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Cervicovaginal microflora isolated from healthy women exhibited probiotic properties and antimicrobial activity against pathogens isolated from cervical cancer patients

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Abstract

Abnormal cervicovaginal microflora plays an important role in HPV persistence and progression to cervical cancer. The present study aimed at isolating and identifying probiotics from vaginal swabs of healthy women and evaluating their activity against vaginal pathogens isolated from cervical cancer patients. Based on probiotic, acid-bile tolerance and antimicrobial properties, 13 lactic acid bacteria (LAB) from the healthy group were identified by MALDI TOF MS. Among these, four strains, *Lactobacillus gasseri* P36Mops, *Limosilactobacillus fermentum* P37Mws, *Lactobacillus delbrueckii* P31Mcs and *Enterococcus faecium* P26Mcm, exhibited significant antimicrobial activity against 8 vaginal pathogens (*Staphylococcus haemolyticus* P41Tcs, *Escherichia coli* P30Tcs, *E. coli* P79Bcm, *Enterococus faecalis* P29Mops, *E. faecalis* P50Tws, *E. faecalis* P68Tcb, *S. haemolyticus* P48Bcb and *S. haemolyticus* P58Bcb) isolated from precancerous and cervical cancer patients. 16S rRNA sequencing of four potential probiotics revealed congruency with the MALDI-TOF MS (Matrix Assisted Laser Desorption and Ionisation, Time Of Flight Mass Spectrometry) identification and phylogenetic analysis showed genetic relationship with previously reported LAB strains. The selected LAB showed strain specific hydrophobicity (35.88-56.70%), auto-aggregation (35.26-61.39%) and antibiotic susceptibility. Interestingly, *L. gasseri* P36Mops was resistant to five standard antibiotics routinely used against urogenital or vaginal infections. LCMS (Liquid Chromatography Mass Spectrometry) analyses of the CFS (cell-free supernatant) of the four probiotics revealed presence of metabolites such as N-(1-deoxy-1-fructosyl)valine, hygroline, acetoxy-2-hydroxy-16-heptadecen-4-one, avocadyne 4-acetate, avocadyne 2-acetate, taraxinic acid glucosyl ester, 6-hydroxypentadecanedioic acid, with reported antimicrobial activity. The overall data suggests the bio-therapeutic potential of the identified vaginal probiotics against cervical cancer associated pathogens.

Introduction

Cervical cancer is the second leading cause of cancer death among Indian women (Siegel et al. 2018). Infection with high-risk human papilloma viruses (HPVs) such as HPV16 and 18 is the major cause for developing cervical cancer (Siegel et al. 2018). Majority of such infections do not progress to cancer, but instead get cleared spontaneously. Recent evidences suggest that the local cervico-vaginal microbiota play a critical role in development of pre-cancerous cervical intraepithelial neoplasia (CIN) and progression to invasive cervical carcinoma (ICC).

Healthy vaginal microflora is mostly dominated by beneficial microbes such as *Lactobacillus* and opportunistic pathogens. Changes in the vaginal microflora could modulate immune responses and favour the growth of pathogens, thereby, facilitating development of several diseases (Kalia et al. 2020; Mitra et al. 2020). Depletion of beneficial *Lactobacillus* could increase infection with diverse aerobic (*Staphylococcus sp*, *Pseudomonas sp. E coli*, and *E. faecalis)* and anaerobic (*Gardnerella* sp., *Atopobium* sp., *Eggerthella* sp., *Sneathia* sp. and *Prevotella* sp.) bacteria. Such vaginal dysbiosis can lead to either bacterial vaginosis (BV) and/ or invasive cervical carcinoma (ICC) (Biswal et al. 2014; Mitra et al. 2020). The exact role of pathogenic bacteria in cervical carcinogenesis is unknown, however, it is anticipated that they produce toxins and carcinogenic metabolites (nitrosamines) that could trigger increased production of proinflammatory cytokines and DNA adducts, which could lead to CIN and ICC (Kyrgiou et al. 2017).

The current standard of care (SOC) drugs for treating vaginal infections during cervical cancer therapy usually include metronidazole or clindamycin, norfloxacin and cotrimoxazole (Mulu et al. 2015; Petrina et al. 2017; Thulkar et al. 2012; Turovskiy et al. 2009). The indiscriminate use of antibiotics mostly results into development of resistant micro-organisms (Ahire et al. 2021), thereby increasing the risk of secondary infections in the cervical cancer patients (Gao et al. 2020). Use of antibiotics may also be associated with vaginal pain, metrorrhagia, nausea, vomiting, diarrhoea and renal failure (Mubarak and Kazi 2014). To overcome such issues, probiotics are being used as adjuncts during treatment of cervical cancer associated bacterial infections (Mejía-Caballero et al. 2021) or BV (Happel et al. 2020; Mejía-Caballero et al. 2021). In the present study, we have isolated and identified vaginal *Lactobacillus* and non-*Lactobacillus* (*Enterococcus)* species from healthy women. The isolates were evaluated for probiotic potential and antibacterial activity against vaginal pathogens isolated from cervical cancer patients.

Materials And Method

Subject selection and sampling

The study was approved by Institutional Ethics Committees of Bharati Vidyapeeth (Deemed to be) University Medical College (BVMC) (Ref: BVDU/MC/57), and B. J. Government Medical College and Sassoon General Hospitals (BJGMCSGH), Pune (Ref No. BJGMC/IEC/Pharmac/ND-Dept 0119007-007). Vaginal swabs from healthy (n=45); low grade squamous intraepithelial lesion, LSIL (n=1); high grade squamous intraepithelial lesion, HSIL (n=1) and invasive cervical carcinoma ICC (n=6) patients (aged between 18-55 years) were collected by the clinicians at the respective clinical sites. All the patients provided informed written consent. The patients were screened for eligibility criteria based upon Pap test, vaginal cytology or colposcopy findings and were categorized under healthy, LSIL, HSIL and ICC groups. Post collection, the samples were immediately placed on ice and taken to IRSHA for further analysis within 2 hours of the collection.

Culture and reagents

The clinical strain, *Pseudomonas aeruginosa* MCC 2081, was procured from National Centre for Microbial Resource (NCMR), NCCS, Pune. All the media components such as de Man, Rogosa and Sharpe (MRS), Tryptone Soya (TSA) or Brain Heart Infusion (BHI), L-cysteine, Bile, Sheep blood agar plates were purchased from HiMedia Laboratories, Mumbai, India. AnaeroGasPak, antibiotics discs and sterile cotton swabs were procured from Himedia Laboratories, Mumbai, India. Pepsin and pancreatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasticware was purchased from Tarson Products Private Limited, India. Ethanol (99% v/v) was procured from Changsu Hongsheng Fine Chemical Co. Ltd. (China).

Isolation and cultivation of microorganisms

For isolation of microflora, aliquots from the collected swabs were plated on de Man, Rogosa and Sharpe (MRS), Tryptone Soya (TSA) or Brain Heart Infusion (BHI) media. Based on the morphology and biochemical tests, *Lactobacillus* and non-*Lactobacillus* colonies were separated and purified. All the *Lactobacillus* strains grown on MRS media, were supplemented with 0.05% L-cysteine at 37°C for 48 h in anaerobic jars, supplemented with anaeroGasPak (Himedia, Mumbai). Non-*Lactobacillus* strains were grown either on TSA or BHI media and incubated at 37°C for 24 h. The pure colonies were stored in 20% glycerol at -80°C until further use.

Hemolysis assay

All the isolates were determined for hemolytic activity by streaking the strains on sheep blood agar plates (HiMedia, Mumbai) and incubated at 37°C for 24-48 h. The hemolytic activity was detected by observing either a clear zone of hydrolysis around the colonies (beta hemolysis), or a greenish zone (alpha hemolysis), or no zone (gamma hemolysis).

Acid and bile tolerance

The tolerance assay was done as reported earlier (Azat et al. 2016; Ehrmann 2002). Briefly, the isolates were grown, harvested and the pellet was washed and resuspended in 1X PBS (pH 3) with a final concentration of 8-9 logCFU/ml or PBS (pH 7.4) containing 0.3 % bile, followed by incubation at 37°C for 3 h. Appropriate dilutions from each sample were spread plated on the respective agar plates and incubated anaerobically using GasPack system at 37°C for 24-48 h. The tolerance was evaluated by total viable count method (log CFU/ml) after 3 h of incubation. The survival rate (%) was calculated as per the following equation:

Survival rate (%) = $\frac{N_1}{N_0} \times 100$,

where, N_0 and N_1 are the total viable counts of the selected strains before and after treatment, respectively.

