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NEW INSIGHTS INTO VAGINAL ENVIRONMENT DURING PREGNANCY: A MULTI-OMICS APPROACH

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Thesis summary

Thesis Abstract

The present thesis focused on the study of the vaginal environment during pregnancy. Pregnancy is a period of significant changes in women's bodies, including alterations in the vaginal microbiome.

Healthy pregnancies are characterized by decreased microbial diversity, increased stability, and a higher presence of *Lactobacillus* spp. Conversely, low levels of lactobacilli and increased bacterial diversity are associated with pregnancy-related complications and preterm births. These microbial changes are accompanied by significant modifications in vaginal metabolites. The project aimed to provide a thorough comprehension of the vaginal ecosystem of pregnant women and enhance the knowledge of pregnancy pathophysiology.

The first study (**paper I**) highlighted the role of pre-pregnancy body max index and diet in shaping the vaginal environment during pregnancy. It pointed out the importance of limiting protein intake from animal sources to maintain a healthy vaginal environment dominated by lactobacilli. Conversely, higher consumption of total carbohydrates before pregnancy appeared to be protective for vaginal health. Additionally, women starting pregnancy overweight exhibited a greater presence of vaginal dysbiosis during pregnancy and dysbiosis-related vaginal metabolites. In particular, women with bacterial vaginosis showed higher levels of biogenic amines and organic acids, while healthy vaginal status was characterized by higher levels of phenylpropionate and several amino acids.

The second paper (**paper II**) explored the vaginal environment in pregnant women at different gestational ages, at puerperium and in women experiencing a first-trimester miscarriage. The vaginal microbiota underwent significant changes during pregnancy, with reduced diversity, increased *Lactobacillus* abundance, and decreased bacterial vaginosis-related genera. In the puerperium, lower *Lactobacillus* levels and elevated counts of *Gardnerella, Prevotella, Atopobium,* and *Streptococcus* were observed. *Lactobacillus* abundance correlated with higher levels of lactate, sarcosine, and amino acids, while bacterial vaginosis-related genera were associated with amines, formate, acetate, alcohols, and short-chain fatty acids. Women receiving intrapartum antibiotic prophylaxis for Group B *Streptococcus* had a higher vaginal abundance of *Prevotella*. Finally, women experiencing a first-trimester miscarriage displayed a higher abundance of *Fusobacterium*.

The third study (**paper III**) aimed to explore the presence of antibiotics resistance genes in the vaginal environment of pregnant women. Antibiotic use during pregnancy is a complex issue, balancing maternal health and potential fetal side effects. To this purpose, macrolide and

tetracycline resistance markers were analyzed and related to vaginal microbiota composition. Different vaginal microbiota types were associated with distinct resistance profiles, with lactobacilli-dominated ecosystems showing fewer or no resistance genes. Samples positive for resistance genes were characterized by an increase in bacterial vaginosis-related genera, particularly when *Megasphaera* was present.

The last two papers aimed to identify potential biomarkers of vaginal health or disease status.

Firstly, in paper fourth (**paper IV**) we assessed the presence of Torquetenovirus, a non-pathogenic virus proposed as an indicator of immune system activation, in the vaginal ecosystem of pregnant women. Positivity for Torquetenovirus decreased from the first to the third trimester, and it was more prevalent in women with higher vaginal leukocyte counts. Torquetenovirus-positive samples showed higher levels of cytokines IL-6 and IL-8, propionate, and cadaverine. *Lactobacillus* species decreased in Torquetenovirus-positive samples, while *Sneathia* and *Shuttleworthia* increased. Torquetenovirus titer positively correlated with the concentrations of 4-hydroxyphenyllactate, isoleucine, and phenylalanine.

The last reported work (**paper V**) aimed to investigate the role of *Gardnerella vaginalis*, a key bacterium in the pathogenesis of bacterial vaginosis, in order to find biomarkers capable of predicting the severity of bacterial vaginosis and preventing pregnancy-related complications. The distribution of *G. vaginalis* clades genes in pregnant women's vaginal ecosystems were studied and correlated with vaginal microbiota composition.

The presence of clade 2 in vaginal samples was associated with a typical bacterial vaginosis composition. Moreover, as the number of simultaneously detected *G. vaginalis* clades increased, bacterial vaginosis-associated bacteria also tended to increase. Additionally, the distribution of the sialidase A gene, was assessed. Sialidase gene levels negatively correlated with *Lactobacillus* and positively correlated with *Gardnerella, Atopobium, Prevotella, Megasphaera,* and *Sneathia*.

In summary, this thesis covers a wide range of topics related to the vaginal environment during pregnancy, providing insights into how various factors can impact maternal health and pregnancy outcomes.

The obtained data could help implement “prognostic” criteria (*e.g.,* evaluation of the risk of spontaneous miscarriage based on the microbiome/metabolome profiles), as well as strategies for the prevention of early pregnancy loss, based on the “manipulation” of the vaginal microbiota.

Thesis theoretical part

1. How to study vaginal microbial niche

1.1 Culture-dependent microbial characterization

The initial characterization of vaginal bacteria relied on observable features such as their ability to grow in various media, their morphology, and their staining properties. These investigations yielded significant insights, including the identification of lactobacilli as predominant members of the vaginal ecosystem in most women.

The earliest known study dates back to 1892, when the German gynecologist Albert Döderlein isolated a bacterium from vaginal secretions of pregnant women on a blood agar plate. The "Döderlein's bacillus", 40 years later classified as *Lactobacillus acidophilus*, was able to produce lactic acid, inhibithing the growth of pathogens both in laboratory settings and in living organism (Thomas, 1928). Subsequently, Menge and Kronig described the isolation of vaginal anaerobic bacteria distinct from lactobacilli (Menge and Kronig, 1899). In 1920, Curtis and Schroeder were the first to associate the "syndrome of vaginal white discharges" with the presence of Gram-variable coccobacilli, along with a relative deficiency of Döderlein's bacillus (Curtis, 1914; Schroder, 1921). These Gram-variable coccobacilli were initially called *Haemophilus vaginalis* by Gardner and Dukes, but it will be later renamed *Gardnerella vaginalis* (GV) (Gardner and Dukes, 1955). Then, the syndrome associated with these changes in vaginal bacterial composition was renamed "Bacterial Vaginitis" and later changed to "Bacterial Vaginosis" (BV), because few inflammatory cells were observed microscopically in the vaginal fluid (Spiegel *et al.,* 1983).

As research has advanced, from cultures established from vaginal fluid samples, an increasing number of microbial species have been isolated, characterized by both anaerobic metabolism (such as bacteria of the genera *Lactobacillus*, *Bacteroides*, *Prevotella*) and aerobic metabolism, including bacteria belonging to the genera *Staphylococcus* and *Streptococcus*, and the family Enterobacteriaceae (Tashjian *et al.,* 1976).

Depending on the type of sampling and isolation methods used, such as the types of culture media, incubation times, and conditions, the composition of detected microorganisms has shown significant variability in terms of frequency and proportions in different studies.

Over time, microscopic analysis became integral to clinical practice and their use has persisted until today. First, Amsel and colleagues in 1983 have identified clinical markers of BV, that were used to differentiate symptomatic women whit abnormal microbiota from asymptomatic women with vaginal microbial composition rich of lactobacilli (Amsel *et al.,* 1983).

Amsel's criteria are the most widely accepted clinical criteria still used today to identify BV and are based on the presence of real-time clinical indicators. In particular, this clinical diagnosis requires that three of the following four criteria must be satisfied to determine BV-positive patients: (i) the presence of thin, milky, homogeneous vaginal discharge; (ii) the release of an amine "fishy" odor after adding a 10% potassium hydroxide (KOH) solution to vaginal fluid ("whiff test"); (iii) vaginal pH>4.5; (iv) presence of "clue cells", identified as vaginal epithelial cells with such a heavy coating of bacteria, observed under wet mount microscopy.
Shortly after Amsel, in 1991 Nugent and colleagues had revised the vaginal Gram-stain criteria for BV, which were first described by Spiegel, by developing a numerical score based on bacterial semi-quantization (Nugent *et al.,* 1991).
Indeed, the **Nugent score** (NS) is based on the assessment of bacterial morphotypes using Gram staining of a vaginal swab smear. The score can be calculated from the average number of three different morphotypes seen per microscopy field (counted at 1000× using immersion in oil): *Lactobacillus* morphotype (Gram-positive stick bacteria), *Gardnerella* or *Bacteroides* morphotype (Gram-variable or Gram-negative rods), and curved Gram-variable rods. A score of 0-3 indicates a healthy composition dominated by lactobacilli, while 4-6 is considered intermediate, and 7-10 is defined as BV (Fig. 1) (Wang *et al.,* 2021).

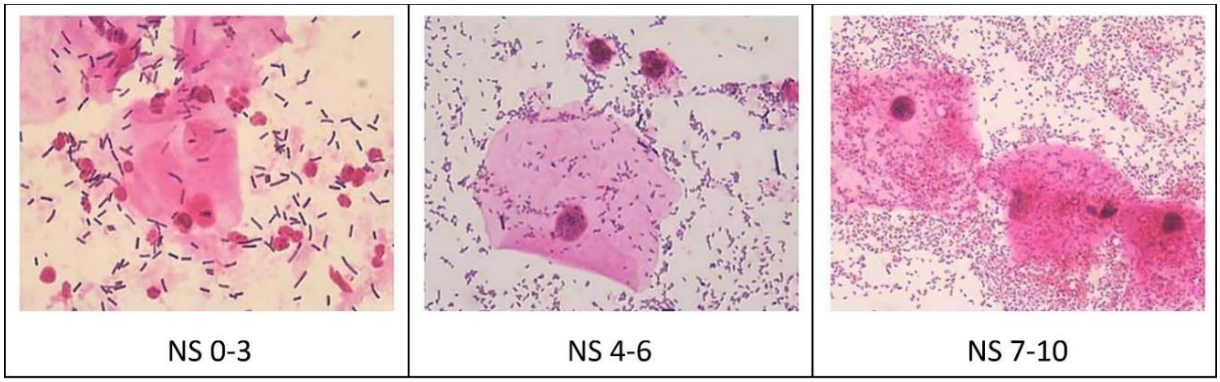

Figure 1. NS category (Wang *et al.,* 2021).

Even if the NS is considered the gold standard for vaginal microbial composition, it has some critical points. Firstly, the category of intermediate flora is perplexing and lacks clear characterization, which presents a diagnostic challenge for BV. Secondly, the identification of morphotypes is subjective and technician-dependent, so the diagnosis can be influenced by individual skills and experience (Chawla *et al.,* 2013).

Therefore, combining the Amsel and Nugent approaches with the results of clinical and microbiological morphology, can lead to a more accurate and reliable diagnosis.

More recently**,** new criteria **(Hay-Ison criteria)** were proposed for BV diagnosis. This procedure is easier and faster to use, categorizing the vaginal microbial composition into three different groups: normal (Group 1), intermediate (Group 2), and BV (Group 3), based on the relative abundance of *Lactobacillus* morphotypes versus *Gardnerella* morphotypes seen at microscopy after Gram-stained (Sherrard *et al.,* 2018).

In Table 1 the comparison between criteria was reported.

	Amsel's Criteria	**Nugent's Criteria**	**Hay/Ison Criteria**
Type	Clinical and laboratory diagnosis	Laboratory diagnosis	Laboratory diagnosis
Diagnosis duration	Fast	Long	Long
Laboratory requirement	Low	High	Moderate
Grading system	Diagnosis is confirmed when 3/4 criteria are fulfilled a) presence of thin grayish-white homogenous discharge b) vaginal pH>4.5 c) KOH or the positive whiff-amine test d) at least 20% of clue cells observed on a saline wet mount	Score 0-3: Normal flora Score 4-6: Intermediate Score 7-10: BV	Group 1: Normal flora (*Lactobacillus* only) Group 2: Intermediate (*Lactobacillus* = *Gardnerella*) Group 3: BV (*Lactobacillus* < *Gardnerella*)

Table 1. Comparison among Amsel's, Nugent's, and Hay/Ison criteria (Abou Chacra *et al.,* 2022).

However, classical microbiological approaches are labor-intensive, slow, subject to operator errors, and require the use of culture media, varying temperatures, and nutrients to isolate and characterize the species. Furthermore, it is well-known that less than 20% of the organisms comprising the human microbiota can be cultured. Thus, working with a complex sample like a vaginal swab is challenging, and culture-dependent methods are very useful for a preliminary assessment of the vaginal flora composition, but are inadequate for accurately describing the true complexity of vaginal flora (Sharma *et al.,* 2021).

1.2 Molecular microbial characterization

Decades – and several technological advancements – later, scientists are complementing microscopy and cultivation methods with modern DNA sequencing techniques to characterize the vaginal microbial composition.

Historically, in 1975, Sanger introduced the concept of the DNA sequencing method, marking the beginning of genomic research (Sanger *et al.,* 1975). In subsequent years, a new approach, known as the metagenomic approach, was introduced to analyze the microbial composition of different body sites. Metagenomics refers to the study of DNA sequences present in complex samples. In this context, the term "microbiota", which refers to "the ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body space", was also introduced (Lederberg and McCray, 2001).

With the advent of the Next Generation Sequencing (NGS), Sanger was rapidly supplanted. NGS is a term that broadly captures several related technologies that enable massively parallel or deep sequencing coverage for a selected region or the entire genome of an organism, reducing analysis times and costs (Sanschagrin and Yergeau, 2014).

Today, NGS techniques were become a fundamental tool in genetic research laboratories and is well known that to determine the composition of the Vaginal Microbiota (VMB), a precise workflow must be used.

The process begins with the isolation of DNA from a given sample (*i.e.* vaginal swab), followed by the sequencing of genetic regions and bioinformatic analysis, with the aim to identify individual microbial community members (Fig. 2).

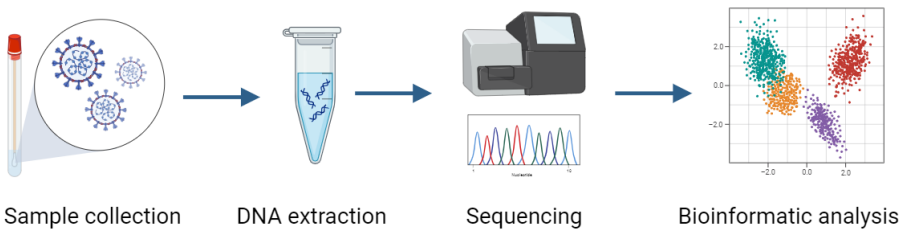

Figure 2. VMB analysis process (Figure created using Biorender.com).

Among sequencing techniques, amplicon sequencing offers the advantage of being relatively cost-effective, fast, and capable of generating reads from a single genomic region, which can generally be aligned. The most widely used method is certainly amplicon sequencing of two or more variable regions (For the VMB, the most used are V3/V4) of 16S rRNA gene (gene encoding the 16S ribosomal RNA subunit), although others have utilized amplicon sequencing

of the chaperonin-60 gene region (cpn60) (*i.e.* this gene was used to classify *Gardnerella* in clades) or a series of taxa-specific qPCR tests, often used in routine diagnostics (Fredricks *et al.,* 2005).

However, a limitation of the sequencing of the former gene regions is that this approach does not detect members of the microbiota belonging to other domains, such as viruses, parasites, and fungi, since they lack a copy of the 16S rRNA gene. Indeed, their characterization requires targeted sequencing of other genetic markers, such as the internal transcribed spacer (ITS) gene for fungi (Woese *et al.,* 1977).

For this reason, the total collection of vaginal viruses, the vaginal "virome", has only been sparsely investigated and only recently it was discussed its potential role in gynecological health and disease. For example, higher vaginal viral diversity was found to be associated with pre-term birth (PTB) and reproduction failure (Madere and Monaco, 2022).

Interestingly, Torquetenovirus (TTV) has been recently proposed as unique and sensitive method to assess the level of immunosuppression, and in the context of VMB Tozetto-Mendoza and colleagues showed that the TTV titer in the vagina of pregnant women was associated with VMB composition and different compounds (Focosi *et al.,* 2015; Tozetto-Mendoza *et al.,* 2020 and 2022).

Overall, the metagenomic analysis allows to study the "within-subject diversity" (α-diversity), that reveals which and how many species are present in a biological sample and their relative abundance. Also "between-subject diversity" (β-diversity), which measures the similarity or dissimilarity between two biological samples, could be calculated. Diversity can be quantified using specific indices, such as Shannon, Chao, or Simpson indices for α-diversity and the Sorensen index for β-diversity (Walters and Martiny, 2020).

1.3 Vaginal microbial metabolome

The study of the metabolome, which encompasses all the metabolites present in a complex sample, is a fundamental method for detecting and identifying molecules produced by the microbiota members, allowing to gain insights into the functional roles of these microbial metabolites (Bauermeister *et al.,* 2022). In the context of VMB, knowing the metabolic targets associated with a state of health or dysbiosis could be a useful diagnostic tool.

In healthy women of reproductive age, the VMB consists of lactobacilli, which are the main contributors to the production of lactic acid (LA). In contrast, BV is characterized by a drastic loss of LA and higher concentrations of short-chain fatty acids (SCFAs), including acetate,

propionate, butyrate, and succinate, indicating their potential as disease biomarkers and/or susceptibility markers (Mirzaei *et al.,* 2023). In addition, while a healthy vaginal microflora showed an increase in maltose derived from the metabolism of glycogen by lactobacilli, BV is characterized by a decrease in maltose in favor of glucose (Vitali *et al.,* 2015).

Srinivasan and collaborators have demonstrated that women affected by BV showed metabolic profiles characterized by lower concentrations of amino acids and dipeptides, along with higher levels of polyamines and amino acid catabolites (Srinivasan *et al.,* 2015). Consistent with these findings, Vitali and colleagues observed an increase in amines, including trimethylamine, trimethylamine, and cadaverine, responsible for the fishy odor of vaginal discharge in BV-women (Vitali *et al.,* 2015).

About pregnant women, Ansari and colleagues have found that specific vaginal metabolites (acetone, ethylene glycol, formate, glycolate, isopropanol, methanol, and trimethylamine N-oxide) were significantly increased in women with PTB compared to women with terminal birth. Additionally, the correlation analysis of these metabolites showed a strong negative correlation with gestational age at birth and cervical length (Ansari *et al.,* 2020). Moreover, a higher level of vaginal metabolites typical of *Lactobacillus* (inosine, fumarate, xanthine, benzoate, ascorbate) seemed to be predictors of a lower risk of spontaneous miscarriage (Xu *et al.,* 2020).

1.3.1 Nuclear magnetic resonance in vaginal metabolome analysis

Nuclear Magnetic Resonance (NMR) is one of the most widely used spectroscopic techniques for the structural analysis of molecules in complex samples. In fact, it has been employed for many years in metabolomics research, as it yields resonance spectra specific to a compound present within a complex sample (Dunn *et al.,* 2005).

The basic principle behind NMR is the phenomenon of resonance of atomic nuclei in a magnetic field. In fact, when nuclei are placed in a magnetic field, they can absorb energy, transitioning to an excited state. The nuclei's return to the ground state gives rise to a measurable phenomenon known as relaxation. When the energy is transferred to the solution in which the molecule is immersed, it is referred to as spin-lattice relaxation (T1), while if it is transferred to nearby nuclei, it is called spin-spin relaxation (T2). These two parameters shape the resulting NMR spectrum. Metabolites are thus represented in a spectrum consisting of a set of peaks positioned differently based on the atomic nucleus structure and with intensities proportional to their concentration in the solution (Macomber, 1998).

Between NMR types, the Proton nuclear magnetic resonance (H-NMR), the application of NMR spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, is using to determine the structure of molecules (Krivdi, 2019).

1.4 Vaginal microbial resistome

The introduction of antibacterial drugs into clinical practice has had a crucial impact on the prognosis of many infectious diseases, but it was quickly followed by the emergence and spread of antibiotic microbial resistance (AMR).

AMR is a natural process, and bacteria can be intrinsically resistant, but resistance could also be acquired by spontaneous mutation or by acquisition of resistance genes through intercellular plasmid exchanges or conjugative transposons (Munita *et al.,* 2016).

Nowadays, is well known that the "resistome", defined as the totality of resistance genes in the microbial genomes, can acting as a reservoir of AMR genes that can reside in commensals or opportunistic pathogens and can be acquired by pathogens via horizontal gene transfer. Consequently, the resistome *reservoir* has the potential to interfere with therapeutic options following infection (Wright, 2007).

Although some resistomes were very well characterized during years (*i.e.,* intestinal resistome), the vaginal resistome (VR) is still little studied (Ma *et al.,* 2021). A study about resistance genes distribution in primates reported that tetracycline resistance genes, in particular *tet(M)* and *tet(W)*, were very widespread in primate's vagina, while other resistance genes (*i.e.,* macrolides resistance genes) were present in lower quantity. Indeed, tetracycline resistance genes are often found in the Firmicutes and Bacteroidetes, both prominent members of VMB of humans and animals (Jeters *et al.,* 2009). Recently, Roachford and colleagues assessed the VR in a cohort of non-pregnant Afro-Caribbean women, showing a great abundant of resistance determinants related to tetracyclines and macrolides (Roachford *et al.,* 2021).

Additionally, Alicea-Serrano and colleagues have demonstrated the presence of tetracycline resistance gene in the oral microbiota of newborns and in the vagina of their mothers at the time of birth, showing a great presence of these genes in both considered populations, although information about resistance genes distribution in pregnant women during pregnancy are still lacking (Alicea-Serrano *et al.,* 2013).

2. Overview of the vaginal microbiota

The VMB is an intricate and dynamic ecosystem that constantly undergoes fluctuations during woman's entire life.

