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Vaginal microbiota dysmicrobism and role of biofilm-forming bacteria

Giuseppina Campisciano[1], Nunzia Zanotta[1], Vincenzo Petix[1], Lucia Corich[1], Francesco De Seta[1,2], Manola Comar[1,2]

[1]Institute for Maternal and Child Health - IRCCS Burlo Garofolo, Via dell'Istria 65/1, Trieste, 34137 Italy, [2]Department of Medical Sciences - University of Trieste, Piazzale Europa 1, Trieste, 34127 Italy

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1. ABSTRACT

Bacterial vaginosis involves the presence of a polymicrobial biofilm on the vaginal epithelium, guaranteeing immune escape and spread of antibiotic resistance. To spot known biofilm-forming bacteria, we profiled the vaginal microbiome of sixty-four symptomatic women suffering from a different grade of vaginal disorders and sixty asymptomatic healthy women. Specific microbial profiles distinguished symptomatic from asymptomatic women and characterized the grade of dysmicrobism within the symptomatic group. *Lactobacillus crispatus* and *iners* predominated on the healthy vaginal mucosa, while *Lactobacillus gasseri* predominated in the intermediate dysmicrobism. Furthermore, the intermediate grade of dysmicrobism was characterized by other lactic acid-producers species than *Lactobacilli*, able to rescue the microbial imbalance, and *Ureaplasma parvum*-serovar 3. The vaginosis group exhibited the overgrowth of *Prevotella bivia*, which is known to enhance the biofilm formation by *Gardnerella vaginalis*, and the presence of *Streptococcus anginosus,* which is emerging as a new cooperating player of the vaginal biofilm. Identifying specific microorganisms promoting or preventing the biofilm formation could increase the accuracy for a better definition of the vaginal dysmicrobism concept and therapeutic intervention.

2. INTRODUCTION

The environment of the vagina is dynamic and it is influenced by factors such as hormonal fluctuations, menstruation, douching, hygiene, pregnancy, breastfeeding and sexual practices (1–4). A plethora of microbial species co-exists in the vaginal niche, 70%–90% of which are *Lactobacilli* (5). Their dominance is pivotal in maintaining the vaginal health, thanks to their production of hydroxyl radicals, lactic acid, bacteriocins, hydrogen peroxide and probiotics (6). Indeed, *Lactobacilli* are reported to be significantly decreased in bacterial vaginosis (BV) (7), which is a non-specific (predominantly anaerobic) polymicrobial biofilm infection, where the predominant bacteria in the biofilm are not the resident *Lactobacilli* (8–11). Above all, the most effective mechanism by which the *Lactobacilli* protect the vaginal niche is the production of a thick, protective biofilm on the vaginal epithelium, which is utilized to counteract the harmful microbe proliferation. The *Lactobacilli* biofilm is responsible for maintaining a healthy and stable condition in the vagina (12).

The ability of *Lactobacilli* species to exert the protective roles previously mentioned is highly dependent on the species involved. The development of the high throughput techniques has led to the discovery that five vaginal microbiota groupings exist, termed community state types (CSTs). CSTs I, II, III and V are dominated by *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners,* and *Lactobacillus jensenii*, respectively. The CST IV contains the most diverse species communities, including the highest proportions of obligate anaerobes. To note, the latter CST, which contains high proportions of anaerobic bacteria, resembles BV (13).

The investigation of the vaginal microbial structure by the deep-sequencing technology has provided new clues to the aetiology of BV, reporting that causative species are associated in a structured-functional polymicrobial biofilm which is dominated by *Gardnerella vaginalis* and often includes *Atopobium vaginae* and several *Lactobacilli* (14, 15).

G. vaginalis is on 90% of the vaginal epithelium of women diagnosed with BV and represents the initial colonizer, playing a central role in the early adhesion stage and providing a scaffold for different microorganisms in the mature biofilm (16–19). Nevertheless, *G. vaginalis* can be detected also in healthy women, confirming the peculiarity of certain subspecies that are characterized by additional virulence factors, including fimbriae, and the ability to produce sialidase and vaginolysin which facilitate biofilm formation (19, 20). BV biofilm contains consolidate core organisms highly specialized for propagation, although it is unclear which are individual symbionts or accidental beneficiaries and which microorganisms belong to the essential core of biofilm (21, 22). *G. vaginalis* induces different symbiotic relationship with other BV-associated bacteria. Specifically, *G. vaginalis* and *A. vaginae*, rarely present in the healthy vaginal microbiome, have been often co-detected in BV, suggesting an effective cooperation in biofilm formation (22, 23).

