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# Isolation of probiotic from human breast milk and study of its functional characteristics

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**Abstract :** With the increasing interest in probiotics having potential application in health benefits and disease prevention, the aim of this study was to isolate and identify bacteria from human breast milk with probiotic potential. Bacteria isolated from human breast milk, was evaluated for their ability to survive *invitro* simulated conditions of gastrointestinal stress, their resistance to different pH, bile concentrations and resistance and susceptibility to different antibiotics.

Molecular identification showed that the strain was *Lactobacillus sp.* which showed maximum resistance at pH 3, tolerated 0.5-4.0% bile concentrations and had potential to survive in low simulated gastric juice of pH 1. Moreover, the strain showed susceptibility to antibiotics like erythromycin, rifampicin, ampicilin, and resistivity to streptomycin and gentamycin.

Therefore, the isolated bacteria may be considered as a potential probiotic that can be mass cultivated *in vitro* for the pharmaceutical and food industry.

***Keywords:*** *Probiotics, Lactobacillus sp., human breast milk.*

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## Introduction:

The probiotic, literally meaning "for life" was first addressed by Lilly and Stillwell (1965) and was used to describe substances produced by protozoa to stimulate the growth of other organisms. Nowadays, the term refers to viable nonpathogenic microorganisms (bacteria and yeast) that when ingested, are able to reach the intestine in sufficient numbers to confer health benefits to the host (Schrezenmeir and De Vrese, 2001). Probiotics stimulates the growth of beneficial microorganisms and reduces the amount of pathogens (Fuller, 1989; Cross, 2002; Chiang and Pan, 2012), also help to alleviate lactose intolerance, enhance nutrients bio availability, and prevent or reduce the prevalence of allergies in susceptible individuals (Chiang and Pan, 2012). Probiotics being safe for human consumption

and resistant to bile and acidic environment survive in the intestine, colonize the human gut and show bacteriocine production to block the invasion of the intestinal cells by enteroinvasive bacteria (Parvez et al., 2006).

The increasing global problems with the traditional disease management strategies have prompted the investigators to hunt for new and better alternatives to deal with the health issues. Probiotics, the foods with "live cultures", have come up as one of the most promising alternatives to traditional disease management. ElieMetchnikoff first suggested the possibility of colonizing the gut with beneficial flora in the early 20$^{th}$century.

A microbial strain has to fulfill a number of specific properties or criteria for it to be regarded as a probiotic (Gibson & Fuller, 2000). The probiotics strain must be of human origin; they should be non-pathogenic and must not deconjugate bile salts. They should carry no antibiotic resistant genes that can be transferred to pathogen (Collins et al., 1998; Saarela et al., 2000). The host must be immunotolerant to the probiotic. With respect to their performance, potential probiotic strains should be acid tolerant and therefore survive human gastric juice and bile. They must be able to survive in sufficient numbers and adhere to the intestinal mucosal surface in order to endure the GIT (gastro intestinal tract).

Human breast milk consists of high amounts of necessary nutrients for infants, including carbohydrates, essential fatty acids, proteins, vitamins and minerals, due to which this has been recognized as gold standards of infant feeding (Sherman et al, 2005). Breast milk has been shown to be a continuous source of commensal, mutualistic and/or probiotic bacteria to the infant gut, including lactic acid bacteria, *Bifidobacteria, Streptococci, Staphylococci*. The genera *Lactobacillus, Lactococcus* and *Pediococcus* belong to the lactic acid bacteria (LAB), and the strains of these genera are frequently used on a large scale in the production and preservation of many foods or as probiotics for humans and animals. The isolation of probiotics with beneficial effects for the host is provided by scientific support of studies over pH and bile salts, cell tolerance to acidity and susceptibility to antibiotics. Thus it serves as potential for probiotic application.

Hence, keeping this in view, the present work was aimed at isolating and characterizing the *Lactobacillus* from human milk sample. This probiotic can be produced *in vitro* under favorable conditions on commercial scales and can be marketed.

**Materials and Method:**

The present research work was conducted in the Department of Industrial Microbiology, Patna Women's College, Patna during the period July to September 2016.

**Collection of Sample:** Human milk used for this study was donated by Jyoti Punj Maternity Hospital, Boring Road, Patna. Sample was collected from the lady with normal full term pregnancy and absence of any pre-natal problems.

