Phenotypic identification and technological attributes of native lactic acid bacteria present in fermented bamboo shoot products from North-East India

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Abstract Fermented bamboo shoots such as *Soibum*, *Soidon*, *Eup*, *Hirring*, *Hecche* and *Ekung* etc. are non-salted acidic products obtained by natural fermentation predominantly with lactic acid bacteria (LAB). In this study, we have characterized 11-representative LAB that includes, *Lactobacillus* sp. ($n=2$), *Lactobacillus plantarum* ($n=3$), and one each of *Lactobacillus fermentum*, *Lactococcus* sp., *Lactobacillus brevis*, *Lactobacillus curvatus*, *Leuconostoc* sp. and *Lactobacillus xylosus*. Subsequently, these cultures were studied for their technological and functional properties. Different isolates exhibited variation in their activities. *L. brevis* showed maximum phytic acid degradation ability (19.33 U ml^{-1}). *L. xylosus* had highest protease activity (64.2 nmol/ml) and also exhibited lipolytic activity. In addition, degree of cell hydrophobicity among these cultures ranged between 12.5 and 93.48 % with *L. plantarum* (SM2) showing highest degree of activity. *Lactobacillus plantarum* was the most common species found in the product studied. Results indicated that most of the LABs showed putative probiotic as well as antagonistic properties against the selected pathogenic bacteria. Characteristic aroma, flavour and texture in the fermented bamboo shoot products could be attributed to presence of these new LAB isolates.

Keywords Fermented bamboo shoots · Probiotics · *Lactobacillus brevis* · *Lactobacillus curvatus*

Introduction

The fermented bamboo shoot products are significant source of protein, carbohydrates, fibre, many essential micronutrients

such as cobalt, copper, lead, manganese, iron, selenium, zinc and cadmium (Nirmala et al. 2007). They are rich in polyphenols like, coumaric acid, synergic acid, ferulic acid, chlorogenic acid, catechin and hydroxybenzoic acid which are responsible for antioxidant activity. Lactic acid bacteria (LAB) play significant role in bamboo shoot fermentation. Involvement of LAB in fermentation results in the enhancement of flavour and aroma and assist in the detoxification of certain undesirable compounds (tannin and phytates) which may be present in the raw material (Das and Deka 2012). The microbial population in fermented bamboo shoot is reported to be in the range of 10^8 cfu g^{-1} that comprised of the LAB and *Bacillus* (Jeyaram et al. 2010; Tamang et al. 2012).

At present, most of the fermented bamboo shoots are prepared with a traditional method allowing natural fermentation by back-slopping. There is no organized sector for large scale production. In order to develop a pilot scale production technology it is essential to identify a dominant microflora associated with fermentation. Previous studies indicated characterization of mixed lactic microflora involved in bamboo fermentation (Jeyaram et al. 2010). However, based on the starter inoculum used for back-slopping and the raw materials, there is possibility in the variation in microflora. Recently, we have reported that, there are varieties of organic acids and flavouring compound produced during the fermentation of bamboo shoots (Sonar et al. 2014), suggesting involvement of different LAB. New bacterial isolate, that has not been reported before could potentially contribute for the development of desirable end product. Fermented bamboo shoot constitutes a unique ecological niche wherein a plethora of microorganisms co-exist (Jeyaram et al. 2010). Hence it can be conceived that these indigenous acidic non-salted fermented products could act as a natural repository of diverse LAB with unique properties. This premise formed the rationale of the present investigation, wherein the primary objective of study was to obtain representative lactic acid bacteria from

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fermented bamboo shoots and screening for the desirable functional and technological attributes.

