

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.908.208>

## Screening of Yoghurt Cultures for their Potential Proteolytic, Antioxidant and Probiotic Properties

Jagrani Minj<sup>1</sup>, Brij Pal Singh<sup>2</sup> and Shilpa Vij<sup>3\*</sup>

<sup>1</sup>Dairy Microbiology Division, ICAR-National Dairy Research Institute, Karnal, India

<sup>2</sup>Department of Food Science and Technology, University of Nebraska-Lincoln, USA

<sup>3</sup>Department of Microbiology, School of Science, RK University Rajkot, India

\*Corresponding author

### ABSTRACT

#### Keywords

Probiotic,  
Proteolytic,  
Yoghurt culture,  
Antioxidant

#### Article Info

Accepted:  
18 July 2020  
Available Online:  
10 August 2020

All the yoghurt cultures (10 *Streptococcus thermophilus* and 10 *Lactobacillus bulgaricus* ssp. *delbrueckii*) have been seen for the proteolytic activity with highest value ST 144 ( $113.47 \pm 0.91 \mu\text{g serine mL}^{-1}$ ) and LB 144 ( $201.11 \pm 0.74 \mu\text{g serine mL}^{-1}$ ). Among all the yoghurt cultures, *S. thermophilus* 144 (ST 144) and *L. bulgaricus* 144 (LB 144) have shown good viability at low pH (1.0) and survivability in bile salt concentration up to 3%. Cell surface hydrophobicity has been noted significantly ( $P < 0.05$ ) higher in n-hexadecane ( $52.02 \pm 0.13\%$ ) for ST 144 and in o-xylene ( $61.91 \pm 0.44\%$ ) for LB 144. The best results for antimicrobial activity against test pathogens and positive bile salt hydrolase activity in the presence of sodium taurodeoxycholate have also been observed in these cultures. Yoghurt cultures ST 144 and LB 144 had the highest antioxidative activity  $78.30 \pm 0.12$ , and  $76.13 \pm 0.12 \text{ U mL}^{-1}$ , respectively. In conclusions, yoghurt cultures ST 144 and LB 144 exhibited the promising probiotic properties with additional functional properties such as antioxidant and proteolytic activity.

### Introduction

Now-a-days probiotic bacteria are widely used for the development of probiotic-enriched fermented milk like yoghurt, dahi, milk beverages etc. The presence of probiotic bacteria in any product enhances the functional value of that product. However, fermented milk products made with well proven probiotic bacteria may have many bio-functional properties like antimicrobial, ACE-inhibitory, antioxidant activity etc. As per the definition by FAO/WHO (2002), “probiotics

are live microorganisms that when administered in adequate amounts confer health benefits to the host”. It is well known that all lactic acid bacteria are not probiotics. Therefore, isolation, screening and well characterization is very important for the assessment of probiotic bacteria. As per FAO/WHO (2002) guidelines, initial screening is an important step to study the probiotic properties of any bacteria which includes strain identification, functional characterization and safety assessment. In this study well identified NCDC yoghurt strains

were subjected for their functional characterization using *in vitro* tests. All the strains were analysed for their low pH and bile salt resistance, cell surface hydrophobicity, antimicrobial action, cell auto-aggregation, bile salt hydrolase activity and antibiotic susceptibility/resistance test. Additional functional properties of NCDC yoghurt cultures were also screened out for their proteolytic and antioxidant activity. These functional properties of any probiotic bacteria could further enhance their therapeutic value. Some lactic acid bacteria (LAB) have the ability to produce proteinases which hydrolyze the milk proteins and release small bioactive peptides. The proteolytic lactic acid bacteria have not only the technological significance in fermented milk for their ability to produce peptides and amino acids for the rapid growth of LAB in milk but these cultures can also liberate small bioactive peptides in fermented milk with additional health benefits (Seppo *et al.*, 2003). However, these biologically active peptides involve in various physiological processes and provides therapeutic benefits to the host. Though, some of these peptides are being used by the other bacteria but some of them remains accumulated in the medium (Leclerc *et al.*, 2002). Bioactive peptides have been identified and characterised in many fermented dairy products such as cheese, yoghurt, and fermented sour milk (Seppo *et al.*, 2003). These bioactive peptides are generated by the proteolytic LAB used in the fermentation processes.

Functional properties such as antioxidant activity of any lactic acid bacteria and probiotic bacteria could have additional benefits to the final product. Free radical scavenging activity of LAB and probiotic bacteria could be helpful in the reduction of abnormally high levels of reactive oxygen species (ROS) generated in the body by the various factors. When these ROS did not get

counteracted, they lead to tissue damages and further complete cell death. Oxidative stress also induces various diseases and disease like conditions. Though natural antioxidant of human body cells continuously fights with it but regular exposure to the oxidative stress weakens the natural antioxidant systems. Many food products are rich in natural antioxidants but when it comes to the development of functional fermented dairy products, screening of bacterial cultures for their antioxidant activity makes them more useful. Kim *et al.*, (2005) have reported that microbial cells have many antioxidant defense mechanisms which protect the biological system by removing or inactivating the ROS. Many lactic acid bacteria are able to exert an antioxidant action, therefore, the toxicity of ROS might be eliminated or counteracted on consumption of such fermented dairy product like yoghurt (Kim *et al.*, 2005). Consumption of yoghurt provides many health benefits including improvement of lactose intolerance, control of gastrointestinal infections, reduction of serum cholesterol, enhancement of bioavailability of calcium and other essential nutrients, stimulation of immune cells, anti-carcinogenic effect and longevity (Mckinley, 2005). A well proven probiotic bacteria along with functional properties like antioxidant activity promote the therapeutic value. Probiotic proven strains have known for their better survivability in the challenging environment of gastrointestinal tract; however, they maintain the host health (Elli *et al.*, 2006). Therefore, the functionality of plain yoghurt can be enhanced by incorporating well proven probiotic bacteria along with their functional properties such as proteolytic and antioxidant properties. Hence, this study has been designed to screen out the probiotic properties as well as functional properties of NCDC yoghurt cultures by *in vitro* methods for the further *in vivo* study and product developments.

## Materials and Methods

### Bacterial cultures

Ten yoghurt cultures NCDC 142, 144, 145, 146, 183, 260, 262, 263, 264 and 300 were procured as a frozen stock cultures from National Collection of Dairy Cultures (NCDC), Karnal. *Lactobacillus bulgaricus* ssp. *Delbrueckii* and *Streptococcus thermophiles* were separated from mixed yoghurt cultures and grown in their respective growth medium such as *L. bulgaricus* ssp. *delbrueckii* strains were grown in MRS broth (Hi-Media, Mumbai) at 37°C for 16-18h and *Streptococcus thermophilus* in M-17 broth (Hi-Media, Mumbai) at 42°C for 16-18 h. All the strains were grown and serially transferred at least three times before use in this study. For the antimicrobial study, pathogenic bacteria as an indicator organisms such as *Bacillus cereus* NCDC 240, *E. coli* NCDC 135, *Enterococcus faecalis* NCDC 115, *Listeria monocytogenes* ATCC (American Type Culture Collection) 15313, *Salmonella enterica* subsp. *enterica* NCTC (National Collection of Type Cultures) 6017, *Shigella dysenteriae* NCDC 107 and *Staphylococcus aureus* NCDC 133 were also procured from NCDC. All the pathogenic bacteria were grown in brain heart infusion (BHI) broth medium (Hi-Media, Mumbai) at 37°C for 16-18 h. Stock cultures were also prepared and kept in 40% glycerol stock for pathogens and yoghurt cultures were stored in litmus milk for further studies.

