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IN VITRO SCREENING OF THE PROBIOTIC POTENTIAL OF *LACTOBACILLUS* AND *ENTEROCOCCUS* STRAINS ISOLATED FROM HUMAN BREAST MILK, FECES OF BREAST-FED INFANTS AND ANIMAL MILK (GOAT, COW AND BUFFALO)

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ABSTRACT

This study sought to investigate the probiotic potent of lactic acid bacteria isolated from animal raw milk, human breast milk and infant fecal matter. A total of 200 LAB strains have been isolated, among this four Lactobacillus spp and four Enterococcus spp. have been screened for their functional properties, among these Lactobacillus para casei NB16 isolated from human breast milk was capable to survive at 1% bile salt, pH 2.0 and SGJ for 4 h without losing viability and ability to grow in a range of temperatures at15- 50°C, pH 3-9 and salt concentration up to 8 %. All LAB strains exhibited inhibitory activity towards wide range of food borne pathogens, in addition, NB12, and NB16 have been found to be resistant to 16 antibiotics out of 17 except Chloramphenicol and fermented 17 sugars out 20. Adhesion percentage of 8 isolates to Hydrocarbons up to (96%), auto-aggregation up to (90%) and co-aggregation with Escherichia coli MTCC 40 up to (62%) was observed and 16S rDNA sequence confirmed NB12, NB 14, NB 113 as Lactobacillus para casei, NB16 as Lactobacillus casei, NB10, NB44, NB94 as Enterococcus faecium and NB7 as Enterococcus faecalis respectively. Probiotic functional properties of isolates have been characterized and isolates were identified by using molecular methods.

KEY WORDS

Enterococcus faecalis, Feces of breastfed infants, Human breast milk, Lactobacillus casei.

I. INTRODUCTION

Human beings and animals use probiotics as a part of the healthy diet to have safe, natural and effective health-promoting benefits [1, 2, 3]. According to the definition by the World Health Organization (WHO), "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [4, 5, 6]. The genera of *Lactobacillus, Lactococcus, Bifidobacteria, Streptococcus, Enterococcus, Saccharomyces* and numerous strains of yeast have been considered as probiotics [1, 7, 8]. However, lactic acid bacteria are considered as the main group of probiotics. Several species of these genera are "Generally Recognized as Safe (GRAS)" by the FDA (US food and drug administration) and they are technologically appropriate for industrial approaches [1, 9].

To date, several lactic acid bacterial species have been isolated from the dairy products. The investigations have revealed that the infectious disorders decreased

among breastfed infants and rural population who consumed unpasteurized milk in contrast to pasteurized milk products and also indicated that certain unique elements are existing in raw milk to provide protection towards infectious diseases by producing organic acids and hydrogen peroxide [10, 11, 12, 13]. Due to these observations, raw milk and infant feces are recognized as one of the attractive sources and natural habitats of lactic acid bacteria, which play an essential role in the prevention of infectious disorder in the host [14]. Numerous studies have assessed the probiotic potential of the isolated lactic acid bacteria such as their tolerance to bile salt, acidic pH, aggregation, immunity modulation characteristics, survival ability when co-administered with antibiotics, inhibitory activity towards pathogens, adherence potential to intestinal epithelial cells to form barriers in preventing colonization by pathogens [15, 16, 17, 18, 19, 20, 21]. Probiotics were recommended for several health benefits in human beings and animals such as promoting proper digestion, enhancing immunity and amplifying resistance to infection [22]. Removal of carcinogens, reducing cholesterol level, synthesis and improving the bioavailability of nutrients, relief from lactose intolerance [22, 23] control of diarrhea [24] and inflammatory bowel illnesses [25] and anti-mutagenic effect [26, 27]. The objective of the current study was isolation, identification and characterization of potent probiotic lactic acid bacteria from human breast milk and milk from goat, cow, buffalo and infant fecal matter.

