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Exploration of Probiotic Attributes of Lactobacillus Sps. From Vagina and Its Rapid Identification by Multiplex Pcr Assays Using Group Specific Primers

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Abstract

Human vagina contains lactobacilli that are the part of vaginal microbiota. The identification and assessment of the unique properties of usual Lactobacillus species in the vagina of healthy women in various topographical locations will assist in selecting the best species. In the current study we have carried out the isolation and identification of Lactobacillus species from the vagina of healthy women to explore and evaluate their probiotic and antimicrobial efficacy. The research comprises of 30 women and bacteriawas isolated from the healthy individual were purified. The Lactobacillus was identified by the Phenotypic and biochemical tests. Lactobacillus species were identified by molecular method that is the amplification of the full length of the 16S rDNA of the isolated bacteria using polymerase chain reaction. In this study, eight vaginal lactic acid bacillus isolates manifested plethora of in vitro probiotic characteristics and showed potential activity against the most prevalent bacterial pathogens.

Keywords – *Vaginal Bacteria, Lactobacillus, antibacterial, polymerase chain reaction.*

Introduction

The vaginal ecosystem's homeostasisget benefited by the complex interactions and synergies between the host and various microbiota that colonize the mucosa of the vagina. Lactobacillus spp is typically

the dominant bacterial flora found in the healthy vaginal microbiota (1). By developing antimicrobial compounds, or by competition for the attachment to the epithelium of vagina, these bacteria exhibits pivotal defense mechanism against deadliest pathogens. There is a growing interest in their use of probiotic formulations for the prophylaxis and treatment of many vaginal infections because of the favorable effects of lactobacilli on the health of the women'sgenital tract (2).In the last decade lactic acid bacillus, along with bifidobacteria, have been the most commonly examined probiotics. It is important to test these probiotic microorganisms for the existence of probiotic properties that include anti-microbial efficacy against specific pathogens and the development of anti-microbial compounds (3). Recently, several attempts have been developed to search for possible human-isolated probiotic strains of Lactobacillus. Simple and reliable identification techniques are needed for this reason (4).

Conventional method of biochemical and physiological studies apparently has certain drawbacks in distinguishing against highest numbers of isolates with homologues physiological properties (5).Hence the use of molecular biology techniques in the rapid detection of lactobacilli has been the subject of several studies. The DNA-DNA hybridization technique has enhanced our taxonomic knowledge that deciphers the connections among the species of Lactobacillus, but this process is still time-dependent and requires intense man power (6).

In the recent years, rRNA genes were widely recognised as the future targets for bacteria phylogenetic study and identification. The respective Lactobacillus species were successfully detected and identified using 23S rDNA - or 16S rDNA -targeted primers by PCR. In addition, multiplex PCR techniques for the concurrent detection and discrimination of many microorganisms have been implemented (7). Primers based on the species-specific sequences of the 16S rRNA or 16S-23S rRNA intergenic spacer region (ISR has its 23S rRNA or16S rRNA flanking region) were used in the multiplex PCR.Still, the restriction of the accession of extra priming sites from each rRNA gene won't allow several organisms to be discriminated against simultaneously (8, 9).

In this analysis, we evaluated unique primers by comprising the sequences according to the 16S genes and their ISR, and evaluated the isolated Lactobacillus Sps. via a multiplex PCR method. In addition, their probiotic efficacy and antimicrobial characteristicshave been measured.

2.0. Materials and methods

2.1. Sampling and study area

A cross-sectional experimental study was carried out at the Department of Microbiology, MelmaruvathurAdhiparasakthi Institute of Medical Sciences and Research, Tamilnadu, India. The study was approved by the Institutional ethical committee. Prior approval has been received from all the participants. The study was done from the period of January 2015 to December 2018. A systematic proforma was obtained to get details from patients who were pursued with Clinical

examination. Subjects were excluded if they have the consequences such as history of hysterectomy, evidence of vulval, genitalor cervical dysplasia over the last 5 years, history of candidiasis, urinary tract infections, or active sexually transmitted disease, history of regular urinary incontinence**,** menstruation, contraceptives and intrauterine devices. Any apparent vaginal or cervical lesions, like genital warts (or papillomas), vaginal discharge, symptoms of vulvar/vaginal inflammation, or indications of vulvar/vaginal irritation have been tested for clinical review (10,11).

2.2. Collection of vaginal swab for the isolation of vaginal microbiota

The labia was propagated directly after the hymenal ring/tissue to visualise vaginal introitus. A sterile collection swab was placed at the vaginal introitus posterior to the hymenal ring/tissue and the swab was rotated in a circular motion for five times along the vaginal lumen. The speculum was used without any lubricant or water. The cervix and posterior fornix was visualised. In the posterior fornix, a sterile swab was placed and the swab was rotated five times around the lumen with a circular movement. The same was performed in the area of the mid vagina. Sample was collected from the vaginal posterior fornix, introitus, and mid-vagina. Precautions were taken during sample collection to avoid affliction in the region where the speculum touched. Three specimens were collected from each participant (12, 13).

