PROBIOTIC EFFICACY AND LOGISTIC GROWTH MODELING OF LACTIC ACID BACTERIA FROM FERMENTED RICE BEVERAGES FOR NOVEL FUNCTIONAL STARTERS FORMULATION

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Abstract
This study aims to isolate, characterize, assess the probiotic potentials, and determine the growth characteristics of Lactic acid bacteria (LAB) from fermented rice beverages of West Garo Hill, Meghalaya, India, that can be used to formulate novel functional starter cultures. LAB isolates were characterized on morphological and biochemical grounds and identified using 16S rDNA sequencing and computational analysis. In addition, the strains were tested for potential probiotic characteristics, including pH and bile tolerance, cell surface hydrophobicity, auto-aggregation, antibacterial activity and antibiotic susceptibility. Growth characteristics of LAB that showed probiotic potential were determined using spectrophotometric and spot analysis. This study revealed that most isolates, in particular Lactobacillus brevis, were tolerant of wide pH ranges (1.0, 2.0, and 3.0) and high bile salt concentrations (0.3%, 0.5%, and 1%) and showed high levels of surface hydrophobicity and auto-aggregation properties. most isolates, showed the broad antibacterial activity specifically against Gram positive bacteria. In general, isolates were susceptible to protein synthesis inhibitors with L. brevis being highly susceptible (60%) to ampicillin, rifampicin, gentamicin, erythromycin, and tetracycline. A careful review of all the probiotic observations led to the conclusion that strain of L. brevis can be used as an additive to functional rice beverage starter cultures. Its logistic growth at 32°C showed a sigmoid growth curve with OD_{max} of 1.3 after 9 hours. The doubling time was determined to be 1.99 hours and the spot test at OD_{600} of 0.5 revealed 1 x 109 log_{10} CFU/ml.

Keywords: fermentation, beverage, lactobacillales, probiotics, growth characterization

Received: 23.11.2021 Reviewed: 07.02.2022 Accepted: 08.02.2022

1. INTRODUCTION

The order *Lactobacillales* comprise heterogeneous spherical (cocci) or rod-shaped (bacilli) gram-positive bacteria that play an important role in fermentation. The common name lactic acid bacterium (LAB) comes from their production of lactic acid, the major metabolic byproduct of carbohydrate fermentation. LAB are typically present in starter cultures used for fermentation of dairy products, meat, vegetables, fruit, fish, and cereals. They have gained prominence in the preservation of food and probiotics industries due to their contribution to taste, texture, and nutrition, as well as providing health benefits to the host by promoting microbial balance in the system when consumed in adequate amounts. Nevertheless, LAB have historically been used to preserve food, particularly in traditional fermented drinks. Cereal-based fermented beverages made from cassava, rice, and other starchy resources. are popular in most Asian countries, such as India, Thailand, China, Korea, Vietnam, etc. (Jeyaram et al., 2008; Wang et al., 2014; Kim et al., 2011). In addition to several volatile compounds that contribute to a complex blend of flavors, these beverages can be an excellent source of minerals, antioxidants, dietary fiber, and probiotics (Kreisz et al., 2008; Gupta and Abu-Ghannam, 2010).

A variety of cereal-based traditionally fermented beverages especially from rice (*apong*, *bhaatijaanr*, *haria*, *jou*, *zutho*, *judima*, and *chubitchi*) are customarily consumed in India, primarily by the indigenous tribal communities of the north-eastern states. This study focuses on fermented rice beverages of the indigenous tribes of West Garo Hills

district of Meghalaya, India namely the *Garos*, *Hajongs*, and *Rabhas* for whom these beverages are an essential part of their culture and have a strong socio-cultural significance. The beverage is prepared by mixing starter culture (made from locally available medicine plants) with cooked rice that is allowed to ferment at room temperature for 5-7 days in an earthen pot so that LAB, molds, and yeasts in the starter can facilitate the saccharification of rice (Muruganet et al., 2018). While a few studies have been conducted on the role of yeasts and molds in the fermentation of cereal-based beverages, there has been little research on the diversity, importance, and probiotic properties of LAB. Accordingly, it may be of benefit to isolate LAB from traditional fermented beverages and to characterize these strains according to their probiotic properties to obtain useful strains with potentially valuable functional properties for future applications in the food and beverage industries. Hence, this study aimed to explore not only the composition of LAB communities in dried starters and fermented rice beverages but also their probiotic efficacy and growth characteristics during fermentation, which will enable us in developing a functional starter that could find potential use in the foresaid industries in the future.

2. MATERIALS AND METHODS

Sample collection, enrichment and isolation

Samples of starter cultures (100 g) and fermented rice beverages (250 ml) were collected from different areas of West Garo Hills in sterilized sample bottles (Thermo Scientific) and transported under refrigeration to the laboratory for further analysis. LAB strains were isolated according to the methods described in Lab manual 14 (2012), with some modifications.1ml of each sample was enriched in M17 and de Man, Rogosa, and Sharpe (MRS) broth and incubated at 37°C for 24 hours under anaerobic conditions. Further dilutions were performed in phosphate buffer saline (PBS), followed by plating on an MRS agar medium and incubation at 37°C for 24 hours. Colonies with distinct morphological characteristics were sub-cultured on MRS agar plates, and viable cultures were stored in MRS slants at 4°C, and the culture stocks were maintained in 15 % glycerol at -20°C.

Identification and characterization

The culture characteristics of distinct LAB colonies (color, shape, surface texture, elevation, margin, and size) were noted, and Gram staining was performed. Presumptive LAB isolates were tested for Catalase activity (3 % hydrogen peroxide) as well as growth on MRS media at 45^{o}C and 6 % NaCl according to standard procedures (Sharpe, 1979). Molecular characterization involved obtaining total genomic DNA (HiPurATM genomic DNA isolation Kit; Himedia, India), amplification of 16S rDNA by polymerase chain reaction (PCR), Sanger sequencing, and computational analysis. The 16s rDNA region was amplified using the forward primer 27F-5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 1492R-5' GGTTACCTTGTTACGACTT 3' (Eurofins Scientific India Pvt. Ltd.). The reaction mixture consisted of 1.5 µl of each primer (0.45 µM), 5 µl of dNTPs (10mM), 2 µl of genomic DNA(15 picogram/µl), 5 µl PCR buffer (10X) and 0.025 U/µl of Taq DNA polymerase (Sigma-Aldrich) in a final volume of 50 µl. Initial denaturation of the PCR was carried out for 4 minutes at 94°C, proceeded by 30 cycles of denaturation for 30 seconds each at 94°C, annealing for 1 minute at 55°C and an extension for 1 minute at 68°C and a further final extension for 10 minutes at 68°C. The PCR products were separated by agarose gel electrophoresis (1.2% w/v) and visualized by staining with ethidium bromide.