MALDI-TOF MS analysis

The identification of isolates by MALDI-TOF MS was carried out at National Centre for Microbial Resources (NCMR), National Centre for Cell Sciences (NCCS), Pune. Sample preparation for MALDI-TOF MS analysis was performed as described earlier (Kurli et al. 2018). The samples were loaded onto the MALDI-TOF MS instrument (AUTOFLEX speed, Bruker Daltonics, GmbH, Germany) and MALDI biotyper software 3.1 (Bruker Daltonik GmbH, Germany) was used to identify the isolates and visualize the mass spectra. The strain showing ≥1.7 log value with the strain in the database were confirmed as the member of that genus and strains showing ≥2.0 log values were confirmed to be the member of that species.

Preparation of cell-free supernatant (CFS)

The LAB strains were grown for 24-48 h and centrifuged at 3000 rpm for 15 min. The CFS was collected and filter-sterilized with 0.22 µm syringe filter. The resultant CFS was used for antimicrobial assays and for LCMS, lyophilized powder was stored until further use.

Antibacterial activity

The antibacterial activity of CFS of the selected isolates was determined by agar well diffusion method (Reuben et al. 2019). Briefly, cultures of overnight grown indicator pathogens were adjusted to OD_{600nm} of 0.5 McFarland. 100 µL adjusted pathogen culture was aseptically spread on the nutrient agar plates. Wells of 8 mm diameter were cut out with sterile cork borer and 150 µl CFS was loaded. The plates were kept at 4ºC for diffusion and incubated at 37°C for 24 h. After incubation, the diameter (mm) of the zone of inhibition (ZOI) around the well was measured. *Pseudomonas aeruginosa* MCC 2081 was used as the standard clinical pathogen. The pathogens (with beta and alpha hemolysis) used in this study were isolated from cervical cancer patients and identified by MALDI TOF MS method. Antibacterial activity of the eight standard antibiotics [carbencillin (100μg), cefoxitin (30μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15μg), metronidazole (5μg), penicillin G (10 unit) and tetracycline (30 μg)] was also evaluated against pathogens by disc diffusion method (Bayer et al. 1966).

Liquid chromatography mass spectroscopy (LCMS)

LCMS analysis of the lyophilized CFS of the selected probiotics was carried out at Center for Applications of Mass Spectrometry (CAMS), Venture Centre, Pune. Samples were analyzed by non-targeted LCMS QTOF using Agilent 1290 HPLC system as described previously (Aphale et al. 2018). Briefly, 8 µl of sample was injected onto an Agilent 1290 HPLC system having Zorbax Eclipse Plus C18 column (2.1 mm × 50 mm, 1.8 µm particle sizes). The mobile phases consisted of (A) water and (B) acetonitrile (LCMS grade, J. T. Baker) with flow rate of 0.3 ml/min and 95:5 acetonitrile/water. Both mobile phases were modified with 0.1% (v/v) formic acid for MS analysis in positive mode and with 5 mm ammonium acetate for analysis in negative mode. The chromatographic conditions included first 18 min run of B from 95% to 5% gradient, applied from 18 to 30 min, followed by 3 min isocratically at 100%. MS analysis was performed on an Agilent 6530 Quadrupole time-of-flight spectrometer fitted with an electrospray ionization source in both positive and negative mode. Data were analysed by using Mass Hunter Qualitative Analysis Software Package (Agilent Technologies) and online database Metlin. Compound lists were screened against online mass databases; METLIN Metabolomics Database and MassBank Database.

16S rRNA gene sequencing and phylogenetic analysis

The identification of isolates was carried out at the sequencing facility of NCMR, Pune. The genomic DNA was isolated by the standard phenol/chloroform extraction method (Sambrook et al.1989), followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. The amplified 16S rRNA PCR product was purified by PEG-NaCl precipitation and directly sequenced on ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per the manufacturer's instructions. Assembly was carried out using Lasergene package followed by identification using the EzBioCloud database (Yoon et al. 2017). The phylogenic trees were constructed using the neighbor-joining method (Tamura et al. 2013) with MEGA6 software.

Tolerance to simulated gastrointestinal conditions

The ability of Lactobacilli to tolerate the simulated gastric conditions was determined as described previously (Pino et al. 2019; Pithva et al. 2014). Briefly, simulated gastric juice (SGJ) consisting of 0.3% pepsin in NaCl solution was adjusted to pH 2.5 by adding 1 M HCl. On the other hand, simulated intestinal fluid (SIF) comprising of 0.1% (w/v) pancreatin and 0.3% (w/v) bile in NaCl solution was adjusted to pH 8 by adding 1 M NaOH. Both the solutions were prepared immediately before use and sterilized using 0.22 µm filter. Probiotics were grown for 48 h, centrifuged for 3000 × g for 15 min. The pellet was washed with saline, resuspended in gastric fluid and incubated at 37°C for 3 h. After incubation, the cells were pelleted down by centrifugation, washed with 1X PBS and resuspended in SIF, followed by incubation at 37 °C for 3 h. The cells were washed with 1X PBS, serially diluted and plated on MRS agar for determining viability by total viable count method. The survival rate (%) was calculated as described for the acid and bile tolerance test.

Hydrophobicity and auto-aggregation properties

Hydrophobicity (H) (Kang et al. 2018) and auto-aggregation (A) (Juárez Tomás et al. 2005) of the selected probiotic strains were determined as described earlier. Overnight grown bacterial suspensions in MRS broth were harvested by centrifugation at 3000 rpm for 15 min at room temperature. The cells were washed twice with 1X PBS and adjusted to an optical density (OD) of 0.5 ± 0.1 (A_0) at 600 nm. To determine the hydrophobicity of the cell surface, xylene was used as a solvent. It was added to each bacterial suspension in the ratio of 1:1 and the mixtures were vortexed for 1 min and incubated for 5 h at 37°C. After phase stabilization and separation, the aqueous phase was removed, followed by measurement of absorbance (A_t) at 600 nm. Hydrophobicity percentage was calculated from the formula, $H(\%) = A_0 - A_t/A_0 \times 100$, where, A_0 and A_t are the optical densities before and after extraction with xylene, respectively. For the auto-aggregation assay, each bacterial suspension (initial OD_{600nm} = 0.5 ± 0.1) was vortexed for 10 s and incubated at 37°C for 5 h without agitation. The absorbance (A_t) was measured at 600 nm in microplate reader (Epoch, BioTek Instruments, Inc, USA). The percentage of auto-aggregation was calculated from the formula $A(\%) = A_0 - A_t/A_0 \times 100$, where, A_0 is the OD at initial time (0 h) and A_t is the OD at final time (5 h) of the assay.

Biofilm formation

For biofilm formation (Terraf et al. 2012), the selected probiotic strains were inoculated in MRS media at 37°C for 24 h. Around 200 µl of the grown bacterial culture (OD 0.5) was added into the each well of 96-well plate and incubated at 37 °C for 72 h. The biofilm formed in the wells was washed twice with 200 µl 1X PBS and dried for 30 min at 37°C, followed by incubation with 200 µl of 0.1% (w/v) crystal violet for 30 min at room temperature (RT). The well was washed twice with 200 µl distilled water, dried for 10 min at RT. The residual crystal violet was dissolved in 200 µl solution containing 95% ethanol and 0.1% acetic acid in water, followed by measurement of the absorbance at 570 nm in multiplate reader.

Antibiotic susceptibility

Susceptibility of the selected probiotics to antibiotics was determined by the agar diffusion method by using two different antibiotics disks (Combi II and Combi III, Himedia, Mumbai). A total of 16 antibiotics were tested that included carbencillin (100μg), cefoxitin (30μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15μg), metronidazole (5μg), penicillin G (10 unit), tetracycline (30 μg), ampicillin (10 μg), norfloxacin (10 μg), nitrofurantoin (300 μg), nalidixic acid (30μg), gentamicin (10 μg), cotrimoxazole (25μg), cefalotin (30 μg), and cefotaxime (30μg).

Statistical analysis

The data has been presented as mean ± standard deviation (SD) and was analyzed for statistical significance by one-way and two-way analysis of variance (ANOVA) by using Tukey's multiple comparisons test. Statistical significance level was defined at p value < 0.05. All the statistical analyses were performed by using Graph Pad Prism version 9 (GraphPad, San Diego, USA).

Accession number

The nucleotide sequences of 16S rRNA of the four strains were deposited at the GenBank database under the following accession numbers: *Lactobacillus delbrueckii* P31Mcs (OM049479), *Lactobacillus gasseri* P36Mops (OM049480), *Limosilactobacillus fermentum* P37Mws (OM049481) and *Enterococcus faecium* P15Mcm (OM049482).

