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Methods of investigation of human microbiome in obstetrics and gynecology

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Abstract Microbiota of the vaginal epithelium undergoes significant changes associated with hormonal status, each change being characterized by a specific prevalence of *Lactobacillus* species. Because the vaginal microbiome varies, a systemic analysis of its metagenomics combined with integrated multi'omics, will help to determine the microbial profile (including the uncultured microorganisms) associated with normal and pathological conditions. A plethora of bacterial species with pro-inflammatory characteristics can induce vaginosis, preterm birth and other adverse outcomes in pregnancy. In order to improve health by manipulating the microbiome, all the factors involved in its plasticity have to be known. New technologies allow to examine the microbiome, highlighting the interaction between its components and the host epithelium. Data brought by next generation sequencing technique will open new perspectives on the role of microorganisms in the pathology of the female genital tract, in association with host genetic factors and different physiological conditions.

Keywords Microbiome, gynecology, methods

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Introduction

Recent studies on the human microbiome demonstrated its high variability and impact in both prevention of diseases and human pathology (AMIR [1]). The term "microbiome", first used by Lederberg and McCray, includes an "ecological community of commensal, symbiotic and pathogenic organisms" that inhabits the human body during normal or pathological conditions (LEDERBERG [2]). About 10-100 trillion symbiotic microbial cells exist on every human body (URSELL [3]), thus making the microbiome more diverse and significantly higher than human genome. The human microbiome project has aimed the sequencing of genome microbiota, targeting in particular the populations that colonize the skin, oral cavity, nasal passages, the digestive tract and vagina.

Human microbiome's mapping was publicly announced by NIH director Francis Collins on June 13, 2012 and the results were published in the journal "Nature" (THE HUMAN MICROBIOME PROJECT CONSORTIUM [4]) and (GEVERS [5]). Recent findings have led to new approaches in the field which can determine both the stability of an individual microbiota and the ability of a community to live together with more microbiomes in a single individual (URSELL [3]).

Different studies showed significant composition differences between the human microbiota and free living microorganisms despite the fact that the human body is populated with bacteria acquired from the environment (LEY [6]). Therefore, a co-evolution of commensally microbiota with the human body led to the selection of specialized bacterial populations. Each community of the microbiome has its own characteristics. For instance, a high biodiversity of intestinal microbiota is associated with health while an increasing number of microorganisms is common in patients with vaginosis. A high variability in vaginal microbiota (*Lactobacillus* genus found predominant) with significant differences in the clustered pattern between subjects with and without bacterial vaginosis was reported (BV) (XIAO [7]).

Microbiota in obstetrics and gynecology

Vaginal microbiota (normal and pathologic)

Microbiota of the vaginal epithelium undergoes significant changes associated with hormonal status, each of these changes being accompanied by a different prevalence of lactobacilli species. The abundance of *Lactobacillus* species was considered a hallmark of vaginal health (a decreasing in its content being associated with menopause) (BOURNE [8]) because their production in lactic acid maintains a pH <4.5 which prevents the increase in neutrophils and provides protection against pathogen invasion (TUROVSKIY [9]). However, new molecular techniques do not confirm this species to be predominant in the vaginal microbiome of healthy women of reproductive age (RAVEL [10]); the healthy vaginal ecosystem was found to vary widely according to the studied population (MARTÍNEZ-PEÑA [11]). On the other hand, bacterial vaginosis (BV) "can be provoked by a plethora of bacterial species with pro-inflammatory characteristics, coupled to an immune response driven by variability in host immune function" (ONDERDONK [12]). BV develops due to changes in the vaginal microbiota profile, from species that produce lactic acid to a great diversity of species that changes the local pH. Some *Lactobacillus* species may display different stability and ability to protect the vagina against the colonization with anaerobic species associated BV, by producing hydrogen peroxide and/ or bacteriocins (TAMRAKAR [13]). For instance, *L. crispatum* is the most stable species giving a 5 times lower risk of developing bacterial vaginosis (BV) versus *L. inertia*, which is the least stable and protective (VERSTRAELEN [14]). BV may leads to development of pelvic inflammatory disease (NESS [15]), increase the risk of acquisition and transmission of HIV or other sexually transmitted diseases (CHERPES [16], COLEMAN [17]), or leads to pathological evolution of pregnancy (intrauterine infection, miscarriage) (HILLIER [18], Nelson [19]).

