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ORIGINAL ARTICLE

In situ fermentation dynamics during production of *gundruk* and *khalpi*, ethnic fermented vegetable products of the Himalayas

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Abstract *Gundruk* is a fermented leafy vegetable and *khalpi* is a fermented cucumber product, prepared and consumed in the Himalayas. *In situ* fermentation dynamics during production of *gundruk* and *khalpi* was studied. Significant increase in population of lactic acid bacteria (LAB) was found during first few days of *gundruk* and *khlapi* fermentation, respectively. *Gundruk* fermentation was initiated by *Lactobacillus brevis, Pediococcus pentosaceus* and finally dominated by *Lb. plantarum*. Similarly in *khalpi* fermentation, heterofermentative LAB such as *Leuconostoc fallax*, *Lb. brevis* and *P. pentosaceus* initiated the fermentation and finally completed by *Lb. plantarum*. Attempts were made to produce *gundruk* and *khalpi* using mixed starter culture of LAB previously isolated from respective products. Both the products prepared under lab condition had scored higher sensory-rankings comparable to market products.

Keywords Fermentation dynamics · LAB · *Gundruk* · *Khalpi*

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Introduction

Gundruk is a fermented and acidic vegetable product commonly prepared by the Nepalis of the Himalayan regions of India, Nepal and Bhutan during winter when perishable leafy vegetables are plenty. During preparation of *gundruk*, fresh leaves of local vegetable known as 'rayo-saag' [*Brassica rapa* L. ssp. *campestris* (L.) Clapham variety *cumifolia* Roxb.], leaves of mustard [*Brassica juncea* (L.) Czern], leaves of radish (*Raphanus sativus* L.), leaves of cauliflower (*Brassica oleracea* L. variety *botrytis* L.) and leaves of cabbages (*Brassica oleracea* L. variety *capitata*) are wilted for 1–2 days [1]. Wilted leaves are crushed mildly and pressed into a container or earthenware pot, made air tight and fermented naturally for about 15–22 days. After desirable fermentation, freshly prepared wet *gundruk* is sun dried for 2–4 days, which can be kept for a year or more at room temperature. *Gundruk* is eaten as soup and pickles with boiled rice. It is similar to the Korean *kimchi* and the German *sauerkraut*. Unlike *kimchi* and sauerkraut, freshly prepared wet *gundruk* is normally not preferred. *Khalpi* is a fermented cucumber (*Cucumis sativus* L.) product, also prepared and consumed by the Nepalis. During preparation, matured and ripened cucumber is cut into suitable pieces, sun dried for 2 days and then put into a bamboo vessel, locally called 'dhungroo' and made air-tight. It is fermented at room temperature for 4–7 days. *Khalpi* is eaten as pickle by adding mustard oil, salt and powdered chillies.

The safekeeping and preservation of food have been part of mankind's struggle for survival throughout the cultural history of the human being [2]. Different preservation methods have been reviewed by several authors such as low oxygen and high carbon dioxide for cucumber

[3], acidified sodium chloride and peroxyacetic acid for leafy salad species [4], grapefruit extract on minimally processed vegetables [5], applications of bacteriocins [6], etc. Nevertheless, lactate fermentation is one of the popular preservation methods and still serves as substitutes where refrigeration and other means are not available for the safe-keeping of food [7]. Some of the LAB strains previously isolated and identified from fermented vegetable products, possesses protective and functional properties, which can be used as starter culture for controlled and optimized production of fermented vegetable products of North-East India [8].

The aim of this paper is to study fermentation dynamics *in situ* of *gundruk* and *khalpi* along with the study of optimisation of fermentation process using starter culture.

Materials and methods

Fermentation dynamics in situ: Gundruk was prepared in the laboratory from leaves of 'rayo-saag' [*Brassica rapa* L. ssp. *campestris* (L.) Clapham variety *cumifolia* Roxb.], following the traditional method. Leaves were wilted in the sun for 1 day, then crushed and soaked in hot water. About 400 g of crushed leaves were put into sterile 500 ml-bottle, pressed with sterile pestle to remove excess water. Then, bottles were tightly capped and fermented at room temperature (20–25°C) for 16 days. Samples were taken at every one-day interval till 16th day for analyses.