2.3. Microscopy of the obtained samples

2.3.1. Grams staining

The sample collected from the swab was used for smear preparation. Contents present on the swab were transferred to a clean grease free slide by rolling (half rotation) the swab on the slide. Precautions were taken to spread the collected material on the slide without interrupting the morphology of bacteria and other structures. Smears were fixed by air drying or heat. Then the grams' staining was performed (14).

2.3.2. Nugent's scoring of the isolates

The score of Nugent's grading is based on the classification of Scale's value ranges between 4-6 in the presence of Clue Cells or >7 are considered bacterial vaginosis, while scores from 0 to 3 and 4 to 6 (without Clue Cells) are considered normal and intermediate, respectively. The scale's value is obtained by the addition of three different scores; in turn, every score represents the quantity of a specific bacterial morphotype which can be evaluated through the optical microscope. In the vaginal smears, microorganisms counted large, tiny Gram-negative rods, Gram-positive rods and curved Gram-variable rods (15, 16).

2.4.Isolation of bacteria from the swabs

Rogosasheep blood agar medium have been used as a culture medium for the growth of Lactobacilli from vaginal swabs. The culture plates were incubated at 37 ^{0}C for the time period of 24–48 h with 5% CO_2 in an anaerobic jar containing AnaeroPack (Himedia). Plates containing 10–20 CFUswere further studied. Ten colonies of each plate were randomly selected for processing.The colonies with Gram-positive rods were inoculated in the culture medium containing de Man-Rogosa-Sharpe (MRS) broth. Individual colonies were grown in 2.5 ml of MRS broth incubated overnight in 5 % CO_2 at 37 ^{0}C. Glycerol (10 %) stocks were prepared in 200 µL of the broth culture and stored at − 80 °C.one set of swab was kept in Blood agar (Himedia) and Sabraud's dextrose agar (Himedia) which was used to culture facultative aerobic bacteria and fungi respectively from a vaginal swab (17, 18).

2.5.Physiological and biochemical characteristics for identification of Lactobacillus

2.5.1. Determination of sugar fermentation

The fermentation assays for sugarswere carried out using 1 percent (w/v) sugar in the MRS broth. In this test, Glucose, Fructose, Sucrose, Xylose And Lactose were used. As an indicatorsolution, 0.01 % Phenol Redwas used. 10 ml of media was distributed in each of the test tubes and the Durham tubes were inserted invertably. The culture (overnight inoculated culture) was inoculated and incubated for 24 hours at 37 ° C. An uninoculated sterile media was maintained as a negative control. Results were noted according to the color change and gas formation (19).

2.5.2. Biochemical parameters

The isolated pure strains were streaked on MRS culture medium and incubated at 30°C for 48 h, the size and shape of the colony were observed and investigated by various methods that includes using a microscope (Olympus, Tokyo, Japan), the gram staining study. Oxidasetest, Catalasetest, gelatin liquefaction test, Nitrate reduction test, litmus milk test, hydrogen sulfide test, Methyl Red test, V.P. test, Indole test, growth temperature test (10°C, 15°C, 45°C, 60°C for 30 min), 6.5% NaCl growth test, pH gradient test and sugar alcohol fermentation test were also performed (20).

2.6. Molecular characterization by 16 srRNA sequencing

By the CTAB-NaCl process, the genomic DNA was extracted. PCR analysis was performed using the 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′), 1492R (5′-CGG TTA CCT TGT TAC GAC TT-3′) primer combination for 16S rRNA gene amplification. NCBI BLASTn (https:/blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the 16S rRNA gene sequence obtained from the isolates and ClustalW 1.83 (https:/www.ebi.ac.uk/cgi-bin/clustalw/) performed multiple sequence alignment. Phylogenetic trees were built by neighbour joining (NJ) and minimum evolution (ME) in MEGA 6.0 (https:/wwwmegasoftware.net) (21).

2.7. Multiplex PCR assay for the determination of *Lactobacillus sps.*,

A multiplex PCR assayswere carried out with the primers which are listed in table 3. PCR master mix (50 µl) which contains 0.25mM of primers, 1× Taq DNA polymerase buffer, 50 ng of genomic DNA, 0.2 mM of each dNTP, 1 U of Taq DNA polymerase, and 1.5 mM MgCl2 was used. PCR was employed with an initial denaturation for 3 min at 94 °C, 30 cycles of denaturation for 30 s at 94 °C, annealing for 10 s at 56 °C, and elongation for 30 s at 72 °C , and a final extension for 5 min at 72 °C. PCR products were observed in 1.5 %agarose gel after electrophoresis and the scale was compared with a 100 bp ladder.

2.8. Probiotic attributes of Lactobacillus isolates form vaginal environment

2.8.1. Acid and bile salt tolerance of the lactobacillus isolates

Using MRS broth, tolerance of the bacterial strains to acid (pH 3.0) and bile salt (0.3 percent w/v) was investigated. With 1.5 ml of NaCl (0.5 w/v) and 5 ml of simulated gastric ,1 ml of washed Lactobacillus cell suspension was incubated for 3 h,. Simulated gastric juice was freshly prepared everyday by suspending pepsin (3 g/L) in sterile saline (0.5 per cent w/v) and the pH was adjusted to 2.0 with concentrated HCl (Charteris et al., 1998). Aliquots of 0.1 ml were then extracted to assess the total viable count. Dilutions were made (up to 10−4) and the cells were duplicated on MRS agar. Plates were incubated for 72 h at 37 °C before counting(22).