### Analysis of proteolytic activity

The proteolytic activity was measured by the o-phthalaldehyde (OPA) method as described by Church *et al.*, (1983) and Donkor (2007). A free NH<sub>3</sub> group released during the reaction was measured. During fermentation, the proteolytic bacteria degrade the proteins and release the small molecules

of peptides. The extent of proteolysis was measured by taking an aliquot of 2.5 mL culture medium and mixed with 5mL of 0.75% trichloroacetic acid (TCA), and then the mixture was filtered using whatmann filter paper number one. In next step 150µL of the filtrate was taken in a test tube and 3mL of freshly prepared OPA reagent was added and incubated at room temperature for 2 min. Absorbance at 340 nm was measured using the spectrophotometer (UV-1800, Shimadzu). The proteolytic activity of yoghurt cultures was expressed as equivalent to free amino acid groups. Control was performed in broth without any culture addition. All the experiments were performed in triplicate.

### Preparation of cell-free extracts (CFE)

The CFE of yoghurt cultures were prepared for antioxidant activity analysis. *S. thermophilus* strains and *L. bulgaricus* strains were grown in their respective broth medium M-17 and MRS. After 18-24 h of incubation at 42°C for *S. thermophilus* and 37°C for *L. bulgaricus*, the cells were harvested by centrifugation at 10,000 rpm for 5 min at 4°C (Mikro 22 R, Hettich) to make cell-free extract (CFE). Then, these CFE samples were subjected to assess the antioxidant capacity by 1, 1-diphenyl- 2-picrylhydrazyl (DPPH) method.

### Antioxidant activity of yoghurt cultures

The free radical scavenging activity was determined by DPPH assay. The antioxidant activity of yoghurt cultures CFEs were determined by the method Lin and Chang (2000) with slight modification. Volume 1 mL of CFE and a freshly prepared DPPH (Sigma-Aldrich, St. Louis, MO) solution (0.2 mM, 1 mL), were mixed properly by vigorous shaking. Then the sample mixture was kept in the dark at room temperature to react for 30 min. The control sample contained deionized

water instead of CFE. The scavenged DPPH was measured by taking absorbance at 517 nm using microplate reader (Spectrostar<sup>nano</sup> BMG Labtech). The radical scavenging activity was quantified as unit mL<sup>-1</sup> or U mL<sup>-1</sup> by using the following formula (Gazi *et al.*, 2007).

$$\text{DPPH activity (U mL}^{-1}\text{)} = (A_c - A_s)/S \times 100$$

Where,

A<sub>c</sub> = Absorbance of the control at 517 nm

A<sub>s</sub> = Absorbance of the test sample at 517 nm

S = Volume of sample in mL

### Probiotic attributes study

#### Resistance to acidic conditions

Resistance to low pH conditions were analysed by the method of Clark *et al.*, (1997). Briefly, M-17 and MRS broth (adjusted to pH 1.0, 2.0 and 3.0) were prepared and inoculated with 1% (~12x10<sup>6</sup> CFU mL<sup>-1</sup>) of overnight grown bacterial cultures. All the inoculated sets of pH broths were incubated at their respective temperature for 0, 1, 2 and 3 h. Viable cell counts were determined by pour plate technique by taking one millilitre aliquot from each inoculated tube at 0, 1, 2, and 3 h of incubation in their respective incubation temperature and growth media.

#### Bile salt tolerance

Gilliland and Walker (1990) method was used for the bile salt tolerance study. Different concentrations (1%, 2% and 3%) of bile salts were added to the M-17 and MRS broth to check the survivability of all yoghurt bacteria, *S. thermophilus* and *L. bulgaricus* ssp. *delbrueckii*, respectively. Viable cell counts were analysed after different time intervals i.e. 1 h, 2 h and 3 h of exposure to bile salts.

Serial dilutions were prepared and pour plate method was used for taking the bacterial cell counts by incubating at their respective temperatures for 24-48 h. The viable cell counts were expressed as colony forming unit or CFU mL<sup>-1</sup>.

#### Cell surface hydrophobicity assay

Cell surface hydrophobicity assay was done to see the ability of yoghurt bacteria to adhere to the hydrocarbons. Microbial adhesion to the hydrocarbon method described by Rosenberg *et al.*, (1980) was followed using n-hexadecane, n-octane and o-xylene and this method was supposed to consider as the potential of adherence to the epithelial surface layer in the gastrointestinal tract (GIT).

To check the cell surface hydrophobicity, overnight grown cultures were centrifuged and bacterial cell pellets were resuspended in equal volume of MRS and M-17 medium for *L. bulgaricus* ssp. *delbrueckii* and *S. thermophiles* respectively. After that cultures were harvested by centrifugation (2000 × g, 15 min, 4°C), washed twice in PUM buffer (K<sub>2</sub>HPO<sub>4</sub>: 22.2 g/L; KH<sub>2</sub>PO<sub>4</sub>: 7.26 g/L; urea: 1.8 g/L; MgSO<sub>4</sub>: 0.2g/L; pH 7.1 ± 0.2) and finally suspended in the same buffer. The absorbance (A<sub>0</sub>) was initially taken at 600 nm of the suspension and adjusted to 0.70±0.02. Five mL of cell suspension in PUM buffer was taken in clean and dry round bottom test tubes followed by addition of one mL of hydrocarbons i. e. n-hexadecane, n-octane and o-xylene. Vortexed for 2 min. The tubes were left undisturbed for 1 h at 37°C for the phase separation. The upper aqueous phase was carefully removed with a sterile Pasteur pipette and the lower aqueous phase was separated for the O.D. measurement. Absorbance (A<sub>1</sub>) was taken at 600 nm and percent hydrophobicity (H %) was calculated using the following formula:

$$H\% = (1 - A_1/A_0) \times 100$$

Where,

A<sub>0</sub> = Initial absorbance

A<sub>1</sub> = Final absorbance

### **Antimicrobial activity**

The yoghurt cultures were assessed for antimicrobial activity by Herreros *et al.*, (2005) with slight modifications. Agar well assay was performed by an overlay of indicator bacteria namely *B. cereus* NCDC 240, *E. coli* NCDC 135, *E. faecalis* NCDC 115, *Listeria monocytogenes* ATCC 15313, *S. enterica* NCTC 6017, *Sh. dysenteriae* NCDC 107 and *Staph. aureus* NCDC 133 on the solidified nutrient agar plate. Fresh overnight grown bacterial cultures in MRS and M-17 broth were taken, and supernatants of the yoghurt cultures were collected by centrifugation (10,000 g, 15 min, 4°C). The pH of the supernatant was adjusted to 6.5 and filtered through sterilised filter (0.22 µm). This cell-free culture supernatants (CFCS) of the yoghurt cultures were screened for inhibitory activity using the agar well diffusion technique. An initial inoculum of approximately 1x10<sup>6</sup> CFU mL<sup>-1</sup> of the test strains was mixed with 7 mL of soft agar (0.7% agar) and overlaid on the nutrient agar plates. The CFCS (100 µL) was transferred in holes (6 mm diameter) drilled into the agar. The plates were incubated at 37<sup>0</sup> C, and the antimicrobial activity was noted as clear zones (in diameter) around the well. A clear zone of inhibition of 1 mm or more was considered as a positive inhibition against indicator test organism.

### **Bile salt hydrolase activity**

The ability of deconjugation to bile salts was determined according to the bile salt hydrolase assay described by Taranto *et al.*,

(1995). According to this method, bile salt plates were prepared by adding 0.5% (w/v) of sodium salts such as sodium taurodeoxycholate (TDC), sodium taurocholic acid (TC), sodium tauroglycocholate (TGC) and sodium deoxycholate (DC) to M-17 and MRS agar for *S. thermophilus* and *L. bulgaricus* ssp. *delbrueckii*, respectively. The individual yoghurt cultures were streaked on the bile salt supplemented media and the plates were anaerobically incubated at 42°C and 37°C for 72 h respectively. The presence of the precipitated bile acids around colonies as white opaque halo were considered as a positive result.

