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Original Article

Cross sectional analysis of vaginal *Lactobacillus* in asymptomatic women of reproductive age in Mumbai, India

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Abstract

Introduction: *Lactobacillus* dominated vaginal microenvironment is associated with lower risk of genital infections. Numerous studies have reported geographic and ethnic variations in vaginal microbiome structure between healthy individuals from different race and ethnicity. India has a great diversity, so it is intriguing to find out if such divergences exist in vaginal lactobacilli. The present study aimed to investigate predominant *Lactobacillus* species in vaginas of healthy Indian women and screen isolates for lactic acid and H_2O_2 production.

Methodology: 203 premenopausal women asymptomatic for any vaginal complaints were recruited. The lactobacilli isolates on MRS agar were identified by Multiplex-PCR and 16sRNA gene sequencing. RAPD was used to differentiate strains of same species. H_2O_2 and lactic acid was evaluated on TMB-HRP MRS agar and BCP-MRS agar respectively.

Results: Lactobacilli were recovered from 107/109 (98.2%) women with normal microflora. *L. iners* 64.7% (68), *L. crispatus* 26.7% (28), *L. reuteri* 21.9% (23), *L. jensenii* 16.2% (17) and *L. gasseri* 15.2% (16) were the most frequently occurring vaginal lactobacilli in normal women. The vaginal microflora was dominated by either by a single (80%, n = 84) or a combination (20%, n = 21) of *Lactobacillus* species. Though most frequently identified, *L. iners*, coexisted only with other *Lactobacillus* species. All isolates were acid producers but H_2O_2 was produced by 94.2% isolates.

Conclusions: Our study reports prevalent vaginal lactobacilli which could be explored as probiotics. Presence of heterogeneous *Lactobacillus* population highlights the cumulative effects of different lactobacilli maintaining vaginal health. Contrasting observations about *L. iners* reiterates its puzzling role in vaginal immunity, advocating further research.

Key words: Lactobacillus; vagina; diversity; lactic acid; hydrogen peroxide; probiotic.

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Introduction

The microbial inhabitants of the vaginal tract play an important role in maintaining the vaginal health by protecting against a number of urogenital infections [1]. This protective microbiota mainly includes *Lactobacillus* that plays a crucial role in maintaining homeostasis of vaginal microbiota (VMB) through the production of metabolites such as lactic acid, hydrogen peroxide and bacteriocins secreted in the cervicovaginal fluid [2]. Dysbiosis in the VMB can lead to depletion in the lactobacilli population, resulting in bacterial vaginosis (BV) [3]. BV is associated with genital tract infections such as urinary tract infections, increased risk of acquisition of STIs including HIV and pelvic inflammatory disease [4,5]. Bacterial vaginosis can lead to adverse pregnancy outcomes such as intra-amniotic infection, preterm birth and infertility [6-8]. Considering antibiotic therapy to control these infections has associated antimicrobial resistance and recurrences, seeking natural methods for rehabilitation of vaginal microbiota may provide better substitute for treatments of vaginal infections [9]. Lactobacilli being one such alternative can attenuate the infection causing microbiota. These lactobacilli dominate the VMB during the reproductive phase as compared to prepubertal or menopausal phase where greater abundance of other taxa is reported [10,11].

Studies have presented the existence of different species and strains of *Lactobacillus* prevalent in vaginal isolates of women from different countries and ethnicities [12,13]. Previous reports show Ugandan women were mainly colonized by *L. reuteri, L. crispatus, L. vaginalis* and *L. jensenii* [14]. The prevalent vaginal lactobacilli studied in South African women were *L. crispatus, L. iners, L. gasseri* [15] whereas *L. acidophilus*, *L. iners, L. gasseri* were identified in Mexican women [16]. Reports on distribution of *Lactobacillus* in the healthy vaginas of

Indian women are conflicting. A study from North India reported prevalence of different species of vaginal *Lactobacillus* in comparison to other countries [17], on the other hand, observed vaginal lactobacilli from women in South India were similar to that of other countries, but different from those in North India. It appears there is variation in vaginal *Lactobacillus* species among different population in India due to its diverse lifestyle and geographic segmentation [18]. Also, despite having similar *Lactobacillus* diversity, the metabolic properties of these *Lactobacillus* species may differ between countries [19]. Identifying and evaluating prevalent lactobacilli in the population could be useful for designing probiotics for vaginal health. The aim of the current study is to identify the diversity of vaginal *Lactobacillus* isolated from women visiting a tertiary care centre in Mumbai and assess these colonized Lactobacilli for their biochemical properties.

