Volume 9, Issue 6, 2019, 4591 - 4597 ISSN 2069-5837

Biointerface Research in Applied Chemistry

www.BiointerfaceResearch.com

https://doi.org/10.33263/BRIAC96.591597

Original Research Article Open Access Journal

Received: 25.09.2019 / Revised: 10.11.2019 / Accepted: 15.11.2019 / Published on-line: 20.11.2019

Molecular identification of native lactic acid bacteria isolated from curd samples with probiotic potential

Prasandeep Biswal [1], Abhisek Pal [2], Alok Prasad Das [3,*]

[1]Department of Pharmacology, School of pharmaceutical sciences, Siksha O Anusandhan (Deemed to be University), Khandagiri Square, Bhubaneswar, Odisha, India
[2]GITAM School of Pharmacy, GITAM (Deemed to be University), Patancheru Mandal, Hyderabad, Telangana, India
[3]Department of Life Science, Rama Devi Women's University, Bhubaneswar, Odisha, India
*corresponding author e-mail address: ***alok1503@gmail.com*** | Scopus ID 36472870200

ABSTRACT

Fermented foods are high in nutrient content than any other category of foods due to the presence of live microorganisms called probiotics. Its application in manufacturing of dairy foods and role in different types of disease prevention ranks it as the most exuberant. The aim of the present study was to isolate and identify lactic acid bacteria from native curd samples collected from dairy farms of Odisha state of India and explore its probiotic potential. Three morphologically distinct bacterial strains were isolated using MRS agar plates. The biochemical study confirmed that all the isolates were gram-positive. The molecular approaches were used to analyze the taxonomical diversity of isolates. 16 S rRNA sequencing was carried out and the bacterial isolates were taxonomically classified as *Lactobacillus sp., Lactobacillus plantarum, Lactobacillus casei* with NCBI Gene bank accession number [MG573071], [MG573072] and [MG573073] respectively. Further, the isolated bacterial strains were screened for their acid and bile tolerance competence as a principal criterion for probiotic. Among the isolates *Lactobacillus casei* (MG573073) was found to be highly tolerant of low pH and bile salts, posed strongest cell surface hydrophobicity of 75%. However, the maximum zone of inhibition was observed against Amoxilline/clavunic acid 44mm. The cell growth was found higher in presence of 2% inulin with cell viability 9.11 log $_{10}$ CFU/ml. In conclusion, based on the obtained results, *Lactobacillus casei* can act as a suitable probiotic candidate.

Keywords: *Fermented foods; Molecular identification; Lactic acid bacteria; Probiotics.*

1. INTRODUCTION

Fermented foods are the oldest known and the most dominantly consuming food worldwide. The application of fermentation in the preparation of food and beverage is most exuberant due to the involvement of cost-effective techniques and Lactic acid bacteria. Fermented foods reinforce the strong bioavailability and nutritional value compare to other foods, because of the association of fermentation technique which has greater impact on bio-processing, bio-preservation, pathogen biocontrol and advanced product quality [1,2,3]. At present, the gross economic values of fermented food products are rising to encounter the market demand, with more than 5000 different fermented foods being served to the mankind worldwide [4]. Fermented food products associated with dairy sector have an annual economic growth of $54.2 billion of global turnover alone. By the end of 2022, the worldwide sales of fermented goods are expected to generate greater revenue approximately US$ 40 Billion, according to Persistence Market Research. India is the world's biggest dairy producer and producing nearly 163.6 million tonns a year which is approximately 16% of world's total dairy production and with largest consumer of dairy products according to the survey report of financial express magazine. While the country is expecting bigger production level of more than 163.6 million tonne by 2019, the estimated demand for dairy fermented food products by 2021-22 is 185 million tonne which implies that production has to be doubled in coming future.

Curd is globally the best known of all fermented dairy products and the most popularly consumed nutritive food with rich source of riboflavin, thiamine, niacin, zinc, magnesium, calcium and proteins [3]. It is beneficial for serving people with lactose intolerance, low gastric juice secretion problems and also reduces risk of pathogen transit [5] Lactic acid bacteria (LAB) are the most diverse groups of bacteria on earth crust and a major source of natural curd fermentation. Due to well documentation fermentative and to be useful as a health adjunct, LAB has high demand in various fermented food producing industries [6]. Basically, they are added to foods as the delivery mechanism and feed raw material, where they can contribute to refining the flavour, texture while relaying on nutritional values and pharmacological ethics of the fermented food products. Typically, LAB is long been used as starter cultures for manufacturing of a different dairy foods that may lead to lesser chance of fermentation failure and higher productivity [7,8,9]) *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus helveticus, Leuconostoc mesenteroides* were characteristically reported starter cultures used in industrial fermentation process. While many varieties of microbes can be found in different types of fermented foods, *Lacbacillus* is by far so common such as *Lactobacillus* species obtained from various legume based fermented foods, *Propionibacterium and Bifidobacterium* from cheese and *Hafnia* species in fermented meats were reported [10,11].

Moreover, fermented foods are highly healthy than any other foods, because of the presence of live microorganism called probiotics. According to food and agriculture organization (FAO) of United Nations Probiotics are defined as "live micro-organisms that provide promotional health aid towards the host when administered in appropriate quantities". Lactobacillus species are

one the first probiotics used and are acknowledged as generally recognised as safe (GRAS) status by Food and Drug Administration (FDA) of United States [12]. These are established non-pathogenic commensal microbes hence, the most common species included in various food products worldwide. But still it is huge challenge maintenance of bacterial viability in natural hassle conditions of human digestive system. Hence, describing the essential merits of probiotic bacteria based on their acid-bile adaptive mechanism is most crucial. However, the diverse applications of probiotic in bioremediation, biofilm-associated infections, non-alcoholic fatty liver disease, cystic fibrosis, depression, Parkinson's disease and atopic dermatitis are the current research interest [13,14,15]. Despite the use of *Lactobacillus* species as probiotics, still, there is a need to protect the consumers from any risk involved. Antibiotic resistance is one of the pronounced factors in commensal microbes due to their ability to root the disease on their own.

