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Vaginal lactobacilli profile in pregnant women with normal & abnormal vaginal flora

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***Background & objectives*: Lactobacilli species that are better adapted to vaginal environment of women may colonize better and offer protection against vaginal pathogenic bacteria. In this study, the distribution of common *Lactobacillus* species was investigated in pregnant women.**

***Methods*: Sixty seven pregnant women were included in the study and vaginal samples were collected for Gram staining. Women were classified as normal vaginal flora, intermediate flora and bacterial vaginosis (BV) based on Nugent's score. Vaginal samples were also collected for the identification of *Lactobacillus* spp. by multiplex polymerase chain reaction (PCR) profiling of 16S rDNA amplification method.**

***Results*: *Lactobacillus crispatus* (100%) was the most predominant *Lactobacillus* spp. present in pregnant women with normal flora, followed by *L. iners* (77%), *L. jensenii* (74%) and *L. helveticus* (60%). While, *L. iners* was commonly present across groups in women with normal, intermediate or BV flora, *L. crispatus*, *L. jensenii* and *L. helveticus* decreased significantly as the vaginal flora changed to intermediate and BV. In women with BV, except *L. iners* other species of lactobacilli was less frequently prevalent. Species such as *L. rhamnosus*, *L. fermentum*, *L. paracasei* and *L. casei* were not detected in any vaginal sample.**

***Interpretation & conclusions*: *L. crispatus*, *L. jensinii* and *L. helveticus* were predominant species in women with normal flora. *L. crispatus* alone or in combination with *L. jensinii* and *L. helveticus* may be evaluated for probiotic properties for the prevention and treatment of BV.**

Key words Bacterial vaginosis - *Lactobacillus crispatus* - multiplex polymerase chain reaction - Nugent score - pregnant women - vaginal *Lactobacillus*

The beneficial microbiota such as *Lactobacillus* spp. in vaginal ecosystem is thought to have been adapted and coevolved by mutualistic association with its human host[1]. It is essential to explore the *Lactobacillus* diversity of vagina in health and disease and to understand whether changes in the individual vaginal *Lactobacillus* spp. can be correlated with changes in vaginal infections[2]. Bacterial vaginosis (BV) is a common vaginal infection caused by imbalance of indigenous microbiota[3,4]. Disturbance in the vaginal flora with overgrowth of bacteria that are present in the vagina in small numbers such as *Gardnerella vaginalis*, *Prevotella, Mobiluncus, Peptostreptococcus* and decrease in the number of *Lactobacillus* spp. often associated with high *p*H and clue cells is generally described as BV[5,6]. BV is known to increase predisposition to sexually transmitted diseases, including gonorrhoea, chlamydia, syphilis,

trichomoniasis, human immunodeficiency virus and human papilloma virus[7,8]. In pregnancy, BV increases the risk of post-abortal sepsis, early miscarriage, recurrent abortion, late miscarriage, preterm premature rupture of membranes, spontaneous preterm labour and histologic chorioamnionitis[9-11].

Therapy of BV involves oral or local administration of metronidazole or intravaginal clindamycin and varies in efficacy[3]. The long-term cure rate is low, and BV recurs in up to 40 per cent of women within three months after initiation of antibiotic therapy and in up to 50 per cent of women after three months[12]. There are several side-effects and disadvantages associated with these therapies, including superinfections by pathogenic microorganisms and disturbance of gut flora when treated by oral supplementation[13]. Moreover, vaginal opportunistic pathogens, particularly *G. vaginalis* and anaerobic bacteria show increasing drug resistance. In this context, *Lactobacillus* spp. administered orally or locally may be an effective alternative therapy which would re-establish the indigenous *Lactobacillus* and prevent BV as well as associated complications[2].

In humans, about 120 *Lactobacillus* species have been identified and more than 20 species have been found in the vagina[14]. Based on the previous molecular-based vaginal microbiome studies, three or four species (mainly *Lactobacillus crispatus*, *Lactobacillus iners, Lactobacillus jensenii* and *Lactobacillus gasseri*) normally predominate[14-16]. Colonization by lactobacilli ensures low *p*H in the genital tract (*p*H 4.5), which protects against colonization by other microbes[7]. *Lactobacillus* species also protect vaginal health by producing antimicrobial compounds such as hydrogen peroxide and bacteriocins[17]. This study was undertaken to identify and study the vaginal lactobacilli profile of pregnant women with normal, disturbed (intermediate flora) and BV flora.