Methodology

Study Population

The use of human subjects in this study had prior approval of NIRRH Ethics Committee for Clinical Studies (Protocol Number 215/2012) and Institutional Ethics Committee of Seth G.S. Medical college and KEM hospital (Protocol No EC/GOV-5/ 2012) and was carried out in accordance with the guidelines of Good Clinical Practice and in compliance with the Helsinki Declaration. Women who satisfied the inclusion criteria and signed an informed consent were recruited from the Gynecology Outpatient Clinic of Seth G.S. Medical College and KEM Hospital, Mumbai from August 2013 to Jan 2015. About 424 women were evaluated for their eligibility to be included in the study. The recruited 203 women were healthy premenopausal between 18–45 years of age, not taking contraceptive steroids or antibiotics in the last six weeks and without complaints of urogenital infections.

Sample collection

Two vaginal swabs were collected from each individual by rolling the swab across the upper lateral wall of the vagina. One swab was used for determination of vaginal pH using pH paper (HiMedia, Mumbai, India), Nugent scoring [20] and wet mount microscopy; the other was used for bacterial culture and molecular analysis. The presence of *Trichomonas vaginalis* and *Candida albicans* were assessed by wet mount and growth on HiChrome Candida Differential agar (HiMedia, Mumbai, India) respectively. Further study was carried out on women with normal microbiota.

Culture conditions and isolation

The bacteria retrieved on swabs were suspended in 1 ml sterile PBS and inoculated on DeMan-Rogosa-Sharpe (MRS) agar and HiChrome Candida differential agar (HiMedia, Mumbai, India). As MRS is not supportive of *L. iners*, Brain Heart Infusion broth (HiMedia, Mumbai, India) was used to enrich its growth. After incubation in candle-jar at 37°C for 48 hours, each isolated colony was observed for Gram's reaction and morphology. Gram-positive rods and catalase negative colonies were selected for further study.

Identification of Lactobacillus

Lactobacillus species identification by Multiplex-PCR

Bacterial genomic DNA was extracted using QIAamp Kit (Qiagen, Hilden, Germany , Cat. No. 69504) following manufacturer′s instructions and stored at -20ºC. Gram-positive isolates from MRS agar medium were identified to the genus level by amplification with primers LbLMA-rev (5' CTC AAA ACT AAA CAA AGT TTC 3') and R16-1 (5' CTT GTA CAC ACC GCC CGT TCA 3') [21]. Taxonomic grouping was carried out by employing multiplex PCR-G using the protocol described previously [22] (Table 1). PCR programme included initial denaturation at 95°C for 1 minute followed by 35 cycles of denaturation at 95ºC for 20 seconds, annealing and extension at 55°C for 2 minutes; and final extension at 74ºC for 5 minutes. *Lactobacillus* species were identified by multiplex PCR for species as mentioned elsewhere [22-24] (Table 1). PCR programme and reaction mix was same as for PCR-G except annealing temperature which was 68ºC for PCR II-1, 65ºC for PCR II-2, 62ºC for multiplex PCR III and 60ºC for PCR IV. PCR generated products were analysed by electrophoresis in 2 per cent agarose gel containing ethidium bromide.

16s rRNA gene sequencing

Partial sequencing of the 16S rRNA gene was carried out to confirm representative isolates identified by species PCR. Isolates with uncertain identity were also identified by sequencing [25] (Table1). Isolates that generated sequences with < 98% homology to *Lactobacillus* species on analysis using BLAST were not included for the study.

