CHAPTER 3

ASSESSMENT OF PROBIOTIC DIVERSITY IN DIFFERENT FERMENTED FOODS OF ASSAM AND ARUNACHAL PRADESH

Assessment of probiotic diversity in different fermented foods

3.1. Abstract

Microorganisms isolated from selected fermented foods of Assam and Arunachal Pradesh were characterized using biochemical techniques and clustered with known strains. Out of total 210 strains isolated, 99 were presumptively characterized as lactic acid bacteria, 52 as *Bacillus* and 31 were characterized as yeasts. Further, strains were characterized for probiotic properties and it was found that 5 isolates, i.e. D6, DS1, NK7, ARDMC1, NL6 and DK6 fulfilled the prerequisite criteria for probiotics and were clustered together with known probiotics.

3.2. Introduction

North-East India comprises seven contiguous states sharing more than 1400 km international border with China, Bhutan, Myanmar and Bangladesh [1]. Among all the North- Eastern states Arunachal Pradesh and Assam share maximum area and substantial population diversity. Approximately 82.89% and 13.67% people of Arunachal Pradesh and Assam respectively belong to different tribal communities [2] gifted with unique culture and identity. This cultural diversity is also reflected on their food habits in spite of sharing the common mode of livelihood, the agrarianism. As a result of close relationship with nature, these people have extensive knowledge about the flora and fauna of this region and the production of fermented foods and beverages utilizing natural resources are ubiquitous in their culture. Due to the perishable nature of most of the cultivated fruits and vegetables, people adopt fermentation as a measure of preservation. Apart from that, people realized the usefulness of fermentation as a flavour enhancer which led to the invention of different fermented food products with enhanced organoleptic properties. The common people may be oblivious towards the science of fermentation, but they know how to provide favourable conditions for fermentation and thus to allow beneficial microorganisms to grow rapidly [3]. More than 250 different types of fermented foods are prepared in the North-East region of India [4]. According to Tamang [5], based on the substrates used the fermented foods prepared in the North- East can be divided into the following groups: fermented soybean and non- soybean legume foods, fermented vegetables, bamboo shoots, fermented cereals and pulses, fermented fish

and meat products, milk- based fermented products, non-food mixed amylolytic starters, and alcoholic beverages. These fermented products contain a plethora of microorganisms ranging from yeasts and filamentous fungi, *bacilli*, lactic acid bacteria to *micrococci* with putative health- beneficial effects. [6]. In this chapter, the microbial diversity of selected fermented foods collected from different areas of Assam and Arunachal Pradesh were assessed using conventional phenotypic, biochemical and molecular techniques. Microorganisms showing comparable characteristics were grouped into similar clusters. The probiotic properties of the isolates were assessed and compared with the reference probiotic strains.

3.3. Materials and methods

3.3.1. Collection of samples

Survey was done in some selected rural areas covering the states of Assam and Arunachal Pradesh. After thorough discussion with the local people who prepares the fermented foods process of fermentation was documented. The indigenous methods of preparation of different types of samples and their use, sample age etc. were also documented. All samples were collected in sterile sample containers, sealed, labeled with appropriate code numbers and immediately stored at -20 ºC for further studies.

3.3.2. Microbial analysis

3.3.2.1. Isolation of microorganisms

Adequate amount of sample (1gm) was homogenised with 9 ml of 0.85% normal saline. A serial dilution was made in the same buffer and spread on de Man Rogosa and Sharpe (MRS) agar, nutrient agar, yeast and mould agar (YMA) plates for the selective isolation of lactic acid bacteria, non lactic acid bacteria and yeasts. Plate count agar was used for total mesophilic count and performed by pour plate method. For bacteria, incubation was performed at 37 ºC for 24-48 h and for yeasts at 28 ºC for 24- 48 h. After the incubation period total number of colonies was counted and square root of the total number of colonies were randomly picked [7] and streaked on to the respective media. Pure microbial cultures were stored in 20% (w/v) glycerol at -80 ºC.

3.3.2.2. Reference strains used

Lactobacillus plantarum MTCC 1407, *Lactobacillus rhamnosus* MTCC 1408 were obtained from Microbial Type Culture Collection (MTCC), IMTECH, India, *Pediococcus pentosaceus* NCDC 273 was obtained from National Collection of Dairy Cultures (NCDC), NDRI, India, *Bacillus subtilis* strain AMS6 (GenBank: KP723361), *Lactobacillus paracasei* strain AMD5 (GenBank: KJ867174), *Bacillus amyloliquefaciens* strain AMS4 (GenBank: KJ162396), *Enterococcus faecalis* strain AMS5 (GenBank: KJ162395), *Leuconostoc mesenteroides* subsp. *mesenteroides* strain AMD20 (GenBank: KC617923), *Lactococcus lactis* strain AMD17 (GenBank: KF113841) and *Lactobacillus fermentum* strain AMD1 (GenBank: KC759404) were isolated and characterized in the Industrial and Applied Microbiology Laboratory, Tezpur University.

