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ORIGINAL ARTICLE

Lactic acid bacteria in Hamei and Marcha of North East India

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Abstract *Hamei* and *Marcha* are mixed dough inocula used as starters for preparation of various indigenous alcoholic beverages in Manipur and Sikkim in India, respectively. These starters are traditionally prepared from rice with wild herbs and spices. Samples of *Hamei* and *Marcha*, collected from Manipur and Sikkim, respectively, were analysed for lactic acid bacterial composition. The population of lactic acid bacteria (LAB) was 6.9 and 7.1 Log cfu/g in *Hamei* and *Marcha*, respectively. On the basis of phenotypic and genotypic characters, LAB strains isolated from *Hamei* and *Marcha* were identified as *Pediococcus pentosaceus*, *Lactobacillus plantarum* and *Lactobacillus brevis*. Technological properties of LAB such as antimicrobial properties, effect on acidification, ability to produce biogenic amines and ethanol, degree of hydrophobicity and enzymatic activities were also performed. *Pediococcus pentosaceus* HS: B1, isolated from *Hamei*, was found to produce bacteriocin. None of the strains produced biogenic amines. LAB strains showed a strong acidifying ability and they also produced a wide spectrum of enzymes.

Keywords LAB · Hamei · Marcha

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Introduction

Use of mixed starters in the form of dry ball-like cakes containing amylolytic and alcohol-producing yeasts, starch-degrading moulds and lactic acid bacteria is common in many Asian countries[1]. *Hamei* and *Marcha (Murcha)* are not foods but dry, round to flattened, solid ball-like mixed dough inocula used as starter cultures for preparation of various indigenous alcoholic beverages in North East India. Traditionally *Hamei* is prepared from crushed raw rice mixed with powdered bark of 'yangli' (*Albizia myriophylla*) and a pinch of previously prepared powdered *Hamei*. The dough is pressed into flat cakes and is kept over rice husk in a bamboo basket for 2–3 days at room temperature (20–30° C), and then sun dried for 2–3 days. *Hamei* is used to prepare a rice-based beverage locally called *Atingba* and a distilled clear liquor called *Yu* in Manipur.

During *Marcha* preparation, soaked glutinous rice is mixed with roots of 'guliyo jara' (*Plumbago zeylanica*), leaves of 'bheemsen paate' (*Buddleja asiatica*), flowers of 'sengreknna' (*Vernonia cinerea*), ginger, red dry chili and a pinch of powdered old *Marcha*. The mixture is then made into paste by adding water and kneaded into flat cakes of varying sizes and shapes, and placed individually on the ceiling floor made up of bamboo stripes above the kitchen, bedded with fresh fronds of ferns, locally called 'pire uneu' (*Glaphylopteriolopsis erubescens*), and covered with dry ferns and jute bags. These are left to ferment for 1–3 days, the longer period being used under the colder condition. Finally, cakes of *Marcha* are sun dried for 2–3 days[2]. *Marcha* is used to prepare alcoholic beverages such as *Kodo ko jaanr* (fermented finger millet beverage), *Bhaati jaanr* (fermented rice beverages) and *Raksi* (distilled liquor) in

Sikkim and the Darjeeling hills[3]. Rural women sell these starter-cakes in local markets in Manipur and Sikkim, respectively for their livelihood. *Hamei* and *Marcha* are similar to other Oriental starters such as *Ragi* of Indonesia, *Nuruk* of Korea, *Bubod* of the Philippines, *Loogpang* of Thailand and *Chiuyueh* of China and *Men* of Vietnam.

Pediococcus pentosaceus was the only lactic acid bacterium along with yeasts and moulds reported from *Marcha* samples of Nepal[4,5], and from the Darjeeling hills and Sikkim in India[6]. The identification of the reported *Pediococcus pentosaceus* was based on the limited phenotypic characteristics, without genotypic characterisation. To our best knowledge microbial composition of *Hamei* has not been reported yet. The aim of this paper was to identify lactic acid bacteria present in *Hamei* and *Marcha* based on both phenotypic and genotypic characteristics and also to study some technological properties of the LAB strains.

Materials and Methods

Collection of samples: Ten samples of *Hamei* were purchased from different shops in Ima market of Imphal in Manipur. Twelve samples of *Marcha* samples were collected directly from their places of preparation in different *marcha*-making villages in Sikkim. All samples were collected aseptically in sterile containers, and transported to laboratory for analyses.