Phylogenetic analysis

PCR amplified products were subjected to Sangers sequencing followed by computational analysis. Nucleotide Basic Local Alignment Search Tool (BLASTN) was employed against the nucleotide collection database (nr/nt) using the default parameters for the Megablast algorithm for identifying the different isolates

(Zhang et al., 2000; Morgulis et al., 2008). MEGA6 was used to assess phylogenetic relationships among LAB isolates as determined by BLASTN and their best matches (Tamura et al., 2013). MUSCLE was used to align the sequences using the default parameters for non-coding nucleotides (Edgar 2004), and the Kimura 2-parameter model was used for molecular phylogenetic analysis (Kimura 1980). The initial trees for the heuristic search were constructed using the Neighbor-joining method applied to the pairwise distance matrix derived by the Maximum Composite Likelihood method (Saitou and Nei, 1987). The tree generation was limited to 5% of alignment gaps, missing data, and the tree's reliability was tested by bootstrapping with 2000 replications (Hall 2013) and the tree with the highest log-likelihood was reported (Figure 1).

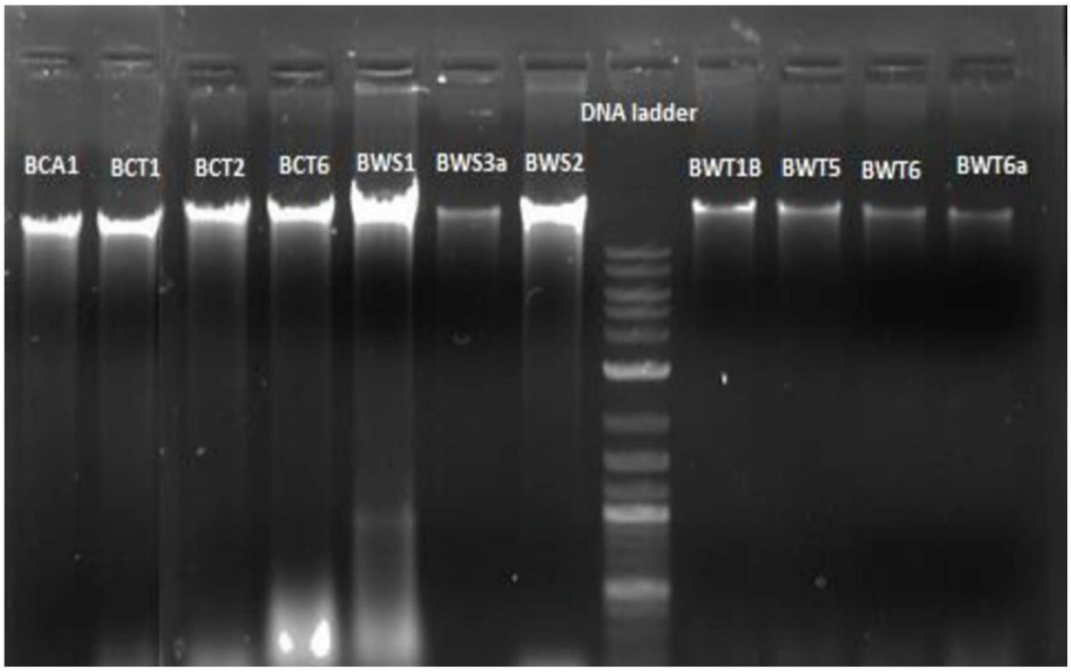


Figure 1. Gel image of DNA isolation of bacterial isolates

Assessment of probiotic efficacy

***pH and Bile tolerance*.**

Tolerance to pH and bile salt was evaluated using the method of Pennacchia et al., (2008) and Sourabh et al., (2011) with slight modification. MRS broth was adjusted to different pH values (6.5, 1, 2, and 3) using Hydrochloric acid (HCl). Each broth was thoroughly mixed, and aliquots of 10 ml of each were added to LAB cultures for 12 hours at 2 % of MRS broth. The pellets were then centrifuged at 10,000 rpm for 10 minutes at 4°C (Eppendorf centrifuge, US) and were rinsed twice in phosphate buffer saline (PBS) before being re-suspended in PBS. The re-suspended cultures were then added at a rate of 2 % to tubes containing 10 ml of MRS broth, adjusted to pH 1.0, 2.0, 3.0, and 6.5, mixed, and incubated at 37^{o}C anaerobically. As for bile tolerance, 2 % of each suspension of bacterial cultures was added to 10 ml of MRS broth with different bile salt concentrations [(0.5%, 0.3%, and 1 %) w/v of bile salt (Himedia) and to the control tube (with no bile salt)]. All the tubes were vortexed before incubating anaerobically at 37^{o}C. 1ml of the sample in both above cases was drawn at intervals of 0, 1, 2, and 3 hours. Further dilution of the samples was achieved with 10 ml of PBS buffer, and appropriate dilutions were poured onto MRS agar plates for anaerobic incubation at 37°C for 24 - 48 hours, after which the colony-forming units (CFU) were determined.

Cell surface hydrophobicity and Auto-aggregation

These analyses were carried out according to the protocol outlined by Syal and Vohra (2013) with some modifications. Bacterial cell suspensions in PBS were prepared as described earlier and adjusted to OD_{600} 0.5 (A_0). 1.5 ml of each cell suspension was mixed with 1.5 ml of n-hexadecane, vortexed vigorously for 2 minutes, and incubated at 37°C in an anaerobic incubator in undisturbed condition for 30 minutes allowing the organic and the aqueous phases to separate. The aqueous phase was removed (1 ml) and its optical density (OD A_1) was recorded against blank prepared in the same manner. Repetitions of the experiment were conducted, and the hydrophobicity percentage (H %) was determined using the following formula as follows:

$$H\ (\%) = \frac{A_i - A_f}{A_i} \times 100$$

In which, A_i = Initial $O.D_{600}$ and

A_f = Final $O.D_{600}$

For auto-aggregation, 4 ml of each cell suspension was vortexed for 1 minute and the upper phase was removed (0.1 ml), and the OD was determined at a wavelength of 600 nm. The suspension was then incubated anaerobically for five hours at 37°C following which 0.1ml of the upper phase was again removed for OD measurement. The percentage of auto-aggregation (% Aa) was measured using the following formulae:

$$Aa\ (\text{per cent}) = 1 - \frac{A1}{A0} \times 100$$

Where, A0= Initial OD_{600} at zero hour

and A1= OD_{600} at five hours

Antibacterial activities

Cell-free supernatants of bacterial cultures were tested for antibacterial activity using the agar well diffusion method according to Fakruddin et al., (2017) and Mezaini and Bouras (2010) with slight modifications. Overnight cultures of LAB were centrifuged (10 minute at 15000 g, 4°C; Eppendorf Centrifuge, US) and the cell-free supernatant was split into two equal parts, with one part neutralized with 6N sodium hydroxide (NaOH). In each case, sterile supernatant was extracted using a Millex-HP syringe filter with a 0.45 m pore-size hydrophilic polyethersulfone (PES) membrane (Merck Pvt. Ltd.). The test strains used included two gram-positive bacteria [Bacillus cereus (ATTC 14459), Staphylococcus aureus (MTCC 114)] and two gram-negative bacteria [Salmonella typhi (NCTC 5017) and Escherichia coli (ATCC 25922)]. Test cultures were grown up to a McFarland density of 0.5 (1.5 x 108 approximate bacterial suspension/ml) and the active cultures (100 µl) of each indicator strain were mixed with nutrient agar (100 ml, 1.6 %) and poured into a plate. As the agar solidified, the plates were refrigerated at 4°C for 10 to 15 minutes before several wells (6 mm diameter, 4 mm deep, and 2 cm apart) were punched out with a sterile 6 mm cork borer (HiMedia). 100 µl of filter-sterilized LAB supernatants were added to each well, and the plates were again refrigerated (4°C) for 12 hours to aid the diffusion of supernatant before being incubated for 24 - 48 hours at 37°C. The presence of a clear zone surrounding the agar wells indicates that the cell-free supernatants are inhibiting the test bacterial strains. The inhibition zones were measured by placing a metric ruler across the zone of inhibition, at the widest diameter, and measuring from one edge of the zone to the other edge. To obtain the average mean diameter of the inhibitory zone, the experiments were conducted in triplicate.