Results

Isolation and characterization of vaginal microflora

From the vaginal swabs of 45 healthy women, 111 microflora were isolated and characterized for their microscopic structure, Gram staining and catalase activity (Supplementary Table S1). From 40 healthy vaginal swabs, most of the isolates showed *Lactobacillus* related morphology (rods, Gram positive and catalase negative) (88.89%). Other 5 vaginal swabs showed presence of cocci and non-*Lactobacilli.* All the isolates were further evaluated for hemolysis. 79 isolates revealed non-hemolytic nature (Supplementary Table S1), out of which four were omitted that showed morphological features similar to yeast and Gram negative cocci. From the vaginal swabs of LSIL (1), HSIL (1) and ICC (6) patients (Supplementary Table S2), 29 microflora were isolated. Among these, 17.24% isolates showed *Lactobacillus*-related morphology, rest were cocci with either catalase negative or positive activity, and few were Gram-negative rods. Out of 29 isolates, 13 exhibited beta hemolysis and 5 showed alpha hemolysis (Supplementary Table S2) while the remaining 11 showed gamma hemolysis.

Non-hemolytic microflora exhibited acid and bile tolerance

The non-hemolytic strains (75) from healthy controls were evaluated for acid bile tolerance. Around 90.66% strains showed tolerance to acidic pH (Table 1). After incubation with bile (0.3%), the viability of 40 isolates was reduced by approximately more than 2-log with survival rates less than 75% (Table 1). Another 22 isolates were totally inhibited in the presence of bile. Interestingly, only thirteen isolates showed tolerance to both acid and bile with less than 2-log reductions and were taken further for antibacterial studies.

MALDI TOF MS identification of the isolates

Table 1
Acid and bile tolerance activity of non-hemolytic isolates

Sr. no.	Isolates	Acid (pH 3.0)			Bile (0.3%, w/v)		
		Log CFU/ml (0 h)	Log CFU/ml (3 h)	Survival rate (%)	Log CFU/ml (0 h)	Log CFU/ml (3 h)	Survival rate (%)
1.	P1Mcs	9.22 ± 0.05	9.03 ± 0.05	97.94	9.18 ± 0.03	8.44 ± 0.09	91.94
2.	P1Bws	8.97 ± 0.04	7.05 ± 0.12	78.59	8.91 ± 0.04	5.28 ± 0.31	59.26
3.	P2Mct	8.91 ± 0.05	0	0	8.90 ± 0.03	0	0
4.	P2Mcs	8.90 ± 0.03	8.24 ± 0.11	92.58	8.89 ± 0.02	6.64 ± 0.05	74.69
5.	P3Bws	8.93 ± 0.03	7.44 ± 0.04	83.31	8.91 ± 0.03	0	0
6.	P3Bwm	9.20 ± 0.04	8.18 ± 0.13	88.91	9.15 ± 0.07	5.18 ± 0.21	56.61
7.	P3Bwb	8.98 ± 0.04	8.63 ± 0.06	96.10	9.01 ± 0.03	0	0
8.	P5Mcs	8.83 ± 0.04	8.47 ± 0.09	95.92	8.81 ± 0.07	6.23 ± 0.07	70.71
9.	P5Bws	8.73 ± 0.07	7.13 ± 0.21	81.67	8.75 ± 0.04	5.16 ± 0.25	58.97
10.	P6MS	8.69 ± 0.05	8.31 ± 0.09	95.27	8.71 ± 0.05	5.8 ± 0.22	66.59
11.	P6Bops	8.82 ± 0.05	8.25 ± 0.14	93.51	8.83 ± 0.03	0	0
12.	P7Bop	8.63 ± 0.06	7.38 ± 0.33	85.51	8.70 ± 0.05	5.00 ± 0.00	57.47
13.	P7Mcs	9.27 ± 0.04	9.26 ± 0.03	99.89	9.34 ± 0.02	5.18 ± 0.21	55.46
14.	P8Mcs	9.24 ± 0.03	9.06 ± 0.05	98.05	9.25 ± 0.01	5.13 ± 0.21	55.45
15.	P9Mcs	8.57 ± 0.06	0.00	0	8.59 ± 0.09	0.00	0
16.	P10Tct	9.21 ± 0.02	9.15 ± 0.02	99.34	9.14 ± 0.04	5.00	54.70
17.	P10Mcb	9.06 ± 0.04	8.71 ± 0.05	96.13	9.07 ± 0.02	5.10 ± 0.16	56.22
18.	P11Mcb	9.00 ± 0.03	8.91 ± 0.04	99.00	8.97 ± 0.02	0 ± 0.00	0
19.	P11Bws	8.89 ± 0.03	8.40 ± 0.09	94.48	8.86 ± 0.04	5.25 ± 0.43	59.25
20.	P12Bops	8.78 ± 0.04	8.06 ± 0.08	91.79	8.80 ± 0.05	5.27 ± 0.26	59.88
21.	P13Bops	8.87 ± 0.05	0	0	8.89 ± 0.04	0	0
22.	P13Mcs	8.17 ± 0.18	8.00 ± 0.14	97.91	8.39 ± 0.08	7.05 ± 0.12	84.02
23.	P14Mcs	8.61 ± 0.06	8.17 ± 0.11	94.88	8.64 ± 0.05	7.05 ± 0.12	81.59
24.	P15Mcm	9.20 ± 0.02	8.98 ± 0.04	97.60	9.29 ± 0.02	8.75 ± 0.04	94.18
25.	P15Mcs	9.10 ± 0.03	8.67 ± 0.06	95.27	9.04 ± 0.03	6.48 ± 0.09	71.68
26.	P16Mws	8.93 ± 0.06	8.61 ± 0.07	96.41	8.92 ± 0.05	7.88 ± 0.10	88.34
27.	P17Mws	9.16 ± 0.03	0	0	9.12 ± 0.03	0	0
28.	P18Mops	8.99 ± 0.08	0	0	8.90 ± 0.07	0	0
29.	P18Topb	8.75 ± 0.08	0	0	8.70 ± 0.06	0	0
30.	P18Mct	9.04 ± 0.03	8.76 ± 0.04	93.19	9.06 ± 0.03	0	0
31.	P18Mcs	9.03 ± 0.03	8.77 ± 0.04	97.12	9.01 ± 0.03	0	0
32.	P19Mws	8.90 ± 0.11	7.89 ± 0.12	88.65	8.88 ± 0.09	0	0
33.	P20 Mop	9.01 ± 0.05	8.31 ± 0.18	92.23	8.96 ± 0.04	0	0
34.	P21Mcb	8.58 ± 0.19	7.42 ± 0.33	86.48	8.79 ± 0.05	6.5 ± 0.11	73.94
35.	P22Mws	8.50 ± 0.11	0	0	8.63 ± 0.06	0	0
36.	22Mops	9.18 ± 0.03	8.94 ± 0.04	97.38	9.15 ± 0.04	7.15 ± 0.16	78.14

The data has been presented as mean ± SD of three independent experiments, each performed in duplicates.