### **Antibiotic susceptibility/resistance test**

This test assay was done to check the sensitivity/ resistance pattern of yoghurt bacteria in the presence of various antibiotics at breakpoint concentration. Recommended method of Clinical and Laboratory Standards Institute CLSI, (2007) was followed to interpret the results in terms of resistance or susceptibility by taking the zone of inhibition in diameter in mm. In this study, antibiotic disk (Hi-media Laboratories Pvt. Ltd. Mumbai, India) were used on MRS/ M7 agar plates. The antibiotic discs were placed on agar surface and incubated at their respective temperature for 24 h.

The antibiotics and their concentrations used in the study were ampicillin (10 mcg), amoxycillin (10 mcg), cefadroxil (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), clindamycin (2 mcg), erythromycin (15 mcg), gentamicin (10 mcg), nalidixic acid (10 mcg), penicillin (10 units), Tetracycline (30 mcg) and vancomycin (30 mcg). The diameter of inhibition zones (in mm) was measured to see the sensitivity/resistance of yoghurt cultures. The zone of clearance (mm) was observed to interpret the susceptibility/ resistance of yoghurt bacteria.

## Cell Auto-aggregation

Cell auto-aggregation assay was performed by previously described method Kos *et al.*, (2003) with slight modifications. In this assay, the overnight grown bacterial cells were harvested by centrifugation and phosphate buffered saline (PBS) having pH 7.2 was used to wash twice. Cells with  $10^8$  CFU mL<sup>-1</sup> were vortex for 10 s and re-suspended in 4 mL of PBS and incubated for 4 h at room temperature. The upper suspension volume of 5 µL was carefully removed at times 0, 1, 2, 3 and 4 h and transferred to the microplate which containing 195 µL of PBS, and then absorbance was measured at 620 nm. The auto-aggregation percentage was calculated using equation below:

$$\% \text{ Cell auto-aggregation} = 1 - (A_t/A_0) \times 100$$

Where,

$A_t$  denotes the absorbance at time  $t= 1, 2, 3, 4$  h

$A_0$  denotes the absorbance at time  $t=0$

## Statistical analysis

All the data pertaining to this study were statistically analyzed using prism 8.0 software. Mean and standard error were also used for the statistical representation of data followed by a comparison at  $P<0.05$  levels of significance between means.

## Results and Discussion

### Proteolytic activity

Lactobacilli utilise milk proteins as their prime source of essential and growth-stimulating amino acids. The proteolytic bacteria may generate the biologically active peptides during the preparation of yoghurt via

fermentation process. These bioactive peptides could have many functional roles in the health management. So, analysis of proteolytic activity could be the representative of the generated bioactive peptides. In this study, the proteolytic activity of 10 NCDC yoghurt cultures (10 *S. thermophilus* and 10 *L. bulgaricus* ssp. *delbrueckii*) was investigated and almost all the yoghurt cultures have exhibited for their proteolytic activity (Fig. 1). Although some yoghurt cultures were high in proteolytic activity and some were little low. The highest proteolytic activity (in µg serine mL<sup>-1</sup>) has been observed in ST 144 (113.47±0.91), LB 144 (201.11±0.74), LB 260 (205.42±0.42), LB 262 (110.28±0.66) and LB 263 (208.61±0.37). It has been noted that *Lactobacillus* strains LB 144, LB 260 and LB 263 have shown higher proteolytic activity than *S. thermophilus* yoghurt cultures. Usually *Lactobacillus* species have been known to exhibit more proteolytic activity. The proteolytic activity of 15 *Lactobacillus* strains has also been reported for the maximum proteolytic activity in 48 h of incubation (Haq and Muktar, 2009). Similarly, Kholifet *al.*, (2011) also reported the protease activity of lactobacillus species such as *L. rhamnosus*, *L. delbrueckii*, *L. plantarum* and *L. helveticus*. The proteolytic action of lactobacillus not only promotes the growth of other bacterial species, but it has a huge impact on the consumer health. Related research conducted by Donkor *et al.*, (2007) suggested that milk cannot supply all essential amino acids needed for *Lactobacillus* species to grow in free form, therefore, these microorganisms have developed their capability to degrade milk proteins, mainly caseins by their proteolytic system into peptides and amino acids essentially needed for their growth. *In vitro* screening of yoghurt bacteria for their proteolytic activity could be an important aspect for the development of functional yoghurt because all the yoghurt

bacteria have not been screened for their proteolytic activity. Shakerian *et al.*, (2015) reported that yoghurt bacteria *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus* or *B. animalis* BB-12 and *L. acidophilus* La5 have considerably higher proteolytic activity which has been seen in the yoghurt samples. Similarly, Raveschot *et al.*, (2020) have been studied the peptide producing ability of dairy products isolated 170 lactobacillus strains and their initial screening on skim milk agar for their proteolytic activity have been reported only in 15 lactobacillus strains. In yoghurt, *L. delbrueckii* is known for the initiation of proteolysis (Pailin *et al.*, 2001). This has indicated the acceptable results with our study in terms of proteolytic activity. It has been explained that *Lactobacillus* proteinases can hydrolyse more than 40% of the peptide bonds of  $\alpha$ -S1-CN and  $\beta$ -CN, and thus form more than 100 different oligopeptides. The complex peptidase system present in lactobacillus actively degrades them to small bioactive peptides (Choi *et al.*, 2012).

### **Antioxidative activity by DPPH radical scavenging activity**

The NCDC yoghurt cultures were studied for their antioxidant potential by measuring the 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging activity. The highest results for DPPH radical scavenging activity have been observed in ST 144 ( $78.30 \pm 0.12$  U mL<sup>-1</sup>), ST 183 ( $65.87 \pm 0.23$  U mL<sup>-1</sup>), ST 263 ( $66.63 \pm 0.30$  U mL<sup>-1</sup>), LB 144 ( $76.13 \pm 0.12$  U mL<sup>-1</sup>), LB 145 ( $52.93 \pm 0.20$  U mL<sup>-1</sup>), LB 260 ( $72.10 \pm 0.10$  U mL<sup>-1</sup>) and LB 263 ( $72.90 \pm 0.29$  U mL<sup>-1</sup>) along with significant p-value ( $P < 0.05$ ) (Fig. 2). Liu and Pan, (2010) also reported the similar pattern of results on DPPH radical scavenging activity of 12 *Lactobacillus* strains and among them 10 tested *Lactobacillus* strains exhibited radical scavenging activities. Similarly, Xing *et al.*, (2015) have also studied the antioxidant

activity of 10 lactobacilli CFEs and found that CFEs of CCFM8661 exhibited strong DPPH radical scavenging activity ( $75.94 \pm 1.05$  U mL<sup>-1</sup>) which was not significantly different from the positive control LGG ( $77.29 \pm 1.51$  U mL<sup>-1</sup>). Huang and co-workers (2005) have also analysed the radical scavenging activity of *L. delbrueckii* subsp. *bulgaricus* LJJ and *L. casei* subsp. *casei* SY13 with their intracellular cell-free extracts and intact cells by DPPH assay. The radical scavenging activities differ from strain to strain because different strains have different capability and mechanisms might be involved in the radical antioxidant reactions. Kim *et al.*, (2005) have reported that among several commercial yogurt starter cultures, *L. bulgaricus* LB207 had exhibited the highest antioxidant activity which might be due to its strong hydroxyl radical scavenging activity and reducing power. Similarly, free radical scavenging capacity of *L. delbrueckii* subsp. *bulgaricus* LJJ and *L. casei* subsp. *Casei* SY13 have been significantly reported by Zhang *et al.*, (2011). The antioxidant activity of LAB has also been reported by the Suet *et al.*, (2015).

