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Isolation and *In vitro* characterization of anti-*Gardnerella vaginalis* bacteriocin producing *Lactobacillus fermentum* HV6b isolated from human vaginal ecosystem

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Abstarct

Background & Objective : Vaginal isolate HV6b is an agent that could be used to combat growing prevalence of sexually transmitted microbial infections and viral diseases. Therapeutic application of this probiotic strain to protect against gastrointestinal infections may be of great importance for future medicinal use. **Methodology:** Bacteriocin producing strains of lactic acid bacteria were isolated from vaginal swabs of healthy and fecund females and evaluated for their antimicrobial activity against pathogens causing important human diseases such as gastrointestinal infections, nosocomial and skin diseases. **Results:** Bacteriocin HV6b shows maximum inhibition against bacterial vaginosis causing *G. vaginalis.* It was identified as *Lactobacillus fermentum* on the basis of biochemical testing and 16S rDNA sequencing. **Conclusion**: Based on the antibiotic sensitivity profiles vaginal LABs, HV6b was suggested as a strain for formulating topical personal care therapeutics aimed at prevention and treatment of many human diseases.

Keywords: *Lactic acid bacteria, Bacterial vaginosis, Gardnerella vaginalis, Bacteriocin*

1. Introduction

Bacterial vaginosis (BV) is an inflammatory disease of vagina that occurs when natural balance of the vaginal microflora gets disturbed due to microbial infections or trichomoniasis. Various studies across the world have shown association of *Bacteroides* spp. *(Prevotella* spp.), *Escherichia coli, Gardnerella vaginalis, Mobiluncus* spp., *Mycoplasma hominis*, *Peptostreptococcus* spp., *Staphylococci, Streptococci*, and/or viruses with BV[1-5]. BV is characterized by a milky or gray vaginal discharge with foul odor, presence of "clue cells" and an increase in pH of the vagina to >4.5[6-7]. Counts of anaerobic bacteria including *G. vaginalis*, *M. hominis* and others are drastically higher in diseased vagina. BV can cause adverse outcomes of pregnancy, including preterm delivery, premature labor, premature birth, infection of the amniotic fluid, infection of the uterus after delivery and death of the fetus or newborn[8]. An increased susceptibility to viral infections by HIV[9], HSV type 2[4] and other sexually transmitted diseases was also reported in BV patients.

G. vaginalis, a Gram-variable facultative anaerobe, is one of the key pathogen involved in BV and is characterized by fastidious, beta-hemolytic, non motile, unencapsulated, nature of bacilli[10]. It produces a pore forming toxin, vaginolysin, which affects only human cells. Microscopic analysis revealed that *G. vaginalis* biofilms grow quickly in the diseased vagina[11] and biofilms are also resistant to some forms of medical treatment[12].

Currently, preventive therapies for BV rely almost exclusively on the use of antibiotics such as metronidazole and gives initial cure rates of approximately 90% or better[13-15]. Metronidazole becomes widely distributed in the body and undergoes oxidative metabolism in the liver, with the formation of several metabolites[16]. High concentration of metronidazole, could partially suppress healthy microflora of vagina[17]. Metronidazole antibiotic therapy has been reported to impose several side effects such as diarrhea, dizziness, headache, loss of appetite, nausea or vomiting, stomach pain or cramps[18]. A number of reports have emerged that indicates emergence of drug resistance trait in *G. vaginalis*[19-21]. That's why more effective and safe therapeutics are desired to control BV.

Lactic acid bacteria (LAB) play a major role in maintaining a healthy vaginal ecosystem and prevent overgrowth of pathogenic bacteria in vaginal ecosystem[22]. Through the production of H_2O_2, organic acids and antimicrobial proteins called bacteriocins,

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probiotic LAB prevent many pathogenic bacteria from overgrowing and thereby creating a condition called BV[23-25]. Several investigators have isolated and partially purified bacteriocins from various strains of lactic acid bacteria[11, 26-28]. Mostly such investigations were conducted with non-human strains, predominantly isolated from food[29]. Moreover, emergence of antibiotic resistant phenotype in BV associated pathogenic bacteria, it has become essential to develop alternative therapeutics/prophylactic measures against these pathogens. Present study was undertaken with the aim to isolate and characterize anti-*Gardnerella vaginalis* bacteriocin producing isolate from human vaginal ecosystem that remains unexplored.

2. Materials and Methods

2.1 Sample collection and isolation of lactic acid bacteria:

Vaginal swabs from healthy and fecund females of reproductive age group were collected with informed consent and were transferred immediately to sterilized saline (0.85% NaCl). 1 ml sample was inoculated into sterile MRS broth (containing peptone 10 g/l, yeast extract 5g/l, beef extract 10g/l, dextrose 20g/l, ammonium citrate 2g/l, sodium acetate 5g/l, $MnSO_4$ 0.05g/l, $MgSO_4$ 0.01g/l, dipotassium phosphate 2g/l, tween-80 1ml/l, pH 6.5) for propagating vaginal LAB microflora and incubated for 18 to 24 h at 37°C. Samples were sub-cultured three times before proceeding with bacterial isolation and activity assays. LAB strains were isolated on MRS agar plates using pure culture techniques.