Materials and methods

Isolation of LAB Bacterial cultures included in this study were isolated from different types of fermented bamboo shoots. These include, four samples of *Soibum* obtained from Assam, three sample of *Soidon* from Manipur; one each of *Eup*, *Hirring*, *Hecche* and *Ekung* obtained from Arunachal Pradesh states of India. About 10 g of each sample was transferred to 90 ml of 0.85 % (w/v) sterile saline and homogenised and initial mother stock was prepared. From the initial stock, 1 ml of sample was serially diluted into 9 ml of saline and plated on MRS (deMann, Rogosa and Sharpe) agar (Hi Media, Mumbai, India) depending upon the species and incubated at 30 °C for 24–48 h. Based on the colony morphology, 2–5 colonies were subjected for microscopic examination and CO₂ production (using Durham tube) in glucose. Representative cultures of LAB were selected, pour-plated to obtain pure colonies. The purified isolates were preserved at –20 °C in MRS media with glycerol (20 % v/v) for further studies.

Characterization and identification Cultures were processed for Gram-staining and were examined by phase contrast microscopy (DP50 digital camera, Olympus Optical Co. Ltd., Tokyo, Japan). Cell morphology and purity was visualised further by scanning electron microscopy (SEM) employing 0.3 % glutaraldehyde as cell fixative, using the instrument Leo 435 Electron microscope (Cambridge, UK) under 20X magnification. Catalase test was conducted using 20 % H₂O₂. The different physiological conditions of growth studied includes, temperature at 45 °C, pH at 3.9 and 9.6, NaCl concentration of 6.5, 10 and 18 %. Modified MRS broth containing bromocresol purple as an indicator was studied for acid production. Sugars such as sucrose, fructose, mannitol, sorbitol, xylose and maltose (obtained from Hi Media) were added at 2 % level to the sterilised MRS broth (devoid of glucose and beef extract), inoculated with 18–24 h grown LAB cultures (1 % v/v) and incubated at 37 °C for 2–3 days. A colour change in the broth observed was considered positive for acid production by fermentation of sugars.

Acidification and coagulation Acidification and coagulation abilities of isolated cultures were assayed using 10 % skim milk which was inoculated with 18–24 h grown LAB cultures (1 % v/v) and incubated at 37 °C for 3 days. Observation was made for coagulation and the pH was measured at the end of 72 h (Tamang et al. 2009).

Bile salt tolerance Ability of the cultures to grow in the presence of bile salt was tested by inoculating 10 µl of

overnight grown bacterial culture, on MRS broth medium supplemented with 0.5 % of bile salts (Hi Media). Change in optical density was recorded at 600 nm at 4 h intervals (du Toit et al. 1998).

Proteolytic activity and protease assay Ability of the isolates to produce protease and degrade casein was tested by clearance zone formation around the growth of organism. The inoculum from log phase (18–24 h) was streaked on milk agar plate (2 % Skim milk powder in 1.5 % bacteriological agar) (Hi Media) incubated at 37 °C for 3–5 days and plates were observed for formation of zone of clearance (Gordon et al. 1973). Additionally, Protease assay was carried out according to Thapa et al. (2004) with minor modifications. The protease activity was obtained from standard plot of tyrosine. One unit of protease activity was defined as the amount of enzyme required to produce one nmol of tyrosine under standard assay conditions.

Amylolytic activity Overnight grown culture was streaked on starch agar plate (2 % starch in 1.5 % bacteriological agar) (Hi Media) and incubated at 37 °C for 3–5 days. After incubation the plates were flooded with iodine solution for 15–30 min and examined for the formation of clear zone which indicate amylolytic activity (Gordon et al., 1973).

Lipolytic activity To check the presence of lipolytic activity, plates of tributyrin agar were streaked with 18–24 h culture and incubated at 37 °C for 4 days. Lipolytic activity was detected by a clear zone formation surrounding the culture in the plate (Jini et al. 2011).

Phytase activity Phytase activity of LAB was estimated by ferrous sulphate molybdenum blue method, as modified in our laboratory. The phytase activity in the cell suspension (prepared as described by Raghavendra and Halami 2009) was obtained from standard plot of inorganic phosphate. One unit of phytase activity was defined as the amount of enzyme required to release one µmol of phosphate min^{–1} at 37 °C ml^{–1}.