### **Probiotic attributes study**

#### **Resistance to acidic conditions**

Top survival NCDC yoghurt cultures at low pH have been given in Table 1. It has been observed that at pH 1.0, ST 144 ( $5.29 \pm 0.02$  log CFU mL<sup>-1</sup>) and LB 144 ( $5.44 \pm 0.01$  log CFU mL<sup>-1</sup>) showed significant ( $P < 0.05$ ) survivability after 2 h of incubation. When the survivability of all the NCDC yoghurt cultures were analyzed at pH 2.0 for 2 h, ST 144 and LB 144 showed good survivability i. e.  $5.47 \pm 0.02$  and  $5.57 \pm 0.00$  log CFU mL<sup>-1</sup> respectively, which indicated a significant log cycle reduction after 3 h incubation. Similarly, the survivability of NCDC yoghurt cultures were checked at pH 3.0. The log count has been significantly higher than the

pH 1.0 and pH 2.0. The overall highest log counts in pH 3.0 for 2 h of incubation have been observed in ST 144 ( $6.58 \pm 0.03$  log CFU mL<sup>-1</sup>) and LB 144 ( $6.63 \pm 0.00$  log CFU mL<sup>-1</sup>) in comparison to pH 1.0 and pH 2.0. Therefore, it could be promising yoghurt cultures for the study to some other techno-functional attributes and *in vivo* study as well. Better survivability at low pH of yoghurt cultures (ST 144 and LB 144) could be beneficial to survive in the adult human stomach where the same pH condition has been seen. Although rest of the NCDC yoghurt cultures has also shown the appreciable growth at pH 1.0, pH 2.0 and pH 3.0 but lower than the ST 144 and LB 144. Interestingly, almost similar growth pattern of log count has been found in ST 260, ST 263, LB 260 and LB 263. Therefore, it could be predicted that yoghurt cultures ST 144 and LB 144 could be the best to tolerate low pH (pH 2.0) without any significant loss in cell count during passage through the stomach. This study has found comparable with the study done by Hoque and co-workers (2010), where they suggested that lactobacilli isolated from regional yoghurt of Bangladesh had maximum survivability at pH 2.22. The basic characteristics of any probiotic bacteria is their ability to survive in the low pH. Therefore, many research studies have been done to check whether any lactic acid bacteria can survive in the low pH and simulated gastric condition or not. Namdari and Netaji, (2016) reported that wild isolates of *L. helveticus* have shown the promising result even after the simulated gastrointestinal digestion. Similarly, Netaji and Oelschlaeger, (2016) have reported that *L. lactis* SPT2 and AS2 have survived in the simulated GIT juices which were quite comparable with the probiotic strain *L. rhamnosus* GG. The ability to resist low pH ( $\leq 3.0$ ) by the Indian traditional fermented food idli batter isolated *L. plantarum* (IB-1) and *L. lactis* (IB-2) have also been reported for their potential probiotic

attribute in terms of viability 88.13% and 89.85% respectively (Iyer *et al.*, 2013). Comparative study on resistance to intestinal conditions and gastric juice environments have been done and concluded that 21 *L. plantarum*, 11 *Pediococcus ethanolidurans* and 7 *L. brevis* have shown less resistance to the intestinal conditions than gastric juice environments (TokatJ *et al.*, 2015). Similarly, cheese isolate *L. plantarum* C6 have been reported for their very good probiotic potential by exhibiting tolerance to low pH 3.0 and pH 2.0 in 2 h of incubation time (Hati *et al.*, 2014). The *in vitro* screening for probiotic potential of various indigenous LAB isolated from yoghurt and milk have also been studied for their ability to resist low pH and reported that PL5 (*L. paracasei*), PL8 (*Enterococcus faecium*), PL13 (*L. delbrueckii*) and PL14 (*L. saecki*) have shown the most promising results at low pH conditions. Achi and Halami, (2019) have studied the probiotic and functional characteristics along with their safety of indigenous bifidobacterial isolates and reported that *B. breve* NCIM5671 exhibited the better tolerance to low pH for the qualifying of probiotic attributes. The legal requirements to prove the probiotic potential have also been considered as necessary while studying the probiotic attributes to further product development. Cunha *et al.*, (2013) have reported that *Lactobacillus* spp. present in trade Belo Horizonte was probiotic in nature in accordance with the legal requirements. Recently, Bhat and Bajaj, (2020) have reported that human breast milk isolated LAB *L. casei* M5 exhibited the highest survival rate 98.82% in the simulate gastric juice than other isolates M2, M3, M6, M7, M8, M10 and M37.

### **Bile salt tolerance**

The resistance to bile acids of yoghurt cultures has been assessed in 1%, 2% and 3%

bile salt concentration. Ability to resist bile acids is one of the most important criteria for the characterization of any probiotic bacteria. In this study, 3 different concentrations of bile acids were added to the M-17 and MRS agar to check the survivability of all NCDC yoghurt cultures. Current study indicated that 1% of bile salt displayed the highest survivability in ST 144 ( $5.45 \pm 0.01 \log \text{CFU mL}^{-1}$ ) and LB 144 ( $5.36 \pm 0.01 \log \text{CFU mL}^{-1}$ ) with significant p-value ( $P < 0.05$ ) in 3 h of incubation time. Further, 2% of bile salt supplemented media also supported the better growth of ST 144 ( $5.42 \pm 0.01 \log \text{CFU mL}^{-1}$ ) and LB 144 ( $5.34 \pm 0.01 \log \text{CFU mL}^{-1}$ ) in 3 h of incubation (Table 2). When survivability of these yoghurt cultures has shown better results then again 3% of bile salt supplemented media have been taken to see their survivability. Although survivability of yoghurt cultures has seen decreased by one log count, but results were still promising.

The viable counts were noted  $4.37 \pm 0.01$  and  $4.34 \pm 0.01 \log \text{CFU mL}^{-1}$  for ST 144 and LB 144 respectively in 3 h of incubation time. Here we have highlighted only highest grown yoghurt cultures. Other yoghurt cultures such as ST 260, ST 263, LB 260 and LB 263 have also showed good survivability at above mentioned bile salt concentrations (Table 2). The gastrointestinal tract (GIT) contains a significant amount of bile acids which are released by the liver and go through the GIT. However, it is considered as an important aspect for the characterization of any probiotic bacteria. Resistance to bile salts varies among species and strains. In this study, the difference in bile salt tolerance of NCDC yoghurt cultures has been observed. Among 10 *S. thermophilus* cultures, ST 144 was highly resistant to bile salts (up to 3%) followed by ST 260 and ST 263. *L. bulgaricus* ssp. *delbrueckii* cultures have also shown the survivability in presence of bile salts up to 3%. Yoghurt culture LB 144 has

exhibited the highest resistance to bile salts followed by LB 260 and LB 263. These yoghurt cultures have survived in 5–6fold higher concentration (1.5–2.0% oxgall bile) than the usual bile salt concentration (0.3%) present in the human stomach. On the other side, some of the yoghurt cultures were observed very sensitive in the presence of bile salts even at low concentration (1%). Many similar research studies have been conducted to see the bile salt tolerance ability of lactic acid bacteria *in vitro*. Probiotic strains *B. catenulatum*, *B. longum* and *B. pseudocatenulatum* have been reported for their ability to grow in the presence of 0.25% and 2% of bile salts (Delgado *et al.*, 2008). Similarly, probiotic potential of two folate producing yoghurt cultures *S. thermophilus* NCIM 2904 and *L. helveticus* NCIM 2733 have been studied for the bile salt tolerance and reported that *S. thermophilus* NCIM 2904 did show more viable counts  $7.15 \pm 0.2 \log \text{CFU mL}^{-1}$  in comparison to *L. helveticus* NCIM 2733 in 2% bile salts (Deep and Kundu, 2015). *L. plantarum* ZDY 2013 a novel isolate from Chinese traditional fermented acid beans had exhibited better survival ability under bile salt stress 0.45% for 3 h (Huang *et al.*, 2015).