II. MATERIALS AND METHODS

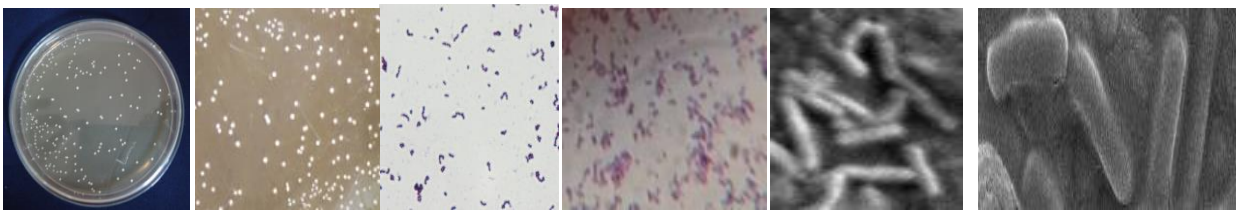
Isolation of Lactic acid bacteria

Samples were collected aseptically from the habitats about lactic acid bacteria (Table 1), stored at 4±1°C and enumerated within an hour of sampling through serial dilution method with sterile saline (NaCl-0.85%) pH 7.0. Aliquot (100 µl) of the samples were placed on MRS agar (pH 6.8 and pH7.0) by spread plate method then plates were incubated at 37°C for 24–48 h in anaerobic condition [28]. Based on the colony color (white and creamy) and morphology, colonies were randomly selected and transferred to MRS agar plates by using streaking methods, pure colonies were preserved on MRS broth with 40 % sterile glycerol at –20°C. A total of 200 pure cultures were isolated and evaluated for probiotics morphological and biochemical characterization as described by Bergey's manual of systematic bacteriology [29, 30, 31]. Eight strains were carefully selected as probiotics from the screened 200 isolates based on morphological and biochemical characteristics (morphological characteristics - Gram-positive, catalase-negative, non-motile, non-spore-forming, irregular short rods and cocci) and biochemical characteristics (growth at 15- 50°C, tolerance of pH 2-9, tolerance of NaCl concentrations upto 8%, sugars fermentation (17 sugars fermented out of 20 tested sugars). These 8 isolates were further tested for probiotic functional properties and genotypic characterization (Figure 1).

Table 1. Sources of lactic acid bacteria.

Isolation Sources	No. of samples	No. of isolates	Location
Colostrum	2	15	Shridevi Institute of Medical Sciences& Research Hospital, Tumkur, Karnataka, India
Fore milk	4	22	Shridevi Institute of Medical Sciences& Research Hospital, Tumkur, Karnataka, India
Goat milk	2	16	Private farms, Mysore, Karnataka, India
Cow milk	2	16	Mysore, Karnataka, India
Kefir grains	1	26	Mysore, Karnataka, India
Buffalo milk	2	12	Mysore, Karnataka, India
Breast feed Infant fecal matter	8	87	Shridevi Institute of Medical Sciences& Research Hospital, Tumkur, Karnataka, India
Total	21	200	

Figure 1.: Lactic acid bacteria



a: Lactic acid bacteria from Breast milk,b: Cocci, c: Bacilli Gram-positive LAB observed under phase contrast microscope d & e: Field Emission Scanning Electron Microscopy (FESEM) image (2000X and 7000X) of *Lactobacillus.*

Probiotic Properties:

Acid, Bile and Synthetic Gastric Juice tolerance test

Selected isolates were tested for tolerance to acidic pH, bile and synthetic gastric juice as described by previous studies [10, 26, 32, 33]. Active culture (18 h) with 0.28 optical density value at 600 nm was inoculated (10% v/v) on to MRS broth adjusted to acidic pH- 2.0 with 0.1 N HCl, MRS medium was enriched with 1% ox bile, in Synthetic Gastric Juice {(8.3 g of protease peptone , 3.5g of glucose, 2.05g of NaCl, 0.6 of KH_2PO_4,0.37g of KCl , 0.11 g of $CaCl_2$, 0.05 g of bile ,13.3 mg of pepsin and 0.1g of lysozyme ; as per liter adjusted to pH- 2.5). The medium was filter sterilized using 0.22 µm membrane filters and incubated at 37°C for 4 hours. Survivability of the cells from 0 h to 4 h was determined by serial dilutions of samples in (0.85% NaCl) physiological saline then placed on MRS agar, incubated for up to 48 h at 37°C. The rate of survivability was calculated by using the formula [34].

% Survival= log number of survived cells (CFU/ml)/log number of inoculated cells (CFU/ml) × 100 --------- (1)

The cell survival was determined, and the results are tabulated.

Bacterial Adhesion to Hydrocarbons Assay

The bacterial adhesion to hydrocarbons (BATH) test was carried out to assess the adherence ability of LAB isolates using hydrocarbon- xylene, toluene, chloroform, and ethyl acetate as described by previous reports. [10,35,36,37,38]. 1ml of 18 h cultures were harvested by centrifugation at 6500 rpm for 5min at 4°C, washed twice with phosphate buffered saline (PBS; 140mm NaCl, 3Mm KCl, 8Mm Na_2HPO_4, 2Mm KH_2PO_4, pH7.2) then re-suspend in the same PBS buffer. The cell suspension was adjusted to obtain an absorbance of 1.0 at 600 nm, 200µl of hydrocarbon was added to 200µl of bacterial suspension then mixed thoroughly by using vortex for 2 min then allowed to stand for 1 h at 37° C for phase separation. The bottom aqueous phase was removed carefully then its absorbance was measured at 600 nm. The decrease in optical density (OD) correlates with the measurement of the cell surface hydrophobicity (H %) calculated by the formula,

Cell surface hydrophobicity H% = $[(A_0 - A)/A_0] \times 100$ ---------- (2)

Where, A_0 and A are the absorbances before and after extraction together with hydrocarbons.