Sr. no.	Isolates	Acid (pH 3.0)			Bile (0.3%, w/v)		
		Log CFU/ml (0 h)	Log CFU/ml (3 h)	Survival rate (%)	Log CFU/ml (0 h)	Log CFU/ml (3 h)	Survival rate (%)
37.	P23Mcs	9.76 ± 0.01	9.68 ± 0.04	99.18	9.71 ± 0.03	6.56 ± 0.15	67/56
38.	P24Mwt	9.11 ± 0.03	8.86 ± 0.05	97.25	9.06 ± 0.02	7.05 ± 0.12	77.81
39.	P25Mws	9.41 ± 0.05	8.89 ± 0.08	94.47	9.5 ± 0.06	6.56 ± 0.09	69.05
40.	P26Mcm	8.96 ± 0.05	8.59 ± 0.08	95.87	9.29 ± 0.04	8.55 ± 0.12	92.03
41.	P26Mct	9.04 ± 0.05	8.64 ± 0.09	95.57	9.01 ± 0.06	0	0
42.	P28Mws	8.92 ± 0.00	8.10 ± 0.24	90.80	9.10 ± 0.05	5.35 ± 0.12	58.79
43.	P31Mcs	9.32 ± 0.023	9.07 ± 0.05	97.37	9.33 ± 0.03	8.49 ± 0.10	90.37
44.	P33Mcm	9.19 ± 0.03	8.40 ± 0.11	91.40	9.16 ± 0.03	5.37 ± 0.31	55.93
45.	P34Mws	9.03 ± 0.03	8.91 ± 0.03	98.67	9.04 ± 0.04	5.43 ± 0.16	60.06
46.	P34Mcs	8.24 ± 0.2	7.96 ± 0.15	96.60	8.38 ± 0.11	6.23 ± 0.12	74.34
47.	P35Mws	8.88 ± 0.06	8.54 ± 0.07	96.17	8.94 ± 0.03	5.18 ± 0.21	57.94
48.	P36Mops	8.61 ± 0.08	8.32 ± 0.10	96.63	8.63 ± 0.06	7.81 ± 0.11	90.49
49.	P36Mws	9.40 ± 0.05	9.18 ± 0.05	97.65	9.36 ± 0.03	6.66 ± 0.12	71.15
50.	P37Mcb	9.22 ± 0.01	8.40 ± 0.11	91.10	9.23 ± 0.02	0	0
51.	P37Mws	9.29 ± 0.03	8.87 ± 0.06	95.47	9.26 ± 0.04	8.55 ± 0.08	92.33
52.	P38Mops	8.80 ± 0.07	8.29 ± 0.10	94.20	8.78 ± 0.05	5.10 ± 0.16	58.08
53.	P39Mops	8.79 ± 0.03	8.52 ± 0.09	96.92	8.78 ± 0.04	5.3 ± 0.27	60.36
54.	P42Mcm	8.85 ± 0.06	7 ± 0.00	79.09	8.84 ± 0.04	0	0
55.	P43Mops	8.72 ± 0.05	8.41 ± 0.09	96.44	8.64 ± 0.07	6.65 ± 0.08	76.96
56.	P44Mcm	9.18 ± 0.02	8.62 ± 0.10	98.73	9.07 ± 0.03	5.38 ± 0.03	59.31
57.	P44Mcs	9.08 ± 0.05	8.99 ± 0.09	99.00	9.07 ± 0.04	6.04 ± 0.10	66.59
58.	P44Tcs	9.02 ± 0.03	8.43 ± 0.08	93.45	9.15 ± 0.33	5.10 ± 0.16	55.73
59.	P45Bws	9.13 ± 0.03	8.09 ± 0.11	88.60	9.13 ± 0.02	5.13 ± 0.21	56.18
60.	P45Tcm	9.21 ± 0.03	9.10 ± 0.02	98.91	9.18 ± 0.03	0	0
61.	P46Mopb	8.74 ± 0.07	8.44 ± 0.09	96.56	8.65 ± 0.11	5.21 ± 0.24	60.23
62.	P46Mops	8.72 ± 0.09	8.30 ± 0.11	95.18	8.75 ± 0.10	6.27 ± 0.10	71.65
63.	P47Mops	8.91 ± 0.04	8.42 ± 0.07	94.50	8.79 ± 0.03	6.74 ± 0.05	76.67
64.	P47Bws	9.05 ± 0.03	8.48 ± 0.11	93.90	9.06 ± 0.02	5.10 ± 0.16	56.29
65.	P49Mopb	8.87 ± 0.06	8.16 ± 0.09	91.99	8.90 ± 0.05	5.23 ± 0.19	58.76
66.	P51Mcm	9.16 ± 0.02	8.16 ± 0.12	89.08	9.11 ± 0.04	5.18 ± 0.21	56.86
67.	P51Mwt	9.17 ± 0.03	9.03 ± 0.03	98.47	9.18 ± 0.02	6.23 ± 0.08	67.86
68.	P51Tops	8.97 ± 0.03	8.39 ± 0.07	93.53	8.97 ± 0.03	5 ± 0.00	55.74
69.	P52Mcm	9.20 ± 0.02	9.05 ± 0.02	98.36	9.16 ± 0.02	5.48 ± 0.20	59.82
70.	P52Tcb	9.16 ± 0.03	9.02 ± 0.03	98.47	9.16 ± 0.02	0	0
71.	P53Mcs	9.02 ± 0.02	8.47 ± 0.06	93.90	8.97 ± 0.04	5.19 ± 0.45	57.85
72.	P53Tcm	9.2 ± 0.03	8.98 ± 0.04	97.60	9.18 ± 0.03	0	0
73.	P54Mcs	9.18 ± 0.02	9.08 ± 0.03	98.91	9.16 ± 0.03	5.15 ± 0.16	56.22
74.	P54Tcb	9.17 ± 0.05	9.10 ± 0.02	99.23	9.12 ± 0.04	0	0

The data has been presented as mean ± SD of three independent experiments, each performed in duplicates.

Sr. no.	Isolates	Acid (pH 3.0)			Bile (0.3%, w/v)		
		Log CFU/ml (0 h)	Log CFU/ml (3 h)	Survival rate (%)	Log CFU/ml (0 h)	Log CFU/ml (3 h)	Survival rate (%)
75.	P54Tcm	9.16 ± 0.04	9.09 ± 0.05	99.23	9.14 ± 0.04	6.78 ± 0.07	74.17
The data has been presented as mean ± SD of three independent experiments, each performed in duplicates.							

All the thirteen isolates showing acid and bile tolerance were further identified by MALDI-TOF MS (Table 2). For each LAB strain, the score value was in the range of 1.7-2. Among the 13 identified LAB from healthy group, *Lactobacillus gasseri* (53.85%) was found to be dominant, followed by *Limosilactobacillus fermentum* (15.38%), *Enterococcus faecium* (15.38%), *Lactobacillus reuteri* (7.69%) and *Lactobacillus delbrueckii* (7.69%). Out of 18 pathogenic isolates, 8 strains were identified by MALDI TOF MS (score value above 2.0) (Table 3), *S. haemolyticus* P41Tcs and *E. coli* P79Bcm were isolated from the swabs of LSIL and HSIL patients, respectively. *E. coli* P30Tcs, *E. faecalis* P29Mops, *E. faecalis* P50Tws, *E. faecalis* P68Tcb, *S. haemolyticus* P48Bcb *and S. haemolyticus* P58Bcb were isolated from the swabs of 6 cervical cancer patients. Among these, *S. haemolyticus* (37.5%) and *E. faecalis* (37.5%) were found to be the dominant strains, followed by *E. coli*(25%). The identified pathogenic strains were used as indicators for further antimicrobial studies.

Table 2
Identification of thirteen isolates from healthy individuals by MALDI-TOF MS

Sr. no.	Isolate from healthy individuals	Organism(best match)	Score
1.	P1Mcs	*Limosilactobacillus fermentum* DSM 20055	1.745
2.	P13Mws	*Lactobacillus gasseri* DSM 20243T	1.843
3.	P14Mcs	*Lactobacillus gasseri* DSM 20243T	2.314
4.	P15Mcm	*Enterococcus faecium* DSM 2146	2.302
5.	P16Mws	*Lactobacillus gasseri* DSM 20604	1.771
6.	P22Mops	*Lactobacillus gasseri* DSM 20243T	2.439
7.	P24Mwt	*Lactobacillus reuteri* DSM 20053	1.729
8.	P26Mcm	*Enterococcus faecium* 20218_1	1.989
9.	P31Mcs	*Lactobacillus delbrueckii* DSM 20073	1.888
10.	P36Mops	*Lactobacillus gasseri* DSM 20243T	1.856
11.	P37Mws	*Lactobacillus fermentum* DSM 20055	2.020
12.	P43Mops	*Lactobacillus gasseri* DSM 20243T	2.442
13.	P47Mops	*Lactobacillus gasseri* CIP 101909	2.304

Table 3
MALDI-TOF MS Identification of pathogenic isolates from precancerous (LSIL and HSIL) and ICC patients

Sr. No.	Isolate	Organism	Score	Patient group
1.	P41Tcs	*Staphylococcus haemolyticus*	2.02	LSIL
2.	P79Bcm	*Escherichia coli*	2.08	HSIL
3.	P30Tcs	*E. coli*	2.20	ICC
4.	P29Mops	*Enterococcus faecalis*	2.43	ICC
5.	P50Tws	*E. faecalis*	2.03	ICC
6.	P68Tcb	*E. faecalis*	2.12	ICC
7.	P48Bcb	*S. haemolyticus*	2.10	ICC
8.	P58Bcb	*S. haemolyticus*	2.05	ICC

CFS of selected strains from healthy group exhibited antimicrobial activity

The cell free supernatant (CFS) of the acid-bile tolerant isolates (13) from healthy individuals were evaluated for antimicrobial activity against standard (commercially obtained) pathogen, *P. aeruginosa* 2081 and eight isolated pathogenic strains from cervical cancer patients (Table 4). Among the thirteen isolates, *L. fermentum* P37Mws exhibited significantly high ($p < 0.05$) antimicrobial activity against four pathogenic strains, *P. aeruginosa*, *E. coli*

P79Bcm, *S. haemolyticus* P48Bcb and P58Bcb. *L. gasseri* P36Mops also showed antibacterial activity ($p < 0.05$) against four pathogens, *E. coli* P30Tcs and P79Bcm; *S. haemolyticus* P41Tcs and P58Bcb. *L. delbrueckii* P31Mcs showed significant ($p < 0.05$) zone of inhibition against (three pathogens) *E. coli* P30Tcs, *S. haemolyticus* P48Bcb and 58Bcb. Interestingly, *E. faecium* P15Mcm showed significantly higher zone of inhibition (> 18 mm) against three pathogenic strains of *E. faecalis* P29Mops, P50Tws and P68Tcb. Thus, overall data showed that four strains (*L. fermentum* P37Mws, *L. delbrueckii* P31Mcs, *L. gasseri* P36Mops and *E. faecium* P15Mcm) exhibited probiotic characteristics and antibacterial activity against more than two pathogens. The vaginal pathogens were also tested for their susceptibility to eight standard antibiotics that included carbencillin, cefoxitin, clindamycin, chloramphenicol, erythromycin, metronidazole, penicillin G and tetracycline. Tetracycline exhibited antibacterial activity against all the eight vaginal pathogens whereas metronidazole did not show any activity against the pathogens (Supplementary Table S3). On the other hand, carbencillin displayed antibacterial activity only against the strains of *E. faecalis*.Rest of the antibiotics displayed varied spectrum of antibacterial activity against the pathogens.

