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Original Article

Microbiology Section

Diversity of Vaginal Lactic Acid Bacterial Microbiota in 15 Algerian Pregnant Women with and without Bacterial Vaginosis by using Culture Independent Method

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ABSTRACT

Introduction: Bacterial Vaginosis (BV) is the most common lower genital tract disorder among women of reproductive age (pregnant and non-pregnant) and a better knowledge of *Lactobacillus* species richness in healthy and infected vaginal microbiota is needed to efficiently design better probiotic products to promote the maintenance of normal flora which will help prevent bacterial vaginosis.

Aim: To evaluate and compare the diversity of lactic acid bacterial species in pregnant women with and without BV.

Materials and Methods: A pilot study was carried out during November-2014 to March-2015 in University Badji Mokhtar, Annaba, Algeria. Vaginal swabs were collected from 15 pregnant women aged between 19 and 35 years (mean 27.6 years; n=15) living in the East of Algeria visiting Gynecology service, hospital Abdallah Nouaouria- El bouni, Annaba. Vaginal samples were gram-stained, and scored by the Nugent method. The cohort included cases of women with healthy "normal" vaginal flora, infected flora with bacterial vaginosis and women with "intermediate" flora. The vaginal LAB community from pregnant women was identified by culture independent method based on Denaturing Gradient Gel Electrophoresis (DGGE), with the 16S rRNA gene sequencing.

Results: A majority of LAB affiliated to the genus *Lactobacillus* was found in "normal" and "intermediate" flora (87.5% and 43.75% respectively), while a majority of LAB affiliated to the genus *Enterococcus* was identified in women with bacterial vaginosis and intermediate flora (60% and 46.75% respectively).

Our results showed that the presence of *Lactobacillus iners* and *Lactobacillus delbruekii* promotes stability of the vaginal microbiota.

Conclusion: This result confirms the findings of previous studies suggesting that the occurrence of predominant *Lactobacillus* negatively correlates with bacterial vaginosis incidence and their current use as probiotics. *Lactobacillus iners* and *Lactobacillus delbruekii* can be defined as critical for defense of the vagina. In addition, *Enterococcus feacalis* can be considered as an indicator of imbalance of the vaginal ecosystem.

Keywords: DGGE, *Enterococcus*, *Lactobacillus*, Lactic acid bacteria, Vaginal infections

INTRODUCTION

The female vagina is a complex habitat for micro-organisms. Lactobacilli are the most dominant bacteria inhabiting this environment, as they play a major role in the maintenance of a healthy urogenital tract [1].

The microbial ecosystem in healthy "normal" vagina can be disrupted by drugs or by local devices with the concomitant decrease of the Lactobacilli community and overgrowth of anaerobic and facultative aerobic bacteria and yeasts [2].

Bacterial Vaginosis (BV) is one of the most common vaginal syndromes affecting women (pregnant or non- pregnant) in their reproductive age. It has been associated with a significant number of obstetric and gynaecologic complications.

Pregnant women with BV are more likely to have a preterm labour and delivery, preterm premature rupture of membranes, post-caesarean delivery wound infections, spontaneous abortion, chorioamnionitis, postpartum endometritis and subclinical pelvic inflammatory disease [3,4]. The mainstay treatment of BV consisted of metronidazole, clindamycin or tinidazole [5].

Many studies have considered probiotics as an adjunct to antimicrobial treatment [6-8]. Others proposed the use of probiotics in preventing the recurrence of BV after an initial treatment [8,9]. In addition, the prophylactic use of probiotics in healthy women with a history of recurrent BV has been confirmed [10].

Culture independent methods based on 16S rRNA-encoding gene sequence community profiling have been used previously to overcome culture-dependent limitations [11] and offer the potential to be rapid and reliable for characterizing the vaginal flora in clinical studies [12].

Our purpose was to assess a potential correlation between the taxonomy of the Lactic Acid Bacterial (LAB) species colonizing the vagina and a balanced healthy vaginal flora by comparing the vaginal LAB taxa found in healthy women and women with bacterial vaginosis by using Polymerase Chain Reaction (PCR) targeting universal and LAB specific 16S rRNA primer sets in conjunction with denaturing gradient gel electrophoresis (DGGE).

To our knowledge, this is the first report describing the vaginal lactic acid bacteria in Algerian pregnant women using this approach.