Material & Methods

Pregnant women were selected from Government Maternity Hospital, Hyderabad, India; from January 2014 to March 2015. Sixty seven women were selected for the study after obtaining written informed consent. The study was approved by the Institutional Ethical Committee (IEC), ICMR-National Institute of Nutrition, Hyderabad. The sample size was calculated with preliminary data on vaginal lactobacilli in normal women; based on which, 18 per group were found to be sufficient to detect significance at 5 per cent between groups with 80 per cent power.

At the first study visit, weight, age, height and blood sample (0.2 ml) for haemoglobin levels were collected. Gestational age was calculated based on the last menstrual period, and birth weight was recorded. Vaginal samples (vaginal exudates of lateral wall) were collected for the identification of *Lactobacillus* spp. from all the 67 pregnant women. The women were classified into BV, intermediate and normal according to the Nugent score (NS) criteria based on vaginal smear Gram staining scores[18] using Microscope (Olympus B202, Japan). NS of 1-3 is considered normal vaginal flora or normal microbiota (BV negative), NS of 4-6 is considered as intermediate vaginal flora or intermediate microbiota, and 7-10 is considered as BV positive[18]. All women diagnosed with BV were treated with local antibiotic (Clindamycin 2% vaginal cream) for one week as per the WHO guidelines[19]. The first swab was used to prepare a smear on a glass slide for the purpose of grading[18]. The second swab was transferred to a sterile phosphate buffer saline (PBS) tube for DNA extraction.

DNA Extraction from vaginal swabs and Lactobacilli identification by Multiplex PCR: DNA extraction from vaginal swab samples was carried out as described by Kumar *et al*[20]. The polymerase chain reaction (AESTAC, Japan) was carried out for isolated DNA samples. Each sample was initially identified to the genus level by amplification with genus-specific primers, [forward primer (F) CTCAAAACTAAACAAAGTTTC-F and reverse primer (R), CTTGTACACACCGCCCGTTCA-R] [250 base pairs (bp) product size]. PCR programme included initial denaturation at 94°C for five minutes, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C and extension at 72°C for five minutes. Amplified product was identified on Low EEO agarose gel using Geldoc (Syngene, UK). Samples positive for *Lactobacillus* genus were subjected to species identification using species-specific primers[21-23] (Table I). Multiplex PCR was used for the identification of 17 *Lactobacillus* spp. as given in Table I. Species was identified on Low EEO agarose gel using Geldoc based on product size and 1 kb ladder DNA. For each sample, PCR reaction was carried out independently in duplicates.

Cultivation of lactobacilli bacteria from vaginal swabs in MRS broth: The vaginal swabs were vortexed in 1 ml sterile PBS (*p*H 7.4) to prepare bacterial suspensions and 100 µl of sample was inoculated in freshly

Table I. Sequences of polymerase chain reaction primers used for the identification of lactobacilli by multiplex polymerase chain reaction

Multiplex PCR group	Species name	Primer sequence (5'-3')	Product size (bp)
1	*Lactobacillus cripatus*	AGGATATGGAGAGCAGGAAT-F CAACTATCTCTTACACTGCC-R	522
	L. jensenii	AAGAAGGCACTGAGTACGGA-F CCTTTCCCTCACGGTACTG-R	700
	L. gasseri	AGCGACCGAGAAGAGAGAGA-F TGCTATCGCTTCAAGTGCTT-R	360
2	*L. delbrueckii*	ACAGATGGATGGAGAGCAGA-F CCTCTTCGCTCGCCGCTACT-R	450
	L. acidophilus	TGCAAAGTGGTAGCGTAAGC-F AAGAAGGCACTGAGTACGGA-R	210
3	*L. iners*	GTCTGCCTTGAAGATCGG-F ACAGTTGATAGGCATCATC-R	158
	L. johnsonii	TCGAGCGAGCTTGCCTAGATGA-F TCCGGACAACGCTTGCCACC-R	527
	L. helveticus	GCAGCAGAACCAGCAGATTT-F GCATCATTGCCTTGGTAAGC-R	219
4	*L. reuteri*	CAGACAATCTTTGATTGTTTAG-F GCTTGTTGGTTTGGGCTCTTC-R	303
	L. fermentum	ACTAACTTGACTGATCTACGA-F TTCACTGCTCAAGTAATCATC-R	192
	L. vaginalis	GCCTAACCATTTGGAGGG-F CGATGTGTAGGTTTCCG-R	550
5	*L. bravis*	CTTCTGGATGATCCCGCGGCG-F ACCGCCTGCGCTCGCTTTAC-R	369
	L. salivarius	AATCGCTAAACTCATAACCT-F CACTCTCTTTGGCTAATCTT-R	411
	L. plantarum	ATTCATAGTCTAGTTGGAGGT-F CCTGAACTGAGAGAATTTGA-R	248
6	*L. paracasei*	GGCCAGCTATGTATTCACTGA-F CTAGCGGGTGCGACTTTGTT-R	312
	L. casei	TGCACTGAGTTCGACTTAA-F CCCACTGCTGCCTCCCGTAGGAGT-R	500
	L. rhamnosus	GCGATGCGAATTTCTATTATT-F CTAGCGGGTGCGACTTTGTT-R	113