2.2 Bacterial strains and culture media used to study antimicrobial spectrum:

The inhibitory spectra of bacteriocin producing human vaginal LAB isolates was evaluated against important human pathogens, using spot-on-lawn[30] and well-diffusion methods[31]. Growth requirements of indicator microorganisms are specified in Table I. Indicator strains were revived and maintained in growth media as prescribed by culture banks.

2.3 Bacteriocin activity assays:

Isolated strains were subcultured thrice in MRS medium (pH 6.5) at 37°C for 24 h before proceeding with bacteriocin activity assays. 1ml aliquot of broth culture was centrifuged at 10,000 rpm for 10 min and cell free supernatant (CFS) was collected in sterile micro-

Table I: Growth media and conditions of indicator microorganisms

Microorganism	Gram Nature	Growth Medium	Nature	Temp. (°C)/pH	Incubation time
General Human Pathogens					
Bacillus subtilis ATCC 6633	+ve	NB	Aerobe	37/7.4	24h
Clostridium perfringens MTCC 450	+ve	RCB	Anaerobe	37/6.8	48h
Escherichia coli BL21 DE3 MTCC 1679	-ve	LB	F. anaerobe	37/7.2	24h
E. coli DH5α MTCC 1652	-ve	LB	F. anaerobe	37/7.2	24h
E. coli KL16 MTCC 1650	-ve	LB	F. anaerobe	37/7.2	24h
Enterococcus faecalis (Lab isolate)	+ve	MRS	F. anaerobe	37/6.5	24h
E. faecalis (NDRI isolate)	+ve	MRS	F. anaerobe	37/6.5	24h
E. faecalis ATCC 29212	+ve	MRS	F. anaerobe	37/6.5	24h
Gardnerella vaginalis ATCC 14018	+ve	CB	Anaerobe	37/7.2	24h
Klebsiella pneumoniae NCIM 2883	-ve	NB	F. anaerobe	30/7.2	24h
K. pneumoniae MTCC 4030	-ve	NB	F. anaerobe	30/7.2	24h
Leuconostoc mesenteroides MTCC 107	+ve	MRS	Aerobe	25/6.5	48h
Listeria monocytogenes MTCC 657	+ve	BHI	Aerobe	37/7.4	24h
N. mucosa MTCC 1772	-ve	NB	F. anaerobe*	37/7.2	24h
Pseudomonas aeruginosa ATCC 10662	+ve	NB	Aerobe	30/7.4	24h
Salmonella typhi NCTC 5760	-ve	BHI	F. anaerobe	37/7.4	24h
Vibrio cholera ATCC 14104	-ve	NB	Aerobe	37/7.4	24h
Yersinia enterocolitica MTCC 861	-ve	BHI	F.anaerobe	30/7.2	12h
Non-Pathogenic Microorganisms					
Lactobacillus brevis MTCC 1750	+ve	MRS	F. anaerobe	30/7.4	24h
L. bulgaricus NCDC 253	+ve	MRS	F. anaerobe	37/6.5	24h
L. casei NCIM 2651	+ve	MRS	F.anaerobe	37/6.5	24h
L. helveticus NCIM 2126	+ve	MRS	F.anaerobe	37/6.5	24h
L. leichmanni NCIM 2027	+ve	MRS	F. anaerobe	37/6.5	24h
L. pentosus NCIM 2669	+ve	MRS	F. anaerobe	37/6.5	24h
L. plantarum NCIM 2912	+ve	MRS	F. anaerobe	37/6.5	24h
Lactococcus lactis subsp. *cremoris* MTCC 1484	+ve	TSY	Aerobe	20/6.5	24h
Pediococcus acidilactici LB 42	+ve	MRS	Aerobe	30/6.5	24h

F. anaerobe- Facultative anaerobe*

centrifuge tube. CFS was heat treated in boiling water bath for 20 min and allowed to cool at room temperature. Bacteriocin activity was assayed using spot-on-lawn[30] and agar well diffusion methods[31].

2.4 Investigation of antibiotic susceptibility: Antibiotic susceptibility of human vaginal LAB isolates was studied against different concentrations (2 to 8 mg/ml) of some commonly prescribed antibiotics to BV patients including ampicillin, amoxicillin, amoxicillin and clavalanic acid, azithromycin, ciprofloxacin, co-trimoxazole, erythromycin, gentamycin, metronidazole, nalidixic acid, ofloxacin, penicillin, rimphicin, tetracycline, tinidazole and vancomycin by spot-on-lawn method using[30].