MATERIALS AND METHODS

Swab Sampling, Nugent Score and pH Measurement

Vaginal samples from fifteen women were collected. Three swabs were collected from each subject after insertion of an un-lubricated speculum into the posterior fornix of the vaginal tract. Women were pregnant at various months of pregnancy and under no antimicrobial or prescribed therapy, aged between 19 and 35 years (mean 27.6 years; n=15). Swabs were vigorously

agitated in 1mL sterile distilled water. One drop was deposited on microscope slide smears, Gram-stained, and scored by the Nugent method [13]. Nugent results were graded as 0–3 (Normal vaginal state; N), 4–6 (Intermediate-grade bacterial colonization; I), and 7–10 (Bacterial vaginosis; BV). The pH was determined using a pH indicator paper (Merck).

DNA Extraction and PCR Amplification

Microbial cell suspensions from vaginal swabs were pelleted by centrifugation (10,000 g, 5 min) previous to DNA extraction using ABIO pure Genomic DNA blood/cell culture (Alliance Bio Laboratories) according to manufacturer's instructions. DNA was quantified using the NanoDrop microvolume sample retention system (Thermo Scientific NanoDrop Products). PCR reactions were carried out in a thermocycler (Thermal Cycler; Biometra) using the universal primers of bacteria 357F (5'-119 TACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3') as described by Muyzer [14] and the specific lactic acid bacteria primers Lac1 (5'-AGCAGTAGGGAATCTTCCA-3'), Lac2 (5'-ATTYCACCGCTACACATG-3') and Lac3 (5'-AGCAGTAGGGAATCTTCGG-3'). A 44-bp GC-rich clamp sequence was added to the 5' ends of primers 357FGC and Lac 2GC [15,16].

The PCR reaction was obtained by mixing 0.6 µL of each primer (25 mM), 10 µL buffer 5X with 20 mM $MgCl_2$, 0.5 µL dNTPs (25 mM), 1 U/ µL of Taq polymerase (Thermo Scientific), 1µL of the genomic DNA, and ultrapure water (Millipore) to a final volume of 50µL. Amplified DNA was analysed after electrophoresis in 1.5% agarose gel followed by staining with ethidium bromide (5µg/ml) using Gel Compare (Version 4.1, Applied Maths, Kortrijk Belgium).

Taxa Identification using DGGE and 16S RNA Sequencing

PCR products generated with primers set N°1, 2 and 3 [Table/Fig-1] were separated by DGGE according to the specifications of Muyzer [15] with the following modifications: The concentrations of polyacrylamide, denaturant (7M urea, 40% formamide) and Tris-acetate buffer were 8%, 35–45% and, 50X respectively. A 20 µL of amplified PCR products mixed with 2X loading buffer were deposited into each well. Gel was run for 16 hour at 100V in TAE 1X buffer at 60°C in INGENYphorU system.

After electrophoresis, the gel was stained for 20 minutes in 5 µg/mL of ethidium bromide, washed with deionized water. Under UV light, DNA bands were cut from the gel, and placed in sterile 50 µL distillated sterile water overnight at 4°C.

A 5 µL of the solution containing fragments from the previously excised bands was used as template for PCR re-amplification as described above for DGGE except that primer sets lacking the GC clamps were used. The resulting PCR products were subsequently sequenced using the BigDye Terminator cycle sequencing kit V3.1 (Applied Biosystems) and an Applied Biosystems 3130 Capillary DNA Sequencer. Analysis of the partial 16S rRNA sequences was conducted using the GenBank DNA database NCBI and the BLAST algorithm [17].

Results of PCR and DGGE fragments in samples from women with Nugent ratings of N, I, or BV were presented as mean±SD.

Cycle stage	Primer set N°1 907R-357F GC	Primer set N°2 Lac1-Lac2 GC	Primer set N°3 Lac3-Lac2 GC
Initial denaturation	94°C; 3 min	94°C; 2 min	94°C; 2 min
Cycles of denaturation	10 cycles; 61°C; 1min 25 cycles; 56°C; 1min	35 cycles; 51°C; 1min	35 cycles; 61°C; 1min
Elongation	72°C; 1min	72°C;1 min	72°C; 1min
Final extension	72°C; 7 min	72°C; 7 min	72°C; 7 min

[Table/Fig-1]: PCR conditions prior to DGGE community profiling.