Autoaggregation

Auto-aggregation assay was carried out according to the previous studies [39, 26, 32, 40]. Isolates were grown at 37ºC for 24 to 48 hrs on MRS broth. The cells were harvested through centrifugation for 10 min at 7000 rpm, washed twice and re suspended in the PBS, pH 7. A cell suspension (4 ml) was vortexed for 10 sec then auto-aggregation was determined after 3 hrs and 5 hrs of incubation at 37ºC. An aliquot (0.1 ml) of the upper layer (suspension) of PBS after incubation was transferred to another tube with 3.9 ml of PBS absorbance (A) was determined at 600 nm. The percentage of auto-aggregation was calculated by the usage of the equation [41]:

$1-(At/A0) \times 100$---------- (3)

Where, A_t and A_0 signify the absorbance of at time 5 h and 0 h, respectively.

Co-aggregation assay

Co-aggregation assays have been executed in accordance with the previous reports [39, 42, 26, 40]. The cells were harvested by centrifugation for 15 min at 5000 rpm, washed twice and re suspended in phosphate buffered saline to assign viable counts of approximately 10^8 CFU/ml. Equal volumes (2ml) of each cell suspension was combined together in pairs through vortexing for 10 s. The absorbance of the cell suspensions at 600 nm was measured after 5 h of incubation at 37°C. The percentage about co aggregation was calculated using the equation [41]:

Coaggregation (%) = [(Ax+Ay)/2)-A(x+y)/ (Ax+Ay) /2] X 100

Where, x and y signify each of the pair strains within the control tubes, then (x + y) the mixture.

Antibacterial assay

The inhibitory effect of selected LAB isolates was determined using the well-diffusion approach [43, 26, 19, 44]. To determines the inhibitory capability of the selected strains towards pathogens, an overnight culture of the pathogenic strain (*Escherichia coli MTCC 40& ATCC 10536, Bacillus aureus MTCC1306, Salmonella Para typhi ATCC9150, Salmonella Typhi murium MTCC91, Salmonella Arizonae ATCC 13314* and *Shigella Boydii ATCC 9207*) was inoculated to BHI (Brain Heart Infusion) media and incubated at 37°C (approximately 100µl for 1ml BHI broth). Wells about 5mm diameter were cut into MRS agar plates and 50 µL of LAB culture supernatant neutralized with 0.1 N NaOH was added to all MRS agar wells. The indicator pathogens (1ml) in nutrient broth were mixed in 7ml of 0.7% soft agar over layered immediately on MRS agar plates and the culture filtrate was inoculated. The inhibitory zone of lactic acid bacteria was observed after 24-48h of incubation about 37°C.

Antibiotics susceptibility test

Antibiotics resistance of each selected isolates were assessed through the paper disc method (HiMedia Pvt. Ltd., Mumbai, India). The antibiotic discs were placed on MRS agar plates previously seeded with18h culture, incubated at 37°C for 24-48 hr, then the diameter of the zone of the inhibition was observed, measured and expressed in mm. The susceptibility of strains used was expressed as described by previous reports [45, 46, 47, 26, 48, 49].

Genotypic characterization

Qualitative and Quantitative determination of DNA

Isolation of chromosomal DNA using the Conventional method

In the genetic characterization, isolation, extraction then purification of genomic DNA of isolates was carried out as per the previously used methods [26, 50]. Where 5-ml of bacterial cultures were centrifuged at 1,5000rpm for 5 min to collect the cells, the cells were re- suspended in one ml of Tris-EDTA buffer (pH 8.0) and further subjected to phenol-chloroform extraction and ethanol precipitation and the thus precipitated DNA was analyzed on 1% agarose gel. DNA concentration was determined by recording the absorbance at 260 nm (A260) in a Nanodrop spectrophotometer (Bio-Tek Instruments, Inc.).

Polymerase chain reaction (PCR)

DNA integrity was evaluated using PCR technique [2, 22, 51, 52]. The nucleotide sequence of DNA of the isolates was carried out with 16SrDNA primer being used for the PCR amplification.

PCR primers* [53].

P3 (forward primer) 5' -AGAGTTTGATCATGGCTCAG- 3'

P13 (reverse primer) 5'-GGTTACCTTGTTACGACTT- 3', this primer amplifies approximately 1500 bp length of 16S rDNA gene of any bacterium.

