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In *Vitro* Evaluation of Probiotic Potential of Lactic Acid Bacteria Isolated from human faeces

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ABSTRACT: LAB helps in nourishment, proper growth and development in stages of life. The main objective of the study was to characterize and evaluate the probiotic potential of LAB from HF. LAB species were isolated from 50 individuals. The complete investigation was performed by using traditional microbiological approaches isolated from the feces of human. They were cultured anaerobically. They were gram- positive bacteria. The strain were catalase-positive and oxidase negative. It shows that good populations of LAB in gut microbiome under anaerobic conditions. Several LAB showed with probiotic potential. The isolated LAB shows growth at low pH, different temperatures, tolerance against bile salts, resistance against antibiotics and antimicrobial activities against common pathogens 8 isolates of the study were found to be very promising in showing resistance against antibiotics and antimicrobial response against enteric pathogens such as *Escheria coli* ATCC 25922, *Shigella* ATCC 23354, *Staphylococcus aureus* ATCC 25922, *Salmonella typhi* ATCC 733. On the basis of biochemical characterization, the isolates were identified as Bifidobacterium *recteri*, Lactobacillus *brevis*, Bifidobacterium *longum*, Lactobacillus *sakei*, Lactobacillus *agilis*, Lactobacillus *nagelii*, Lactobacillus *helviticus and* Lactobacillus *plantarum*. The present papers deal with the isolation, characterization and evaluation of probiotic potentials of LAB isolated from human faeces.

Key words: Human Faeces (HF), Lactic Acid Bacteria, Probiotics, Gut Microbiome, Anaerobic

INTRODUCTION: The Lactic Acid Bacteria (LAB) generally known for their health benefits like nourishment, vitality that helps in growth and development of human adult and especially for the infants during their early stages of development. Usually Human Faeces (HF) is considered as solid waste material that cannot be digested inside the small intestine of human body. Faeces contain large number of bacteria, many of which may be Lactic Acid Bacteria (LAB). Several research investigations were performed with some specific objectives by the researchers. The achieving goals in the studies were dealing with the human health and for the animal health. The role of prebiotics in maintenance of probiotics population has attracted

the attention of scientific community **(Gibson, 2004).** Lactic acid is one of the most important organic acids that is widely used in food industry. Recent trends show that lactic acid production through microbial fermentation is advantageous over chemical processes because of its environmental concern **(Ghaffar *et al.*, 2014)**. Lactic acid bacteria are safe microorganisms that can provide health benefits. They can inhibit those microorganisms which are harmful. Lactic acid bacteria as potential probiotics can be considered as alternative at place of antibiotics. To enhance growth and development and to control several bacterial infections LAB can be used as potential probiotics. Due to their capability to adapt the environment in which they are delivered as probiotics they are very well suits for livestocks **(Gaggia *et al.*, 2010; FAO, 2016)**. LAB is a large group of bacteria used worldwide as a probiotic. This group of bacteria involves the microorganisms of genera Lactobacillus, Lactococcus, Aerococcus, Enterococcus, Pediococcus, Leuconostoc, Streptococcus, Sporolactobacillus, Vagococcus and Cornobacterium (**Pavli *et al.*, 2018 and Neha 2019).** LAB have innumerable health benefits such as blood pressure lowering **(Robles- Vera *et al.*, 2017),** prevention of colon cancer (Rafter 2003), reduction of allergic symptoms (**Cuello- Garcia *et al.*, 2017**), reduction of cholesterol (**Agerholm- Larsen *et al.*, 2002**), boosting of immune system (**King *et al.*, 2014)** prevention of urinogenital infections (**Shoetliffe *et al.*,** 2013), reduction of Helicobacter pylori infections (**Hamilton, 2003),** Intestinal inflammation **(Jin-Sil *et al.*, 2018),** antimicrobial effects on pathogens **(Tankoano *et al.*, 2019)** and many more. The present study deals with isolation, characterization and evaluation of probiotic potential of HF. *Escherichia coli* is Gram-negative, facultative anaerobic, and rod-shaped bacterium of the genus *Escherichia*. This is a large diverse group of bacteria commonly found in the lower intestine of warm-blooded organisms. Most of them are commensals inhabiting the lower gastrointestinal tract (GIT) of mammals. The other strains that are pathogenic are categorized into two groups, according to the site of infection. *E. coli* that infects and cause disease syndromes in the gastrointestinal tract are intestinal pathogenic *E. coli*. Most pathogenic *E. coli* are transmitted by faecal-oral route from food materials, water, animals, and environment. Its Depend on the pathotype and the system, *E. coli* predominantly inhabit the gastrointestinal tract of mammals and are shed to the environment through feces.