Isolation of lactic acid bacteria: Ten gram of sample were mixed with 90 ml of 0.85 % sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min and were serially diluted in the same diluent. Lactic acid bacteria (LAB) were isolated on MRS agar (M641, HiMedia) supplemented with 1 % $CaCO_3$, and incubated at 30°C in an Anaerobic Gas-Pack system (LE002, HiMedia) for 48–72 h. Purity of the isolates was checked by streaking again and sub-culturing on fresh MRS agar plates, followed by microscopic examinations. Identified strains of LAB were preserved in MRS broth (M369, HiMedia) using 15 % (v/v) glycerol at −20°C.

Phenotypic characterization: The cell morphology and motility of LAB strains were observed in a phase contrast microscope (CH3-BH-PC, Olympus, Japan). Isolates were Gram-stained and tested for catalase production. Isolates were identified by testing for phenotypic properties such as gas production from glucose, ammonia production from arginin, growth at different temperatures (10, 15 and 45°C) and at different pH (3.9 and 9.6), as well as the ability to grow in different concentrations of NaCl (6.5, 10 and 18 %) in MRS broth[7]. 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 on cellulose plates[8]. Sugar fermentation patterns of LAB were determined using API 50 CHL test strips (bioMérieux, France) and the result was obtained using the APILAB PLUS database identification software (bioMérieux, France).

Genotypic characterisation: The DNA was extracted from overnight grown cultures in MRS broth[9]. The primer M13 (5'-GAG GGT GGC GGT TCT-3') was used for RAPD-PCR[10]. The PCR amplification was conducted[11] with a Primus 96 Plus thermal cycler (MWG Biotech, Ebersberg, Germany). For identification of *Lactobacillus brevis*, a species-specific PCR was applied using the oligonucleotide primer 5'-CTT GCA CTG ATT TTA ACA-3' and 5'-GGG CGG TGT GTA CAA GGC-3' as forward and reverse primers, respectively[12]. To verify the identity of the PCR product, amplified fragments were digested with P*st*I in a 15 µl reaction mixture containing 11.5 µl of the PCR product, 1.5 µl incubation buffer and 10 U P*st*I (1 h, 37°C). For identification of *Lactobacillus plantarum*, rep PCR with the primer GTG_5 (5'-GTG GTG GTG GTG GTG-3') was carried out using reaction and amplification conditions[13]. Amplification products were subjected to electrophoresis in 1.8 % agarose gels in TBE buffer[14].

Antagonism and bacteriocin activity: Isolates were screened for antagonistic activity by the agar spot method[15]. The indicator strains used for antagonisms were *Listeria innocua* DSM 20649, *Listeria monocytogenes* DSM 20600, *Bacillus cereus* CCM 2010 and *Staphylococcus aureus* S1. Cell-free neutralized supernatants of LAB isolates were screened for bacteriocin production by the agar spot test method[16]. Bacteriocin activity was quantified by two-fold serial dilutions of the neutralized supernatants, expressed as the reciprocal of the highest dilution exhibiting a zone of inhibition and were reported in activity unit (AU) per ml[17].

Acidification and coagulation: The acidification and coagulating abilities of the LAB strains were assayed by inoculating 10 % skim milk (RM1254, Hi Media) at 1 % level and incubation at 30°C. Observation was made of the commencement of clotting and the pH was measured[18].

Amylolytic activity: Surface-dried plates of starch agar were streaked with 24 h-old cultures, incubated at 30°C for 48 h. After incubation, the plates were flooded with iodine solution for 15-30 min and examined the clear zone underneath (after the growth was scrapped off) for amylolytic activity.

Enzymatic profile by API-zym system: The enzymatic profile of lactic acid bacteria were assayed using commercial 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 fucosidase.

Biogenic amine production: The ability of LAB strains to produce biogenic amines was determined qualitatively using a 'cocktail' of four precursor amino acids (histidine, lysine, ornithine and tyrosine)[19]. Change of the bromocresol purple indicator to purple was considered as an index of significant amino acid decarboxylase activity[18].

Hydrophobicity assay: Adhesion of LAB strains to hydrocarbons was determined[20] with slight modification. Isolates were grown in MRS broth at 30°C for 24 h, centrifuged at 8000 *g* for 5 min, and the pellet was washed three times with 9 ml of Ringer solution (Merck, Germany) and thoroughly mixed in a vortex. 1 ml of the suspension was taken and the absorbance at 580 nm was measured. Then, 1.5 ml of the suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia) in duplicate and mixed thoroughly in a vortex. The phases were allowed to separate for 30 min at room temperature, after which the aqueous phase was carefully removed and absorbance at 580 nm was measured. The percentage hydrophobicity was expressed as follows: hydrophobicity % = $[A_0\text{-}A/A] \times 100$, where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane. The percent hydrophobic index greater than 70 % was arbitrarily classified as hydrophobic[21].