3.3.2.3. Physiological and biochemical characterization

Isolates were pre- grown in their respective medium and overnight cultures were used for further work. Gram staining and catalase production test were performed according to Norris et al. [8]. CO_2 production was evaluated according to Muller [9]. Ammonia production was assessed in media containing arginine as a sole nitrogen source (3% w/v). After 5 days of incubation, few drops of Nessler's reagent were added to the culture [10].

The isolates were also evaluated for temperature and salt tolerance. For this, isolates were grown at different temperatures (15 and 45 ºC) and different salt concentrations (40, 65 and 80 gL^{-1} sodium chloride) as described by Sánchez et al. [10].

For the carbohydrate utilization tests, carbohydrate discs such as glucose, fructose, lactose, cellobiose, sorbitol, raffinose, rhamnose, ribose, mannose, trehalose, arabinose, mannitol, melezitose, xylose, melibiose, amygdalin, galactose, salicin, saccharose, maltose, esculin were used. Phenol red broth base containing these discs were inoculated with the isolates and observed for colour change. Positive reaction was confirmed by change of the media colour from red to yellow.

3.3.2.4 Cluster analysis

Cluster analysis based on physiological and biochemical characteristics was performed using Ward's method in SPSS (SPSS Inc. No.15, Chicago, IL, USA), version 18. Isolates with percentage disagreement in terms of those were taken as a measure of proximity among them.

3.3.3. Probiotic characterization

3.3.3.1. Acid tolerance

Tolerance to acidic conditions was evaluated by a method described by Lee et al. [11] with a modification. Briefly, 1% (v/v) overnight grown cultures of different isolates were inoculated into their respective media adjusted to pH 2.5 and incubated at 37 ºC. After 2 h, total viable count was measured by spread plate method. % viability was calculated according to the formula

$$\% \text{ viability} = \frac{\text{viability at 2 h}}{\text{Viability at 0 h}} \times 100 \quad (1)$$

3.3.4.2 Bile tolerance

Bile tolerance for the isolates were measured according to the protocol of Sabir et al. [12] Overnight cultures were inoculated into the respective media containing 0.3% (w/v) oxgall bile and incubated for 37 ºC. After 24 h, viable count was taken on agar plates. % viability was calculated according to the formula

$$\% \text{ viability} = \frac{\text{viability at 24 h}}{\text{Viability at 0 h}} \times 100 \quad (2)$$

3.3.4.3 Hydrophobicity

The hydrophobicity of isolate was assessed by following the method of Rosenberg [13]. Cells from a previously grown culture were harvested and washed twice with PBS, pH 7.4. Cell count was adjusted approximately to 10^9 CFU/mL. 2 mL of cell suspension was mixed with equal volume of n-hexadecane by vortexing for 2 minutes. The aqueous and the organic phases were allowed to separate by keeping the mixture undisturbed for 1 h. After that, the aqueous layer was gently pipetted out and OD_{600} was measured. The cell surface hydrophobicity was calculated as:

$$\text{Hydrophobicity}(\%) = \left(\frac{\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}}}{\text{Abs}_{\text{initial}}}\right) \times 100 \quad (3)$$

Where $\text{Abs}_{\text{initial}}$ represents initial absorption before mixing and $\text{Abs}_{\text{final}}$ represents final absorption after mixing with n-hexadecane.

3.3.4.4. Cluster analysis

For the determination of most promising probiotic strains, the Matrix Hierarchical Cluster Analysis (normalized data, pearson distance, and average linkage UPGMA method) was performed using PermutMatrix program v. 1.9.3 (LIRMM, France). [14].

3.4. Results and discussions

3.4.1. Sample collection

Sample collection was done from various accessible areas of Assam and Arunachal Pradesh (Fig. 3.1). The methods of preparation of different fermented food samples are listed in the appendix.

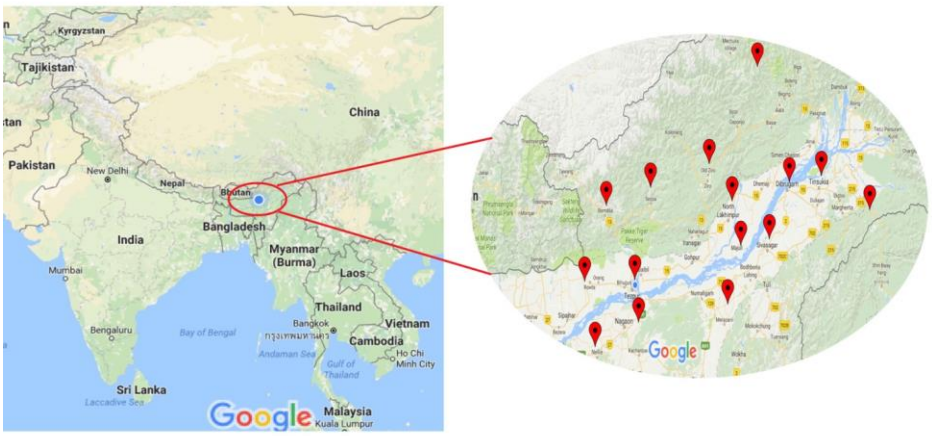

Fig. 3.1. Sample collection sites (Map source: https://www.google.co.in/maps)