MATERIALS AND METHODS:

Sample Collection:

The samples of Human Faeces (HF) were collected from 50 different individual volunteers aseptically and were brought to the laboratory immediately in the School of Biological Engineering and Life Sciences, Department of Biotechnology, Shobhit Institute of Engineering and Technology, Meerut (UP) India.

Isolation of LAB:

The isolation of LAB from Human Faeces (HF) was quickly processed after completion of samples collection. The HF sample were diluted (up to 10^{-6}) using sterile peptone water. The last three dilutions were inoculated on MRS agar plates using Spread Plate Technique. The inoculated plates were incubated at 37°C±1 for 48h under anaerobic conditions using anaerobic jar.

Biochemical characterization of Isolates:

Gram's Staining:

A single drop of sterile water was placed on a clean plain glass slide and a pure colony of isolate was picked from the plate and was mixed gently to prepare a smear. The smear was heat fixed carefully and stained as per standard Gram's stain method. The slide was observed under oil emulsion lens 10 x 100x of compound light microscope (Carl Zeiss Microscopy GmbH). As LAB are Gram positive in nature, all the isolates which showed purple (positive) color were further processed for physiological and biochemical tests.

Catalase Test:

Catalase test was performed for all the isolates which were Gram's positive. Catalase is a type of enzyme which is produced by several microorganisms that breaks down hydrogen peroxide into water and oxygen and forms bubbles of gas. The 3% hydrogen peroxide solution was mixed gently on the surface of clean glass slide containing test microbial culture and was observed for formation of bubbles. As LAB are catalase negative, all isolates that showed negative results were further tested for oxidase test.

Oxidase Test:

Cytochrome C oxidase is an enzyme found in several bacterial electron transport chain. Presence of cytochrome c oxidase the reagent called tetramethyl-phenylenediamine into indophenols (purple colour) end product. In the absence of this enzyme, the reagent remains reduced and colorless. To perform this test, a filter paper soaked with the substrate tetramethyl-p-phenyl diamine dihydrochloride was taken which was moistened with sterile water. The colony from MRS agar plate was picked up using sterile nichrome wire loop and was smeared on the paper. The paper was observed for change in color within 30 second. As LAB are oxidase negative.

Sugar Fermentation Test:

Carbohydrate when fermented by microorganisms form an acid or acid with gas at the end. Depending on the microorganisms involved, the end products may vary. All the isolates which were Gram positive and catalase and oxidase negative were tested for their sugar fermentation activity. Sugars were prepared using standard protocol (Hi Media) and each tube of sugar contained Durham's tube in inverted position. Each isolate was inoculated in all different sugars (Glucose, lactose, Dextrose, Maltose, Fructose, Sucrose) to note down the breakdown of sugars into acid or acid/gas. Incubation for 48h at 37°C were given to all the sugars. Results were recorded after completion of incubation period. On the basis of sugar fermentation activity, the isolates were identified using Bergey's Manual of Systematic Bacteriology **(Hammes P *et al*., 2009).**

Determination of probiotics potential:

After biochemical characterization, all the isolates were tested for their probiotic potential by testing their growth at low pH, different temperatures, tolerance against bile salts, resistance against common human pathogens and resistance to antibiotics.

Growth at low pH:

The pH of human stomach ranges between 2 to 3. It is also believed that food eaten by us in stomach for at least 4h **(Bistha N *et al.*, 2019).** Therefore, it is the necessary for the isolate to survive at low pH for more than 4h. To check the growth of isolates at low pH, all the isolates were inoculated in peptone water prepared with different pH (6,5,4,3,2) for a period of 6h. After incubation period, the isolates were inoculated on MRS agar plates and were incubated under anaerobic conditions to check their survival at different pH. All the isolates were further checked for their tolerance against bile salts.

Tolerance against Bile Salts:

The concentration of bile salts in the intestine is believed to be 0.3% (w/v) and the food eaten stays in small intestine is suggested to be 4h **(kumari A *et al.*, 2008).** Therefore, all the isolates were examined for their growth at different bile salts concentration. Peptone water with different bile salts concentration was prepared using oxoid and active cultures of isolates were inoculated in the medium for 6h. after incubation, the isolates were inoculated on MRS agar plates for its viable count. The isolates which showed best growth at both high as well as low temperatures were further screened for their resistance against antibiotics.