Some of the anaerobes involved in BV are listed below:

- anaerobes typically identified through conventional medium cultivation: *Gardnerella vaginalis*, *Prevotella bivia*, *Atopobium vaginae*, *Fusobacterium nucleatum*, *Mobiluncus mulieris*, *Ureaplasma urealyticum*, and *Mycoplasma hominis* (LIVENGOOD [20]).
- anaerobes detected by molecular techniques: *Eggerthella*, *Bifidobacterium*, *Leptotrichia*, *Megasphaera*, *Dialister*, and *Slackia* organisms, as well as other bacteria related to *Arthrobacter*, *Caulobacter*, and *Butyrivibrio* organisms (ROMERO [21]).

G. vaginalis has the capacity to adhere to vaginal epithelial cells, establish biofilm with higher tolerance to hydrogen peroxide and lactic acid comparing with planktonic cells (PATTERSON [22]), and has cytotoxic activity (Patterson [23], MACHADO & CERCA [24]). Co-infection of *Gardnerella vaginalis* and *Mycoplasma hominis* is very common in BV (60.7 %) (COX [25]).

Changes in vaginal microbiota may be a marker of HPV (human papillomavirus) infection and development of CIN (cervical intraepithelial neoplasia) lesions. HPV positive women show a higher diversity of the microbial vaginal flora (a small percentage of *Lactobacillus* and an increased presence of *Prevotella* and *Leptotrichia*) (DARENG [26]) when compared with HPV negative

subjects, and the diversity of microbial vaginal flora is in relationship with the severity of CIN. According to Mitra (MITRA [27]), high grade squamous intraepithelial lesions (HGSIL) are associated with low prevalence of *Lactobacillus* and increased levels of *Sneath sanguinegens* ($p < 0.01$), *Anaerococcus tetradus* ($p < 0.05$), *Peptostreptococcus anaerobius* ($p < 0.05$), in contrast to the low grade (LGSIL) lesions. Commercial kits based on vaginal microbiota changes (like Invitrogen®Pure Link®, DNA Purification microbiome Kit®) are under investigation in order to detect women at high risk of developing cervical cancer.

Bacterial vaginosis itself is a risk factor for premature birth, although Brocklehurst (2013), in a Cochrane meta-analysis, found a non-significant decrease in the risk of preterm birth after treatment with antibiotics (BROCKLEHURST [28]). Recently, Gille showed no improvement of vaginal microbiota when testing the effect of oral administration of probiotics in Q2 of pregnancy (GILLE [30]). Instead, in Pregnancy Study (PiP Study), the administration of probiotics during pregnancy (probiotic *Lactobacillus rhamnosus* HN001) and breastfeeding, showed a reduced incidence of eczema and atopic dermatitis in the infant and a diminished incidence of gestational diabetes, of VB and vaginal colonization with *Streptococcus* B before birth, of postpartum depression and anxiety.