Based on the substrates used, the collected fermented food samples were divided into five broad classes: fermented bamboo shoot, fermented mustard seeds, fermented soybean seeds, fermented fruits and vegetables and alcoholic products as shown in the table 3.1.

Table 3.1. Different types of fermented food collected from various places of Assam and Arunachal Pradesh

Sl no	Substrate used	Local names of fermented food prepared	Community	Places
1	Bamboo shoot	*Khorisa*	Assamese	Nagaon, North Lakhimpur, Dibrugarh; Assam
		Henoop	*Karbi*	Erdangkte, Karbi Anglong, Assam
		Danglong	*Tiwa*	Komarkuchi, Morigaon, Assam
		Ekung	*Nishi*	Lichi, A.P.
		Eup	*Apatani*	Ziro, A.P.
		Hirring	*Apatani*	Ziro, A.P.
		Mesu	*Nepali*	Bhalukpung, A.P.
2	Mustard seeds	*Kharoli, Panitenga*	*Assamese*	Golaghat,Nagaon,; Assam
3	Soybean seeds	*Kinema*	*Nepali*	Sessa, Arunachal pradesh
		Libi churpi	*Monpa*	Bomdila, A.P.
		Peruyan	*Apatani*	Ziro, A.P.
4	Fruits and vegetables	*mango pickle, lemon pickle etc*	*Assamese*	Tezpur,Dibrugarh; Assam
		Gundruk, sinki	*Nepali*	Bhalukpung, A.P.
5	Rice/ finger millet wine and alcoholic starter culture	*Apong*	*Mishing*	Dhemaji, Assam
		Chang	*Monpa*	Mechuka, A.P.
		Pona	*Apatani*	Ziro, A.P.
		Xajpani	*Ahom*	Golaghat, Assam

3.4.2. Microbial analysis

Total 210 strains were isolated, from which 179 were found to be bacteria and 31 were yeasts. From the 179 of the bacteria isolated, 151 were gram positive and 28 were gram negative. All the gram positive bacteria were tested for catalase activity and spore formation. Total 99 strains were catalase negative and non-spore forming growing in MRS media were grouped as lactic acid bacteria. Another 52 strains showing catalase positive reactions and spore formation were characterized as *Bacilli*.

Based on cell shape, lactic acid bacteria were divided into rods and cocci. Rod-shaped *Lactobacilli* were further divided into three groups: facultative heterofermentative and arginine negative (group I), obligately heterofermentative and arginine negative (group II) and obligately heterofermentative and arginine positive (group III) as described by Axelsson [15]. Cluster analysis which also included the reference strains, resulted in 10 clusters or subgroups (cluster A-J) as shown in the Fig. 3.2A. Group I was the largest with 55 isolates which included the subgroups B, G, H, I and J. Among them, subgroup H or the *Lactobacillus plantarum* cluster was the largest with 34 isolates which showed differences in the fermentation of sugars (table 3.2).

Based on gas production, cocci were differentiated into two groups: homofermentative cocci (group IV) and heterofermentative cocci (group V) [16]. Based on the position of the reference strains used, the isolates were clustered into 9 subgroups (K-S) as shown in the Fig. 3.2B. Subgroup L or the *Pediococcus pentosaceus* group was the largest with 11 isolates followed by *Enterococcus fecalis* group with 8 isolates as shown in the table 3.3.

Table 3.2. Physiological and biochemical characterization of lactic acid bacteria (*Lactobacilli*)