2.5 Biochemical characterization of Human vaginal LAB isolate:

Preliminary identification was preceded according to Bergey's Manual of Determinative Bacteriology-9th Edition[33] and Methods for General and Molecular Bacteriology[34]. Overnight grown cultures of bacteriocin producing isolates were Gram stained and examined microscopically for morphological and phenotypic properties. Growth of isolates was observed in MRS broth at 10 and 45°C temperature and at pH 9.6 by incubating at 37°C. Salt tolerance was tested by incorporating 6.5% NaCl (w/v) in MRS broth. Bile test was performed by adding 30% bile salt to culture medium. Catalase activity was tested in 3% hydrogen peroxide and oxidase test was performed by adding tetramethyl paraphenyl diamino dihydrochloride to a test tube containing 24h old culture. The sugar fermentation profile of isolate was checked for glucose (1%), lactose (5%), mannitol (1%), sucrose (1%), dulcitol (1%), salicin (1%) by adding 1 ml of Andrades indicator in MRS broth containing inverted Durham's tube. Indole test was performed using peptone water. Peptone water was inoculated and incubated overnight. After 24h Kovac's reagent was added and color was observed. Methyl red test and Voges-Proskaeur test were performed by inoculating in glucose phosphate medium. Production of ammonia from arginine was assessed in arginine broth. Arginine deamination was detected using Thornley's semi solid medium. Lactic acid production was determined by HPLC on C_{18} reverse phase column by maintaining flow rate of 0.8ml/min using phosphate buffer (pH 2.4) in PDA 214 nm detector and compared with C-18 reverse phase chromatogram of pure lactic acid. Lactic acid production was also analyzed by gas chromatography using polyethylene glycol (PEG) column.

2.6 Molecular Characterization:

The 16SrRNA gene sequencing of HV6b isolate was carried out by Bangalore Genei Private Limited, Bangalore, India. 16SrRNA gene sequence was uploaded to Genbank database and the strain was deposited with MTCC culture bank, India.

2.7 Statistical analysis of data:

QI Macros ANOVA was used to calculate all statistical parameters. Statistical tools like ANOVA one factor was used to determine significance of the results obtained in duplicate experimental sets.

3. Results and Discussion

3.1 Isolation of bacteriocin producing LAB strains:

In the present study, more than 100 bacterial strains were isolated from vaginal swabs and further screening was done on the basis of bacteriocin producing activity. Bacteriocin production was observed in only eight isolates designated as HV6, HV6b, HV75, HV76, HV54A, HV59A, HV59C, HV59D and HV69. Their antibacterial activity was tested against many indicator strains implicated in BV, gastrointestinal, skin and lung diseases in humans (Table I). Isolate HV6b possess extremely attractive antimicrobial spectrum which was highest among the tested indicators especially *G. vaginalis* ATCC14018 and therefore, was selected for further detailed study.

3.2 Antimicrobial spectrum of the isolated LAB strains:

Antimicrobial activities of isolated LAB strains were comparatively investigated against a panel of microorganisms and highly significant results were obtained in the study (Table II; Figure I and II). A number of pathogenic and non-pathogenic tested Gram-positive bacterial strains were inhibited by the bacteriocin producing LAB isolates except species like *B. subtilis, C. perfringens, E. faecalis* (NDRI isolate), *L. casei* NCIM 2651, *L. leichmanni* NCIM 2027, *L. plantarum* NCIM 2912, *L. pentosus* NCIM 2669, *L. lactis* subsp. *Cremoris.* Among non-pathogenic lactic acid bacteria *L. brevis, L. bulgaricus, L. helveticus* and *P. acidilactici* LB42 were strongly inhibited by isolate HV59D and HV69 respectively. Isolates produced anti-listerial bacteriocins as they successfully inhibited *L. monocytogenes* that causes spectic abortion, newborn and adult septicemia, listeriosis, meningitis and meningo-encephalitis in immune-deficient persons[35]. Other pathogens including food spoilage causing *L. mesenteroides* was successfully inhibited by most of the vaginal LAB isolates. *L. mesenteroides*, an epiphytic bacterium that plays an important role in several industrial food fermentations, is also responsible for nosocomial infections[36,37]. It is of interest to the food and pharma industry that vaginal isolates exhibited broad inhibitory spectrum against food borne pathogens as well as spoilage organism. Bacteriocins produced by human vaginal isolates are effective against *E. faecalis* that can cause life-threatening infections in humans, especially in the nosocomial (hospital) environment, where the naturally high levels of antibiotic resistance contribute to its pathogenicity[38,39] . In accordance with earlier reports, the utility of bacteriocins to control these human diseases can be regarded as the best alternative to antibiotic therapy[40-41].