Table 4
Antimicrobial activity of selected isolates from healthy group against pathogens

Sr. no.	Isolates (CFSs)	Zone of inhibition (mm) against different pathogens								
		P. aeruginosa 2081	E. coliP30Tcs	E. coliP79Bcm	E. faecalis P29Mops	E. faecalis P50Tws	E. faecalis P68Tcb	S. haemolyticus P41Tcs	S. haemolyticus P58Bcb	S. haemolyticus P48Bcb
1.	*L. fermentum* P1Mcs	13.56 ± 0.73^{e}	14.56 ± 0.53^{a}	12.56 ± 0.53^{e}	-	-	-	-	15.33 ± 0.50^{a}	14.89 ± 0.60^{b}
2.	*L. gasseri* P13Mcs	11.56 ± 0.53^{e}	12.44 ± 0.73^{e}	-	-	-	-	-	12.89 ± 0.60^{e}	13.56 ± 0.53^{e}
3.	*L. gasseri* P14Mcs	14.00 ± 0.71^{e}	13.44 ± 0.53^{e}	-	-	-	-	-	14.44 ± 0.88^{c}	12.78 ± 0.44^{e}
4.	*E. faecium* P15Mcm	13.78 ± 0.44^{e}	-	-	21.00 ± 1.32^{a}	18.79 ± 0.97^{a}	19.67 ± 0.50^{a}	-	-	-
5.	*L. gasseri* P16Mws	15.33 ± 0.5^{e}	14.78 ± 0.44^{a}	-	-	-	-	-	15.00 ± 0.71^{a}	14.44 ± 0.53^{e}
6.	*L. gasseri* P22Mops	16.11 ± 0.60^{a}	-	-	-	-	-	-	11.33 ± 0.50^{e}	13.78 ± 0.44^{e}
7.	*L. reuteri* P24Mwt	13.11 ± 0.93^{e}	13.67 ± 0.71^{e}	-	-	-	-	-	11.67 ± 0.50^{e}	11.89 ± 0.33^{e} 4
8.	*E. faecium* P26Mcm	12.56 ± 0.53^{e}	-	-	14.44 ± 0.53^{e}	14.11 ± 0.60^{e}	14.00 ± 0.50^{e}	-	-	-
9.	*L. delbrueckii* P31Mcs	15.67 ± 0.71^{a}	15.11 ± 0.60^{a}	12.89 ± 0.33^{e}	11.67 ± 0.50^{e}	-	-	13.44 ± 0.53^{a}	15.11 ± 0.60^{a}	14.89 ± 0.60^{b}
10.	*L. gasseri* P36Mops	15.11 ± 0.60^{d}	14.44 ± 0.73^{a}	12.00 ± 0.50^{e}	12.67 ± 0.50^{e}	-	-	11.22 ± 0.97^{e}	15.00 ± 0.71^{a}	15.67 ± 0.50^{a}
11.	*L. fermentum* P37Mws	15.56 ± 0.53^{a}	13.78 ± 0.44^{e}	14.00 ± 0.5^{a}	14.33 ± 0.71^{e}	14.89 ± 0.33^{e}	-	11.89 ± 0.33^{e}	15.33 ± 0.50^{a}	15.22 ± 0.44^{a}
12.	*L. gasseri* P43Mops	14.00 ± 0.87^{e}	13.33 ± 0.71^{e}	-	-	-	-	-	13.78 ± 0.44^{e}	14.11 ± 0.6^{e}
13.	*L. gasseri* P47Mops	13.67 ± 0.71^{e}	11.67 ± 0.50^{e}	-	-	-	-	-	14.44 ± 0.53^{c}	14.33 ± 0.50^{e}

"-' no activity observed. The data has been presented as mean ± SD of three independent experiments, each performed in triplicates. Statistically different results within the column were labelled with various lowercase letter (a:p > 0.05; b:p < 0.05; c:p < 0.005; d:p < 0.001; e:p < 0.0001).

The inhibition zones (ZOI) were classified as: no activity (< 9 mm); weak (< 14 mm); good (15–19 mm) and strong (> 20 mm) (Reuben et al 2019).

CFS showed presence of metabolites with reported antimicrobial activity

LCMS analysis (non-targeted) of the CFS from the selected probiotics was carried out to identify the potential antibacterial compounds secreted by the respective strains. Around 119 compounds from *L. gasseri*, 95 from *L. fermentum*, 106 from *L. delbrueckii* P31Mcs and 117 from *E. faecium* P15Mcm were identified. The major metabolites with antibacterial activity have been shown in Table 5 and their respective chromatograms have been included in Supplementary Fig. S1. The antibacterial metabolites from CFSs of *L. gasseri* P36Mops included N-(1-deoxy-1-fructosyl)valine, homoarecoline, 2-

Isopropyl-1,4-benzenediol, 1-Acetoxy-2-hydroxy-16-heptadecen-4-one, avocadyne 4-acetate, avocadyne 2-acetate, grandidentatin, taraxinic acid glucosyl ester and nigellicine. The metabolites detected from *L. fermentum* P37Mws included methylarmepavine, (-)-hygroline, avocadyne 4-acetate, avocadyne 2-acetate and 1-acetoxy-2-hydroxy-16-heptadecen-4-one. The CFS of *L. delbrueckii* P31Mcs showed the presence of metabolites such as (+)-O-methylarmepavine, 6-methylquinoline, quinaldine, 1-acetoxy-2-hydroxy-16-heptadecen-4-one, 3-O-sulfogalactosylceramide. The metabolites such as nepetalactam, hordenine, cuminaldehyde, 4-phenyl-3-buten-2-ol, 6-hydroxypentadecanedioic acid, hygroline, taraxinic acid glucosyl ester, avermectin A2b aglycone were detected from *E. faecium*P15Mcm. Thus, the CFS of the probiotic strains showed presence of antimicrobial metabolites, which could be responsible for their observed antibacterial activity.

Table 5
Antibacterial metabolites present in the CFSs of selected probiotics identified by LCMS

Name of identified compounds	Empirical formula	Observed RT	M/Z ratio	Observed mass	Score
L. gasseri **P36Mops**					
N-(1-Deoxy-1-fructosyl)valine	C11 H21 N O7	0.805	280.1383	279.131	96.74
Homoarecoline	C9 H15 N O2	1.158	170.1172	169.1099	98.96
2-Isopropyl-1,4-benzenediol	C9 H12 O2	1.158	170.1172	152.0834	98.92
(-)-Hygroline	C8 H17 N O	3.191	166.1207	143.1316	93.34
1-Acetoxy-2-hydroxy-16- heptadecen-4-one	C19 H34 O4	6.573	344.2793	326.2451	91.02
Avocadyne 4-acetate	C19 H34 O4	6.573	344.2793	326.2451	91.02
Avocadyne 2-acetate	C19 H34 O4	6.573	344.2793	326.2451	91.02
Grandidentatin	C21 H28 O9	6.584	442.2053	424.1717	92.5
Taraxinic acid glucosyl ester	C21 H28 O9	6.598	442.2056	424.172	93.3
Nigellicine	C13 H15 N2 O3	8.962	930.6324	247.108	94.77
L. fermentum **P37Mws**					
Methylarmepavine	C20 H25 N O3	2.12	328.1897	327.1826	91.85
6-Hydroxypentadecanedioic acid	C15 H28 O5	2.906	289.2015	288.1943	93.50
(-)-Hygroline	C8 H17 N O	4.081	166.1208	143.1317	93.65
Taraxinic acid glucosyl ester	C21 H28 O9	6.803	442.2056	424.1717	92.45
1-Acetoxy-2-hydroxy-16-heptadecen-4-one	C19 H34 O4	6.814	344.2794	326.2452	93.81
Avocadyne 4-acetate	C19 H34 O4	6.814	344.2794	326.2452	93.81
Avocadyne 2-acetate	C19 H34 O4	6.814	344.2794	326.2452	93.81
L. delbrueckii **P31Mcs**					
(+)-O-Methylarmepavine	C20 H25 N O3	2.075	328.1898	327.1826	94.22
6-Methylquinoline	C10 H9 N	2.979	144.0811	143.0737	93.17
Quinaldine	C10 H9 N	2.979	144.0811	143.0737	93.17
1-Acetoxy-2-hydroxy-16-heptadecen-4-one	C19 H34 O4	6.692	344.2796	326.245	95.36
Avocadyne 4-acetate	C19 H34 O4	6.692	344.2796	326.245	95.36
Avocadyne 2-acetate	C19 H34 O4	6.692	344.2796	326.245	95.36
Taraxinic acid glucosyl ester	C21 H28 O9	6.694	442.2056	424.172	92.95
E. faecium **P15Mcm**					
Ephedrine	C10 H15 N O	1.841	166.1219	165.1148	93.25
Nepetalactam	C10 H15 N O	1.841	166.1219	165.1148	93.25
Hordenine	C10 H15 N O	1.841	166.1219	165.1148	93.25
Cuminaldehyde	C10 H12 O	1.841	166.1219	148.0882	93.12
Anethole	C10 H12 O	1.841	166.1219	148.0882	93.12
Estragole	C10 H12 O	1.841	166.1219	148.0882	93.12
6-Hydroxypentadecanedioic acid	C15 H28 O5	2.201	289.2012	288.1941	96.85
Hygroline	C8 H17 N O	3.089	166.1207	143.1316	94.48
Taraxinic acid glucosyl ester	C21 H28 O9	6.598	442.2056	424.172	93.3