Gel electrophoresis

A DNA ladder (1-kb) was used as a molecular size marker. The PCR was conducted through 30 cycles in Eppendorf master cycler gradient thermal cycler at 95°C for 40-sec denaturation, 52°C for 1 min annealing and 72°C with 1min extension. The DNA was denatured for 3 min in the beginning and later extended for 15 min at 72°C. PCR products were analyzed on 1% agarose gel (Figure 2). The amplified PCR products were purified and then amplicons were sequenced using Sanger's method. A BLAST (Basic Local Alignment Search Tool) search was performed using the obtained DNA sequence of 16S rDNA as the query sequence with the NCBI database in accordance with the query sequence similarity together with the NCBI database and the best match of resemblance was selected to identify the isolates.

Statistical analysis

All assays were performed in triplicates and the data are expressed as Mean ± SEM (n=3) and standard deviations stability by the use of Microsoft Excel (Version 7.0).

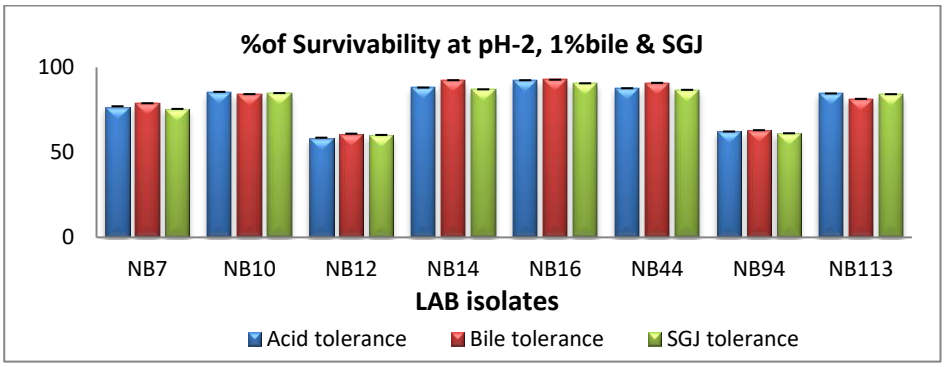
III. RESULTS AND DISCUSSIONS

Probiotic functional Properties

Acid, Bile and Synthetic Gastric Juice tolerance test

In order to consider lactic acid bacteria as probiotic it has to withstand the acidic condition in stomach in order to reach the gut and create suitable conditions for residence, for this it has to survive in the excessive acidic condition similar to that of human stomach pH 2.0 at least for 120 min which is the food transit period through the human stomach [54, 55]. In the present study, six out of eight isolates (NB 7, NB10, NB14, NB16, NB 44 and NB 113) showed greater than 75% and 2 isolates (NB12 and NB 94) showed less than 63% survivability after 4 h of exposing to acidic pH 2. In the present study, survival ability was variable, even within the same species (NB12, 14 &113), (NB10, 44 &94) and all isolates exhibited a significant reduction in survival rate. However, these results correlated with previous reports as stated that Bifidobacteria and L. delbrueckii subsp *bulgaricus* strain has poor survival rates at acidic pH [56, 57]. However, there are reports a better survival of 2 strains at same condition [58]. Due to these, we can conclude survivability is species and strain-specific [19] (Figure-2).

Figure 2: Acid, Bile and Synthetic Gastric Juice tolerance of LAB isolates



Survival of LAB isolates at pH 2.0, 1%bileconcentration and SGJ at 37°C for 4 h, Values were exhibited as mean ± SD in (n=3).

Tolerance to bile salts is considered a prerequisite for probiotics for their viability, colonization and metabolic activity within the host's gut as antimicrobial molecules. The magnitude of tolerance is determined by the concentration of bile salts, which perform an important role in the specific and nonspecific defense mechanism of the gut [59]. The mean intestinal bile concentration is considered to be 0.3 to 0.5% of the intestinal juice and the residence period of food within the digestive tract is considered to be 4 to 6 h [60]. In this study,6 out of 8 isolates (NB 7, NB10, NB14, NB16, NB 44 and NB 113) showed greater than 78% and 2 isolates (NB12 and NB 94) showed less than 63% survivability after 4 h exposure to 1% bile. In the present study, survival capacity was variable even within the same species (NB12, 14 &113), (NB10, 44 & 94) and overall a small reduction has been found in survivability of all isolates. These findings correlate with previous studies [57, 58]. However, there are some reports which have stated as *Weissella* and *Lactobacillus* strains found to be viable at 1% bile, *W. koreensis FKI21* and *L. crispatus GI9* have been tolerant to bile and acid concentrations. Due to these, we can conclude survivability was species and strain-specific [51, 32] (Figure-2).