At present, attention has amplified in the presence of LAB in fermented dairy foodstuffs and as a probiotic bacteria, owing to their biotechnological perspective for providing enhanced and novel bioprocessing techniques of fermented products [16].While a substantial quantity of research has been carried out on commercial application of LAB as probiotics, there is still a lacuna on modern knowledge about the identity of wild LAB implicated in the fermented dairy industries. Possibly the greatest challenge for researchers at the moment is to selecting the right probiotics.

Thus it signifies to use modern techniques to enable molecular characterization and identification of the microbes involved in dairy fermentation. In Odisha state of India, no such studies have addressed and characterized the lactic microbiota from common curd samples; neither there is a report on potential applications to the development of fermented foods and as probiotic. Therefore, the present study was focused on (i) to isolate *Lactobacillus* strain from native curd sample (ii) to molecularly characterize and identify the isolated bacterial species and iii) *In-vitro* assessment of the probiotic potential and iv) Antibiotic resistance patterns

2. MATERIALS AND METHODS

2.1. Sample collection.

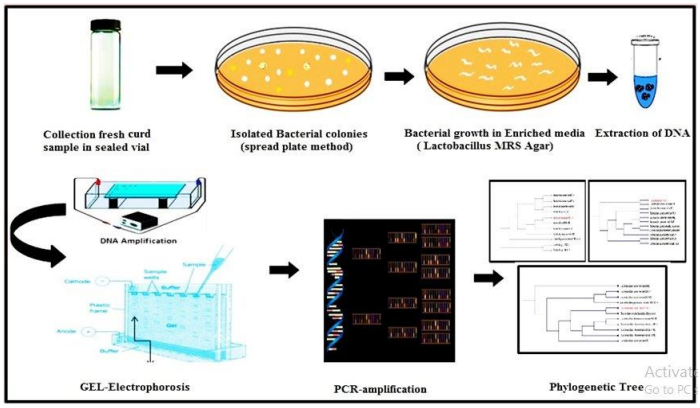
With an aim of isolation and identification native *Lactobacilli* strain with potential probiotic properties, curd samples were collected in a sterile tube from various dairy farms of Khurda district of Odisha state of India and subsequently transferred to the laboratory, in the ice box and stored at 4°C for further investigations. Sterile conditions were maintained during collection and transportation of samples.

2.2. Isolation & medium of growth.

Isolation of bacterial strains was carried out using de Man Rogosa and Sharpe (MRS) agar medium from collected curd samples. Samples were serially diluted 10^{-7} fold with distilled water. An aliquot of 100 µl from dilution was spread onto MRS agar plates using sterile spreader. The inoculated plates were observed for prominent bacterial colonies for 48hr incubation at 35°C. However, to assess the purity isolated individual colonies were sub cultured in the MRS agar medium. The purified isolates were subjected to morphological and biochemical characterizations including gram staining, catalase production, motility and carbohydrate fermentation. The cell morphology was investigated using Phase contrast microscope-BX43 (Olympus, japan). Eventually, the strains were preserved on MRS agar slant in a refrigerated condition [17,18].

2.3. DNA extraction and amplification.

It is the most common preliminary study used to isolate DNA from nucleus of bacteria. The extraction of DNA was carried out following phenol-chloroform method. The amplification of 16S rDNA genes were conducted by using the universal primers 27F (5′-GCCTACGGGNGGCWGCAG-3′) and 1492 R (5′-ACTACHVGGGTATCTAATCC-3′) by following cycle parameters: Pre-heating of DNA at 94^{o}C for 3min, followed by 35 cycles; each cycle consisted of 1min denaturation at 94^{o}C, annealing at 55^{o}C for 45s and primer extension at 72^{o}C for 1min. The final cycle is followed by elongation step at 72^{o}C for 10min. The total volume of the reaction was 50 µl. The PCR products were electrophoresed. The amplified DNA pellet was dissolved in 10 mm TE buffer and 2 µl of the dissolved sample was analyzed using gel electrophoresis at 120 v for 60min to visualize the present DNA fragments(18). However, the purity was assessed by determining A260/280 ratio through spectrophotometric method (UH5000, Hitachi, Japan).



Scheme 1. Diagrammatic representation of molecular identification of the bacterial isolate.

2.4. Molecular identification and evolutionary relationship.

The amplified yields of 16S rRNA gene were sequenced by Sangers' Sequencing method [19]. The sequences were quality checked and trimmed off accordingly. Subsequently, sequences were submitted and compared with the available nucleotide database from the Gen-Bank using the blast program. Alignment was generated with the help of 10 sequences of the adjoining matches by using CLC sequence viewer Scheme 1. In order to illustrate the similarity with the closely allied species, the phylogenetic tree was constructed. A diagrammatic representation of isolation and molecular identification has been shown in Fig. 1.

2.5. Preliminary screening for acid and bile tolerance.

Isolated bacterial strains were screened for their acid and bile tolerance potential. The studies were carried out using in 250ml flasks containing MRS broth and each flasks were supplemented individually with 0.3, 0.5, 1 and 2% (w/v) concentration of bile salts (OX-bile, HiMedia, Mumbai, India) whereas lower acid survivability was examined with pH adjusted

to 1.5, 2.5, 3.5 and 4.5 using 1M HCl in separate flasks. The inoculums of three *Lactobacillus* strains were prepared growing overnight in liquid MRS medium at 35°C. 100 µl of inoculums were grafted to each prepared enriched media and incubated for 24hr at 35°C using shaker incubator RemiRS-24BL (Remi, India). Eventually, centrifuged deposit cells were collected after centrifugation (RemiC-24BL) at 7000× g at 4ºC for 10min and by following appropriate serial dilutions in phosphate buffer saline (PBS), surviving cells were determined in MRS agar plates.

Figure 1. Geographical location of sample collection.