Lactobacillus strain differentiation

Random amplified polymorphic DNA (RAPD) analysis was done to differentiate various isolates of the same species. The primer used was 5' AGT CAG CCA

C 3' (Sigma, Banglore, India) as per Tynkkynen *et al.* [26]. The reaction mix contained 30 ng of template DNA in PCR buffer with 2 mM $MgCl_2$, 0.2 mM of each nucleotide and 2.5U of Taq polymerase (Merck, Mumbai, India) in a total volume of 25 µL. PCR amplification was conducted in an Applied Biosystems Thermal Cycler with the following temperature profiles, initial denaturation at 94° C for 5 minutes, followed by 30 cycles at 94° C for 45 seconds, 32° C for 2 minutes, 72° C for 2 minutes, and final extension at 72° C for 5 minutes. PCR products were visualized on 1.5 per cent agarose gel.

Biochemical Characterization

Screening of acid producers

Lactobacillus isolates were streaked on MRS agar plate containing Bromo-cresol purple (HiMedia, Mumbai, India). The lactic acid producing bacteria were identified by the change in color of colony from purple to yellow on incubation at 37° C for 48 hours. Acid production was further determined indirectly by measuring pH of cultured supernatant.

Screening of Hydrogen peroxide (H_2O_2) producers

Hydrogen peroxide producing ability of the strains was determined using the method described by Parolin *et al.* [27] with slight modifications. Briefly *Lactobacillus* isolates were grown on MRS agar containing 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, St Louis, USA) horseradish peroxidase (HRP) (Sigma-Aldrich, St Louis, USA). After incubation plates were exposed to air for variable period of time before scoring them for blue coloration. On the basis of the time required for the blue coloration to appear, isolates were scored as weak (>60 minutes), intermediate (15–60 minutes) and strong producing strains (< 15 minutes). Isolates not producing blue coloration were scored as non-producers. Since it was difficult to culture *L. iners* on MRS agar medium, *L. iners* isolates were not included in the H_2O_2 production test.

Table 1. List of oligonucleotide primers used in the study.

Target	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Amplicon size (bp)	Annealing Temp. (°C)	References
Lactobacillus Genus PCR	CTT GTA CAC ACC GCC CGT TCA	CTC AAA ACT AAA CAA AGT TTC	250	55	[21]
Multiplex PCR					
Group I *(L. delbrueckii)*	ACAGATGGATGGAGAGCAGA (Ldel-7)	CCTCTTCGCTCGCCGCTACT (Lac-2)	450	55	[22]
Group II	ATTGTAGAGCGACCGAGAAG (LU-1)		300		
Group III	AAACCGAGAACACCGCGTT (LU-3)		400		
Group IV	CTAGCGGGTGCACTTTGTT (LU-5)		350		
Group II-1					
L. acidophilus	TGCAAAGT GGTAGCGTAAGC (Laci-1)	CCTTTCCCTCACGGTACTG (23-10C)	210	68	[22]
L. jensenii	AAGAAGGCACTGAGTACGGA (Ljen-3)		700		
Group II-2					
L. crispatus	AGGATATGGAGAGCAGGAAT (Lcri-3)	CAACTATCTCTTACACTGCC (Lcri-2)	522	65	[22]
L. gasseri	AGCGACCGAGAAGAGAGAGA (Lgas)		360		
Group III					
L. paracasei	CTAGCGGGTGCGACTTTGTT (LU-5)	GGCCAGCTATGTATTCACTGA (Lpar)	312	62	[22]
L. rhamnosus		GCGATGCGAATTTCTATTATT (Lrha)	113		
Group IV					
L. salivarius	AATCGCTAAACTCATAACCT (Lsal-1)	CACTCTCTTTGGCTAATCTT (Lsal-2)	411	60	[22]
L. reuteri	CAGACAATCTTTGATTGTTTAG (Lreu-1)	GCTTGTTGGTTTGGGCTCTTC (Lreu-4)	303		
L. plantarum	ATTCATAGTCTAGTTGGAGGT (Lpla-3)	CCTGAACTGAGAGAATTTGA (Lpla-2)	248		
L. fermentum	ACTAACTTGACTGATCTACGA (Lfer-3)	TTCACTGCTCAAGTAATCATC (Lfer-4)	192		
Species PCR					
L. iners	GTCTGCCTTGAAGATCGG	ACAGTTGATAGGCATCATC	158	55	[23]
L. vaginalis	CTGATATGACGTGCTTGCACTG	CCGAAACCATCTTTGAAACG	168	60	Present study
L. johnsonii	GAGCTTGCCTAGATGATTTTA	ACTACCAGGGTATCTAATCC	770	57	[24]
Universal 16s rRNA gene	AGA GTT TGA TCC TGG CTC AG	CCC ACT GCT GCC TCC CGT AG	350	48	[25]
RAPD PCR	AGTCAGCCAC		variable	32	[26]