Ripened cucumber was collected from Gangtok market (Sikkim) and *khalpi* was prepared following the traditional method. Cucumber was washed and cut into pieces and sun dried for 1 day. About 400 g of cucumber pieces were filled into sterile 250 ml bottles and fermented at room temperature (20–25°C) for 3 days. Samples were taken at every 6 h interval till 72 h for analyses.

Changes in microbial population of major microbial groups were analysed as described in microbial analysis section. Temperature of each sample was recorded using thermometer before taking sample for analysis.

The pH of the sample was determined directly using a digital pH meter (Type 361, Systronics, India) calibrated with standard buffer solutions (Merck). Titratable acidity was expressed as a percentage of lactic acid of sample [9].

Preparation of gundruk using Selected Strains of LAB: 'Rayo-saag' leaves of *Brassica rapa* L. sub-sp. *campestris* (L.) Clapham variety *cumifolia* Roxb. were purchased from Gangtok market. Leaves were washed thoroughly in sterile distilled water and wilted in oven (~30°C) for 6 h. Leaves were crushed, put into sterile warm water (about 90°C) for 5 min and transferred into another sterile glass container. Excess water in the leaves was removed by squeezing and then, about 400 g of crushed leaves of 'rayo-saag' were distributed aseptically into each sterile 500 ml capped-bottles, totalling 6 bottles for samplings. Each bottle was inoculated by a mixture of actively grown culture strains of *Lactobacillus plantarum* GLn: R1 (MTCC 9483) and *Pediococcus pentosaceus* GLn:R2 (MTCC 9484) at the ratio of 10^7 cfu/g, previously isolated from market samples of *gundruk* [1]. Bottles were tightly capped and incubated at 20°, 25° and 30°C, respectively for 6 days. Samplings were done on 3rd and 6th days for organoleptic test followed by determination of pH and acidity.

Preparation of khalpi using Selected Strains of LAB: Ripened cucumber (*Cucumis sativus* L.) was collected from Sadam village in Sikkim. Cucumber was cleaned, washed and cut into pieces and oven dried at ~30°C for 8 h. About 400 g of oven-dried pieces of cucumber were transferred into each sterile 250 ml bottles, totaling 9 bottles for samplings. Each bottle was inoculated by a mixture of pure culture strains of actively grown *Lb. plantarum* KG: B1 (MTCC 9485), *Lb. brevis* KG:B2 (MTCC 9486) and *Leuconostoc fallax* KB:C1 (MTCC 9487) at the ratio of 10^7 cfu/g, previously isolated from traditionally prepared *khalpi* samples [1]. Bottles were tightly capped and incubated at 20°, 25° and 30°C, respectively for 72 h. Samples were taken on 24, 48 and 72 h, respectively for sensory evaluation and also for determination of pH and acidity of the fermenting cucumbers.

Sensory evaluation: Sensory evaluation of *gundruk* and *khalpi*, prepared by selected starter cultures were evaluated in terms of aroma, taste, texture, colour and general acceptability as described by Meilgaard et al. [10]. *Gundruk* and *khalpi* were organoleptically evaluated by a panel of 7 judges with score rate of 1, bad and 5, good considering market *gundruk* and *khalpi* as control with scoring rate of 3, moderate.

Microbiological analysis: Samples (10 g) of each product were mixed with 90 ml of 0.85% (w/v) sterile physiological saline and homogenised in a Stomacher lab-blender (400, Seward, UK) for 1 min. A serial dilution in the same diluents was made. LAB were isolated on plates of MRS agar (M641, HiMedia) supplemented with 1% $CaCO_3$ and incubated at 30°C in an anaerobic gas-jar (LE002, Hi-Media) for 48–72 h. Colonies of moulds and yeasts were examined on potato dextrose agar (M096, HiMedia) and yeast-malt (YM) agar (M424, HiMedia), supplemented with 10 IU/ml benzylpenicillin and 12 μg/ml streptomycin sulphate, respectively, which were incubated aerobically at 28°C for 72 h. Isolated colonies based on colony morphology were selected randomly from the highest diluted plates. Purity of the isolates was checked by streaking again and sub-culturing on fresh agar plates of the isolation media,

followed by microscopic examinations. Purified isolates of LAB were preserved at −20°C in MRS broth (M369, HiMedia) with 15% (v/v) glycerol added. 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 [11].