Similarly, Panicker and Behare, (2014) have found that faecal origin *L. fermentum* BIF-18, BIF-19 and BIF-20 have tolerated up to 1.8% of bile salts and dairy origin NCDC-400 have also exhibited the better tolerance to bile salt. Shahid Riaz *et al.*, (2015) have screened out *in vitro* probiotic potential of various indigenous LABs isolated from yoghurt and milk and they reported that all LAB strains were more resistance at 0.30% than 0.5%, 1.0% and 1.5% of bile salts. In next study Thakkar *et al.*, (2015) have also reported that fermented cabbage (Sauerkraut) isolated *L. rhamnosus* PFC21 have shown good bile salt tolerance of sodium taurocholate at 0.8%.

**Table.1** Highest survival NCDC yoghurt cultures in acidic conditions at low pH 1, 2 and 3

Cultures	pH 1 (Log CFU mL <sup>-1</sup> )				pH 2 (Log CFU mL <sup>-1</sup> )				pH 3 (Log CFU mL <sup>-1</sup> )			
	0 h	1 h	2 h	3 h	0 h	1 h	2 h	3 h	0 h	1 h	2 h	3 h
ST 144	6.53±0.03	6.41±0.02	5.29±0.02	4.40±0.01	7.38±0.01	6.52±0.01	5.47±0.02	4.53±0.02	8.75±0.01	7.66±0.01	6.58±0.03	5.24±0.02
ST 260	6.23±0.01	6.25±0.01	4.90±0.03	4.01±0.01	7.02±0.04	6.32±0.01	5.23±0.01	4.34±0.01	8.51±0.01	7.50±0.02	6.32±0.01	4.77±0.04
ST 263	6.34±0.02	6.02±0.04	4.84±0.04	3.59±0.06	7.07±0.01	6.09±0.03	5.24±0.03	4.25±0.01	8.54±0.01	7.46±0.01	6.29±0.01	4.56±0.04
LB 144	7.65±0.01	6.50±0.02 <sup>b</sup>	5.44±0.01	4.36±0.01	7.67±0.01	6.58±0.00	5.57±0.00	4.59±0.00	8.74±0.00	7.67±0.02	6.63±0.00	5.57±0.01
LB 260	7.39±0.02	6.30±0.01	4.84±0.04	4.15±0.02	7.55±0.01	6.42±0.01	5.39±0.02	4.36±0.01	8.64±0.01	7.52±0.01	6.35±0.02	4.82±0.04
LB 263	7.45±0.01	6.25±0.01	4.80±0.05	4.04±0.02	7.47±0.00	6.25±0.01	5.23±0.01	4.26±0.01	8.55±0.00	7.46±0.02	6.28±0.01	4.69±0.05

Data are given here in Mean ± SEM (3 replicates)

**Table.2** Highest survival NCDC yoghurt cultures in presence of bile salt 1, 2 and 3%

Cultures	1% Bile salt (Log CFU mL <sup>-1</sup> )				2% Bile salt (Log CFU mL <sup>-1</sup> )				3% Bile salt (Log CFU mL <sup>-1</sup> )			
	0 h	1 h	2 h	3 h	0 h	1 h	2 h	3 h	0 h	1 h	2 h	3 h
ST 144	8.65±0.00	7.51±0.01	6.52±0.01	5.45±0.01	8.64±0.01	7.50±0.00	6.44±0.01	5.42±0.01	8.62±0.00	7.47±0.01	5.51±0.01	4.37±0.01
ST 260	8.57±0.01	7.48±0.01	6.14±0.01	4.97±0.02	8.57±0.01	7.38±0.01	5.97±0.01	3.88±0.02	8.56±0.00	6.98±0.32	5.09±0.03	3.63±0.07
ST 263	8.55±0.00	7.43±0.01	6.05±0.05	4.86±0.02	8.54±0.00	7.15±0.03	6.05±0.01	3.90±0.03	8.53±0.01	6.81±0.32	4.71±0.35	3.63±0.03
LB 144	8.66±0.01	8.09±0.33	6.41±0.01	5.36±0.01	8.63±0.01	7.44±0.01	6.37±0.01	5.34±0.01	8.62±0.00	7.44±0.01	5.70±0.32	4.34±0.01
LB 260	8.54±0.00	7.38±0.01	6.15±0.02	5.05±0.01	8.48±0.01	7.19±0.01	6.11±0.02	4.98±0.03	8.45±0.02	7.09±0.01	5.23±0.41	4.04±0.02
LB 263	8.57±0.01	7.37±0.01	6.19±0.01	5.04±0.02	8.49±0.01	7.18±0.02	6.14±0.01	5.03±0.01	8.47±0.01	7.16±0.01	5.41±0.35	4.08±0.02

Data are given here in Mean±SEM (3 replicates)

**Table.3** Antimicrobial activity of yoghurt cultures (Zone of inhibition in mm)

Yoghurt cultures	<i>B. cereus</i> NCDC 240	<i>E. coli</i> NCDC 135	<i>E. faecalis</i> NCDC 115	<i>L. monocytogenes</i> ATCC 15313	<i>S. enterica</i> 6017	<i>Sh. dysenteriae</i> NCDC 107	<i>Staph. aureus</i> NCDC 133
ST 142	14.97±0.03	13.13±0.18	10.80±0.06	10.13±0.03	18.17±0.03	11.17±0.03	12.07±0.03
ST 144	15.13±0.09	11.00±0.35	11.10±0.06	11.23±0.03	17.83±0.03	13.83±3.03	15.37±0.03
ST 145	10.10±0.06	11.27±0.03	11.13±0.07	11.07±0.03	17.07±0.07	9.07±0.03	10.33±0.03
ST 146	11.13±0.09	12.17±0.07	11.17±0.03	10.07±0.03	19.03±0.03	10.07±0.03	9.63±0.07
ST 183	11.03±0.09	9.83±0.03	9.77±0.03	10.07±0.03	13.33±0.07	10.43±0.03	10.17±0.03
ST 260	14.10±0.06	12.07±0.03	14.10±0.06	14.07±0.03	20.03±0.03	10.07±0.03	13.03±0.03
ST 262	12.10±0.06	9.57±0.03	9.80±0.06	11.83±0.09	13.07±0.03	10.03±0.07	11.47±0.03
ST 263	12.00±0.10	13.20±0.06	13.20±0.06	11.23±0.03	17.07±0.03	9.07±0.03	13.40±0.06
ST 264	11.13±0.07	11.50±0.06	12.13±0.09	11.97±0.09	12.10±0.06	11.10±0.06	12.63±0.03
ST 300	9.00±0.06	10.27±0.03	10.13±0.03	11.47±0.23	12.10±0.06	11.20±0.10	10.43±0.03
LB 142	12.67±0.07	12.27±0.09	12.23±0.07	18.10±0.10	10.57±0.13	13.07±0.03	12.57±0.09
LB 144	15.20±0.15	14.60±0.06	15.47±0.03	22.00±0.06	16.47±0.03	18.43±0.03	14.10±0.10
LB 145	10.80±0.00	11.17±0.03	9.67±0.09	16.57±0.13	12.23±0.03	12.03±0.03	11.97±0.09
LB 146	11.10±0.06	10.10±0.00	10.27±0.03	15.53±0.03	11.73±0.03	11.57±0.03	13.53±0.22
LB 183	13.03±0.03	10.90±0.06	12.30±0.10	12.00±0.06	10.47±0.03	9.77±0.07	10.67±0.03
LB 260	14.33±0.03	12.37±0.03	14.53±0.03	19.47±0.07	11.33±0.03	12.77±0.03	13.23±0.09
LB 262	11.37±0.07	8.57±0.03	9.90±0.00	11.63±0.03	12.13±0.03	10.10±0.06	12.10±0.06
LB 263	14.53±0.07	11.57±0.03	10.67±0.03	17.83±0.03	12.57±0.03	13.13±0.03	14.20±0.06
LB 264	11.67±0.03	10.30±0.06	13.47±0.03	13.33±0.07	10.40±0.06	8.83±0.03	10.07±0.09
LB 300	11.83±1.02	9.87±0.03	10.77±1.00	12.50±0.10	11.37±0.73	9.90±0.15	10.30±0.06