Statistical analysis

To achieve sample size of 100 asymptomatic women for the study, considering prevalence of reproductive tract infections of 52% among women, sample needed to screen the spectrum of vaginal lactobacilli in healthy Indian women was 403 (5% level of significance, 5% precision and 5% non-response rate). The proportion of women with a particular *Lactobacillus* was analysed using SPSS (version 16.0). Data of culture supernatants pH was summarized in the form of Mean and Standard deviation.

Results

Characterization of vaginal samples by Nugent scoring and culture based methods

Of the 424 women screened, vaginal swabs were sampled from 203 asymptomatic healthy women who satisfied the inclusion criteria of the study.The mean age of the study population was 31.1 years and the mean vaginal pH was 4.1 ± 0.5. Nugent scoring of vaginal swabs obtained from these women was done to exclude 57 participants with altered vaginal flora (Nugent score > 4) (Figure 1a). Additional 37 women with Nugent score of 0-3 showing presence of trichomonas and yeast determined by wet mount and/or growth on Candida differential agar were excluded from the study. Vaginal specimens from the rest 107 women with Nugent score 0-3 were processed for *Lactobacillus* identification by culture on MRS and BHI medium (Figure 1b). Cultivable lactobacilli from vaginal specimens were recovered from 107/109 (98.2%) women. All 244 isolates from MRS agar plates were Gram-positive, non-spore forming, catalase-negative, rods with variable morphology characteristic of *Lactobacillus*. Cell morphology observed by Gram-staining varies widely from short, long, straight or slightly crescent shaped rods to coryneform coccobacilli; though colony characteristics were similar (Figure 1c and d).

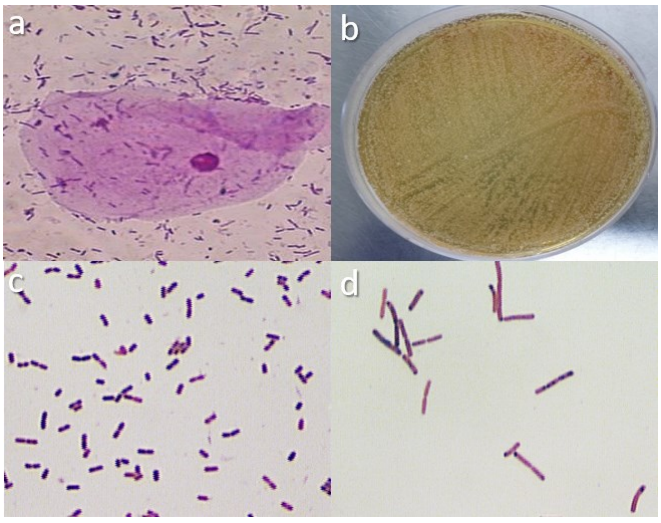
Identification of lactobacilli

Genus PCR could identify *Lactobacillus* from 105 women (Figure 2a). Multiplex species PCR identified *L. iners* (n = 68; 64.7%), *L. crispatus* (n = 28; 26.7%), *L. reuteri* (n = 23; 21.9%), *L. jensenii* (n = 17; 16.2%) and *L. gasseri* (n = 16; 15.2%) as the prevalent species in our studied population (Figure 2b, c and 3). The other lactobacilli identified were *L. vaginalis* (n = 13; 12.4%), *L. rhamnosus* (n = 11; 10.5%), *L. johnsonii* (n = 11; 10.5%), *L. salivarus* (n = 10; 9.5%), *L. plantarum* (n = 6; 5.7%), *L. fermentum* (n = 6; 5.7%), *L. acidophilus* (n = 3; 2.9%), and *L. delbruckii* (n = 2; 1.9%) (Figure 3). *L. paracasei* was not detected in any tested isolates. Representative strains of each species were confirmed by DNA sequencing. RAPD analysis indicated the presence of different strains within the same species (Figure 2d).