The vaginal mucosa is made up of a stratified squamous nonkeratinized epithelium covered by cervicovaginal secretion. The mucosa acquires oxygen, glucose, and other nutrients from underlying submucosal tissues through diffusion due to the limited blood supply, establishing a relatively anaerobic habitat condition where mainly bacteria, but also viruses and fungi, are present (Linhares *et al.,* 2011).

The VMB has a key role into vaginal dynamics. First, the sharp co-operative relationship of microbes with the host provides first line of defense against the migration of opportunistic vaginal pathogens. Moreover, a mutual relationship exists between woman reproductive physiology and VMB (Kroon et *al.,* 2018).

If comparing with all the other human body sites, the VMB represent a unique microbiota site (Fig. 3) (Barrientos-Durán *et al.,* 2020). In fact, the vagina is characterized by the lowest α-diversity, and β-diversity is relatively low at the phylum and genus level, primarily due to the dominance of the *Lactobacillus* genus in most women (Zhou *et al.,* 2021). However, the β-diversity becomes significantly higher if *Lactobacillus* species dominance, which varies among different women, is considered (Verstraelen H *et al.,* 2022).

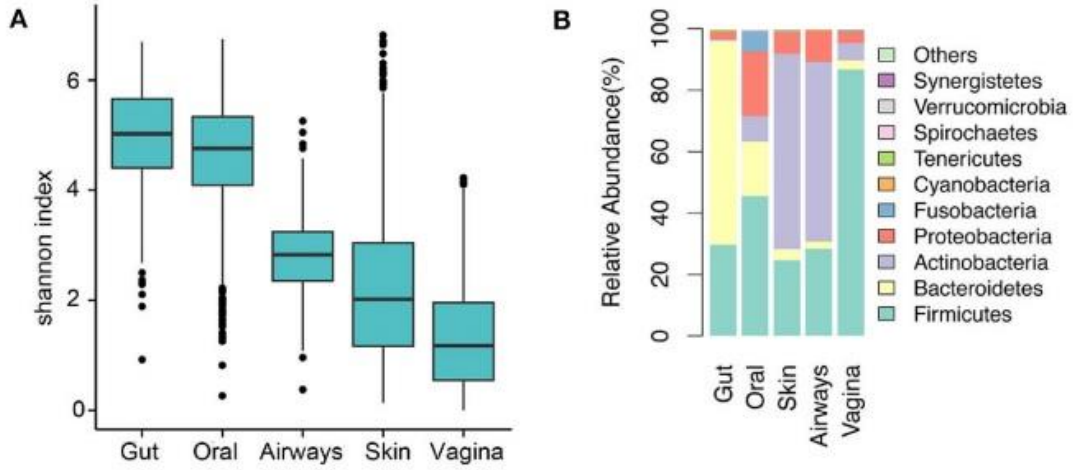

Figure 3. Diversity and abundance of bacteria from different anatomical sites obtained from healthy subjects in Human Microbiome Project (HMP). (A) Bacterial α-diversity according to the Shannon diversity index; (B) Microbial composition at the phylum level (Zhou *et al.,* 2021).

In what is arguably the most cited study of VMB communities, Ravel and colleagues revealed the presence of five VMB community state types (CSTs), divided on composition and abundance of vaginal bacterial species (Ravel *et al.,* 2011). This study was carried out on a cohort of 396 North American healthy reproductive aged women, using a high throughput method based on pyrosequencing of barcoded 16S rRNA genes (Fig. 4). CTSs can be readily distinguished based on two criteria: (i) whether the constituent communities are dominated by lactobacilli; (ii) the *Lactobacillus* spp. present.

Communities in group I, which occurred in 26.2% of the women sampled, were dominated by *L. crispatus*, whereas groups II (6.3%), III (34.1%), and V (5.3%) were dominated by *L. gasseri*, *L. iners*, and *L. jensenii*, respectively. The most diverse communities were those of group IV (27%), characterized by higher proportions of strictly anaerobic bacteria including *Prevotella, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Sneathia, Eggerthella, Aerococcus, Finegoldia, and Mobiluncus.* A similar study conducted by Gajer and colleagues confirmed these previous observations, splitting the group IV into two subgroups, IV-A and IV-B (Gajer *et al.,* 2012). IV-A consists of a moderate amount of *L. iners* or other lactobacilli, and lower proportions of various anaerobic bacteria species (such as *Anaerococcus, Corynebacterium, Finegoldia or Streptococcus*). In contrast, CST IV-B is characterized by higher proportions of the genus *Atopobium, Prevotella, Parvimonas, Sneathia, Gardnerella, Mobiluncus and Peptoniphilus*.

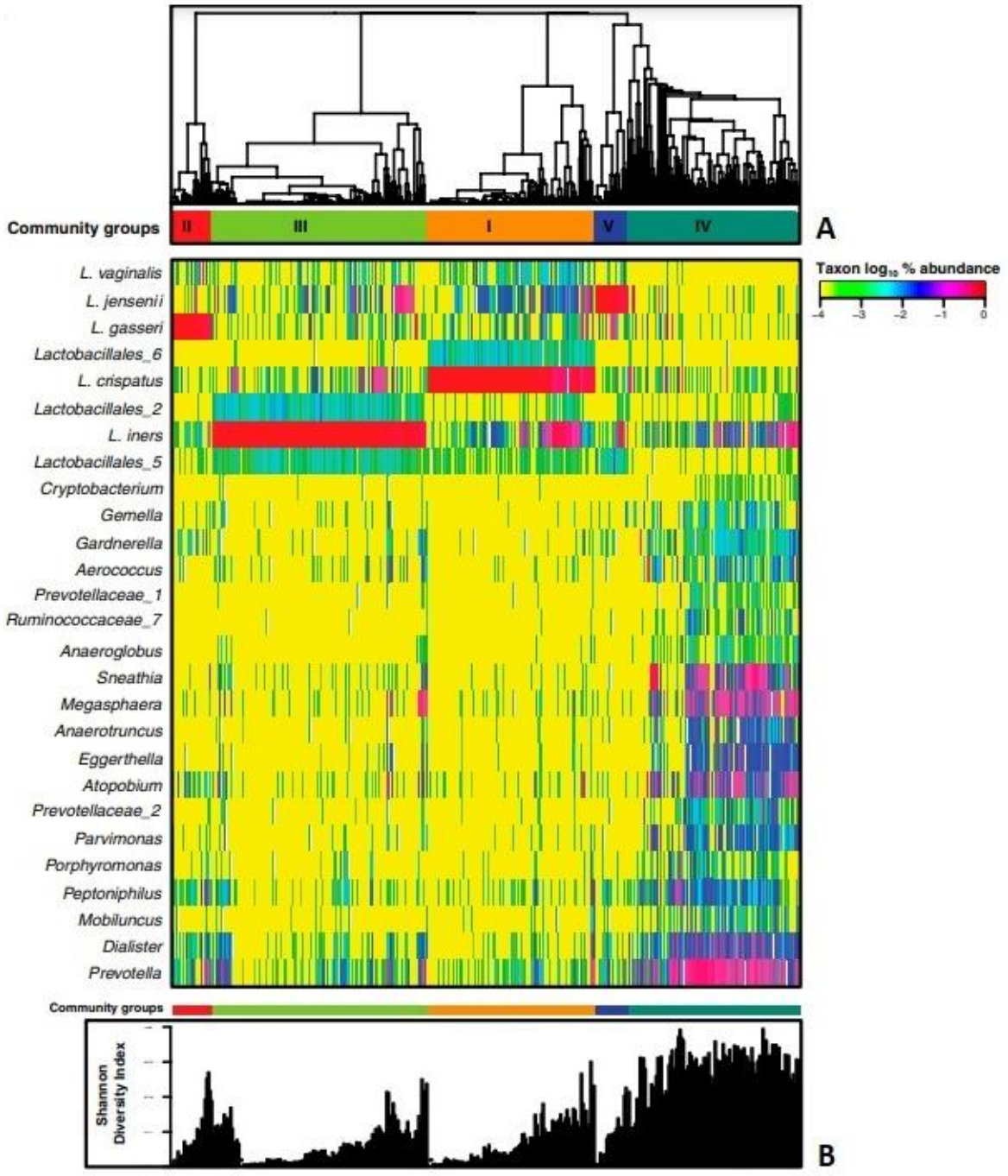

Figure 4. Heat map of microbial taxa found in the vaginal bacterial communities of 394 reproductive age-women. (A) Complete linkage clustering of samples based on the species composition and abundance of vaginal bacterial communities that define community groups I to V. (B) Shannon diversity indices (Ravel *et al.,* 2011).

2.1 Eubiotic vaginal microbiota

Over the years, the definition of an eubiosis status to be used as a reference for microbiota analysis has been much debated. Regarding the VMB, unlike many other sites in the body, the challenge in defining its due to the substantial dynamics of the microbiota, which are marked by temporal shifts influenced by sexual development, sexual activity, personal hygiene, menstruation, and hormone levels (Gajer *et al.,* 2012).

Consequently, it seems not realistic to establish a universally applicable eubiotic VMB status that applies to all women. In the context of Human Microbiome Project, the microbiota from adults considered "relatively healthy" has been used as the reference microbiota (Human Microbiome Project, 2007).

In the past decade, there has been a significant increase in investigations focused on the VMB, which have revealed the various microbial communities that shape the distinct composition of vaginal microflora in women (Ravel *et al.*, 2011; Li *et al.,* 2019). The collective findings from these studies suggest that a *Lactobacillus*-dominated community is commonly observed in a healthy vagina. Typically, *L. crispatus, L. gasseri, L. iners,* and *L. jensenii* have been identified as the predominant species within the VMB of healthy reproductive-aged women.

However, additional studies also reported that some women's vaginal ecosystems can be healthy without a *Lactobacillus*-dominant VMB. In these cases, other species belonging to the lactic acid bacteria (LAB) such as *Atopobium vaginae, Megasphera* and/or *Leptotrichia* spp., can be identified as dominant vaginal phylotype (Zhou *et al.,* 2007; Srinivasan *et al.,* 2012). The same species were also detected by Ravel and colleagues in group IV, suggesting that some key functions of vaginal community, like LA production, must be preserved to guarantee an adult healthy vaginal ecosystem (Ravel *et al.,* 2011).

In addition, an interested study conducted by Hyman and colleagues on healthy and asymptomatic premenopausal women, showed great variability in vaginal ecosystem composition among women with very similar epidemiological and clinical characteristics, highlighting challenges in classification (Hyman *et al.,* 2005).

2.1.1 *Lactobacillus* spp.

Since their first description, lactobacilli have been considered the dominant inhabitants of healthy vaginal community. Today, it is well known that *Lactobacillus* spp. generally constitute more than 80% of total VMB and are present at a concentration of 10^7-10^8 CFU/g of vaginal fluid. Lactobacilli belonged to Firmicutes phylum, and are characterized as Gram-positive, microaerophilic, acid-tolerant, non-sporulating bacteria (Borges *et al.,* 2014).

Vaginal lactobacilli exert important health-promoting effects to maintain the homeostasis of the host and their dominance in the vaginal environment. This is accomplished by various direct and/or indirect mechanisms, first the LA production (Fig. 5) (Borges *et al.,* 2014).

LA is the main metabolite present in the vaginal environment, with physiological concentrations of 110 mM, produced from glycogen-derived products metabolized under anaerobic conditions. As results, the low pH (4-4.5) of the vagina creates a hostile environment that inhibits the growth of many pathogenic organisms. Indeed, LA can lyse bacteria, causing bacterial cell death by acidifying the cytosol and increasing the permeability of the cell membrane to other antimicrobial compounds like H_2O_2. For these reasons, the acidic environment is highly protective against infections or colonization of the vaginal mucosa by opportunistic (*i.e. Escherichia coli*) or non-indigenous (*i.e. Neisseria gonorrhoeae*, *Chlamydia trachomatis*, Human Immunodeficiency Virus (HIV)) pathogens (Barrientos-Durán *et al.,* 2020; O'Hanlon *et al.,* 2013).

Given that VMB is dominated by distinct *Lactobacillus* species, the vaginal acidification can raise different pH levels, with *L. crispatus* normally achieves the highest acidification level (Tachedjian *et al.,* 2017). Moreover, LA acid exists in two isomers, L(+)- and D(-)-, with the first primarily produced by lactobacilli and less than 1.5% by vaginal epithelial cells (Boskey *et al.,* 2001). Several research have shown that the D(-)-LA isomer is the most effective in terms of microbicidal effects *in vivo* and *in vitro* against uropathogens, as well as in preventing adverse outcomes in pregnancy, and is produced only by some *Lactobacillus* species (Zalambani *et al.,* 2023; Jang *et al.,* 2019).

Both D(-)- and L(+)-LA can enhance vaginal epithelial cell survival by facilitating the repair of damaged DNA through the inhibition of histone deacetylase activity leading to increased acetylation of histones on the surface of DNA (Zalambani *et al.,* 2023).

Additionally, LA protonated form performs immunomodulatory actions on the genital mucosa, creating an anti-inflammatory state by inhibiting the production of inflammatory cytokines and

chemokines, like interleukin 6 (IL-6), interleukin 8 (IL-8), α-tumor necrosis factor (TNFα), and macrophage inflammatory protein-3α (MIP-3α) (Hearps *et al.,* 2017).

There are additional mechanisms through which lactobacilli maintain the of vaginal health.

Some *Lactobacillus* spp. produce **hydrogen peroxide (H_2O_2)**, which inhibits the growth of catalase-negative anaerobic organisms by production of hydroxyl free radicals. To protect himself from the radicals, lactobacilli seem to produce Fe^{3+}-activated extracellular peroxidase. It was reported that approximately 80% of the strains of vaginal origin produced H_2O_2 and there are striking differences between the produced amounts (of 1.01–15.50 μg/mL) (Reid *et al.,* 2011). The H_2O_2-producing microorganisms have been suggested to maintain ecologic balance, protecting against the development of BV during pregnancy and may have a role in the pathophysiology of PTB (Kim *et al.,* 2006).

Lactobacilli can also secrete **bacteriocins**, that induces cytoplasmatic membrane pore formation and permeabilization, interfering with cellular enzymatic reaction and nuclease activity of pathogens (Zhang *et al.,* 2018).

Furthermore, through the production of **biosurfactants**, amphipathic molecules that emulsify aqueous solutions, lactobacilli can reduce surface tension, making it more difficult for bacteria to adhere to host epithelia (Foschi *et al.,* 2021).

Some *Lactobacillus* spp., including *L. acidophilus, L. gasseri* and *L. jensenii*, have been shown to possess the ability to **co-aggregate** with *E. coli*, vaginal *Staphylococci* and *Candida albicans*. This mechanism allows the homeostasis of the vaginal tract to be restored because it creates a biochemically hostile microenvironment for growth and prevents access to the epithelial tissues, hindering harmful bacteria proliferation in the vaginal niche (Younes *et al.,* 2012).

Additionally, the **adherence** of lactobacilli to the vaginal epithelium involves occupying or masking potential binding sites of pathogens in the mucosa through steric hindrance (Borges *et al.,* 2014). Furthermore, they manage to inhibit biofilm formation by pathogens such as *C. albicans* and *N. gonorrhoeae* (Haque *et al.,* 2016; Foschi *et al.,* 2017).

Moreover, some lactobacilli like *L. reuteri*, have been found to produce anti-virulence **signaling molecules**, which are able to suppress toxin production by pathogenic bacteria (Laughton *et al.,* 2006).

Again, lactobacilli significantly contribute to maintaining the **integrity of the epithelial barrier** influencing the cytoskeleton, stimulating mucus production, and promoting the phosphorylation of tight junction proteins (Zhang *et al.,* 2018).

To conclude, the influence of the microbiota on the **regulation of the immune response** can yield several beneficial outcomes including host-factors production such as defensins,

lactoferrin and lysozyme, which possess pathogen-killing properties. Lactobacilli can also generate alkaline phosphatases that bind to lipopolysaccharide, neutralizing its toxicity, and can modulate the signaling of nuclear factor-kB (NF-kB) in host epithelial cells, leading to the regulation of immune responses (Reid *et al.,* 2011).

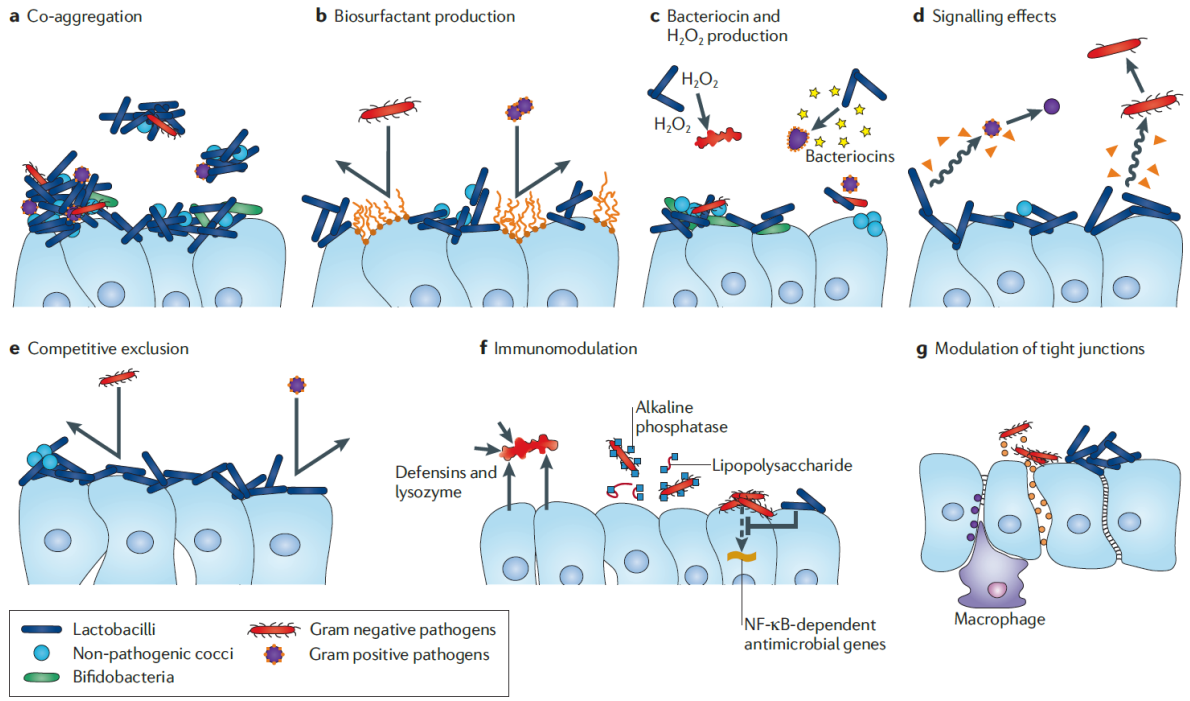

Figure 5. The lactobacilli-associated protective mechanisms in the vagina (Reid *et al.,* 2011).

As described above, the various protective mechanisms are species-specific or even strain-specific.

In particular, whether *L. iners* is beneficial or pathogenic still remains controversial. In fact, this species had escaped scientists' attention for a long time due to its inability to grow on Man-Rogosa-Sharpe (MRS) agar under the same culture conditions as other lactobacilli, as well as for the apparent Gram-negative morphology and the inability to produce D-LA and H_2O_2. Aditionally, if compared with other lactobacilli like *L. crispatus*, *L. iners* seems to be less effective in promoting the stability of the VMB, thereby predisposing individuals to dysbiosis. *L. iners* presence has been related to higher levels of pro-inflammatory factors. Supporting by these evidences, has been suggested that the prevalence of *L. iners* may contribute to the onset and maintenance of BV, being a risk factor for sexually transmitted infections (STIs) and adverse pregnancy outcomes (Zheng *et al.,* 2021).

2.2 Vaginal dysbiosis

In healthy reproductive-age women, the VMB is characterized by low bacterial diversity, often dominated by various species of *Lactobacillus.* However, when this balanced microbiota is disrupted, due to endogenous or exogenous perturbations, a dysbiosis can arise. Vaginal dysbiosis is generally defined as a polymicrobial condition marked by a low prevalence of *Lactobacillus* spp. and an increase in anaerobic microorganisms (Ravel *et al.,* 2011).

This imbalance results in an increase in the production of pro-inflammatory cytokines, a decrease in the levels of glycogen and antimicrobial peptides, and vaginal epithelial barrier deterioration (Dabee *et al.,* 2021). An alteration of the vaginal ecosystem can also expose individuals to cervicovaginal infections, primarily STIs. Moreover, the shifting of vaginal microbial communities can lead to severe gynecological issues such as pregnancy loss, PTB, and decreased conception rates (Abou Chacra *et al.,* 2022).

The most common forms of dysbiosis include BV, aerobic vaginitis (AV), and vulvovaginal candidiasis (VVC).

2.2.1 Bacterial vaginosis

BV is the most common vaginal disorders in women of reproductive age (Abou Chacra *et al.,* 2022). It is characterized by a shift in the vaginal microbial composition, with a dramatic depletion of lactobacilli and a significant overgrowth of obligate or facultative anaerobes such as *Gardnerella, Atopobium, Ureaplasma, Mycoplasma, Prevotella, Peptoniphilus, Megasphaera, Mobiluncus* (Abou Chacra *et al.,* 2022; Romero *et al.,* 2014).

The reduction of lactobacilli disrupts host physiology through multiple pathways, including the depletion of essential nutrients required for the growth of other resident bacteria within the vagina, the destruction of the vaginal barrier through hydrolytic enzymes (sialidase and prolidase), and the promotion of pro-inflammatory cytokines release (IL-6, IL-8, IL-1α, IL-1β, TNF-α).