2.6. Transit tolerance assay.

All the strains were tested against simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) with modifications. Overnight grown culture broths were collected by centrifugation at 7000× g at 4ºC for 15min. The obtained cell pallets were washed with PBS and re-suspended. The bacterial suspensions were inoculated to SGF with subsequent composition (45 mm $NaHCO_3$, 7 mm KCL, 125 mm NaCl, 3g/l pepsin, and pH adjusted to 2.5 1M HCL) and were incubated at 35ºC for 4hr. For the intestinal fluid tolerance assay the cell suspensions were inoculated to SIF comprising (0.1g pancreatin and 0.1g bile salts and pH adjusted to 8.0 using 1m NaOH) for 24hr at 35ºC. After incubation, cell viability was determined by plate-count method in MRS agar plates [20].

2.7. Cell surface hydrophobicity.

Cell surface hydrophobicity of the bacteria was assessed to measure the degree of cell adhesion to the hydrocarbons. Here, the selected strain was subjected to toluene, xylene and chloroform to determine the cell affinity towards three organic solvents in two phase system. Overnight grown cell culture was harvested by centrifugation at 7000× g at 4ºC for 15min, washed twice in PBS and suspended separately in the same buffer. The absorbance of the cell suspension was measured at (OD_{600}). 1000 µl of solvent was added to 3 ml of bacterial suspension and allowed to stand at room temperature for 10min [21]. Then the mixture was vortexed for 5min to visualize the bi-phase [22]. The aqueous phase was withdrawn carefully to determine the absorbance at (OD_{600}) using spectrophotometer. The degree of hydrophobicity was calculated by following equation:

$$\text{Hydrophobicity (\%)} = [OD_{600} \text{ after 5min} - OD_{600} \text{ at 0min} / OD_{600} \text{ after 5min}] \times 100$$

2.8. Growth in presence of inulin.

Microbial growth is substantially enriched by utilizing carbon as a sole source of energy. The bacterial growth proficiency of in presence of different concentration inulin was investigated. The MRS broth medium was prepared by incorporating three variable concentrations 0.5% (w/v), 1% (w/v) and 2% (w/v) of inulin whereas medium without inulin used as control. The selected strain *Lactobacillus casei* was grown overnight in MRS medium at 35°C. The cultures were then centrifuged at 7000×g at 4°C for 15min. Then cell biomasses were washed two times in PBS and mixed separately in the same buffer. 100 µl lactobacillus suspensions were poured into two different flasks comprising supplemented media and incubated 40hr at 35°C. After incubation, the number of bacterial colony was determined by serial dilution and plate-count method in in MRS agar plates [23].

2.9. Antibiotic resistance pattern.

The selected potential strain was examined to establish antibiotic resistance patterns by Kirby Bauer's method (disc diffusion method) on (MHA)/Mueller-Hinton agar as mentioned in the guidelines of Clinical and Laboratory Standards Institute [24]. 100 µl of freshly prepared bacterial cell suspensions were spread onto MH agar plates. Subsequently, pre-selected group such as (sulphonamides, aminoglycosides, beta lactams, quinolones, macrolids, carbapenms, penicillines etc.) of antibiotic discs were positioned after drying up for 10min and incubated for 24hr at 35ºC. The following antibiotics were tested: 300 mcg of sulphatriad, 10 mcg of ampicillin, 10 mcg of streptomycin, 30 mcg of ciprofloxacin, 1 unit of penicillin G, 10 mcg of gentamicin, 25 mcg of co-trimoxazole, 30 mcg of nalidixic acid, 30 mcg of tetracycline, 30 mcg of chloramphenicol, 30 mcg of amoxyclav, 30 mcg of amikacin, 30 mcg of kanamycin, 10 mcg of imipenem, 15 mcg of erythromycin, 30 mcg of cefotaxime, 5 mcg of rifampicin, 75 mcg of cefoperazone, 300 mcg of nitrofurantoin, 10 mcg of ampicillin/cloxacillin, 30 mcg of cefepime, 10 mcg of tobramycin, 100 mcg of piperacillin, 30 mcg of netillin, 75 mcg of ticarcillin, 5 mcg of levofloxacin, 10 mcg of ceftazidime, 10 mcg of meropenem.

2.10. Chemicals and instruments used.

The analytical grade *Lactobacillus* MRS media procured from (HiMedia, Mumbai, India) for isolation of bacteria colonies. Pepsin 1000NF (HiMidia, Mumbai, India), Pancreatin 8NF (HiMidia, Mumbai, India) were purchased from HiMedia. Spectrophotometer (UH5300, Hitachi, Japan), and Shaker-incubator (RemiRS-24BL) were used for carrying out cell growth study whereas centrifugation was done in (RemiC-24BL). All the chemicals obtained to accomplish the experimental work were all of analytical grade. Thermal-cycler from Applied Biosystems was used for 16S rRNA gene amplification. All the glass wares used for experimental work were made of Borosil.

3. RESULTS

3.1. Isolation and characterization. Three bacterial strains were isolated from collected curd samples on MRS agar plates following 48hr incubation at 35°C. All strains showed typical appearance of *Lactobacilli* (yellowish white, round and 0.5–

0.8mm in diameter) on MRS agar plates were assayed for biochemical and physiological properties. The best growth of isolated strains yielded after 48hr of incubation at 35°C. The biochemical investigation of the three strains gram positive, catalase negative, non-motile and short rod shape were shown in Table 1. The sugar fermentation resulted fructose positive for all the strains namely *Lactobacillus plantarum* (MG573071), *Lactobacillus sp.* (MG573072), *Lactobacillus casei* (MG573073).