Microbiota during pregnancy

Pregnancy involves hormonal and metabolic changes, immune system's modulation (KUMAR & MAGON [31]) and changes in microbiome composition that can affect host hormones, metabolism and immunity [NURIEL-OHAYON [32]). In pregnancy, the levels of secreted hormones (like progesterone and estrogens) increase dramatically (KUMAR & MAGON [31]). The immunity changes during different pregnancy stages: implantation and parturition (inflammatory stage), fetus growing (anti-inflammatory stage) (MOR & CARDENAS, [33]). On the other hand, metabolic changes associated to pregnancy are similar to metabolic syndrome's changes like weight gain, increase in blood-glucose levels, insulin resistance, glucose intolerance, low-grade inflammation, and changes in metabolic hormones' levels (EMANUELA [34], NEWBERN & FREEMARK [35], KUMAR & MAGON [31]). Also, during pregnancy the diet influences the diversity of microbiota at different body sites. The gut's microbiota of pregnant women is similar to those of non-pregnant women (healthy microbiota) in the first trimester, but towards the third trimester it is characterized by an increase of *Actinobacteria* and *Proteobacteria* phyla, and decrease of alpha diversity, including decrease of the levels of *Faecalibacterium*, a butyrate-producing bacterium with anti-inflammatory activities (COLLADO [36], KOREN [37]). Regarding the oral microbiota, studies show that pregnancy promotes the proliferation of microorganisms, especially in the early stages. The colonization of periodontal pathogens is facilitated in early and middle pregnancy, the incidence of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in gingival sulcus being significantly higher, without changes in the levels of *Prevotella intermedia* and *Fusobacterium nucleatum* (FUJIWARA [38]). On the other hand, during middle and late pregnancy the levels of *Candida* species are increased (FUJIWARA [38], BORGO [39]). The naturally fluctuant vaginal microbiota undergoes significant changes during pregnancy. Some changes were noted in the vagina: 1) increased vascularity and hyperemia; 2) increase in mucosa thickness, cervical secretions and hypertrophies of epithelium; 3) decrease of pH (acidity) due to the metabolism of glycogen into lactic acid (increases in glycogen levels can be based on increase of epithelial cells crowding, estrogen rises across gestation (GREGOIRE [40], PAAVONEN [41]), and, more importantly, based on lactic acid produced by vaginal lactobacilli (BOSKEY [42]). It was observed thatthe pregnancy microbiome was enriched in *L. iners, L. crispatus, Lactobacillus jensenii, and Lactobacillus johnsonii* (AAGAARD [43 which are primary sources of lactic acid in the vagina,. The alpha diversity of the vaginal microbiome decreased with gestational age in African-American (ROMERO [21]) and also in Caucasian gravidae (WALTHER-ANTÓNIO [44]). Also, the dominant *Lactobacillus* species differs among ethnic groups: the vagina of pregnant Asian and Caucasian women is predominantly populated by *L. jensenii* compared to Black women in which *L. gasseri* is absent (MACINTYRE [45]). The predominance and low variability of *Lactobacillus* species in vagina is based on their bactericidal activities, which assure protection against infections during pregnancy (SPURBECK & ARVIDSON [46]). The placenta contains a unique microbiome, with major phylum *Proteobacteria*, and including species such as *Prevotella tannerae* and *Neisseria* (AAGAARD [47]) at very low density. A correlation between the microbiota and the health of the pregnancy seems to exist (MYSOREKAR & CAO [48]) and dysbiosis produced by bacterial infections may be associated with pregnancy complications (SEONG [49]).

Maternal microbiota and neonatal outcome

The abnormal changes of vaginal microbiota during pregnancy can cause uterine contractions and disruption of the fetal membranes (PARK [50], LAJOS [51]). Following preterm rupture of membranes (PPROM), the vaginal microbiota is highly diverse, sequences from *Megasphaera* type 1 and *Prevotella* spp. being ubiquitously detected in all vaginal samples while those derived from *Mycoplasma* and/or *Ureaplasma* are found in 81% of cases (PARAMEL JAYAPRAKASH [52]). The colonization of vagina with *Candida albicans* (FARR [53]) and higher abundance of *Streptosporangium*, *Burkholderia* and *Anaeromyxobacter* in the placentas (ROMERO [21]) were correlated to higher rates of preterm birth.

Recent research has shown that neonatal outcome may be influenced by the maternal microbiota during pregnancy. Thus, the microbiota of vaginally born babies is

different from that of children born by caesarean section which means they display similar bacteria species with maternal vaginal microbiota instead of microbial flora similar to maternal skin ones (DOMINGUEZ-BELLO [54]). This rises the hypothesis of the potential role of a normal vaginal microbiota to influence fetal characteristics. In this regard, Dominguez-Bello reported partial restoration (30 days) of oral, intestinal and skin microbiota in babies born by caesarean section and exposed to maternal vaginal flora at birth (DOMINGUEZ- BELLO [54]). Exposure to microbiota in the genital tract during birth is thought to contribute to the maturation of immune system of the new born (CLAUSEN [55]).