Furthermore, the decreased production of LA prevents the vaginal pH from being maintained within the physiological range, rendering the host more susceptible to infections (O'Hanlon *et al.,* 2013).

2.2.1.1 Epidemiology

Globally, the prevalence of BV in the general population ranges from 23% to 29% (Europe and Central Asia, 23%; East Asia and Pacific, 24%; Latin America and the Caribbean, 24%; Middle East and North Africa, 25%; Sub-Saharan Africa, 25%; North America, 27%; South Asia, 29%). The estimated annual global economic burden of treating symptomatic BV in United States (US) is 4.8 billion $ (Peebles *et al.,* 2019).

2.2.1.2 Clinical features

According to data from the National Health and Nutrition Examination Survey in US, most women diagnosed as BV-positive (84% of the total) does not report any symptoms. However, when symptoms are present, the predominant symptom is the presence of thin, homogeneous, white-grayish discharges caused by the exfoliation of vaginal epithelial cells. These discharges often have a typical "fishy" odor, due to amines production (such as trimethylamine, putrescine, and cadaverine from) by anaerobic bacteria. Women have been also reported itching, burning, and pain, mainly during sexual intercourse (Abou Chacra *et al.,* 2022).

2.2.1.3 Etiology

The etiology of BV is still not completely understood. Although BV is considered a polymicrobial condition, bacteria belonged to *Gardnerella* genus are often the predominant and they are frequently detected (up to 95% of cases) in vaginal samples of women with BV (Fredricks *et al.,* 2005).

Gardnerella is a Gram-variable facultative anaerobic bacterium that exhibits notable virulence potential compared to other BV-bacteria. Indeed, *Gardnerella* produce the cytolytic toxin vaginolysin (which specifically targets human cells and activates cell death pathways), the sialidase enzyme (which is associated with degradation of vaginal mucin), and proteolytic enzymes (which are capable of degrading proteins and decarboxylases that convert amino acids). When not degraded, these aminic compounds become malodorous and assume the typical "fishy odor". Therefore, *Gardnerella* seems to be responsible of all the BV-related symptoms (Hardy *et al.,* 2017).

Additionally, *Gardnerella* is capable of degrading vaginal glycogen through extracellular enzymes, which represent an important nutrient source in this body sites (Bhandari and Hill, 2023). Again, due to the presence of fimbriae, *Gardnerella* can adhere to host cells and form

biofilm, acting as a scaffold where other anaerobic species can subsequently attach (Hardy *et al.,* 2017).

The description and taxonomy of *Gardnerella* has been changed during time. Nowadays, species designation has been added, even if the previous classification in clades (identified by cpn60 barcode sequencing) is still widely used. The identified species are: *G. swidsinskii* and *G. leopoldii* (clade 4), *G. piotii* (clade 2), *G. vaginalis* (clade 1), and clade 3 members, which include *G. species* (Shvartsman et al., 2023).

Nevertheless, even if *Gardnerella* is present at higher abundances in women with abnormal vaginal flora, it can also be found in healthy women, suggesting that it might be essential but not sufficient for BV development (Hickey and Forney, 2014).

In this context, Gilbert and colleagues studied the potential synergistic relationship between GV and other BV-typical bacteria: *Prevotella bivia,* and *Atopobium vaginae*. They demonstrated that after exposure to GV*,* vaginal lactobacilli decrease, and biofilm formation occurs on the vaginal epithelium. Proteolysis by GV may promote the growth of *P. bivia*, which degrades the mucin layer of the vaginal epithelium. After the loss of the protective mucosal layer, greater adherence to the polymicrobial biofilm of other bacteria associated with BV, including *A. vaginae*, occurs (Gilbert *et al.,* 2019). Moreover, *A. vaginae* seems to contribute to BV pathogenesis by altering the host's immune system, increasing localized production of IL-6, IL-8, and the antimicrobial peptide β-defensin, but further research are necessary to clarify that (Libby *et al.,* 2008).

2.2.1.4 Diagnosis

The diagnosis of BV is challenging and complex due to its polymicrobial nature and the wide range of clinical features. BV has historically been diagnosed using Amsel's criteria and the NS system, both utilizing clinical and microscope characterizations of vaginal swabs smear (Amsel *et al.,* 1983; Nugent *et al.,* 1991) (Pages 57-58).

With the advent of new technologies, multiple BV nucleic acid amplification tests (NAATs) are available for BV diagnosis among symptomatic women (Coleman and Gaydos 2018).

These tests represent a quantitative, fast, and reproducible approach and are based on detection of specific bacterial nucleic acids and have high sensitivity and specificity for BV (*i.e., G. vaginalis, A. vaginae, BVAB2, or Megasphaera* type 1) and certain lactobacilli (*i.e., L. crispatus, L. jensenii, and L. gasseri*). There are several commercially available assays with a

sensitivity ranging from 90.5% to 96.7% and a specificity ranging from 85.8% to 95% compared to Amsel's and Nugent analysis (Coleman and Gaydos, 2018).

2.2.1.5 BV-correlated sequelae

It has been demonstrated that women with BV are more vulnerable than others. BV can initially cause cervicitis, endometritis, salpingitis, and urinary tract infections (Abou Chacra *et al.,* 2022).

In women with BV, an increase in the production of pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α, TNF-β, IL-8, IL-10) has been observed, contributing to the establishment of a chronic state of inflammation that can lead to pelvic inflammatory disease (PID), damaging the endometrium, Fallopian tubes, ovaries, and pelvic peritoneum. PID is often associated with infertility, abdominal inflammations, and ectopic pregnancies (Dabee *et al.,* 2021).

Moreover, it is well known that women with BV are at increased risk of acquiring STIs like herpes simplex virus (HSV), human papillomavirus (HPV), HIV and transmission of pathogens causing syphilis, chancroid, gonorrhea, trichomoniasis, and chlamydia (Abou Chacra *et al.,* 2022).

During pregnancy, BV increases the risk of PTB, intrauterine fetal death, preterm rupture of membranes, amniotic fluid infections, chorioamnionitis, miscarriages and postpartum infections (Nelson *et al.,* 2016).

2.2.1.6 Therapeutic options

The first-line therapy recommended by World Health Organization (WHO) for BV is 500 mg of oral metronidazole twice daily for one week. As a pro-drug, metronidazole is reduced to its active state under anaerobic conditions, and this mechanism seems to spare beneficial *Lactobacillus* spp. Other proposed therapeutic regimens include oral or intravaginal clindamycin, and both antimicrobial agents exhibit almost comparable effectiveness, with cure rates ranging from 58% to 92% after 1 month of treatment (WHO, 2021). However, these outcomes are often temporary and result in recurrences or reinfections at rates exceeding 50% within 6-12 months after treatment. The reasons of this reoccurrence are not clear. Certainly, the presence of a complex microbial community dynamics governs the collective response to treatment, and the complete removal of BV-associated bacteria is complicated by the presence of biofilms that prevent antibiotics action, as well as the presence of antibiotic resistant bacteria (Abou Chacra *et al.,* 2022).

Given the substantial body of evidence linking PTB and BV, the use of antibiotics during pregnancy as a prophylactic measure or for BV treatment has been proposed. However, there is still limited consensus on this matter. In a review of 15 trials, the authors concluded that despite successful BV eradication during pregnancy, antibiotic treatment did not reduce the risk of PTB (McDonald *et al.,* 2007). Conversely, it has been demonstrated that treatment programs in pregnant women may reduce the risk of PTB (Swadpanich *et al.,* 2008).

Moreover, probiotic preparations have been proved to be a safe alternative to antibiotics for restoring the microecological balance of female reproductive tract. Specific strains of *Lattobacillus*, administered orally or intravaginally, can colonize the vagina of women with symptomatic or asymptomatic BV, reduce the colonization of pathogens, and reduce symptoms and/or signs of BV (Borges *et al.,* 2014). Some studies have relived that combination of antibiotics and probiotics significantly reduces the recurrence rate of BV compared to treatment with antibiotics alone, although other researchers have produced controversial results (Mastromarino *et al.,* 2013) (Table 2). It has been suggested that probiotics could also play a role in the prevention of PTB, due to the associations between PTB and BV (Jarde *et al.,* 2018).

Authors	Size	Type of study duration	Intervention	BV cure rate
Anukam *et al.,* 2006a	125	R, DB, PC 30 days	Oral metronidazole 500 mg for 7 days and oral capsule containing *L. rhamnosus* GR-1 (10^9 CFU) and *L. reuteri* RC-14 (10^9 CFU) or placebo for 30 days starting on day 1 of metronidazole treatment	88% compared to 40% control ($p<0.001$)
Petricevic and Witt, 2008	190	R, OB, PC, 4 weeks	Oral clindamycin 300 mg for 7 days, then vaginal capsules containing 10^9 CFU of *L. casei rhamnosus* for 7 days	83% compared to 35% control ($p<0.001$)
Larsson *et al.,* 2008	100	R, DB, PC 6 menstrual periods	Vaginal 2% clindamycin cream followed by vaginal capsules containing *L. gasseri* EB01-DSM14869 (10^8-10^9 CFU) and *L. rhamnosus* PB01-DSM14870 (10^8-10^9 CFU) for 10 days, probiotic treatment repeated for 10 days after each menstruation during 3 menstrual cycles	65% compared to 46% control ($p=0.0042$)
Eriksson *et al.,* 2005	187	R, DB, PC 2 menstrual periods	Vaginal 100 mg clindamycin ovules for 3 days, then tampons containing 10^8 CFU of *L. gasseri, L. casei rhamnosus, L. fermentum* or placebo tampons during the next menstrual period	56% compared to 62% control (p= non-significant)

Authors	Size	Type of study duration	Intervention	BV cure rate
Bradshaw *et al.,* 2012	268	R, DB, PC 6 months	Oral metronidazole 400 mg for 7 days followed by vaginal pessary containing *L. acidophiuls* KS400 ≥ 10^7 CFU and 0.03 mg estriol for 12 days	72% compared to 73% control (p= non-significant)

Table 2 - Clinical trials on probiotics use combined with antibiotic treatment for BV. R=randomized; DB=double blind; PC=placebo controlled; OB=observer blind; CFU=colony forming units (Mastromarino *et al.,* 2013).

2.2.1.7 Vaginal microbiota transplantation

Another exciting approach, which has been recently proposed for treating human diseases, is microbiota transplantation. This practice is based on a holistic view of the entire microbiota rather than targeting a single bacterial species as in the case of probiotics. The most known is the fecal microbiota transplantation (FMT), that involves the administration of fecal material from a healthy donor into the intestines of a diseased individual. FMT was initially used to eradicate recurrent *Clostridium difficile* infections, but researchers are now exploring its potential therapeutic effects in several diseases (Wang *et al.,* 2019).

Inspired by FMT, Vaginal Microbiota Transplantation (VMT) aims to restore the VMB to a healthy state, by implanting vaginal secretions from a healthy individual into the recipient with BV (Fig. 6).

In the first preliminary study, Lev-Sagie and colleagues recruited a total of five patients with recurrent BV for VMT procedures. Four of them have showed symptom improvements and were associated with complete long-term remission until the end of the follow-up period. Notably, these patients exhibited rapid changes in bacterial composition as early as one month after VMT (correlated with recovery in Amsel criteria) and became significantly more similar to the respective donor's VMB configuration (Lev-Sagie *et al.,* 2019).

VMT could presents an innovative method to reintroduce balance to the VMB, especially for women who have persistent and recurrent BV that hasn't responded to antibiotic treatment. However, developing VMT remains a challenge as donor selection involves strict criteria, and the ethical issue of using vaginal fluids remains open for discussion (Lev-Sagie *et al.,* 2019).

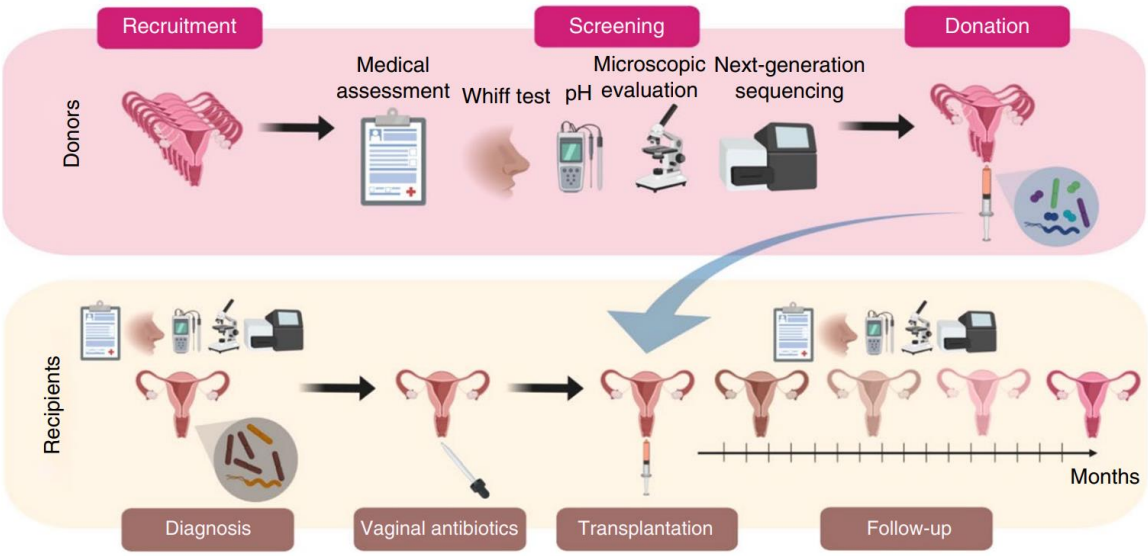

Figure 6. Schematic depiction of VMT (Lev-Sagie *et al.,* 2019).

2.2.2 Aerobic vaginitis

AV is the name given by Donders and colleagues to a vaginal infection which was not recognized as such before. AV is characterized by dysbiotic vaginal microflora containing aerobic and enteric bacteria such as *E. coli*, *Staphylococcus aureus*, coagulase-negative *Staphylococci* such as *S. epidermidis*, group B *Streptococcus* and *Enterococcus faecalis* (Donders *et al.,* 2017).

AV is still poorly known, and it is often confused with BV, leading to incomplete and imprecise diagnostic workouts and erroneous management of patients in both clinical and research settings. Indeed, AV and BV share some characteristics such as a diminished number or absence of lactobacilli, discharges and increased pH, but there are also striking differences between them. Firstly, there is no inflammation in women with BV, whereas AV is characterized by the presence of a marked inflammatory response, accompanied by a strong recruitment of leukocytes, neutrophils, and pro-inflammatory cytokines, especially IL-6 and IL-1β. The vagina of women with AV often appears red and edematous and may even display small erosions or ulcerations (Ma *et al.,* 2022).

Moreover, the color of the discharge in BV is usually whitish or grey and of a watery consistency, whereas in AV it is yellow to green and rather thick and mucoid.

In addition, women with BV do not have dyspareunia (pain during sexual intercourse), while some women with severe AV do. Finally, the microscopic appearance differs in various aspects,

such as the presence of leucocytes and parabasal or immature epithelial cells in AV and the absence of the granular aspect of the microflora, typical of BV (Donders *et al.,* 2017).

The frequency of AV varies from 12% to 23% in not-pregnant women, and 4 to 8% during pregnancy. AV has been associated with increased risk of STIs (such as HPV, HIV, *Trichomonas vaginalis* and *C. trachomatis*) and cervical dysplasia. In pregnant women, AV has been associated with spontaneous abortion, PTB, puerperal sepsis and infection of the urinary system (Donders *et al.,* 2017).

AV diagnosis involves the use of wet mount microscopy to determine the lactobacillary grade (quantity of lactobacilli or coccoid bacteria), presence of inflammation, proportion of toxic leukocytes, microflora characteristics, and presence of immature epithelial cells. Each of these five aspects is assigned a score ranging from 0 to 2, and the scores are combined to determine an overall composite score, classified as follows: a score of 0-2 indicates no AV; a score 3-4 suggests mild AV; a score between 5-6 signifies moderate AV; and a score from 7-10 indicates severe AV. The precise causative agent of AV is still unknown, and consequently also the best treatment (Ma *et al.,* 2022; Donders *et al.,* 2017).

2.2.3 Vulvovaginal candidiasis

VVC is the second most common cause of vaginal imbalance, as well as the most common fungal infection in the vaginal region. It is estimated that around 70-75% of women, especially those of reproductive age, will experience VVC at least once during their lifetime, with a very common recurrence (Sobel, 2007).

The causative agents of VVC are *Candida* species, with *C. albicans* accounting for 85-95% of total vaginal fungal infections. *C. albicans* is a dimorphic fungus, capable of growing in both yeast and filamentous forms. It is a normal resident of the VMB, colonizing approximately 20% of women without causing particular symptoms. However, VVC can also be associated with non-albicans species, and among these *C. glabrata* is the most linked species to candidiasis with a worldwide prevalence of 10-20% (Corsello *et al.,* 2003).

Several factors contribute to the transition from asymptomatic colonization to symptomatic infection, including intrinsic factors within the host, environmental factors, host behavior, and characteristics of the organism itself. For instance, conditions such as pregnancy, prolonged use of broad-spectrum antibiotics, contraceptive usage, frequent sexual intercourse, diabetes, and immunosuppression can all contribute to *Candida* colonization and infection. VVC has various clinical manifestations, including vaginal itchiness, dyspareunia, external dysuria (pain during

urination), vulvar burning, irritation, and vaginal aching. The classic symptom of a cottage cheese-like discharge is typically mild and may sometimes be absent (Sobel, 2007).

The primary approach to treating VVC revolves around the use of azole drugs, administered orally or topically. Moreover, the use of *Lactobacillus* probiotics has been proposed (*L. rhamnosus, L. casei* and *L. acidophilus*), which are able to *in-vitro* reduction the virulence of *Candida* and may provide additional benefits to antifungals (Shenoy and Gottlieb, 2019).

3. Conditions and factors influencing the vaginal microbiota composition

Since vaginal bacterial communities differ in species composition, they are likely to differ in how they respond to changes and disturbances. In fact, the composition of the vaginal ecosystem is not static but changes rapidly over time and in response to endogenous and exogenous factors (Fig. 7) (Eschenbach *et al.,* 2000).

Endogenus factors encompass age, stages of life (*i.e.,* pregnancy) and ethnicity.

Exogenus factors involve diet, smoke, hygiene practices, probiotics, stress, contraceptives use, sexual behaviors, antibiotics use.

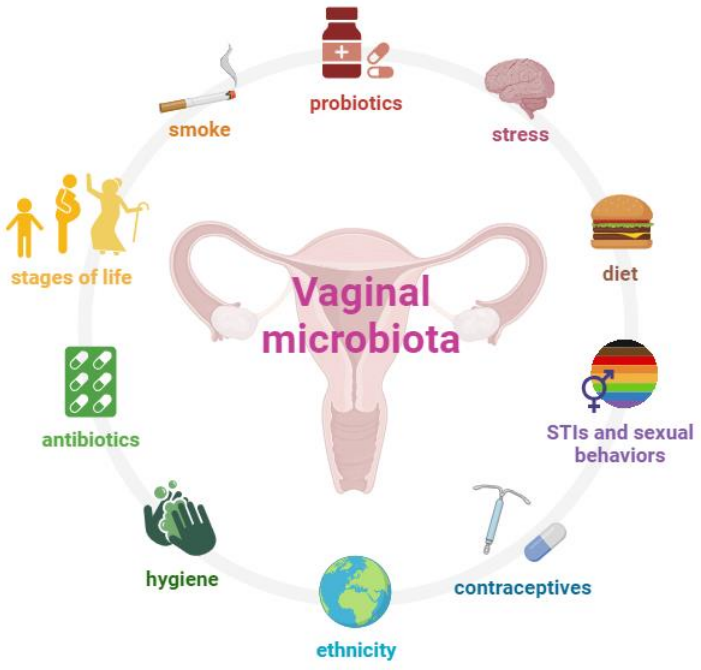

Figure 7. Determinants that influence the composition of the VMB (Figure created using Biorender.com).

3.1 Vaginal microbiota through ages

The VMB is a complex and dynamic ecosystem that constantly undergoes fluctuations during women's life (Fig. 8). The composition and abundance of VMB are influenced by physiological characteristics such as sex, hormone levels, vaginal pH and glycogen production (Verstraelen *et al.,* 2022).

It is believed that a healthy human fetus develops in a bacteria-free environment, and the method of delivery may lead to variations in the development of the microbiota. Indeed, the initial microbiota at all body sites after vaginal delivery was found to originate mainly from the maternal VMB, often dominated by lactobacilli. In the case of caesarean delivery, the lack of vaginal exposure leads to initial microbial communities resembling the human skin microbiota, with an abundance of *Staphylococcus, Corynebacterium* and *Propionibacterium* spp. (Dominguez-Bello *et al.,* 2010).

In the firsts 2-4 weeks after birth, maternal-derived estrogen induces thickening of the infant's vaginal epithelium and regulates glycogen deposition, as well as the pH decrease, promoting the growth of *Lactobacillus* spp. (Boskey *et al.,* 2001).

However, this process is temporary, and in early childhood and up to pre-puberty, maternal estrogens are no longer present and there is a thinning of the mucosa, as well as a decrease in glycogen levels. This results in a consequent reduction in glycogen-fermenting bacteria and an increase in vaginal pH, allowing the proliferation of a wide range of anaerobic Gram-negative (*Veillonella, Bacteroides, Fusobacteria*) and Gram-positive (*Actinomyces, Bifidobacteria, Peptococcus, Peptostreptococcus* and *Propionibacterium*) bacteria, resembles a BV typical bacterial composition (Randelovic *et al.,* 2012).