RT: Retention time

Name of identified compounds	Empirical formula	Observed RT	M/Z ratio	Observed mass	Score
Avermectin A2b aglycone	C34 H50 O9	7.009	603.3528	602.3459	93.29
Avermectin B2a aglycone	C34 H50 O9	7.009	603.3528	602.3459	93.29
RT: Retention time					

16s rRNA sequencing and phylogenetic analysis

The selected probiotic strains were subjected to molecular identification and phylogenetic analysis by the Sanger sequencing method and EzBioCloud database, respectively (Table 6). A comparative 16S rRNA gene based phylogenetic analysis of the four vaginal strains, P36Mops, P37Mws, P31Mcs and P15Mcm revealed their closest similarity (ranging from 100 to 99%) to the sequences of the type strains, *L. gasseri* ATCC 33323 (Azcarate-Peril et al. 2008), *L. fermentum* CECT562 (Zheng et al. 2020), *L. delbrueckii* DSM 20072 (Schoch et al. 2020) and *E. lactis* BT159 (Morandi et al. 2012), respectively, from GeneBank database (Supplementary Fig. S2-S5).

Table 6
Molecular identification of selected vaginal probiotics by 16S rRNA sequencing

Sr. No	Strain	Closest Neighbour %		% Similarity
		Taxonomic Designation	Accession no.	
1	P36Mops	*Lactobacillus gasseri* ATCC 33323(T)	CP000413	99.86
2	P37Mws	*Limosilactobacillus fermentum CECT 562(T)*	AJ575812	99.85
3	P31Mcs	*Lactobacillus delbrueckii subsp. lactis DSM 20072(T)*	AEXU01000148	99.92
4	P15Mcm	*Enterococcus faecium LMG 11423(T)*	AJ301830	99.78

Probiotics survived the simulated gastric juice (SGJ) and intestinal fluid (SIF) conditions

All the selected strains displayed > 80% survival in 0.3% pepsin (Table 7), representing SGJ. *L. fermentum* P37Mws exhibited significant ($p < 0.0001$) survival (96.3%) with viable count of 8.86 ± 0.09 log CFU/ml, followed by *E. faecium* P15Mcm (94.16%). On the other hand, *L. gasseri* P36Mops and *L. delbrueckii* P31Mcs showed survival upto 85.94 to 84.86%, respectively. All the four probiotics showed 60.09–76.30% survival upto 3 h in the presence of pancreatin enzyme. The viable count for *L. fermentum* P37Mws and *L. gasseri* P36Mops did not differ significantly ($p > 0.05$) under SIF environment. The viable count was decreased to approximately 6 logs CFU/ml from the initial count (approx. 8–9 logs CFU/ml) in all, except in *L. delbrueckii*P31Mcs that showed viable count of 5.24 log CFU/ml. Thus, the probiotic strains survived the gastrointestinal (GI) transit, which is a prerequisite for colonization to the host epithelial cells for providing health benefits.

Table 7
Effect of simulated human gastric (SGJ) and intestinal fluid (SIF) on the survival of selected vaginal probiotics

Probiotic strains	(log CFU/ml) 0 h	SGJ (log CFU/ml) 3 h	SR (%)	SIF (log CFU/ml) 3 h	SR (%)
L. gasseri P36Mops	8.89 ± 0.04^{A}	7.64 ± 0.19^{A}	85.94	6.15 ± 0.12^{A}	69.18
L. fermentum P37Mws	9.20 ± 0.02^{AcB}	8.86 ± 0.09^{AcB}	96.30	6.18 ± 0.18^{AaB}	67.17
L. delbrueckii P31Mcs	8.72 ± 0.10^{AaBcC}	7.40 ± 0.30^{AbBcC}	84.86	5.24 ± 0.19^{AcBcC}	60.09
E. faecium P15Mcm	9.07 ± 0.04^{AaBaCc}	8.54 ± 0.11^{AcBcCc}	94.16	6.92 ± 0.09^{AcBcCc}	76.30
The data has been presented as mean ± SD of three independent experiments, each performed in triplicates. Statistically (Tukey's multiple comparisons test) different results within the column were labelled with a various lowercase letter (a:p > 0.05; b:p < 0.005; c:p < 0.0001)					

Probiotics exhibited properties of hydrophobicity, auto-aggregation and biofilm formation

All the selected probiotics, after 5 h of incubation with the xylene, showed moderate hydrophobicity (35.88–56.70%). *L. gasseri* P36Mops showed highest (56.70%±1.63) hydrophobicity (Table 8), followed by *L. delbrueckii* P31Mcs (55.92%±2.41), *E. faecium* P15Mcm (39.69%±2.83) and *L. fermentum* P37Mws (35.88%±1.97). There was no significant difference ($P > 0.05$) in the hydrophobicity between *L. gasseri* P36Mops and *L. delbrueckii* P31Mcs. The tested probiotics showed highest auto-aggregation with *E. faecium* P15Mcm (61.39%±1.49; $p < 0.0001$), followed by *L. fermentum* P37Mws (52.20%±0.93; $p < 0.0001$), *L. delbrueckii* P31Mcs (43.12%±2.27; $p < 0.0001$) and *L. gasseri* P36Mops (35.26%±2.03; $p < 0.0001$) (Table 8). All the four probiotic strains showed higher optical density (OD_T) at 570 nm that was 4 times greater ($4ODc < OD_T$; $p < 0.0001$), than the OD of control media (ODc = 0.321 ± 0.04). Based on biofilm production, the strains were divided into non- ($OD_T \leq ODc$); weak ($ODc < OD_T \leq 4ODc$); and strong ($4ODc < OD_T$) producers, where ODc is OD of control media (Terraf et al. 2012). It was noted that *L. fermentum* P37Mws produced more biofilm, (3.25 ± 0.15; p <

0.0001), which was followed by *E. faecium* P15Mcm (2.57 ± 0.43; p < 0.0001), *L. delbrueckii* P31Mcs (2.40 ± 0.41; p < 0.0001) and *L. gasseri* P36Mops (1.41 ± 0.35; p < 0.0001) (Fig. 1). Thus, the selected strains exhibited probiotic properties of hydrophobicity, auto aggregation and biofilm production.

Table 8
Hydrophobicity and auto-aggregation properties of selected probiotics

Probiotic strains	Hydrophobicity (%)	Auto-aggregation (%)
L. gasseri P36Mops	56.70 ± 1.63^{A}	35.26 ± 2.03^{A}
L. fermentum P37Mws	35.88 ± 1.97^{AcB}	52.20 ± 0.93^{AcB}
L delbrueckii P31Mcs	55.92 ± 2.41^{AaBcC}	43.12 ± 2.27^{AcBcC}
E. faecium P15Mcm	39.69 ± 2.83^{AcBbCc}	61.39 ± 1.49^{AcBcCc}
The data has been presented as mean ± SD of three independent experiments, each performed in triplicates. Statistically (Tukey's multiple comparisons test) different results within the column were labelled with a various lowercase letter (a:p > 0.05; b:p < 0.05; c:p < 0.0001)		

Probiotics exhibited resistance towards standard antibiotics

The selected probiotics were evaluated for their resistance towards 16 different antibiotics (carbencillin, cefoxitin, clindamycin, chloramphenicol, erythromycin, metronidazole, penicillin G, tetracycline, ampicillin, norfloxacin, nitrofurantoin, nalidixic acid, gentamicin, cotrimoxazole, cefalotin and cefotaxime), mostly prescribed for bacterial vaginosis or urogenital infections. *L. gasseri* exhibited resistance towards five antibiotics that included nalidixic acid, norfloxacin, nitrofurantoin, metronidazole and cotrimoxazole (Table 9). *L. fermentum* P37Mws showed resistance to four antibiotics namely, cefoxitin, metronidazole, nalidixic acid and cotrimoxazole. *E. faecium* P15Mcm exhibited resistance to four antibiotics (carbencillin, metronidazole, nalidixic acid and cotrimoxazole) and *L. delbrueckii* showed resistance towards two antibiotics (nalidixic acid and cotrimoxazole).