Antibiotic susceptibility

The disc diffusion method was used to study the patterns of resistance or susceptibility to antibiotics in selected LAB cultures, based on the guidelines from the Clinical and Laboratory Standards Institute (CLSI) (2007) and Charteris et al., (2001). In total, 12 antibiotic discs were used (HiMedia Ltd, Mumbai, India) namely, Ciprofloxacin (5 µg), Ampicillin (10 µg), Azithromycin (15 µg), Rifampicin (5 µg),

Gentamicin (120 µg), Tetracyclin (30 µg), Streptomycin (10 µg), Erythromycin (15 µg), Kanamycin (30 µg), Nalidixic acid (30 µg), Vancomycin (30 µg), and Norfloxacin (10 µg). Petri plates containing 15 ml of MRS agar were set, and then 4 ml of soft MRS agar was overlaid with 200 µl of active cultures. The Petri plates were left at room temperature for 15 minutes before dispersing the antibiotic discs (HiMedia) on the agar using a disc dispenser. The agar plates were incubated anaerobically at 37ºC for 24 hours and the zone of inhibition around the antibiotic discs was measured.

Growth modeling

Spectrophotometric measurements and spot analyses were used to model the growth characteristics of selected LAB according to Stamer, et al., (1971) and Fleet, (1993) with necessary modifications. A single colony of LAB was inoculated into 15 ml of MRS broth overnight at 32°C. 1 ml of LAB inoculum was transferred to 100 ml of MRS broth the next day, and the OD values were determined by spectrophotometric quantification every hour. A spot test was also conducted to determine the number of viable cells at every interval using 10 µl serially diluted LAB culture on MRS plates. The plates were incubated for 24 hours at 32°C, and the viable cell counts were recorded as $\log_{10}$ CFU/ml.

Statistical analysis

All measurements were all carried out in triplicate, and the mean and standard deviation were calculated using Microsoft Excel 2010. Further, the results incurred were subjected to one-way ANOVA with multiple comparisons by Scheffe's test at $p \leq 0.05$ using XLSTAT.2016. GraphPadPrism 7.04 was used to generate microbial growth curves using data collected in duplicate.

3. RESULTS AND DISCUSSION

Isolation and preliminary identification

Eleven strains were selected on MRS and M17 media (Table 1), all of which were non-motile, non-spore-forming, and microaerophilic. Most colonies were white punctiform or small creamy white or yellow with smooth surfaces and entire margins. While few colonies were glistening, others appeared opaque except isolate BWT5 which developed large colonies that were slightly iridescent and flat with irregular edges. Except for BWT5, all isolates were Gram-positive and negative for catalase, indicating that they were fermenters without catalase enzyme and possibly were LAB. On microscopic observation (100x), isolates appeared as elongated rods, short chains, or pairs of rounds to oval cocci cells. Other authors have also reported these characteristics in LAB. Patil et al., (2010) observed that LAB colonies on MRS agar were circular, cream-colored, and low convex, with the entire margin forming tetrads, whereas non-pigmented circular colonies with low convex and the entire margin formed chains or pairs. Similarly, Akinola and Osundahunsi (2017) found that lactobacillus appeared under the microscope as short rods or clusters, long rods or networks, and as cocci. Additionally, the test for growth at 45ºC and in 6.5 % NaCl found visible turbidity in MRS broth for some strains, indicating their survival. In general, LAB are mesophilic, but some genera have species that are psychrotrophic, thermoduric, or thermophilic (Hutkins, 2006).

Molecular characterization

The total genomic DNA of eleven presumptive lactobacillus isolates were extracted from 24 hours old cultures using HiPurATM's bacterial and yeast DNA Isolation Kit (Himedia, India). In all cases, the extraction method produced good yield of DNA (Figure 1). Amplification of the 16s rDNA gene with primers 27f and 1492r produced informative and reproducible fingerprint profiles for all strain and fragments of 1500 bp corresponding to almost the entire 16S rDNA was obtained (Figure 2).

Table 1: Macroscopic and Microscopic identification of isolates of Lactic acid bacteria from traditional starter cultures and fermented rice beverages

Isolate/ source	Media	Colony characteristics	Microscopic characteristics	Gram's staining	Catalase test	6.5% NaCl	45^{0}C
BCA1/ rice beverage	MRS	White punctiform colonies, convex and smooth surface with entire margin	small elongated rod-like bacilli assembled in pairs or in chains of variable length	+	-	+	-
BCT1/ rice beverage	MRS	small round creamy-yellow colonies	Short rods observed in single or in chains	+	-	+	+
BCT2/ rice beverage	MRS	small white opaque colonies, entire margins, convex smooth glistening	Short rods observed with round ends in single or in short chains.	+	-	-	-
BCT6/ rice beverage	MRS	White punctiform colonies, convex and smooth surface with entire margin	small elongated rod-like bacilli assembled in pairs or in chains of variable length	+	-	+	-
BWS1/ Starter culture	MRS	Circular, smooth, cream colonies with entire edges	Spherical to oval Cocci cells observed in pairs or short chains	+	-	+	-
BWS2/ Starter culture	MRS	Circular, smooth, cream colonies with entire edges	Spherical to oval Cocci cells observed in pairs or short chains	+	-	+	-
BWS3a/ Starter culture	MRS	small white opaque colonies, entire margins, convex smooth glistening	Short rods observed with round ends in single or in short chains.	+	-	-	-
BWT1B/ Starter culture	MRS	Colonies appear smooth, round, and with cream coloring	Spherical cells observed forming short chains or tetrads	+	-	-	+
BWT5/ Starter culture	M17	Large colonies, slightly iridescent and flat with irregular edges	Straight rod-shaped cells	-	+	+	+
BWT6/ Starter culture	MRS	Colonies appear smooth, round, and with cream coloring	Spherical cells observed forming short chains or tetrads	+	-	-	+
BWT6a Starter culture	M17	Small rounded smooth, white colonies with entire edges	Cocci shaped cells observed in pairs (diplococci) or short chains,	+	-	-	+

Further, Sanger sequencing and computational analysis had shown significant similarities (97-99 %) to the sequences of known lactobacillus species using Basic Local Alignment Search Tool (BLASTN) (Table 2). Further, phylogenetic analysis linked BCA1 and BCT6 to *Lactobacillus plantarum*, BCT1 with Lactobacillus *paracasei*, and BWT1B and BWT6 with *Pediococcuspentosaceus*. BWS3a and BCT2 were identified as being *Lactobacillus brevis*, whereas BWS1 and BWS2 were ascertained to be *Enterococcus durans*, and BWT6a was verified as *Enterococcus lactis.*