The breast milk sample was obtained between 4 and 7 days after the infant's birth in a sterile tube after manual expression using sterile gloves. To collect milk sample nipple and mammary areola of the left breast was cleaned with soap and sterile water, and then chlorhexidiene was applied. First two drops were discarded to avoid chlorhexidiene contamination. The sample was stored in refrigerator at 4ºC till use.

**Isolation of** *Lactobacillus sp.* **from milk sample:** The breast milk sample was inoculated on MRS media (dextrose-20gm, peptone-10gm, beef-extract-10gm, yeast-extract-5gm, sodium acetate-5gm, dipotassium-hydrogen phosphate-2gm, ammonium chloride-1gm, sodium citrate-1gm, tween 80-1gm, $MgSO_4.7H_2O$-0.1gm, $MnSO_4.7H_2O$ -0.05gm, agar-15gm, distilled water-1lit., pH-6.2). 250ml of prepared media was autoclaved at 121ºC for 15 minutes. The slightly cooled media was then poured in the sterilized petri plates and after solidification, the milk sample was inoculated using spread plate technique after which the petri plates were incubated at 37ºC for 48-78 hrs (de Man, Rogosa and Sharpe, 1960).

On establishment of growth, each culture plate was examined for distinct colony from which sub-cultures were made on fresh MRS media and incubated. When there was new growth, they were examined for uniformity as a mark of purity. The resulting pure culture was used for characterization and subsequent identification (Dave and Shah, 1996).

**Phenotypic characterization of the isolated strain:** The morphological characteristics of the isolated colony that grew on MRS media was observed by viewing under microscope and performing Gram Staining.

**Functional characterization of the isolated strain:** The colony was subjected to biochemical tests catalase and oxidase, employing methods proposed by (Morrow, 2004).

The isolated colony was evaluated for its tolerance to pH levels 3,5,7,9 and 11 (S. K. Hood and E. A. Zottola, 1987); bile concentrations 0.5, 1.0, 2.0, 3.0 and 4.0% concentrations of oxgal bile salt (Aneja, 2003); survival at pH 1, 2, 3 and 4 of gastric juice (Kim et al., 2007); and its susceptibility towards antibiotics Erythromycin, Gentamycin, Rifampicin, Ampicillin and Streptomycin with concentration of 50mg/ml (Kirby-Bauer Disk Diffusion Method, 1966).

**Molecular identification of the isolated strain:** Genus and species specific detection of the isolated colony was accomplished using the primers and PCR at Rajendra Memorial Research Institute of Medical Science, Agamkuan, Patna.

Each PCR assay included DNA extraction from the isolated colony by using DNA Extraction Kit. After which the amplification was carried out in the thermal cycler with appropriate reaction mixtures and conditions reported by Collado et al. (2008).

DNA fragments were amplified as follows: initial denaturation at 95ºC for 5 minutes, followed by 20-30 cycles consisting of denaturation at 95ºC for 30 seconds, annealing at 52ºC, 55ºC and 58ºC for 30 seconds, extension at 72ºC for 1 minute and a 10 minutes final extension step at 72ºC. The products were stored at 4ºC until analysis.

Aliquots (15 µl) of the amplified products with loading dye (2µl) were subjected to electrophoresis in 1% agarose gels for genus and 0.8% for species. Then, the gels were visualized under UV light and the results were noted down.

Finally, phylogenetic tree was constructed employing sequences with ~ 250 bp obtained in a FASTA file, the sequences were edited and compared with reported sequences in the GeneBank data base (http://www.ncbi.nlm.nih.gov/BLAST) available at the National Centre for Biotechnology Information web-site. Sequences representing the best matches were retrieved and aligned using the clustal method.

**Results and Discussion :**

**Isolation of *Lactobacillus* sp. from milk sample:** For isolation of *Lactobacillus* from the human breast milk, the sample was cultured on the MRS medium which is a selective and differential medium for the *Lactobacillus* species.

Numerous bacterial colonies were obtained on sterilized MRS plates (Fig. 1).

**Phenotypic characterization of the isolated strain:** For macro morphology, the physical appearance of the colonies like the color, texture, elevation, margin and number of prevailing colonies were noted down. For micro morphology, Gram stained slides of bacteria were prepared and viewed under microscope at 40X. All the colonies were amber, slimy and slightly elevated; Gram positive rods. One colony was taken for further characterization and isolation of strain. The microscopic view of the colony after Gram staining is shown in Figure 2.