Table 9
Antibiotics susceptibility of selected probiotics

Antibiotics	Antibiotic susceptibility			
	L. gasseri P36Mops	L. fermentum P37Mws	L. delbrueckii P31Mcs	E. faecium P15Mcm
Carbecillin	S	S	S	R
Cefoxitin	S	R	S	S
Clindamycin	S	S	S	S
Chloramphenicol	S	S	S	S
Erythromycin	S	S	S	S
Metronidazole	R	R	S	R
Penicillin G	S	S	S	S
Tetracycline	S	S	S	S
Gentamycin	S	S	S	S
Nalidixic acid	R	R	R	R
Nitrofuramion	R	S	S	S
Norfloxacin	R	S	S	S
Ampicillin	S	S	S	S
Cefotaxime	S	S	S	S
Cephathnin	S	S	S	S
Cotrimoxazole	R	R	R	R
Note: S: susceptible, R: resistance				

Discussion

In the present study, microflora isolated from the vaginal swabs of healthy women exhibited probiotic properties along with antimicrobial activity against pathogenic bacteria isolated from vaginal swabs of precancerous and cervical cancer patients. Depletion of beneficial vaginal *Lactobacilli* with increased load of pathogenic bacteria is one of the high-risk factors for cervical cancer progression (Adebamowo et al. 2017; Mitra et al. 2020). Vaginal

microflora of cervical cancer patients is predominantly colonised with different anaerobic (*Ureaplasma parvum, Atopobium vaginae, Megasphaera, Prevotella, Gardnerella, Sneathia sanguinegens, Fusobacteria, Fastidiosipila* and *Dialister*) and aerobic (*S. epidermidis, E. faecalis, E. coli*) pathogens (Adebamowo et al. 2017; Caselli et al. 2020; Kalia et al. 2020; Mitra et al. 2020). Such microflora produce toxins and carcinogenic metabolites (nitrosamines) that trigger oxidation of DNA and production of pro-inflammatory cytokines, which together aid in the progression of cervical cancer (Chen et al. 2019; Mitra et al. 2020).Thus, a balanced vaginal microbiome may not only prevent vulvo-vaginal infections but could also regulate cancer development (Godoy-Vitorino et al. 2018; Hatta et al. 2021).

The present data showed that out of 75 non-hemolytic isolates from healthy group, 13 strains survived gastro-intestinal transit (GIT) with more than 75% survival, which is an important criteria for development of oral probiotics. For oral delivery, probiotics should a) be non-pathogenic; b) tolerate the unfavourable conditions of the gastrointestinal tract (acidic pH and bile acid concentrations) and c) reach the intestine in a viable state (Dinçer and Kıvanç 2021; Oh et al. 2018). The tolerance of other vaginal probiotics such as *L. rhamnosus*, *L. helveticus* and *L. salivarius* (Pithva et al. 2014), *L. gasseri* and *L. plantarum* (Bouridane et al. 2016), *L. fermentum* (Brandt et al. 2020) to simulated human gastrointestinal tract conditions has been reported earlier. Acid-bile tolerance for *L. gasseri, L. fermentum* has been reported with above 70–80% survival (Archer and Halami 2015; Oh et al. 2018). Thus, the thirteen strains from healthy individuals exhibited probiotic properties of survival in GI tract.

Initially, MALDI-TOF MS analysis was used to identify the thirteen stains, among which *L. gasseri* was found to be the dominant strain, followed by *L. fermentum*, *E. faecium*, *L. reuteri* and *L. delbrueckii*. The species level identification of the LAB strains was further confirmed by 16S rRNA identification, with scores in the range 1.7–2.0. Similar observations have been reported earlier with vaginal LAB (Anderson et al. 2014). The isolates from LSIL, HSIL and ICC vaginal swabs were either cocci or Gram negative rods and only few had Lactobacilli-like morphology. Among these isolates, three strains of *E. faecalis* exhibited alpha hemolytic activity whereas three strains of *S. haemolyticus* and two strains of *E. coli* exhibited beta hemolytic activity, thereby confirming their pathogenic nature. *E. faecalis* isolated from patients with intestinal infection has been reported to exhibit alpha hemolysis (Bello Gonzalez et al. 2017). Beta hemolysis has been reported for several clinical strains of *E. coli* (Navidinia et al. 2012; Navidinia 2014; Toval et al. 2014) and *S. haemolyticus* (Pinheiro et al. 2015), isolated from patients with urinary tract and blood infections, respectively.

In the current study, the CFSs of all the thirteen strains inhibited the growth of the standard pathogen, *P. aeruginosa* MCC 2081. CFS of four isolates, *L. fermentum* P37Mws, *L. gasseri* P36Mops, *L. delbrueckii* P31Mcs and *E. faecium* P15Mcm exhibited good to strong antibacterial activity against vaginal pathogens isolated from vaginal swabs of LSIL, HSIL and ICC patients. The antibacterial activity of vaginal probiotic *L. fermentum*, isolated from healthy Algerian women was reported against the vaginal pathogens *E. coli, Staphylococcus* sp., *Enterococcus* sp., and *Candida* sp. (Ouarabi et al. 2019). CFS from Lactobacillus strain VLb3 has shown antibacterial activity against the vaginal pathogen *G. vaginalis* ATCC14018 (Andreeva et al. 2016). Bacteriocin extracted from vaginal probiotic *Lactobacillus* has shown activity against *Salmonella, Gardnerella, Chlamydia, Trichomonas* and *Neisseria*, isolated from patients with cervicovaginal infection (Dasari et al. 2014). The healthy vaginal microflora is mostly dominated by *Lactobacillus* species and opportunistic pathogens (Kyrgiou et al. 2017). However, *Lactobacillus* dominant communities protect the host against genital pathogenic infections through the production of antimicrobial compounds and short chain fatty acids (SCFA) that acidify the local microenvironment by keeping vaginal pH below 4.5 (Kyrgiou et al. 2017). Depletion of beneficial *Lactobacillus* could increase vaginal pH, thereby increasing its susceptibility to infection with diverse aerobic (*Staphylococcus* sp, *Pseudomonas* sp. *E. coli*, and *E. faecalis*) and anaerobic (*Gardnerella* sp., *Atopobium* sp., *Eggerthella* sp., *Sneathia* sp. and *Prevotella* sp.) bacteria. Such dysbiosis could modulate immune responses and thus lead to pathogenesis of several diseases including cervical cancer.

The CFS from *L. gasseri* P36Mops was found to be rich in antimicrobial compounds such as N-(1-deoxy-1-fructosyl)valine (organic acid) (Fuochi et al. 2019); alkaloids, homoarecoline (Matsumoto et al. 2012) and hygroline (Jurica et al. 2017), 2-isopropyl-1,4-benzenediol (hydroquinone) (Cretton et al. 2021); long chain fatty alcohols, 1-acetoxy-2-hydroxy-16-heptadecen-4-one, avocadyne 4-acetate and avocadyne 2-acetate (Rodríguez-Sánchez et al. 2019); grandidentatin (cinnamate ester) (Tyśkiewicz et al. 2019); taraxinic acid glucosyl ester (sesquiterpene lactone); (Cartagena et al. 2008) and alkaloid, nigellicine (Mohammed et al. 2019). *L. fermentum* P37Mws CFS showed presence of methylarmepavine (benzylisoquinolines) with reported anti-Leishmanial and antibacterial activities (Do Nascimento et al. 2015); and antimicrobial compounds such as 6-hydroxypentadecanedioic acid (long-chain fatty acid) (Rocchetti et al. 2020), hygroline (Jurica et al. 2017), avocadyne 4-acetate, avocadyne 2-acetate and 1-acetoxy-2-hydroxy-16-heptadecen-4-one (Rodríguez-Sánchez et al. 2019). CFS from *L. delbrueckii* P31Mcs showed the presence of antimicrobial metabolites such as (+)-O-methylarmepavine (Do Nascimento et al. 2015), 1-acetoxy-2-hydroxy-16-heptadecen-4-one, avocadyne 4-acetate, avocadyne 2-acetate (Rodríguez-Sánchez et al. 2019) and quinoline derivatives, 6-methylquinoline and quinaldine (Bawa et al. 2009; Jeon et al. 2009). *E. faecium* P15Mcm CFS had antimicrobial compounds such as alkaloids, ephedrine (Tulgar et al. 2018) and hordenine (Zhou et al. 2018), nepetalactam (tetrahydropyridine) (Aridoss et al. 2008); cuminaldehyde (benzaldehyde) (Wongkattiya et al. 2019); anethole (phenylpropanoid) (Esfandyari-Manesh et al. 2013); estragole (olefinic compound) (Song et al. 2016); 6-hydroxypentadecanedioic acid (Rocchetti et al. 2020); hygroline (Jurica et al. 2017); taraxinic acid glucosyl ester (Cartagena et al. 2008; Tyśkiewicz et al. 2019); oxane and tertiary allylic alcohol derivatives, avermectin A2b aglycone and avermectin B2a aglycone (El-Saber Batiha et al. 2020). Some antimicrobial metabolites were common in CFS of all the four probiotics. Antimicrobial activity of *L. fermentum* TcUESC01 having valine and benzeneacetic acid as metabolites have shown activity against *Streptococcus mutans* UA159 (de Souza Rodrigues et al. 2020). Different strains of *L. fermentum* and *L. gasseri*, isolated from human (oral and vaginal) samples, have shown antibacterial activity against *P. aeruignosa* (Fuochi 2016) and *Legionella pneumophila*. Hydroquinone has shown antibacterial activity against *E. faecalis* (Jurica et al. 2017). Different species of *Lactobacillu*s producing metabolites such as 2,4-hexadienoic acid and hydroxypentadecanedioic acid have shown antimicrobial activity against *Candida vini* (Lipinska-Zubrycka et al. 2020). *L. rhamnosus and L. salivarius*, producing valine, acetate, ethanol, 2-3-butanediol, uridine, 3 hydroxyphenylacetate have shown antibacterial activity against *L. pneumophila* (Fuochi et al. 2019). *L. plantarum*, producing organic acids such as 1,2-