Group	Group I					Group II		Group III	
Subgroup	B	G	H *(L. plantarum)*	I *(L. paracasei)*	J *(L. rhamnosus)*	A	E	D	F *(L. fermentum)*
No. of isolates	2	1	34	9	9	4	2	1	5
CO_2 from glucose	–	–	–	–	–	+	+	+	+
NH_3 from arginine	–	–	–	–	–	–	–	+	+
Growth at									
15°C	–	–	+	+	+	–	+	–	–
45°C	–	–	–	+	+	–	+	+	+
Growth in									
4 g/l NaCl	–	–	+	+	+	–	+	–	+
6.5g/l NaCl	–	+	–	–	–	–	+	–	–
8g/l NaCl	–	+	–	–	–	–	+	+	–
Sugar fermentation									
Sucrose	+	+	+	+	+	+	+	+	+
cellobiose	–	–	+	+	+	–	–	–	+
sorbitol	–	–	+	+	+	–	–	+	+
raffinose	–	+	+	–	–	–	–	+	+
ribose	–	+	+	+	+	–	+	+	+
Gluconate	–	–	+	+	+	–	+	+	+
arabinose	–	–	+	–	+	–	+	+	+
mannitol	+	–	+	+	+	+	+	+	–
melezitose	+	–	+	+	+	+	–	+	–
xylose	+	–	+	+	–	+	–	+	+
melibiose	+	–	+	–	–	+	–	+	+
amygdalin	+	–	+	+	+	+	–	+	+
esculin	+	–	+	+	+	+	–	+	–

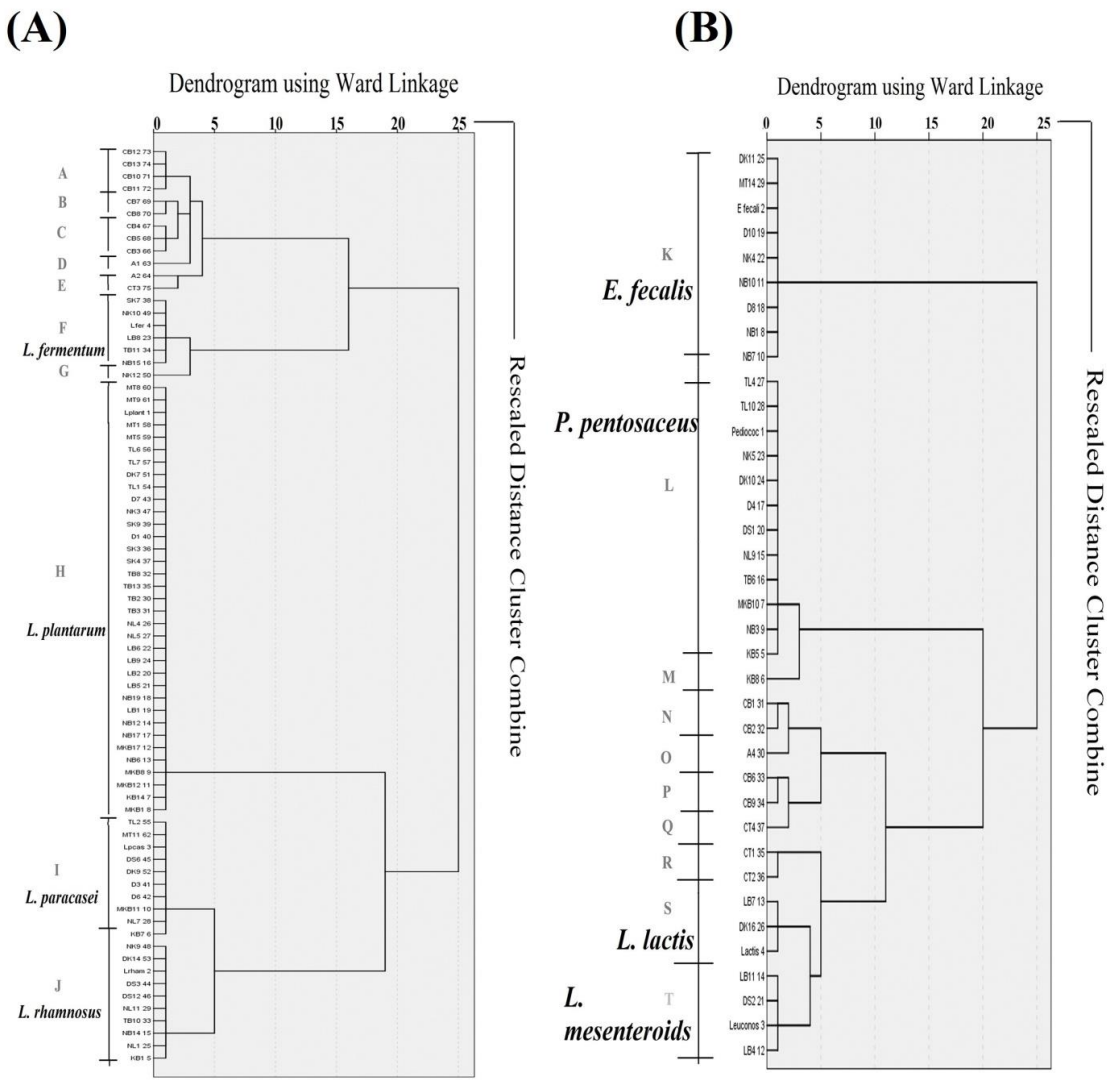

Fig. 3.2. Clustering structure of lactic acid bacteria isolates and indicator strains. Dendrogaram constructed using Ward Linkage, SPSS; version 18.