When the gonads mature and puberty begins, the levels of sex hormones gradually increase, leading to the thickening of the vaginal epithelium once again. As previously mentioned, this glycogen-rich environment favors the growth of glucose-fermenting microorganisms, resulting in increased acidity in the vaginal environment. Indeed, the evidence suggests that the VMB gradually transitions to an adult-like microbiota dominated by lactobacilli (Hickey *et al.,* 2015). This ecosystem is predominant until menopause, when the marked decrease in circulating estrogen changes the composition of the VMB that is replaced with a microflora like the one found prior the menarche.

Indeed, in menopausal women the VMB is dominated by *G. vaginalis* and *Candida* spp., and characterized by a lower abundance of *L. iners, Mobiluncus* spp., *Staphylococcus* spp., *Sneathia* spp., and *Bifidobacterium* spp. (Łaniewski and Herbst-Kralovetz 2022).

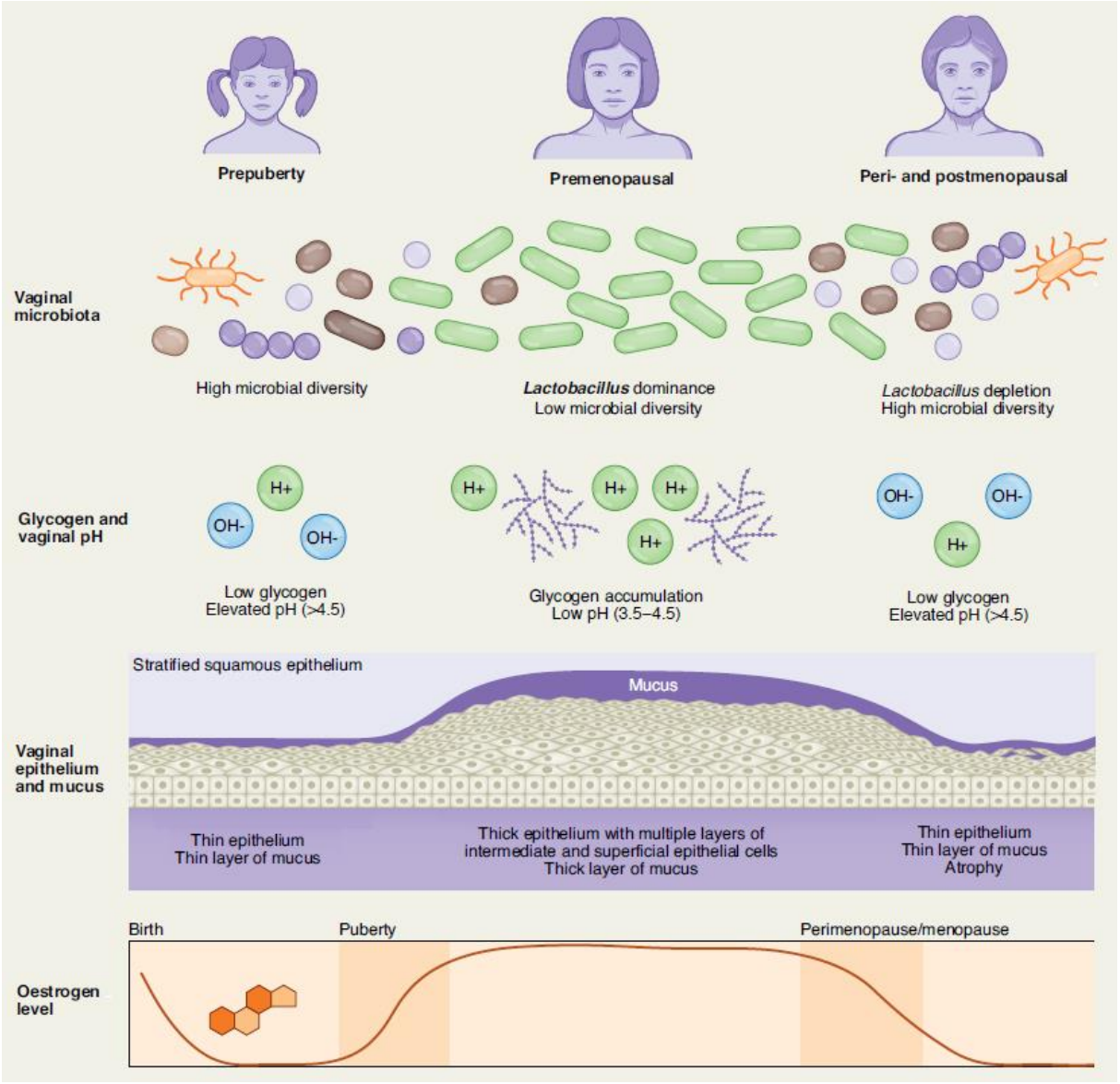

Figure 8. Changes in the vaginal environment over time life (Łaniewski and Herbst-Kralovetz, 2022).

3.1.1 Vaginal microbiota during menstrual cycle

During the menstrual cycle**,** the cyclic fluctuation of estrogen and progesterone levels affects the vaginal epithelial cell surface receptor expression, the amount and viscosity of cervical mucus, the amount of vaginal transudate, the glycogen level, vaginal oxygen and carbon dioxide tension, the reduction-oxidation potential, the pH, and the vaginal innate immune response (Holdcroft *et al.,* 2023). Consequently, also the VMB is subject to cyclical changes during the menstrual cycle (Fig. 9). Although the total number of microorganisms does not change during the menstrual cycle, the composition of the vaginal flora changes significantly during the days of menstruation (Johnson *et al.,* 1985).

The luteal phase represents the peak of sex hormones in the female body. In particular, this phase is characterized by the thickening of the endometrium and vaginal epithelial cells, as well as the accumulation of glycogen, in preparation for prospective embryo implantation. If fertilization does not occur, sex hormone levels decrease rapidly, resulting in the detachment of the endometrium from the uterine wall and the initiation of menstruation.

During menses, the composition of the VMB resembles that one before adolescence, and BV prevalence increases (Gajer *et al.,* 2012). During menstruation, the relative abundance of *G. vaginalis* and *L. iners* increased, resulting in a general rise in α-diversity and a decrease in the abundance of *Lactobacillus* spp. Additionally, there is an elevation in vaginal pH, which facilitates the growth of anaerobic microorganisms like *Clostridium*, *Aspergillus*, and *Actinobacteria* (Eschenbach *et al.,* 2000). With the transition from the menstrual phase to the follicular phase, sex hormones gradually start to increase, and the epithelial cells of the vaginal wall thicken and secrete more glycogen, which is broken down to produce lactic acid and hydrogen peroxide. This process lowers the vaginal pH and promotes the growth of *Lactobacillus* spp., while reducing the abundance and diversity of other anaerobic bacteria (Eschenbach *et al.,* 2000).

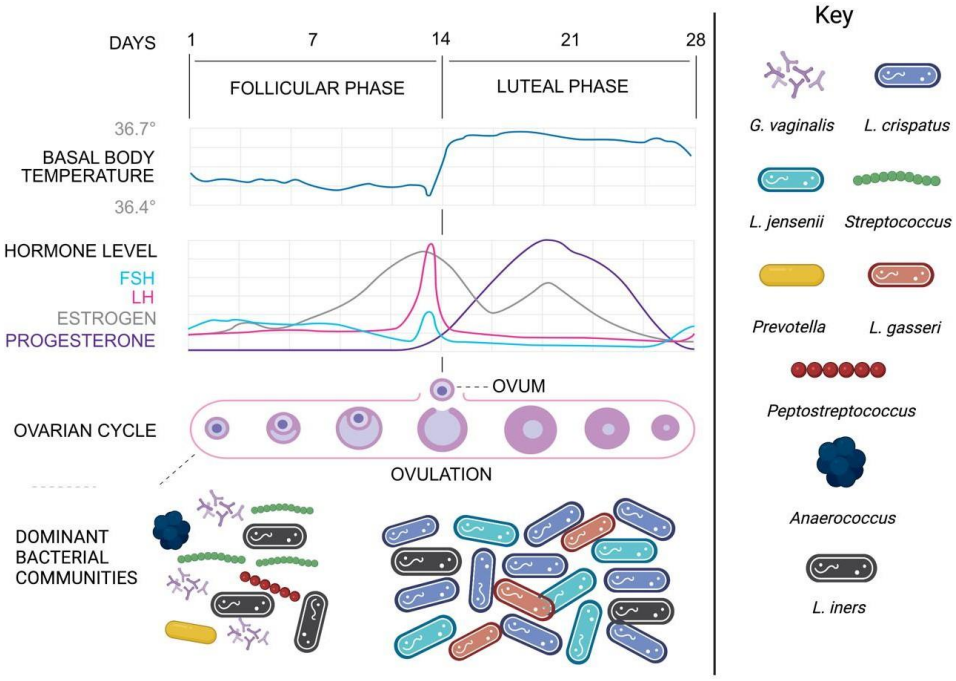

Figure 9. Bacterial dynamics of major bacterial genera and species throughout the menstrual cycle (Holdcroft *et al.,* 2023).

3.2 Vaginal microbiota during pregnancy

Pregnancy is a time of significant metabolic, immunological, and endocrine changes for women's body, which also affect the composition of the VMB.

During pregnancy, the VMB is predominantly populated by lactobacilli and is more stable than in a non-pregnant state. Serrano and colleagues showed that VMB transitions during pregnancy follow predictable patterns, and that pregnant women have a significantly higher prevalence of the four most common *Lactobacillus* spp. (*L. crispatus, L. iners, L. gasseri* and *L. jensenii*) and a proportionally lower prevalence of other taxa (Serrano *et al.,* 2019). About stability, it was attributed to the high levels of estrogen and increased vaginal glycogen deposition. Some believe that the absence of menstruation and changes in sexual habits during this period lead to a more stable vaginal flora (Gajer *et al.,* 2012).

However, hormonal changes during pregnancy change the vaginal bacterial balance. In general, as gestational age advances, estrogen levels increase, resulting in greater microbial diversity during the first trimester of pregnancy, followed by a decrease during the second and third trimesters. After delivery, the microbial diversity usually increases again due to the decline in estrogen production. Conversely, the levels of lactobacilli increase from the first to the third trimester and then decrease after delivery (Fig. 10). These changes occur independently of the mode of delivery, ethnicity, or microbiota composition during pregnancy and can persist for up to one year after delivery (Di Giulio *et al.,* 2015).

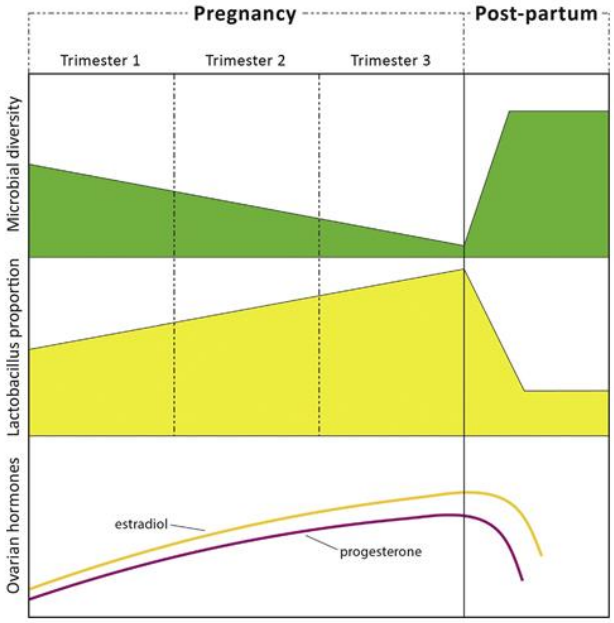

Figure 10. Hormonal changes and corresponding changes in VMB composition during pregnancy (Kroon *et al.,* 2018).

A longitudinal study conducted by Romero and colleagues was the first to compare the VMB of pregnant women (who delivered at term) with non-pregnant healthy women. The study has found that most pregnant women were classified into CST I and Ill, while non-pregnant women were more likely to have CST III or CST IV-B microbiota. It was observed that the VMB of women were dynamic and could transition between CSTs, with non-pregnant women being more likely to persist in CST IV-B compared to pregnant women (Romero *et al.,* 2014).

3.2.1 The role of vaginal microbiota in pregnancy disorders

The composition of the vaginal flora appears to play a role in the occurrence of PTB, which is defined as birth before 37 weeks of gestation and is the leading cause of neonatal mortality and morbidity. In the presence of dysbiotic microbiota, the VMB increases the levels of vaginal inflammatory cytokines, thereby increasing the risk of spontaneous PTB. In this context, BV has been associated with a 40% increased risk of PTB and it occurs in approximately 16% of the untreated female population (Di Simone *et al.,* 2020).

Petricevic and colleagues have examined the diversity of *Lactobacillus* spp. in a subset of women delivering at term versus preterm and found that *L. iners* alone was detected in 85% of PTBs, but only 16% of term births (Petricevic *et al.,* 2014a). In fact, CST-III promotes the development of dysbiosis and is prevalent in older pregnant women and African-American women On the other hand, CST-I, dominated by *L. crispatus*, promotes the maintenance of a stable and normal microbiota, protects against PTB, and is commonly found in early pregnancies (Walther-António *et al.,* 2014). Additionally, it was recently reported that the abundance of *L. iners* is associated with an increased infertility rate (Campisciano *et al.,* 2020). Another study by Ansari and colleagues have reinforced this concept, showing that *L. crispatus* dominance seems to characterize full-term pregnancies, while the prevalence of *L. iners* in the second trimester increases the risk of early spontaneous PTB (Ansari *et al.,* 2021). Research conducted by Tabatabaei and colleagues also showed that, during the first trimester, a VMB composed of *L. gasseri, L. jenseni, L. crispatus, L. acidophilus, L. iners, Ralstonia solanacearum, Bifidobacterium longum,* and *Bifidobacterium breve* might represent a lower risk of early spontaneous PTB compared to *G. vaginalis, A. vaginae,* and *Veillonellaceae bacterium* (Tabatabaei *et al.,* 2019).

However, it has not been possible to identify a specific microbial community composition that allows for the a priori identification of PTBs, as genetic and ethnic factors also influence the interaction between the microbiota and the host, leading to different results from the same

microbial community. For example, the association between a VMB with high bacterial diversity, low presence of lactobacilli, and PTB is valid for Caucasian women, but not for African women. Interestingly, some studies suggested that that *L. crispatus* is a protective factor against PTB in all ethnicities, independently from the total VMB composition (Freitas *et al.,* 2018).

PTB may also be influenced by exogenous or endogenous ascending genital infections. Common bacterial species identified in PTB-associated infections include *Ureaplasma urealyticum, Mycoplasma hominis, Bacteroides* spp., *G. vaginalis,* and *Fusobacterium nucleatum* (Mysorekar and Cao, 2014). For example, *F. nucleatum*, a usually nonpathogenic oral anaerobe, has been suggested to spread hematogenously to the placenta and alter vascular endothelium permeability, potentially allowing for the colonization of other potentially pathogenic organisms, such as *E. coli* (Fardini *et al.,* 2011).

Considering the other vaginal dysbiosis, also AV, mainly caused by *Klebsiella, E. coli, Enterococci, Staphylococcus* spp., and group B *Streptococci,* has been related to spontaneous PTB (Ma *et al.,* 2022).

Moreover, several studies have investigated the association between vaginal bacterial composition and miscarriages. First trimester miscarriage seems to be associated with reduced prevalence of *Lactobacillus* spp. and higher α-diversity compared to healthy pregnancies, and it has been shown that the vaginal levels of IL-2 was higher and IL-10 lower in women with embryonic miscarriages compared to controls (Xu *et al.,* 2020). One of the causes of this phenomenon is ascending infections of the genital tract, which are facilitated by an altered vaginal flora. Most intra-amniotic infections appear to be caused by microorganisms entering the amniotic fluid via the lower genital tract in ascending infections. Consequently, these infections disrupt the immune tolerance mechanism established by the mother to support fetal development, leading to the production of pro-inflammatory cytokines, activation of the adaptive immune response, and alteration of the function of cells at the maternal-fetal interface. Ultimately, this cascade of events can result in spontaneous abortion (Makrigiannakis and Gravanis, 2008).

This correlation has been observed not only in isolated cases but also in recurrent miscarriages (RM). In women with RM, a significant increase in *Atopobium, Prevotella* and G. *vaginalis*, as well as an increasing α-diversity and a lower abundance of lactobacilli, was described (Giannella *et al.,* 2023).

In women suffering to gestational diabetes (GD), which represents the most common metabolic disorder in pregnancy, the VMB evaluation reveled a dysbiotic condition, together with an

increase of inflammatory cytokines (Taddei *et al.,* 2018). Cortez and colleagues found a significantly higher abundance of *Bacteroides, Veillonella, Klebsiella, Escherichia, Shigella, Enterococcus,* and *Enterobacter* in GD patients, while the healthy control group presented significantly higher levels of *Varibaculum, Prevotella, Porphyromonas,* and *Ezakiella* (Cortez *et al.,* 2019).

Despite numerous studies, our understanding of the VMB, its function, and its impact during pregnancy remains incomplete. There are conflicting opinions regarding its variation based on gestational age, as some authors have reported no significant changes and propose a perspective of greater stability in the composition of the vaginal ecosystem. This contrasts with claims about changes in the microbial community related to estrogen production during pregnancy (Di Giulio *et al.,* 2015; Kroon *et al.,* 2018). Furthermore, the mechanisms underlying the involvement of the VMB in pregnancy outcomes still require elucidation to identify parameters for predicting potential adverse outcomes at an early stage.

3.3 Ethnicity

Several epidemiology studies have reported that VMB composition varies depending on the ethnicity. Ravel and colleagues had investigated differences between CSTs distribution based on ethnicities in White, Black, Hispanic, and Asian women. Notably, Asian and White women were more likely to have *Lactobacillus*-dominant vaginal bacterial communities such as CST I, II, III, and V than Black or Hispanic women. Additionally, CST IV, which is dominated by anaerobes, was overrepresented in Black and Hispanic women (Ravel *et al.,* 2011) (Fig. 11).

According to these results, Fettweis and colleagues discovered that the *Lactobacillus*-depleted CST-IV profile was four times more common in Black women than Caucasian women. This study also suggested that African-American ethnicity was associated with a VMB that more closely resembles BV, characterized by an increase in species diversity and a decrease in lactobacilli. In addition, Caucasian and Asian women tend to have high levels of *L. crispatus* and lower levels of *L. iners* compared to African women (Fettweis *et al.,* 2014).

These findings were also consistent with Zhou *et al.,* who reported an incidence of vaginal communities not-*Lactobacillus*-dominated higher in Black women (33%) compared to Caucasian women (7%) (Zhou *et al.,* 2007).

Interestingly, has been shownthat in Sub-Saharan populations, *Lactobacillus* dominance is less than 40% of healthy women, further indicating the controversy around whether *Lactobacillus* dominance should be considered “normal” in all populations.

Nevertheless, it remains possible that VMB configurations, which are associated with elevated baseline genital inflammation, may be advantageous in specific contexts, contributing to the high prevalence of diverse VMB in certain populations (Verstraelen *et al.,* 2022).

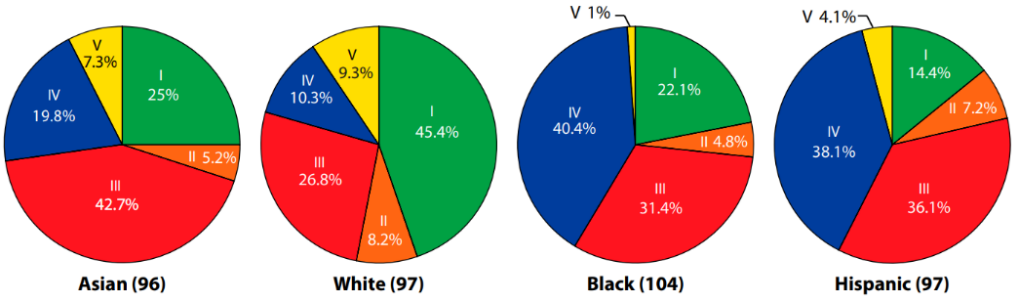

Figure 11. Frequency of the different CSTs in the different ethnic groups (Ravel *et al.,* 2011).

Given that ethnicity is a social construct, the factors that drive VMB differences are multifaceted and it has been hypothesized that socioeconomic, cultural and/or behavioral factors, as well as inequalities in healthcare, could also be responsible of these differences (Ravel *et al.,* 2011). In this context, in an interesting recent work, Dixon and colleagues have evaluated the effects of individual socioeconomic status on VMB composition. Factors such as higher maternal education, private insurance, and private hospital were associated with higher prevalence of *Lactobacillus*-dominant typically associated with better health outcomes (Dixon *et al.,* 2023).

Additionally, variations among pregnant women of different ethnicities have also been documented. Serrano and colleagues studied changes in VMB in African, Hispanic, and European pregnant women. The study revealed minimal differences in the VMB profiles of pregnant and non-pregnant women of European ancestry, while women of African and Hispanic ancestry showed a higher prevalence of *Lactobacillus* spp. dominance compared to their non-pregnant counterparts. Regarding African women, the prevalence of VMB dominated by *Lactobacillus*, specifically by *L. iners*, was lower in the first trimester compared to the subsequent ones. In the first trimester, the α-diversity in African women was greater than others and tended to decrease as through pregnancy. In European women, a higher prevalence of *Lactobacillus*-dominated VMB during pregnancy was found, and the microbial composition did not show significant changes over the trimesters (Fig. 12) (Serrano *et al.,* 2019).

