Microbiological Analyses of Traditional Alcoholic Beverage (Chhang) and its Starter (Balma) Prepared by Bhotiya Tribe of Uttarakhand, India

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Abstract Present article depicts microbiology of starter (Balma) used in traditional solid-state fermentation of alcoholic beverage (Chhang) by Bhotiya tribe of Uttarakhand. It also highlights the importance of herbs in Balma preparation and kinetics of lactic acid and ethanol fermentation under Chhang preparation using Balma. Balma contains 214×10^6 cfu/g yeasts, 2.54×10^6 cfu/g lactic acid bacteria (LAB) and 1.4×10^6 cfu/g other mesophilic bacteria. ITS sequence analysis revealed a rich diversity of yeast comprising of *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera* and *Saccharomycopsis malanga* in Balma. 16S rDNA sequence analysis revealed *Lactobacillus pentosus* and *Pediococcus pentosaceus* among LAB, while amylolytic *Bacillus subtilis* and *B. aerophilus* among other bacteria in Balma. Based on the results, it is speculated that herbs such as *Inula cuspidata*, *Micromeria biflora*, *Origanum vulgare*, *Rubus* sp. and *Thymus linearis* used earlier by Bhotiya in Balma preparation contribute as a source of yeasts, LAB and amylolytic bacilli. Study also demonstrates that Bhotiya tribe is rational in preparation of starter as they have circumvented the need of plants by using previous year Balma as inoculum and possibility of deficient quality of Balma due to weak colonization of

phyllosphere and rhizosphere microbiota. Results suggest that simultaneous saccharification and lactic acid–ethanol fermentation take place in traditional cereal based Chhang fermentation system of Bhotiya.

Keywords Traditional fermentation system · Balma · Keem · Bhotiya · Jaunsari

Introduction

Fermented foods and beverages have been the integral part of human civilization and fermentation is the first signature of human–microbes relationship [1]. Fermentation enhances the organoleptic property and preservation time of food stuff. In India, numerous traditional fermented food types belonging to tribal communities have been documented [2–6]. Bhotiya and Jaunsari are Indian tribes which inhabit the highlands of Uttarakhand state in Indian Himalayan Region (IHR). They have a long legacy of alcohol fermentation using traditionally prepared starter known as Balma (Bhotiya) and Keem (Jaunsari).

In the present study, extensive survey was carried out in the Bhotiya and Jaunsari dominated villages of Chamoli and Dehradun district and information on the traditional methods of alcohol preparation was acquired through semi-structured interviews, field observations, participation in their social life and events. These tribal communities prepare starters using dough of indigenous cereals with added herbs of Himalayas. They incubate it under bed of *Cupressus torulosa*, *Cannabis sativa* or *Pinus roxburghii* leaves for 2 weeks or until they get dehydrated and turned white after microbial colonization. Jaunsari use leaves, bulbils or roots of various herbs in every new batch of Keem preparation [7]. In contrast, field observation

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revealed an interesting feature where Bhotiya have stopped the use of plants and started to use previous year Balma in every new batch of its preparation. Some studies have, however, documented the use of leaves, roots or fruits of various herbs such as *Humulus lupulus*, *Inula cuspidata*, *Micromeria biflora*, *Origanum vulgare*, *Rubus* sp. and *Thymus linearis* etc. [8] and *Cinnamomum zeylanicum*, *Amomum subulatum*, *Piper longum* and seeds of *Ficus religiosa* [2, 9] in preparation of Balma. These starters are used round the year in solid-state fermentation of alcoholic beverages. Chhang of Bhotiya and Gaingti or Paakuyi of Jaunsari tribes are types of wine prepared using boiled indigenous cereals as substrates whereas Balmaa and Keem, respectively as starter. Distilled alcohol such as Daru by Bhotiya and Soor by Jaunsari are also prepared using jaggery, fruits and boiled cereals as sole substrate and these starters.

Despite their immense ethnic values, a diminishing trend of traditional fermentation practices has been observed in these tribal areas. Enhanced accessibility to urban areas after road construction, adoption of modern life style and inclination towards commercial liquor are some of the plausible reasons behind it. As stated, Bhotiya have altered the traditional method of Balma preparation by not using herbs in it. It is also observed that fragmentary information persists on microbial composition and function of Balma; that too is devoid of any information on microbial succession, colonization and importance of added herbs during its preparation. Therefore, in view of traditional non-standardized starter preparation as well as fermentation practice, microbiology of Balma, role of plants in microbial succession during its preparation and kinetics of Chhang fermentation mediated by Balma as model system was studied. Microbial diversity of Keem was also studied for comparative analysis of microbial composition of these starters. This study may be helpful in selection of functionally important strains, optimization of microbial composition of starters and quality control of traditional fermentation practice.