Ethanol production: The ethanol yield of LAB strains, and reference yeast strains (*Saccharomycopsis fibuligera* MSD, *Pichia anomala* MAP, *Saccharomyces cerevisiae* MJS, and *Candida glabrata* MSY, previously isolated from *Marcha*)[22] was determined after growth at 30°C for 48 h in MRS broth and 25°C for 4 days in YM broth containing 10% glucose, respectively. Ethanol concentration in the culture supernatant was determined by an enzyme assay (F-Kit Ethanol; Roche Diagnostics Co., Ltd.).

Results and Discussion

Samples of *Hamei* and *Marcha*, collected from different places of Manipur and Sikkim, respectively were analysed

Table 1 Phenotypic characteristics of LAB isolated from *Hamei* and *Marcha.*

Parameter	Strains				
	HB:B1	HB:B4	HS:B1	MA:R5	MA:C1
Cell morphology	Rod	Coccus	Coccus	Rod	Coccus
CO_2 from glucose	–	–	–	+	–
NH_3 from arginine	–	+	+	–	+
Growth in 10 % NaCl	–	–	–	+	+
DAP	+	–	–	–	–
Isomer of lactate	DL	DL	DL	DL	DL
Sugars fermented:					
Ribose	–	–	+	+	+
Xylose	–	+	–	+	+
Galactose	–	–	–	+	+
Sucrose	+	+	–	–	–
Trehalose	–	–	–	–	+
Mannose	+	–	–	–	+
Amygdaline	–	+	+	–	+
Esculin	+	–	–	–	–
Salicin	+	–	+	–	+
Cellobiose	–	–	–	–	+
Maltose	–	+	–	+	+
Lactose	–	+	+	–	–
Melibiose	+	–	+	+	–
Identity	***Lb. plantarum***	*P. pentosaceus*	*P. pentosaceus*	*Lb. brevis*	*P. pentosaceus*

All strains are Gram positive and catalase negative; all grew at 10^0 C and 15^0 C, in pH 3.9 and 6.5 % NaCl; no growth was observed at 45^0 C, in pH 9.6 and 18 % NaCl.

None of the strains fermented starch, arabinose, rhamnose, sorbitol, raffinose and melezitose.

Table 2 Acidification and degree of hydrophobicity of LAB strains from *Hamei* and *Marcha.*

Strains	Acidification[a] and Coagulation[b] (30^0 C)	% Hydrophobicity[c]
Control (Skim milk)	– (6.8)	10.1[d] ± 0.92
Lactobacillus plantarum HB:B1	+ (4.2)	63.7 ± 1.95
Pediococcus pentosaceus HB:B4	– (5.7)	3.7 ± 1.06
Pediococcus pentosaceus HS:B1	– (5.4)	1.3 ± 0.51
Lactobacillus brevis MA:R5	– (4.3)	3.8 ±1 .96
Pediococcus pentosaceus MA:C1	– (5.7)	3.8 ± 0.38

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

[a]Numbers in brackets represent the pH values for acidification.

[b]Coagulation occurred at 22 h; +, indicates coagulation; –, indicates non-coagulation.

[c] % Adherence ≥ 75 %, hydrophobic; 26–74 %, intermediate; < 25 %, hydrophilic.

[d] % Hydrophobicity of *Lb. rhamnosus* GG (BFE 752) as a control.

for lactic acid bacterial load. The average population of LAB in *Hamei* was 6.9 Log cfu/g and *Marcha* was 7.1 Log cfu/g, respectively. All bacterial isolates from samples of *Hamei* and *Marcha*, were considered lactic acid bacteria based on their positive Gram reactions, nonmotality, absence of spore formation and catalase activity. Out of 45 isolates, 29 isolates were cocci and 16 were lactobacilli on the basis of cell morphology, gas production and arginine hydrolysis tests (data not shown). Five representative strains were selected randomly for further phenotypic characterisation including determination of the sugar fermentation pattern using the API system, lactate configuration and meso-diaminopimelic acid (Table 1). Based on the phenotypic characteristics and interpretation of APILAB PLUS database and also following the taxonomical keys of Wood and Holzapfel[23], homofermentative *Lactobacillus* strain HB:B1 (isolated from *Hamei*) was identified as belonging to the *Lactobacillus plantarum* group; the heterofermentative strain MA:R5 (*Marcha*) was identified as *Lactobacillus brevis*; and the tetrad-forming cocci strains HB:B4 and HS:B1 (*Hamei)* and MA:C1 (*Marcha*) were identified as *Pediococcus pentosaceus*.