Table 3.3. Physiological and biochemical characterization of lactic acid bacteria (*Cocci*)

Group	Group IV					Group V			
Subgroup	K (*E. fecalis*)	L (*P. pentosaceus*)	R (*L. lactis*)	N	M	O	S (*L. mesenteroides*)	Q	P
No. of isolates	8	11	2	2	1	2	3	2	1
CO_2 from glucose	–	–	–	–	–	+	+	+	+
NH_3 from arginine	–	–	–	+	+	+	+	+	–
Growth at									
15°C	+	–	+	–	–	–	–	–	–
45°C	+	+	–	–	–	–	–	–	–
Growth in									
4 g/l NaCl	+	+	+	+	+	+	+	+	+
6.5g/l NaCl		+	–	–	+	–	–	–	+
8g/l NaCl	–	+	–	+	+	–	–	–	+
Sugar fermentation									
Sucrose	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	–	+	–	–
Sorbitol	+	–	–	+	–	–	–	–	–
Raffinose	–	+	–	–	–	–	+	+	–
Ribose	+	+	+	–	–	–	+	+	–
Gluconate	+	–	–	–	–	–	–	+	–
Arabinose	–	–	–	+	–	+	+	–	–
Mannitol	+	–	–	–	–	–	+	+	+
Melezitose	+	–	–	+	–	+	+	+	+
Xylose	–	+	+	+	–	+	+	+	+
Melibiose	+	–	–	+	–	+	–	+	+
Amygdalin	–	+	–	+	–	+	+	+	+
Esculin	+	–	+	+	–	+	–	+	+

For *Bacillus* isolates, the largest cluster was found to belong to *Bacillus subtilis* group with 20 isolates (Fig. 3.3). Different biochemical characteristics of the isolates are given in the table 3.4.

Table 3.4. Physiological and biochemical characterization of spore forming *Bacilli*

Groups	A	B	C (*Bacillus amyloliquifaciencs*)	D (*Bacillus subtilis)*	E
No. of isolates	11	7	13	20	1
Pigmentation	–	+	–	–	+
Motility	+	+	+	+	–
Voges proskauer	–	+	+	+	+
Acid from					
Arabinose	–	+	+	+	+
Glucose	+	+	+	+	+
Glycogen	+	–	+	+	–
Mannitol	–	–	+	+	+
Mannose	–	+	+	+	+
Salicin	+	–	+	+	–
Starch	–	+	+	+	+
Xylose	+	+	+	+	–
Utilization of					
Casein	+	–	+	+	+
Gelatin	–	+	+	+	+
Starch	+	+	+	+	+
Nitrate reduction	+	–	+	+	+
NaCl conc.					
2%	+	+	+	+	+
5%	+	+	+	+	+
10%	–	–	–	+	–
Growth temp					
10°C	+	–	+	+	+
30°C	+	+	+	+	–
50°C	–	+	–	+	–