Material and Methods

Collection of Samples

Samples of Balma and Chhang were collected from Bhotiya inhabited villages of Neeti valley of Chamoli district (1900–2400 m above msl) while, Keem were collected from Jaunsari inhabited villages of Tons valley of Dehradun district (800–2000 m above msl). To study importance of plants in Balma preparation, samples of leaves of *I. cuspidata*, *M. biflora*, *O. vulgare*, *T. linearis* and roots of *Rubus* sp. were collected, shade dried in field itself and stored in autoclaved polythene bags.

Estimation of Chemical Parameters in Chhang

The important chemical properties of Chhang such as pH, ethanol, L-lactic acid, total reducing sugars, phenolics and furfurals content were analyzed using its cell free suspension. Ethanol concentration was analysed using Gas Chromatograph (Clarus 500, Perkin Elmer) with an elite-wax (cross bond-polyethylene glycol) column (30.0 m × 0.25 mm) at oven temperature of 85 °C and flame ionization detector (FID) at 200 °C. The ethanol standards were prepared using commercial grade ethanol (Merck, Germany). Nitrogen with a flow rate of 0.5 ml/min was used as the carrier gas. Total reducing sugars [10] and phenolics and furfurals [11] were estimated by the Spectrophotometric methods. L-lactic acid content in Chhang was estimated using commercial kit (Megazyme) following the manufacturer's protocol.

Microbiological Study of Balma and Keem

Isolation of microbes from Balma and Keem was carried out using serial dilution and spread plate technique. Aliquot of serially diluted sample was spread on to nutrient agar plate spiked with 10 µg/ml of cycloheximide to isolate bacteria and on yeast extract peptone dextrose (YEPD) agar plate spiked with 50 µg/ml of chloramphenicol to isolate yeast at 28 °C. To isolate Lactic acid bacteria (LAB), 0.1 ml of diluted sample was spread onto de Man, Rogosa, Sharpe (MRS) agar and incubated at 37 °C under anaerobic condition (Anaerobic Jar, HiMedia). All the plates were incubated for 48 h and the total viable count (TVC) was calculated and expressed as colony forming unit per gram (cfu/g). The discrete colonies of different morpho-types were picked and purified by repeated streaking on respective medium, single colony isolation and microscopic observation. The purified bacterial isolates were streaked over starch agar (1 % soluble starch, 0.5 % peptone, 0.5 % yeast extract, 1.5 % agar) followed by incubation at 37 °C for 48 h. The plates were flooded with Gram's iodine solution in order to select amylase producing isolates showing clear zone.

Molecular Characterization of Bacterial and Fungal Diversity

Genomic DNA was isolated from bacterial and yeast cells using commercial kit (RBC, Taiwan). 16S rRNA gene sequences were amplified using primer pair 27F and 1492R described earlier [12]. ITS region of yeasts was amplified using primer pair ITS1 and ITS4 [13]. The amplified PCR products were resolved on 1 % agarose gel amended with 0.5 µg/ml EtBr and amplicons were purified using DNA gel extraction kit (RBC, Taiwan). The sequencing of

purified PCR product was done at DNA sequencing facility at University of Delhi, South Campus. The bacterial and fungal isolates were identified on the basis of 16S rRNA and ITS sequence data, respectively using the EzTaxon and EzFungi server (<http://www.ezbiocloud.net/>). Further, the identified 16S rDNA and ITS sequences in the study were deposited in the GenBank.