Characterization and identification: Cell morphology of all bacterial isolates and their motility were determined using a phase contrast microscope (Olympus CH3-BH-PC, Japan). LAB isolates were Gram-stained and tested for catalase production by placing a drop of 10% hydrogen peroxide solution on isolates, and were preliminarily identified on the basis of carbon dioxide production from glucose, ammonia production from arginine, growth at different temperatures (10°C, 15°C, 45°C), the ability to grow in different concentrations of sodium chloride (6.5% , 10% , 18%) and pH (3.9, 9.6) in MRS broth (M369, HiMedia, India) following the methods of Schillinger and Lücke [12] and Dykes et al. [13]. The configuration of lactic acid produced from glucose was determined enzymatically using D-lactate and L-lactate dehydrogenase test kits [1]. The presence of *meso*-diaminopimelic acid (DAP) in the cell walls of LAB was determined on cellulose plates using a thin layer-chromatography [14]. Sugar fermentation of LAB isolates were determined by the API 50 CHL test strips (bioMérieux, France) and the identifications were interpreted using APILAB PLUS software (bioMérieux, France). Taxonomical keys of Simpson and Taguchi [15], Wood and Holzapfel [16] were followed for identification of LAB isolates. Yeast isolates were identified upto genera according to the criteria laid down by Kurtzman and Fell [17].

Statistical Analysis: Data were analyzed by determining the standard deviation (SD), the standard error of measurement and the analysis of variance (ANOVA) [18].

Results and Discussion

Fermentation dynamics *in situ*

Successional studies of LAB and other non-lactics, and their effect on pH and acidity of *gundruk* and *khalipi* was studied separately as described in material and method section. The temperature of fermenting 'rayo-saag' leaves remained around 18–20°C (Fig 2). As expected in a typical lactate fermentation, the pH of the fermenting substrates decreased significantly ($p<0.05$) from 6.6 to 3.7 (Fig. 2), due to growth of LAB which, converts fermentable sugars into lactic acid [19]. Significant increase in titratable acidity% (as lactic acid) was observed during fermentation. Exponential increase in LAB population was significant ($p<0.05$) till 3rd day detecting at the level of almost 10^9 cfu/g (Fig. 1). The LAB population after 3rd day, gradually decreased to a level of 10^7 cfu/g till the end of fermentation period. Yeasts were detected only in the raw leaves and during initial stage of fermentation. During lactate fermentation, the contaminating bacteria such as *Staphylococcus aureus* and enterobacteriaceae disappeared by the end of 8th day of fermentation process because of dominance of LAB. The LAB produced sufficient acid for inhibition of pathogenic microorganisms in foods [20]. During initial stage of fermentation of *gundruk*, *Lb. brevis* and *P. pentosaceus* were dominant. As the fermentation progressed, indigenous LAB changed spontaneously and at the end mainly *P. pentosaceus* and *Lb. plantarum* were involved. Spontaneous change in LAB population during several vegetables fermentation involving lactobacilli was reported [21, 22]. *Saccharomyces* sp,

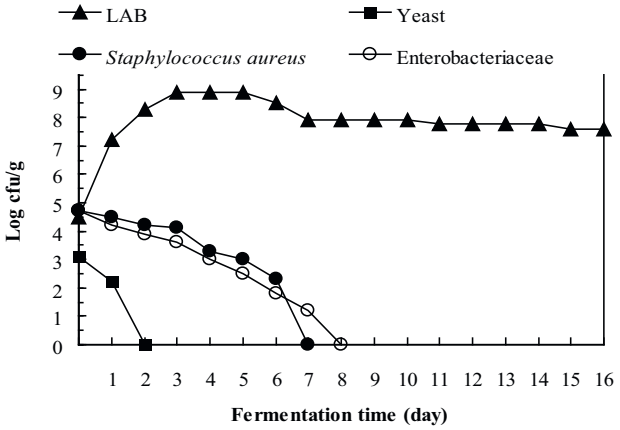

Fig. 1 Changes in microbial load during *gundruk* fermentation.