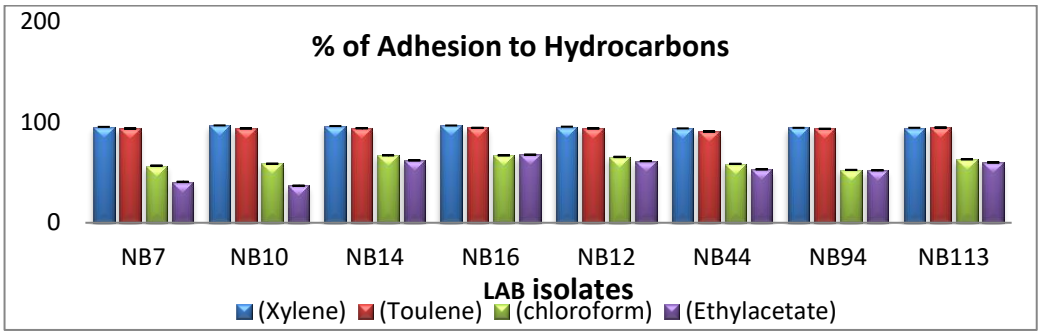
In order to work as a probiotic, the bacteria should survive in gastric juice (low pH) in the stomach and digestive enzymes and then bile acids in the duodenum [61]. Generally, 2.5 L of the gastric juice [62] and 1 L of the bile [63] are produced in the human gastrointestinal tract per day. In the current study, 6 out of 8 isolates (NB 7, NB10, NB14, NB16, NB 44 and NB 113) showed greater than 75% and 2 isolates (NB12 and NB 94) showed less than 61% survivability. The viability of probiotic strains in gastric juice dependents upon their intrinsic tolerance to the adverse environment, the availability of food materials with fat and certain proteins increases the survival rate of microorganisms in gastric transit [56,64] (Figure-2).

Bacterial Adhesion to Hydrocarbons (BATH) assay

The probiotics adherence potential to the gastrointestinal epithelial cells is considered a prerequisite to colonize in the human digestive tract and exert health benefits and for the exclusion of enteropathogenic bacteria [65, 66]. Adhesion is a non-specific physical interaction between two surfaces;

adhesins (proteins) and corresponding receptors [67, 68]. The bacterial adhesion ability depends on hydrophobicity and the intestinal mucus [35]. Generally, 30% of hydrophobicity is considered low/less for adhesion, 30–60% is medium, and more than 60% is high for adhesion [69]. In the present study all the tested 8 isolates (NB7, NB10, NB12, NB14, NB16, NB44, NB94 and NB113) showed a significant (>93.72%) hydrophobic nature (adherence potential) towards xylene and toluene (>90.86%), NB12 and NB16 showed a significant adherence (more than 62%) to all hydrocarbons and can be considered as potent probiotics as they have the ability to colonize in the digestive tract and establish a barrier and modulate the gut immune system to provide protection towards pathogenic microbes[35,36](Figure3).

Figure 3: Bacterial Adhesion to Hydrocarbons

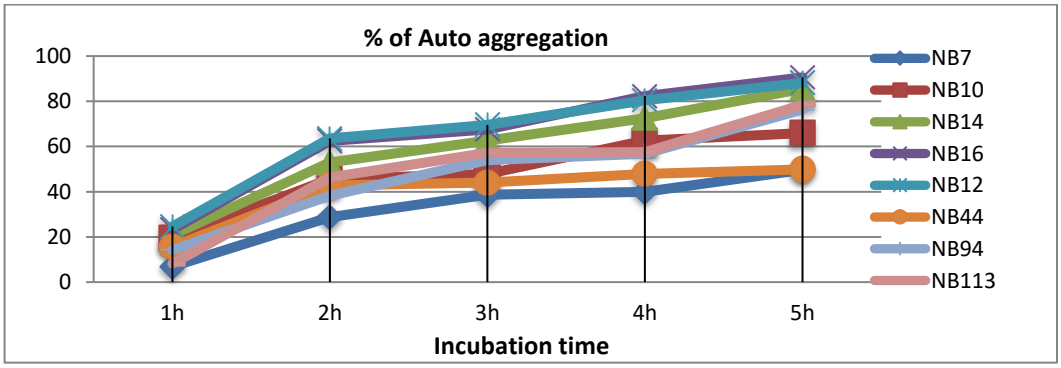


Percentage of adhesion to hydrocarbon, Values are expressed as mean ± SD in (n=3).

Auto aggregation assay

Auto-aggregation is recommended as a major property to considered lactic acid bacteria as probiotics for adherence to gut epithelial cells by forming bio films to protect the host from colonization of pathogens, it has been reported that above 80% of aggregation is considered to be strong auto-aggregation [70]. In the present study, 5out of 8 isolates (NB12, NB14, NB16, NB94 &NB113) exhibited more than 76% and 3 isolates (NB7, NB10& NB44) exhibited lower than 50% about auto aggregation ability. Overall NB16 and NB12 strains exhibited 90.56% and 88.14% of auto aggregation. Our findings co-relate with the previous results of LAB isolates [2, 45, 40] (Figure-4).