Recent experimental evidences have demonstrated that BV can be accompanied by a bacterial colonization of the upper genital tract (24, 25). As argued by Swidsinski *et al.*, both *G. vaginalis* and *A. vaginae* seem to play a central role in this ectopic colonization by ascending from the vagina to the endometrium and, in turn, organizing a completely interdependent biofilm polymicrobial communities that seem responsible for several adverse health outcomes (26).

Nowadays, there are still a lot of controversial studies on the aetiology of BV and its efficient management in the clinical setting. In the present study, we discuss, at the light of recent literature data, our experience on the characterization of vaginal microbiome in women with different grade of vaginal dysmicrobism, with a particular focus on the pathogenic role of biofilm.

3. MATERIALS AND METHODS

3.1. Patients and samples

One hundred and twenty-four consecutive women, attending as outpatients the Gynaecology Service of the Institute for Mother and Child Health, IRCCS Burlo Garofolo, Trieste, Italy were enrolled in this study. The inclusion eligibility criteria were defined as follows: Caucasian women, of reproductive age (age range, 32–40 years), not pregnant, no current use of hormonal or barrier contraceptive products, vaginal douching, tobacco or alcohol abuse, not hospitalized or systemic use of medication for chronic diseases or antibiotics/probiotics (oral or topical) within the 6 months prior to sampling collection, and no intercourse in the day prior to sampling.

Sixty-four women were symptomatic, suffering from vaginal disorders including discharge, malodourous leucorrhea (fishy-like), burning and dysuria. Of these, 34 were diagnosed with BV (Vaginosis) and 30 with an intermediate Nugent score (Intermediate). Sixty women were asymptomatic and showed a healthy vaginal microenvironment, hereafter referred to as Healthy. All women were without symptoms of recent sexually transmitted infections and tested negative for sexually transmitted infection including HPV and HIV infection.

Vaginal samples were collected 7 days before the first day of the menstrual period, using a 200 mm polyethylene Cervex brush device37 (Rovers Medical Devices B.V., The Netherlands) by a single gentle 360° rotation of the cytobrush at the cervical os, under speculum examination. Swabs were suspended in 1.5. ml of TE buffer. Each sample was divided into 3 (500 µl) aliquots and stored at −80 °C.

3.2. Sample processing, Ion Torrent sequencing

DNA extraction was performed by the automatic NucliSENS® easyMAG® system (bioMèrieux, France, Europe), according to the protocol, with a final elution volume of 50 µL.

A real-time quantitative EvaGreen® dye (Biotium, California, USA) PCR was performed with the degenerated primer 27FYM and with the primer U534R, which target the V1–V3 region of 16S rRNA gene. Template preparation was performed using the Ion PGM Hi-Q View kit on Ion OneTouch™ 2 System (Thermo Fisher Scientific, Massachusetts, USA) and the sequencing of the V1-V3 region of bacterial 16S rRNA using the Ion PGM Hi-Q View sequencing kit (Thermo Fisher Scientific, Massachusetts, USA) by the Ion PGM™ System technology as recently described (25).

3.3. Data analysis

Quantitative insights into microbial ecology (QIIME) 1.8.0.1. was used to process the sequence data (27). High quality (Q>25) sequences were demultiplexed and filtered by quality using split_libraries_fastq.py with default parameters, except for the length parameter (150 bp). Operational taxonomic units (OTUs) were defined at 97% similarity and