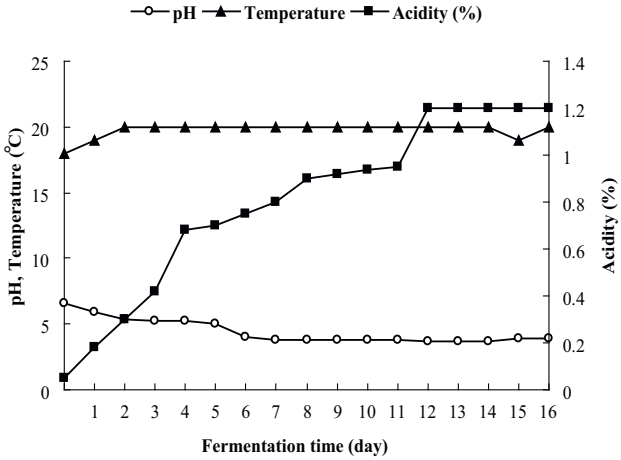

Fig. 2 Changes in pH, acidity and temperature during *gundruk* fermentation.

Pichia sp. and *Zygosaccharomyces* sp. were found during initial stage of *gundruk* fermentation.

Difference in temperature of the fermenting cucumbers during *khalpi* fermentation was not significant ($p<0.05$). The pH decreased significantly ($p<0.05$) from 5.6 to 3.2 and acidity% increased significantly ($p<0.05$) from 0.28% to 1.24 at end of fermentation (Fig. 4). Population of LAB in raw cucumber was very small (10^3 cfu/g) which increased significantly ($p < 0.05$) to 10^8 cfu/g within 36 h, and then remained at the level of 10^7 cfu/g in the final product (Fig. 3). Microbial load of yeast in raw cucumber was 10^4 cfu/g, which disappeared after 48 h. Load of *Staphylococcus aureus* and enterobacteriaceae reduced significantly ($p<0.05$) and disappeared during fermentation. Acidity, pH and buffer capacity greatly influence establishment and extent of growth of LAB during cucumber fermentation [23].

The predominant LAB involved in *khalpi* fermentations were *Leuc. fallax*, *P. pentosaceus, Lb. brevis* and *Lb. plantarum. Leuconostoc* is the major bacterial genus in the initial phase of lactate fermentation of vegetables [24]. Heterofermentative LAB such as *Leuc. fallax, Lb. brevis* and *P. pentosaceus* were isolated from the initial fermentation stage of *khalpi*. As the fermentation progressed, more acid producing homofermentative lactobacilli mainly *Lb. plantarum* remained dominant. Yeasts detected in initial stage of *khalpi* fermentation were *Pichia* sp., *Candida* sp. and *Saccharomyces* sp. Pathogenic contaminants disappeared during fermentation because of dominance of LAB. By averting the invasion of these potential contaminants, lactic acid fermentation imparts attributes of robust stability and safety in the product like *gundruk* and *khalpi*. There has been no report of any food poisoning or infectious disease infestation by consuming *gundruk* and *khalpi*.

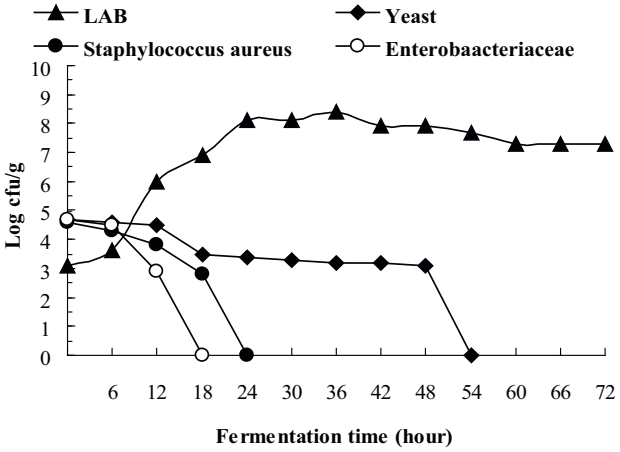

Fig. 3 Changes in microbial load during *khalpi* fermentation.

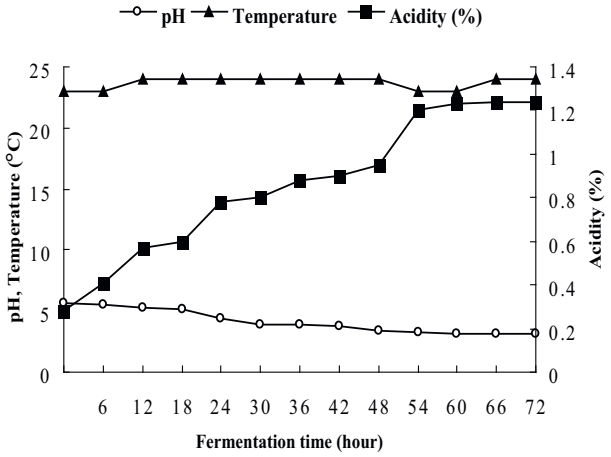

Fig. 4 Changes in pH, acidity and temperature during *khalpi* fermentation.