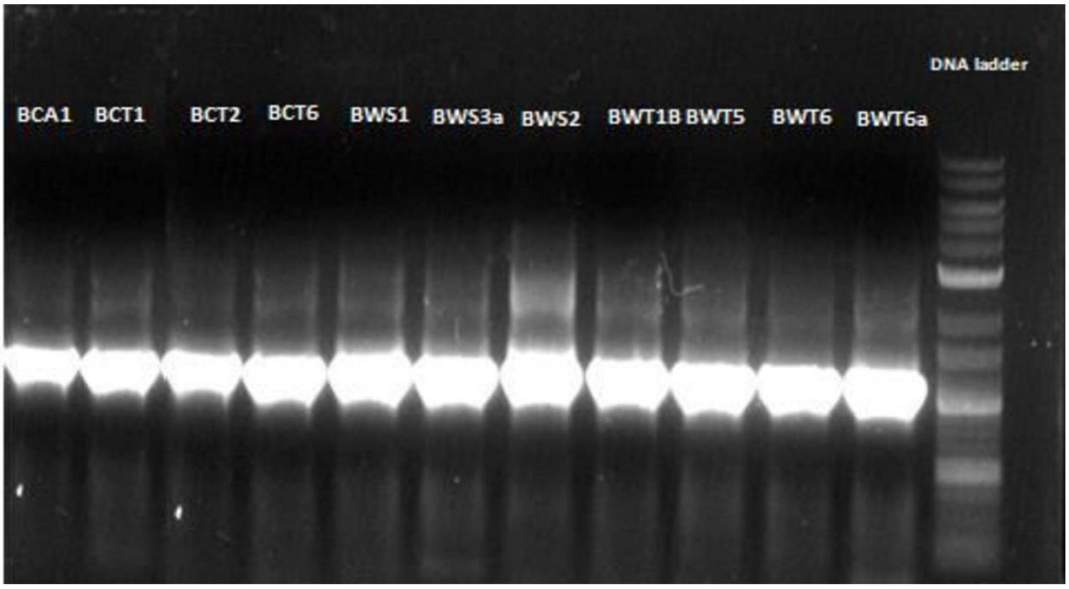
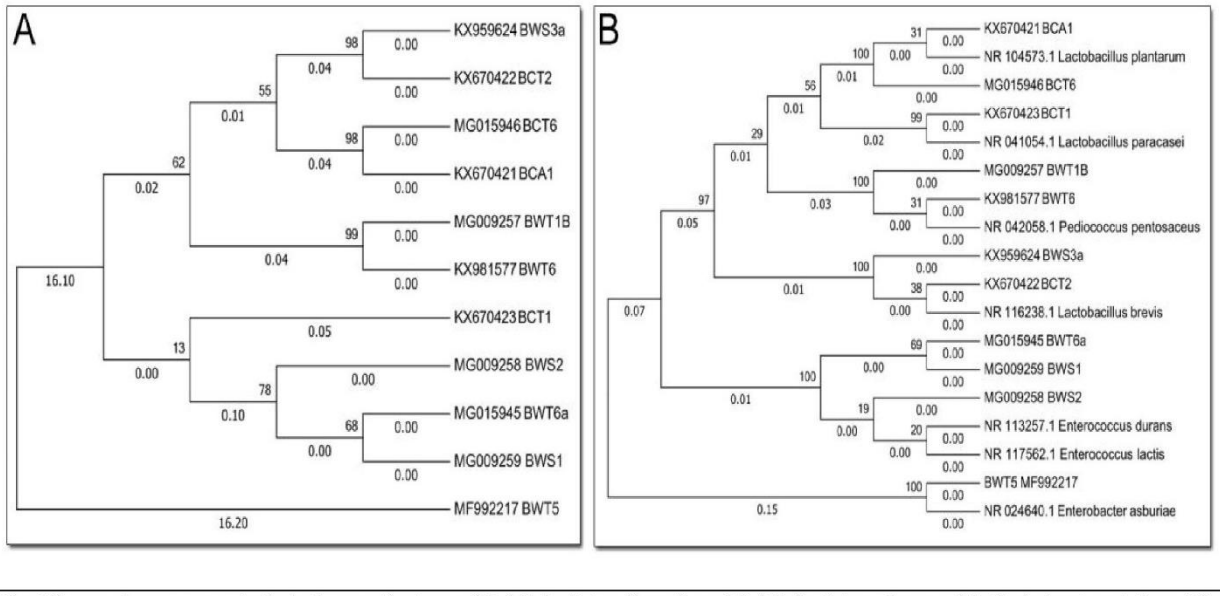


Figure 2. Gel image of PCR amplification of 16s rDNA fragment of bacterial isolates

Table 2: Identification of bacterial isolates based on 16S rRNA gene sequence homology

Bacterial Isolates		Best Match	
Identifier	**GenBank Accession No.**	**Organism**	**NCBI Accession No.**
BCA1	KX670421	*Lactobacillus plantarum*	NR_115605.1
BCT1	KX670423	*Lactobacillus paracasei*	NR_041054.1
BCT2	KX670422	*Lactobacillus brevis*	NR_116238.1
BCT6	MG015946	*Lactobacillus plantarum*	NR_104573.1
BWS1	MG009259	*Enterococcus durans*	NR_113257.1
BWS2	MG009258	*Enterococcus durans*	NR_113257.1
BWS3a	KX959624	*Lactobacillus brevis*	NR_116238.1
BWT1B	MG009257	*Pediococcus pentosaceus*	NR_042058.1
BWT5	MF992217	*Enterobacter asburiae*	NR_024640.1
BWT6	KX981577	*Pediococcus pentosaceus*	NR_042058.1
BWT6a	MG015945	*Enterococcus lactis*	NR_117562.1

Furthermore, BLASTN results also show high similarity (99.03 %) for three isolates with the two Enterococcus species (Figure 3A and 3B). Interestingly, these results were like those reported by Chiang et al., (2006) who reported that *tapai*, the indigenous fermented beverage of *Kadazan-Dusun-Murut* tribes of East Malaysia, predominately contained *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus paracasei*.



The Figure shows unrooted phylogenetic tree of LAB isolates (inset) and LAB isolates along with their best matches. The branch lengths and the bootstrap percentages are shown next to the branches and nodes respectively.