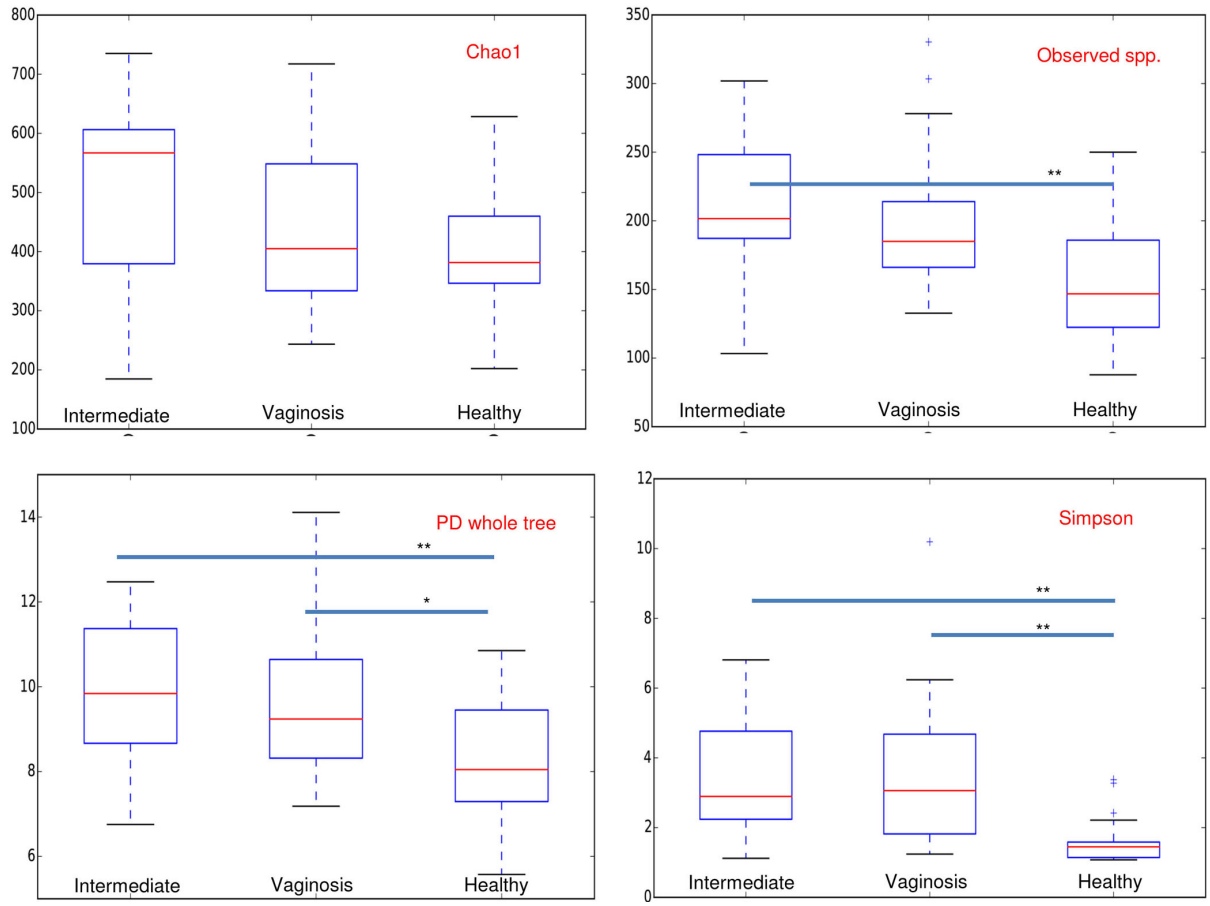

Figure 1. Comparison of bacterial diversity between cohorts. Chao1, PD whole tree, observed species and Simpson reciprocal metrics were used to assess the alpha diversity. The values were compared by means of a non-parametric t-test using the compare_alpha_diversity.py script of QIIME. ** p-value < 0.01, * p-value < 0.05.

clustered against the Vaginal 16S rDNA Reference Database constructed by Fettweis *et al.* (28) using open-reference OTU picking with a uclust clustering tool (29). Differences in community composition between cohorts were investigated by the Kruskal-Wallis test, using the P value corrected by the Benjamini-Hochberg False Discovery Rate (FDR).

Chao1, PD whole tree, observed species and Simpson reciprocal metrics were used to assess alpha diversity (within-sample diversity). The alpha diversity values were compared by means of a non-parametric t-test using the compare_alpha_diversity.py script of QIIME.

4. RESULTS

In our cohort, the vaginal microbiota of symptomatic women consisted of a highly heterogeneous number of microbial species (Figure 1). More precisely, according to the three alpha diversity (within-sample bacterial heterogeneity) metrics, the intermediate dysmicrobism group showed the highest microbial heterogeneity ($p < 0.0.5$) when compared to the vaginosis and the healthy cohorts.

Although none of the bacterial species showed statistical differences across cohorts according to the FDR p-value, we observed that *Lactobacilli*, which are known to be responsible for the formation of a protective biofilm against the invasion by pathogens, differentially dominated the vaginal microbiome. We observed different *Lactobacillus* spp. not only comparing symptomatic and asymptomatic women but also based on the grade of dysmicrobism within the symptomatic group. More precisely, *L. crispatus* and *L. iners* were the most abundant species colonizing the healthy vaginal mucosa, the latter identified also in the vaginosis group (Figure 2A) (9, 30–32). *L. gasseri* was the most abundant *Lactobacillus* in women with an intermediate dysmicrobism (Figure 2A).