Opportunistic pathogens of humans including *P. aeruginosa* also gets moderately inhibited by LAB isolates obtained from human vaginal swabs. *P. aeruginosa* sometimes exists as a part of normal flora of humans. However, in immune-deficient persons, it causes chronic lung infections, burn and eye infections,

Table II: Antimicrobial spectrum of human vaginal LAB isolates

Microorganism	Diameter of inhibition zone (mm)								
	6	6b	75	76	54A	59A	59C	59D	69
General Human Pathogens									
B. subtilis ATCC 6633	-	-	-	-	-	-	-	-	-
C. perfringens MTCC 450	-	-	-	-	-	-	-	-	-
E. coli BL21 DE3 MTCC 1679	-	-	-	-	-	-	-	-	-
E. coli DH5α MTCC 1652	12	16	18	16	11	14	19	20	18
E. coli KL16 MTCC 1650	18	17	16	18	16	14	16	20	17
E. faecalis (Lab isolate)	23	24	22	24	23.5	24	12	13	12
E. faecalis (NDRI isolate)	-	-	-	-	-	-	-	-	-
E. faecalis ATCC 29212	-	-	12	-	-	-	19	18	15
G. *vaginalis* ATCC 14018	22	23	19	22	18	22.5	14	15	13
K. pneumoniae NCIM 2883	16	17	14	-	-	-	19	20	13
K. pneumoniae MTCC 4030	16	14	13	14	15	14	13	12	14
L. mesenteroides MTCC 107	13	18	-	-	12	10	21	25	18
L. monocytogenes MTCC 657	15	13	14	13	14	14	13	12	11
N. *mucosa* MTCC 1772	15	18	16	8	15	14	15	16	17
P. aeruginosa ATCC 10662	16	15	16	14.5	13	14	13	13	12
S. typhi NCTC 5760	12	14	18	17	15	15	13	15	17
V. cholerae ATCC 14104	12	12	12.5	11	14	14	13	13.5	12
Y. enterocolitica MTCC 861	-	-	-	-	-	-	-	-	-
Non-Pathogenic Microorganisms									
L. brevis MTCC 1750	-	13	-	15	-	-	16	18	17
L. bulgaricus NCDC 253	-	-	-	-	-	-	12	11	14
L. casei NCIM 2651	-	-	-	-	-	-	-	-	-
L. helveticus NCIM 2126	17	19	18	17	16	13	17	12	14
L. leichmanni NCIM 2027	-	-	-	-	-	-	-	-	-
L. plantarum NCIM 2912	-	-	-	-	-	-	-	-	-
L. pentosus NCIM 2669	-	-	-	-	-	-	-	-	-
L. lactis subsp. *cremoris* MTCC 1484	-	-	-	-	-	-	-	-	-
P. acidilactici LB 42	22	22	21	21	23	21	12	13	14

Each data is an average of two samples; P value<0.05; Fcrit (1.7452)<F value (26.629)

(-* negative reaction) Each data is an average of two samples; P value<0.05; Fcrit (2.0698)<F value (33.614)

