
Carbohydrate, % DM	35.8 ± 2.78	30.0 ± 1.25	6.3 ± 2.2	21.1 ± 2.07
Minerals, mg/100 g DM				
Calcium	103.8 ± 1.45	111.0 ± 0.4	76.9 ± 0.65	89.0 ± 0.73
Magnesium	39.7 ± 0.85	64.3 ± 0.65	34.7 ± 0.85	50.1 ± 1.24
Manganese	1.2 ± 0.1	3.1 ± 0.25	1.0 ± 0.12	1.5 ± 0.25
Iron	1.7 ± 0.1	4.5 ± 0.3	1.0 ± 0.12	2.7 ± 0.12
Zinc	57.7 ± 0.75	87.6 ± 1.1	49.1 ± 1.34	56.6 ± 0.68

% DM = g/100 g dry matter; Data represent the means (± SD) of 5 samples

Proximate composition (Table 4) of raw milk and Chhu shows that Chhu was acidic (pH 4.3-4.8) in nature, due to lactic acid fermentation. Like in typical cheese products, high contents of protein and mineral were observed in Chhu.

Conclusions

Indigenous knowledge of the people of Sikkim for production of fermented milks is sparse outside the region. Chhu is an important dairy food in local diet, which has not been documented yet. This study showed that strains of LAB play an important role in fermentation processes by their functional properties related to partly wide enzyme spectrum, antagonistic activities (though bacteriocin production was not detected in the applied methods), coagulation and acidification ability, probiotic properties (adherence potential indicated by a high degree of hydrophobcity), and even non-producers of biogenic amines. Some of these LAB strains possess protective and functional properties, which render them interesting candidates for use as starter culture for controlled and optimized production of fermented milk products.

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