Assessment of Importance of Herbs Used in Starter Preparation

Balma was prepared in the laboratory with infusion of shade dried plants following the traditional practice of Bhotiya and examined the TVC and diversity of microorganisms. A composite sample of approximately equal amount of herbs (leaves of *I. cuspidata*, *M. biflora*, *O. vulgare*, *T. linearis* and roots of *Rubus* sp.) was prepared and powered in pre-sanitized mixer-grinder with ethanol. The infusion was prepared by soaking 25 % of this composite powder in autoclaved distilled water for overnight. Flour of *Triticum aestivum* (Wheat), *Fagopyrum esculentum* (Phafar), *F. tataricum* (Koda or Kotu) and *Hordeum vulgare* (Jau) was used as substrate to prepare Balma. Each substrate was first baked and then autoclaved before kneading to avoid the contamination. All the sets of Balma balls were kept under the bed of *C. torulosa* needles and incubated in Kozi-room at 22 °C and 80 % humidity. Following the 5 days incubation when Balma dehydrated, they were powdered and used to prepare a new set of Balma with infusion of herbs. This process was repeated for four times until Balma were turned white due to microbial colonization. Analysis of TVC was done applying spread plate technique and microbial diversity was assessed using API system (BioMerieux Vitek 2, France) for yeast and BIOLOG (Biolog, USA) for bacteria in Central Instrumentation Facility at University of Delhi, South Campus.

Kinetics of Traditional Alcohol Fermentation

Kinetics of lactic acid and ethanol fermentation was studied in 100 ml flask at pH 7.0 containing Glucose (12 %) and Soyabean meal (1.2 %) in which Balma (1 g) collected from Lata village of Bhotiya community was used as inoculum. The flasks were incubated at 30 °C at 200 rpm and sampling was done at 4 h of interval. A cell-free solution was obtained by centrifuging (8000 rpm, 10 min and 4 °C) the culture broth at various intervals and used for further analyses. Concentration of ethanol and total reducing sugar was analyzed as mentioned earlier. Titratable acidity expressed as percent lactic acid, was determined by titrating the samples with 0.1 N NaOH using phenolphthalein as indicator [14]. Appearance of pink

colour was considered as the end point of the titration. The percent lactic acid was calculated as follow:

$$\text{Lactic acid (\%)} = 0.009 \times \text{volume of NaOH used (ml)} \times 100 / \text{volume of the sample (ml)}$$

Results and Discussion

Bhotiya and Jaunsari have developed a typical system of traditional starter preparation and alcohol fermentation using indigenous resources. Analyses of chemical constituents in traditionally prepared Chhang showed ethanol (65 g/l) and lactic acid (2 g/l) with relatively low reducing sugars, phenolics and furfurals (Table 1). Based on molecular taxonomy, yeasts and bacteria (LAB and amylase producing bacilli) have been observed to be the functional microbiota in Balma and Keem (Table 2). A high density of yeast cells was noted in the Balma ($>214 \times 10^6$ cfu/g). ITS sequence based identification of isolated yeasts revealed three yeast species in Balma i.e. *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera* (former synonym, *Endomyces fibuligera*) and *Saccharomycopsis malanga* (former synonym, *Hansenula malanga*). Earlier also, *Saccharomyces* sp., *Saccharomycopsis fibuligera*, *Kluyveromyces marxianus* and amylase positive bacilli have been reported from Balma samples of Johar valley of Pithoragarh district in Uttarakhand [9]. *S. cerevisiae* and *S. fibuligera* have also been reported in Phab, starter prepared by tribal community of Lug valley in Kullu district of Himachal Pradesh [15]. Balma was observed to contain 2.54×10^6 cfu/g of LAB comprising of two prominent species, i.e. *Lactobacillus pentosus* and *Pediococcus pentosaceus*. The *L. plantarum*, *L. Brevis*, *L. casei*, *L. pentosus* and *P. pentosaceus* have also been reported in starters used by tribal communities in Himachal Pradesh and North-East states of India [15, 16]. Balma samples were estimated to contain 1.4×10^6 cfu/g of bacteria comprising amylolytic strains of *B. subtilis* and *B. aerophilus*.

The microbial load in Keem collected from different villages of Tons valley varied significantly. Only the Keem samples of Birmau village showed a high TVC of yeasts

Table 1 Chemical composition of Chhang

Parameters	Value
pH	4.1
Ethanol	65.0 g/l
L-lactate	2.0 g/l
Phenolics	0.40 g/l
Furfurals	0.03 g/l
Reducing sugars	7.65 g/l

Table 2 Identification of isolates according to 16S rDNA (for LAB, other Bacteria) and ITS rDNA (for yeasts) sequencing