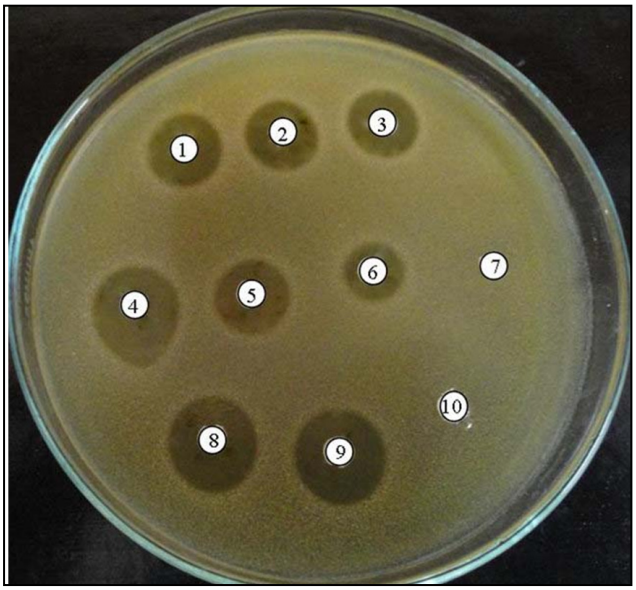

Figure I: Spot-on-lawn bacteriocin activity assay

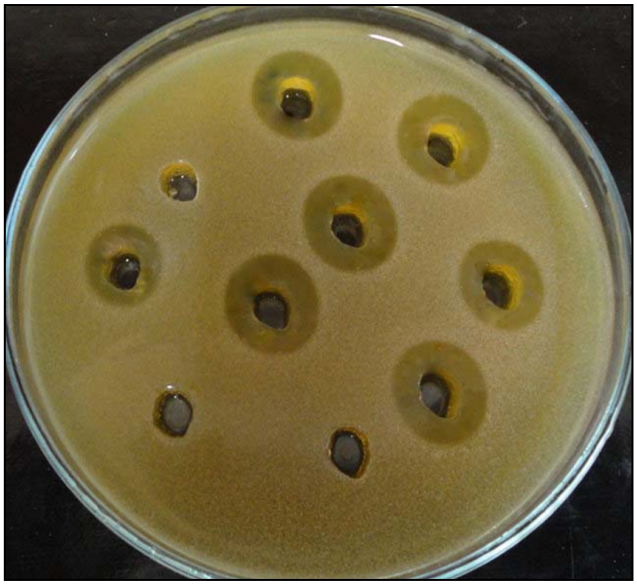

Figure II: Well diffusion bacteriocin activity assay

pneumonia; thus being a serious problem in patients hospitalized with cancer, cystic fibrosis and burns[43]. Isolated bacteriocins have a great potential in formulating nasal and oral sprays and can be studied to

control such infections in model systems. Growth inhibition of UTI pathogen *N. mucosa* by bacteriocins of vaginal LAB isolates makes them potential ingredient of anti-neisserial skin/mucosal formulations to eradicate such opportunistic UTI pathogens[44].

G. vaginalis causing bacterial vaginosis in humans was strongly inhibited by human vaginal isolates 6, 6b, 76 and 59A. Several investigators have isolated and partially purified bacteriocin from different species of lactobacilli. Most of them were with nonhuman strains, predominantly isolated from food[27,29,45]. A heat-resistant peptide was extracted from a vaginal *Lactobacillus salivarius,* which inhibited growth of important UTI pathogens such as *E. faecalis, E. faecium,* and *N. gonorrhoeae*[26]. Bacteriocin HV219 produced by *L. lactis* isolated from human vaginal secretions was found to inhibit Gram-positive and Gram-negative bacteria[46]. Aroutcheva and coworkers[29] purified an antimicrobial protein produced by endogenous vaginal *L. acidophilus* 160 against *G. vaginalis*. Recent study reveals that the molecular mechanisms of action for the lactocin 160 target the cytoplasmic membrane of *G. vaginalis*, causing the reflux of ATP molecules and dissipation of the proton motive force or PMF of sensitive cells[47].

Antibiotic resistance is being increasingly reported around the World. Goldstein and others[48] had demonstrated resistance of *G. vaginalis* to metronidazole. Recurrence rates of upto 30% within three months after treatment have been reported in literature[19]. Bacteriocin produced by LAB isolate HV6b has a great potential to control *Gardnerella* associated BV in humans. The potential use of human lactobacilli as probiotics assigned to restore and maintain a healthy urogenital tract represents a promising alternative to conventional chemotherapy[40,49-50]. Colonization of the vaginal epithelial cells with *Lactobacillus* successfully thwarts the subsequent colonization of the cell surface with harmful bacterial, yeast infections[51]. Present study therefore aimed at investigating *in vitro* efficacy of a human vaginal LAB isolates in inhibiting growth of BV associated pathogens and further characterization of the isolate itself.

Antimicrobial spectrum of the isolated LAB strains was also studied against a battery of Gram-negative pathogenic as well as non-pathogenic bacteria. LAB isolates inhibited growth of many bacterial species up to its maximum extent except species like *E. coli* BL21 and *Y. enterocolitica* (Table II). All the tested strains of *Bacteroides* spp. were inhibited by isolate HV6b. *B. fragilis* is reported to cause obstetric and gynecologic infections. *B. ovatus* infections can develop in all body sites, including the CNS, the head, the neck, the chest, the abdomen, the pelvis, the skin, and the soft tissues[52]. *B. vulgatus* is commonly found in patients with ulcerative colitis. Inadequate therapy against these anaerobic bacteria may lead to clinical failure, thus probiotic therapy consisting of bacteriocin producing *Lactobacilli* is being suggested here[53]. *K. pneumonia*, an opportunistic pathogen for patients with chronic nosocomial disease, enteric pathogenicity, nasal mucosa atrophy, and rhinoscleroma, is highly sensitive to bacterioins secreted by vaginal LAB isolates[54]. *C. albicans, Mobiluncus, M. hominis, Peptostreptococcus* spp. and *P. bivia* are some other BV associated pathogens of urinary tract. Inhibition of *C. albicans* was also studied which indicated moderate to high inhibition by bacteriocins of LAB origin especially isolate HV6b. Some strains of *E. coli* were also inhibited by the isolated bacteriocin producers. *Lactobacilli* have been recommended as GRAS bio-therapeutic agents for cure as well as prevention of human gastrointestinal and vaginal diseases. Colonization of the infected tissue by health promoting LAB particularly prevents infection by synthesizing a variety of antagonistic factors such as bacteriocins, diacetyl, H_2O_2, fungicidal agents etc[22-23,25] and by competing for available nutrients and mannose sugar and by interfering pathogen attachment to cell surface receptors[55-56]. An acidic pH of vagina alone is not sufficient to inhibit vaginal pathogens and to prevent bacterial vaginosis[22-23]. Thus, bacteriocin based therapeutics are urgently desired to cure such diseases and to overcome problems associated with antibiotic therapy such as diarrhea, poor compliance and recurrence of vaginal infections. There is increasing body of evidence that indicate potential of GRAS lactic acid bacteria in maintaining and restoring gut homeostatis[57]. Use of live probiotic bacteria may have prophylactic applications, but use of purified bacteriocins appears to be more attractive for eradicating an established infection[58]. Ideally, anti- BV or anti-diarrheal therapeutics should specifically target disease causing microorganism and should have least interference with health promoting commensal microflora. In fact, the spectrum of activity of *L. fermentum* HV6b may be extremely well suited for targeting specific pathogens in vivo. In contrast to it, antibiotic prescribed frequently to cure gut and vaginal infections, strongly inhibit most of these beneficial microorganism at much lower concentrations[59].