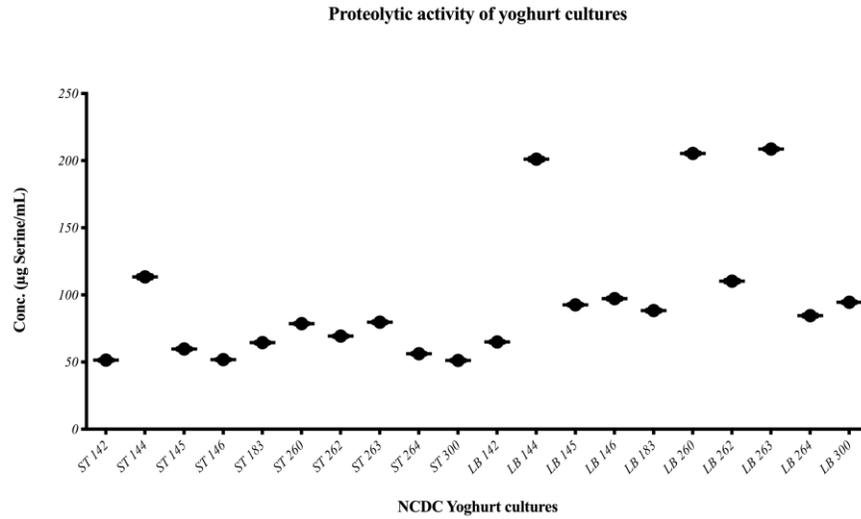
Data are given here in Mean ± SEM (3 replicates)

**Table.4** Bile salt hydrolase activity of NCDC yoghurt cultures

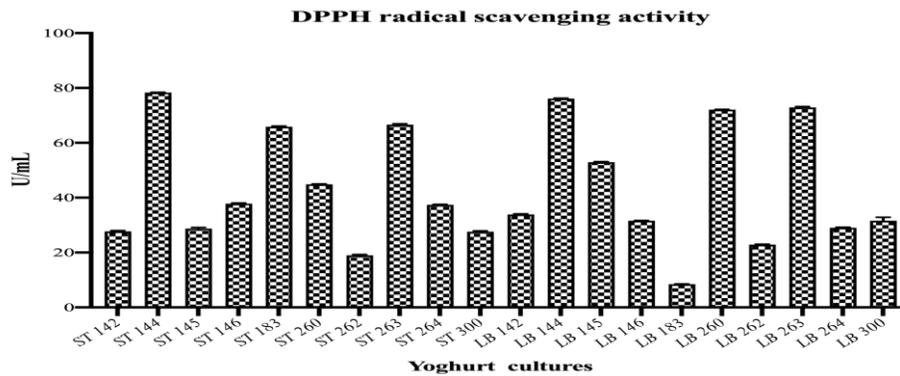
Yoghurt cultures	Sodium Deoxycholate (SD)	Sodium Taurodeoxycholate (STD)	Sodium Taurocholate (STC)	Sodium Tauroglycocholate (STG)
ST 142	-	+++	+	+
ST 144	-	+++	++	+
ST 145	-	+++	+	+
ST 146	-	+++	+	+
ST 183	-	+	+	++
ST 260	-	++	++	++
ST 262	-	++	+	++
ST 263	-	++	+++	++
ST 264	-	+	+	+
ST 300	-	+	++	+
LB 142	-	+	+	+
LB 144	-	+++	++	+
LB 145	-	++	+++	+
LB 146	-	+++	+++	+
LB 183	-	+	++	++
LB 260	-	++	+	+++
LB 262	-	+	+	+++
LB 263	-	+++	++	+++
LB 264	-	+	+	+
LB 300	-	+	+	++

-: no precipitation; +: slight precipitation;  
++: moderate precipitation; +++: intense precipitation

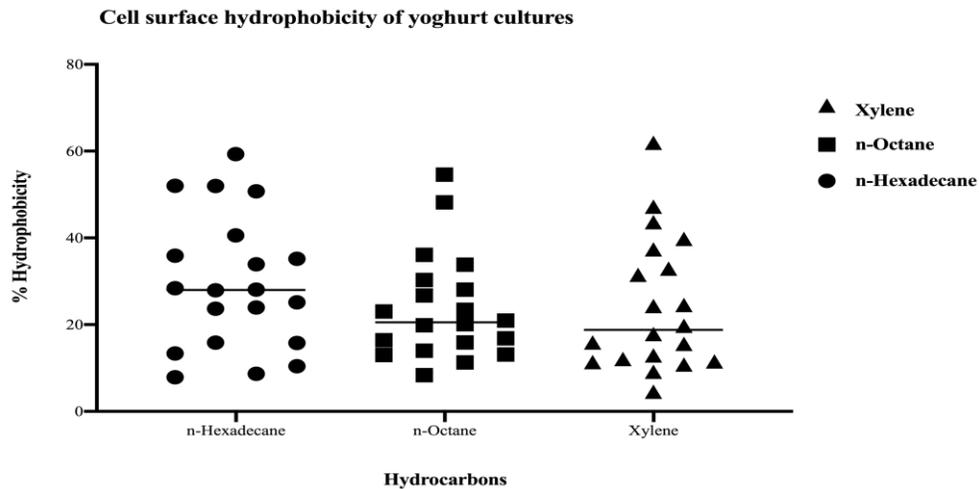
**Fig.1** Proteolytic activity of NCDC yoghurt cultures



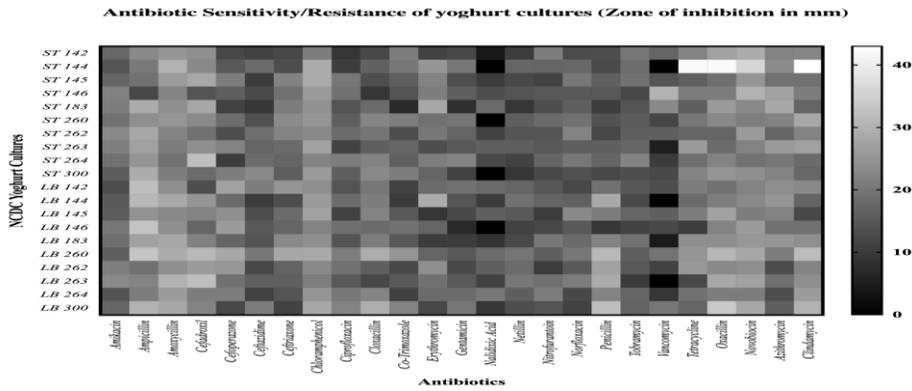
**Fig.2** DPPH radical scavenging activity of NCDC yoghurt cultures



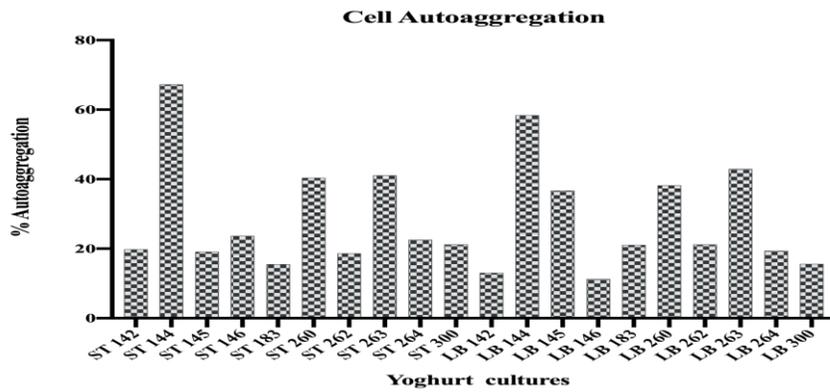
**Fig.3** Cell surface hydrophobicity of NCDC yoghurt cultures