The MRS broth containing bile was prepared by adding 0.3 (v/v) of bile salt.Cells were collected by centrifugation from 100 ml (20 h MRS tested culture) (3400RPM, 10 min), washed two times in saline (8.5 g NaCl/L) and resuspended in 10 ml MRS broth. This suspension was inoculated into MRS broth (1%) that lacked or contained bile salt.After incubation at 37 °C for 0, 1, 2 and 3 h, viable counts on MRS agar plates and culture absorption at 625 nm were taken and noted (23).Acid and bile tolerance assay was performed trice with duplicates.

2.8.2. Hydrogen peroxide production

Lactobacillus sps., cells (or cell-free extracts) were resuspended with or without 55.5 mM glucose as needed in a cold phosphate buffer (0.2M, pH 6.5) and incubated at 5°C for the desired period. Cells were extracted after incubation by centrifugation and the supernatant was used for the test of hydrogen peroxide level. The hydrogen peroxideproduction assay was performed with the culture supernatant in a test tube containing 1 ml of 0.1 percent aqueous peroxidase solution (Horseradish Type VI-A; Sigma Chemical) and 100 µl of 1 percent aqueous o-dianisidine solution (Sigma Chemical), respectively. Instead of a test sample, 5 ml of sodium phosphate buffer was taken in blank. Tubes were incubated at 37 ° C for 10 min. The reaction was halted by adding 200 µl of 4N HCl to each test tube. Absorbance (A400 nm) was taken for each sample and the peroxide amount was calculated by comparing the A_{400} nm absorbance with the standard curve (24).

2.9. Determination of antimicrobial potential of the lactobacillus isolates

The lactic acid bacillus strains' antibacterial activity spectrum was analyzed by a well-diffusion assay. In soft nutrient agar of 20 ml (0.8 percent, w/v), 100 µl- of early stationary phase test bacterial culture was added. On the lawn of hardened soft agar in Petri dishes, Wells was bored. Aliquots (100 µl) of the overnight culture supernatant (16-18 h) were poured into the wells. The plates were left in sterile conditions at room temperature for 1 h, and then incubated for the growth of the test micro-organism at 37 ^{o}C.A clear zone (at least 2 mm in diameter) of inhibition was (25).

3.0. Results and Discussion

3.1. Sampling and study area

The current research was conducted in pre-menopausal Indian women (n=15, aged 15-45 years) who did not have anysymptoms of vaginal or urinary tract infection. For the study, female who were not menstruating and were not undergoing local or oral antimicrobial therapy within the previous 2 weeks were selected.Swab was collected from the mid vaginal secretion and the swab sample was immediately processed for bacteria isolation. The specimens were coded to maintain the inconspicuousness throughout the study. Based on their age participants were divided into groups. More participants were (n= 55) from the age group of 41-45. Vaginal bacterial communities were isolated and characterized from women'swho attended the OPD due to difficulties during menstruation, antenatal issues, and postnatal issues and attended for family planning and contraception.

3.2. Nugent's score of the isolates

Nugent's score was manifested and the scoring was done based on the Gram's stain of vaginal smear. Out of 15 patients 11 (80%) patient's smear has the score between 0-3 which depicts they are vaginosis negative. 3 (20%) smear has a score of 7 which was reported as bacterial vaginosis positive. Gram stained smears of all the 15 women were scrutinized using Nugent's scoring system. Majority, 82.7% of the women had normal vaginal flora corresponding to a score between 0-3. Depending on the smear findings, the study populations were delineated as those having normal (Grade I), intermediate (Grade II) and abnormal or BV flora (Grade III) as tabulated.

3.3. Gram's Stain

A gram positive bacterial cell appears as violet in color, while Gram negative bacterial cells appear as pink. In addition, the cell morphology was also recorded. The gram's staining results were enlisted.

3.4. Biochemical characteristics for identification of Lactobacillus

3.4.1. Sugar fermentation and Biochemical characterization

Among various sugars examined both isolates fermented the following sugars that includes glucose, fructose, sucrose, mannitol, maltose, mannose, galactose, d-xylose, trehalose, salicin and cellobiose,

but they are failed to ferment D-Arabinose, Rhamnose, Sorbitol and starch. The isolates were identified as members of lactic acid bacteria based on the sugar fermentation results.

Phenotypic characteristics of isolated bacteria: The morphology and results of Grams' reaction, oxidase, catalase, MRVP, and other biochemical tests for the isolates were enlisted. Most of the cultures which were catalase negative were also negative for citrate utilization and Indole test. The majority of the cultures were motile except for a few isolates.

The gram's staining results, depicts that the non-motile isolates showed clear cocci structure. Based on the Haemolytic test on Blood agar medium, isolate 15P1B2, 17P1B2 showed a slight transparent zone on the blood agar medium, which delineates that it hydrolyses RBCs. Majority of the isolates were positive for starch hydrolysis since it belongs to the lactobacilli group, those isolates showed effective lactose fermentation. The presence of exoproteolytic and exosaccharolytic activity was observed in samples and some bacterial isolates also carry sugar fermentation.