Growth at Different Temperatures:

To examine the growth of isolates at different temperature, the active cultures of isolates were inoculated on MRS agar plates and were incubated at different temperatures (28 and 37°C) in anaerobic conditions. The isolates which showed best growth at both high as well as low temperatures were further screened for their resistance against antibiotics.

Resistance to Antibiotics:

The isolates which gave best growth at high as well as low temperature were tested for their resistance against common antibiotics using Kirby Bauer Method. The isolates were spreaded on the entire surface of MH Agar Plate and the discs of Antibiotics with different concentrations were placed on the surface of agar and gently pressed. The plates were allowed to incubate at room temperature for 24-48h. the isolates which did not give appropriate zone of inhibition around the discs of antibiotics according to standard chart were further examined for their antimicrobial activities against common human pathogens.

Antimicrobial activity:

All the isolates which fulfilled the above-mentioned criteria were further tested for their antimicrobial activities against common human pathogens using agar well diffusion method. The indicator pathogenic microorganisms were spreaded on the entire surface of Muller Hilton (MH) agar plates and using a sterile core borer of 7mm diameter. 5 different wells of same size were made by puncturing the MH agar plates. Using micropipettes 50µl of overnight grown culture of isolate were inoculated carefully in the wells. The plates were incubated for 24h in upright position. Therefore, the zone of inhibition were measured. The isolates which showed greater zones of inhibition were considered having good probiotic potential.

RESULTS:

A total of 50 LAB were isolated from the HF. The isolates were identified on the basis of physiological and biochemical characteristics. On the basis of Bergey's Manual of Systematic Bacteriology, 25 different species of LAB were identified. Of these, 8 isolates of LAB were found to be very promising with the potential of probiotics. The isolates were initially confirmed by using biochemical test such as Catalase, oxidase, Gram's Staining, Arginine Hydrolysis test and Sugar Fermentation test. All the isolates in the present study were found grams positive, catalase and oxidase negative and also had the capacity to breakdown sugars into acids and gas. On the basis of their Sugar Fermentation activity and Gram's morphology (figure 1), the isolates were identified using Bergey's Manual of Systematic Bacteriology **(Hammes P et al.,2019).**