Lactobacillus communities harboured in vaginal milieu

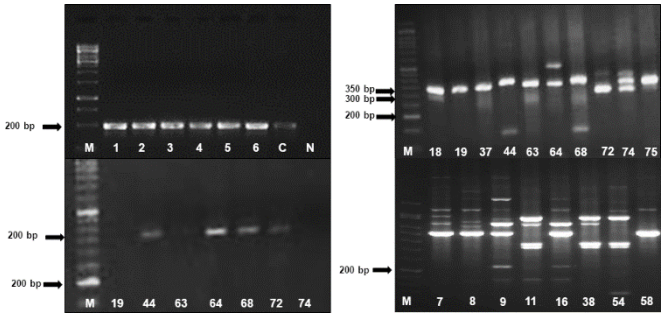
On further analysis we categorized 21 women (20%) colonized with a single species of vaginal lactobacilli and 84 women (80%) colonized with two or more *Lactobacillus* species simultaneously. Of these 84

Figure 1. Characterization of vaginal samples.



a) Gram-stained image of vaginal specimen with Nugent grade I. b) *Lactobacillus* colonies obtained from vaginal samples when grown on MRS agar. c) and d) Variable cell morphology of different species of *Lactobacillus* observed by Gramstain.

Figure 2. Molecular identification of Lactobacillus isolates.



Representation of agarose gel electrophoresis of PCR products from (a) Genus PCR products of isolates (1-6) showing 200bp DNA band (b) Multiplex PCR for grouping of lactobacilli showed presence of Group II by isolates (19,44,72) having 300bp; 350bp produced by isolates (44,63,64,68,72,74,76,79) indicated presence of Group IV, 400bp produced by isolates (18,19,37) indicated presence of Group III and 450bp amplicon was seen in isolate no. 75 showing presence of Group I *Lactobacillus* (c) Species specific PCR products for Group II, where isolates (6,19,44,72) showing presence of *L. gasseri* generating amplicon of 360bp d) RAPD of *L. reuteri* isolates (7,8,9,11,16,68,74,85) generating variable amplicon patterns Key: M: DNA Marker of 50bp, C: Positive control, N: Negative control. The numbers in the parenthesis denote different isolates.

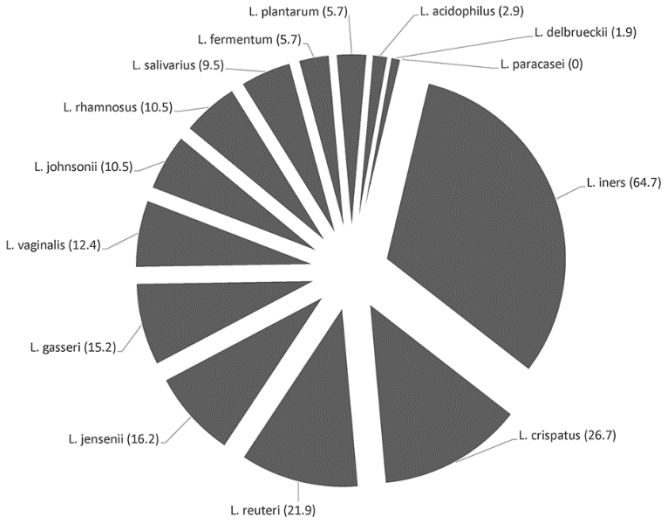
women, 48.6% harboured two species, 28.6% had three species and 2.9% women presented with four species. *L. iners* (n = 68; 80.9%,), *L. crispatus* (n = 21; 25%), *L. reuteri* (n = 18; 21.4%), *L. jensenii* (n = 16; 19.0%) were the prevalent species of heterogenous population whereas *L. crispatus* (n = 7; 29.2%), *L. reuteri* (n = 5; 20.8%), *L. gasseri* (n = 2; 8.3%) and *L. vaginalis* (n = 2; 8.3%) formed homogenous population (Table 2). None of the women with homogenous *Lactobacillus* population harboured *L. iners*. The species was found be co-isolated majorly with *L. crispatus* (n = 17; 16.2%), *L. reuteri* (n = 13; 12.4%) and *L. jensenii* (n = 12; 11.4%). Other pairs co-isolated were *L. gasseri* and *L. reuteri* (n = 5; 4.7%), *L. jensenii* and *L. crispatus* (n = 4; 3.8%), *L. crispatus* and *L. vaginalis* (n = 4; 3.8%).