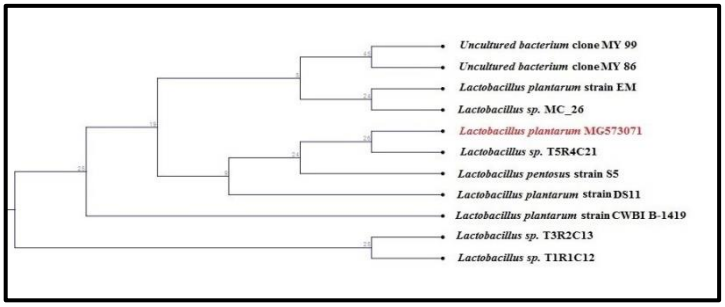


Figure 3a

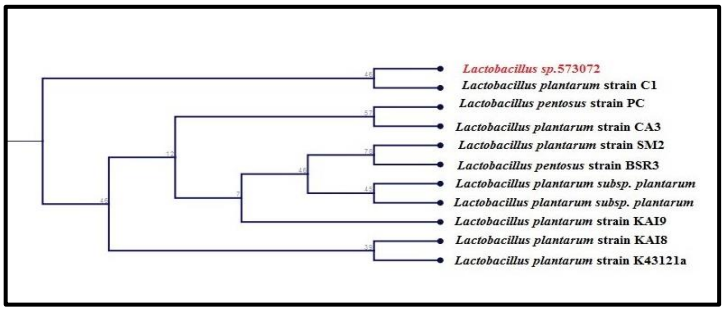


Figure 3b

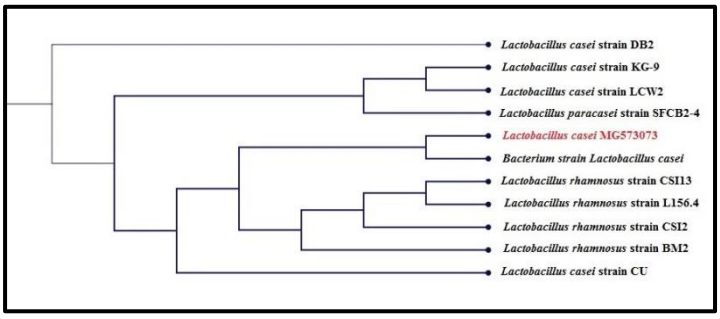


Figure 3c

Figure 3. Phylogenetic tree of 3 identified strains (a) phylogenetic tree of *Lactobacillus plantarum* [MG573071], (b) phylogenetic tree of *Lactobacillus sp.* [MG573072], (c) phylogenetic tree of *Lactobacillus casei* [MG573073] based in the 16S-rRNA sequences was constructed using the tree builder program of CLC- Sequence viewer 7.6.

3.2. Molecular identification and phylogenetic analysis.

Molecular characterizations were carried out for the three isolated strains and were identified as *Lactobacillus plantarum*, *Lactobacillus sp.* and *Lactobacillus casei.* The isolated native curd microorganisms were disclosed a greater percentage of similarity in blast search. The sequence of the 16s rRNA gene of strains isolated in this study was deposited to NCBI Genbank with accession numbers *Lactobacillus plantarum* (MG573071), *Lactobacillus sp.* (MG573072) and *Lactobacillus casei.* (MG573073). All the sequences from isolated strain showed maximum closeness between (99% -100%) to the reference strain available in Gen bank database. The phylogenetic relationship of the sequence of *L. plantarum* MG573071 (932bp), revealed that it belongs to Family-Lactobacillaceae, Order-Lactobacillales, Class-Bacilli, Phylum-Firmicutes and Genus-*Lactobacillus.* Its closest phylogentic relative is *Lactobacillus plantarum* strain Gt6 with sequence closeness of more than 99% and the other relatives are *Lactobacillus plantarum* strain Cys5-4, *Lactobacillus plantarum* strain CA3, *Lactobacillus plantarum* sub sp. Similarly, *Lactobacillus sp.* MG573072 (705bp), was seen to belongs the *Lactobacillus* family. It was observed that the closest match of the *Lactobacillus sp.* are *Lactobacillus plantarum strain TC69* (100%) and *Lactobacillus sp.* complete genome strain D1501 (100%). However, *Lactobacillus pentosus* strain PC (100%) and *Lactobacillus plantarum* strain CSI9 (100%) are the other closely related among them. *L. casei* MG573073 (697bp) belongs to *Lactobacillus* group and its closest relatives being, are *Lactobacillus rhamnosus strain* BM2, *Lactobacillus casei strain* CU and *Lactobacillus casei* strain LCW2.

3.3. Transit simulated gastric and intestinal fluid tolerance assay.

An *in-vitro* methodology was applied to determine the tolerance capacity bacterial strain to the traumatic instance of gastrointestinal tract and access the viable state of isolated strain before reach to colon. Studies were carried out in a premade artificial environment, simulated gastric fluid (SGF), pH 2.0 following 4hour incubation. Only one isolate *L. casei* showed high tolerance following 4hr incubation whereas *L. plantarum* and *Lactobacillus sp.* had shown more than 90% of cell viability loss during simulated gastric transit. The maximum cell count was 6.33 $\log_{10}$ CFU/ ml^{-1} as compare to other two lactobacilli isolates. Similarly, in simulated intestinal juice (SIF) *L. casei* viability after incubation was 7.12 $\log_{10}$ CFU/ ml^{-1}.

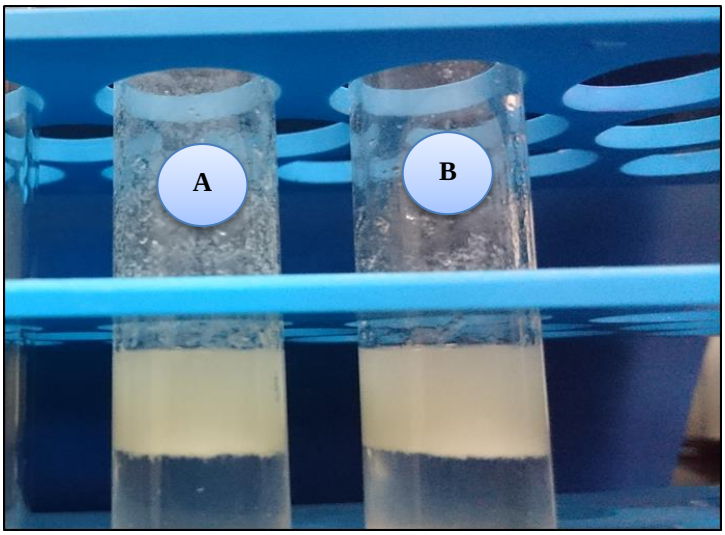


Figure 4. Study of cell surface hydrophobicity. (A) Toluene and (B) Xylene.