Among risk factors contributing to spontaneous preterm birth (SPTB) may be the vaginal microbiome as BV is associated with the development of chorioamnionitis, an intra-amniotic infection found in the placenta of near half of all preterm births (AGGER [56]). Although several reports prove the contribution of some infectious agents to SPTB (PAYNE & BAYATIBOJAKHI [57]), the role of vaginal and placental microbiota in this pathology remains to be elucidated through studies focused on sequentially changes of microbiota that occur during the pregnancy (AGGER [56]).

Methods for highlighting the human microbial genome in obstetrics and gynecology

Traditional methods

Traditional diagnostic methods based on microscope examination (Nugent score and Amsel's diagnostic criteria) have several disadvantages raised by the limited information on the vaginal microbiota (mainly in the case of patients with negative results) or on the risk of developing a disease (in the case of bacterial resistance) (XIAO [7]). Culture based methods can offer valuable information about the bacterial metabolism and growth requirements but have the disadvantages of growing microorganisms outside their natural habitat, thus limiting the investigation of the strains that are difficult to cultivate and, subsequently, the bacterial biodiversity in a specific area (TYLER [58]).

Culture-independent targeted polymerase chain reaction (PCR) methods were developed to identify species that are not cultivatable *in vitro*. The methods are based on the analysis of 16S rRNA sequences that allows the identification of a much large number of taxa (MENDZ [59]). 16S rRNA gene belongs to prokaryotic DNA that is found in all bacteria and used to identify bacteria as apart from other type of DNA (animal, plant, fungal). PCR method uses a pre-amplification step with 8F-1492R universal bacterial primers (derived from 16S rDNA) followed by an amplification step with primers specific for each bacteria taxa (WEN [60]). Amplification methods can be combined with molecular fingerprinting approaches like denaturing gradient gel electrophoresis or DNA cloning followed by sequencing.

To understand the different properties of microbiota and the mechanisms governing its changes leading to diseases, new technologies are used. These approaches are more reliable as they allow to evaluate different microbial communities (including those that cannot be cultivated) and their abundance in various cavities. The most sensitive molecular techniques for investigating the microbiota use non-culture based approaches to study genes or genomes from mixed populations of microbes (MENDZ [59]) and include shotgun analysis (using mass genome sequencing), genomic sequence studies (for phylogenetic analysis) and next generation sequencing technologies (COX [61]).

Next-generation sequencing (NGS) technology is also known as high-throughput sequencing and its performance, compared to other sequencing techniques, is due to the capability to sequence millions of DNA fragments in parallel. Microbiome analysis using NGS is based on comparing the genomic regions of experimental samples with reference data. For this purpose, genetic markers are used. The best known marker is 16S ribosomal RNA (rRNA) gene (present in all bacteria and Archaea) that is about 1,550 bp and contains nine regions with high variability (hypervariable regions), flanked by conserved regions. Therefore, different bacterial species from a sample can be identified and quantified using NGS that targeted 16S rRNA gene (MENDZ [59]). To eliminate some of the disadvantages of 16S rRNA (multiple number of copies, lack of specificity for some bacterial strains), alternative markers like 23S rRNA and cpn60 were proposed, but incomplete databases have limited their usefulness in practice (TYLER [58]). Generally, sequencing of long regions enables an accurate framing of investigated samples into the taxonomic groups, but current NGS technology provides only short lengths sequences. Since the whole 16S rRNA gene cannot be used as marker (due to its length), short regions derived from conserved (C) and hypervariable (V) regions of this gene are targeted. Conserved regions are used to design the primers, while hypervariable regions allows for specific taxonomic distinction (TAO [62]). Variable regions like V1–V3, V4, and V4–V5 are most frequently targeted as they can provide a good genus-level sequence resolution (KIM [63]).

NGS workflow includes DNA isolation, library preparation, sequencing and data analysis. Different experimental techniques performed in each step can alter the information provided (TYLER [58]) because various factors such as sampling methods, DNA isolation and purification as well as sequencing depth can affect NGS results.