Biochemical characterization

All 244 isolates of *Lactobacillus* were acid producers as determined on BCP-MRS agar (Figure 4a). H_2O_2 producing Lactobacilli were present in 103(98.2%) women (Figure 4b). Hydrogen peroxide was produced by 94.2% of lactobacilli isolates. Detectable H_2O_2 was produced by 93% of *L. crispatus*, 96.7% of *L. reuteri*, 96.0% of *L. jensenii* and 94.6% of *L. gasseri* isolates. Of the total *Lactobacillus* studied more than half of each species were strong producers of H_2O_2 (Table 3).

The acidification ability of the different *Lactobacillus* isolates was determined by growth in MRS for 48 hours. The mean pH of the culture supernatants was 4.09 ± 0.6. *L. crispatus* and *L. reuteri* acidified the pH of the medium to 4.2 whereas *L. jensenii* and *L. gasseri* reduced the pH to 4.0. Isolates of *L. plantarum, L. delbreuckii, L. johnsonii* and *L. salivarius* showed pH below 4.0 (Table 3).

Figure 3. Distribution of Lactobacillus species colonized in the vaginas of Indian women.



Number in the parenthesis denotes percentage of women harboured with *Lactobacillus* species.

Discussion

In our study of 105 women *L. iners, L. crispatus, L. reuteri L. jensenii and L. gasseri* are identified as the most frequently occurring native species of the vaginal microenvironment. These species have been reported as the most prevalent vaginal species in other studied populations [14,15]. Predominant vaginal lactobacilli in Indian women were reported to be different from other

Table 2. Distribution of colonizing *Lactobacillus* species in 105 asymptomatic women at a tertiary care centre in Mumbai and types of *Lactobacillus* population harboured in vagina.

Species	Women colonized n (%)	Type of population	
		Homogenous n (%)	Heterogenous n (%)
L. iners	68 (64.7)	0 (0)	68 (80.9)
L. crispatus	28 (26.7)	7 (29.2)	21 (25)
L. reuteri	23 (21.9)	5 (20.8)	18 (21.4)
L. jensenii	17 (16.2)	1 (0.9)	16 (19.0)
L. gasseri	16 (15.2)	2 (8.3)	14 (13.3)
L. vaginalis	13 (12.4)	2 (8.3)	11 (10.5)
L .johnsonii	11 (10.5)	0 (0)	11 (10.5)
L. rhamnosus	11 (10.5)	1 (4.8)	10 (9.5)
L. salivarius	10 (9.5)	1 (4.8)	9 (8.6)
L. fermentum	6 (5.7)	0 (0)	6 (5.7)
L. plantarum	6 (5.7)	0 (0)	6 (5.7)
L. acidophilus	3 (2.9)	1 (4.8)	2 (1.9)
L. delbrueckii	2 (1.9)	1 (4.8)	1 (0.9)
L. paracasei	0 (0)	0 (0)	0 (0)
Total	**105**	**21 (20)**	**84 (80)**

Numbers in the parenthesis denotes percentage. n indicates the number of women showing presence of that particular *Lactobacillus.*

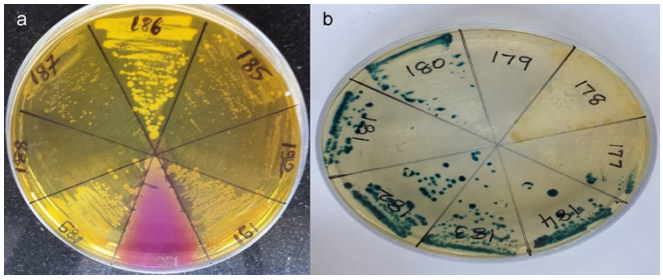
populations [17]. However, findings from our study and that in South India depicts different scenario of VMB among women within India [18]. Likewise, three different studies reported contrasting observations of vaginal lactobacilli within South Africa [15,28,29]. This leads to the possibility of variations in vaginal lactobacilli among women from different parts of the country due to ethnic and geographic diversity [18].