benzenedicarboxylic, palmitic, oleic, pentadecanoic acid, inhibited the growth of *E. coli* (Kanjan and Hongpattarakere 2016). *Lactiplantibacillus plantarum* producing the different metabolites showed antibacterial activity against *S. aureus* (Ray Mohapatra et al. 2022).

The selected four probiotic strains showed moderate hydrophobicity (35.88–56.70%) towards xylene (apolar solvent), with *L. delbrueckii* P31Mcs (56.70%) showing highest hydrophobicity. Cell surface hydrophobicity and auto-aggregation properties correlate with adhesion of the probiotics to the epithelial cells for a longer time, a prerequisite for preventing colonization of pathogens at the epithelial surface and for ensuring health benefits (Krausova et al. 2019). Hydrophobicity of any probiotic is measured by evaluating their affinity to hydrocarbon solvent by microbial adhesion to hydrocarbon (MATH) method. Hydrophobicity can be divided into low (< 33%), moderate (33–66%), or high (> 66%) (Fonseca et al. 2021). Bacteria with higher hydrophobicity can bind better to epithelial cells and thus prevent colonization of pathogens. Interestingly, *E. faecium* P15Mcm exhibited strong auto-aggregation and others showed moderate auto-aggregation. Different vaginal *L. fermentum* species have shown 60–80% auto-aggregation, whereas vaginal *L. gasseri* UBLG36 has shown 32.98% auto-aggregation (Ahire et al. 2021). The selected probiotic strains produced strong biofilm on the plastic surface of the 96-well microplate with higher production by *L. fermentum* P37Mws. Biofilm formation by probiotic bacteria is considered to be a beneficial property because it prevents the colonization of pathogenic bacteria (Salas-Jara et al. 2016). Biofilm producing probiotics such as *L. rhamnosus* and *L. reuteri* have been successfully used in adjuvant treatments of bacterial vaginosis (Ventolini 2015). Earlier, two strains of *L. plantarum*, LSC3 and LSC22 have shown biofilm formation, whereas three strains of *L. plantarum* LSC did not produce biofilm on plastic surfaces (Gheziel et al. 2019). *L. delbrueckii* HY5 and three strains of *L. fermentum* (RGM3, RCM11 and RCM13) have been reported to produce strong biofilm (Aziz et al. 2019). Our results are in agreement with the reported biofilm production by *L. plantarum* and *L. fermentum* (Gheziel et al. 2019; Aziz et al. 2019).

L. gasseri P36Mops, *L. fermentum* P37Mws and *E. faecium* P15Mcm showed resistance to most of the antibiotics such as norfloxacin, nitrofuramion, metronidazole, nalidixic acid and cotrimoxazole. These antimicrobials are generally used for the treatment of BV (Bradshaw and Sobel 2016; Larsson et al. 2011; Schwebke and Desmond 2007) and urinary tract infections (Anger et al. 2019). Most of the vaginal *Lactobacillus* sp. has been reported to show resistance towards norfloxacin, nitrofuramion, nalidixic acid (Fonseca et al. 2021) and co-trimoxazole (Salas-Jara et al. 2016) and metronidazole (Mastromarino et al. 2002; Pithva et al. 2014). Resistance to antibiotics is considered to be a positive feature in vaginal microbiota restoration therapy (Wiik et al. 2019). Decrease in Lactobacilli load favours the growth of HPV and microorganisms in bacterial vaginosis (Happel et al. 2020). A significant correlation has been reported between BV and cervical cancer (Muñoz et al. 2006). Recent evidences have suggested that besides HPV infection, cervical microflora play an important role in the development of cervical cancer (Muñoz et al. 2006). Thus, the resistant probiotics strains, *L. gasseri* P36Mops, *L. fermentum* P37Mws, *L. delbrueckii* P31Mcs and *E. faecium* P15Mcm could be used to restore the normal cervico-vaginal microflora during the vaginal infections.

Probiotics work through different mechanisms through 1) production of organic acids (lactic acid and short chain fatty acids) that maintain vaginal pH below 4.5 and antimicrobial agents such as hydrogen peroxide, bacteriocins and peptides (Tachedjian et al. 2017); 2) increase in the mucosal viscosity in the vagina (Di Cerbo 2016); 3) stimulation of the immune system (Di Cerbo 2016); and 4) formation of biofilms at the epithelial layer, thus inhibiting colonization of pathogens at the epithelial surface of the cells (Di Cerbo 2016; Mitra et al. 2020). Probiotic supplementation in cervical cancer patients has been reported to reduce the radiotherapy associated side effects such as diarrhoea, vaginal dryness, itching and increased risk of vaginal infections (Linn et al. 2019). The present probiotics hold a great promise in managing cervical cancer-associated bacterial infections and may also help in preventing chemo/radio therapy-associated side effects in cervical cancer patients.

Conclusion

The present study indicated that healthy vaginal ecosystem is an excellent source of *Lactobacillus* and *Enteroccocus* sp. with promising probiotic characteristics that could be explored to manage cervical cancer-associated infections. Probiotic LAB supplementations in cervical cancer patients can help in restoration of abnormal vaginal flora, prevent vaginal dysbiosis and help in attenuating therapy associated side effects. However, extensive studies need to be done to identify the most beneficial and safe probiotic strains for the treatment of cervical cancer associated pathogens as well as management of cervical cancer.

Declarations

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Data availability

The data generated in this study has been included in the article and in the supplementary information files.

Compliance with ethical standards

The study was conducted according to the guidelines of the Helsinki Declaration and all the procedures involving human patients was approved by the Institutional Ethics Committee (IEC)s of Bharati Vidyapeeth (Deemed to be) University Medical College (Ref: BVDU/MC/57) and B. J. Government Medical College Sassoon General Hospitals (Ref No. BJGMC/IEC/Pharmac/ND-Dept 0119007-007], Pune.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figures

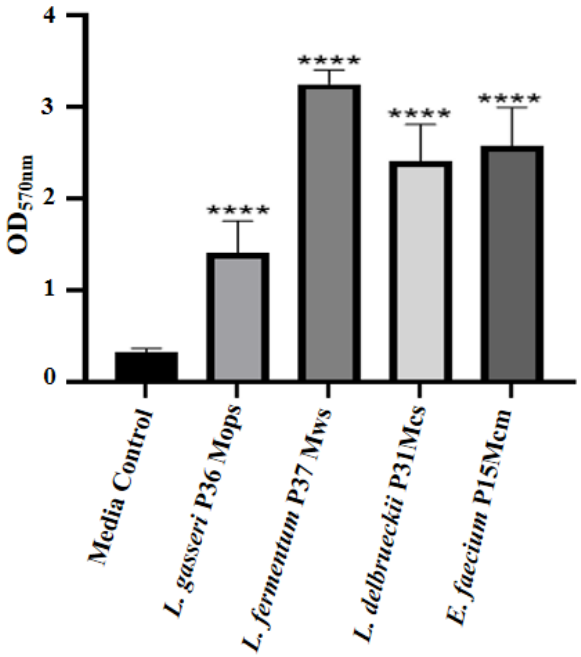

Figure 1

Probiotics exhibited property of biofilm formation. The figure shows biofilm property of the selected probiotics. The data has been presented as mean±SD of three independent experiments, each performed in triplicates. ****p<0.0001 indicates statistically significant differences compared to the media control.

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