RESULTS

Nugent Score and pH Measurement of Vaginal Swabs

Among 15 pregnant women tested Nugent ratings for intermediate flora (8 cases), bacterial vaginosis (4 cases) and healthy flora (3 cases). Among the vaginal swabs of women with intermediate Nugent ratings, the pH was comprised between 4 and 5, while the pH of swabs of women with normal flora and bacterial vaginosis were of pH 4 and pH 5, respectively [Table/Fig-2].

Taxonomical Identification of Vaginal Swabs

DGGE analysis of PCR products using *Lactobacillus*-specific primers and universal primers showed that samples typically yielded 2.4 ± 0.95 dominant fragments per sample. It was possible to identify 12 bacterial species.

In women with Nugent scores rated normal flora, 2.66 ± 0.94 dominant DNA fragments were observed, and those fragments, when sequenced, were homologous to *Lactobacillus iners*, *Lactobacillus delbrukii* and *Streptococcus agalatiae*.

In women with Nugent scores rated intermediate flora, 2.12 ± 0.92 dominant DNA fragments were observed, and those fragments, when sequenced, were homologous to *Aerococcus urinae, Enterococcus feacalis, Enterococcus hirae, Enterococcus* spp., *Lactobacillus acidophilus, Lactobacillus iners, Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus crispatus*, and *Streptococcus anginosus* with predominance of *Enterococcus feacalis* (33.25%).

In women with Nugent scores rated bacterial vaginosis, 2.75 ± 0.82 dominant DNA fragments were observed, and those fragments, when sequenced, were homologous to *Enterococcus feacalis, Enterococcus feacium, Enterococcus hirae, Lactobacillus acidophilus*, uncultured *Lactobacillus* spp. and *Streptococcus anginosus*, with predominance of *Enterococcus feacalis* (40%).

The homology between the sequence obtained from the DGGE bands and the closest species from the database are given in [Table/Fig-3].

Several species of *Lactobacillus* were detected: *Lactobacillus iners* and *Lactobacillus delbrueckii* in the case of normal flora, *Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus crispatus, Lactobacillus acidophilus* and *Lactobacillus iners* in the case of intermediate flora and *Lactobacillus acidophilus*, uncultured *Lactobacillus* spp. in the case of bacterial vaginosis [Table/Fig-4].

Subject	Age (years)	Nugent score	Nugent rating	Vaginal pH
W01	24	6	Intermediate flora	4
W02	26	3	Normal flora	4
W04	21	5	Intermediate flora	5
W05	35	7	Bacterial vaginosis	5
W06	25	3	Normal flora	4
W09	26	4	Intermediate flora	4
W11	33	6	Intermediate flora	5
W12	25	8	Bacterial vaginosis	5
W13	27	3	Normal flora	4
W14	22	4	Intermediate flora	5
W15	19	5	Intermediate flora	4
W16	32	6	Intermediate flora	5
W17	27	5	Intermediate flora	4
W18	24	7	Bacterial vaginosis	5
W19	34	7	Bacterial vaginosis	5

[Table/Fig-2]: Phenotypic characteristics of the vaginal flora of 15 pregnant women.
The phenotypical characterization was carried out by determining the pH and the Nugent score for each swab.