The RAPD-PCR characterisation of all 5 strains of LAB was performed (data not shown) and revealed the close relationship of the two *Pediococcus* isolates from *Hamei*. The application of the rep-PCR with GTG_5 primer confirmed the homofermentative *Lactobacillus* HB:B1 as *Lactobacillus plantarum* and the species-specific PCR confirmed the heterofermentative *Lactobacillus* MA:R5 as *Lactobacillus brevis*. Occurrence of *Lactobacillus plantarum* and *Pediococcus pentosaceus* in *Hamei* is reported for the first time in this paper. *Pediococcus pentosaceus* was more predominant than lactobacilli in both *Hamei* and *Marcha*.

Antagonistic properties of LAB strains were tested against *Listeria innocua* DSM 20649, *Listeria monocytogenes* DSM 20600, *Bacillus cereus* CCM 2010 and *Staphylococcus aureus* S1. All three strains of LAB isolated from *Hamei* showed strong inhibition zones against these bacteria in the agar spot assay, whereas in *Marcha* only *P. pentosaceus* MA:C1 showed strong inhibition zones. This suggests that antimicrobial properties of functional LAB can reduce the number of other undesired microorganisms in this traditional starter. However, using culture supernatants only *P. pentosaceus* HS:B1 (*Hamei*) was found to produce bacteriocin in the applied method. Quantification of bacteriocin produced by *P. pentosaceus* HS:B1 (*Hamei*) against *Listeria innocua* DSM 20649 and *Listeria monocytogenes* DSM 20600 was calculated as 128 AU/ml and 32 AU/ml, respectively (data not shown). It is interesting to note that *Hamei* contained bacteriocin-producing pediococcus, whereas no such activity was observed in *Marcha*.

Strains of LAB were screened for their ability to produce biogenic amines. None of the strains of LAB produced biogenic amines in the applied method (data not shown). Inability of all the LAB strains to produce biogenic amines is a good indication for starter cultures[24]. All LAB strains showed acidification of milk at 30º C with a significant drop in pH (Table 2). However, none of them showed coagulation properties except *Lb. plantarum* HB:B1 (*Hamei*). Most of the tested strains did not show strong hydrophobic properties (Table 2). However, the degree of hydrophobicity in *Lb. plantarum* HB:B1 (*Hamei*) was 63.7 % indicating its intermediate hydrophobic nature.

Enzymatic profiles of LAB strains were assayed using the API zym (bioMérieux, France) galleries (Table 3). Each of the predominant LAB strain produced a wide spectrum of enzymes. All strains showed strong arylamidase and glucosaminidase activities. However, they showed no detectable esterase, lipase, phosphatase and proteinase activity in the method applied. Absence of proteinases (trypsin and chymotrypsin) and presence of strong peptidase (leucine-arylamidase) activities produced by the LAB isolated from *Hamei* and *Marcha* are possible traits

Table 3 Enzymatic profiles using API zym system of LAB from *Hamei* and *Marcha.*

Enzyme	Activity (nanomoles)				
	HB:B1	HB:B4	HS:B1	MA:R5	MA:C1
Control (without enzyme)	0	0	0	0	0
Alkaline phosphatase	0	0	0	0	0
Esterase (C4)	0	0	0	0	0
Esterase lipase (C8)	0	0	0	0	5
Lipase (C14)	5	5	5	5	5
Leucine arylamidase	>40	>40	>40	>40	>40
Valine arylamidase	>40	>40	>40	>40	>40
Cystine arylamidase	10	10	10	10	5
Trypsin	0	0	5	0	0
α-chymotrypsin	5	0	0	0	0
Acid phosphatase	10	5	5	20	10
Napthol-AS-BI-phosphohydrolase	5	10	10	10	10
α-galactosidase	0	0	0	0	0
β-galactosidase	10	0	0	0	0
β-glucuronidase	0	0	0	0	0
α-glucosidase	0	0	0	0	0
β-glucosidase	>40	5	5	10	5
N-acetyl-β- glucosaminidase	30	20	20	30	20
α-mannosidase	0	0	0	0	0
α-fucosidase	0	0	0	0	0

Data represents the means of 3 sets of experiments.

HB:B1 *Lb. plantarum* (*Hamei*); HB:B4 *P. pentosaceus* (*Hamei*); HS:B1 *P. pentosaceus* (*Hamei*); MA:R5 *Lb. brevis* (*Marcha*); MA:C1 *P. pentosaceus* (*Marcha*).

for production of typical flavour in traditional alcoholic beverages. The ability of LAB strains present in *Hamei* and *Marcha* to produce ethanol was tested along with yeasts, previously isolated from *Marcha* using the enzyme assay kit (Figure 1). Data showed that reference yeast strains mostly *Saccharomyces cerevisiae, Pichia anomala* and *Saccharomycopsis fibuligera* produced a high amount of alcohol comparable to that of LAB strains which was negligible. This confirms the strains of LAB present in *Hamei* and *Marcha* have little or no role in alcohol production.