**Functional characterization of the isolated strain:**

(a) **Biochemical characterization:** The strain was biochemically characterized by performing catalase test and oxidase test. The strain showed negative results for catalase (Fig. 3) and oxidase (Fig. 4) tests. Control for catalase test was used for comparison.

(b) **Tolerance to acidic pH: -** The isolated strain was evaluated for its tolerance to acidic conditions and their survival potential was tested separately in MRS broth having pH 3,5,7,9 and 11. The strain was able to grow on all the specified pH and showed best positive results for pH 3 (Fig. 5).

(c) **Tolerance to Bile salts: -** The isolated strain was evaluated for its survival in MRS broth having bile concentrations 0.5%, 1.0%, 2.0%, 3.0% & 4.0%. The strain showed positive results for tolerance to all the specified bile concentrations. The observations are tabulated in Table 1.

(d) **Tolerance to gastric juice: -** The isolated strain was evaluated for its survival in MRS broth having SGJ (Simulated Gastric Juice) at pH 1, 2, 3 & 4. The strain showed positive results for tolerance to gastric juice at all the specified pH (Fig.6) with comparatively better result at pH 2. The result showed similarity with the work of Charteris et al., (1998), who found better results at pH 2-3.

(e) **Tolerance to Antibiotics:-** Isolated colony cannot be considered as probiotic until it is tested for its resistivity and sensitivity against and for different antibiotics. This is the most important test as probiotic should not be affected by the intake of certain antibiotics which would be taken for other harmful microbes or pathogen present in one's body (Argyri et al., 2013; Pisano et al., 2014).

The isolated strain was tested against different antibiotics such as Rifampicin, Ampicillin, Streptomycin, Erythromycin and Gentamycin. The strain showed maximum susceptibility towards Rifampicin [fig. 7(a)] and minimum against Streptomycin [fig. 7(b)].

**Molecular identification of the isolated strain: -** The aim of genetic characterization in our study was to identify the isolated strain at the genus and species level. The genus and species specific primer used in the work were obtained from Eurofins Genomics India Pvt Ltd.

For colony PCR the specific primer for genus identification were:-

| S. No. | Oligo Name | Sequence (5'– >3') |
|---|---|---|
| 1. | LBLMA1-rev | CTCAAAACTAAACAAAGTTTC |
| 2. | R16-1 | CTTGTACACACCGCCCGTCA |

For colony PCR the specific primer for species identification were:-

| S. No. | Oligo Name | Sequence (5'– >3') |
|---|---|---|
| 1. | pA | AGAGTTTGATCCTGGCTCAG |
| 2. | pB | AAGGAGGTGATCCAGCCGCA |

Amplification of the gene was carried out in Gradient PCR with known conditions and the results are shown in Figure 8.

The primer used at genus level was specifically of genus *Lactobacillus*, which provided a positive result in the PCR with bands having ~250 bp. The specific primer used at species level was of *Lactobacillus salivarius*, which however gave a negative result with the absence of band after the PCR was performed and gel electrophoresis was done.

Thus, the test confirmed the presence of *Lactobacillus* species to be present as a probiotic in mother's milk.

**Conclusion:**

In this study, the breast milk sample was used as an inoculum and the strain which could serve as probiotic was isolated on MRS medium.

To evaluate the potential use of isolated strain as probiotic, studies on the effect of gastric juice, pH and bile salts were conducted. The isolated strain showed tolerance to wide range of SGJ and Bile salt concentrations. The strain was found to be susceptible to antibiotics like Erythromycin, Rifampcin, Ampicilin and resistant to Streptomycin and Gentamycin. The morphological characteristics and biochemical tests of the isolated strain showed its resemblance with *Lactobacilli.* Molecular identification by DNA amplification and sequencing using genus specific primer confirmed the isolated strain to be *Lactobacilli*. Thus, the present studies showed that breast milk contain *Lactobacilli* species that constitute healthy microbiota and which have potential of probiotics.

**Phylogenetic tree: -** Phylogenetic tree was constructed (Fig. 9) by comparing the obtained sequences with the reported sequences in the GeneBank data base. Sequences representing the best matches were retrieved and aligned using the clustal method.