3.3 Antibiotic susceptibility of vaginal LAB isolates:

Vaginal isolates displayed variations in their sensitivity to commonly prescribed antibiotics for treating BV. The safety investigation of the bacterial vaginal isolates revealed that they were sensitive to antibiotics viz. ampicillin, amoxicillin and clavalanic acid and penicillin but express a natural resistance phenotype to amoxicillin, azithromycin, erythromycin, gentamycin, metronidazole, nalidixic acid and tinidazole. Maximum inhibition zone was observed in case of isolate HV6 against ampicillin (20 mm) followed by amoxicillin and clavalanic acid (19 mm). Antibiotic susceptibility of vaginal isolates against various antibiotics is given in table 6. HV6b, HV54A and HV75 exhibited resistance to ampicillin. Strain HV69 shown its resistance to amoxicillin and clavalanic acid and its sensitivity to amoxicillin, azithromycin and metronidazole (Table III).

3.4 Biochemical characterization of human vaginal LAB isolate:

The selected isolate HV6b was identified biochemically as per Bergey's Manual of Determinative Bacteriology 9th Edition. The strain consisted of Gram-positive rods arranged singly or in pairs and small chains. The strain

Table III: Antibiotic susceptibility of vaginal LAB isolates

Antibiotics used	Antibiotic susceptibility of bacterial isolates (mm)							
	6	6b	54A	59A	59C	59D	69	75
Ampicillin	20	R	R	12	13	15	16	R
Amoxicillin	R	R	R	R	R	R	13	R
Amoxicillin + Clavalanic acid	19	15	14	12	12	12	R	13
Azithromycin	R	R	R	R	R	R	14	R
Erythromycin	R	R	R	R	R	R	R	13
Gentamycin	R	R	R	R	R	R	R	R
Metronidazole	R	R	R	R	R	R	13	R
Nalidixic acid	R	R	R	R	R	R	R	R
Penicillin	15	12	12	13	12	13	R	14
Tinidazole	R	R	R	R	R	R	R	R

Each data is an average of two samples; P value<0.05; Fcrit (2.0166)<F value (9.468)

R: Resistance

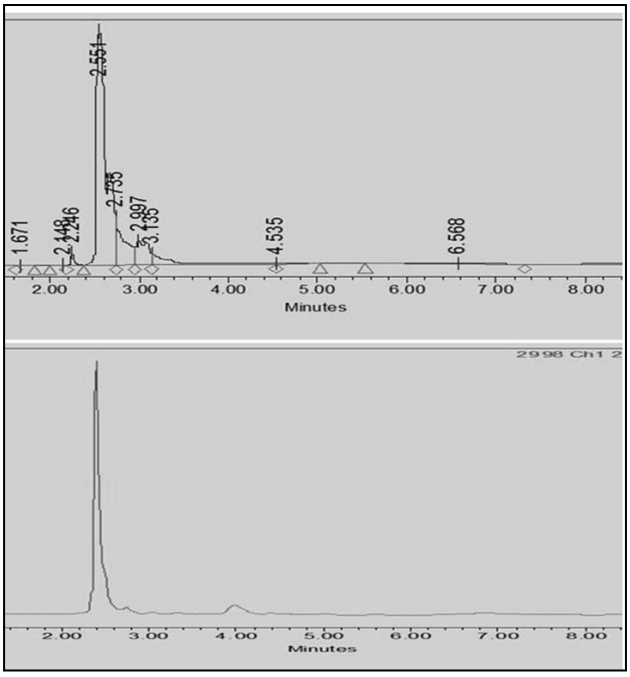

Figure III: C-18 Reverse phase chromatogram of **a)** cell free supernatant; **b)** pure lactic acid