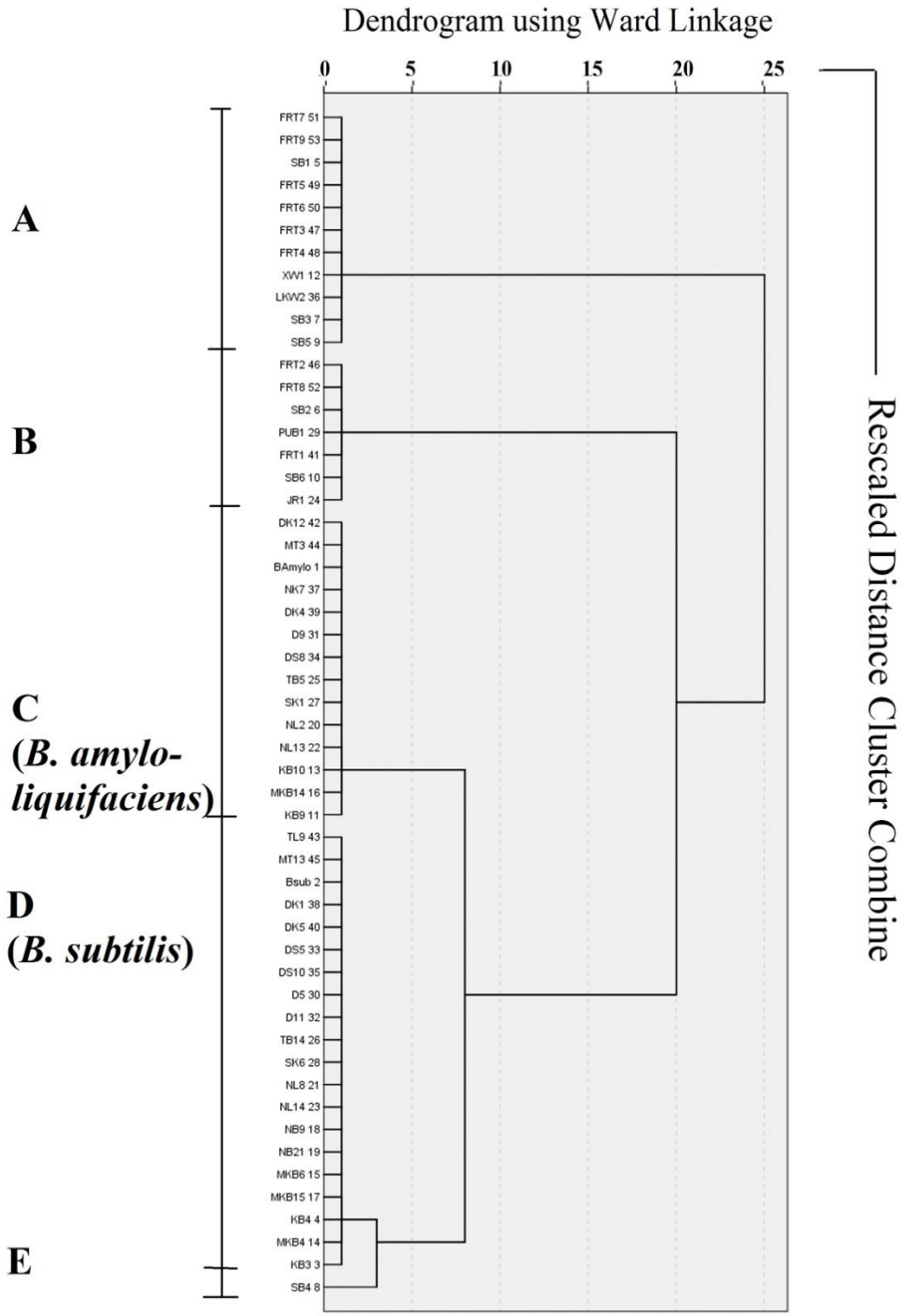

Fig. 3.3. Cluster analysis of *Bacilli* based on biochemical characteristics. Dendrogaram constructed using Ward Linkage, SPSS; version 18.

The overall results of the biochemical tests presumptively suggested that maximum 23.94% of the isolates belong to *Lactobacillus plantarum* group, followed by *Bacillus subtilis* group (13.38%) as shown in the pie chart (Fig. 3.4).