Among the whole isolates, 84 isolateshas the ability to ferment the following sugars which include Arabinose, Fructose, Glucose, Lactose, Galactose, Mannose, Maltose, Rhamnose, Cellobiose, Salicin, Starch, Amygdalin, Sucrose and positive for esculin hydrolysis. Detailed biochemical test results were observed for the lactobacilli.

3.5. Molecular characterization by 16 srRNA sequencing

3.5.1. 16 srRNA sequencing

For phylogenetic study in microbial ecology, the 16S rRNA gene encodes the small subunit of the ribosomal RNA molecule that has been shown to be a universal and accurate marker gene. It comprises nine hyper-variable regions (V1-V9) which has been used for the assessment of bacterial populations. Over the last few decades, sequence analysis of cloned 16S ribosomal RNA genes (16S rDNA) has evolved as the most consistent tool for analyzing the microbial diversity of environmental samples.

16S loci in 8 different species of Lactobacillus were amplified successfully. A concentration of 2.5mM of MgCl2, 2.5mM of dNTP, 20µM of primer, 3 units of Taq DNA polymerase and 30ng/µl of template DNA was found to be optimal for the amplification. Optimum annealing temperature for each primer was standardized by gradient PCR. Using universal primers 27F and 1492R, the 16S rRNA gene of the isolates was amplified. A single band with a length of about 1500 bp was obtained for all amplified items. Figure 1 and Figure 2 demonstrate the agarose gel electrophoresis of the 16S rRNA gene amplicon product using PCR.

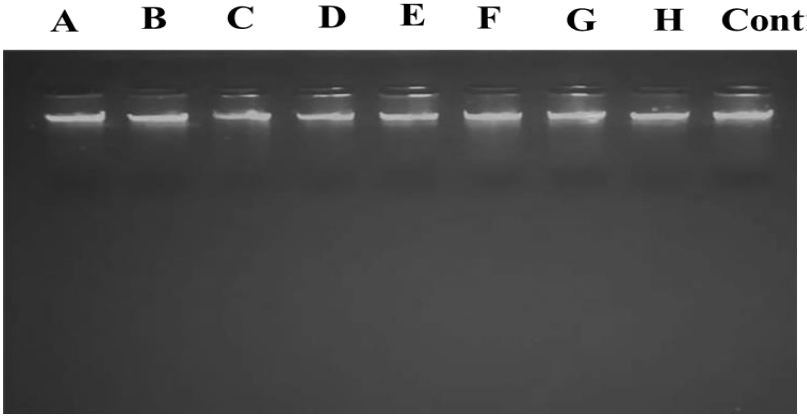

Figure 1 – Genomic DNA isolation bands of selected lactobacillus strains such as (A) 15P1B1, (B) 22P1B1, (C) 22P5B2, (D) 23P1B1, (E) 27P2B1, (F) 29P2B1, (G) 29P3B1 and (H) 44P2B1.

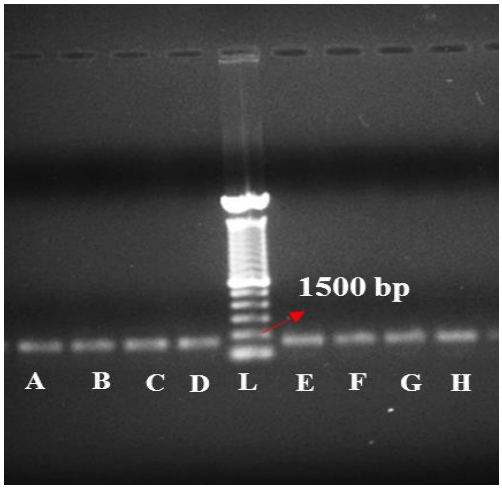

Figure 2 – Amplified PCR product and 16 S rRNA amplification bands (A) 15P1B1, (B) 22P1B1, (C) 22P5B2, (D) 23P1B1, (E) 27P2B1, (F) 29P2B1, (G) 29P3B1 (H) 44P2B1 and (L) molecular weight marker ladder DNA .

3.5.2. Sequence alignment of Lactobacillus spp.

To check the homology among the isolates and with others, the sequences were scrutinised by multiple sequence alignment. The similarity between the sequences varied from 91 to 98% among the isolates. Minimum of 91% homology was found in 15P1B1 with *Lactobacillus delbrueckii* and a maximum of 98% similarity was found between 22P5B2 and *L. reuteri*. None of these isolates were shown 99% or more similarity among themselves. Vaginal swab isolates 29P3B1 and 44P2B1 showed 95% similarities.