Source: Refs 21-23

prepared sterile MRS broth (BD Difco™, USA). After incubation for 48 h under anaerobic condition (anaerobic workstation, N_2 80%, CO_2 10%, H_2 10%) at 37°C, samples positive for growth were used for DNA extraction. DNA extraction and PCR procedures followed were similar to those mentioned above.

Statistical analysis: ANOVA was performed to compare means of NS and *p*H in women with normal, intermediate and BV flora and Chi-square was used to compare proportions between groups using SPSS version 19.0 software (SPSS, Chicago, IL, USA). Heatmap was created using R-programme software package (G-PLOT HEATMAP 2) to depict the frequency of the lactobacilli species.

Results & Discussion

Mean age, weight, height, body mass index and haemoglobin concentration were similar in women with normal, intermediate and BV flora. Of the 67 pregnant women, 15 had low birth weight babies (birth weight <2.5 kg) and five had preterm deliveries (gestational

Table II. Demographic and clinical characteristics of pregnant women and neonates

Characteristics	Overall (n=67)	Normal (n=27)	Intermediate (n=21)	BV positive (n=19)	*P*
Age (yr)	22.24±2.17	22.50±2.21	22.05±2.42	22.11±1.84	0.742
Height (cm)	152.43±5.00	151.91±4.46	154.23±5.68	150.99±4.42	0.097
Weight (kg)	52.76±8.06	51.60±7.20	53.45±8.62	53.58±8.77	0.649
BMI (kg/m^2)	22.7±2.7	22.5±3.7	21.8±2.1	23.9±3.7	0.444
Gestational age at recruitment (wk)	23.20±5.06	23.88±6.07	23.16±4.46	22.33±4.21	0.627
*p*H	5.02±0.47	4.77±0.38	5.09±0.29	5.31±0.57	0.001
Nugent's score	4.47±2.37	2.31±0.74	4.18±0.50	7.94±0.64	0.001
Amsel's criteria (%)	40.9 (27)	7.7 (2)	45.5 (10)	83.3 (15)	0.001
Gestational age at delivery (wk)	38.84±1.43	38.43±1.59	39.05±1.32	39.11±1.28	0.232
Birth weight (kg)	2.64±0.50	2.68±0.54	2.61±0.45	2.60±0.52	0.842
Low birth weight (%)	24.2 (15)	26.1 (6)	28.6 (6)	16.7 (3)	0.664
Pre-term deliveries	5.9 (5)	11.1 (3)	4.7 (1)	5.2 (1)	0.540

Values mean±SD. BMI, body mass index; BV, bacterial vaginosis; SD, standard deviation