Subjects	Nugent rating	DGGE fragments†	Primer set	Band number	Most closely related bacterial sequence	GenBank accession no.	Identity, %
W01	I	3	Lac3-Lac2	12	*Enterococcus faecalis*	JUNL01000323	98%
			Lac3-Lac2	13	*Enterococcus hirae*	ASVZ01000003	97%
			Lac1-Lac2	39	*Lactobacillus crispatus*	AY335500	99%
W02	N	2	Lac1-Lac2	38	*Lactobacillus iners*	KU726667	99%
			357F-907R	48	*Lactobacillus iners*	ADHG01000003	99%
W04	I	2	Lac3-Lac2	11	*Aerococcus urinae*	NC_015278	98%
			Lac1-Lac2	35	*Lactobacillus iners*	ADHG01000003	98%
W05	BV	4	Lac3-Lac2	22	*Streptococcus anginosus*	AZMF01000010	97%
			Lac3-Lac2	23	*Enterococcus faecalis*	KX073796	99%
			Lac3-Lac2	25	*Enterococcus faecalis*	JUNL01000323	99%
			357F-907R	44	*Uncultured Lactobacillus* spp.	KM250388	99%
W06	N	2	357F-907R	45	*Lactobacillus iners*	ADHG01000003	99%
			Lac3-Lac2	10	*Streptococcus anginosus*	AZMF01000010	97%
W09	I	1	Lac3-Lac2	20	*Enterococcus faecalis*	JUNL01000323	99%
W11	I	2	Lac1-Lac2	32	*Lactobacillus acidophilus*	AYUA01000121	98%
			Lac3-Lac2	19	*Enterococcus faecalis*	KR137541	99%
W12	BV	2	357F-907R	53	*Lactobacillus crispatus*	KU991819	99%
			Lac3-Lac2	9	*Streptococcus* spp.	JMBI01000046	96%
W13	N	4	Lac1-Lac2	29	*Lactobacillus delbrueckii subsp. delbrueckii*	BALP01000125	97%
			Lac1-Lac2	30	*Lactobacillus delbrueckii subsp.delbrueckii*	BALP01000125	99%
			Lac1-Lac2	31	*Lactobacillus delbrueckii subsp. bulgaricus*	NC_014727	99%
			Lac3-Lac2	18	*Streptococcus agalactiae*	JMBI01000046	98%
W14	I	2	Lac1-Lac2	28	*Lactobacillus jensenii*	GG704690	97%
			Lac3-Lac2	7	*Enterococcus* spp.	KB029381	80%
W15	I	4	Lac3-Lac2	8	*Enterococcus faecalis*	KX073789	99%
			Lac1-Lac2	26	*Lactobacillus iners*	ADHG01000003	100%
			Lac1-Lac2	27	*Lactobacillus iners*	KU726667	100%
			Lac3-Lac2	5	*Streptococcus anginosus*	KU726683	99%
W16	I	1	Lac3-Lac2	17	*Enterococcus faecalis*	JUNL01000323	99%
W17	I	2	357F-907R	55	*Lactobacillus johnsonii*	KU991818	99%
			Lac3-Lac2	14	*Enterococcus* spp.	KX079817	99%
W18	BV	3	Lac3-Lac2	15	*Enterococcus faecium*	KB946737	98%
			Lac3-Lac2	16	*Enterococcus faecalis*	JUNL01000323	99%
			Lac3-Lac2	1	*Enterococcus faecalis*	KK640484	99%
W19	BV	2	Lac3-Lac2	2	*Enterococcus hirae*	ASVZ01000003	97%
			Lac3-Lac2	3	*Uncultured Lactobacillus* spp.	KM250388	99%

[Table/Fig-3]: (Suite): 16S rRNA sequence BLAST analysis corresponding to DGGE gel excised fragments.
BV, bacterial vaginosis; I, Intermediate grade bacterial colonization; N, normal flora; DGGE, denaturing gradient gel electrophoresis.
† Approximate number of dominant fragments observed.

It was observed that the loss in detection of a *Lactobacillus* species by DGGE and sequencing analysis correlated with a higher Nugent score [Table/Fig-4]. In addition, all the women samples were colonized by a single species (or group of closely related strains) of *Lactobacillus*.

Our data suggest the absence of *Enterococcus* species in women belonging to normal flora, in contrast with others women belonging to intermediate flora or to bacterial vaginosis which are predominated by *Enterococcus feacalis*. So, this species can be considered as an indicator of imbalance of the vaginal ecosystem.