Studies have significantly associated *L. crispatus, L. reuteri, L. jensenii* and *L. gasseri* with normal vaginal microbiota suggestively playing protective functions to the FRT by preventing it from urogenital infections [18,28]. Identifying the same species in our study of healthy women supports the importance of these species in maintaining homeostasis of vaginal niche.

Vaginal microbiota in a woman was considered to be dominated by a single *Lactobacillus* species [30]. Majority of our population on the contrary, was inhabited by two or more *Lactobacillus* species, advocating the importance of cumulative effects of *Lactobacillus* species in safeguarding the vaginal ecosystem.

The role of *L. iners* in the female reproductive tract has been overlooked until recently due to its inefficiency in growing on MRS medium. Studying *L. iners* has been possible due to the advent of high throughput methods. The information on distribution of *L. iners* in the healthy VMB of Indian women is scarce. Presence of *L. iners* as the most frequent species in our population suggests its potential in adaptation to the vaginal niche. It was interesting to note the absence of *L. iners* as the single vaginal lactobacilli in women. Despite it being the most frequently identified vaginal

Figure 4. Biochemical Characterization of lactobacillus isolates.



a) Qualitative evaluation of *Lactobacillus* isolates for acid production on MRS agar supplemented with pH indicator dye Bromocresol purple. The acid producing isolates (185-189,191,192) were identified by the change in color of media from purple to yellow. Isolate no. 190 (identified as *Candida* sps.) was used as negative control. b) Semi quantitative evaluation of H_2O_2 production was performed on MRS agar supplemented with TMB and HRP. Isolates (177,180-184) producing blue colour on exposure to O_2 were considered H_2O_2 producers. Isolates (178,179) showing absence of blue colour after exposure O_2 for 60 minutes were assigned as H_2O_2 non-producers.

Lactobacillus species, its co-isolation with other *Lactobacillus* species indicates its minimal protective function alone. In fact presence of *L. iners* as the only *Lactobacillus* species in pregnant women has been indicated as a potential risk in preterm deliveries [31]. Various reports on *L. iners* indicated it to possess having the features of both probiotic and vaginal pathogens [32]. Due to its dual characteristic, the contribution of *L. iners* in vaginal health is ambiguous, necessitating further research.

RAPD analysis of *Lactobacillus* isolates showed considerable genetic diversity among the species. It is well recognized that different isolates of same *Lactobacillus* species exhibit variable functional properties depending on their strain [33,34], hence it is

Table 3. Characterization of biochemical metabolites of *Lactobacillus* isolates obtained from vaginal specimen of asymptomatic women.

Species	Total isolates	Acid producers	pH of cultured medium	H_2O_2 Producers	H_2O_2 production		
	(n)	n (%)	(Mean ± SD)	n (%)	Strong n (%)	Intermediate n (%)	Weak n (%)
L. crispatus	43	43 (100)	4.2 ± 0.63	40 (93)	23 (57.5)	05 (12.5)	12 (30.0)
L. reuteri	30	30 (100)	4.2 ± 0.67	29 (96.7)	19 (65.5)	02 (6.97)	08 (27.6)
L. jensenii	25	25 (100)	4 ± 0.64	24 (96)	08 (33.3)	06 (25)	10 (41.6)
L. gasseri	37	37 (100)	4 ± 0.66	35 (94.6)	14 (40.0)	07 (20.0)	14 (35.0)
L. vaginalis	27	27 (100)	4.2 ± 0.60	26 (96.3)	14 (11.3)	0 (0)	12 (14.6)
L. johnsonii	22	22 (100)	3.7 ± 0.61	21 (95.4)	12 (57.1)	04 (19.0.)	05 (23.8)
L. rhamnosus	18	18 (100)	4 ± 0.60	18 (100)	09 (50.0)	0 (0)	09 (50.0)
L. salivarius	10	10 (100)	3.7 ± 0.67	09 (90)	06 (66.6)	01 (11.1)	02 (22.2)
L. fermentum	16	16 (100)	4 ± 0.60	15 (93.8)	09 (60.0)	03 (20.0)	03 (20.0)
L. plantarum	09	09 (100)	3.4 ± 0.46	09 (100)	07 (77.7	01 (11.1)	01 (11.1)
L. acidophilus	05	05 (100)	4.4 ± 0.64	03 (60)	02 (66.6)	01 (33.3)	0 (0)
L. delbrueckii	02	02 (100)	3.7 ± 0.67	01 (50)	01 (50.0)	0 (0)	0 (0)
Total isolates	**244**	**244 (100)**	**____-**	**230 (94.2)**	**124 (53.9)**	**30 (13.0)**	**76 (33.0)**