Biogenic amines production The ability of LAB isolates to produce biogenic amines was determined qualitatively on decarboxylase screening medium as described by Joosten and Northolt (1989), using a cocktail of four precursor amino acids (histidine, lysine, ornithine and tyrosine). Change of the bromocresol purple indicator to purple was considered as index of significant amino acid decarboxylase activity that corresponding to >350 mg of a particular amino acid L^{–1} (Olasupo et al. 2001).

Antibacterial activity The antibacterial activity of the isolates was determined by the agar well diffusion method using 18–

24 h neutralized cell-free supernatants (Devi and Halami 2011). The indicator strains used included: *Listeria monocytogenes* Scott A, *Staphylococcus aureus* FRI722 and *Micrococcus luteus* ATCC 9341.

Bacterial adhesion to hydrocarbons (BATH)/hydrophobicity test Degree of hydrophobicity of the strains was determined by employing the methods described by Thapa et al. (2004) which is based on adhesion of cells to hexane droplets. The percentage hydrophobicity of strain adhering to hexane was calculated using the equation: Hydrophobicity (%) = (OD580 (initial)—OD580 (hexane)*100)/OD580 (initial).

Results and discussion

Phenotypic characterization of LAB The total LAB count observed in MRS agar plate ranged from 6×10^2 to 15×10^6 cfu gm⁻¹. However, only 11 representative LAB cultures were selected from the fermented bamboo shoots based on their typical colony morphology, gas production etc. These FBS isolates were designated as SM₁, SM₂, SM₃, SM₄ (from *soibum*), SNM₁ and SNP₁ (from *soidon*), SNK₁ (from *soidon*), HE₁, HE₂ (from *hecche*), E1 (from *eup*) and HI₁ (from *hiring*). All the isolated cultures were Gram-positive, and were found to be rods except for SM₃ which was cocci. Additionally, SEM analysis (Fig. 1) was also performed and it was observed that HI₁, HE₂, HE₁ and E1 were short broad rods, whereas SM₁, SM₂, SM₄, SNP₁, SNK₁, SNM₁ were narrow and elongated. The bacterial isolates were catalase negative; eight cultures grew at 45°C, at different pH (3.9 and 9.6) and NaCl concentrations (6.5 %). Except SM₁, none of the culture could grow in presence of 18 % NaCl. Also the ability of the cultures to produce gas from fermentation of glucose was checked. It was found that, seven cultures viz. SM₂, SM₃, SNM₁, SNP₁, HE₂, HI₁ and E1 were found to be homofermentative, whereas four isolates i.e. SM₁, SM₄, SNK₁ and HE₁ were heterofermentative. Out of the seven carbohydrates tested for acid production by these cultures, all the isolates were able to utilize glucose, fructose and maltose. Xylose was least utilized and only isolate (E1) was able to produce acid. Based on the results obtained from morphological, physiological and sugar utilization tests and comparison of the results presented in Bergey's manual of Systematic Bacteriology (Sneath et al. 1999), the isolates were tentatively identified as *L. fermentum* (SM₁), *L. plantarum* (SM₂), *Lactococcus* sp. (SM₃) and *L. brevis* (SM₄) in *soibum*; *L. curvatus* (SNK₁) from *soidon*. Tamang et al. (2007) have also reported the presence of *L. curvatus*, *L. plantarum* and *L. brevis* in *soidon* and *soibum* samples. *L. plantarum* and *L. brevis* were the dominant strains obtained by Jeyaram et al. (2010) in samples of *soidon*. The isolates HE₁ and HE₂

(isolated from *hecche*) were identified as *Leuconostoc* sp. and *L. plantarum*, respectively. E1 (*eup*) and HI₁ (*hiring*) were identified as *L. xylosus* and *L. plantarum*, respectively. Occurrence of *L. plantarum* in most of the products and presence of *L. xylosus* in *Eup* is a new finding reported in this study.