The isolate 29P2B1 showed 96% homology with two different *Lactobacillus* species, *Lactobacillus rhamnosus*. This isolate was identified as *Lactobacillus* spp by the phylogenetic tree. Fascinating fact is that the sources of all the isolates of NCBI database which were used to construct phylogenetic trees were probiotic bacteria. It correlates well with the fact that isolates of present study were also

isolated from traditional food and human gut and possess probiotic attributes. It is noteworthy that isolates 29P3B1 16S rRNA did not show high identity with sequences in the database. The sequences that share an identity below 98% are usually considered to be part of the same genus **Table 1.** On this basis, these isolates described here are probably representing a new member of a known genus (*Lactobacillus*), which has probiotic characteristics. Eight bacterial isolates 16S rRNA gene equences were determined in this study and deposited in the GenBank of NCBI data library.

Table 1 - Sequence alignment results of lactobacillus with other sps.

S.No	Species	Similarity with other isolate
1	*L. delbruckii*	98%
2	*L. reuteri*	99%
3	*L. salivarius*	97%
4	*L. fermentum*	95%
5	*L. plantarum*	98%
6	*L. crispatus*	99%
7	*L. rhamnosus*	98%
8	*L. acidophilus*	96%

3.5.3 Identification of lactobacilli at the species level using multiplex PCR assays

Lactobacilli are classified using multiplex PCR-G, *L. salivarius*, *L. delbrueckii*, *L. reuteri*, *L. plantarum*, *L. crispatus*, *L. fermentum*, *L. rhamnosus*, and *L. acidophilus* were successfully characterized by one of the four second-step multiplex PCR assays at the species level. The specificity of each group- and species-specific primer pair was confirmed using PCR amplification with DNA samples of 8 Lactobacillus prior tothe demonstration of the multiplex PCR assays, **Table 2.**

Table 2 - Discrimination of subspecies

S.No	RAPID type	Species specific PCR	Sequencing of 16S rDNA
1	D1	*L. delbruckii*	*L. delbruckii*
2	S1	*L. salivarius*	*L. salivarius*

3	R1	*L. reuteri*	*L. reuteri*
4	F1	*L. fermentum*	*L. fermentum*
5	P1	*L. plantarum*	*L. plantarum*
6	C1	*L. crispatus*	*L. crispatus*
7	Rh1	*L. rhamnosus*	*L. rhamnosus*
8	A1	*L. acidophilus*	*L. acidophilus*

The PCR assays were carried out with seven Lactobacillus type strains to investigate whether all unique primers are available for identification of the respective species. As anticipated, with the exception of the IDL11F primer, each pair of the specific and preserved primer generated a unique amplicon that differs in length from the others (approximately 0.2-1.1 kb), amplifying PCR products of the same size in *L. rhamnosus*and *L. casei* .Further we investigated whether the set of multiplex primers, comprising of all nine equimolar primers, must be relevant to each species for identificaton. All primers that are not interact with each other and seven Lactobacillus type strains with complete exclusion of unrelated species were explicitly identified by the multiplex PCR. Despite it was hard to discriminate L. *rhamnosus* (ca. 448 bp) from *L. plantarum* (ca. 428 bp) using their species-specific products, the troubles could be undoubtedly solved by the *L. casei* group-specific amplicon from *L. rhamnosus*. In order to assess the possibility of quick and easy detection without DNA extraction, the same experiments were repeated using cell suspensions as a guide. The results were in line with those who use genomic DNA. In addition, the individual strain in the mixed cell suspension of several lactobacilli was also determined. Even in the cases of the mixed sample containing *L. rhamnosus* and*L. plantarum* or other members of *L. casei* group, each species could be clearly differentiate from one another by the double bands respective to *L. casei*species-specific and group-specific amplicon from *L. rhamnosus***Table 3.**

Table 3 - Species identified by multiplex PCR

S.no	**Programme**	**Species**	**Amplicon**	**Primer (5'-3' sequence)**
1	PCR II-1	*L.acidophilus*	210 bp	L-aci-1 (TGCAAAGT GGTAGCGTAAGC) 23-10C CCTTTCCCTCACGGTACTG

2	PCR II-2	*L. crispatus*	522 bp	Lcri-3 AGGATATGGAGAGCAGGAAT Lcri-2 CAACTATCTCTCTTACACTGCC
		L. gasseri	360 bp	Lgas-3 AGCGACCGAGAAGAGAGAGA Lgas-2 TGCTATCGCTTCAAGTGCTT
3	PCR-III	*L. paracasei*	312 bp	Lpar-4 GGCCAGCTATGTATTCACTGA
		L. rhamnosus	113 bp	RhaII GCGATGCGAATTTCTATTATT
		L. salivarius	411 bp	Lsal-1AATCGCTAAACTCATAACCT Lsal-2 CACTCTCTTTGGCTAATCTT
4	PCR-IV	*L. reuteri*	303 bp	Lreu-1 CAGACAATCTTTGATTGTTTAG Lreu-4 GCTTGTTGGTTTGGGCTCTTC
		L. plantarum		Lpla-3 ATTCATAGTCTAGTTGGAGGT Lpla-2 CCTGAACTGAGAGAATTTGA

		L. fermentum	192 bp	Lfer-3 ACTAACTTGACTGATCTACGA Lfer-4 TTCACTGCTCAAGTAATCATC
		L.delbruckii	1,065-bp	Ldel-1 5'AAGGAGGTGATCCAGCCGCA3'