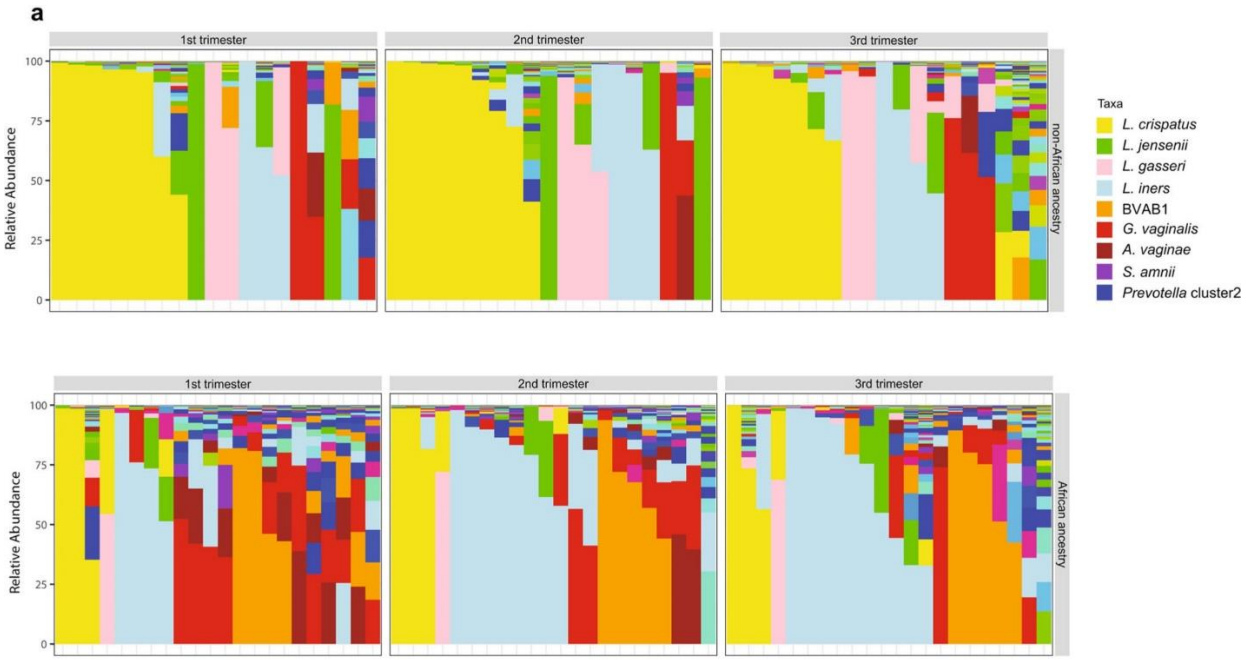

Figure 12. Longitudinal changes in VMB profiles across trimesters during pregnancy. Samples collected by non-African (n=19) or African (n=22) ancestry pregnant women who provided at least 1 sample from each of 3 trimesters (Serrano *et al.,* 2019).

3.4 Diet and BMI

While the correlation between food intake and an individual's health status is widely acknowledged, less is known about how nutrition may impact vaginal homeostasis (Barrientos-Durán *et al.,* 2020).

In the context of the VMB, research have shown that an insufficient intake of micronutrients may increase the risk of BV in both pregnant and non-pregnant women (Tohill *et al.,* 2007; Neggers *et al.,* 2007; Verstraelen *et al.,* 2005).

In addition, high energy and total fat consumption has been associated with a high risk of BV (Thoma *et al.,* 2011). The mechanisms are not completely clear but has been hypothesized that the correlation between fat consumption and BV depends on both the elevation of vaginal pH and their stimulating effect on the mucosal immune system (Tohill *et al.,* 2007).

Moura and colleagues, in a study on 516 brazilian women, reported that women with VMB dominated by *L. iners* who consume milk and/or dairy presented increased abundances of *L. crispatus*. Therefore, they could benefit from *L. crispatus* protective properties conferring greater temporal microbiota stability and, consequently, increased protection against infections (Moura *et al.,* 2023). Similarly, Rosen and colleagues associated the higher consumption of dairy with increased likelihood of membership in the *L. crispatus* group compared to the *L. iners* group in a dose-dependent manner (Rosen *et al.,* 2022).

Among dietary assessment methods used in epidemiological studies, food frequency questionnaires (FFQ) are widely used, due to their lower costs and demanding for the interviewee. FFQ are self-administered questionnaires, designed to gather information about food frequency over a specific period (in years, months, or weeks). For certain foods, portion sizes are estimated based on photographs of dishes of various sizes or by using standard portions like "cups" or "glasses," always accompanied by photographic representations. A list of 100-150 foods is presented, categorized (*e.g.,* meat, fish, pasta, soups) but without delving into the characteristics of the consumed foods or the food combinations in meals. FFQs should be developed specifically for each study group and research purposes because diet may be influenced by ethnicity, culture, an individual's preference, economic status, etc. (Kroke *et al.,* 1999).

Moreover, only a few studies have explored the relationship between pre-pregnancy BMI (body mass index) and BV prevalence, and a consensus on whether BMI is a risk factor for BV has not been reached (Oh *et al.,* 2015; Si *et al.,* 2017; Brookheart *et al.,* 2019).

3.5 Smoke

Cigarette smoking has been strongly associated with an increased prevalence of BV in several epidemiologic studies. In what was the first study evaluating differences in the VMB between smokers and non-smokers, has been suggested that women who smoke cigarettes were significantly more likely to have a VMB characterized by low proportions of lactobacilli. They also hypothesized that smoking cessation could benefit some women struggling with recurrent BV (Brotman *et al.,* 2014). Moreover, Westhoff and colleagues have investigated the antiestrogenic effect of smoking. Interestingly, women who smoke had significantly lower levels of mid-cycle and luteal phase estradiol compared to nonsmokers, which is a predisposing factor for BV (Westhoff *et al.,* 1996).

Several compounds from cigarette smoking are detectable in the cervical mucus of smokers. For example, nicotine metabolites were found significantly higher in the vaginal metabolomes of smokers.

Smokers with CST-IV microbiota had significantly higher levels of biogenic amines, known to impact the virulence of infective pathogens and contribute to vaginal malodor, suggesting that smoking may precipitate increased malodor and predispose women to vaginal infections (Nelson *et al.,* 2018).

Of particular interest, another study has analyzed the VMB of pregnant women for the presence of three target organisms (*Ureaplasma, Mycoplasma,* and *Candida* spp.) previously associated with PTB and found that smoking significantly increased the odds of detecting all three (Payne *et al.,* 2016). A similar phenomenon has been reported previously in relation to smoking and vaginal microbiology in the case of HPV infection.

3.6 Hygiene practices

While the vagina is a self-cleansing organ, in some women natural discharges can be unpleasant, leading to the use of feminine hygiene products and practices to eliminate discharge and odor from their genital area (Holdcroft *et al.,* 2023). The term "hygiene practices" encompasses a wide range of practices used to cleanse in and around the female genital area, which can vary from country to country based on the social and cultural norms of different ethnic groups.

One of the most well-studied vaginal hygiene practices is vaginal douching, which involves the introduction of water and/or cleansing products into the vagina. Vaginal douching is a very common practice for US women, while African women are more likely to use a hand or piece of cloth to wash inside the vagina as often as daily, but using water, household remedies, or soaps (Hilber *et al.,* 2010). In contrast to the perceived benefits, it has been hypothesized that vaginal washing alters the vaginal microbial community, altering the vaginal pH, causing inflammation, and providing an opportunity for pathogenic bacteria to invade and colonize the area. In fact, washings have been associated with increased risks of BV, HIV acquisition and PTB (Hilber *et al.,* 2010).

3.7 Probiotics

During years, the clinical applications of probiotics have included multiple targets, starting from body infections (*i.e.,* gastrointestinal, urogenital, respiratory infections) to stress-related disorders (Jarde *et al.,* 2018). Vaginal probiotics are somministrated to improve the VMB of healthy women, restore the abnormal composition, prevent recurrent urogenital infections, and as adjunct treatment to BV and recurrent-VVC (Borges *et al.,* 2014).

The microbial genera more frequently used as vaginal probiotics belong to the group of LAB and related microorganisms, which use to re-establish a physiological microbial flora was already known in early 1900s. The commonest probiotic used are *Lactobacillus* (*i.e.,* L.

rhamnosus, L. acidophilus, L. casei, L. plantarum, L. reuteri, L. delbrueckii), which oral intake has been shown to reduce pathogenic bacteria in the VMB (Borges *et al.,* 2014).

Vaginal probiotcs could be assumed in oral or local way. It was shown that oral intake of *Lactobacillus* strains improves the microbial pattern in vaginal dysbiosis (De Vrese *et al.,* 2018). In a similar way, Vladareanu and colleagues demonstrated that oral probiotic increased the vaginal colonization of lactobacilli via cross-contamination from the gastrointestinal tract to vagina, indicating an improvement of vaginal conditions, suggesting then the successfully prevention of VVC episodes (Vladareanu *et al.,* 2018).

This also suggested the possibility to deliver probiotics in foods and dietary supplements, although the load of lactobacilli that can be delivered this way is obviously lower than via vaginal administration and depend on viability of the strains as they pass through the stomach and gut (Borges *et al.,* 2014).

Nevertheless, probiotic effects seem to be strain specific and dose dependent, and the lack of standardized manufacturing procedures affect multiple factors such as microbial survival, their growth, and their viability (Barrientos-Durán *et al.,* 2020).

3.8 Stress

Only recently the role of stress in the female lower genital tract has been examined (Nansel *et al.,* 2006). Stress stimulates the hypothalamic-pituitary-adrenal axis, leading to elevated levels of cortisol. This, in turn, results in cortisol-induced inhibition of glycogen deposition in the vagina, which translates into an interruption in epithelial maturation that is crucial for maintaining vaginal homeostasis, leading to shifts towards low-*Lactobacillus* state (Chrousos, 1992). Indeed, stress has been significantly correlated with BV in both non-pregnant and pregnant women, suggesting that BV may be mediated by stress-related dysregulation of immune function rather than behavioral changes associated with stress (Nansel *et al.,* 2006; Culhane *et al.,* 2001).

This phenomenon is especially relevant during pregnancy, where high levels of corticotropin-releasing hormone are locally produced in the decidua, fetal membranes, and placenta. Among pregnant women, usually Black women has significantly higher rates of BV compared to White women. In this, the role of stress could be crucial, and considering that Black women are more likely to be exposed to chronic stressors at personal and community levels than White women, this could explain a significant portion of the racial disparity in BV prevalence (Culhane *et al.,* 2002).

3.9 Contraceptive methods

Nowadays, a myriad of options that allows women to prevent pregnancy exist and is possible to select the available product that suits personal situations and lifestyles. Since contraceptive methods are widely used by women worldwide, it is interesting to study the impact of these methods on the VMB. Several studies suggested that contraceptives may influence the VMB and women's immunity. In particular, some types of hormonal contraceptives seem to negatively alter the VMB, leading to a condition of BV.

The use of intramuscular medroxyprogesterone acetate (DMPA-IM) for twelve months decreased of half percent the proportion of women with a *Lactobacillus*-dominant VMB. Additionally, the use of DMPA-IM appeared to increase inflammation, resulting in elevated concentrations of IL-8, MCP-1, and IP-10 (Molatlhegi *et al.,* 2020). In the same way, the use of copper intrauterine device (IUD) has been led to an increase in BV-associated bacteria and various inflammatory cytokines and chemokines (Brown *et al.,* 2023).

Several studies have shown a "protective" effect of hormonal contraceptive pills (OCPs) against BV, and these results could be attributed to a higher glycogen accumulation stimulated by estrogen induced by OCPs. Moreover, the use of OCPs seemed to increase the prevalence of yeast colonization and the risk of VVC (Aminzadeh *et al.,* 2016).

3.10 Sexual transmitted infections and sexual behaviors

Several studies have reported a strong association between vaginal dysbiosis and STIs. Indeed, alterations in the microbiological environment of the vagina such as those that occur in dysbiosis, like BV, are considered risk factors for the acquisition of STIs (Abou Chacra *et al.,* 2022).

Women with NS of 9-10 were reported at the highest risk, whereas women with NS of 4-8 were at moderate risk of any bacterial STIs (*N. gonorrhoeae, C. trachomatis,* and *T. vaginalis*) (Allsworth and Peipert, 2011).

Compared with women with normal vaginal microbiota, women with BV were 3.5 and 4 fold more likely to detect positive for *N. gonorrhoeae* and *C. trachomatis*, respectively (Wiesenfeld *et al.,* 2003). CST IV-BV is a risk factor for persistent HPV, and the biomarkers of persistent HPV are *Atopobium* spp. and the sialidase gene of *G. vaginalis* (Di Paola *et al.,* 2017). Additionally, BV was linked to a greater risk of HIV and HPV infection (Allsworth and Peipert, 2011).

It is interesting to underline that to being a risk factor for increased acquisition of STIs, however, BV could also be the consequence of prevalent STIs. Indeed, it was suggested that it is likely that other established risk factors for STIs also increase the risk of BV, even if it is unclear whether the mechanism involves the transmission of specific microorganisms. Indeed, sexual behaviors commonly associated with the acquisition of STIs were also associated with the development of BV. In this context, it has been observed that sexual activity has an impact on the composition of the VMB, but changes depend on the nature of the couple and their sexual practices (Vodstrcil *et al.,* 2017).

Epidemiological studies in women who have sex with men (WSM) report that BV prevalence varies between 10-30% (Forcey *et al.,* 2015). Mitchell and colleagues have discovered that penile-vaginal sex can increase the diversity of *G. vaginalis* clades, indicating potential transmission through sexual contact (Mitchell *et al.,* 2012).

Indeed, it is known that semen contains a complex community of microbiota, dominated by *Ralstonia* spp., *Lactobacillus* spp.*, Corynebacterium* spp.*, Staphylococcus* spp.*, Prevotella* spp.*, Finegoldia* spp.*, Ureaplasma* spp.*, Clostridiales* spp., and many other bacterial taxa present during BV (Hou *et al.,* 2013). In addition, male circumcision seems to reduce the risk for BV among women, and unprotected sex with a male partner has been associated with the presence of inflammatory VMB, with a higher prevalence of bacteria associated with BV and a decrease in *Lactobacillus* spp. (Morris *et al.,* 2019; Ratten *et al.,* 2020).

Regarding the number of vaginal intercourses, a higher frequency has been observed to be correlated with a higher risk of suffering from BV. Furthermore, having multiple or new male partners is directly associated with BV. Finally, a direct association has been found between BV and immediate vaginal intimacy after passive anal intercourse (Cherpes *et al.,* 2008). Indeed, due to the proximity between the two anatomical sites, the intestine can act as an extravaginal reservoir for bacterial colonization (Barrientos-Durán *et al.,* 2020).

Although there have been fewer epidemiological studies in women who have sex with women (WSW), BV appears to be common in this population, with prevalence estimates ranging from 25% to 52%. Monogamous WSW relationships have been shown to have high concordance of NS category and to share *Lactobacillus* strains (Evans *et al.,* 2007).

Moreover, sexual contact with a partner with BV or with new partners has been associated with an increased risk of BV or a shift towards a more diverse vaginal CST dominated by *G. vaginalis*. BV has been associated with risk factors implying sexual transmission between women, including an increased number of female sexual partners and receptive oral sex (Forcey *et al.,* 2015).

3.11 Antibiotic use

Antibiotics are widely used worldwide, and medical prescriptions continue to increase. It is well known that antibiotics can shift the microbiota, and the post-antibiotic dysbiosis is generally characterized by a loss of diversity, changes in metabolic capacity, and reduced colonization resistance against invading pathogens. Some broad-spectrum antibiotics also target non-pathogenic strains, leading to an overgrowth of certain taxa and decrease in specific important strains (Chopra and Roberts, 2001). However, the effects on the microbiota have been reported for only a limited number of antibiotics, and most studies focus on changes in bacterial communities in the gut (Munita *et al.,* 2016).

In recent years, there has been significant attention regarding changes in the VMB following antibiotic therapy for vaginal dysbiosis. Currently, the standard treatments for this class of conditions are metronidazole or clindamycin, and although they are effective, BV recurrence is very common (Donders *et al.,* 2017). The main reason for this may be that antibiotics destroy endogenous lactobacilli, and the host is unable to restore it. In this context, a study based on cultures has shown that *L. crispatus* and *L. jensenii* were sensitive to clindamycin, while other lactobacilli like *L. iners* were not. Treatment with clindamycin could theoretically decrease both anaerobic bacteria associated with dysbiosis and *L. crispatus*, promoting the proliferation of the non-optimal *L. iners* species and increasing the risk of dysbiosis recurrence. Furthermore, vaginal candidiasis has been reported as a side effect of systemic antibiotic use, with incidence of 32% after use of antibiotics for non-genital infections (Melkumyan *et al.,* 2015).

Prescribing antibiotics during pregnancy represents a challenge, as mother infections need to be treated but the fetus must be protected from possible drug side effects (Mitchell *et al.,* 2011). Stokholm and colleagues have conducted a study on vaginal samples from pregnant women obtained at the 36th week of pregnancy. Women who received oral antibiotics during any trimester of pregnancy showed an increased rate of colonization by *Staphylococcus* species in the vaginal samples, and women treated with antibiotics (in particular, antibiotics for respiratory infection) in the third trimester were more often colonized by *E. coli* (Stokholm *et al.,* 2014).

Thesis experimental part

I. Pre-pregnancy diet and vaginal environment in Caucasian pregnant women: an exploratory study

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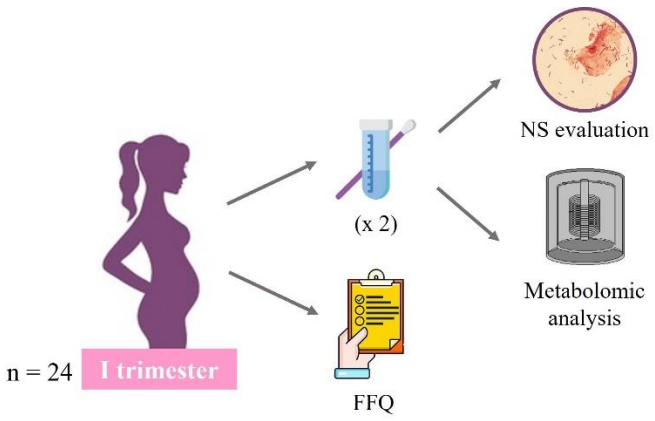

Figure 13. Graphical abstract.

1. Abstract

Vaginal microbes and their metabolic products have crucial functions, affecting local immunity development and maternal-fetal health. The vaginal microbial composition can vary in response to various factors, including BMI and diet.

This study gets new insights into the vaginal ecosystem of Caucasian women (n=24) at the first trimester of pregnancy, assessing whether pre-pregnancy diet can affect the structure of the vaginal environment in terms of bacterial composition and vaginal metabolite concentration.

The following parameters have been characterized: (i) the vaginal bacterial composition (using NS system), (ii) the vaginal metabolic profiles (using ^{1}H-NMR spectroscopy), and (iii) the dietary nutrient intake by means of a validated FFQ.

Pre-pregnancy BMI was negatively related to vaginal health status, indicating that women who begin pregnancy overweight/obese have a greater occurrence of vaginal dysbiosis during pregnancy. A lactobacilli-dominated environment was negatively associated with higher pre-pregnancy intake of animal-sourced protein (ASP). Conversely, a higher pre-pregnancy consumption of total carbohydrates and sugars seemed to be a protective factor for vaginal health.

The vaginal environment of women with BV was characterized by higher levels of biogenic amines and organic acids, whereas higher levels of phenylpropionate and diverse amino acids were fingerprints of a healthy vaginal status.

A significant association between a higher pre-pregnancy BMI and several dysbiosis-related vaginal metabolites was also found.

This study shed light on the role of pre-pregnancy BMI and diet on the vaginal environment during pregnancy, underlining the importance of limiting protein intake from animal foods to maintain a healthy lactobacilli-dominated microbiota.

2. Materials and methods

2.1 Study group and sample collection

From November 2019 to March 2021, all the Caucasian pregnant women attending the Family Advisory Health Centers of Ravenna (Italy) for prenatal care were enrolled for the study.

Exclusion criteria were the following: (i) age <18 years; (ii) HIV status; (iii) medically assisted procreation; (iv) use of any antimicrobial in the past month; (v) use of vaginal douches or topical agents in the previous two weeks; (vi) presence of uncontrolled chronic diseases (*e.g.,* diabetes, autoimmune disorders, malignancies); (vii) drug addiction or heavy smokers (>15 cigarettes/day). Moreover, women with STIs, VVC or AV were further excluded after laboratory testing.

The required sample size of the study was evaluated according to the formula proposed by Viechtbauer and colleagues for pilot studies (Viechtbauer *et al.,* 2015).

During the routine clinical visit at the first trimester of pregnancy (gestational ages 9-13 weeks), demographic, anthropometric, and clinical data were recorded from each patient.

From each woman, two vaginal swabs were collected gently rubbering for ~20 sec against the mid vaginal wall. The first one (E-swab, Copan, Brescia, Italy) was used on the same day of collection for microbiological tests. The second was collected with a sterile cotton bud, resuspended in 1 mL of sterile saline, and stored at -80°C until use. Frozen vaginal swabs were thawed, vortexed for 1 min and the liquid was centrifuged at 10000×g for 15 min. Cell-free supernatants were employed for metabolomic analysis, as described below. All subjects gave written informed consent prior to the study starting, and the protocol was approved by the Ethics Committee of Romagna (CEROM) (n°2032 of 21st February 2018).