Where we observed the loss of the predominance of specific *Lactobacilli* species, we concomitantly identified the overgrowth of opportunistic pathogens. Indeed, the intermediate dysmicrobism

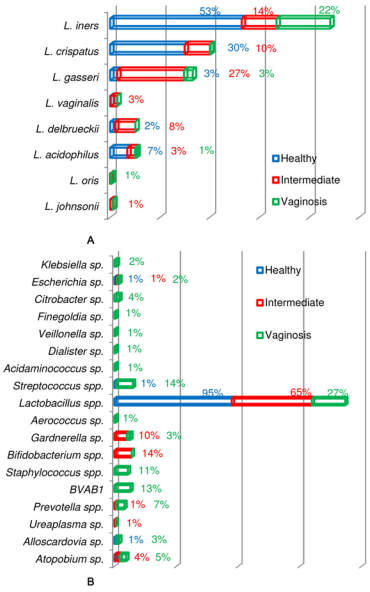

Figure 2. The bacterial composition of the analyzed samples. The plot_taxa_summary.py script of QIIME was used to plot the relative abundance of the *Lactobacillus* spp. (A) and of the bacterial genera (B).

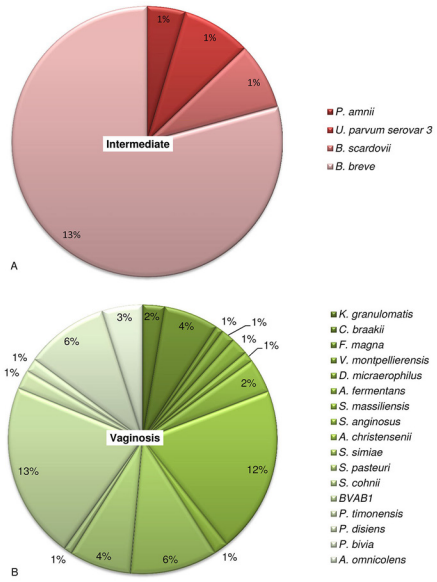

Figure 3. The microbial specific series characterizing the symptomatic cohort. The plot_taxa_summary.py script of QIIME was used to plot the relative abundance of the bacterial species.

and the vaginosis groups showed the presence of a plethora of obligate/facultative anaerobes, such as *Klebsiella*, *Citrobacter*, *Finegoldia*, *Veillonella*, *Dialister*, *Acidaminococcus*, *Aerococcus*, *Gardnerella*, *Staphylococcus*, BVAB1, *Prevotella* and *Atopobium* (Figure 2B).

Some of these, such as *Atopobium vaginae,* and other lactic acid producers, such as *Leptotrichia* and *Megasphaera*, may help in maintaining the acid pH of the vagina in absence or decrease of *Lactobacilli* (33, 34). In our cohort, the intermediate dysmicrobism status was characterized by the presence of *Bifidobacterium breve and Bifidobacterium scardovii*, two acid lactic producers which have been until now hugely overlooked.

To note, peculiar bacterial profiles characterized the women with an intermediate dysmicrobism with respect to the vaginosis group. Precisely, *Prevotella amnii, Ureaplasma parvum* serovar 3*, Bifidobacterium scardovii* and *Bifidobacterium breve* were identified in intermediate dysmicrobism group. Several species, such as *Klebsiella granulomatis, Citrobacter braakii, Finegoldia magna, Veillonella montepellierensis, Streptococcus spp., Aerococcus christensenii, Staphylococcus spp., BVAB1, Prevotella sp., Alloscardovia,* were identified in vaginosis group (Figure 3).

5. DISCUSSION

Recently, a growing awareness of the role of the complex vaginal microbiota in influencing the women's health has spread (9). Particular attention has been paid to the vaginal biofilm formation which plays a pivotal pathogenic role in many urogenital diseases, mainly by counteracting the activity of antibiotics or by allowing microorganisms to escape host defence mechanisms (35, 36) and, in turn, leading to recurrent infections (37).

Efforts have been made in understanding the nature of the polymicrobial BV biofilm, the formation of which seems to depend on several host factors including stress, sexual practices and microbial synergism or antagonism. Despite the increasing efforts and awareness, we currently know little about the relationship between bacteria forming-biofilm and the resident community, and how and if this interaction could influence women's health.

Our results highlighted that the dysbiotic vaginal microbiota is more heterogeneous (as assessed by the alpha diversity measure) comparing to that of healthy women, especially in the intermediate dysmicrobism group. Moreover, we demonstrated that some *Lactobacillus* species characterized the different grade of dysbiosis suggesting their central role in orchestrating the local microbiome composition.

We speculated that the identification of multiple microbial identities in the vaginal dysbiotic microbiome may be the result of a mutual relationship between specific bacterial species and specific *Lactobacillus* species. To ascertain this hypothesis, we looked into the *Lactobacillus* species present in our groups.