The final product is not always consistent in natural fermentation; the use of a mixed lactic starter culture could provide fermentations that are more consistent and products of higher quality [25]. Besides, use of mixed starter culture complements different technological properties to attain better products [26]. On the basis of superior technological properties of LAB strains [8] such as acidification ability, antimicrobial activities, non-production of biogenic amines, ability to degrade antinutritive factors, and even high degree of hydrophobicity, *Lb. plantarum* GLn: R1 (MTCC 9483) and *P. pentosaceus* GLn:R2 (MTCC 9484) were selected as a starter culture for production of *gundruk*, as described in materials and methods. *Gundruk* prepared by the starter culture was evaluated organoleptically. With respect to general acceptability, 6 day-old *gundruk* fermented at 20°C had the highest score with better aroma, acidic taste typical of *gundruk* and thus acceptable to consumers (Table 1). Significant ($p<0.05$) difference in aroma and taste, typical to *gundruk*, was found on 6 day-old *gundruk*.

Lb. plantarum KG:B1 (MTCC 9485), *Lb. brevis* KG: B2 (MTCC 9486) and *Leuc. fallax* KB:C1 (MTCC 9487) previously isolated from *khalpi*, were selected, on the basis of superior technological properties such as acidifying capacity, antimicrobial activities, non-production of biogenic amines and ability to degrade antinutritive factors of the raw materials [8]. *Khalpi* produced with a mixed pure culture at 20°C for 72 h had highest score in aroma (Table 2). There was significant ($p < 0.05$) increase in taste score with time at 20°C and had score of 4.5 at 72 h. The highest score ($p<0.05$) in taste (strongly acidic) was observed at 72 hour fermented at 30°C. General acceptability score was highest in *khalpi* fermented at 20°C for 72 h. *Khalpi* produced at 25°C was also more or less similar within 48 h while at 30°C strongly acidic tastes developed quickly and not preferred by the consumers.

Table 1 Sensory evaluation of *gundruk* prepared from leaves of 'rayo-saag' using a mixed pure culture strains*

Fermentation period (day)	Attribute					pH	Titratable acidity %
	Aroma	Taste	Texture	Colour	General acceptability		
Fermentation at 20^0 C							
3	3.8 ± 0.3^{b}	3.9 ± 0.3^{b}	3.6 ± 0.3^{a}	3.8 ± 0.3^{a}	3.8 ± 0.5^{ab}	4.3 ± 0.29^{c}	0.52 ± 0.09^{b}
6	4.6 ± 0.5^{a}	4.9 ± 0.3^{a}	3.1 ± 0.2^{a}	3.8 ± 0.5^{a}	4.4 ± 0.5^{a}	4.0 ± 0.13^{d}	0.75 ± 0.11^{a}
Fermentation at 25^0 C							
3	3.0 ± 0.4^{c}	2.9 ± 0.5^{c}	3.5 ± 0.4^{a}	3.8 ± 0.5^{a}	2.8 ± 0.3^{c}	4.6 ± 0.20^{ab}	0.40 ± 0.06^{bd}
6	3.9 ± 0.4^{b}	3.5 ± 0.5^{bc}	3.4 ± 0.5^{a}	3.6 ± 0.5^{a}	3.5 ± 0.5^{b}	4.4 ± 0.20^{bc}	0.50 ± 0.10^{bc}
Fermentation at 30^0 C							
3	1.4 ± 0.3^{d}	1.1 ± 0.3^{e}	3.6 ± 0.3^{a}	3.5 ± 0.4^{a}	1.3 ± 0.3^{d}	4.8 ± 0.12^{a}	0.37 ± 0.10^{cd}
6	1.3 ± 0.5^{d}	1.5 ± 0.8^{de}	3.9 ± 0.4^{a}	2.8 ± 0.9^{b}	1.3 ± 0.5^{d}	4.4 ± 0.20^{bc}	0.48 ± 0.08^{bd}

Data represents the mean scores ± SD (n = 7).
Values bearing different superscripts (^{a,b,c,d,e}) in each column differ significantly ($p < 0.05$).
Market *gundruk* was used as control (score 3), score 1, bad; score 5, good.
*Mixture of *Lb. plantarum* GLn:R1 and *P. pentosaceus* GLn:R2.