3.6. Probiotic attributes of *Lactobacillus* spp.

3.6.1. Microbial adhesion to solvents

The use of three solvents has made it possible to test the hydrophobic/hydrophilic cell surface properties of Lactobacilli and their Lewis acid-base (electron donor and acceptor) properties. As shown in **Table 4,** strains of *L. delbrueckii and L. fermentum* had a minimum percentage of separation in the polar solvent n-hexadecane, which delineates that those strains has a hydrophilic surface. All *L. crispatus, and L.fermentum*strains were distinguished by a maximum affinity to n-hexadecane, designating the nature of surface hydrophobicity.To evaluate the properties of Lewis acid-baseof the surface of bacteria, two solvents (chloroform and ethyl acetate) with the same van der Waals characteristics were applied,to ensure that the affinity of each solvent examined was not due to the forces of van der Waals. The microbial attachment to chloroform resultdecipherthat *L. delbrueckii and L. reuteri strains* had a less affinity for this acidic solvent, whereas all other strains had a potential affinity for it. When ethyl acetate, an electron donor, was employed for determining the microbial adhesion to solvents, attachment values were potentially high (ranging from 17.8 to 69.1%) for *L.delbrueckii*and *L. fermentum* strains, It indicates that there is also an acidic surface character of these bacteria. *L. crispatus*strains also exhibited relatively good adherence value (35%) to ethyl acetate.

Table4– Adhesion of bacterial isolates to solvents (n=3; observations comes from 3 replicative assays. Data represented as mean ± SD)

% Adhesion			
Isolated bacteria	**H-Hexadecane**	**Chloroform**	**Ethyl acetate**
L.delbruckii	53.89 ± 3	47 ± 1.7	51± 3

L. salivarius	45.43 ± 2	33 ± 3.2	41±2
L. reuteri	14.72 ± 2	17 ± 2.2	12±1
L. fermentum	12.24 ± 3	18 ± 1	17±3.4
L. plantarum	62 ± 2.3	67 ± 3.2	69±2
L. crispatus	33 ± 1.7	42 ±1.7	37±1
L. rhamnosus	21 ± 2.2	32 ± 3	35±1
L. acidophilus	17 ± 1.3	12±2	11±2

3.6.2. Hydrogen peroxide production by the isolated Lactobacillus Sps.,

Hydrogen peroxide is another antagonistic compound produced by Lactobacilli. Generally its production is evaluated using quantitative methods. In the current study, the isolate *Lactobacillus delbrueckii* showed maximum H_2O_2 production i.e. 3.18 mg/ml and minimum 0.31mg/ml was observed in *L. acidophilus*, while all other isolates exhibited moderate production of H_2O_2 ranging from 0.4-0.86 mg/ml (**Table 5**). Data also depicts that the production of lactic acid increased along with the incubation period ranging from 24hrs to 72 hrs.

Table 5 - H_2O_2 production by the isolated Lactobacillus species

S.No	Lactobacillus species (no. of isolates tested)	Negative	Strongly positive	Weakly positive
1	*L. delbrueckii (26)*	0	14	12
2	*L. reuteri (20)*	0	9	11
3	*L. salivarius (13)*	0	4	9
4	*L. fermentum (5)*	0	2	3
5	*L. plantarum (4)*	1	3	1
6	*L. crispatus (3)*	0	2	1

7	*L. rhamnosus (4)*	0	3	1
8	*L. acidophilus (5)*	1	3	1

3.6.3. Survival of *Lactobacillus* strains under acidic condition

Similar gastrointestinal tract areas will have different levels of acid. The maximum acidity of the stomach and the regions following the stomach and the pH of these areas can decrease to pH 2.0. Lactobacillus could be able to endure these hard environments and colonize the gut and thus, resistant acidity so that it can be used as abeneficial adjuncts. Lactobacillus must be able to endure these harsh environments and colonize the gut and thus, resistant acidity in order to be used as beneficial adjuncts. Survivals of six different *Lactobacillus* strains under acidic conditions (pH 2.0) are shown in **Figure 3 and 4.**Generally, after 3 hours of incubation, the number of survivors from all cultures decreased under acidic conditions**.** The viable count log CFU/ml substantially reduced at pH 2.0. *Lactobacillus delbrueckii*showed the maximum viability followed by *L. reuteri*, *L. fermentum*, *L. plantarum*, showed moderate activity at pH 2.0. As shown in Figure, *L. salivarius*had lowest viability at pH 2.0.