## LIST OF TABLES

**Table 1: Tolerance to different Bile concentrations on MRS medium**

| S. No. | Bile concentration | No. of colony | Result |
|---|---|---|---|
| 1. | 0.5% | Numerous | Positive |
| 2. | 1.0% | Numerous | Positive |
| 3. | 2.0% | Numerous(small) | Positive |
| 4. | 3.0% | Numerous(very small) | Positive |
| 5. | 4.0% | Numerous(very small) | Positive |

## LIST OF FIGURES

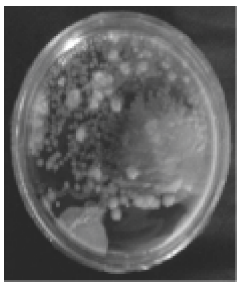
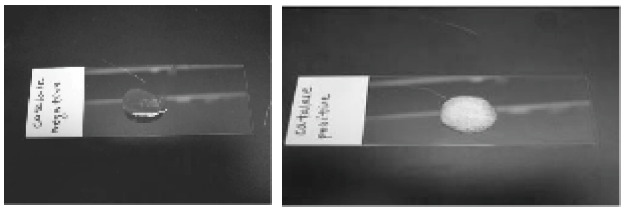


Fig.1. Isolated bacterial colonies on MRS plates

Fig.2. Microscopic view of the colony showing Gram positive *Bacillus*



(a) Isolated strain (b) Control

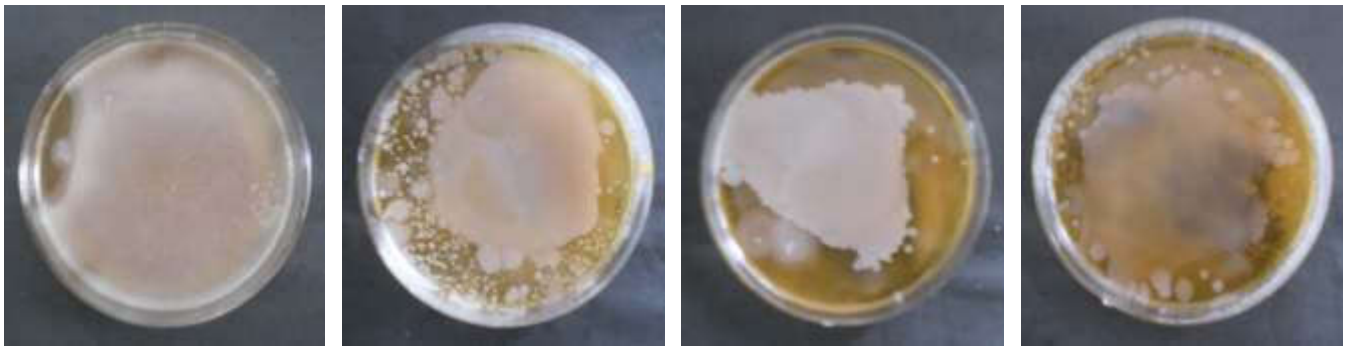
**Fig. 3. Catalase test (a) Isolated strain showing negative result; (b) Control showing positive result**

(a) Filter paper dipped in Oxidase reagent (b) Colony streaked on the dried filter paper

**Fig. 4. Oxidase test showing negative test as the color of the colony did not change.**

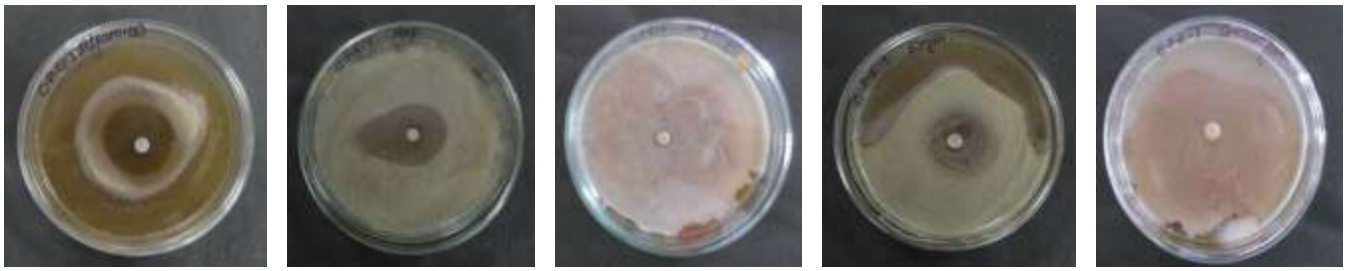
(a) (b) (c) (d) (e)