3.4. Bacterial characterization for acid and bile tolerance.

Out of all isolated microbial strains from native curd samples *Lactobacillus casei* MG573073 was selected due to its effective tolerance ability to acid and bile. Strains showing more than 50% tolerance were considered as bile-tolerant. The maximum percentage of tolerance was reported for *L. casei* as compare to both *L. plantarum* and *Lactobacillus sp.* Though, the acid and bile tolerance efficacy can be described by the fact that it is one of standard measure for survival of probiotic bacteria, the acid tolerance ability of lactobacilli is considered as the presence of steady gradient between cytoplasmic pH and extracellular pH.

The highest colony forming units (CFU/mL) was 5.31 $\log_{10}$ CFU/ ml^{-1} observed for *L. casei* at pH 2.5 whereas *L plantarum* and *Lactobacillus sp.* was not able to grow with viability 1.16

$\log_{10}$ CFU/ ml^{-1} and 1.1 $\log_{10}$ CFU/ ml^{-1} respectively at same pH value. The lower pH 2.5 is attributed to one of the critical concentration of bacterial survival to acidic conditions. Similarly, 0.3% bile used here is considered as acute concentration for screening of bile resistant strain. However, *L.casei* showed greater resistance with 0.3% bile having cell viability 8.42 $\log_{10}$ CFU/ ml^{-1} and the other two strains were unable to grow at similar concentration with cell count 1.39 $\log_{10}$ CFU/ml^{-1} and 1.87 $\log_{10}$ CFU/ ml^{-1}.

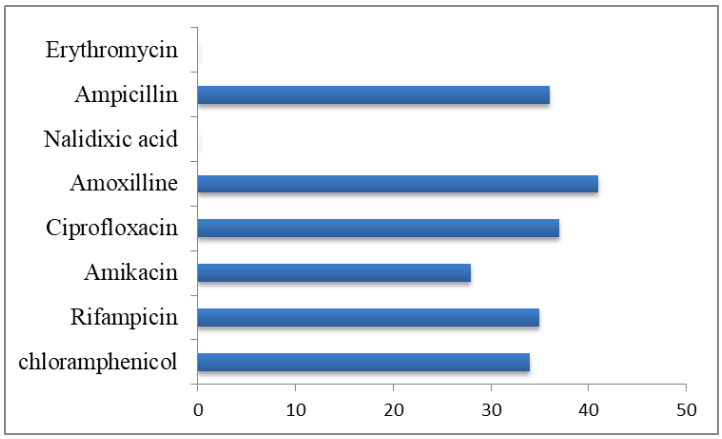


Figure 5 Antibiotic Resistance . **A.** (MRP) Meropenem, (NET) Netillin, (TOB) Tobramycin, (LE)Levo floxacin, (CIP)Ciprofloxacin, (CPZ) Cefoperazone, (AK) Amikacin, (CPM)Cefepirome, (PI) Piperacillin, (TI) Ticarcillin, (GEN) Gentamycin, (CAZ) Ceftazidime; **B.** (S) Streptomycin, (P) Penicilline, (S3) Sulphatriad, (AMP) Ampicillin, (TE) Tetracycline; **C.** (NIT) Nitrofuran, (NA) Naldixic acid , (COT) Co-trimaxozole. **E.** Rifampicin (RIP) and Amicacin(AK) **F.** Nalidixic acid (NA) and Ampicillin /cloxacillin (AX) **G.** Ciprofloxacin (CIP) and Amoxillin (AMC) **H.** Erythomycin(E) and Chloramphenicol (C)

3.5. Cell surface hydrophobicity.

Cell surface hydrophobicity study was performed to evaluate adhesion profile of isolated strains to hydrocarbons. As a result, selected bacterial strain showed remarkable adhesion percentage to three tested hydrocarbons toluene, xylene and chloroform. It is one of the relevant factors that impact bacterial adhesion. The percentage of hydrophobicity indicated that the strain *L. casei* (75%± 1.0) and (70%± 1.0) showed relatively high percentage of hydrophobicity in presence of xylene and toluene (Table 1) whereas less affinity was observed towards chloroform compared to other two hydrocarbons, that is, (64%± 1.23).

Table 1. Percentage of cell surface hydrophobicity of lactobacillus

Bacterial strain	Percentage of hydrophobicity		
L. casei	**Xylene**	**Toluene**	**Chloroform**
	75% ±1.0	70%±1.0	64% ± 1.23

3.6. Effect of inulin on *L.casei.*

Some distinct microbial communities have a significant correlation to carbon as a primary enrichment. Here, inulin was used as a sole source of carbon and is the prime nutrition for probiotic bacteria. The effect of three different concentrations of inulin in growth of *L. casei* was investigated. The maximum growth was observed with 2% inulin. The cell count was of 9.11 $\log_{10}$ CFU/ ml^{-1}compared to medium without carbon source after 24hr incubation while decrease in viability was observed in 0.5% and 1% inulin with cell viability 5.63 $\log_{10}$ CFU/ ml^{-1} and 6.77 $\log_{10}$ CFU/ ml^{-1}, Respectively. Hence we can conclude that inulin as a carbon source is responsible for the improved growth of selected strain *L. casei*.

3.7. Antibiotic susceptibility tests.

The selected strain *L. casei* was inspected for antibiotic resistance pattern against several commonly used antibiotic groups: penicillins, cephems, aminoglycosides, ansamycins, tetracyclines, fluoroquinolones, quinolones, beta lactams, phenicols, nitrofurans, carbapenems, macrolides, and sulphonamides. *L. casei* had shown resistance to majority groups of antibiotics. Only six antibiotics including Amikacin, Ciprofloxacin, amoxicillin/clavulanic acid, Ampicillin/cloxacillin, Rifampicin, and Chloramphenicol were found susceptible to *L. casei*. The maximum zone of inhibition was detected (44 mm) to both amoxicillin/clavulanic acid and Ampicillin/cloxacillin whereas amikacin (27 mm), Ciprofloxacin (35 mm), chloramphenicol (35 mm) and Rifampicin (37 mm), respectively. The bacterial resistant to tested antibiotics were shown in figure 5.