Figure 3. Molecular phylogenetic of LAB isolates by Maximum Likelihood method

Similarly, Endo and Okada (2005) had reported *Lactobacillus brevis* and *Lactobacillus paracasei* to be prevalent in *shochu* (Japanese rice wine). Generally, pH ranges of 3.5 to 4.5 are frequently reported to harbor Lactobacilli during and after fermentation (Carr *et al*., 2002). In contrast, we found greater diversity in the starter cultures. The isolates were identified as *Enterococcus durans, Lactobacillus brevis, Pediococcuspentosaceus, Enterobacter asburiae, and Enterococcus lactis*. *Pediococcus* has been reported to be the more dominant genera in Nepalese starter *marcha*, Chinese starters *daqu* and *hongqu* and in Vietnamese starter *banh men* (Thanh *et al*., 2008; Lv *et al*., 2013) and responsible for acidification of raw materials during fermentation. As for *Lactobacillus* species, several authors have found *Lactobacillus brevis* to be predominant in starter cultures (Tamang*et al*., 2007; Bora *et al*., 2016). Limited data are available, however, on the prevalence of *Enterobacter asburiae* in starter cultures. Thanh et al., (2016) had identified *Enterobacter asburiae* in *banh men*, the Vietnamese starter culture, which produces acid when grown on sugar substrates and is therefore typically regarded as contaminant. Also, previous studies have shown that *Enterococcus durans* and *Enterococcus lactis* are present in the microbiota of the intestines, so they too are considered contaminants (Dubin and Pamer,2014).

Probiotic efficacy

Isolates belonging to six different groups namely *Lactobacillus brevi, Lactobacillus plantarum, Enterococcus lactis, Enterococcus durans, Pediococcuspentosaceus* and *Lactobacillus paracasei* respectively were further tested for probiotic efficacy.

pH and bile tolerance

An important indicator of potential probiotic performance is viability of LAB at pH 2 to 4 (Garcia et al., 2016). Our study revealed that *Lactobacillus* spp. isolated from fermented rice beverages and its starter culture, in general, can survive the acidic environs of stomach without getting degraded. The isolates showed a marked variation in the endurance across different pH gradients (1, 2 and 3) and incubation times (0.1, 2, and 3 hours) (Figure 4).

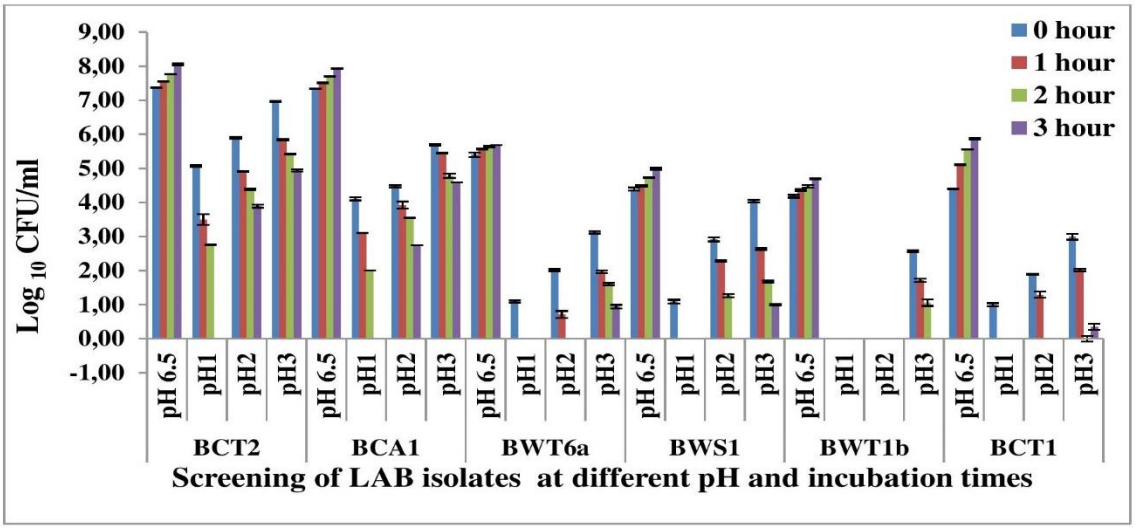


Figure 4. Graph showing screening of LAB isolates at different pH and incubation times

The least tolerant isolate was BWT1B (*Pediococcuspentosaceus*), which survived at pH 3 for only 2 hours (31.16%). While BCT2 (*L. brevis*) and BCA1 (*L. plantarum*) were able to survive the extreme low pH 1 after incubation of 2 hours, showing a survival rate of 36.86 % and 30.25 %, respectively. In addition, these isolates showed good survival rates even after 3 hours of incubation (62.10 % and 48.20 % at pH 2 and 75.34% and 67.29 % at pH 3). The results presented here agree with Gautam et al., (2014), who reported that *Lactobacillus brevis*, at pH 3, had a survival rate of 91.87 % even after 3 hours of incubation.

The results for bile tolerance (Figure 5) indicate that, after three hours of incubation, all bile concentrations were tolerated by the lactobacillus strains. Overall, the degree of resilience varied widely between isolates, and cell viability declined with incubation time in all cases, perhaps as a result of differences in these strains (Chateau *et al.*, 1994). At bile concentrations of 0.3%, 0.5%, and 1% respectively, BCT2 again demonstrated effective resistance with survival rate of 82%, 77%, and 66%. BWS1 had survival rates of 81.4%, 77.2%, and 57.7%, which were similar to isolate BCT2. In few previous studies, lactobacillus strains isolated from fermented rice beverages have been found to be able to survive in 0.3 % bile concentrates (Zhao *et al*., 2017; Giri *et al*., 2018). We observed similar results as Sharma et al. (2016), who evaluated lactobacillus isolates from a variety of dairy products, fermented foods, and human faeces and found 81.22% survival with 0.5 % bile.

Cell surface Hydrophobicity and Auto-aggregation

Hydrophobicity and auto-aggregation properties that determine the ability a probiotic strain to adhere to host cells and form multicellular aggregates, are important for preliminary screening and selection of probiotics (Del Re et al., 1998). A marked difference in hydrophobicity was observed among LAB isolates in this study (Table 3). BCT2 (*L. brevis*) displayed the highest value (91.68±2.81 %), followed by BWS1 (*Enterococcus durans*; 78.60±4.09 %). This indicated that these strains are able to strongly adhere to mucus in the intestines. Isolates, BCT1 (*L. paracasei*) displayed the least hydrophobicity (42.11±4.24 %). In line with our findings, Giri et al., (2018) showed that LAB isolates from fermented rice beverages differed considerably regarding hydrophobicity (13.6± 0.07 % to 61.4±1.9 %). Several factors may account for the different levels of hydrophobicity observed among strains of the same species, including the different type of hydrophobic and hydrophilic appendages of the cell surface membrane as well as the differential expression of the cell surface protein between the strains (Zhao *et al*., 2017).