Technological and functional properties In order to elucidate the role of indigenous LAB found in the fermented bamboo shoots, several technological attributes were studied. Results of these tests are presented in Table 1. Isolates HE₁ and E1 showed lipolytic activity as demonstrated by clear zone formation. None of the other isolates were able to degrade tributyrin. In protease assay, all the cultures showed clear zone of casein hydrolysis on skim milk agar plate and hence, were subjected for quantitative protease assay. *L. xylosus* (E1) also showed highest protease activity (64.2 nmol/ml) and least was *Lactobacillus* sp SNP₁ from *Soidon*. Microorganisms associated with the fermentation of foods produce desirable amounts of enzymes, which may degrade anti-nutritive compounds and thereby convert the substrates into edible products with enhanced flavour and aroma (Tamang and Tamang 2009). Phytic acid is one such anti-nutritive compound that forms an insoluble complex with calcium, zinc, iron and copper and interferes with their absorption in the diet. Hence, the ability of LAB isolates to degrade phytic acid was analysed on the basis of their phytase activity. It was observed that among 11 cultures, *L. brevis* (SM₄) from *soibum* had the maximum phytase activity of 19.33 U ml⁻¹ followed by *L. plantarum* (SM₂). However, *L. plantarum* (HE₂), homofermentative *Lactobacillus* sp. isolated from *soidon* (SNM₁) and heterofermentative *L. fermentum* (SM₁) did not show phytase activity. *L. brevis* isolated from *Hiring*, an ethnic tender bamboo shoot foods of NE India reported for considerable degradation of phytic acid (Tamang et al., 2008; Tamang and Tamang, 2009). Previously, we have reported the production of phytase like enzyme by several LAB cultures obtained from fermented foods and poultry intestinal sources that known to exhibit functional properties (Raghavendra and Halami 2009). It was observed that none of the LAB isolated in the study were amylolytic as they failed to liquify starchy substrate. In this study, amylolytic LAB as expected could not be recovered since the initial screening material was devoid of starch. During preparation of *Hamei* and *Marcha* several amylolytic LAB were reported (Tamang et al. 2007). Such amylolytic cultures could probably be associated with imparting the flavor and sour taste to traditional cereal-based alcoholic beverages of North East India (Tamang et al. 2007). Biogenic amines (histamines, tyramines etc.) are organic basic compounds which occur in different types of foods such as sauerkraut, fishery products, cheese, wine, beer, dry sausages and other fermented fruits and vegetables (Suzzi and Gardini, 2003). Either of the cultures

Fig. 1 SEM image of LAB cultures isolated from FBS samples. (A) HI₁; (B) HE₂; (C) E₁; (D) SM₁; (E) SM₃; (F) SM₄; (G) SM₂; (H) SNM₁; (I) SNP₁; and (J) SNK₁

isolated in the present study failed to produce biogenic amines from lysine, ornithine, tyrosine and histidine. In foods, biogenic amines are mainly generated by decarboxylation of the corresponding amino acids through substrate specific enzymes of the microorganisms present in them (Tamang et al.

2008). The production of biogenic amines is not a desirable property for LAB strains to be selected as starter cultures (Tamang and Tamang, 2009). In parallel, the antibacterial activity of these isolates was screened against *Listeria monocytogenes* Scott A, *Staphylococcus aureus* FRI722 and