3.8. Discussion.

In the present investigation, we described the microbial population dynamics in collected curd samples from Odisha state of India to identify native Lactic acid bacteria with probiotic properties. We screened the bacterial tolerance to low pH and various bile salt concentrations. Further, the investigations carried out for verifying isolated microbial probiotic potential were transit acid and bile tolerance proficiency, cell adhesion capacity, growth in presence of inulin and antibiotic susceptibility.

Culturing in a selective medium has major impact on isolating both pathogenic and beneficial microbes from food and environmental samples [25]. Here, we isolated three prominently growing Gram-positive bacterial strains using selective Lactobacillus MRS agar medium. The molecular characterization was manifested to identify the isolated strains. The study results presented that the curd had *Lactobacillus* communities and were identified as *Lactobacillus plantarum*, *Lactobacillus sp.* and *Lactobacillus casei.* The obtained sequences were deposited to NCBI Gen Bank with accession number MG573071, MG573072 and MG573073, respectively. The phylogenetic analysis confirmed that the isolated bacterial strains possessed more than 99 percent of similarity to the reference strains of NIH genetic sequence database. *L. casei* has the maximum similarity to *Lactobacillus rhamnosus strain* BM2 whereas *Lactobacillus plantarum* and *Lactobacillus sp.* were shown the highest closeness to *Lactobacillus plantarum* strain Cys5-4 and *Lactobacillus plantarum* strain CSI9 (100%), respectively.

Bile salts change the bacterial membrane properties to induce cell death. Interaction of carbon dioxide with the membrane increases the membrane permeability to bile [26]. The bile and acid tolerance screening was carried out to check the resistance ability of bacterial strains and *L. casei* was found to highly tolerant while other two *Lactobacillus* strains shown deficient growth. Several studies have reported that Gram positive bacteria are more sensitive to bile compared to Gram negative bacteria [27]. The cell viability of isolated bacterial strains to bile salt was 8.42 $\log_{10}$ CFU/ ml^{-1} *L. casei*, 1.39 $\log_{10}$ CFU/ ml^{-1}*L. plantarum* and 1.87 $\log_{10}$ CFU/ ml^{-1}*Lactobacillus sp.*' respectively. The declined cell count is due to the fact that the bacterial cell membranes composed of lipids and fatty acids that are susceptible to bile salts.

Every micro-organism has a standard range of pH tolerance and it differs depending on pH concentration. If the pH drops below the acceptance range, cell death will occur. Generally, Low

pH causes ions disturbances because the cell engulfed the hydrogen ions and expelled the sodium ions and dissociation can alter the osmotic effects. The lowest pH of the stomach is near about 1.5, it increases when mixed with food particles during digestion and raises up to pH4 [28, 29]. Therefore we investigated with four variable pH. However, the maximum tolerance capacity was observed with *L. casei* with cell viability 5.31 $\log_{10}$ CFU/ ml^{-1} at pH 2.5 which is a critical concentration whereas deficient growth 1.16 $\log_{10}$ CFU/ ml^{-1} and 1.1 $\log_{10}$ CFU/ ml^{-1} was shown by the other two *Lactobacillus* strains at same pH.

Resistance to transit gastric fluid (SGF) and intestinal fluid (SIF) is associated with extended tolerance to gut microbiota. The bile secreted from gall bladder contains approximately 0.7% bile acids. The pH of GI tract varies depending on site: pH 1.5 to 3.5 for stomach; pH of small intestine, inclusive of duodenum, jejunum and ileum are 6 to 7.4 and pH 5.5 to 7 for large intestine [30]. Among all the strains *L casei* was found more tolerant to SGF and SIF with cell count 6.33 and 7.12 $\log_{10}$ CFU/ ml^{-1}, respectively. It is clearly evident that *L. casei* could survive the stress condition of GI tract which may allow it reach large intestine in viable state.

Bacterial adhesion to hydrocarbons is rapid method to measure the surface hydrophobicity. This emphasizes the separation of hydrocarbon phase and water phase, which indicates that the bacterial adherence to surface can affect the retention time in the GI-tract. Hence, contributes to strengthening the interaction between intestinal bacteria and host. Here xylene showed the strongest percentage (75%) of affinity with *L. casei* as compare to toluene (70%) and chloroform (64%). It suggests that the adaptation eminence of commensal bacteria can confer protection to mucosal tissue injury and effectively reverse the many harmful consequences.

On the other hand, most antibiotics are not pathogen specific, they also destroyed commensal bacteria of gut microbiome. Hence, the commensal microorganisms should possess resistance to most commonly used antibiotics up to a limited dosage to favor their own survival and human physiological processes. The varying antibiotic resistance patterns of tested bacteria *L. casei*, isolated from curd were shown in Figure-5. The highest frequency of resistance was observed against Erythromycin, penicillin G, gentamicin, Nalidixic acid, co-trimoxazole, anamycin, imipenem, piperacillin, cefoperazone, sulphatriad, nitrofurantoin, levofloxacin, cefepime, ticarcillin, tobramycin, meropenem, netillin and ceftazidime whereas Amikacin, Ciprofloxacin, amoxicillin/clavulanic acid, Ampicillin/cloxacillin, Rifampicin and Chloramphenicol were highly susceptible to *L. casei*. Inulin is the naturally occurring carbohydrates [31]. Its use as carbon source is because the metabolism in large bowel favors the fermentation of complex sugars. The upper GI-tract isn't digests inulin and faster the transit allows it to pass through small intestine and ferment the large intestine [32, 33]. In our experiment, the concentration of carbon source had progressive effect on biomass growth of bacteria. We tested three variable concentrations of inulin. The result showed that in presence of 2% inulin stimulated the cell biomass with viability 9.11 $\log_{10}$ CFU/ ml^{-1}which indicates the increase in biomass of native bacterial isolate, is directly proportional to inulin concentration used. Hence suggesting that inulin had an innate capacity to modulate the *L. casei* richness by rapid fermentation which can help to colonize the gut microbiome.