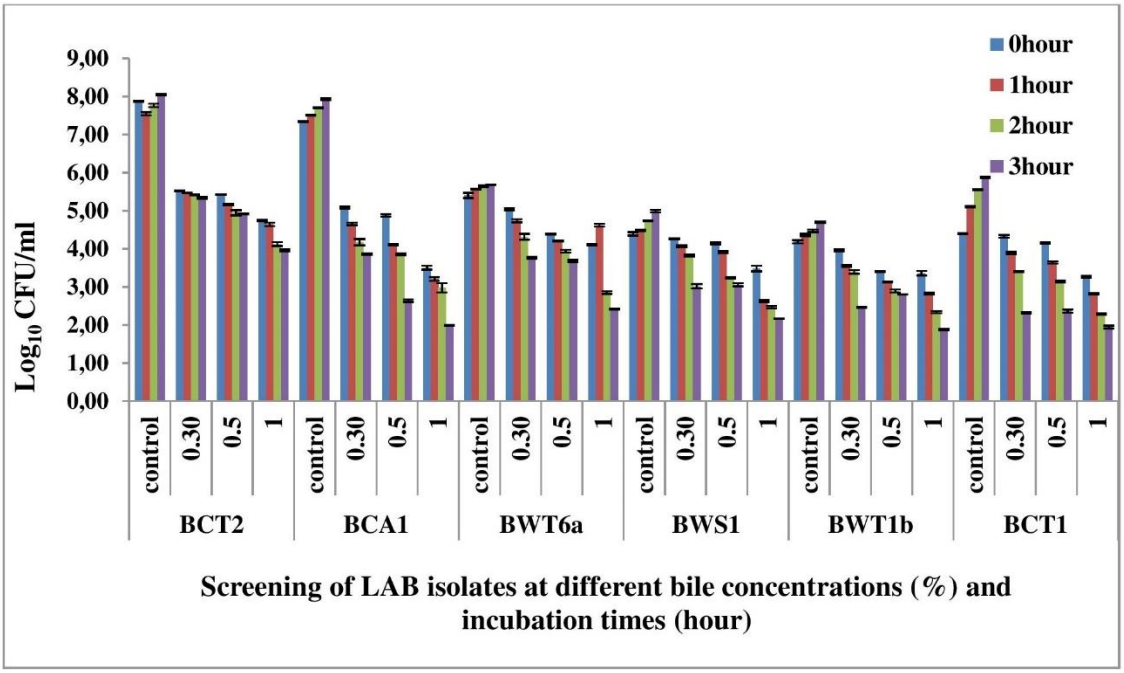


Figure 5. Graph showing screening of LAB isolates at different bile concentrations and incubation times

Table 3. Hydrophobicity and auto-aggregation tests of LAB isolates

Isolate code/strain	Hydrophobicity (percent)	Auto-aggregation (percent)
BTC2/*L. brevis*	91.68±2.81^{a}	89.75±3.68^{a}
BCA1/*L. plantarum*	61.57±4.69^{d}	54.99±4.38^{c}
BWT6a/*Enterococcus lactis*	65.46±5.42^{cb}	64.80±3.16^{b}
BWS1/*Enterococcus durans*	78.60±4.09^{ab}	62.88±4.80^{b}
BCT1/*L. paracasei*	42.11±4.24^{d}	26.31±3.41^{d}
BWT1B/*Pediococcus pentosaceus*	51.76±4.56^{cd}	33.26±3.42^{cd}
F Value	55.34**	111.27**

Values expressed as mean±SD. (n -3). Mean data with **different superscript lower-case letters in the individual row** are significantly different at $p \leq 0.05$, according to Scheffe's test (*$p \leq 0.05$,**$p \leq 0.01$)

When it comes to auto-aggregation, a percentage greater than 80 is beneficial for competition and colonization (Holzapfel et al., 1998). BCT2 exhibited an excellent auto-aggregation ability of 89.75±3.58 % (Table 3). BCA1, BWT6a, and BWS1 isolates showed moderate auto-aggregation of 54.99±4.38% 64.80±3.16% and 62.88±4.80% respectively. Our results were found to be far higher than those reported by Giri et al., (2018) whose LAB isolates from fermented rice beverages *bhaatijaanr* exhibited average degree of auto-aggregation (22.4±1.03 - 41.4±0.93 %). However, according to Handa and Sharma (2016), a strain of *L. plantarum* isolated from *chhang* (a traditional fermented beverage from Himachal Pradesh, India) had relatively high auto-aggregation values of 79.5 %. It is reported that lactobacilli auto-aggregate due to proteins or lipoproteins located on the cell surface, and their differential expression may account for the variation observed between strains and species of lactobacillus (Reniero *et al*., 1992).

Additionally, we predict a correlation between cell surface hydrophobicity and auto-aggregation abilities in LAB isolates. It is interesting to note that BCT2, which showed the highest hydrophobicity (91.68±2.81 %), also exhibited the highest auto-aggregation (89.75±3.68 %) property. Similarly, isolate BCT1, which exhibited the lowest hydrophobicity (42.11±4.24 %), also showed the lowest auto-aggregation (26.31±3.41 %). Although these traits are independent of one another, they have been likewise correlated in several previous publications. In a similar study, Li et al., (2015) found that strains with higher auto-aggregation had higher hydrophobicity.

Antibacterial activities

The results showed that the cell-free supernatants (before and after neutralization) of the six selected LAB strains showed varying inhibitory zones for all the test pathogens (Table 4). An insignificant decrease in antibacterial activity was observed in the cell free supernatants after neutralization. However, while four LAB isolates [BCT2 (*L. brevis*), BCT1 *(L. paracasei*), BWT6a (*Enterococcus lactis*) and BCA1 (*L. plantarum*)] demonstrated best antibacterial activity against *B. cereus*, the supernatant of isolate BCA1 (*L. plantarum*) exhibited the highest zones, 39.00±1.00 mm and 37.17±0.76 mm before and after neutralization respectively. It has been noted that several *L. plantarum* strains produce a variety of bacteriocins and are therefore highly antibacterial (Diep et al., 2009). In addition, our results are consistent with those obtained by Mezzaini and Bouras (2010) finding that the cell free supernatants of isolated LAB were broadly active against gram-positive microbes (*Bacillus cereus, Bacillus subtilis*) even after adjusting the pH to 7. Further, the isolate BWT1B (*Pediococcuspentosaceus*) showed the least inhibitory activity against all four pathogenic strains tested. Numerous studies have demonstrated the antimicrobial activity of cell-free extracts of LAB isolates from different sources (Dunne *et al.,* 2001; Obadina *et al*., 2006).

Antibiotic susceptibility test

From a safety perspective, antibiotic susceptibility is a key criterion for potential probiotic bacteria, since they sometimes become carriers of antibiotic resistance genes, which are then passed on to pathogens. Several authors have documented lactobacillus sp. susceptibility including strain-specific, species-specific, antibiotic-specific, and even aliment-related variations (Charteris et al., 2001; Klayraung et al., 2008). The antibiotic susceptibility patterns in our study varied widely among LAB isolates. Generally, all the isolates were resistant to nalidixic acid (30 µg) a phenomenon that has been previously reported by Charteris et al., (2001). This indicated that our LAB isolates in general were resistant to group 3 antibiotics which inhibit nucleic acids, and that the resistance was chromosomally encoded. In addition, all LAB isolates tested were azithromycin-susceptible, which is consistent with the findings of Sharma et al., (2018). Overall, most isolates tested were sensitive to protein synthesis inhibitors, such as azithromycin, tetracycline, and gentamicin, which seems to align with findings of James et al., (2016). On the other hand, all isolates except BCT2 were resistant to kanamycin, and none were completely susceptible to streptomycin, both of which are on the list of protein inhibitors (Table 5). It has been largely shown that lactobacilli species are resistant to Gram-negative antibiotics such as kanamycin and streptomycin attributed mostly due to high rate of spontaneous chromosomal mutations (Danielsen et al., 2003). Therefore, the use of such strains as food additives has been advocated because of their low likelihood of horizontal spread of their acquired resistance (Georgievaa et al., 2015). The isolate BCT 2 notably (*L. brevis*) was highly susceptible (60 %) to ampicillin, rifampicin, gentamicin, erythromycin, and tetracycline. Interestingly, the isolate was resistant to norfloxacin, vancomycin, ciprofloxacin, and nalidixic acid, all of which inhibit nucleic acid, indicating that the resistance might be intrinsic and not transmittable horizontally (Fabrega et al., 2009).