Figure 4: Auto-aggregation of LAB



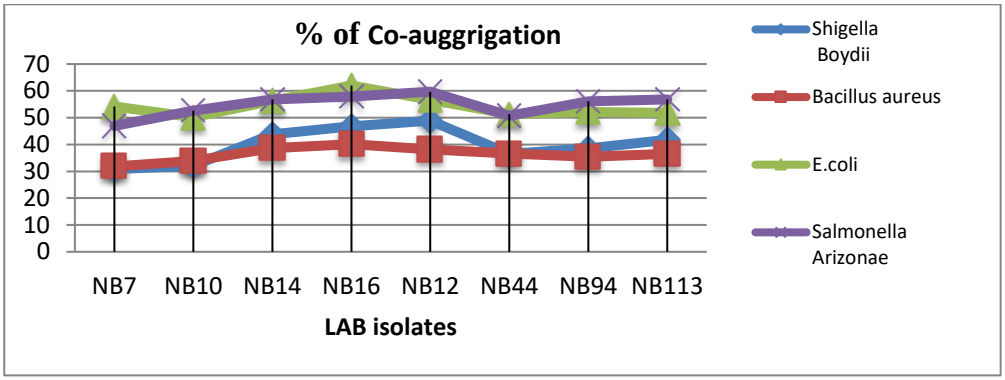
Co-aggregation assay

Coaggregation of bacterial strains plays a significant role in control of pathogenic environment and increases the concentration of excreted antimicrobial substances in the human gastrointestinal and urogenital tracts [68, 71]. It has been stated that co-aggregation ability above 50% is considered to be a strong co-aggregation [72]. In the present study, 5 out of 8 isolates (NB12, NB14, NB16, NB94 and NB113) exhibited more than 55% co-aggregation ability after 5 hours of incubation. NB16 showed a strong co-aggregation with pathogens. Previous studies reported that it depends on strain, species, incubation period, structure composition and forces of the interactions between carbohydrate-lectin and proteinaceous elements existing on the cell surface

[65, 73, 74, 40]. Our studies co-related with previous reports [6, 74, 75] (Figure-5).