4. CONCLUSIONS

The growing consumer interest in the fermented foods has been increasing every passing day. The use of probiotics has shown to have greater future potential due to its beneficial influence on improving a number of disease conditions. The molecular approach through 16s rRNA sequencing has been employed in this study to identify the potential strain and possibly will helpful in-depth analysis of microbes that eventually play a major role in future microbial medicines. The present investigation aimed to isolate lactic acid bacteria with probiotic potential from native fermented food curd confirmed that *L. casei* has the ability tolerates gastric fluid and bile. However the growth modulation in presence of inulin indicates the direct influence of *L. casei* that can colonizing digestive system. It also showed strong adherence capacity to hydrocarbons and posed the resistance spectra against commonly used antibiotics.

Particularly, the use of molecular technique in field of probiotics not only stretch new opportunities to solve the complex relationship between host and bacteria but also would avail better characterization and understanding of microbe that lives within us and its industrial application. Our investigational results collectively suggest that the isolated native strain *L. casei* from curd can be a potential probiotic candidate.

5. REFERENCES

1. Castellano, P.; Perez, M.I.; Blanco, M.M.; Fontana, C.; Vignolo, G.M. Strategies for Pathogen Biocontrol Using Lactic Acid Bacteria and Their Metabolites: A Focus on Meat Ecosystems and Industrial Environments. *Microorganisms* **2017**, *5*, 38, https://doi.org/10.3390/microorganisms5030038.
2. Garcia, P.; Rodriguez-Rubio, L.; Rodriguez, A.; Martinez, B. Food biopreservation: Promising strategies using bacteriocins, bacteriophages and endolysins. *Trends in Food Science & Technology* **2010**, *21*, 373-382, https://doi.org/10.1016/j.tifs.2010.04.010.
3. Sanlier, N.; Gokcen, B.B.; Sezgin A.C. Health Benefits of Fermented Foods. *Critical Reviews in Food Science and Nutrition* **2017**, 1-22, https://doi.org/10.1080/10408398.2017.1383355.
4. Adolfsson, O.; Meydani, S.N.; Russell, R.M. Yogurt and gut function. *The American journal of clinical nutrition* **2004**, *80*, 245-256, https://doi.org/10.1093/ajcn/80.2.245.
5. Ray, M.; Ghosh, K.; Singh, S.; Mondal, K.C. Folk to functional: An explorative overview of rice-based fermented foods and beverages in India. *Journal of Ethnic Foods* **2016,** *3*, 5-18, https://doi.org/10.1016/j.jef.2016.02.002.
6. Othman, M.; Ariff, A.B.; Rios-Solis, L.; Halim, M. Extractive Fermentation of Lactic Acid in Lactic Acid Bacteria Cultivation: A Review. *Frontiers in Microbiology* **2017,** *8*, 2285, https://doi.org/10.3389/fmicb.2017.02285.
7. Chen, C.; Zhao, S.; Hao, G.; Yu, H.; Tian, H.; Zhao, G. Role of lactic acid bacteria on the yogurt flavour: a review. *International Journal of Food Properties* **2017,** *20*, 316-330, https://doi.org/10.1080/10942912.2017.1295988.
8. Mugula, J.; Narvhus, J.; Sorhaug, T. Use of starter cultures of lactic acid bacteria and yeasts in the preparation of Togwa, a Tanzanian fermented food. *International journal of food microbiology* **2003**, *83*, 307-318, https://doi.org/10.1016/S0168-1605(02)00386-0.

9. Kavitake, D.; Kandasamy, S.; Devi, P.B.; Shetty, P.H. Recent developments on encapsulation of lactic acid bacteria as potential starter culture in fermented foods–a review. *Food Bioscience* **2017**, *21*, 34-44, https://doi.org/10.1016/j.fbio.2017.11.003.

10. Kubo, Y.; Rooney A.P.; Tsukakoshi, Y.; Nakagawa, R.; Hasegawa, H.; Kimura, K. Phylogenetic analysis of bacillus subtilis strains applicable to natto (fermented soybean) production. *Appllied Environmental Microbiology* **2011**, *77*, 6463-6469, https://doi.org/10.1128/AEM.00448-11l

11. Bourdichon, F.; Casaregola, S.; Farrokh, C.; Frisvad, J.C.; Gerds, M.L.; Hammes, W.P.; Harnett, J.; Huys, G.; Laulund, S.; Ouwehand, A.; Powell, I.B.; Prajapato, J.B.; Seto, Y.; Schure, W.T.; Boven, A.V.; Bankerckhoven, V.; Zgoda, A.; Tuijtelaars, S.; Hansen, E.B. Food fermentation: microorganisms with technological beneficial use. *International Journal of Food Microbiology* **2012**, *154*, 87-97, https://doi.org/10.1016/j.ijfoodmicro.2011.12.030.

12. Cano-Garrido, O.; Seras-Franzoso, J.; Garcia-Fruitos, E. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. *Microbial cell factories* **2015**, *14*, 137, https://dx.doi.org/10.1186%2Fs12934-015-0313-6.

13. Biswal, P.; Pal, A.; Das, A.P. A New Light of Therapy for Non-Alcoholic Fatty Liver Disease: Symbiotic. *International Journal of Pharmaceutical Sciences Review and Research* **2015**, *33*, 326-336.

14. Das, A.P.; Ghosh, S.; Mohanty, S.; Sukla, L.B. Consequences of manganese compounds: a review. *Toxicological & Environmental Chemistry* **2014**, *96*, 981-997, https://doi.org/10.1080/02772248.2015.1005428.

15. Huang, R.; Ning, H.; Shen, M.; Li, J.; Zhang, J.; Chen, X. Probiotics for the Treatment of Atopic Dermatitis in Children: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Frontiers in cellular and infection microbiology* **2017,** *7*, 392, https://doi.org/10.3389/fcimb.2017.00392.