was found to be catalase negative and identified as *Lactobacillus*. The isolate was able to grow under alkaline environment (pH 9.6) at 10 and 45°C. The optimum temperature for growth of the organism is 35 to 38°C. It was able to ferment mannitol, lactose, sucrose and glucose. *Lactobacillus* isolate HV6b did not show oxidase activity and indole production. Isolate HV6b was unable to grow in the presence of 6.5% and 18% NaCl but is salt tolerant as showed growth on addition of 30 % bile salt in the culture medium. It gave a positive methyl red test, whereas a negative Voges-Proskaeur reaction. Strain was capable of producing ammonia from arginine that reflects presence of arginine deaminase activity[63-64]. Lactic acid production was analyzed by HPLC. Retention time of pure lactic acid on C-18 reverse phase column was 2.45 min (Figure IIIb) and retention time of sample was 2.55 min (Figure IIIa). Results confirmed production of lactic acid by the isolated strain. It was a homo-fermentative lactic acid bacterium as there is no additional peak in the reverse phase chromatogram pertaining to other organic acids.

3.5 Molecular characterization of LAB isolate:

Preliminary biochemical identification was confirmed and validated by molecular characterization. For molecular typing 16SrRNA sequencing was done by Bangalore Genei Private Limited, Bangalore, India. The genotypic analysis confirmed the isolated strain HV6b as *Lactobacillus fermentum*. This sequence was deposited in NCBI's Genbank database vide Accession no. HQ214673 and deposited with MTCC culture bank, Chandigarh vide MTCC No. 10770. The phylogenetic standing of the isolated strain of *Lactobacillus fermentum* on the basis of 16S rDNA is represented in the clades (Figure IV). Phylogeneic analysis revealed that the isolated *L. fermentum* HV6b had a close homology with other strains of *Lactobacillus fermentum*[64].

3.6 Statistical analysis of data:

QI Macros ANOVA statistical tools like ANOVA one factor was used to determine significance of the results obtained in duplicate experimental sets. Level of significance was adjusted at 0.05 and results were said to be significant if their P value<0.05 and Fcrit<Fvalue according to one way ANNOVA.

4. Conclusion

In the last two decades, a variety of antagonistic bacteriocins, mostly produced by lactic acid bacteria, have attracted the attention of food and pharmaceutical sector for their potential use as natural food biopreservatives, probiotic formula foods and health care products[49-50,65]. Most of the LAB bacteriocins show a relatively narrow inhibitory spectrum, while only few of them could inhibit diverse groups of Gram-positive and Gram-negative bacteria[50,66]. A highly potent anti-*Gardnerella vaginalis* bacteriocin producing isolate from human vaginal ecosystem was characterized for its antimicrobial spectrum. Bacteriocin production trait of *L. fermentum* HV6b isolate was studied by spot-on-lawn