Sample collection. For gynecologic investigations, vaginal swabs and tissue can be used, but microbial profile of these samples is substantially different even in the

same individual. However, a Chinese study shows little heterogenity across microbial community at the three sampling sites (cervix, posterior fornix and vaginal canal) harvested from pregnant women at different gestational ages (HUANG [64]). Samples' storage at −80°C is recommended to prevent alterations in microbial community structure, although some authors consider RNAlater (Qiagen) as a good preservative for microbiome. Since biologic samples contain both Gram-negative and Gram-positive species (that require gentle or hard lysis, respectively), DNA isolation must be performed with optimized protocols which can provide nucleic acids suitable for molecular techniques. The purification and quantification procedures for nucleic acids can also influence NGS results and the study of Sinclair *et al* is suggestive in this regard: up to five-fold difference in sequence counts was noted when different methods for these purposes were used (SINCLAIR [65]).

Library preparation. This step consists in random fragmentation of DNA/cDNA samples and sizing the target sequences at the required length, followed by 5' and 3' adapter ligation. DNA fragmentation is accomplished by physical (sonication), enzymatic (DNase I) and chemical procedures. Subsequently, adapter-ligated fragments are amplified by PCR with specific primers for the adaptors. For cluster generation, the fragments are separated using complementary oligos to the library adapters and each fragment is then amplified into clonal clusters used for sequencing. DNA libraries might generate biases that compromise NGS datasets leading to incorect interpretation. Several kits for library preparation are provided, some of them using the classical protocol of DNA fragmentation, adaptor ligation and amplification. Illumina's Nextera tagmentation technology uses a transposase enzyme that simultaneously fragments DNA and inserts adapter sequences.

Sequencing can be performed with several NGS platforms:

Illumina NGS technology performs sequencing by synthesis (SBS). It is similar to capillary electrophoresis sequencing and is based on the ability of DNA polymerase to add fluorescently labelled dNTPs into growing DNA strands during sequential cycles of their synthesis. Labelled nucleotides are identified at the point of incorporation through fluorophore excitation. Briefly, a fluorescently labeled reversible terminator added to each dNTP is subsequently split to allow incorporation of the next base, thus resulting in the detection of single bases during their incorporation into growing DNA strands. Virtually, errors and missed calls associated with repeated nucleotide sequences are eliminated (www.illumina.com). The best known Illumina platforms are Illumina MiSeq (lower output, longer reads) and HiSeq (higher output, shorter reads).

454 GS-FLX (Roche) platform, that was on market till 2016. The principle is based on the incorporation of complementary nucleotides during sequencing with eliberation of a light signal generated by the activity of specific enzymes that use molecular byproducts as substrates. The light signal is captured and subsequently converted into base-space by the instrument's software. 454 pyrosequencing is able to read longer sequences thus allowing a better taxonomic analysis.

Ion Torrent NGS technology, based on converting the chemical signals from a semiconductor chip into digital information. This technique uses a single species of dNTP, one NTP at a time. Basically, incorporating a nucleotide into DNA molecule is accompanied by the release of a proton with local change of pH which is subsequently detected by an ion sensor. Two platforms are known: Ion Torent PGM (Personal Genome Machine) and Proton, whose disadvantages include more hands-on time.

Next-generation sequencing (NGS) technique can perform the sequencing of the whole population of microorganisms from many samples in a single run and provides data including for low abundance strains. However, differences in microbiota composition were reported using V1-V2 amplicons in different platforms (LOMAN [66]) due to the ability of these systems to perform full- length reads for certain microbes. The quality of sequencing is dependent ~~of~~ on the selected regions of the gene 16S rRNA, V4-V5 primer being a better option than V3-V4, independent of the NGS platform (CLAESSON [67]). Due to different degrees of diversity between bacteria, selected hypervariable regions allow to distinguish specific bacteria in a sample. Some studies suggest V4-V6 regions for the design of universal primers because they offer a significant phylogenetic resolution for bacterial phyla (YANG [68]).

Each type of NGS platform displays error rates either through introducing mismatches or through insertions/deletions, but several methods are recommended to minimize the undesirable effect (standardized controls, error-corrected base callers). DNA isolation and sequencing platform type are reported to be the steps that generate systematic biases thus resulting in microbiota differences across studies (LOZUPONE [69]).