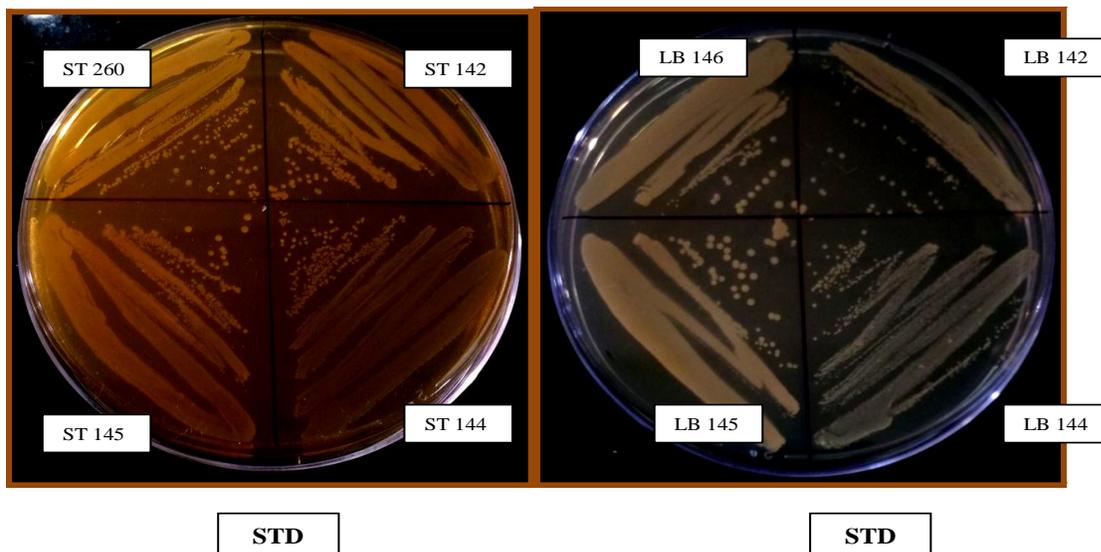
**Fig.4** Antibiotic sensitivity/Resistance pattern of NCDC yoghurt cultures



**Fig.5** Cell auto-aggregation of NCDC yoghurt cultures



**Plate.1** Bile salt hydrolase activity of NCDC yoghurt cultures



### Cell surface hydrophobicity

Ability to attach and adhere to the intestinal epithelial cells is another important criteria for the selection of potential probiotic strain. All the probiotic proven strains must have to adhere to the intestinal epithelial layer. By the attachment to an epithelial cell surface, these probiotic bacteria exhibit the competitive exclusion towards the harmful and pathogenic bacteria. The competitive exclusion may be due to the production of various antimicrobial compounds such as biologically active peptides, bacteriocins, metabolites and some organic acids. In this study, cell surface hydrophobicity of all the NCDC yoghurt cultures were assessed in presence of three different hydrocarbons such as n-hexadecane, n-octane and o-xylene (Fig. 3). Among all the NCDC yoghurt cultures, ST 144 have exhibited the significant ( $P<0.05$ ) results in terms of % hydrophobicity (Fig. 3). Cell surface hydrophobicity for ST 144 have been observed  $52.02 \pm 0.13\%$ ,  $48.21 \pm 0.16$  and  $47.20 \pm 0.74\%$  in presence of n-hexadecane, n-octane and o-xylene respectively. Similarly, *L. bulgaricus* ssp. *delbrueckii* LB 144 have displayed significantly ( $P<0.05$ ) higher % hydrophobicity such as  $61.91 \pm 0.44$ ,  $59.32 \pm 0.28$  and  $54.60 \pm 0.42\%$  in presence o-xylene, n-hexadecane and n-octane, respectively (Fig. 3). This study indicated that yoghurt cultures ST 145 and ST 264 have shown the lowest % hydrophobicity (7–20%) in all the three hydrocarbons. Many compatible hydrocarbons could be used in the study of cell surface hydrophobicity such as chloroform, xylene, ethyl acetate, n-hexadecane and n-octane. Xing *et al.*, (2017) have reported that Tibetan kefir grains isolate *Lactobacillus kefranofaciens* XL10 have exhibited 79.9% hydrophobicity in presence of xylene. The best performed yoghurt culture ST 144 and LB 144 have shown  $47.20 \pm 0.74\%$  and  $61.91 \pm 0.44\%$  hydrophobicity which seems like moderate hydrophobicity.

Similarly, Bhat and Bajaj, (2020) have performed the cell surface hydrophobicity in presence of chloroform, xylene and ethyl acetate with results 29.04%, 74.73% and 54.26% hydrophobicity respectively for *L. casei* M5. The large differences in the cell surface hydrophobicity could be due to variation in the level of expression of cell surface proteins among strains of a species as well as due to environmental conditions which is mainly responsible for the expression of particular surface proteins. However, present study indicates that some of the yoghurt cultures had good hydrophobicity and may play a role in the cell adhesion and interaction as well.

### Antimicrobial activity

The antimicrobial action of any lactic acid bacteria and probiotic bacteria is responsible for the inhibitory action of intestinal pathogenic bacteria. In this study, the antimicrobial activity of all NCDC yoghurt cultures have been studied against the common pathogenic bacteria such as *B. cereus* NCDC 240, *E. coli* NCDC 135, *E. faecalis* NCDC 115, *L. monocytogenes* ATCC 15313, *S. enterica* NCTC 6017, *Sh. dysenteriae* NCDC 107 and *Staph. aureus* NCDC 133 and their inhibitory actions were measured in terms of zone of inhibition (mm). It has been observed that antimicrobial activity was significantly ( $P<0.05$ ) higher in yoghurt cultures ST 144 and LB 144 cultures (Table 3). Yoghurt culture ST 144 have shown the maximum antimicrobial activity against *S. enterica* ( $17.83 \pm 0.03$ ) and minimum ( $11.00 \pm 0.35$ ) against *E. coli* whereas, LB 144 have exhibited maximum antibacterial activity ( $22.00 \pm 0.06$ ) against *L. monocytogenes* and minimum ( $14.10 \pm 0.01$ ) against *S. aureus*. The different diameter of zones of inhibition have been observed for different test pathogens such as *B. cereus* ( $9.00 \pm 0.06$  to  $15.20 \pm 0.15$ ); *E. coli* ( $9.57 \pm$

0.03 to  $14.60 \pm 0.06$ ); *E. faecalis* ( $9.67 \pm 0.09$  to  $15.47 \pm 0.03$ ); *L. monocytogenes* ( $10.07 \pm 0.03$  to  $22.00 \pm 0.06$ ); *S. enterica* ( $10.40 \pm 0.03$  to  $20.03 \pm 0.03$ ); *Sh. dysenteriae* ( $8.83 \pm 0.03$  to  $18.43 \pm 0.03$ ); and *Staph. aureus* ( $9.63 \pm 0.07$  to  $15.37 \pm 0.03$  mm) (Table 3). All these yoghurt cultures have inhibited the growth of all the pathogens at neutral pH of cell-free supernatant which means the antimicrobial activity might be due to the production of some antimicrobial compounds. Hati *et al.*, (2014) have also reported that cheese isolate *L. plantarum* C6 have possessed the antibacterial activity against some common pathogens. *B. cereus* have been reported for the most sensitive to *L. plantarum* C6 ( $23.67 \pm 0.25$ mm) followed by *S. aureus* ( $22.67 \pm 0.86$  mm); *L. monocytogenes* ( $22.43 \pm 0.48$  mm) and *E. coli* ( $19.67 \pm 0.62$  mm) (Hati *et al.*, 2014).