Preliminary screenings of amylolytic activities of LAB isolates were tested in the starch agar plates (data not shown). None of the lactic acid bacteria showed amylolytic activity indicating they have no role in saccharification and liquefaction of starchy substrates. The inability to utilize starch by LAB (Table 1) indicates that they are not significant contributors to the breakdown of starch of substrates during preparation of *Hamei* and *Marcha* itself or any beverage; probably their role is to give flavour and impart mild sour-taste to *Aitanga, Kodo ko jaanr* and *Bhaati jaanr*, traditional cereal-based alcoholic beverages of Manipur and Sikkim, respectively[25,26].

Hamei and *Marcha* making technology reflect an indigenous knowledge of sub-culturing of desirable inocula from previous batch to a new culture using rice as a base substrate. This indigenous technique of 'microbiology' preserves the microbial diversity essential for alcoholic beverage productions in the Himalayas. *Hamei* and *Marcha* retain their potencies *in situ* for over a year or more. Like other starter cultures of Asia, *Marcha* showed coexistence of mixed population of filamentous moulds, yeasts and lactic acid bacteria[22]. Reports showed that *Saccharomycopsis fibuligera* played the main roles in amylase production in Asian mixed starter cultures, and *Rhizopus* seemed to supplement the saccharification[26,27]. It can be concluded that LAB present in *Hamei* and *Marcha* play role in imparting flavour, antagonism and acidification of the substrates.

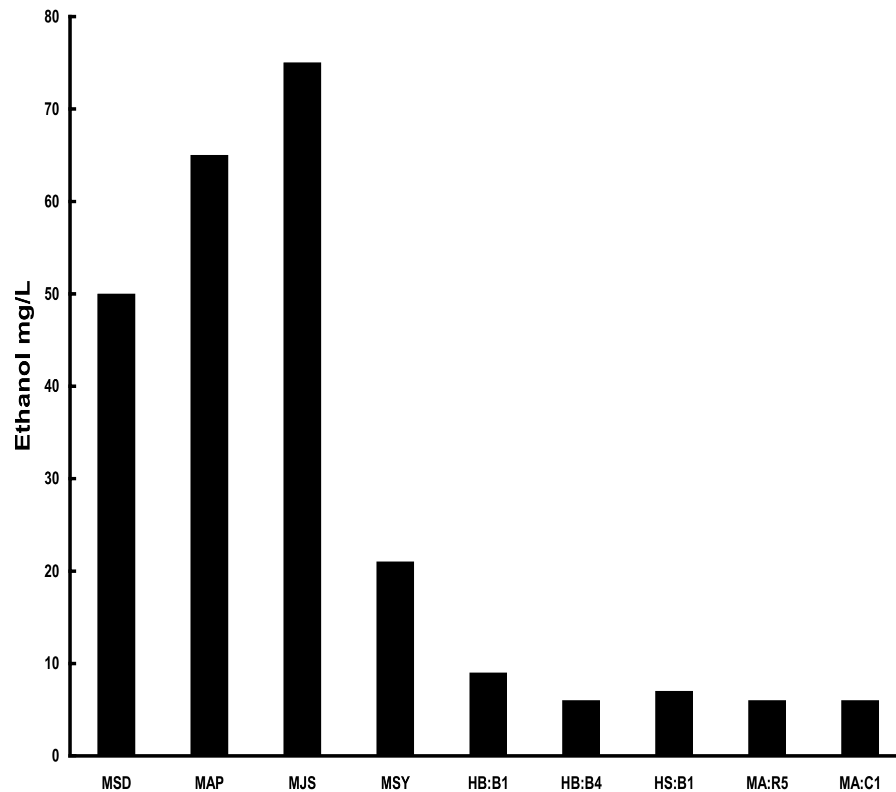

Fig. 1 Ethanol production by yeast strains (MSD, *Saccharomycopsis fibuligera*; MAP, *Pichia anomala*; MJS, *Saccharomyces cerevisiae* and MSY, *Candida glabrata*, previously isolated from *Marcha*) and LAB strains (HB:B1, *Lb. plantarum*; HB: B4, *P. pentosaceus*; HS:B1, *P. pentosaceus* all isolated from *Hamei*; MA:R5, *Lb. brevis*; MA:C1, *P. pentosaceus* isolated from *Marcha*.

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