**Fig. 5. Growth of the isolated strain at pH 3 (a), at pH 5 (b), at pH 7 (c), at pH 9 (d), at pH 11 (e)**



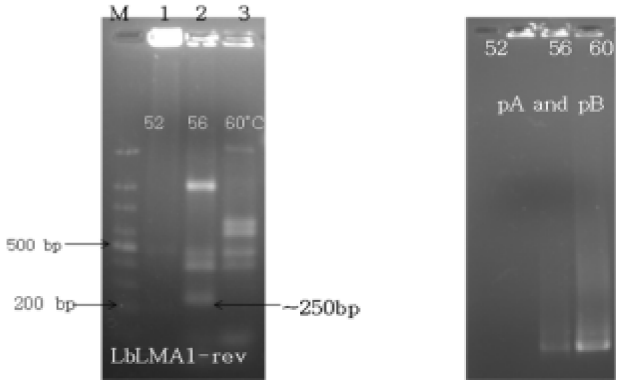
(a) (b) (c) (d)

**Fig. 6. Growth of the isolated strain on MRS broth having SGJ (Simulated Gastric Juice) at pH 1 (a), pH 2 (b), at pH 3 (c) at pH 4 (d)**



(a) (b) (c) (d) (e)

**Fig. 7. Isolated strain showing susceptibility towards Rifampicin (a), Ampicillin (b) and Erythromycin (d); and resistivity against Streptomycin (c) and Gentamycin (e)**




(a) Bands obtained from LbLMA1-rev. (b) Bands did not obtain from pA & pB.

**Fig. 8. Gel image of 16S rRNA Amplicon band of the isolate visualized under UV light**

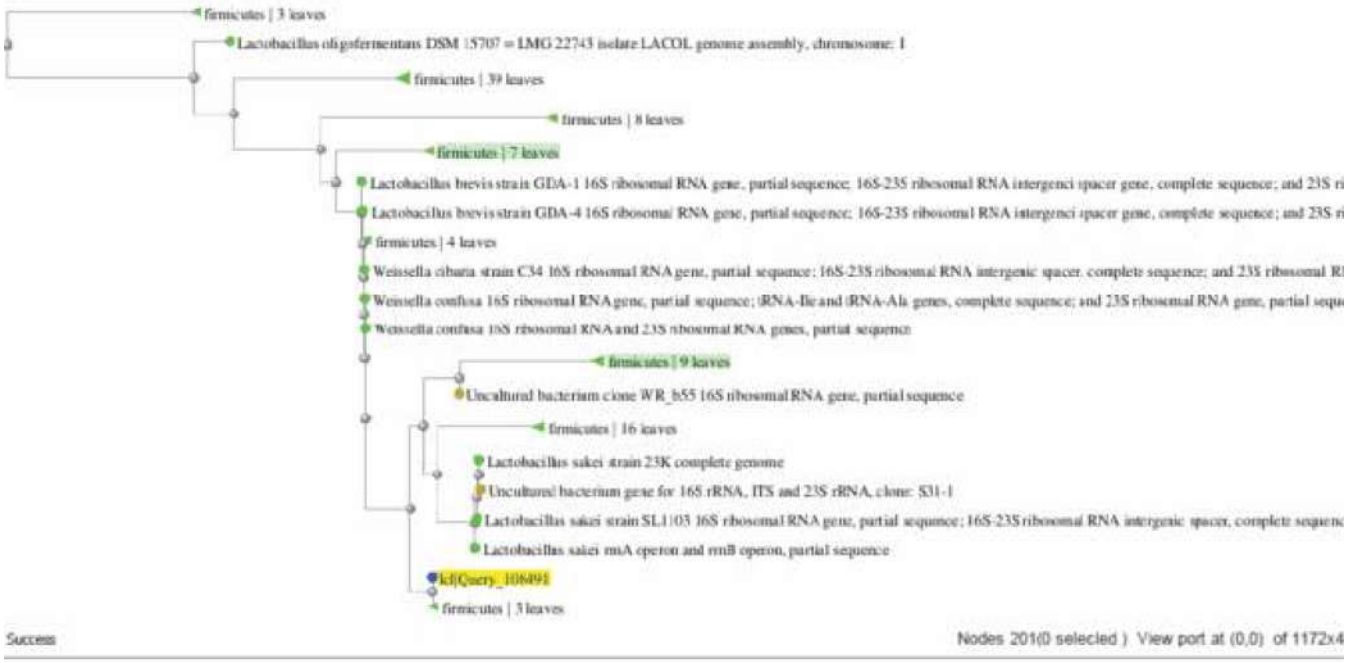



**Fig. 9. Phylogenetic Tree**

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