Isolate number	Source	% Homology with the GeneBank sequence	GeneBank accession number of corresponding sequence	Identification	GeneBank accession number of isolates under present study
K1	Balma	100	U10409	<i>Saccharomycopsis fibuligera</i>	JQ716924
K2	Balma	100	AB180473	<i>Saccharomyces cerevisiae</i>	JQ768412
K4	Balma	100	U10409	<i>Saccharomycopsis fibuligera</i>	JQ716926
K5	Balma	99.7	JX464196	<i>Saccharomycopsis malanga</i>	JQ716927
LAB1	Balma	100	D79211	<i>Lactobacillus pentosus</i>	JQ716920
LAB2	Balma	99.54	AJ305321	<i>Pediococcus pentosaceus</i>	JQ716921
LAB3	Balma	99.46	AJ305321	<i>Pediococcus pentosaceus</i>	JQ716922
LAB4	Balma	100	D79211	<i>Lactobacillus pentosus</i>	JQ716923
KB1	Balma	98.87	AMXN01000021	<i>Bacillus subtilis</i>	KC597818
KB3	Balma	99.81	AMXN01000021	<i>Bacillus subtilis</i>	KC597825
KB4	Balma	99.61	AJ831844	<i>Bacillus aerophilus</i>	KC597826
KB7	Balma	99.82	ABQL01000001	<i>Bacillus subtilis</i>	KC597819
KY1	Keem	99.01	AF270936	<i>Wickerhamomyces anomalus</i>	KC597821
KY2	Keem	100	EF192214	<i>Candida tropicalis</i>	KC597822
KY4	Keem	97.32	GU199447	<i>Candida glabrata</i>	KC597823
KY7	Keem	100	JQ768048	<i>Candida tropicalis</i>	KC597824
KL1	Keem	99.46	ACKU01000017	<i>Weissella paramesenteroides</i>	KC597827
KL2	Keem	99.44	ACKU01000017	<i>Weissella paramesenteroides</i>	KC597820
KL5	Keem	99.79	AJAM01000006	<i>Enterococcus casseliflavus</i>	KC597828
KL10	Keem	99.01	AJ420801	<i>Enterococcus durans</i>	KC597829
KL11	Keem	99.63	ACKU01000017	<i>Weissella paramesenteroides</i>	KC597830

(>320 × 10⁶ cfu/g) comprising *Wickerhamomyces anomalus* (former synonym, *Pichia anomala* and *Hansenula anomala*), *Candida tropicalis*, and *Candida glabrata*, while samples from Magrauli and Tiuni were observed relatively low in TVC of yeasts (<0.8 × 10⁶ cfu/g). In contrast, each Keem sample showed presence of more than 57 × 10⁶ cfu/g of LAB comprising *Weissella paramesenteroides*, *Enterococcus casseliflavus* and *E. durans* as identified on the basis of 16S rDNA sequence analysis. *Weissella* strains have also been reported from fermented soybean of North-East India [17] and Korean fermented food, Kimchi [18]. Keem samples were observed to contain 250 × 10⁶ cfu/g of amylolytic bacilli. These results suggest that colonization of microflora of plants phyllosphere or rhizosphere sometime fail and result in low quality of starter.

Further, reports indicate that variety of herbs have been used as essential ingredients in starter preparation by prominent tribal communities of India [6–8, 16, 19, 20]. In order to understand importance of plants in terms of microbial inoculum and diversity, Balma was prepared in the laboratory following the traditional practice (Fig. 1). The Balma which was prepared with infusion of herbs showed high TVC of yeasts and LAB comparable to microbial load of field samples collected from Bhotiya inhabited villages (Tables 2, 3). Each substrate was first

Fig. 1 Balma prepared in laboratory with various substrates following the traditional practice

baked and then autoclaved before kneading to avoid contamination. Bhotiya also bake the flour until it turns brown before kneading and making Balma [8]. Further, BIOLOG

Table 3 TVC of Balma prepared in laboratory following the traditional practice

Substrate used	TVC (cfu/g)		
	Yeasts	LAB	Bacteria
Wheat (<i>Triticum aestivum</i>)	3.2×10^8	1.3×10^6	Uncountable
Phafar (<i>Fagopyrum esculentum</i>)	1.96×10^8	1.0×10^6	Uncountable
Jau (<i>Hordeum vulgare</i>)	1.40×10^8	1.4×10^6	Uncountable
Koda (<i>F. tataricum</i>)	1.27×10^8	1.0×10^6	Uncountable