16. Holzapfel, W.H. Appropriate starter culture technologies for small-scale fermentation in developing countries. *International journal of food microbiology*, **2002**, *75*, 197-212, https://doi.org/10.1016/S0168-1605(01)00707-3.

17. Das, A.P.; Mishra, S. Biodegradation of the metallic carcinogen hexavalent chromium Cr (VI) by an indigenously isolated bacterial strain. *Journal of carcinogenesis* **2010**, *9*, https://doi.org/10.4103/1477-3163.63584.

18. Ghosh, S.; Mohanty, S.; Nayak, S.; Sukla, L.B.; Das, A.P. Molecular identification of indigenous manganese solubilising bacterial biodiversity from manganese mining deposits. *Journal of basic microbiology* **2015**, *56*, 254-262, https://doi.org/10.1002/jobm.201500477.

19. Sanket, A.S.; Ghosh, S.; Sahoo, R.; Nayak, S.; Das, A.P. Molecular Identification of Acidophilic Manganese (Mn)-Solubilizing Bacteria from Mining Effluents and Their Application in Mineral Beneficiation. *Geomicrobiology Journal* **2016**, *34*, 71-80, https://doi.org/10.1080/01490451.2016.1141340.

20. Bao, Y.; Zhang, Y.; Zhang, Y.; Liu, Y.; Wang, S.; Dong, X.; Wang, Y.; Zhang, H. Screening of potential probiotic properties of Lactobacillus fermentum isolated from traditional dairy products. *Food Control* **2010**, *21*, 695-701, https://doi.org/10.1016/j.foodcont.2009.10.010.

21. Xu, H.; Jeong, H.S.; Lee, H.Y.; Ahn, J. Assessment of cell surface properties and adhesion potential of selected probiotic strains. *Letters in applied microbiology* **2009**, *49*, 434-442, https://doi.org/10.1111/j.1472-765X.2009.02684.x.

22. Rosenberg, M. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. *FEMS microbiology letters* **2006**, *262*, 129-134, https://doi.org/10.1111/j.1574-6968.2006.00291.x.

23. Adebola, O.O.; Corcoran, O.; Morgan, W.A. Synbiotics: the impact of potential prebiotics inulin, lactulose and lactobionic acid on the survival and growth of lactobacilli probiotics. *Journal of functional foods* **2014**, *10*, 75-84, https://doi.org/10.1016/j.jff.2014.05.010.

24. Patel, J.B.; Cockerill, F.R.; Alder, J.; Bradford, P.A.; Eliopoulos, G.M.; Hardy, D.J.; Hindler, J.A.; Jenkins, S.G.; Lewis, J.S.; Miller, L.A.; Powell, M.; Swenson, J.M.; Traczewski, M.M.; Turnidge, J.D.; Weinstein, M.P.; Zimmer, B.L. Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. *Clinical Laboratory Standards. Institute* **2014**, *34*.

25. Kawanishi, T.; Shiraishi, T.; Okano, Y.; Sugawara, K.; Hashimoto, M.; Maejima, K.; Komatsu, K.; Kakizawa, S.; Yamaji, Y.; Hamamoto, H.; Oshima, K. New detection systems of bacteria using highly selective media designed by SMART: selective medium-design algorithm restricted by two constraints. *PloS one* **2011**, *6*, e16512, https://doi.org/10.1371/journal.pone.0016512.

26. Merritt, M.E.; Donaldson, J.R. Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J Med Microbiol* **2009**, *58*, 1533–1541, https://doi.org/10.1099/jmm.0.014092-0.

27. Begley, M.; Gahan, C.G.; Hill, C. The interaction between bacteria and bile. *FEMS microbiology reviews* **2005**, *29*, 625-651, https://doi.org/10.1016/j.femsre.2004.09.003.

28. Zhu, H.; Hart, C.A.; Sales, D.; Roberts, N.B. Bacterial killing in gastric juice–effect of pH and pepsin on Escherichia coli and Helicobacter pylori. *Journal of medical microbiology* **2006**, *55*, 1265-1270, https://doi.org/10.1099/jmm.0.46611-0.

29. Kambayashi, A., & Dressman, J. B. Predicting the Changes in Oral Absorption of Weak Base Drugs Under Elevated Gastric pH Using an In Vitro–In Silico–In Vivo Approach: Case Examples—Dipyridamole, Prasugrel, and Nelfinavir. *Journal of pharmaceutical sciences*, **2019**, *108*, 584-591. https://doi.org/10.1016/j.xphs.2018.11.008.

30. Eriksen, E.K.; Holm, H.; Jensen, E.; Aaboe, R.; Devold, T.G.; Jacobsen, M.; Vegarud, G.E. Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes. *British journal of nutrition* **2010**, *104*, 374-381, https://doi.org/10.1017/S0007114510000577.

31. Biswal, P.; Pal, A.; Das, A.P. Current Trends and Future Prospective of Prebiotics as Therapeutic Food. In: *Handbook of Food Bioengineering.* Holban, A.M.; Grumezescu, A.M. (Eds.), 2017; pp. 57-88, https://doi.org/10.1016/B978-0-12-811520-6.00003-9.

32. Seedorf, H.; Griffin, N.W.; Ridaura, V.K.; Reyes, A.; Cheng, J.; Rey, F.E.; Smith, M.I.; Simon, G.M.; Scheffrahn, R.H.; Woebken, D.; Spormann, A.M. Bacteria from diverse habitats colonize and compete in the mouse gut. *Cell* **2014**, *159*, 253-266, https://doi.org/10.1016/j.cell.2014.09.008.

33. Ashwini, A., H. N. Ramya, C. Ramkumar, Kakarala Raghava Reddy, Raghavendra V. Kulkarni, V. Abinaya, S. Naveen, and Anjanapura V. Raghu. Reactive mechanism and the applications of bioactive prebiotics for human health. *Journal of microbiological methods* **2019**. https://doi.org/10.1016/j.mimet.2019.02.019.