Table 4. Antibacterial activities of cell free supernatants of selected LAB isolates before neutralization (BN) and after neutralization (AN) against four common test pathogens

Samples	Diameter of Zone of Inhibition (mm)							
	B. cereus		***S. typhi***		***S. aureus***		***E. coli***	
	BN	**AN**	**BN**	**AN**	**BN**	**AN**	**BN**	**AN**
BTC2/ *L. brevis*	36.50±0.50^{ab}	30.40±1.25^{b}	27.33±1.04^{b}	26.33±0.58^{b}	20.33±0.57^{b}	19.50±0.50^{b}	8.73± 0.64^{ab}	6.16±0.28^{a}
BCA1/ *L. plantarum*	39.00±1.00^{a}	37.17 ±0.76^{a}	36.67±0.58^{a}	32.17±1.23^{a}	25.63±0.32^{a}	24.93±0.12^{a}	9.7±0.98^{a}	7.03±0.25^{a}
BWT6a *Enterococcus lactis*	37.33±0.58^{ab}	35.33± 0.58^{a}	25.13±0.23^{bc}	23.86±0.42^{c}	26.43± 0.40^{a}	25.16±0.29^{a}	9.43±0.51^{ab}	6.60±0.36^{a}
BWS1/ *Enterococcus durans*	22.4±1.78^{c}	16.33±1.15^{c}	23.40±1.22c	19.53±0.50^{d}	9.87±0.42^{d}	4.66±0.58^{c}	6.33± 0.29^{cd}	0.000±00^{c}
BWT1B/ *Pediococcus pentosaceus*	17.83± 0.76^{d}	15.73±0.64^{c}	15.46±0.50^{d}	12.93±1.01^{e}	7.40±0.53^{c}	4.93±0.12^{c}	5.50 ±0.56^{d}	0.000±00^{c}
BCT1/ *L. paracasei*	34.80± 1.20^{b}	30.33±1.52^{b}	26.83±0.29^{b}	24.83±0.35^{cb}	19.37±0.51^{b}	18.00±0.00^{b}	8.07±0.12^{d}	4.96±0.50^{b}
F value	210.02**	238.50**	256.84**	223.30**	831.61**	2,237**	25.43**	363.34**

Values expressed as mean±SD. (n -3). Mean data with **different superscript lower-case letters in the individual column** are significantly different at $p \leq 0.05$, according to Scheffe's test (*$p \leq 0.05$, **$p \leq 0.01$)

Table 5. Antibiotic susceptibility of selected LAB isolates by Disc diffusion method

Antibiotic (Concentration)	BTC2	BCA1	BWT6a	BWS1	BWT1B	BCT1	F value
Ampicillin (10µg)	38.67±0.58^{a}(S)	0.00±0.00^{b}(R)	45.33±0.58^{c}(S)	10.67±0.58^{d}(R)	12.76±0.68^{e}(MS)	0.00±0.00^{e}(R)	4,683**
Ciprofloxacin (5µg)	7.33±0.58^{e}(R)	25.40±0.69^{b}(S)	6.50±0.50^{f}(R)	20.73±1.10^{c}(S)	21.83±0.76^{c}(S)	33.93±0.12^{a}(S)	710**
Rifampicin (5 µg)	44.33±0.58^{a}(S)	32.46±0.50^{b}(S)	0.00±0.00^{c}(R)	0.00±0.00^{c}(R)	0.00±0.00^{c}(R)	0.00±0.00^{c}(R)	12,497**
Azithromycin (15 µg)	26.50±0.50^{d}(S)	35.33±0.58^{a}(S)	24.53±0.50^{f}(S)	35.00±1.00^{b}(S)	25.33±0.58^{e}(S)	31.10±0.55^{c}(S)	170.94**
Nalidixic acid (30 µg)	0.00±0.00^{b}(R)	0.00±0.00^{b}(R)	0.00±0.00^{b}(R)	0.00±0.00^{b}(R)	0.00±0.00^{b}(R)	13.10±0.79 ^{a} (R)	817.19**
Tetracyclin (30 µg)	25.53±0.50^{a}(S)	14.67±0.58^{b}(MS)	15.33±0.58^{b}(MS)	24.73±0.46^{a}(S)	14.40±0.53^{cb}(R)	13.03±0.45^{c}(R)	352**
Gentamicin (120 µg)	24.66±0.58^{b}(S)	0.00±0.00^{d}(R)	32.66±0.58^{a}(S)	0.00±0.00^{d}(R)	23.33±1.15^{b}(S)	15.46±0.50^{c}(S)	1,468**
Streptomycin (10 µg)	12.17±0.29^{b}(MS)	9.53±0.50^{c}(R)	11.67±0.58^{b}(MS)	9.40±0.53^{c}(R)	14.23±0.25^{a}(MS)	11.97±0.25^{b}(MS)	54.96**
Erythromycin (15 µg)	32.970.45^{c}(S)	0.00±0.00^{e}(R)	35.470.50^{b}(S)	20.500.50^{d}(S)	0.00±0.00^{e}(R)	40.170.45^{a}(S)	6,331**
Kanamycin (30 µg)	15.16±0.29^{a}(MS)	0.00±0.00^{d}(R)	6.80±0.35^{c}(R)	10.67±1.15^{b}(R)	11.33±0.57^{b}(R)	0.00±0.00^{d}(R)	377.92**
Norfloxacin (10 µg)	0.00±0.00^{c}(R)	8.33±0.58^{b}(R)	0.00±0.00^{c}(R)	8.33±0.58^{b}(R)	8.40±0.53^{b}(R)	20.77±0.67^{a}(S)	752.60**
Vancomycin (30 µg)	0.00±0.00^{c}(R)	14.76±0.25^{ab}(MS)	0.00±0.00^{c}(R)	14.2±30.25^{b}(R)	15.33±0.31^{a}(MS)	0.00±0.00^{c}(R)	5,370**
Susceptibility %	**60**	**25**	**33.3**	**33.3**	**25**	**41.6**	

Values expressed as mean±SD. (n -3). Mean data with **different superscript lower-case letters in the individual row** are significantly different at $p \leq 0.05$, according to Scheffe's test (*$p \leq 0.05$, **$p \leq 0.01$)

In addition, studies have shown that resistance to ciprofloxacin may be caused by either intrinsic characteristics of the cell wall, permeability of the cell membrane or an efflux mechanism, all of which confers no imminent threat to the host organism. Also, the vancomycin-resistant trait is well known in the lactobacillus, and there is a consensus that this characteristic may not be a problem since it differs from the inducible resistance observed in enterococci and no determinants have yet been identified in *L. brevis* (Fukao et al., 2009).