Percentage of Auto-aggregation of LAB

Figure 5: Co-aggregation of the LAB

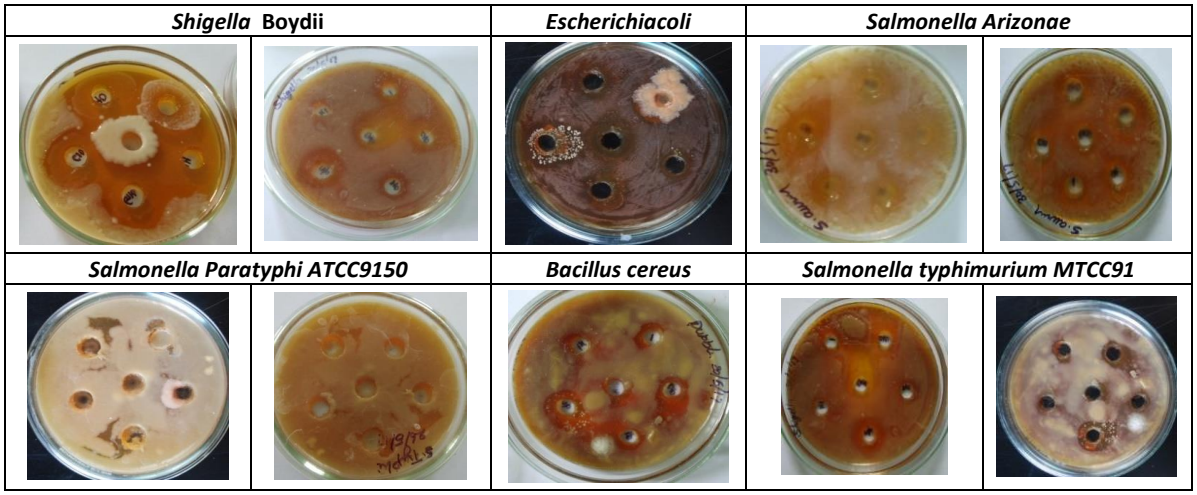


Co-aggregation of the LAB with food borne pathogens

Anti-microbial activity

Antimicrobial activity considered as a major property to prevent the growth of the pathogenic microorganism in the digestive tract by producing antimicrobial compounds such as bacteriocins, organic acids, hydrogen peroxide and diacetyl as well as their competition for the nutrients [76, 77]. In the existing study, all 8 isolates (NB7, NB10, NB12, NB14, NB16, NB44, NB94 and NB113) showed resistance against *Salmonella Para typhi ATCC9150*. 4 isolates (NB7, NB10, NB44 and NB94) showed resistance against *Bacillus aureus*, 4 isolates (NB12, NB14, NB16 and NB113) showed resistance against *Salmonella typhimurium MTCC91*. From the selected 8 isolates, all 8 confirmed good *in vitro* inhibitory activity on *Escherichia coli ATCC10536 & MTCC 40, Salmonella Arizonae ATCC 13314 and Shigella boydii ATCC 9207* however, inhibitory activity towards pathogens varied within the species (NB12, 14 &113), (NB10, 44 &94), which might be species and strain-dependent [45, 70]. Our findings were correlated with the previous studies [5, 75]. All isolates of lactic acid bacteria were capable of preventing the growth of pathogenic microorganisms and its effect was particularly evident towards pathogens (Figure. 6) (Table 2).

Figure 6: Antibacterial activity of LAB isolates



LAB isolates zone of inhibition against food borne pathogen

Table 2. Antibacterial activity of selected probiotic LAB against food borne pathogen

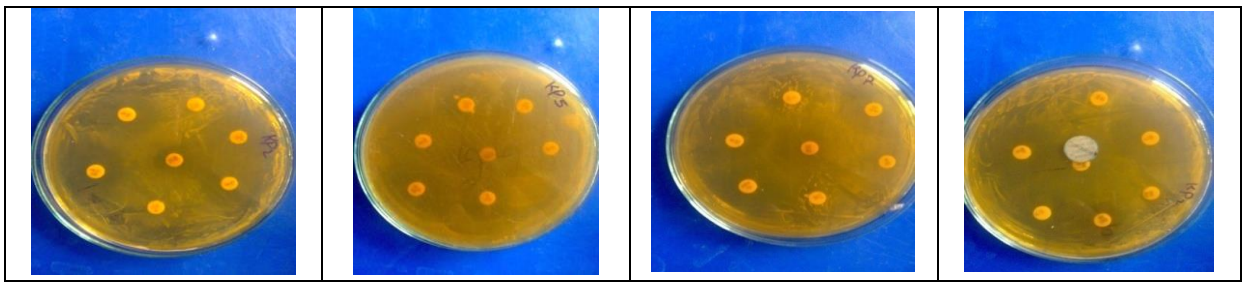
Probiotic LAB	Pathogenic bacteria (Zone of inhibition)						
	Escherichia coli ATCC 10536	*Escherichia coli MTCC 40*	*Bacillus cereus MTCC1306*	*Salmonella Para typhi murium ATCC9150*	*Salmonella typhi murium MTCC91*	*Salmonella Arizonae ATCC 13314*	*Shigella Boydii ATCC 9207*
NB7	++	+++	-	-	+++	+	+++
NB10	+	+++	-	-	++	++	+++
NB 12	+++	+++	++	-	-	+++	++
NB14	+++	+++	+	-	-	++	+
NB16	+++	+++	++	-	-	+++	+++
NB44	++	+++	-	-	+++	++	+++
NB94	+++	+++	-	-	+++	+	+++
NB113	+++	+++	+	-	-	+	-

Symbols refer to size of inhibition zone diameter observed with growing cells: -, no inhibition zone; +, 1mm to 3 mm (weak); ++, 3.1 mm to 6.0 mm (good); +++,>6.0 mm (strong).

Antibiotics susceptibility assay

Antibiotic treatment is the major method followed in the healthcare sector to fight bacterial infections; antibiotic resistance analysis helps to assure the absence of transferable antibiotic resistance genes in any of the probiotic strains and bacterial products which may use as food additives for human consumption, that have to exhibit multidrug-resistant to survive with co-administering of antibiotics [78, 79, 80, 81]. Such resistance characteristic is generally intrinsic and non-transmissible [82]. In the existing study, 2out of 8 isolates (NB12, and NB16) were found to be resistant to all the tested antibiotics except *Chloramphenicol*. All 8 isolates were found to be resistant towards *Cefixime, Co-Trimoxazole, Trimethoprim, Nalidixic Acid* and *Ampicillin*. The susceptibility of strains was expressed as described by [49,48]. Our results were correlated with the previous studies [83]. In conclusion, NB12 and NB16 can be recommended as safe for usage for animals and humans (Figure. 7) (Table3).