Table 2 Sensory evaluation of *khalpi* prepared from cucumber using a mixed pure culture strains*

Fermentation period (day)	Attribute					pH	Titratable acidity %
	Aroma	Taste	Texture	Colour	General acceptability		
Fermentation at 20^0 C							
24	1.5 ± 0.6^{c}	1.5 ± 0.6^{d}	2.1 ± 0.3^{fg}	3.8 ± 0.5^{a}	1.1 ± 0.3^{e}	4.3 ± 0.05^{a}	0.57 ± 0.01^{fg}
48	4.3 ± 0.5^{ad}	2.8 ± 0.5^{bc}	2.5 ± 0.6^{def}	3.5 ± 0.6^{a}	2.3 ± 0.5^{c}	4.1 ± 0.10^{b}	0.68 ± 0.01^{d}
72	4.8 ± 0.5^{a}	4.5 ± 0.6^{a}	3.5 ± 0.6^{b}	3.5 ± 0.6^{a}	4.8 ± 0.5^{a}	3.8 ± 0.02^{c}	0.95 ± 0.02^{a}
Fermentation at 25^0 C							
24	1.5 ± 0.6^{c}	2.5 ± 0.6^{c}	2.1 ± 0.3^{fg}	3.8 ± 0.5^{a}	1.3 ± 0.5^{de}	4.3 ± 0.05^{a}	0.56 ± 0.01^{g}
48	4.3 ± 0.5^{ad}	4.3 ± 0.5^{a}	2.3 ± 0.5^{eg}	3.8 ± 0.5^{a}	4.5 ± 0.6^{ab}	3.8 ± 0.04^{c}	0.70 ± 0.04^{cd}
72	3.3 ± 0.5^{bd}	4.5 ± 0.6^{a}	3.1 ± 0.3^{bcde}	3.8 ± 0.5^{a}	3.5 ± 0.6^{b}	3.8 ± 0.04^{c}	0.78 ± 0.02^{b}
Fermentation at 30^0 C							
24	3.8 ± 0.5^{ab}	2.8 ± 0.5^{b}	2.1 ± 0.3^{fg}	3.5 ± 0.6^{a}	2.3 ± 0.5^{c}	4.1 ± 0.03^{b}	0.58 ± 0.02^{efg}
48	3.8 ± 0.5^{ab}	4.5 ± 0.6^{a}	3.5 ± 0.6^{b}	3.5 ± 0.6^{a}	3.5 ± 0.6^{b}	3.6 ± 0.0^{d}	0.94 ± 0.02^{a}
72	3.8 ± 0.5^{ab}	4.8 ± 0.5^{a}	4.5 ± 0.6^{a}	3.8 ± 0.5^{a}	2.3 ± 0.5^{cd}	3.6 ± 0.03^{d}	0.97 ± 0.01^{a}

Data represents the mean scores ± SD (n = 7).
Values bearing different superscripts (^{a,b,c,d,e,f,g}) in each column differ significantly ($p < 0.05$).
*Mixture of *Lb. plantarum* KG:B1, *Lb. brevis* KG:B2 and *Leuc. fallax* KB:C1.
Market *khalpi* was used as control (score 3), score 1, bad; score 5, good.

Conclusion

In the Himalayas, most of the ethnic fermented foods are prepared by spontaneous fermentation [27], except production of ethnic alcoholic beverages by using mixed starter cultures [28]. Use of starter cultures may appear appropriate in *gundruk* and *khalpi* production at household level since it is cost-effective and may contribute to effective control and safeguarding of the fermentation process. Interesting there was no inter-antimicrobial activities among the selected pure cultures [8]. *Gundruk* and *khalpi* prepared by using mixture of pure starter cultures had thus advantages over the conventional method, which resulted in a lesser fermentation period that may eliminate non-lactic contaminants, may ensure the hygienic conditions, maintaining consistency with better quality and flavour.

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