This study was supported by "Fondazione del Monte di Bologna e Ravenna" (Prot. N°329bis/2017).

2.2 Microbiological analysis

Starting with the first collected swab, the presence of STIs (*i.e., C. trachomatis, N. gonorrhoeae, T. vaginalis,* and *M. genitalium*) was excluded by using a commercial NAAT (Seeplex STI Master Panel 1; Seegene, Seoul, KR) (Zozaya-Hinchliffe *et al.,* 2010).

VVC was excluded by the microscopic presence of fungal buds and a significant growth of *Candida* colonies by culture (Yano *et al.,* 2019). AV was diagnosed by means of a microscopic examination (*i.e.,* diminished/absent lactobacilli, presence of leukocytes, parabasal cells, small

coliform bacilli, cocci, or chains), combined with the growth of aerobic microorganisms, mainly of intestinal origin, by culture (Donders *et al.,* 2011).

The NS system was used to assess the vaginal microbial composition (Nugent *et al.,* 1991).

In particular, a Gram staining was performed as following (Smith AC and Hussey MA, 2005). The vaginal swab was smeared on a slide, and the slide was fixed by heating. Subsequently, the slide was immersed in Crystal violet stain for 1 min. Then, the slide was immersed in the mordant iodine solution (Lugol's solution) for 1 min, followed by rinsing with tap water. Next, a 70% ethanol solution was added drop by drop to slide until decolorizing agent running from the slide run clear. Finally, the slide was immersed in safranine for 1 min and then washed in a gentile and indirect stream of tap water until no color appears in the effluent and then the slide was air-dry (Fig.14). Once dried, the slide was examined under a microscope using the 1000× immersion objective.

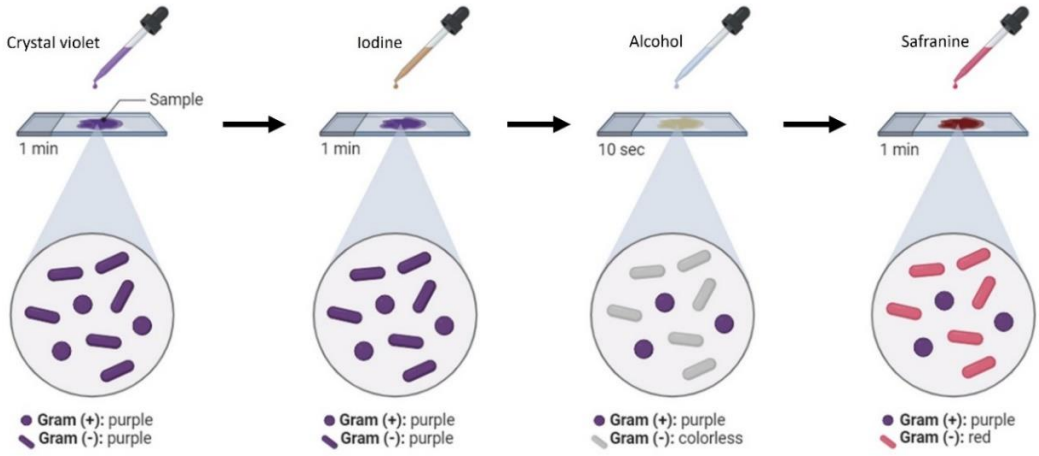

Figure 14. Gram staining procedure (Figure created using Biorender.com).

Finally, NS was calculated using the reference table (Table 3). Morphotypes were scored as the average number seen per field. Total score was calculated as = number of lactobacilli + number of *G. vaginalis* and *Bacteroides* spp. + curved rods. Based on this score, women were divided into three groups: "H" (NS 0-3; healthy lactobacilli-dominated microbiota), "I" (NS 4-6; intermediate microbiota), "BV" (NS 7-10; BV composition) (Nugent *et al.,* 1991).

Score	*Lactobacillus* morphotypes	*Gardnerella* and *Bacteroides* morphotypes	Curved Gram-variable morphotypes
0	>30	0	0
1	5-30	<1	1-5
2	1-5	1-5	>5
3	<1	6-30	-
4	0	>30	-

Table 3. NS system reference table (Nugent et al., 1991).

An example of stained slides and NS calculation is reported in Fig. 15.

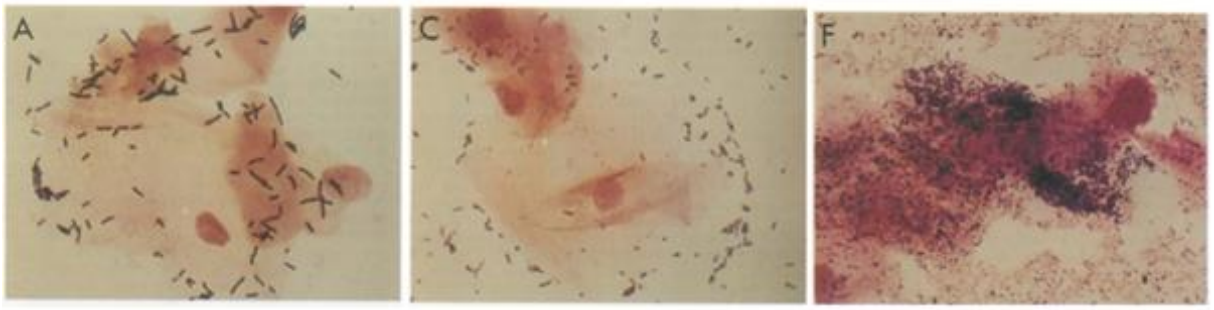

Figure 15. Images of vaginal smears from women grouped in H (A), I (C), or BV (F). [A] 4+ morphotypes of lactobacilli, absence of Gram-negative or Gram-variable cocci (score=0); [C] 3+ morphotypes of lactobacilli, 3+ Gram-variable cocci (score=4); [F] absence of lactobacilli, 4+ Gram-negative and curved cocci, presence of *Mobiluncus* spp. morphotypes on the central clue cell (score=10). (Figure modified by Nugent *et al.,* 1991).

2.3 Metabolomic analysis

Metabolomic analysis was performed by means of a ^{1}H-NMR spectroscopy starting from 700 µL of the cell-free supernatants of the vaginal swabs, added to 100 mL of a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM set to pH 7.0 (Foschi *et al.,* 2018).

^{1}H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz, equipped with Topspin software (Ver. 3.5) (Ventrella *et al.,* 2016; Foschi *et al.,* 2018). The signals originating from large molecules were suppressed by a CPMG filter of 400 spin-echo periods, generated by 180° pulses of 24 µs separated by 400 µs (Ventrella *et al.,* 2016). To each spectrum, line broadening (0.3 Hz) and phase adjustment were applied by Topspin software, while any further spectra processing, molecules quantification and data mining step were performed in R computational language (R:

A Language and Environment for Statistical Computing, R version 4.0.5) by means of scripts developed in house.

The spectra were aligned towards the TSP signal, set at −0.017 ppm in agreement with Chenomx software data bank (version 8.3, Chenomx Inc., Edmonton, Alberta, Canada). The spectra were then baseline-adjusted by means of peak detection according to the "rolling ball" principle implemented in the "baseline" R package (Liland *et al.,* 2010).

The signals were assigned by comparing their chemical shift and multiplicity with the Chenomx software database. Molecules were quantified in the first sample acquired using the added TSP as an internal standard, and all other samples were normalized to this sample using probabilistic quotient normalization (Dieterle *et al.,* 2006). Integration of the signals was carried out for each molecule using rectangular integration.

As example, portions of ^{1}H-NMR spectra representative of all the spectra obtained in this study, were reported in Fig 16.

Metabolic analyses were conducted by Professor Luca Laghi from the Department of Agri-Food Sciences and Technologies, University of Bologna, Cesena Campus.

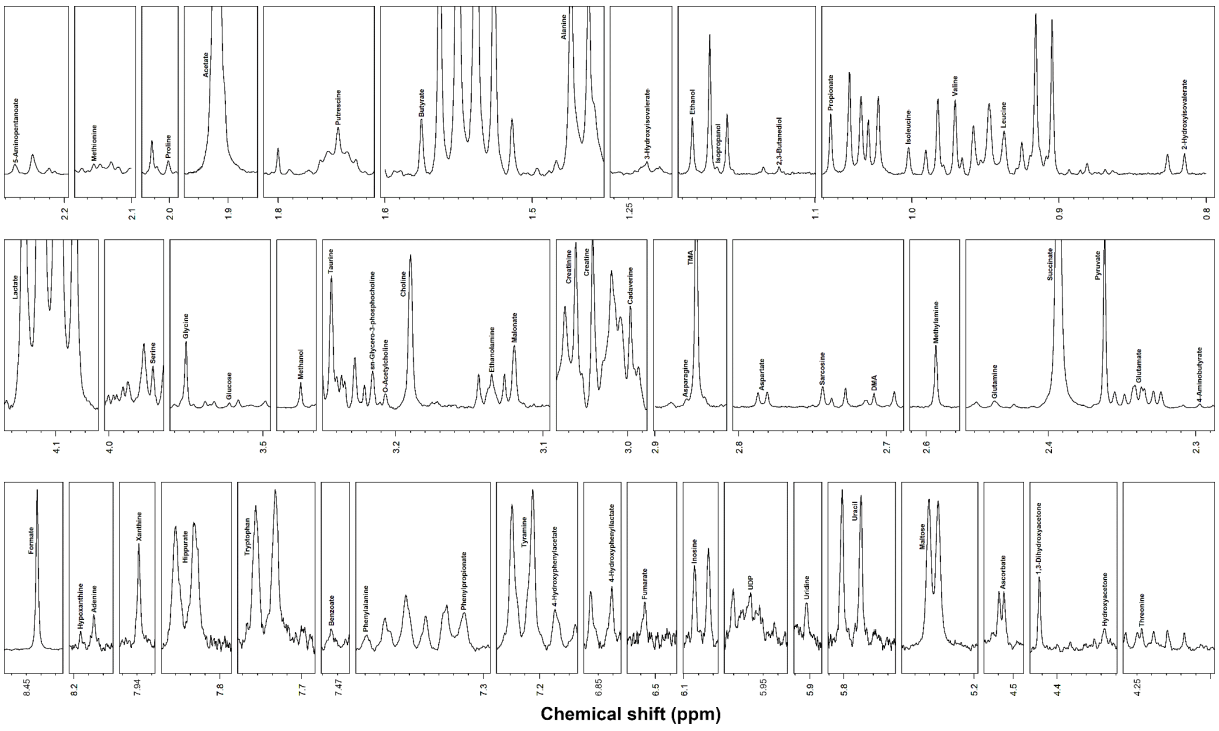

Figure 16. Portions of 1H-NMR spectra, representative of all the spectra obtained in this study. The name of each molecule appears over the signal employed to quantify it. To optimize the visualization of each portion, a different spectrum with a convenient signal-to-noise ratio was selected.

2.4 Anthropometric measurements and dietary assessment

At recruitment, enrolled women provided information about their body weight (BW) before the start of pregnancy, and their height was recorded during the first visit. BMI was calculated as weight(kg)/height(m^2) and categorized according to the World Health Organization's cut-points for underweight (18.5-24.99 kg/m^2), overweight (25-29.99 kg/m^2) or obesity (≥30 kg/m^2) (WHO, 2000).

To assess long-term nutritional habits (over a 1-year period), participants were asked to complete a FFQ developed in the European prospective investigation into cancer and nutrition (EPIC) study with the contribution of the Italian Association for Cancer Research (AIRC) and validated in the Italian population. EPIC questionnaire can assess the frequency of food consumption and is enriched with figures to define portion sizes and indications about dietary patterns (Pala *et al.,* 2003).

The presence of mis-reporters was assessed by evaluating the ratio of reported energy intake to estimated basal metabolic rate according to the protocol developed by the European Food Safety Authority (EFSA) (Ambrus *et al.,* 2013). All subjects resulted plausible reporters. The declared dietary habits were subsequently converted into nutrients using software developed by the National Cancer Institute of Milan, which calculates adherence to the Mediterranean diet based on consumption criteria for 11 foods (Giampaoli *et al.,* 2015).

Individual intakes of nutrients were compared with current dietary reference values (DRVs) for macronutrients, minerals, and vitamins (WHO/FAO, 2003; FAO, 2010; EFSA, 2017) (Table 4). In addition, moderate alcohol drinking (one drink or less in a day) was considered as an acceptable intake (WHO/FAO, 2003).

To assess overall diet quality, the collected dietary data were used to compute the MD adherence score (MEDI-LITE) (Sofi *et al.,* 2017). The MEDI-LITE, ranging from 0 (minimal adherence) to 18 (maximal adherence), includes food and nutrient indicators of diet quality, such as nine components focusing on the consumption of whole grains, legumes, fruit, vegetables, nuts, and olive oil (positive points), dairy, red and processed meat (negative points), and alcohol (points according to the consumption).

The dietary data evaluation was conducted by Professor Margherita Dall'Asta from the Catholic University of Piacenza and Professor Francesca Danesi from the Department of Food Science and Technology University of Bologna, Cesena Campus.

Dietary variables	DRVs	Type of DRV	Source
Energy	18–29 y: 7.9 (PAL=1.4) – 9 (PAL=1.6) MJ/day 30–39 y: 7.6 (PAL=1.4) – 8.7(PAL=1.6) MJ/day	AR	EFSA, 2017
Total carbohydrates	45–60 E%	RI	EFSA, 2017
Sugars (monosaccharides and disaccharides)	<10 E%	RI	WHO/FAO, 2003
Dietary fiber	25 g/day	AI	EFSA, 2017
Protein	0.66 – 0.83 g per kg BW per day	AR – PRI	EFSA, 2017
Total fat	20–35 E%	RI	EFSA, 2017
SFA	<10 E%	RI	FAO, 2010
MUFA	15–20 E%	RI	FAO, 2010
PUFA	6–11 E%	RI	FAO, 2010
ALA	0.5 E%	AI	EFSA, 2017
LAC	4 E%	AI	EFSA, 2017
Calcium	18–24 yo: 1000 mg/day ≥25 yo: 950 mg/day	PRI	EFSA, 2017
Iron	16 mg/day	PRI	EFSA, 2017
Phosphorus	550 mg/day	AI	EFSA, 2017
Potassium	3500 mg/day	AI	EFSA, 2017
Sodium	2 g/day	Safe intake	EFSA, 2017
Zinc	LPI 600 mg/day: 9.3 mg/day	PRI	EFSA, 2017
Thiamin	0.1 mg/MJ	PRI	EFSA, 2017
Riboflavin	1.6 mg/day	PRI	EFSA, 2017
Niacin	1.6 mg NE/MJ	PRI	EFSA, 2017
Vitamin B6	1.6 mg/day	PRI	EFSA, 2017
Folate	330 µg DFE/day	PRI	EFSA, 2017
Vitamin C	95 mg/day	PRI	EFSA, 2017
Vitamin A	650 µg RE/day	PRI	EFSA, 2017
Vitamin E (as α-tocopherol)	11 mg/day	AI	EFSA, 2017
Vitamin D	15 µg/day	AI	EFSA, 2017

Table 4. Set of reference values used to assess energy and nutrient intakes of the study population. Macronutrients are reported in yellow, Minerals are reported in green, and Vitamins are reported in blue. Abbreviations: ALA, α-linolenic acid; AI, adequate intake; AR: average requirement; BW, body weight; DFE, dietary folate equivalents; E%, percentage of energy intake; LAC linoleic acid; LPI, level of phytate intake; MJ, megajoule (1 MJ=238.83 kcal); MUFA, monounsaturated fatty acids; NE, niacin equivalent; PAL, physical activity level (1.4 low active, sedentary lifestyle; 1.6 moderately active lifestyle); PRI, population reference intake; PUFA, Polyunsaturated fatty acids; RE, retinol equivalents; RI, reference intake range for macronutrients; SFA, saturated fatty acids; y, years.

2.5 Data analysis and statistics

The distribution of clinical parameters was evaluated using the D'Agostino-Pearson test. Student's t-test for normally distributed data and Mann-Whitney U test for non-normally distributed data were used to compare the dietary intakes of the study population to the reference values.

For total fat and total carbohydrates, RI is a range given as a percentage of total energy intake and the 50th percentile of the RIs was used to compare with each individual intake. $\chi 2$ test was used to test for differences in BMI and MEDI-LITE score between the age groups 20-29 and 30-39. To find correlations between the vaginal microbiota composition and nutrient intake, NS was related to anthropometric/dietary data. Correlations were searched by calculating Spearman correlation coefficient (r) after correction for multiple comparisons (*i.e.,* Bonferroni-Holm correction). A p-value<0.05 was considered statistically significant.

Metabolomic data were analysed with R computational language (ver. 4.0.5). Vaginal metabolite concentrations were correlated to clinical (*i.e.,* vaginal health), anthropometric (*i.e.,* BMI), and dietary data (*i.e.,* energy and nutrient intake, MEDI-LITE score). Trends encompassing the overall metabolome were highlighted with Principal component analysis (PCoA) models. To reduce influences of potential outliers, this was done by employing its robust version (rPCoA) according to Hubert and colleagues (Hubert *et al.,* 2005). Correlation between each molecule's importance over principal components and its concentration were assessed according to Pearson.

3. Results

3.1 Study population

A total of 24 Caucasian pregnant women with a mean age of 30.8±4.9 years (min-max: 21-39) were enrolled for the study. Most women showed a NS ranging between 0 and 3 (18/24; 75%), indicating a normal lactobacilli-dominated flora. The remaining subjects were characterized by a NS 4-6 (2/24; 8.3%) or ≥7 (4/24; 16.7%), indicating a progressive shift towards a condition of dysbiosis (Fig. 17).

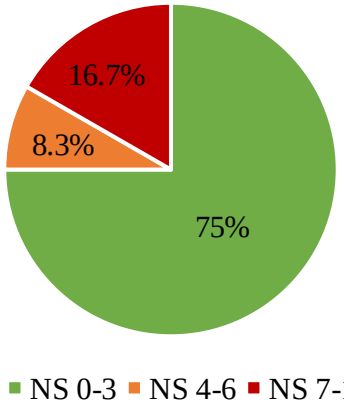

Figure 17. Distribution of participating women based on their assigned NS.

3.2 Anthropometric and nutritional data

Anthropometric characteristics of the subjects are presented in Table 5. No differences in BMI distribution were observed between the age groups (p=0.17), and the overall trend in BMI in this study was similar when compared to the national distribution in 2019 (Statista - The Statistics Portal, 2019).

Age	Median BMI (IQR) [kg/m^2]	Underweight (n)	Normal weight (n)	Overweight (n)	Obese (n)
20-29 y (n=11)	22.04 (21.15-22.40)	1	9	1	0
30-39 y (n=13)	22.76 (21.64-26.84)	0	8	5	0

Table 5. Prevalence of underweight, normal weight, overweight, and obesity in the study population based on self-reported pre-pregnancy body weight and height measurements.

Daily intake of energy, nutrients, and alcohol are presented in Table 6, while Table 7 reported the distribution of intake adequacy of each dietary variable.

Overall, the trend resulted was similar to other studies previously reported in the literature (Elmadfa and Freisling, 2009).

An inadequate intake of energy from total carbohydrates and an excessive intake of protein was observed in the study population. A high intake of energy from total fat, SFA, and sugars was evidenced in most of the women. The intake of energy from PUFA and AL was lower than the DRVs. Almost all the subjects did not reach the recommended goal of 25 g/day of dietary fiber. The prevalence of inadequacies was generally high for vitamins and minerals. Specifically, most of the study population did not meet the daily requirement for calcium, iron, potassium, riboflavin, folate, and vitamin D. Differently, more than two-thirds of the study population presented an adequate intake of vitamin B6, vitamin C, and vitamin A. Alcohol consumption was below the maximum intake level (one drink a day) for almost all the subjects.

Dietary variables	DRVs	Value in the study population*	p-value**
Energy	18–29 y: 7.9 (PAL=1.4) - 9 (PAL=1.6) MJ/day 30–39 y: 7.6 (PAL=1.4) - 8.7(PAL=1.6) MJ/day	8.05 (6.73-9.72) MJ/day	0.69
Total carbohydrates	45–60 E%	47.14 (41.18-51.82) E%	**<0.001**
Sugars	<10 E%	21.00 (16.93-23.04) E%	**<0.001**
Dietary fiber	25 g/day	17.98 (13.79-22.16) g/day	**<0.001**
Protein	0.66-0.83 g per kg BW per day	75.04 (68.94-83.31) g/day	**<0.001**
Total fat	20-35 E%	37.75 (35.12-40.47) E%	**<0.001**
SFA	<10 E%	11.53 (10.34-12.35) E%	**<0.001**
MUFA	15-20 E%	17.86 (15.53-18.75) E%	0.74
PUFA	6-11 E%	5.37 (4.46-6.17) E%	**<0.0001**
ALA	0.5 E%	0.45 (0.40-0.52) E%	**0.0469**
LAC	4 E%	4.10 (3.53-5.04) E%	0.2864
Calcium	18–24 y: 1000 mg/day; ≥25 y: 950 mg/day	710.25 (575.26-832.43) mg/day	**0.0002**
Iron	16 mg/day	10.21 (8.52-11.35) mg/day	**<0.0001**
Phosphorus	550 mg/day	1226.31 (1100.95-1346.06) mg/day	**<0.0001**
Potassium	3500 mg/day	3146.39 (2401.40-3296.50) mg/day	**<0.001**
Sodium	2 g/day	2088.30 (1894.28-2372.93) mg/day	0.36
Zinc	LPI 600 mg/day: 9.3 mg/day	9.86 (8.71-10.84) mg/day	0.4056
Thiamin	0.1 mg/MJ	0.93 (0.83-1.24) mg/day	**0.0255**
Riboflavin	1.6 mg/day	1.51 (1.34-1.65) mg/day	0.14

Dietary variables	DRVs	Value in the study population*	p-value**
Niacin	1.6 mg NE/MJ	20.39 (16.19-25.35) mg NE/day	**<0.0001**
Vitamin B6	1.6 mg/day	1.92 (1.52-2.24) mg/day	**0.0027**
Folate	330 µg DFE/day	262.18 (209.69-297.40) µg DFE/day	**<0.001**
Vitamin C	95 mg/day	113.42 (86.81-142.21) mg/day	**0.03**
Vitamin A	650 µg RE/day	794.96 (628.47-920.25) µg RE/day	**0.006**
Vitamin E	11 mg/day	12.43 (10.53-15.80) mg/day	**0.0269**
Vitamin D	15 µg/day	2.66 (2.34-3.90) µg/day	**<0.001**
Alcohol	-	3.64 (0.95-9.94) g/day	**0.0366**

Table 6. Pre-pregnancy median intakes of energy, nutrients, and alcohol in the study population and their comparison to the reference values (as reported in Table 4). Significant differences with reference values are marked in bold. * Median with interquartile range (Q1 to Q3). ** p-values from Student's t-test for normally distributed variables (energy, total carbohydrates, sugars, dietary fiber, protein, total fat, SFA, MUFA, ALA, iron, potassium, sodium, zinc, thiamin, niacin, vitamin B6, folate, vitamin C, vitamin D) and Mann-Whitney U test for non-normally distributed variables (PUFA, LA, calcium, phosphorus, riboflavin, vitamin A, vitamin E, alcohol).