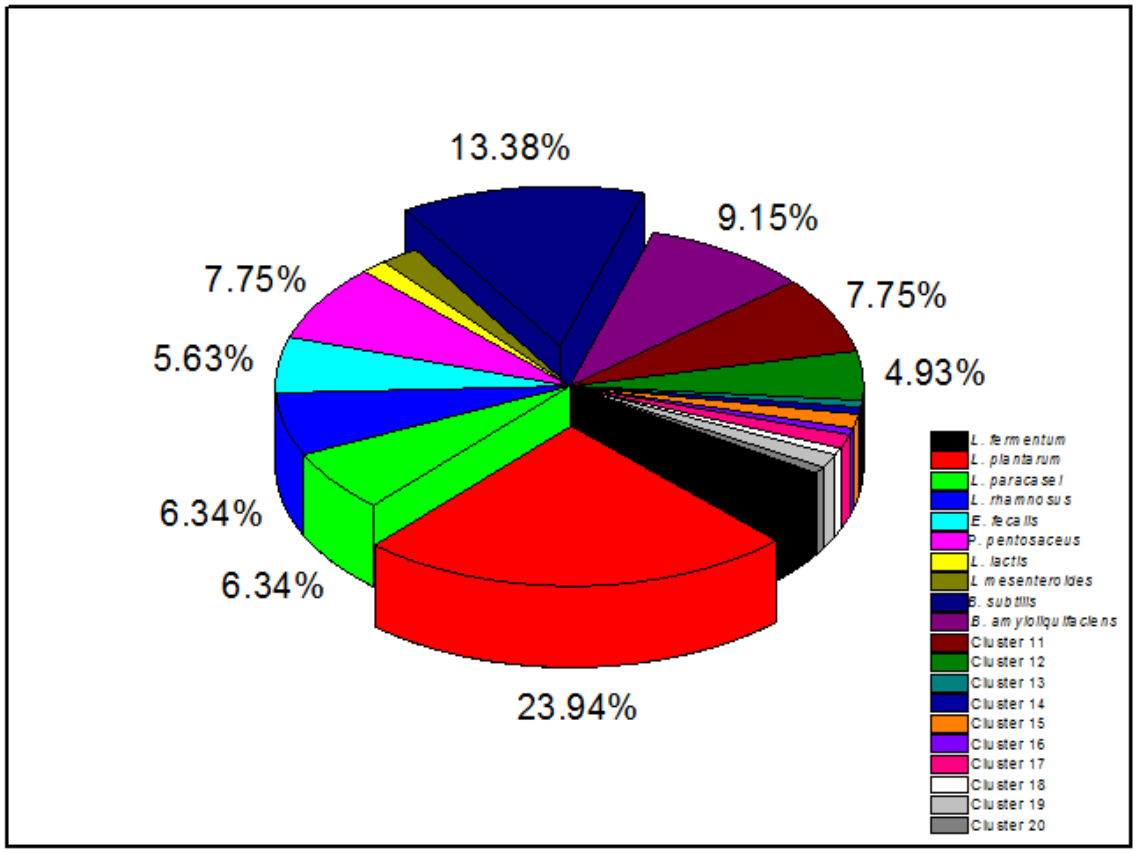

Fig. 3.4. Pie diagram showing microbial diversity of fermented foods based on biochemical characteristics

3.4.3. Probiotic characterization

3.4.3.1. Acid and bile tolerance, hydrophobicity

Tolerance to low pH, high bile salt and showing hydrophobic characteristics are the primary criteria for selecting a probiotic strain. Strains isolated from different sources showed high variability in terms of acid and bile tolerance and hydrophobicity. The average viability of the isolates of alcoholic products was maximum (51.68%) in acidic conditions, whereas the isolates of fermented mustard seeds showed maximum viability (50.73%) in 0.3% (w/v) bile (Fig. 3.5). Maximum hydrophobicity was shown by the

isolates of fermented bamboo shoot products. However, acid, bile tolerance and hydrophobicity were found to be statistically comparable (P <0.05) among isolates from different substrates.

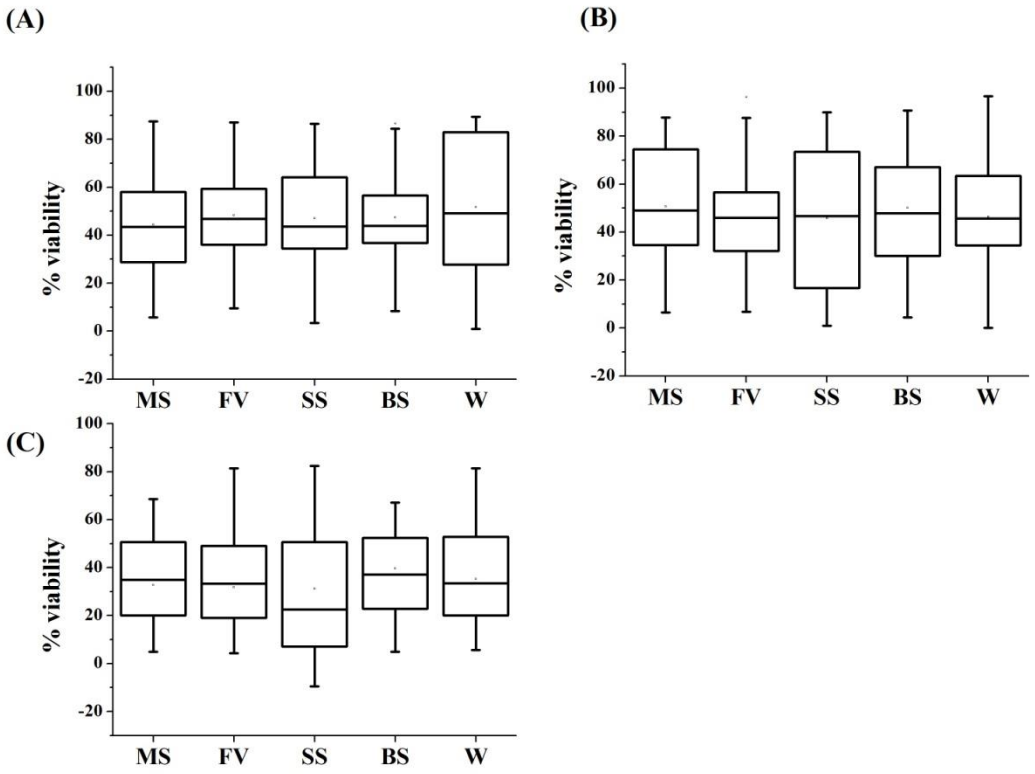

Fig. 3.5. Box plot showing probiotic properties of isolates from different origin: (A) Acid tolerance, (B) Bile tolerance and (c) Hydrophobicity. Abbreviations: MS: fermented mustard seeds, FV: fermented fruits and vegetables, SS: fermented soybean, BS: Fermented bamboo shoot and W: alcoholic products