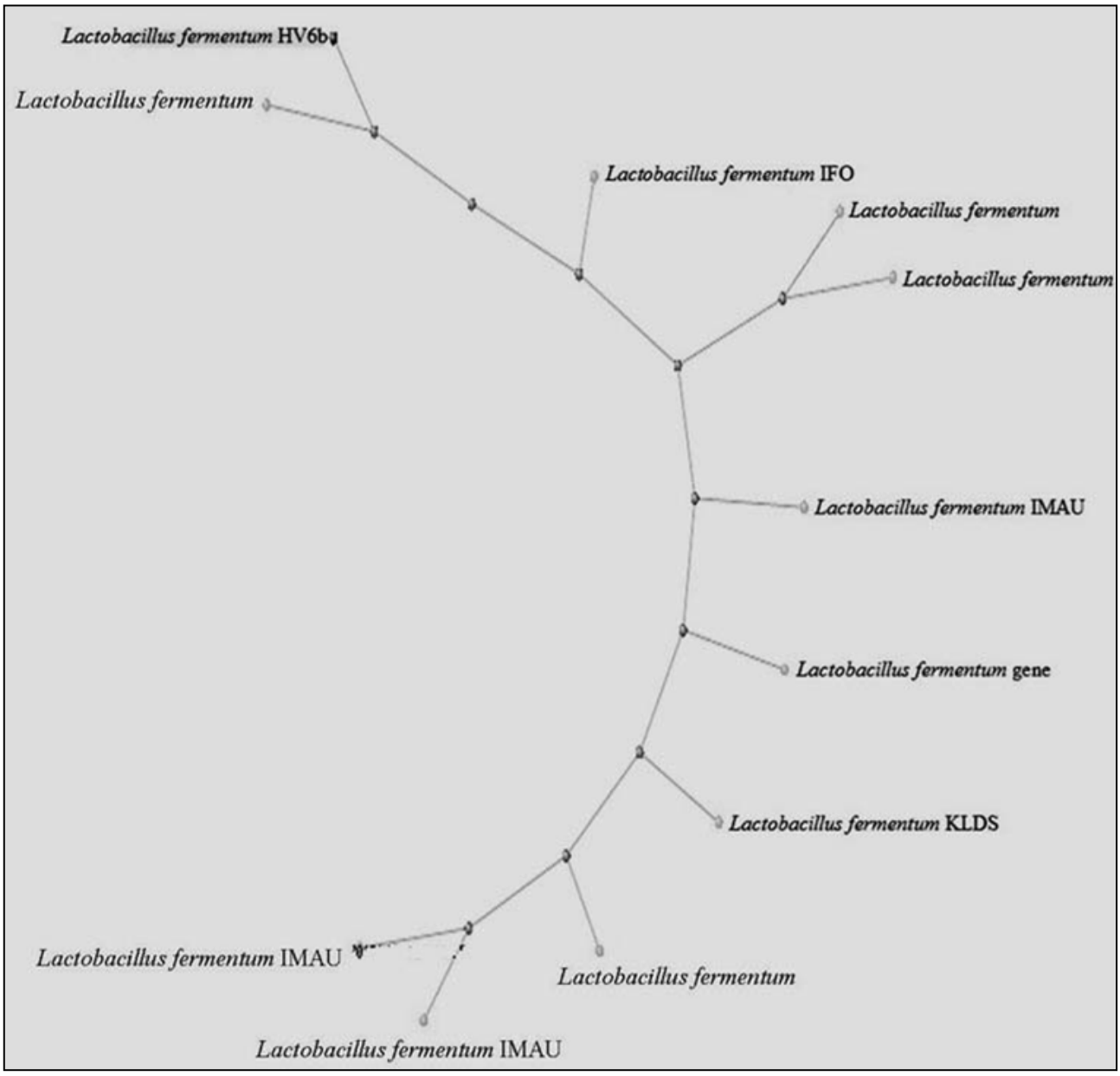

Figure IV: Phylogenetic relationship based on 16S rRNA sequence of *L. fermentum*

and agar well diffusion methods against important human pathogens causing gastrointestinal infections, nosocomial and skin diseases.

Antibiotic susceptibility of the isolated vaginal strains was tested against many commonly prescribed antibiotics. Based on the results obtained in this study, human vaginal LAB isolates are strongly recommended for formulating anti-gardnerella, therapeutics/ointments/ vaginal creams aimed at prophylaxes and treatment of BV and other sexually transmitted diseases in combination with antibiotic therapy that could check recurrence of the disease after termination of antibiotic treatment.

Colonization of the vaginal ecosystem by probiotics LAB can particularly prevents vaginal infection by bacteria and fungi. Moreover, bacteriocin based therapeutics are urgently desired to overcome undesirable side effects of antibiotics therapy. There is also an increasing body of evidence that indicate potential of the probiotics in cure and prevention of gut infections, various forms of diarrhea, enterocolitis, peptic ulcers and inflammatory bowel disease. Growing scientific evidences have proven efficacy of probiotics LAB strains in maintaining and restoring gut as well as vaginal homeostasis. Antimicrobial properties of the selected isolate are supporting their potential therapeutic usage for formulating probiotic dietary supplements, yogurts, drinks, capsules and personal care products. Use of probiotic LAB may have prophylactic application, while the isolated bacteriocin appears to be more attractive for eradicating an established microbial infection. Isolate HV6b showed inhibition of most of the tested pathogens. But it did not interfere with most of the Gram-positive probiotic lactic acid bacteria tested in the study. Therefore, there is a least possibility of its interference with normal human microflora, in contrast to frequently prescribed antibiotics. Isolate HV6b was

identified on the basis of biochemical tests and 16S rDNA sequencing as *Lactobacillus fermentum.* Its 16SrDNA sequence was deposited in NCBI's Genbank database wide accession no. HQ214673 and the culture was deposited in MTCC culture bank, Chandigarh. Preliminary experiments have established bacteriocin produced by *L. fermentum* sp. HV6b as an effective antimicrobial agent, with very impressive market value to formulate personal care products. Continued study of some aspects, especially its toxicity assessment on human tissues in vitro / in vivo and dose formulation etc., is desired in order to exploit its full potential as a pharmaceutical. Further studies are required for proposing a triple therapy formulation including antibiotic, bacteriocin producer / pure bacteriocin and a pH regulator for infections. Statistical approaches could be exploited for cost effective production of pure bacteriocin. This preliminary study has successfully revealed the desirable antimicrobial properties of *L. fermentum* sp. HV6b as a therapeutic. It could be integrated and exploited with the state of art knowledge to fully explore their suitability as in vivo therapeutic.

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