Dietary variables	Adequate intake (n %)	Low intake (n %)	Excessive intake (n %)
Energy	6 (25%)	10 (42%)	8 (33%)
Total carbohydrates	17 (71%)	7 (29%)	0 (0%)
Sugars	0 (0%)	0 (0%)	24 (100%)
Dietary fiber	3 (13%)	21 (88%)	0 (0%)
Protein	3 (13%)	0 (0%)	21 (88%)
Total fat	6 (25%)	0 (0%)	18 (75%)
SFA	5 (21%)	0 (0%)	19 (79%)
MUFA	17 (71%)	5 (21%)	2 (8%)
PUFA	7 (29%)	17 (71%)	0 (0%)
ALA	8 (33%)	16 (67%)	0 (0%)
LAC	13 (54%)	11 (46%)	0 (0%)
Calcium	2 (8%)	22 (92%)	0 (0%)
Iron	0 (0%)	24 (100%)	0 (0%)
Phosphorus	24 (100%)	0 (0%)	0 (0%)
Potassium	1 (4%)	23 (96%)	0 (0%)
Sodium	10 (42%)	0 (0%)	14 (58%)
Zinc	14 (58%)	10 (42%)	0 (0%)
Thiamin	15 (63%)	9 (38%)	0 (0%)
Riboflavin	8 (33%)	16 (67%)	0 (0%)
Niacin	24 (100%)	0 (0%)	0 (0%)
Vitamin B6	16 (67%)	8 (33%)	0 (0%)
Folate	3 (13%)	21 (88%)	0 (0%)
Vitamin C	17 (71%)	7 (29%)	0 (0%)
Vitamin A	17 (71%)	7 (29%)	0 (0%)
Vitamin E	15 (63%)	9 (38%)	0 (0%)
Vitamin D	0 (0%)	24 (100%)	0 (0%)
Alcohol	19 (79%)	0 (0%)	5 (21%)

Table 7. Pre-pregnancy adequacy of dietary intake of the study population in comparison to the reference values.

The MEDI-LITE score ranged from 5 to 15, with most subjects having a moderate to high adherence to MD (Table 8). No difference in adherence distribution between the age groups (p=0.16) was observed. The mean MEDI-LITE score (10.13±2.38) resulted slightly lower than the mean value for women (12.39±2.39) reported in a previous publication specifically analysing the adherence to the MD of Italian adults, using the same tool (Sofi *et al.,* 2017).

Age	Average MEDI- LITE score (IQR)	Low adherence (n)	Moderate adherence (n)	High adherence (n)
20-29 y (n=11)	10.00 (9.50-11.50)	2	8	1
30-39 y (n=13)	10.00 (8.00-13.00)	0	9	4

Table 8. Prevalence of low, moderate, and high adherence to a Mediterranean- type diet before pregnancy in the study population.

3.3 Correlation between vaginal status and anthropometric/dietary data

Significant correlations were explored between NS and several anthropometric data and dietary indices (Table 9). A higher NS (*i.e.,* indicating a shift towards vaginal dysbiosis) was related to a higher BMI (r=0.43; p=0.034) and to a higher intake of ASP (r=0.42; p=0.039).
Conversely, a vaginal health status (*i.e.,* lower NS) was related to a higher intake of total carbohydrates (p=0.041) and sugars (p=0.046). Finally, a trend between alcohol consumption and a condition of vaginal dysbiosis was also found (p=0.055), even if not fully significant.

The Spearman correlation coefficients r for the NS according to the linear regressions with all anthropometric and dietary data are reported in Fig. 18.

Parameter	r	p-value
BMI	**0.43**	**0.034**
ASP	**0.42**	**0.039**
Total carbohydrates	**-0.42**	**0.041**
Sugars	**-0.41**	**0.046**
Alcohol	0.40	0.055
Calcium	0.38	0.068
Riboflavin	0.36	0.083
Phosphorus	0.34	0.105

Parameter	r	p-value
MUFA	0.32	0.133
Total fat	0.30	0.160
Carotene	0.28	0.188
Total protein	0.26	0.215
PUFA	-0.26	0.221
LAC	-0.26	0.226
Zinc	0.23	0.279
Vitamin D	-0.18	0.402
Potassium	0.16	0.452
Vitamin B6	0.16	0.457
Oleic acid	0.16	0.464
Cholesterol	-0.15	0.484
Folate	0.14	0.507
Niacin	0.14	0.508
Dietary fiber	0.14	0.525
Iron	0.14	0.525
Vitamin C	0.12	0.580
ALA	0.10	0.630
SFA	0.09	0.676
Vitamin A	0.08	0.693
Thiamin	0.08	0.694
Vitamin E	0.05	0.822
Sodium	0.04	0.851
Plant protein	0.04	0.861
Energy	0.02	0.937
MEDI-LITE score	-0.01	0.952

Table 9. List of the main pre-pregnancy anthropometric and dietary data for which a correlation with vaginal health status (NS) during pregnancy was investigated.Correlations were searched by calculating Spearman correlation coefficient (r) after correction for multiple comparisons (*i.e.,* Bonferroni-Holm correction). Significant differences with reference values are marked in bold.

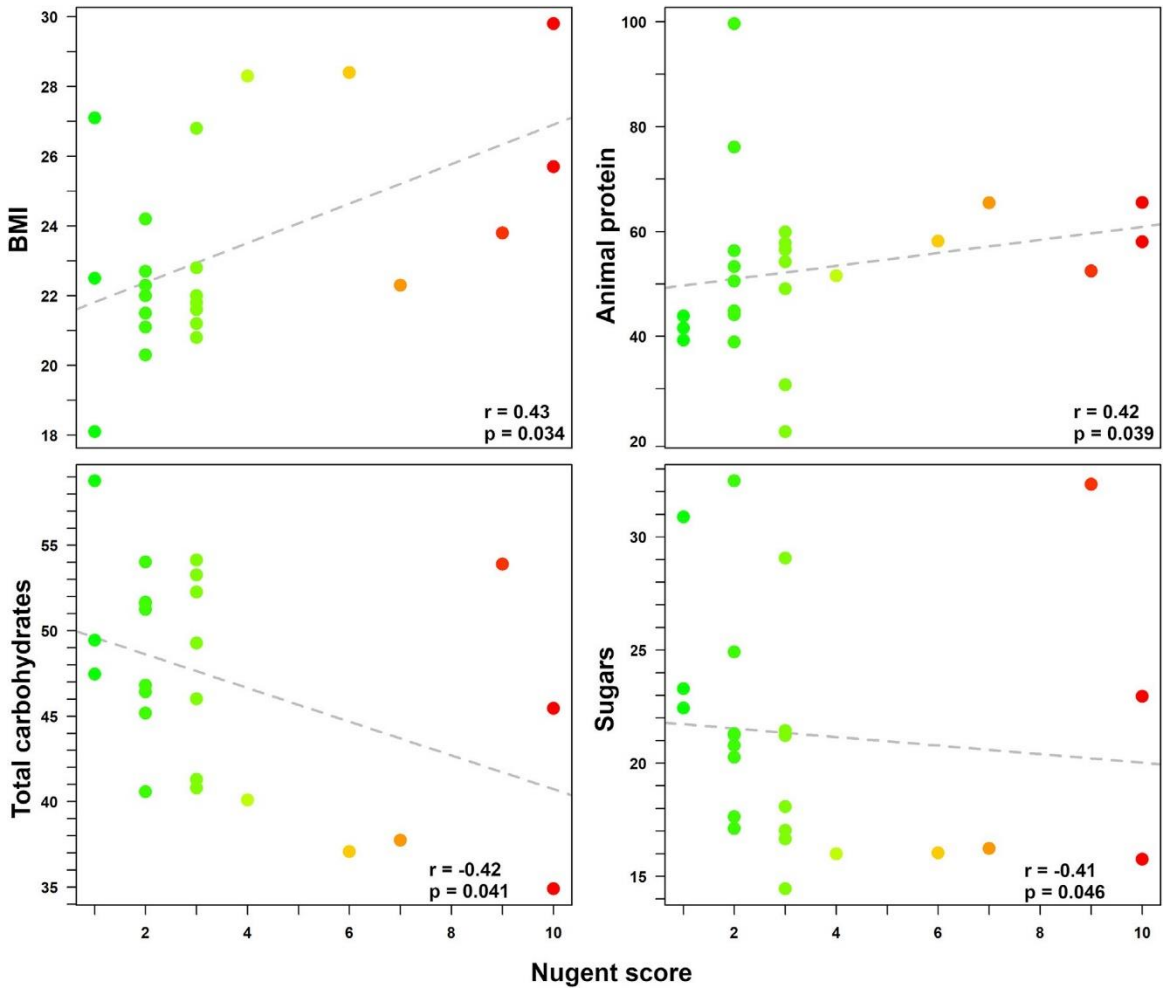

Figure 18. Correlations between NS and various pre-pregnancy anthropometric/dietary data. Statistically significant relationships between NS (0-10) and anthropometric/dietary indices (*i.e.,* BMI, intake of ASP, total carbohydrates, and sugars) are displayed in each box. For each correlation, Spearman coefficient (r) and p-value are shown. Each dot represents a woman enrolled in the study. For easier visualization, dots color goes from green to red as the NS increases.

3.4 Vaginal metabolome

A total of 63 metabolites were detected and quantified by ^{1}H-NMR spectroscopy:

1) Formate
2) Hypoxanthine
3) Adenine
4) Xanthine
5) Hippurate
6) Tryptophan
7) Benzoate
8) Phenylalanine
9) Phenylpropionate
10) Tyramine
11) 4-Hydroxyphenylacetate
12) 4-Hydroxyphenyllactate
13) Fumarate
14) Inosine
15) UDP
16) Uridine
17) Uracil
18) Maltose
19) Ascorbate
20) 1,3-Dihydroxyaceton
21) Hydroxyacetone
22) Threonine
23) Lactate
24) Serine
25) Glycine
26) Glucose
27) Methanol
28) Taurine
29) sn-Glycero-3-phosphocholine
30) O-Acetylcholine
31) Choline
32) Ethanolamine
33) Malonate
34) Creatinine
35) Creatine
36) Cadaverine
37) Asparagine
38) TMA
39) Aspartate
40) Sarcosine
41) DMA
42) Methylamine
43) Glutamine
44) Succinate
45) Pyruvate
46) Glutamate
47) 4-Aminobutyrate
48) 5-Aminopentanoate
49) Methionine
50) Proline
51) Acetate
52) Putrescine
53) Butyrate
54) Alanine
55) 3-Hydroxyisovalerate
56) Ethanol
57) Isopropanol
58) 2,3-Butanediol
59) Propionate
60) Isoleucine
61) Valine
62) Leucine
63) Hydroxyisovalerat

Fig. 19 shows the correlation between NS and the composition of the vaginal metabolome. As visualized in the correlation plot, higher levels of tryptophan, phenylpropionate, leucine, isoleucine, phenylalanine, O-acetylcholine, and sarcosine characterized the vaginal metabolome of women with a lower NS (*i.e.,* lactobacilli-dominated flora). Conversely, higher concentrations of putrescine, tyramine, methylamine, taurine, xanthine, 5-aminopentanoate, proline, creatine, UDP, formate, 2,3-butanediol, and glucose seemed to be fingerprints of women with higher NS (*i.e.,* indicating a shift towards vaginal dysbiosis).

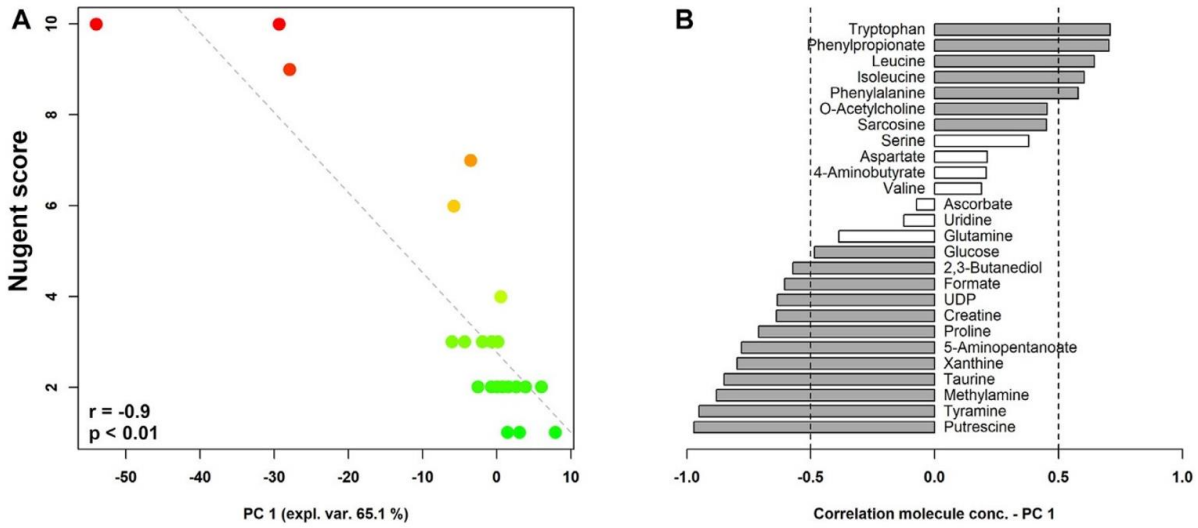

Figure 19. rPCA model built on the centered and scaled concentrations of the metabolites showing significant differences based on NS. In the scoreplot (A1), the color of the dots (each representing a woman enrolled in the study) goes from green to red as the NS increases. Y-axis shows NS values. In the barplot (A2), describing the correlation between the concentration of each molecule and its importance over PC1, dark grey bars highlight statistically significant correlations ($p<0.05$).

When pre-pregnancy BMI was correlated to vaginal metabolic profiles (Fig. 20), a significantly higher levels of methylamine, hydroxyacetone, UDP, creatine, uracil, and ascorbate was found in women who begun pregnancy overweight.

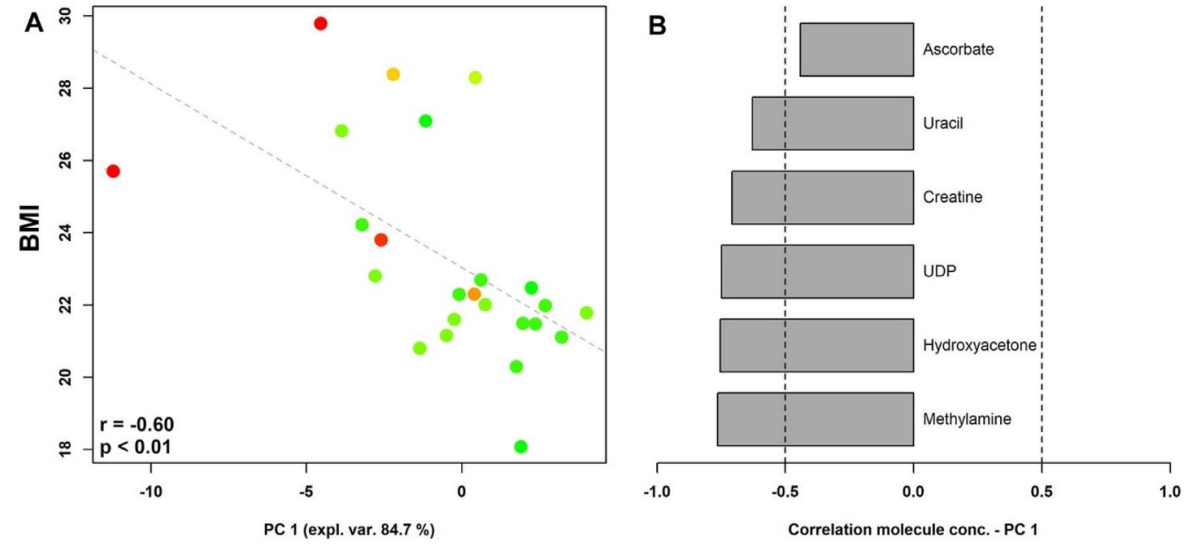

Figure 20. rPCA model showing correlations between pre-pregnancy BMI and vaginal metabolome in pregnant women stratified by the NS. In the scoreplot (B1), the color of the dots (each representing a woman enrolled in the study) goes from green to red as the NS score increases. Y-axis shows the values of BMI. In the barplot (B2), describing the correlation between the concentration of each molecule and its importance over PC1, dark grey bars highlight statistically significant correlations ($p<0.05$).

The vaginal metabolome of women with a higher sugar intake were characterized by higher levels of valine, 4-hydroxyphenylacetate, asparagine, aspartate, and threonine (Fig. 21).
One molecule constituting vaginal metabolome, namely UDP, showed a significant negative correlation with total carbohydrate intake, whereas 5-aminopentanoate concentration was related with ASP intake.

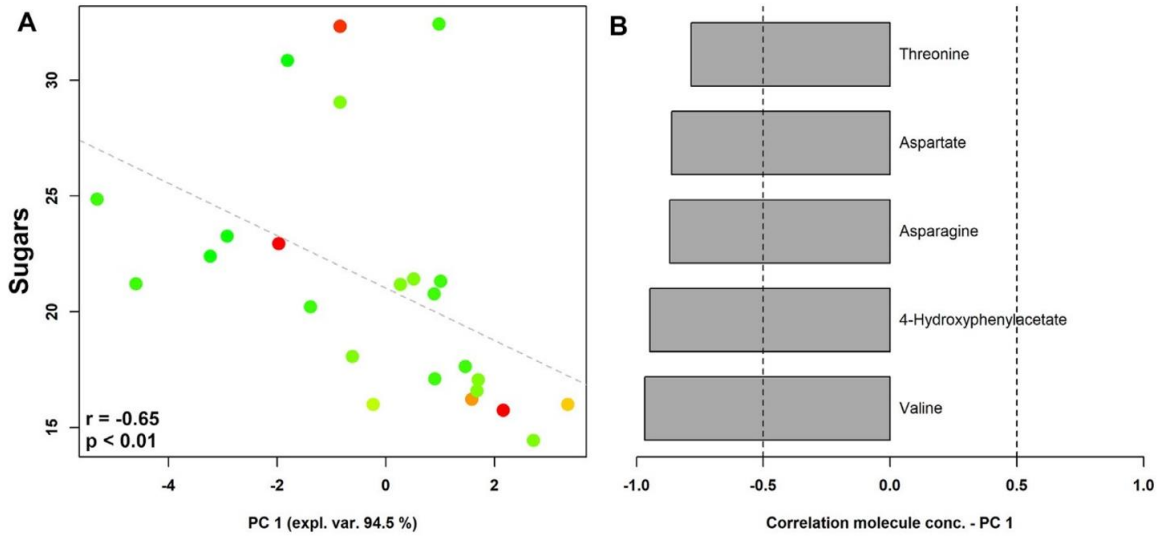

Figure 21. rPCA model showing correlations between pre-pregnancy sugar intake and vaginal metabolome in pregnant women stratified by the NS. In the scoreplot (C1), the color of the dots (each representing a woman enrolled in the study) goes from green to red as the NS increases. Y-axis shows the values of sugar intake. In the barplot (C2), describing the correlation between the concentration of each molecule and its importance over PC1, dark grey bars highlight statistically significant correlations ($p<0.05$).

4. Discussion

At today, this was the first report evaluating the impact of pre-pregnancy anthropometric and nutritional variables on the vaginal environment of Caucasian pregnant women. Pre-pregnancy BMI and daily energy and nutrient intake of 24 women at the first trimester of pregnancy were correlated to the bacterial and metabolomic composition of the vaginal ecosystem.

Since various factors can affect the microbial composition of the vagina, all the women with conditions able to perturb the vaginal microbial composition (*e.g.,* genital infections, recent use of antibiotics, heavy smoking, chronic diseases) were excluded from the study.

At first, analysis shown that that pre-pregnancy BMI was negatively related to vaginal health status, indicating that pregnant overweight/obese women have a greater occurrence of vaginal dysbiosis (*i.e.,* BV and/or reduced number of vaginal lactobacilli).