Different species of *Lactobacilli* were identified in the dysbiotic and eubiotic vaginal niches. *Lactobacillus crispatus* and *iners* were predominant in the healthy group. *L. crispatus,* able to produce many antimicrobial compounds (31, 38, 39), was inversely associated with the amount of BV-associated anaerobes confirming a protective role in maintaining a healthy milieu. On the other hand, high levels of *L. iners* (15) has been measured both in women with BV and in healthy women suggesting a poor ability to protect the vaginal epithelium from pathogens invasion. *Lactobacillus gasseri* was the most abundant *Lactobacillus* in women with an intermediate dysmicrobism (Figure 2A). Several *in vitro* studies have reported that *L. gasseri* is inversely correlated with the presence of both *L. iners* and *A. vaginae*, a microorganism acting in synergy with *G. vaginalis* in biofilm formation (23, 39, 40). Our data confirmed this experimental evidence. In the intermediate dysmicrobism group, both *L. iners* and *A. vaginae* were little represented (Figure 2), highlighting how the massive dominance of *L. gasseri* can effectively inhibit the overgrowth of certain biofilm forming-pathogen bacteria.

A central role in the BV has been highlighted for species associated to biofilm, favouring a persistent local dysmicrobism. Among these species, *G. vaginalis* has demonstrated a strong adherence to vaginal cells, cytotoxicity and the capacity of biofilm production (41, 42). When the growth of *G. vaginalis* is not counteracted, this bacterium is able to establish symbiotic relationships with other BV associated anaerobes and to promote the biofilm growth (23). This mechanism seems more effective in presence of *Prevotella bivia*, showing a higher ability to enhance *G. vaginalis* growth than other BV-associated anaerobes (43). According to these findings, in our study, among the *Prevotella* spp., the overgrowth of *P. bivia* (6%) has been detected exclusively in women with BV, characterizing the severity of dysmicrobism (Figure 2B).

Several Gram-positive anaerobic cocci have been hugely neglected by the studies of the vaginal biofilm despite their pathogenic potential and their ability to produce metabolites which favor the growth of other BV-associated bacteria (44). Thanks to the advancement of molecular techniques, *Streptococcus anginosus*, a pathogen of the vaginal community, emerged as a new cooperating player of the vaginal biofilm (45). Our study demonstrated a massive colonization of *S. anginosus* only in women with BV (Figure 2B). Indeed, as already assessed in the oral cavity, species such as *Klebsiella, Citrobacter, Veillonella,* and *Dialister*, identified in the vaginosis group, are able to cooperate with *Streptococcus* in the biofilm formation (46–48).

It is supposed that a moderate/intermediate vaginal dysmicrobism is the result of a compensatory mechanism exerted by the presence of *Bifidobacteria* (49), species able to counteract the biofilm formation and thus able to enhance the persistence of an eubiotic condition (50). Indeed, in the intermediate dysmicrobism group, *Bifidobacterium breve* and *Bifidobacterium scardovii* were identified, suggesting a partial rescue of the dysbiotic condition (Figure 2B). Nevertheless, a particular attention has to be paid to the role of the resident *Ureaplasma/Mycoplasma* which has the potential of being harmful to the women's health. In our cohort, the intermediate status was uniquely characterized by a low relative abundance of *Ureaplasma parvum* serovar 3. This bacterium is able to cooperate to biofilm formation and able to drive the release of inflammatory factors, that may be causative for the clinical symptomatology and the partial microbiome unbalance characterizing the intermediate dysmicrobism (51).

In conclusion, the assessment of the microbiome composition increases the accuracy for a more realistic concept of the vaginal dysmicrobism and its clinical management. Taken together, data from our study demonstrated that the massive colonization of the vaginal mucosa with different non-resident microorganisms, potentially associated with biofilm formation, could be more informative for a severe dysmicrobism rather than the quantification of a single species such as *G. vaginalis.* Conversely, a moderate dysmicrobism could be evidenced by the concomitant recovery of a low quantity of *Ureaplasma* serovars and specific *Lactobacilli.*

6. ACKNOWLEDGEMENT

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Abbreviations: BV: bacterial vaginosis; BVAB1: bacterial vaginosis-associated bacterium 1; HPV: human papilloma virus; HIV: human immunodeficiency virus; QIIME: quantitative insights into microbial ecology; OTUs: operational taxonomic units; FDR: false discovery rate.

Key Words: Biofilm, Bacterial Vaginosis, Microbiota, Dysmicrobism, Recurrence

Send correspondence to: Manola Comar, Medical Science Department, University of Trieste. Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Via dell Istria 65, 34137 Trieste, Italy, Tel: 39-040-3785527, Fax: 39-040-762623, E-mail: manola.comar@burlo.trieste.it