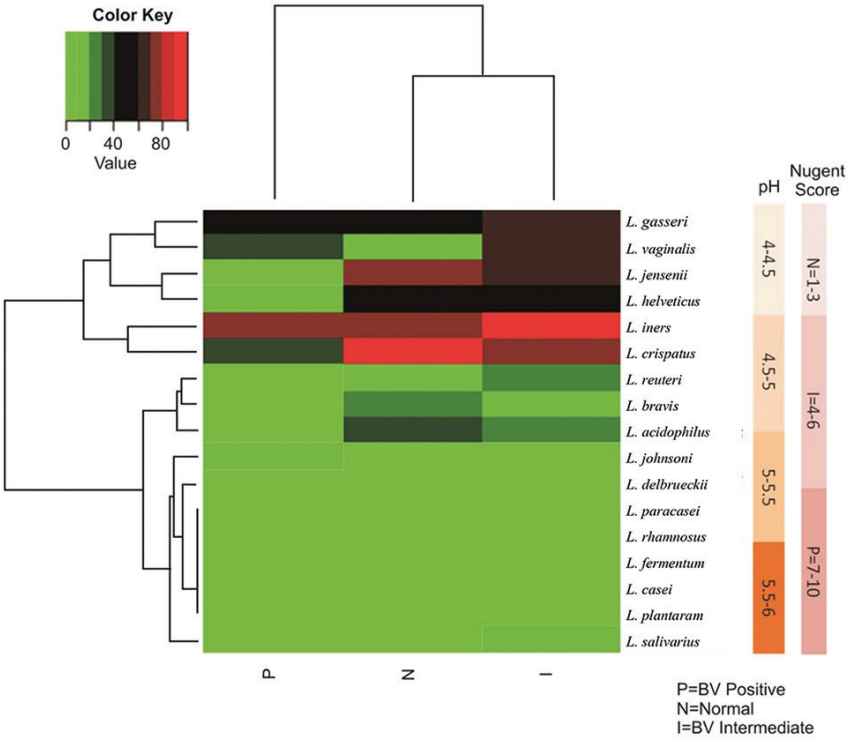

Fig. 1. Heatmap shows proportions of microbial species found in the vaginal bacterial communities of 67 pregnant women. As the colour key indicates, light green colour represents the absence of the organisms, and the darker coloured tiles indicate the presence of percentage of that particular organism. *Lactobacillus crispatus* was the dominant flora in the normal and intermediate group and *L. iners* was the more frequent organism in the positive group. Nugent's score and *p*H bars are shown on the right side of the Figure. The Nugent's score increased with increasing *p*H.

age at delivery <37 wk) (Table II). The mean birth weight and gestational age at delivery were comparable between groups. Of the 67 pregnant women, 27 had normal vaginal flora, 21 had intermediate flora and 19 had BV. The vaginal *p*H and NS means were significantly (P<0.001) higher in women with BV compared to normal.

Only 13 of the 17 lactobacilli species were detected by multiplex PCR. Heatmap (Fig. 1) shows distribution of lactobacilli species in pregnant women

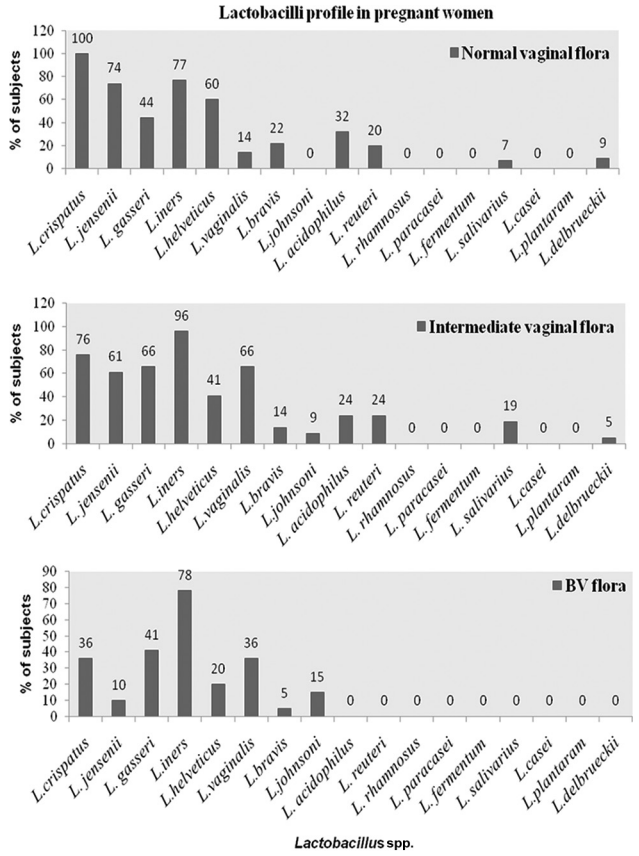

Fig. 2. Percentage of the most frequent vaginal *Lactobacillus* spp. in women with normal, intermediate and bacterial vaginosis flora as determined by multiplex polymerase chain reaction profile.

L. jensenii, *L. vaginalis* and *L. helveticus.* Species such as *L. rhamnosus*, *L. fermentum, L. paracasei* and *L. casei* were not detected by multiplex PCR.

The proportion of pregnant women with *Lactobacillus* spp. in normal, intermediate and BV flora are shown in Fig. 2. *L. crispatus* (100%) was the most predominant *Lactobacillus* spp. present in pregnant women with normal flora, followed by *L. iners* (77%), *L. jensenii* (74%) and *L. helveticus* (60%) (Fig. 2). Significantly (P<0.05) higher proportion of women with normal flora had *L. crispatus* compared to women with intermediate flora and BV. Similarly, *L. jensenii* and *L. helveticus* were significantly (P<0.05) higher in women with normal flora compared to women with BV (Fig. 2). *L. iners* was commonly present across groups in women with normal, intermediate or BV flora. Except *L. iners*, other species of lactobacilli were less frequently prevalent in women with BV.