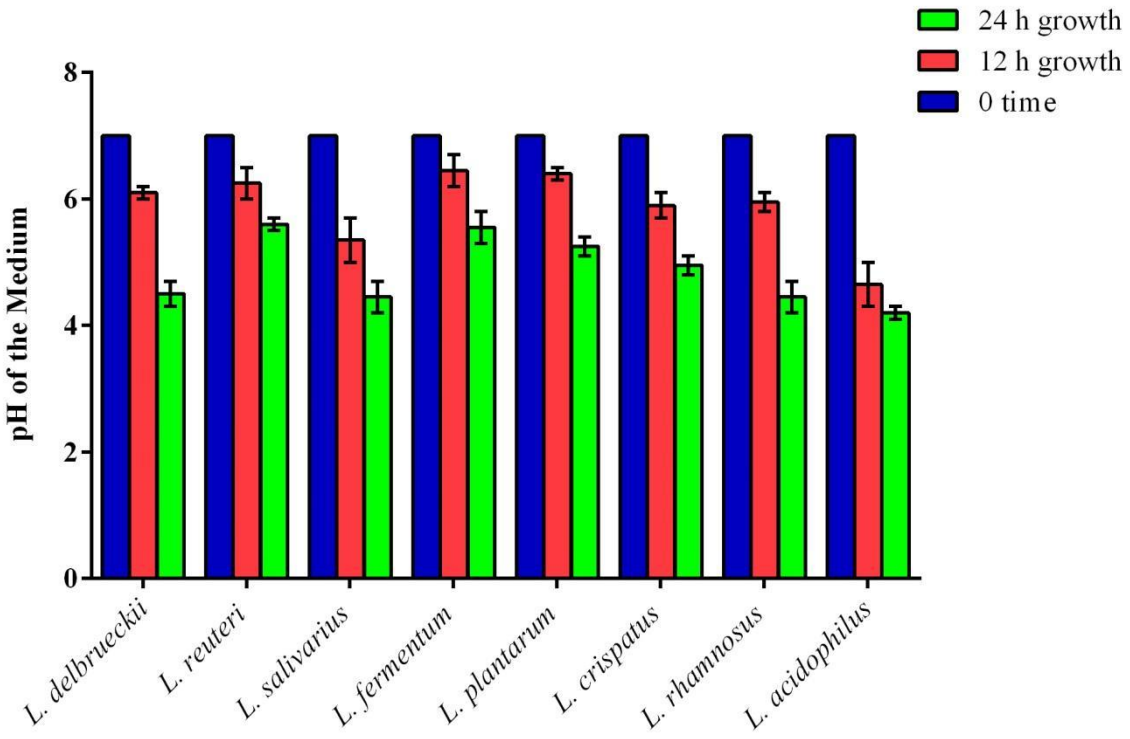

Figure 3 - Acid production by the isolates in 24 h of incubation

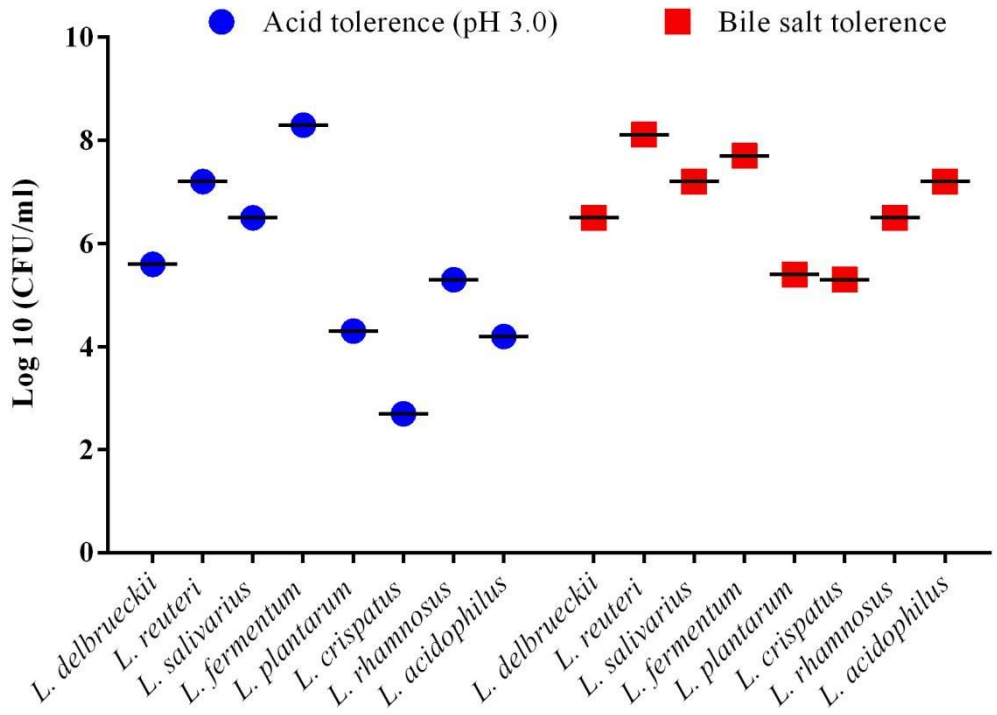

Figure 4 - Acid and bile salt tolerance of the isolates

3.6.4. Survival of *Lactobacillus* strains in the presence of bile

Gastrointestinal systems have different bile concentrations. In various regions of the intestine, the rate of bile secretion and bile concentration varies and depends primarily on the type of food taken, and it may not be possible to quantify the bile concentration of the intestine at any given time. Figure 4 shows the viable count of six separate Lactobacillus strains at bile concentrations of 0.3 percent. All eight strains of Lactobacillus display varying degrees of bile sensitivity for up to 3 h of incubation.*Lactobacillus delbrueckii*deciphered highest growth followed by *Lactobacillus reuteri*and *Lactobacillus crispatus*at growth was observed in 0.3% bile concentration. Left over *Lactobacillus* strains was observed to have better growth up to 3 h of incubation. Results of 0.3% bile concentration were enlisted in (Table 2). The isolate *L. fermentum, L. plantarum* showed sensible growth during incubation.