Figure 7: Antibiotics susceptibility of LAB isolates



antibiotics susceptibility of selected LAB isolates

Table 3. Antibiotics resistance of selected probiotic LAB isolates

Antibiotics	Disc Content	Interpretative zone diameter (mm)							
		NB16	NB7	NB113	NB14	NB10	NB94	NB12	NB44
Ofloxacin	5mcg	00(R)	00(R)	00(R)	20(S)	15(I)	12(R)	10(R)	10(R)
Cefixime	5mcg	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Cefotaxime	30mcg	00(R)	00(R)	20(I)	00(R)	18(I)	15(I)	14(R)	14(R)
Ceftriaxone	30mcg	00(R)	21(S)	19(I)	18(I)	14(I)	16(I)	00(R)	13(R)
Co-Trimoxazole	25 mcg	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Amoxycillin	30 mcg	15(R)	00(R)	15(R)	20(S)	17(R)	15(R)	14(R)	15(R)
Trimethoprim	5 mcg	00(R)	00(R)	10(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Nalidixic Acid	30 mcg	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Ampicillin	2mcg	00(R)	17(R)	17(R)	21(R)	18(R)	20(R)	13(R)	15(R)
Streptomycin	10 mcg	11(R)	14(I)	12(I)	10(R)	20(S)	10(R)	10(R)	10(R)
Oxy tetra cycline	30mcg	14(R)	23(S)	22(S)	25(S)	21(S)	22(S)	14(R)	14(R)
Chloramphenicol	30mcg	17(I)	26(S)	19(S)	22(S)	20(S)	18(S)	17(I)	19(S)

Antibiotics	Disc Content	Interpretative zone diameter (mm)							
		NB16	NB7	NB113	NB14	NB10	NB94	NB12	NB44
Ciprofloxacin	5mcg	00(R)	00(R)	14(R)	22(S)	20(I)	11(R)	00(R)	16(R)
Azithromycin	15 mcg	11(R)	23(S)	19(S)	28(S)	16(I)	22(S)	00(R)	13(R)
Gentamicin	10mcg	12(R)	17(S)	17(S)	17(S)	17(S)	15(S)	11(R)	00(R)

(R)- Resistant; (I) - Intermediate; (S*) - Sensitive,* in accordance to performance of standards for antimicrobial disk susceptibility test.

GENOTYPIC CHARACTERIZATION

DNA isolation and quantification

Genetic characterization of potent probiotic isolates was an important tool to understand the microbial biodiversity of the genus. The extracted genomic DNA of 8 isolates was analyzed by (1%) agarose gel electrophoresis [84] (Figure 8).

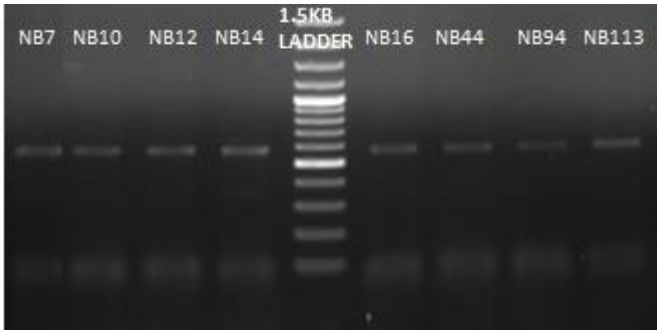
Figure 8: Genomic DNA analysis

Agarose gel (1%) electrophoresis of genomic DNA isolated from the cultures

Polymerase chain reaction (PCR)

Approximately 10 µl of each resultant PCR product was visualized on agarose gel electrophoresis, a single visible and sharp band of 8 isolates was observed (98%) (Figure9).

Figure.9: Polymerase chain reaction (PCR)



Agarose gel (1%) electrophoresis of the 16S rDNA PCR amplicons

Demonstrating the highest similarity to *Lactobacillus casei, Lactobacillus para casei, Enterococcus faecium* and *Enterococcus faecalis* respectively. The 16s rDNA identification showed that NB16 and NB7 strains are 98% identical to *Lactobacillus casei* and *Enterococcus faecalis*, NB12, NB14, NB113 and NB10, NB44, NB94 strains have 98.8% sequence identity to *Lactobacillus para casei* and *Enterococcus faecium*.

IV. CONCLUSION

In the present study, out of 200 strains, eight strains were identified as Lactobacillus para casei (NB12, NB14, NB113), Lactobacillus casei (NB16), Enterococcus faecium (NB10, NB44, NB94) and Enterococcus faecalis (NB7). Out of eight strains Lactobacillus para casei NB16 isolated from breast milk was capable of tolerating high

bile salt, acidic pH and able to survive in synthetic gastric juice and showed the broadest antagonism against a wide extent of food pathogens. In addition, the strain was observed to be resistant to the majority of the antibiotics used, had a strong auto, co-aggregation, hydrophobicity and capable to grow in a range of salt concentration, temperature and pH. Therefore, Lactobacillus para casei NB16 has been proved to remain highly effective overall. Our study indicated that breast milk is an excellent resource to isolate lactic acid bacteria with outstanding probiotic characteristics. However, *in vivo* and therapeutic investigations are still required to assure the beneficial roles about the isolates to human health after that can be encouraged for the improvement of new pharmaceuticals and functional food preparations for public health.

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