Four of 27 pregnant women with normal flora had a combination of *L. crispatus*, *L. jensenii, L. helveticus* and *L. acidophilus* and this combination was not found in women with intermediate or BV flora. In contrast, *L. iners*, *L. gasseri*, *L. vaginalis* and *L. salivarius* combination were found in three women with intermediate and two with BV flora; interestingly, this combination was not found in normal group. Combination of *L. iners*, *L. gasseri* and *L. vaginalis* was found in both intermediate and BV groups, but not in normal flora. However, a combination of *L. iners* along with *L. crispatus*, *L. jensenii, L. helveticus* and *L. reuteri* was found in three of 27 women with normal flora, a similar combination was observed less frequently in women with intermediate and BV microbiota.

By culture-dependant method, 12 lactobacilli spp. *(L. crispatus*, *L. jensenii L. gasseri*, *L. iners*, *L. helveticus*, *L. vaginalis*, *L. bravis*, *L. johnsoni, L. acidophilus*, *L. reuteri*, *L. paracasei* and *L. salivarius*) could be identified from women with normal flora and intermediate flora while, none of the lactobacilli spp. could be isolated from vaginal samples of women with BV flora. *Lactobacillus delbrueckii,* which could be detected by multiplex PCR, could not be isolated by culture-dependent method from any vaginal samples. In culture-dependent method *L. iners, L. jensenii* and *L. crispatus* were detected only in 33, 39 and 61 per cent compared to 78.3, 70.2 and 91.8 per cent by multiplex PCR method. *L. gasseri* (50%) and *L. reuteri* (28%) isolation rates in MRS broth, however, were similar to multiplex PCR (*L. gasseri*, 51%; *L. reuteri*, 22%). *L. paracasei* on the other hand, which was not detected by multiplex PCR was isolated from eight per cent of pregnant women.

Our findings showed that *L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii* and *L. vaginalis* dominated the vaginal microbiota of Indian women, which was similar to those found in European and Brazilian women[2,22]. A similar *Lactobacillus* spp. profile in vagina has been reported from South Africa[24]. In our study, *L. helveticus* was identified more commonly in Indian women with normal flora which was less frequent in several other studies[25,26].

An association between the presence of *L. crispatus* and absence of BV has been shown[27]. Association of *L. crispatus* has been observed with stability of the vaginal microbiota[28]. Several clinical trials have been performed to investigate the efficacy of specific strains of *L. rhamnosus*, *L. fermentum* and *L. reuteri* administered either orally or intravaginally in treating

BV or urogenital infections[27]. *L. fermentum* and *L. rhamnosus* probiotic strains have been used with poor results in preventing BV[26]. Their uncommon presence in the vagina as observed in the current study and uncertain role in vaginal health may be the reason for the failure of efficacy with *L. fermentum* and *L. rhamnosus*.

The presence of *L. gasseri*, *L. vaginalis* and *L. iners* in women with intermediate and BV flora as observed in the current study could be due to their poorer colonization resistance to pathogens or inadequate production of antimicrobial substances, thereby allowing overgrowth of other pathogenic bacteria. Longitudinal studies in pregnant women have also shown that women harbouring *Lactobacillus* spp., particularly *L. gasseri* and *L. iners*, are more susceptible to BV compared to those colonized by *L. crispatus*[26,28]. Similar findings were observed in the present study, but *L. vaginalis* was also found to be commonly associated with intermediate and BV flora.

There are many commercially available probiotic strains for BV treatment. Most of the strains (*L. acidophilus, L. casie, L. plantarum, L. lactis, L. jensenii* and *Bifidobacterium bifidum, Bifidobacterium infantis*, *etc*.) that are available on the market are not frequently found in women with normal vaginal flora[26]. From our observations, it may be speculated that *L. iners*, *L. gasseri* and *L. vaginalis* may become a dominant part of the vaginal microbiota when the microbiota is in a transitional stage from normal to abnormal vaginal flora. Hence, *L. crispatus* individually or in combination with *L. jensenii*, *L. helveticus* and *L. acidophilus,* may be evaluated for probiotic potential to combat BV.

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***Conflicts of Interest*:** None.

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