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VAGINAL LACTOBACILLI PROFILE IN PREGNANT WOMEN WITH NORMAL AND ABNORMAL FLORA

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Abstract:

Background & objectives: *Lactobacilli species that are better adapted to vaginal environment of women may colonize better and offer protection against vaginal pathogenic bacteria. This study deals with the distribution of common Lactobacillus species which were investigated in pregnant women.*

Methods: *Sixty seven pregnant women were included in the study and vaginal samples were collected for Gram staining. Based on Nugent's score the subjects were classified as normal, intermediate flora and bacterial vaginosis (BV). In this study we also collected vaginal samples to identifyLactobacillusspp. by multiplex polymerase chain reaction (PCR) profiling of 16S rDNA amplification method.*

Results:*The most predominant Lactobacillus spp. found in pregnant women (normal flora) was Lactobacillus crispatus(100%), subsequentlyL. iners(77%), L. jensenii(74%) and L. helveticus(60%). L. inerswas found to be common across all groups in women with all three flora, L. crispatus, a significant decrease was presented by L. jenseniiand L. helveticusas the vaginal flora changed to intermediate and BV. Except L. iners, other species of lactobacilli prevalence was less frequent in women with BV. Species such as L. rhamnosus, L. fermentum, L. paracaseiand L. caseiwere not detected in any vaginal sample.*

Interpretation & conclusions: *Predominant species in women with normal florawere L. crispatus,L. jensiniiand L. helveticus. L. crispatusalone or in combination with L. jensiniiand L. helveticuscould be evaluated for probiotic properties to prevent and treat BV.*

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INTRODUCTION:

The beneficial microbiota such as *Lactobacillus* spp. in vaginal ecosystem is thought to have been adapted and coevolved by mutualistic association with its human host1. It is essential to explore the *Lactobacillus* diversity of vagina in health and diseaseand to understand whether changes in the individual vaginal *Lactobacillus* spp. can be correlated with changes in vaginal infections2. Bacterial vaginosis (BV) is a common vaginal infection caused by imbalance of indigenous microbiota3,4. 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 andclue cells is generally described as BV5,6. BV is known to increase predisposition to sexually transmitted diseases, including gonorrhoea, chlamydia, syphilis, trichomoniasis, human immunodeficiency virus and human papilloma virus7,8. 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 chorioamnionitis9-11.

Therapy of BV involves oral or local administration of metronidazole or intravaginal clindamycin and varies in efficacy3. 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 months12. 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 supplementation13. 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 complications2.

In humans, about 120 *Lactobacillus* species have been identified and more than 20 species have been found in the vagina14. Based on the previous molecular-based vaginal microbiome studies, three or four species (mainly *Lactobacillus crispatus, Lactobacillus iners, Lactobacillus jensenii*and *Lactobacillus gasseri*) normally predominate14-16.Colonization by lactobacilli ensures low *p*H in the genital tract (*p*H 4.5), which protects against colonization by other microbes7. *Lactobacillus* species also protect vaginal health by producing antimicrobial compounds such as hydrogen peroxide and bacteriocins17. 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 Gyn and Obs Department of Holy Family Hospital Rawalpindi. Sixty seven women were selected for the study after obtaining written informed consent.

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 scores18 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 positive18. All women diagnosed with BV were treated with local antibiotic (Clindamycin 2% vaginal cream) for one week as per the WHO guidelines19. The first swab was used to prepare a smear on a glass slide for the purpose of grading18. 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 extractionfrom 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 primers21-23 (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 mlsterile PBS (*p*H 7.4) to prepare bacterial suspensions and 100 µl of sample was inoculated in freshlyprepared 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 tocompare 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 22.0 software (SPSS, Chicago, IL, USA). Heat map was created using R-programme software package (G-PLOT HEATMAP 2) to depict the frequency of the lactobacilli species.