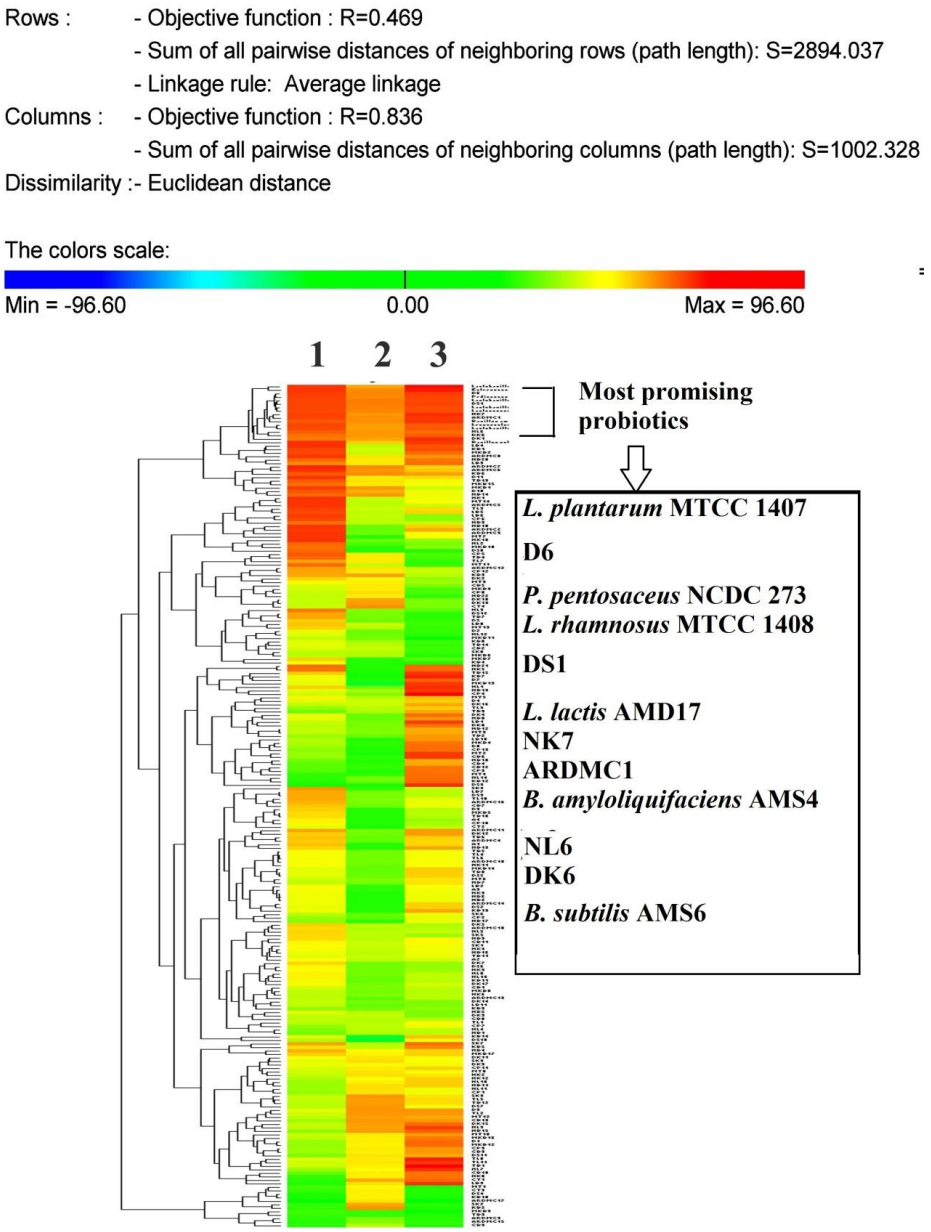

Fig. 3.6. Matrix Hierarchical Cluster Analysis of the isolates and the reference strains. Lane 1: Acid tolerance, lane 2: hydrophobicity and lane 3: bile tolerance.

3.4.3.2. Clustering of strains for determining the most promising probiotic strain

The strains showing maximum tolerance to acid and bile conditions and hydrophobicity are the most promising probiotic strains. As shown in the Fig. 3.6, the isolates D6, DS1, NK7, ARDMC1, NL6 and DK6 showed maximum tolerance compared to other strains.

The UPGMA dendrogram which was constructed using the PermutMatrix program reveals that the abovementioned strains were clustered along with the reference strains which have previously reported probiotic properties. [17, 18, 19, 20, 21, 22, 23].

3.5. Conclusion

The non- dairy fermented foods of Assam and Arunachal Pradesh were found to contain different microorganisms with probiotic properties. There was no significant difference among the isolates from different origins for probiotic properties. Overall five strains, i.e. D6, DS1, NK7, ARDMC1, NL6 and DK6 which showed comparable probiotic properties as the standard probiotic strains were selected for further works.

3.6. Bibliography

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