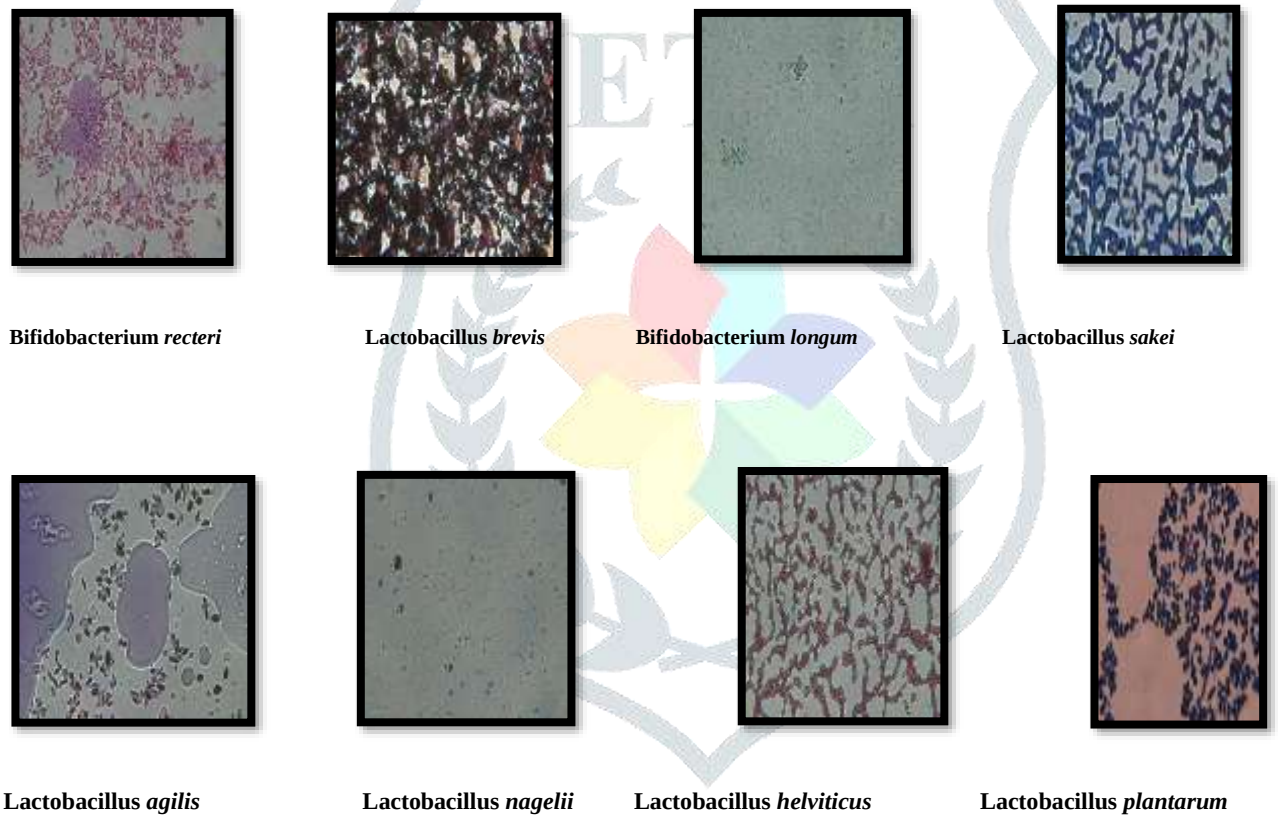

Figure 1: Microscopic observation of Gram's Staining.

Determination of LAB to be potentially probiotic:

All the isolates identified as LAB through biochemical tests were further screened for determining their probiotic potential. Firstly, the growth of isolates checked at low pH. Out of 50 isolates 25 were showed its positive growth at pH 2. Which were further screened for their tolerance against different bile salts concentration. Out of 50 isolates, 25 were found to be prominent against tolerating the 0.3% (w/v) bile salts concentrations. The isolates were further examined for their growth at different temperatures. Out of 50 isolates 25 showed a good growth at 37° and 28° C. on the basis of these three criteria, 25 best isolates were

selected for checking their resistance against antibiotics from which 16 best isolates, only 8 showed very high degree of zone of inhibition against pathogenic bacteria.

TABLE 1. Colony Characteristics of Isolates with Biochemical test.

Isolates No.	Shape	Size	Color	Opacity	Margin	Elevation	Surface	Gram's Reaction	Shape	Catalase test	Oxidase test	Arginine hydrolysis
SUB-101	Circular	**Large**	**Creamy white**	**opaque**	**Irregulr**	**Raised**	**Dry**	+	**Bacilli**	-	-	+
SUB-102	**Circular**	**Small**	**Pale yellow**	**opaque**	**Entire**	**Convex**	**Smooth**	+	**Cocco-Bacilli**	-	-	+
SUB-103	**Circular**	**Medium**	**Off white**	**translucent**	**Entire**	**Flat**	**Smooth**	+	**Coccus**	-	-	+
SUB-104	**Circular**	**Moderate**	**Pale yellow**	**opaque**	**Entire**	**Convex**	**Rough**	+	**Bacilli**	-	-	+
SUB-105	**Circular**	**Medium**	**Off white**	**Opaque**	**Entire**	**Convex**	**Smooth**	+	**Coccus**	-	-	+
SUB-106	**Circular**	**Small**	**Creamy white**	**Opaque**	**Entire**	**Convex**	**Smooth**	+	**Coccus**	-	-	+
SUB-107	**Circular**	**Moderate**	**Pale yellow**	**Opaque**	**Entire**	**Raised**	**Rough**	+	**Bacilli**	-	-	+
SUB-108	**circular**	**Small**	**Off whit**	**Opaque**	**Entire**	**Raised**	**Rough**	+	**Bacilli**	-	-	+

TABLE 2. Fermentation of different sugars for identification of isolates as per the recommendations of Bergey's Manual.