Table 1 Technological properties of LAB isolated from fermented bamboo shoot

Sample	Isolates	Lipolytic activity	Protease activity (nmol ml ⁻¹ min ⁻¹)	Phytase activity (U ⁻¹ ml)	Acidification (pH) and coagulation*	% Hydrophobicity
Soibum	<i>L. fermentum</i> (SM ₁)	–	24.3	0	4.38 (+)	59.81
	<i>L. plantarum</i> (SM ₂)	–	25.1	14.78	3.9 (+)	93.48
	<i>Lactococcus</i> sp. (SM ₃)	–	25.6	13.81	4.34 (+)	26.18
	<i>L. brevis</i> (SM ₄)	–	32.5	19.33	4.70 (+)	70.47
Soidon _m	<i>Lactobacillus</i> sp. (SNM ₁)	–	23.8	0	5.78 (–)	30.98
Soidon _p	<i>Lactobacillus</i> sp. (SNP ₁)	–	21.1	11.81	4.80 (+)	12.5
Soidon _k	<i>L. curvatus</i> (SNK ₁)	–	33.8	14.67	5.60 (–)	40.58
Hecche	<i>Leuconostoc</i> sp. (HE ₁)	+	26.9	13.97	4.87 (+)	39.04
	<i>L. plantarum</i> (HE ₂)	–	30.8	0	3.75 (+)	18.58
Eup	<i>L. xylosus</i> (E ₁)	+	64.2	13.42	5.36 (+)	47.68
Hirring	<i>L. plantarum</i> (HI ₁)	–	24.2	11.94	3.98 (+)	55.91

ND means not detected; *Coagulation reaction/lipolytic activity (+positive; –negative)

Micrococcus luteus ATCC 9341, *L. plantarum* strains (SM1 and HI1) from *soibum* and *hirring*, homofermentative *Lactobacillus* sp. isolated from *soidonm* (SNM2), *Leuconostoc* sp., were lacking antibacterial activity against these pathogens. *L. xylosus* (E1) and (HE1) showed antibacterial activity against *Staphylococcus aureus* FRI722, inhibitory activity was not observed against *Listeria monocytogenes* Scott A and *Micrococcus luteus*. Tamang et al. (2008) have reported the production of a bacteriocin by a *L. plantarum* strain isolated from *inziangsang* which is active against *Staphylococcus aureus*. Previously, we have reported large number of pediocin PA-1 producing intergeneric and interspecific LAB isolated from vegetable sources (Devi and Halami 2011), suggesting fermented vegetables as an important source for obtaining LAB cultures for basic studies. Adherence is one of the most important selection criteria for probiotic bacteria (Tamang and Tamang 2009). LAB isolated from bamboo fermented foods showed moderate degree of hydrophobicity indicating that isolates were not hydrophilic in nature. Although, hydrophobicity is only the indirect indication of hydrophobic interaction between bacterial cell surface and epithelial cells, it provides a preliminary idea of adherence ability of the potential cultures. Previously, LAB obtained from FBS found to possess potential probiotic properties such as adherence to intestinal cell wall, colonize in gastrointestinal tract and able to prevent pathogens from their adherence (Tamang et al. 2009). A high degree of hydrophobicity was observed in LAB isolated from *soibum* i.e. *L. plantarum* (SM2) and *L. brevis* (SM4) indicating strain were not hydrophilic in nature. In addition to above, acidification is an important functional attribute for selecting starter cultures among the LAB. The ability of some species of LAB particularly *L. plantarum* in acidification of the substrates is significant for food preservation (de Vuyst 2000). In the present study, the isolated LAB showed growth in presence of bile salt with 0.5 % concentration. *L. plantarum* strains (SM2, HE2 and HI1) showed the lowest acidification value of pH less than 4.0 along with the coagulation of milk among all tested strains of LAB. *L. curvatus* SNK1 and homofermentative *lactobacillus* sp. SNM1 from *soidonp* were unable to coagulate milk as well as showed the acidification value of pH more than 5.0.

In conclusion, the fermented bamboo product comprising LAB provides a selective characteristic aroma, flavour and texture with desirable environment to fermentative bacteria other than lactics. Results presented in this investigation demonstrated the complexity of LAB microflora especially the species of *L. xylosus* and *L. curvatus* that were not reported before found to possess the protective and functional properties which can be used as starter culture(s) for controlled optimized production of food preservation. Hence, including these species as starter cultures during fermentation process formulation can impart potential health attribute to the consumers.

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