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	522
		CAACTATCTCTTACACTGCC-R	
	L. jensenii	AAGAAGGCACTGAGTACGGA-F	700
		CCTTTCCCTCACGGTACTG-R	
	L. gasseri	AGCGACCGAGAAGAGAGAGA-F	360
		TGCTATCGCTTCAAGTGCTT-R	

2	*L. delbrueckii*	ACAGATGGATGGAGAGCAGA-F	450
		CCTCTTCGCTCGCCGCTACT-R	
	L. acidophilus	TGCAAAGTGGTAGCGTAAGC-F	210
		AAGAAGGCACTGAGTACGGA-R	
3	*L. iners*	GTCTGCCTTGAAGATCGG-F	158
		ACAGTTGATAGGCATCATC-R	
	L. johnsonii	TCGAGCGAGCTTGCCTAGATGA-F	527
		TCCGGACAACGCTTGCCACC-R	
	L. helveticus	GCAGCAGAACCAGCAGATTT-F	219
		GCATCATTGCCTTGGTAAGC-R	
4	*L. reuteri*	CAGACAATCTTTGATTGTTTAG-F	303
		GCTTGTTGGTTTGGGCTCTTC-R	
	L. fermentum	ACTAACTTGACTGATCTACGA-F	192
		TTCACTGCTCAAGTAATCATC-R	
	L. vaginalis	GCCTAACCATTTGGAGGG-F	550
		CGATGTGTAGGTTTCCG-R	
5	*L. bravis*	CTTCTGGATGATCCCGCGGCG-F	369
		ACCGCCTGCGCTCGCTTTAC-R	
	L. salivarius	AATCGCTAAACTCATAACCT-F	411
		CACTCTCTTTGGCTAATCTT-R	
	L. plantarum	ATTCATAGTCTAGTTGGAGGT-F	248

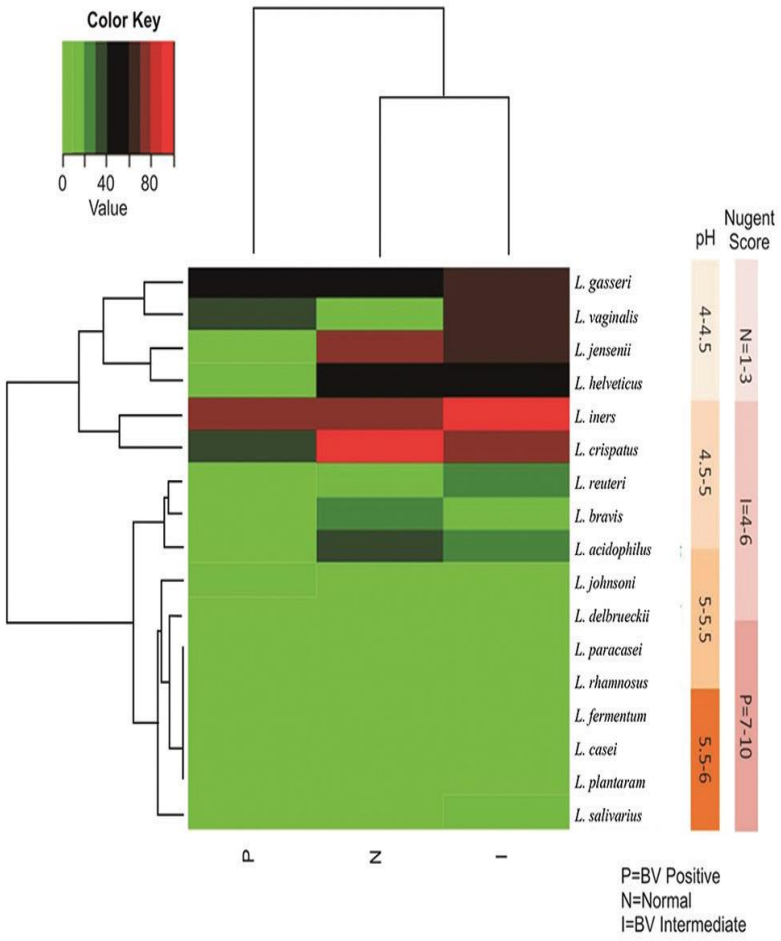
		CCTGAACTGAGAGAATTTGA-R	
6	*L. paracasei*	GGCCAGCTATGTATTCACTGA-F	312
		CTAGCGGGTGCGACTTTGTT-R	
	L. casei	TGCACTGAGTTCGACTTAA-F	500
		CCCACTGCTGCCTCCCGTAGGAGT-R	
	L. rhamnosus	GCGATGCGAATTTCTATTATT-F	113
		CTAGCGGGTGCGACTTTGTT-R	