3.7. Auto-aggregation of *Lactobacillus* strains

Based on the sedimentation features of the strains, auto-aggregation was analysed because when cells accumulate, they clear the supernatant and sediment. Figure 5 showed aggregation values of more than 75% for L. delbrueckii, while *L. Reuteri and L. Crispatus* Auto-aggregated with a value below 30 percent. Even though both the strains were well known for their attachment with epithelial cells of the intestine, an apparent difference in auto-aggregation could be observed as *L.*

rhamnosus manifested highest auto-aggregation values (more than 80%), while those of *L. fermentum* were below 60%. There was no substantial difference in aggregation values obtained using acidic pH 4.0) and neutral (pH 7.4) PBS.Self-aggregation was only intensified for *L. salivarius* strain when analysed in their overnight supernatants and was significantly higher than that obtained in PBS at pH 4.0.

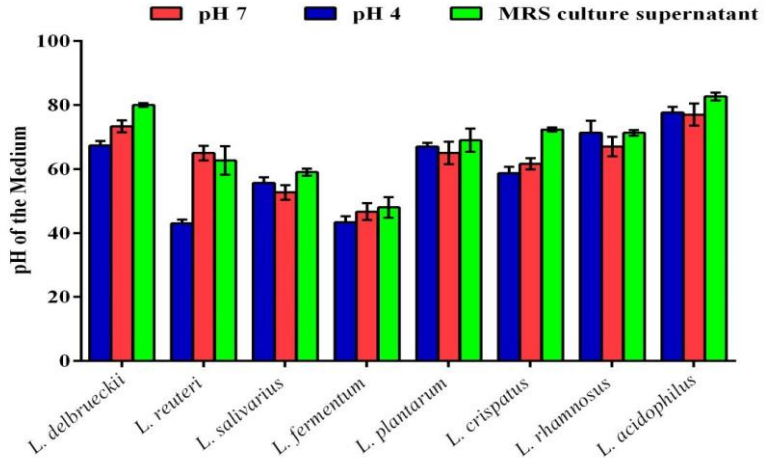

Figure 5 - Auto aggregation potential of the isolates

3.8. Antimicrobial activity of the isolates

The preliminary efficiency of the antimicrobial activity of the strains was evaluated by the agar well diffusion method with 1 M HCl/NaOH to eliminate the acid which can halt the pathogenic bacteria production in the supernatant. Apparent zone inhibition on the plate was observed, and the diameter of inhibition zone was ranged from 10 to 20 mm, which depicts the strength of antimicrobial efficacy of the 8 isolates they are, *L. delbruckii, L. salivarius*, *L. reuteri*, *L. plantarum*, *L. fermentum*, *L. rhamnosus*,*L. crispatus*, and*L. acidophilus* isolates. *Lactobacillus* Sps., displayed the greatest activity against the wide array of pathogens include *Staphylococcus aureus(MSSA) ATCC 25923*, *Staphylococcus aureus(MRSA)ATCC 39592*, *Enterococcus faecalis*ATCC35550, *Streptococcus pyogenes group A*ATCC14289, *E. coli ESBL* MTCC 8934, *Pseudomonas aeruginosa* ATCC25619, *Klebsiella pneumonia* ATCC11298, *Proteus vulgaris*ATCC6380,*Shigella flexneri* ATCC29508, *Proteus mirabilis* ATCC29906 ,*Shigella boydii*ATCC 8700 , *Shigella sony*, *Salmonella para* MTCC 3220and *Salmonella typhi* NCTC50017, with zones of inhibition greater than 15 mm.. With an inhibition zone of more than 14 mm, eleven strains evoked a potential antibacterial impact. In comparison with the other lactobacillus sps., the activity of *L. delbrueckii*against the pathogenic bacterial sps., comprehensively higher according to the zone of inhibition in the treatment group. The greatest activity against *Staphylococcus aureus(MRSA)ATCC 39592*, Staphylococcus *aureus(MSSA) ATCC 25923*, with zones of inhibition greater than or equal to 22 mm, were observed for the 3 isolates. Eventually, we have chosen *L. delbrueckii* for further tests of characterization and

antibacterial activity, because it has significantly higher antibacterial activities when compared to other strains ($p < 0.05$). Among the isolates, *Lactobacillus delbrueckii*exhibits potential antimicrobial activity against most of the tested pathogens, hence it was fixed as an organism of interest with great antimicrobial potential.

4. Conclusion

The results obtained in the current work delineatethat lactobacillus occurrence in the vaginal eco-system. Using PCR of genes and biochemical parameters encoding different putative probiotic markers, we have also characterized the isolates. In comparison to traditional microbiological methods that are laborious and repetitive, such molecular detection is beneficial for the rapid identification and selection of novel probiotic cultures.

Conflict of interest

Authors do not have conflict of interest

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