### **Bile salt hydrolase activity**

The bile salt hydrolase (BSH) activity of any probiotic bacteria reside in the gastrointestinal tract is generally associated with its cholesterol-lowering property. Therefore, BSH activity has been performed to select the most potential BSH active and cholesterol-lowering yoghurt cultures. In this study we found that all the NCDC yoghurt cultures have shown slight to intense precipitation in presence of sodium taurodeoxycholate (STD), sodium taurocholate (STC) and sodium tauroglycocholate (STG) but none of the cultures have shown precipitation in the presence of sodium deoxycholate (SD). An intense precipitation has been observed in presence of STD by ST 142, ST 144, ST 145, ST 146, LB 144, LB 146 and LB 263 (Plate 1). On the STC supplemented plates, intense precipitations have been observed by ST 263, LB 145 and LB 146 whereas LB 260, LB 262 and LB 263 have shown intense precipitation in presence of STG (Table 4). The BSH activity of *Lactobacillus plantarum* EM and

*L. sakei* DC1 have also been reported (Choi *et al.*, 2015). Similarly, BSH activity of *L. casei* M5 and M9 have been reported to have higher BSH activity with 57.63 and 52.12 nmol/mL of glycine per min (Bhat and Bajaj, 2020). In another study, native bifidobacterial isolates have also demonstrated the BSH activity by precipitation zones (Achi and Halami, 2019). Functional properties like BSH activity of probiotic bacteria is also an important aspect with their survivability in the gastrointestinal tract for maintaining the health benefits of host (Elli *et al.*, 2006). The presence of bile salts in small intestine is known as the most detrimental factor for the survival of any probiotic bacteria (Bezkorovainy, 2001). Therefore, any probiotic lactobacilli having BSH enzymes activity to hydrolyse bile salts present in small intestine could be a plus point to better survive in the gastrointestinal tract (Du Toit *et al.*, 1998). Likewise, BSH activity of probiotic bacteria could be the best alternate possibilities to reduce the cholesterol level (Begley *et al.*, 2006). The presence of different bile salts could be the reason for the different BSH activities by the *Lactobacillus* spp. (Ridlonet *et al.*, 2006). Differences between BSH activities for sodium salts of cholytaurine and cholyglycine have also been reported (Sedlackova *et al.*, 2015).

### **Antibiotic sensitivity/resistance pattern**

In this study all the NCDC yoghurt cultures were analyzed for their sensitivity/resistance in presence of a wide range of antibiotics. Most of the NCDC yoghurt cultures have seen sensitive towards a breakpoint concentration of many antibiotics such as ampicillin (10 mcg), amoxicillin (10 mcg), cefadroxil (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), clindamycin (2 mcg), erythromycin (15 mcg), gentamicin (10 mcg), nalidixic acid (10 mcg), penicillin (10 units), tetracycline (30 mcg), and vancomycin (30 mcg). Some yoghurt cultures particularly, ST

144, LB 144 and LB 263 have been seen for vancomycin resistance, which can be seen by the intensity of dark colors (Fig. 4). LAB have been reported for their highly susceptible nature in presence of Beta-lactams except for penicillin (Gad *et al.*, 2014). Streptococci and lactococci have also been reported for very susceptible to vancomycin which was just opposite in case of lactobacilli (Gad *et al.*, 2014). Similarly, many *Lactobacillus* species have been reported for the vancomycin resistance as of intrinsic characteristic of probiotic lactobacilli (Ammor *et al.*, 2007). A broad spectrum of antibiotic have been used to check the sensitivity/resistance of 17 strains including *L. plantarum*, *L. pentosus* and *L. mesenteroides* species and reported as safe and even not cytotoxic to the contacted H4-1 human epithelial cells (Bottaet *al.*, 2014). In another study, cheese isolate *L. plantarum* C6 have also been demonstrated for sensitivity to most of the antibiotics (ampicillin, amoxyclov, chloramphenicol, penicillin-G and gentamycin) except vancomycin (Hati *et al.*, 2014).

Human milk and curd isolated LAB have also been evaluated for antibiotic susceptibility/resistance and reported that *L. plantarum* D7 (curd isolate) and *L. casei* HM-1 (human milk isolate) were the highly resistance (Sharma *et al.*, 2017). Recently, Bhat and Bajaj, (2020) have also been reported for the different pattern of antibiotic sensitive/resistance among LAB isolates. As per FAO/WHO, (2002) guidelines, safety of probiotic organism in food is the prime concern by testing the antibiotic resistance. According to the proposed breakpoint concentration of antibiotics suggested by the European Food Safety Authority (EFSA) and FAO/WHO, (2002) guidelines some of the yoghurt cultures exhibited the promising results and could be considered as safe for the further product development.

### Cell auto-aggregation

Aggregation is the process of reversible clumping of bacterial cells belonging to the same bacterial strain (auto-aggregation) or two different bacterial strains (co-aggregation). Auto-aggregation ability of probiotic bacteria can be correlated with the adhesion, which is a pre-requisite need for the colonisation and protection of gastrointestinal epithelial cells. This characteristic of probiotic bacteria helps to form a strong barrier for the attachment and colonisation of harmful pathogenic bacteria. Though auto-aggregation ability varies among strain to strain and high auto-aggregation ability of bacteria contributes to the potential probiotic. In this experiment, the auto-aggregation ability of all the NCDC yoghurt cultures were investigated for their selection as a potential probiotic.

The auto-aggregation ability of NCDC yoghurt cultures have been observed significantly higher ( $P < 0.05$ ) in ST 144 ( $67.28 \pm 0.51\%$ ) and LB 144 ( $58.42 \pm 0.36\%$ ) in comparison to the other yoghurt cultures (Fig. 5). Some yoghurt cultures including ST 260, LB 260, ST 263, and LB 263 have also been noted for the appreciable cell auto-aggregation ability (Fig. 5). Many research reports on cell auto-aggregation have been documented for the screening of LAB including probiotic bacteria. Tuncer and Tuncer, (2014) have reported that the auto-aggregation value of *S. thermophilus* ST8.01 was recorded as  $49.55 \pm 6.24\%$  which can be comparable with this study and even better results have been shown by the ST 144 and LB 144.

Tamara *et al.*, (2012) have also reported that three strains of *L. plantarum* possessed the ability to auto-aggregation and co-aggregation, which are an important feature in the selection of probiotic bacteria. After 24 h

of incubation almost 80% of *Lactobacillus* have been displayed for aggregation. They have explained that cultivation in broth had better auto-aggregation whereas cultivation on agar had better co-aggregation ability (Tamara *et al.*, 2012). Incubation time is also considered as an important factor for the auto-aggregation (Bhat and Bajaj, 2020). LAB isolates M5 and M8 have been reported for the 47.81% and 68.24% of auto-aggregation after 5 h of incubation time (Bhat and Bajaj, 2020). In this study, almost similar auto-aggregation results have been seen by some yoghurt cultures.

In conclusions, *in vitro* probiotic properties study has revealed that ST 144 and LB 144 could be the potential probiotic yoghurt cultures followed by ST 260 and LB 260 and ST 263 and LB 263. Almost all the parameters such as tolerance to acid and bile, cell surface hydrophobicity, antimicrobial activity and BSH activity for probiotic screening were seemed promising in case of yoghurt cultures ST 144 and LB 144. Safety aspect of yoghurt cultures ST 144 and LB 144 have also seen acceptable. Functional characteristics including proteolytic activity, antioxidant activity and cell auto-aggregation have also been observed significantly higher in case of yoghurt cultures ST 144 and LB 144. However, it can be concluded that yoghurt cultures ST 144 and LB 144 could be a promising probiotic yoghurt cultures for the further *in vivo* study and product development as well. The application of these probiotic yoghurt cultures in product development will increase the functional and therapeutic value of that product which will ultimately benefit to the host health.

### Acknowledgements

The lead author acknowledges UGC-Rajiv Gandhi National Fellowship (UGC-RGNF) for the financial support.

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**How to cite this article:**

Jagrani Minj, Brij Pal Singh and Shilpa Vij. 2020. Screening of Yoghurt Cultures for their Potential Proteolytic, Antioxidant and Probiotic Properties. *Int.J.Curr.Microbiol.App.Sci.* 9(08): 1811-1831. doi: <https://doi.org/10.20546/ijcmas.2020.908.208>