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)	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 keyindicates, 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



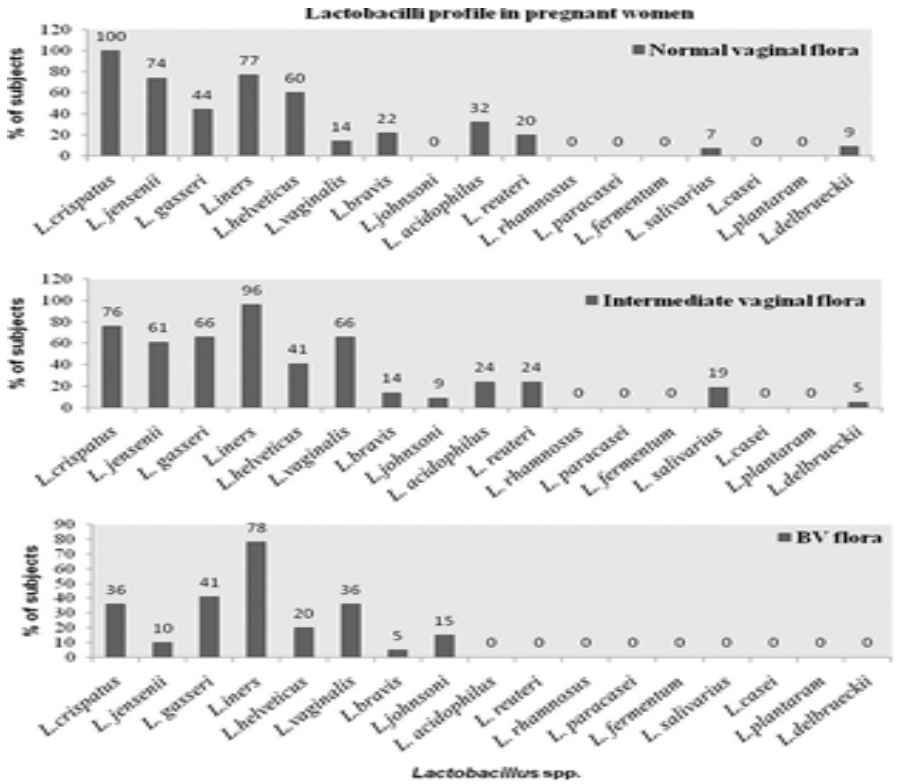


Fig. 2. Percentage of the most frequent vaginal*Lactobacillus*spp.in women with normal, intermediate and bacterial vaginosis flora as determined by multiplex polymerase chain reaction profile.

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 age at delivery <37 week) (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 *L. jensenii*, *L. vaginalis* and *L. helveticus.* Speciessuch 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 floraare 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 combinationwas 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 womenwith 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 centcompared 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 detectedby 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 thevaginal microbiota of Pakistani women, which was similar to those found in European and Brazilian women2,22. A similar *Lactobacillus* spp. profile in vagina has been reported from South Africa24. In our study, *L. helveticus* was identified more commonly in Pakistani women with normal flora which was less frequent in several other studies25,26.

An association between the presence of *L. crispatus* and absence of BV has been shown27. Association of *L. crispatus* has been observed with stability of the vaginal microbiota28. 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 infections27. *L. fermentum* and *L.rhamnosus*probiotic strains have been used with poorresults in preventing BV26. 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 floraas 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 associatedwith 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 onthe market are not frequently found in women with normal vaginal flora26. 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 forprobiotic potential to combat BV.

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