imperative to characterise their biochemical properties. Lactic acid is one of the major metabolites of *Lactobacillus* that plays a crucial role in the vaginal immunity [35]. Considering all the isolated lactobacilli in our study produced acid substantiates the fact that it is one of the major elements, protecting the ecological niche of healthy vagina. The isolates were able to acidify the culture medium to pH 3-4.5, which is also the physiological pH of a healthy vagina. The low pH detected may be however attributed due to other organic acids besides lactic acid. It would be worthwhile to investigate the concentrations of lactic acid isomers in these samples.

Another metabolite of lactobacilli is H_2O_2 which is an essential component of vaginal defence system [36] Nearly all the lactobacilli isolates in our study produced H_2O_2 indicating its possible role in regulating the growth of pathogens. *In vitro* studies have shown antimicrobial and virucidal properties of hydrogen peroxide [33,37]. Still the antimicrobial effect of H_2O_2 *in vivo* has been questionable [38]. On the other hand, our findings show presence of H_2O_2 producers in nearly all women with a normal microbiota suggests its plausible role as a defence molecule. Besides, work by other researchers has also indicated sustained vaginal colonization of H_2O_2 producing lactobacilli over non-H_2O_2 producing isolates in women with normal microbiota [39]. Nonetheless quantifying the H_2O_2 producing ability of our isolates might be helpful in designing a microbicide.

Our study in identifying the vaginal lactobacilli is limited by the fact that only cultivable lactobacilli grown on MRS medium were studied. Studying the cultivable lactobacilli can be rationalized by our motives to evaluate their *in vitro* function that may help to identify potential candidates for microbicides or probiotics. The present investigation also had constraints of being a cross sectional study. Vaginal swabs collected at single time point may not reflect the dynamics of the *Lactobacillus* communities. Nonetheless the information generated from the present study can be authoritative from the reports of other studies that describe *L. crispatus* and *L. gasseri* dominated communities promotes stability over a period of time [40]. The isolated species could be further evaluated for their biotherapeutic properties such as colonization on vaginal epithelial surfaces and inhibitory effects on uropathogens.

Conclusion

Our investigation identified *L. iners L. crispatus, L. reuteri, L. jensenii* and *L. gasseri* as the major indigenous members of normal VMB in women of reproductive age. The diversity in *Lactobacillus* species identified in different parts of the country could be attributed due to the ethnic and lifestyle changes that prevail. Presence of heterogeneous *Lactobacillus* population emphasises the importance of analysing composite strains as probiotics or microbicides. Regardless *L. iners* being the prevalent lactobacilli, its lack of existence as the lone *Lactobacillus* species in the normal vaginal microbiota was conflicting. This depicts the ambiguous role of *L. iners* in vaginal immunity, warranting further research. Almost all women in our study harboured lactic acid and H_2O_2 producing lactobacilli, emphasizing the importance of lactic acid and H_2O_2 in maintaining the healthy vaginal microenvironment. The foremost hydrogen peroxide and lactic acid strains could be evaluated for other functional properties to efficiently design better probiotics for maintenance of normal flora and prevention of bacterial vaginosis.

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