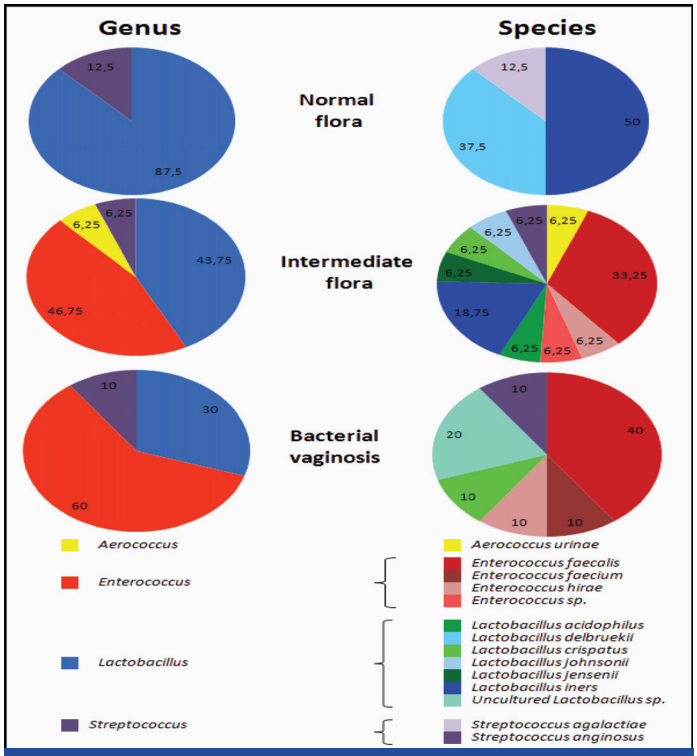
DISCUSSION

The maintenance of a low pH in the vagina through the microbial production of lactic acid is known to be an important defense against infectious disease in reproductive age women [18]. In our study, all communities (women with normal flora, intermediate flora or bacterial vaginosis) contained members that have been assigned to genera known to produce lactic acid (including *Lactobacillus, Streptococcus, Aerococcus* and *Enterococcus*), but the correlation between this bacteria and the maintenance of a low pH has not been well defined.

Communities belonging to normal flora have the lowest median pH (4.0), whereas communities belonging to bacterial vaginosis have the highest median pH (5.0). Clearly, the acidification of vaginal ecosystem is negatively correlated with the incidence of bacterial vaginosis among women. These results are in agreement with those of Zhou et al., [19].

The findings of our study indicate that the structure of vaginal lactic acid bacteria varied between the three categories. The vaginal flora of each women were colonized by a single species of *Lactobacillus*, which is consistent with the findings of other studies [11,20,21].

In particular, *L. delbrueckii* detection in vaginal swabs of healthy women has been previously recorded but as a minor vaginal bacterial species [22] and not as a major flora as observed here (W13). We observed that vaginal swabs of healthy pregnant women were mostly colonized by *L. iners* (W02 and W06). The occurrence of *L. iners* as a member of vaginal communities in healthy women has also been demonstrated by others [19,23-26]. First described



[Table/Fig-4]: Occurrence of Gram positive bacteria in the vagina of Algerian pregnant women.
The vaginal bacterial flora was classified according to the Nugent scores. Two levels of taxonomical classification are shown: at the level of the genus and of the species.

in 1999 [27], *L. iners* does not grow on Man Rogosa and Sharpe media (MRS agar). [14,19] which highlights the usefulness of culture-independent approaches for vaginal flora inventory.

Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus crispatus and *Lactobacillus acidophilus* were recovered from Algerian pregnant women with intermediate flora, in contrast with other studies that have frequently identified these species in vagina of women with normal flora [3,18,25,28-31].

Women belonging to intermediate flora were also characterized by a predominance of *Enterococcus feacalis*. In addition, *Enterococcus hirae, Aerococcus urinae* and *Streptococcus anginosus* have been identified in this category of women. These results are in correlation with other studies which confirmed the vaginal colonization of these women (intermediate flora) by aerobic enteric commensals [32,33].

In contrast with a general opinion that species of *Lactobacillus acidophilus* constitute most of the healthy vaginal *Lactobacillus* flora [25,28], our results recovered the absence of this species in a healthy women and its presence in 6.25 % of women with intermediate flora. These results suggests that this species is not indicative of a normal microflora, but may point to some intermediate condition.

According to research in PubMed, our study is the first to describe the diversity of lactic acid bacteria associated to bacterial vaginosis in contrast with the entire previous studies which describe only the pathogenic bacteria [34-36], or describe only *Lactobacillus* species associated to this category (women with Bacterial vaginosis) [37,38]. In addition, as per our knowledge our study is the first to describe the vaginal flora of Algerian women using culture independent method.

Our results need to be confirmed in a larger cohort preferably using a study design combined with data on subjects' behaviors to avoid the limitations of the present study and properly reflect the diversity of vaginal flora in female population of Algeria.

CONCLUSION

Our data suggest that a predominance of *Lactobacillus* in the vaginal bacterial flora is negatively correlated with the incidence of bacterial vaginosis in 15 pregnant women of Algeria. In addition, *Enterococcus* genus is negatively correlated with the incidence of normal Nugent rating among the tested women. According to our results, *L. iners* and *L. delbruekii* can be considered as critical for defense of the vagina. In addition, *Enterococcus feacalis* can be defined as an indicator of the imbalance of vaginal ecosystem.

Compared to culture-dependent methods, PCR- DGGE technique able a better estimation of species composition of the vaginal community, which is a prerequisite for clinical studies of the vaginal tract. Future development of effective probiotics must take into account the results of this study which gives an idea on the specificity of the vaginal lactic acid bacterial flora of Algerian woman where local perineal hygiene is different from the western world.

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