and BioMerieux analyses showed the presence of *B. subtilis*, *B. pumilus*, *Lactobacillus brevis*, *L. delbrueckii*, *E. casseliflavus*, *E. mundtii*, *Pediococcus pentosaceus* and *L. buchneri* among bacteria, while *Candida tropicalis* and *C. glabrata* among yeasts in Balma prepared using infusion. Balma which were prepared using autoclaved distilled water instead of infusion, get spoiled due to massive bacterial growth and eventually with overgrowth of species of *Rhizopus*, *Aspergillus* and *Penicillium*. On the basis of these observations, it may be speculated that infusion of herbs contain beneficial yeasts and LAB of phyllosphere and rhizosphere origin which colonize in Balma balls and also possess some antimicrobial property which prevent contamination and microbial spoilages of Balma during succession and enrichment process. Natural environment provide conditions which select species of bacteria, fungi and yeasts that are characteristically found with plants [21]. However, direct evidence of microbial source by isolating the same group of microbes from phyllosphere and rhizosphere of herbs is needed to validate it. Herbs such as *I. cuspidata*, *M. biflora*, *O. vulgare*, *Rubus* sp. and *T. linearis* used by Bhotiya community have already been reported elsewhere to possess antimicrobial properties [22–26]. Antimicrobial property of these herbs might be instrumental in preventing the microbial spoilage of Balma balls. Similar observations have also been made elsewhere [27]. Owing to relatively high TVC, *T. aestivum* may be the best substrate for preparation of Balma among others (Table 3). It was also observed during field survey that Bhotiya prefer *T. aestivum* over other cereals such as *Fagopyrum esculentum*, *F. tataricum* and *H. vulgare* for Balma preparation. These results showed that Bhotiya have optimized the process of Balma preparation by substituting the plants with previous year Balma as inoculum and only wheat flour as a substrate. In fact, they adopted a kind of “back-slopping” strategy for preparation of good and stable quality of Balma. This strategy exploits the ecological principle of inoculum enrichment by natural selection [28]. Older Balma contains relatively a high dose of potential microorganisms as compared to the microflora of plants phyllosphere or rhizosphere, which ensure the quality and high TVC in every new batch of starter. They compete with the spoilage causing microorganisms during

Fig. 2 Kinetics of lactic acid and ethanol fermentation of Chhang preparation using Balma of Lata village as inoculum

incubation, and in turn, compensate with antimicrobial activity of plants.

Under study of fermentation kinetics, it was noted that lactic acid fermentation started immediately after incubation and reached at about 0.53 % within 24 h but decreased thereafter (Fig. 2). Simultaneously, ethanol fermentation started after 12 h of incubation and completed at 49.65 g/l after 24 h when the residual sugar was almost utilized (1.1 g/l). Majority of bacilli were observed to produce amylase which suggests that amylolytic fermentation occurs in traditional fermentation systems of Bhotiya where cereals are used as major substrate. Previously, amylolytic *Rhizopus* and *Aspergillus* among filamentous fungi while *S. fibuligera* and *S. malanga* among yeasts have also been reported in traditional starters [15, 29–32]. Amylolytic microorganisms hydrolyze the starchy substrate and liberate the fermentable sugars which are used for lactic acid and ethanol fermentation by LAB and yeasts [33, 34]. Metabolic activity of LAB lowers down the pH which provides an optimal growth condition for yeasts to carryout ethanol fermentation. Similar kinetic model has also been observed in traditional fermented beverage of Eastern and North-Western Indian Himalaya [15, 35]. Rapid sugar utilization by LAB and yeasts eliminates the risk pertaining to growth of potential spoilage microorganisms.

Conclusively, it can be stated that Bhotiya and Jaunsari tribes have developed remarkable technique for long term preservation of potential amylolytic and ethanol-lactic acid fermenting microorganisms. However, as a rational approach Bhotiya have stopped using plants as source of fermenting microorganisms in every new batch of Balma preparation and substituted it with older Balma. In doing so, they successfully escaped the labour cost in plant collection and minimize the probability of failure or phyllosphere or rhizosphere mediated microbial colonization. Traditional alcohol fermentation in these tribal communities comprised of a typical starch hydrolysis followed by lactic acid and ethanol fermentation by virtue of the amylolytic bacilli, LAB and yeasts, respectively. Value added traditional alcoholic beverages may emerge as a potential livelihood option for these underprivileged tribes. However, optimum microbial load of functionally important strains in traditional starter, standardised fermentation kinetics, nutritional profile of final product, probiotic characteristics of LAB and scale-up of production are some of the issues need to be addressed.

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