Isolates No.	Names of Sugar												Identification based on Bergey's Manual Systematic Biology
	Lactose		Maltose		Sucrose		Fructose		Dextrose		Glucose		
SUB-101	A	G	A	G	A	G	A	G	A	G	A	G	Bifidobacterium *recteri*
SUB-102	+	_	+	_	+	_	+	_	+	+	+	_	Lactobacillus *brevis*
SUB-103	+	_	+	_	+	+	+	_	+	+	+	+	Bifidobacterium *longum*
SUB-104	+	+	+	+	+	_	+	+	+	+	+	_	Lactobacillus *sakei*
SUB-105	+	_	+	+	+	_	_	_	+	_	+	_	Lactobacillus *agilis*
SUB-106	+	+	+	_	+	_	_	+	+	+	+	+	Lactobacillus *nagelii*
SUB-107	+	_	+	+	+	_	+	+	+	+	+	+	Lactobacillus *helviticus*
SUB-108	+	+	+	_	+	_	+	+	+	+	+	+	Lactobacillus *plantarum*

TABLE 3. Antimicrobial activity of isolated LAB against common pathogens.

	Name of Pathogens			
Isolate No.	**E. coli ATCC-25922**	***Shigella* ATCC- 23354**	**S.aureus ATCC-25922**	**S.typhi ATCC-733**
Bifidobacterium *recteri*	18 mm	17 mm	18 mm	17 mm
Lactobacillus *brevis*	21 mm	14 mm	16 mm	19 mm
Bifidobacterium *longum*	19 mm	18 mm	18 mm	20 mm
Lactobacillus *sakei*	20 mm	16 mm	15 mm	16 mm
Lactobacillus *agilis*	18 mm	17 mm	18 mm	18 mm
Lactobacillus *nagelii*	20 mm	13 mm	17 mm	20 mm
Lactobacillus *helviticus*	21 mm	17 mm	18 mm	18 mm
Lactobacillus *plantarum*	19 mm	18 mm	15 mm	16 mm

TABLE 4. Evaluation of Resistance of isolates against common antibiotics using disc diffusion method.

Isolates No.	Erythromycin		Tetracycline		Pencillin		Gentamicin		Streptomycin		Amoxocillin		Ciprofloxacin	
Measurement on zone of Inhibition in (mm) and its Resistance (R) or Sensitivity (S) against Antibiotics														
Bifidobacterium *recteri*	13	R	9	R	10	R	11	R	9	R	12	R	13	R
Lactobacillus *brevis*	12	R	10	R	12	R	12	R	8	R	9	R	14	R
Bifidobacterium *longum*	9	R	11	R	8	R	9	R	10	R	10	R	10	R
Lactobacillus *sakei*	14	R	9	R	9	R	13	R	9	R	11	R	12	R
Lactobacillus *agilis*	10	R	10	R	11	R	9	R	11	R	9	R	11	R
Lactobacillus *nagelii*	11	R	13	R	12	R	10	R	9	R	10	R	10	R
Lactobacillus *helviticus*	13	R	11	R	11	R	8	R	13	R	9	R	9	R
Lactobacillus *plantarum*	12	R	9	R	14	R	9	R	10	R	8	R	12	R

DISCUSSION:

Total 8 best species of LAB were found and screened out of 50 1isolates. Identification of LAB was made on the basis of colony morphology, physiology and biochemical tests as per the guidelines mentioned in Bergey's Manual of Systematic Bacteriology. Similar test performed by earlier researchers found 8 species of LAB that were Grams positive, catalase and oxidase negative and also showed active hydrolysis of arginine.

The acidic pH of stomach and the antimicrobial actions of pepsin provide an effective barrier for LAB to survive in gastrointestinal tract. For existing beneficial effects on host, probiotic should be able to maintain its viability along the gastrointestinal transit by surviving under harsh conditions. The survival rate of the isolates of our study were found to be best even at pH of 2.

Tradional techniques of microbiology were used in the study rather than modern techniques because it is more reliable. Modern techniques have some limitations such as the viability of faeces microbes cannot be analyzed, total bacteria counts may be over-or underestimated because of cell-wall composition, DNA extraction methods which may lead to the over or underestimation of bacteria counts. Contamination in DNA extraction kit and reagents was also reported in the past studies. (McGuire MK, 2015)

CONCLUSION:

Human faeces contain of large number of bacteria with probiotic potential and in maintaining the gut microbiome. We have also found that LAB have great potentials of fighting against the common human pathogens. In our present study we have found that some LAB have great efficiency to resist against antibiotics. These such species of LAB should be commercialized and marketed at a global stage so that problems related to imbalance in gut microbiome can be solved. Through our studies, we also came to know that unnecessary consumption of antibiotics reduces the LAB count in HF. Therefore, use of antibiotics used be minimized. There are several other facts which are still not known till date such as existence of LAB in HF is still a mystery. LAB in HF is a wide area of research and still needs lots of genuine studies to be carried out to solved the unknown.

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