Study of growth characteristics of selected LAB strain

L. brevis (BCT2) emerged as the strongest candidate to be included in functional starter cultures, outperforming all other probiotic strains tested. The study by Hong et al. (2016) found substantial amounts of *L. brevis* in good quality beers, indicating that the bacterium is not harmful if it is not dominant during fermentation. The logistic growth model at 32°C demonstrated a sigmoid growth curve with an initial period of slow growth (lag phase) extending up to 3 hours till OD of 0.18 that was attributed to physiological adaptation of cells to their new environment (Figure 6). Following this phase, an exponential phase lasted between ODs of 0.5 to 1 and was characterized by rapid cell growth and doubling, leading to an increase in absorbance as cell number increased.

After achieving an OD_{max} value of 1.3 at 9 hours, the strain entered the stationary phase and subsequently experienced very slow growth for the next 24 hours. The doubling time was determined to be 1.99 hours by using GraphPadPrism7.04 software, and the spot test at OD_{600} of 0.5 detected 1 x 109 $\log_{10}$ CFU/ml.

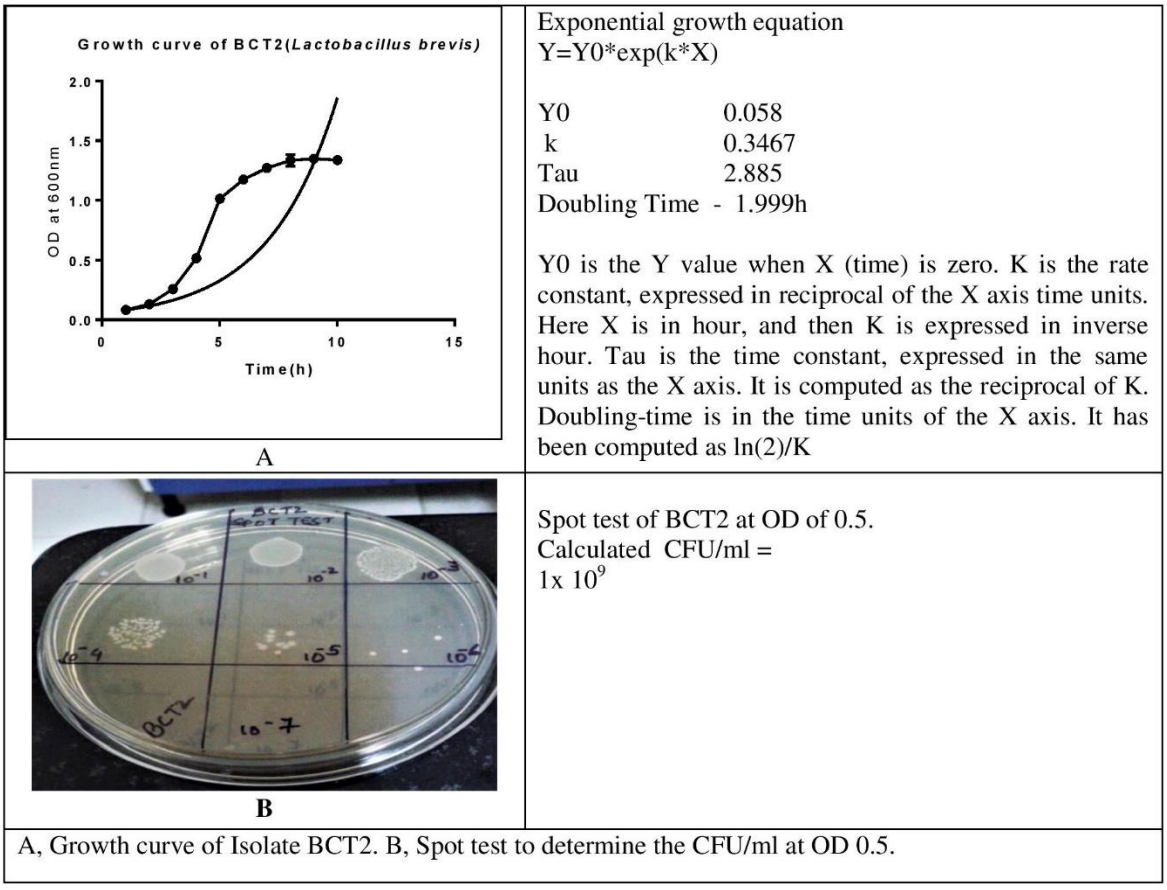


Figure 6. (A). Growth curve of LAB isolate BCT2. (B). Spot test to determine the CFU/ml at OD of 0.5

Accordingly, for further experimental work involving starter culture preparation, we recommended that cells be harvested at half OD_{600} at mid-log phase. These observations corroborate with Monod (1949) findings that microorganisms grow in three distinct phases: lag phase, log phase, and stationary phase. We found that Stamer et al., (1971) estimated the time of generation for *L. brevis* on 37ºC tryptophan yeast extract (TYE) medium to be 81 to 84 minutes, which is lower than our inferred time. As pH and temperature have been shown to affect LAB growth, it's likely that this is due to the temperature and different culture used (Yang *et al*., 2018). The maximum cell concentration reported for *L. brevis* in vegetable juices was reported between 7 to 8 CFU/ml (Champagne et al., 2009), which is consistent with our results. It was also found by Jaiswal and Ghannam (2013) that LAB growth reached its stationary phase within 24 hours, after which there was no significant growth of LAB, which corresponds to our observations.

4. CONCLUSION

In the present investigation, selected strains of LAB isolated from traditionally fermented rice beverage of West Garo Hills of Meghalaya were isolated, characterized, and further assayed for potential probiotic lineaments such as acid tolerance, bile tolerance, hydrophobicity test, auto-aggregation tests, antibiotic tolerance, and antibacterial activities. Generally, most isolates showed good pH tolerance and bile tolerance, and effective Cell surface hydrophobicity and auto aggression activity with *L. brevis* outperforming all others. Most of the isolates showed broad antibacterial activity against Gram-positive bacteria. Overall, most of the strains were susceptible to protein synthesis inhibitors, including azithromycin, tetracycline, and gentamicin. Specifically, *L. brevis* was highly susceptible (60 percent) to antibiotics belonging to the Group 1-Inhibitors of cell wall synthesis, and the Group 2-Inhibitors of protein synthesis. Therefore *L. brevis* was considered to be an effective adjunct in the formulation of functional starter cultures. Consequently, its logistic growth model at 32ºC was determined by spectrophotometric quantification, and spot analysis was executed to ascertain the time and cell concentration (CFU/ml) at its exponential growth phase. Study results showed that the LAB isolates from fermented rice beverages have probiotic lineaments and can be used to formulate novel functional starters useful for food or beverage production.

ACKNOWLEDGEMENTS

The authors extend their gratitude to Professor Arun Goel, Department of Biosciences and Bioengineering, IIT Guwahati, for providing the logistical assistance for DNA extraction and 16s rDNA PCR. Also, the authors acknowledge the help they received from Dr. Subrota Hati and Professor J.B. Prajapati from Anand Agricultural University, Anand, Gujarat in providing logistic support for the probiotic assessments. A special thanks is owed to Dr. S. K. Nandy Research Associate of Bioinformatics Infrastructure Facility, North-Eastern Hill University, Tura Campus, Meghalaya for his relentless assistance in phylogenetic analysis.

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