Data analysis. The results generated by NGS contain noisy data which require a filtering step that eliminates sequences suggestive for errors, sometimes induced by the technology used. This step includes the removal of redundant sequences, low quality paired-end (PE) reads and the alignment of all filtered PE reads in order to eliminate potential human contamination (WANG [70]). The chimeras generated by inefficient ligation to adapters (due to non-proofreading enzymes that add non-templated purines to the 3′ ends of templates, resulting in incomplete template extension) can alter the NGS results and other several filters are required. Sequence assembly is performed

with specific programs and reference genomes are used for the assembly of the most abundant microbial species. The analysis of the sequences assigned to a taxonomic group is performed by comparison with databases (phylotype) but the selection of these databases can affect the assignation. In case of sequences that cannot be included in a taxonomic group, they are grouped based on similarities into operational taxonomic units (OTUs) that are compared with reference databases. In this regard, sequencing technology development was supported by new data analysis methods such as mothur, QIIME etc. Mothur is a software (SCHLOSS [71]) that allows to calculate distances and assigns sequences to OTUs, while QIIME is a platform that import sequences generating inter- and intra-sample measures for an accurately identification of OTUs and a better comparison of results across studies (CAPORASO [72]). QIIME is an useful tool that can supports the MIMARKS (Minimum Information about a MARKer Sequence) adopted by the INSDC (International Nucleotide Sequence Database Consortium, which includes GenBank, EBI, and DDBJ) as standard for metadata (GOODRICH [73]) Data analysis estimates the diversity of microbiota within a sample (alpha diversity) or between samples (beta diversity), and is performed with specific algorithms like Shannon or Simpson diversity index or with principal coordinates analysis (PCoA) (PIRCALABIORU [74]. Recent data showed that Canberra/ Gower distances are more appropriate to discriminate clusters when analyzing beta diversity, while UniFrac method is more robust for comparison analysis of microbes based on the length of the phylogenetic branch tree they share (LOZUPONE [75]).

There are many applications of NGS in gynecology. For instance, the diagnostic of bacterial vaginosis based on clinical criteria (Amsel criteria) and on Gram-stained vaginal smear, with the Hay/Ison or the Nugent criteria (quantification of bacterial morphotypes) are still useful as a cheap and easy method. Recent techniques based on the pyrosequencing of V3-V5 sequences of 16S rRNA gene that quantifies the ribosomal RNA allows the identification of 5 different profiles of vaginal microbiota (community member types - CST), of which 4 are dominated by *Lactobacillus sp.* (RAVEL [76], FETTWEIS [77]). As few databases of 16S rRNA gene sequences for vaginal microbiome are available, Vaginal Human Microbiome Project (Virginia Commonwealth University) developed a new method (STIRRUPS) which, applied to two datasets of V1-V3 16S rDNA reads, can offer an accurate analysis of vaginal samples. (FETTWEIS [78]). Sequencing has the advantages of a high resolution and faster and cheaper high-throughput genomic analysis coupled with a statistical and computational evaluation of enormous generated data (PFEIFER [79]). 16S rRNA pyro-sequencing is valuable to determine the composition of the vaginal microbiota but the research has to be orientated towards identification of specific ecosystems that populate a particular site. Human host responses to different microbiota, especially inflammatory response pathways, are of particular interest (BORGDORFF [80], COSIER [81]) to design successful strategies for prevention and therapy.

Conclusions

In order to improve vaginal health through microbiome manipulation, we first need to understand the factors that govern the microbiome's plasticity. Taking into account the variability of vaginal microbiome across the lifespan of each reproductive healthy women and in disease stages, a systemic analysis of the microbiome metagenomics combined with integrative multi'omics, will help to elucidate the polymicrobial profile in a sample (including uncultivated organisms). New technologies will allow the identification of the microbiome in a specific site, its interaction with others and with the host epithelium, thus leading to new insights into the roles of these microorganisms in the female diseases associated with host genetic factors and various physiological and infectious conditions. Understanding all these aspects will help to design successful strategies for prevention and therapy.

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