These data agree with a previous report, showing a significant association between obesity and BV in White women (Brookheart *et al.,* 2019).

However, only a few studies have explored the relationship between pre-pregnancy BMI and BV prevalence, and a consensus on whether BMI is a risk factor for vaginal dysbiosis has not been reached. Oh and colleagues reported that the vaginal microbial composition of obese fertile women is more likely to be enriched by dysbiosis-related *Lactobacillus* spp. (*i.e., L. iners*), rather than by eubiosis-associated species (*i.e., L. crispatus*) (Oh *et al.,* 2015).

Moreover, obesity significantly increases the diversity of the vaginal microbiota in association with *Prevotella*, an anaerobic microorganism typically found in BV-positive women (Si *et al.,* 2017).

The mechanisms behind the association between obesity and BV are not completely understood. Presumably, disturbances in hormonal, dietary, metabolic and/or immune functions can play a significant role. Moreover, also the gut microbiota can influence the composition of the vaginal environment, acting as an extravaginally reservoir of BV-associated bacteria (Marrazzo *et al.,* 2012).

When looking to the correlations between dietary data and the vaginal bacterial composition, this study found that an increased risk of BV during pregnancy was associated with a higher intake of ASP. Previous studies have mainly investigated the impact of dietary intake on the vaginal environment of American and Afro-American women, with dietary habits different from those of European Caucasian women (Neggers *et al.,* 2007; Tohill *et al.,* 2007; Thoma *et al.,* 2011).

Overall, the risk of BV has been associated with the increased dietary fat intake, higher glycemic loads, and lower concentrations of vitamins A, C, E, and β-carotene (Neggers *et al.,* 2007; Thoma *et al.,* 2011; Tohill *et al.,* 2007). In addition, recently, it has been shown that diets richer in fiber are associated with lower odds of BV (Shivakoti *et al.,* 2020). Nevertheless, other authors failed to find associations between vaginal microbiota profiles and specific nutrient intake, including sugar, dietary fiber, protein, or fat (Song *et al.,* 2020). Thus, it is plausible that long-term dietary habits and energy metabolism can influence the vaginal microbiome composition, but additional large-scale studies are needed to better understand the potential role of different dietary patterns and/or specific dietary components on genital health and eubiosis. The present study demonstrated for the first time that a reduced intake of ASP during the year prior to the pregnancy is crucial in maintaining a normal lactobacilli-dominated vaginal flora.

The "negative" impact of a diet rich in ASP have been previously described for the gut microbiome composition. For instance, a higher intake of plant-sourced protein is associated with greater abundance of "health-related" microorganisms in the gut (*e.g., Bifidobacterium*, *Roseburia*, *Lactobacillus*), as opposed to *Bacteroides* and *Clostridia*, found primarily in ASP (Prokopidis *et al.,* 2020).

It is well known that the vaginal bacterial composition plays a crucial role in maternal-fetal health (Nelson *et al.,* 2016). Healthy pregnancies are usually characterized by a lactobacilli-dominated environment, whereas reduced lactobacilli with increased bacterial diversity are associated with pregnancy-related complications and PTB (Di Simone *et al.,* 2020). Thus, the demonstration of an association between a pre-pregnancy excessive intake of ASP and a status of vaginal dysbiosis is of great importance, opening the way to new strategies for the prevention of negative outcomes during pregnancy.

Additionally, a high prevalence of inadequate intake of calcium, iron, potassium, riboflavin, folate, and vitamin D was shown. It is well known that these micronutrients are important for their roles as molecular regulators, energy substrates, or for the synthesis of molecules involved in the immune response and can consequently alter the control of immune response and inflammation.

In this context, it can be hypothesized that a deficiency in beneficial micronutrients involved in regulating the immune system may compromise the maintenance of a healthy vaginal microbial composition, limiting the ability to restore a eubiotic condition after transient disruptions, such as antibiotic therapy. Although the results of this study didn't show significant correlation between the deficiency of micronutrients and vaginal status, previous studies have associated the low dietary intakes of calcium, folate and vitamin D with BV (Neggers *et al.,* 2007).

Nevertheless, the vitamin D role in vaginal health remain controversial, even if is well know that vitamin D plays various roles in regulating the immune response, promoting monocyte differentiation, enhancing phagocytosis, superoxide production, and bacterial killing by natural killer (NK) cells (Klebanoff and Turner, 2014). Similarly, iron is involved in the proper functioning of the immune response; therefore, a deficiency can alter infection defense mechanisms at the level of the vaginal mucosa (Verstraelen *et al.,* 2005).

Moreover, a higher intake of total carbohydrates and sugars seemed to be associated with a condition of vaginal eubiosis (*i.e.,* lower NS, with a lactobacilli-dominated flora). It has been hypothesized that the high starch content of the human diet can lead to high levels of glycogen in the vaginal tract, creating a suitable environment for the proliferation and dominance of lactobacilli (Song *et al.,* 2020). Therefore, is possible to speculate these results go in this direction: diets including a high intake of total carbohydrates may have led to high levels of glycogen in the vaginal tract, which, in turn, might have created a favorable environment for a lactobacilli-dominated flora (*i.e.,* lower NS).

However, other studies are necessary to investigate the effect of a high carbohydrate diet on vaginal glycogen levels in humans, as well as the impact on the vaginal environment and health.

Notably, a trend between alcohol consumption and vaginal dysbiosis was also found. Evidence from animal studies suggests that very heavy alcohol consumption might contribute to BV susceptibility through direct alteration of the vaginal microbial composition (Loganantharaj *et al.,* 2014). At today, relatively few studies have examined relationships between alcohol consumption and BV. Results from existing literature are conflicting, with some studies finding no association and others finding increased BV risk (Froehle *et al.,* 2021).

The composition of the vaginal microbiome is accompanied by specific fingerprints of the vaginal metabolome (Vitali *et al.,* 2015). In the present study, it was confirmed that the vaginal environment of women with vaginal dysbiosis is characterized by higher levels of biogenic amines (*e.g.,* tyramine, methylamine, putrescine), and organic acids (*e.g.,* formate).

Conversely, higher levels of phenylpropionate, and diverse amino acids (*e.g.,* tryptophan, phenylalanine, isoleucine, leucine) were peculiar elements of a healthy vaginal status (Vitali *et al.,* 2015; Ceccarani *et al.,* 2019).

Interestingly, a significant association between a higher BMI and several dysbiosis-related vaginal metabolites (*e.g.,* methylamine) was found. Thus, this interesting interplay between BMI and vaginal metabolic profile suggests that BMI can represent a potential indicator of vaginal health.

Although the limitations (the low number of women enrolled and the need of more appropriate techniques to in-depth evaluate the composition of the vaginal microbiome (*e.g.,* 16s rRNA sequencing) and metabolome (*e.g.,* LC-MS/MS)), this study sheds light on the role of pre-pregnancy BMI and diet on the vaginal environment during pregnancy.

In particular, the study underlines the importance of limiting protein intake from animal foods to maintain a healthy lactobacilli-dominated vaginal microbiota enriched in eubiosis-related metabolites. Future studies are needed for a thorough comprehension of the mechanisms involved the impact of pre-pregnancy diet on the vaginal environment to set tailored dietary approaches for the maintenance of a healthy vaginal flora during pregnancy.

II. A deep look at the vaginal environment during pregnancy and puerperium

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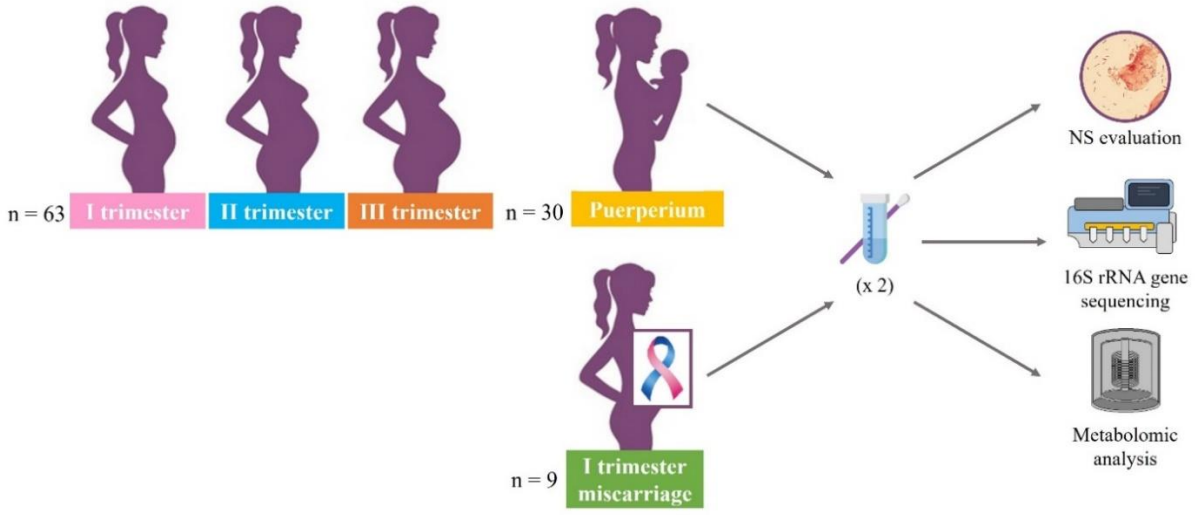

Figure 22. Graphical abstract.

1. Abstract

A deep comprehension of the vaginal ecosystem may hold promise for unravelling the pathophysiology of pregnancy and may provide novel biomarkers to identify subjects at risk of maternal-fetal complications.

In this prospective study, the characteristics of the vaginal environment in a cohort of pregnant women throughout their different gestational ages and puerperium were assessed. Both the vaginal bacterial composition and the vaginal metabolic profiles were analyzed.

A total of 63 Caucasian women with a successful pregnancy and 9 subjects who had a first trimester miscarriage were enrolled. For the study, obstetric examinations were scheduled along the three trimester phases (9-13, 20-24, 32-34 gestation weeks) and puerperium (40-55 days after delivery). Two vaginal swabs were collected at each time point, to assess the VMB profiling (by NS and 16S rRNA gene sequencing) and the vaginal metabolic composition (by ^{1}H-NMR spectroscopy).

During pregnancy, the VMB underwent marked changes, with a significant decrease in overall diversity, and increased stability. Over time, a significant increase of *Lactobacillus* and a decrease of several genera related to BV, such as *Prevotella*, *Atopobium* and *Sneathia,* was found. It is worth noting that the levels of *Bifidobacterium* spp. tended to decrease at the end of pregnancy.

At the puerperium, a significantly lower content of *Lactobacillus* and higher levels of *Gardnerella*, *Prevotella*, *Atopobium*, and *Streptococcus* were observed. Women receiving an intrapartum antibiotic prophylaxis (IAP) for GBS were characterized by a vaginal abundance of *Prevotella* compared to untreated women. Analysis of bacterial relative abundances highlighted an increased abundance of *Fusobacterium* in women suffering a first trimester abortion, at all taxonomic levels. *Lactobacillus* abundance was strongly correlated with higher levels of lactate, sarcosine, and many amino acids (*i.e.,* isoleucine, leucine, phenylalanine, valine, threonine, tryptophan).

Conversely, BV-associated genera, such as *Gardnerella*, *Atopobium*, and *Sneathia*, were related to amines (*e.g.,* putrescine, methylamine), formate, acetate, alcohols, and short-chain fatty-acids (*i.e.,* butyrate, propionate).

2. Materials and methods

2.1 Study group and sample collection

Subjects were enrolled among all the Caucasian pregnant women presenting to the Family Advisory Health Centers of Ravenna (Italy) for prenatal care starting from April 2019 to March 2021.

Exclusion criteria were the following: (i) age <18 years; (ii) HIV status; (iii) BMI>33; (iv) medically assisted procreation; (v) use of any antimicrobial in the past month; (vi) use of vaginal douches or topical agents in the previous two weeks; (vii) presence of uncontrolled chronic diseases; (viii) drug addiction or heavy smokers (>15 cigarettes/day).

Moreover, women with STIs, AV and VVC were further excluded after laboratory testing. At gestational age of 9-13 weeks (first trimester, T1), 20-24 weeks (second trimester, T2), 32-34 weeks (third trimester, T3), and puerperium (40-55 days after delivery, T4) women underwent an obstetric examination.

For all patients, demographic data and information about urogenital symptoms were recorded. Women colonized with GBS at the third trimester of pregnancy received IAP (*i.e.,* penicillin G or ampicillin), following international guidelines (Kolkman *et al.,* 2020).

Two vaginal swabs were collected at each time point. The first one (E-swab, Copan, Brescia, Italy) was used for microbiological diagnostic tests and NS assessment. The second was collected with a sterile cotton bud, re-suspended in 1 mL of sterile saline, and stored at -80°C until use. Frozen vaginal swabs were thawed, vortexed for 1 min and removed from the liquid. After centrifugation (10000×g for 15 min), the cell-free supernatants were used for metabolomic analysis, whereas bacterial pellets were employed for VMB profiling.

A written informed consent was obtained from all subjects and the study protocol was approved by CEROM (n° 2032 of 21st February 2018).

This study was supported by “Fondazione del Monte di Bologna e Ravenna” (Prot. N°329bis/2017).

2.2 Microbiological investigations

A commercial NAAT was used for *C. trachomatis, N. gonorrhoeae, T. vaginalis* and *M. genitalium* detection (Seeplex STI Master Panel 1; Seegene, Seoul, KR).

Microscopic examination and cultures were employed for the diagnosis of VVC and AV as previously reported (Paper I, pages 56-58) and positive women were excluded from the study.

NS system was used for a preliminary assessment of the vaginal flora composition and to classify women in "H" (NS≤3), "I" (NS 4-6) or "BV" (NS≥7) category (Nugent *et al.*, 1991).

2.3 Vaginal microbiota profiling

Nucleic acids were extracted from vaginal swabs by means of the Versant molecular system (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) equipped with a sample preparation module designed for automated sample preparation (Marangoni *et al.,* 2015).

The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified, according to the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). Final indexed libraries were prepared by equimolar (4 nmol/L) pooling, denaturation, and dilution to 6 pmol/L, before loading onto the MiSeq flow cell (Illumina). A 2×300 bp paired-end run was used. Raw sequencing reads were processed, generating a single fragment covering the whole amplicon from the two overlapping pairs, using PandaSeq software (v2.5), keeping 250-900 base long fragments and filtering out those having more than 25% nucleotides with a Phred score ≤3.

The sequencing was performed by Dr. Clarissa Consolandi and Dr. Tania Camboni from the Institute of Biomedical Technologies (National Research Council, Milan, Italy).

Quality filtering, taxonomy assignments, and diversity analyses of the samples were performed using the QIIME suite (release 1.9.0). Filtered reads were de-duplicated and de-noised, creating zero-radius Operational Taxonomic Units (zOTUs), using the unoise3 algorithm provided in the usearch pipeline (v. 11.0.667) and discarding those with less than 5 supporting reads.

Taxonomic assignment was performed against the SILVA 16S rRNA database (release 132, https://www.arb-silva.de/fileadmin/silva_databases/qiime/Silva_132_release.zip accessed on 25 November 2021) through the RDP classifier at 0.5 confidence (Wang, *et al.,* 2007).

Species-level classification for all genera except *Lactobacillus* was performed by using SPINGO46 with default parameters.

Characterization of *Lactobacillus* spp. was performed by BLAST-aligning all reads belonging to that genus to a custom reference database made up collecting all available reference

sequences in NIH-NCBI database (ftp://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prokaryotes.txt) of 17 *Lactobacillus* species commonly found in the vaginal environment, with finishing status of "complete genome", "chromosome" or "scaffold".

Potential matches were filtered to retrieve an unequivocal classification for each read. Since 2020, *Lactobacillus* taxonomy underwent major update, with the re-classification of the genus in 25 different genera (23 of which are novel) (Zheng J. *et al.,* 2020).

Old and new species names used in the present article are available in Table 10.

Taxonomy changed[a]		**Taxonomy unchanged**
Old name	**New name**[b]	
Lactobacillus fermentum	*Limosilactobacillus fermentum*	*Lactobacillus iners*
Lactobacillus plantarum	*Lactiplantibacillus plantarum*	*Lactobacillus gasseri*
Lactobacillus brevis	*Levilactobacillus brevis*	*Lactobacillus crispatus*
Lactobacillus casei	*Lacticaseibacillus casei*	*Lactobacillus jenseni*
Lactobacillus vaginalis	*Limosilactobacillus vaginalis*	*Lactobacillus acidophilus*
Lactobacillus salivarus	*Ligilactobacillus salivarius*	*Lactobacillus delbrueckii*
Lactobacillus reuteri	*Limosilactobacillus reuteri*	*Lactobacillus helveticus*
Lactobacillus rhamnosus	*Lacticaseibacillus rhamnosus*	*Lactobacillus johnsonii*
Lactobacillus paracasei	*Lacticaseibacillus paracasei*	*Lactobacillus iners*

Table 10. Table of the species used for *Lactobacillus* species-level classification. Due to the re-classification of the *Lactobacillus* genus in 25 different genera, the 17 interesting species for the vaginal environment were divided in "Taxonomy changed" or "Taxonomy unchanged". In case of re-classification, new and old species names are provided. [a]Classification according to Zheng J *et al.,* 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerink 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. https://doi.org/10.1099/ijsem.0.004107. [b]Correspondences between new and old names was determined thanks to "Lactotax" webservice: http://lactotax.embl.de/wuyts/lactotax.

Co-abundance network analysis was performed as previously described by Claesson and colleagues using Spearman's correlation between taxa and building hierarchical clusters of co-abundant groups (CAGs) at genus level by Spearman's correlation metric and Ward linkage (Claesson *et al.,* 2012). Cytoscape (v 3.0) was used to graphically represent CAGs, as well as relative abundance of bacterial genera and strength of correlation (Shannon *et al.,* 2003).

α-diversity evaluation was estimated according to several microbial diversity metrics (*i.e.*, chao1, Shannon index, observed species, Good's coverage, and Faith's phylogenetic distance). β-diversity analysis was conducted using both weighted and unweighted Unifrac metrics and through the PCoA.

Survival analysis was performed on bacterial genera selected as among the most representative from the taxonomic classification, and the detection of positivity to one of the resistance genes in the single samples over time was considered as a censoring event.

Several thresholds of bacterial relative abundance were implemented as time-dependent covariates, which started at value 0 and changed to 1 once the relative abundance of the genus in the sample increased above a certain value. Bacterial thresholds were determined from the mean abundances of all 228 samples and pondering the number of samples that would fall in one of the sides.

For a better analysis performance, the original four collection points, divided into trimesters and days after delivery, were subdivided into 20 time points according to the weeks of pregnancy and post-partum, with week 36 as an indicative delivery time point.

Raw sequencing data of 16S rRNA gene are available at NCBI Short-reads Archive (SRA) with BioProject accession number PRJNA766806 (https://www.ncbi.nlm.nih.gov/sra/PRJNA766806).

Microbiota analyses were performed by Dr. Marco Severgnini and Dr. Clarissa Consolandi from the Institute of Biomedical Technologies (National Research Council, Milan, Italy).

2.4 Metabolomic analysis

Metabolomic analysis was performed using ^{1}H-NMR spectroscopy starting from 700 µL of the cell-free supernatants of the second vaginal swabs, as reported at pages 58 and 59.

2.5 Data analysis and statistics

Statistical evaluation of α-diversity indices was performed by non-parametric Monte Carlo-based tests through the QIIME pipeline.

β-diversity differences were assessed by a permutation test with pseudo-F-ratios using the "adonis" function from R package "vegan" (version 2.0-10, Oksanen *et al.,* 2013).

Pairwise relative abundance analysis was performed using a non-parametric Mann-Whitney U test.

For comparing relative abundances across multiple categories, Kruskal-Wallis test, followed by Dunn's post-hoc test for pairwise comparisons, was applied.

Metabolite concentrations were correlated to bacterial composition by calculating Spearman's correlation coefficient between metabolites and bacterial genera present ≥1% in at least 1 sample (n=51). In this analysis, all data points (T1, T2, T3 and T4) were considered.

Spearman's rank-based correlation between genus relative abundances and metabolite quantities was performed, selecting only those with p-value<0.05 (*i.e.,* correlation significantly different from 0).

To better visualize patterns of positively correlated bacteria and metabolites, a heatmap was drawn, clustering correlation coefficients for metabolites and bacteria (using Pearson's correlation as clustering metric and average linkage). For each statistical analysis, unless otherwise stated, p-values<0.05 were considered as significant. Statistical analyses were performed using Matlab software (Natick, MA, USA).

3. Results

3.1 Study population

A total of 63 Caucasian pregnant women with a mean age of 30.8±5.1 years (min: 21, max: 44) and a mean BMI of 23.5±3.5 (min: 16.0, max: 32.5) were enrolled and sampled during all gestational ages. Among these 63 women, only for 30 of them, due to the Covid-19 pandemic period, clinical and microbiological data were available for the puerperium. In addition, 9 women (mean age: 33.8±6.4 years; mean BMI: 24.5±4.7) who had a spontaneous miscarriage at the first trimester of pregnancy (gestational age: 11-13 weeks) during the study were included.

From the first to the third trimester of pregnancy, a significant decrease of BV cases, together with an increase of samples characterized by a normal microbiota, was noticed (p=0.001; Table 11). During the puerperium, only one third of the women (33.3%) showed a lactobacilli-dominated flora, being most of them characterized by an alteration of the vaginal bacterial composition (26.6% intermediate flora, 40.0% BV-condition). Ten out of the 30 (33.3%) women with puerperium data available received an IAP to prevent GBS neonatal infection.

Most cases of spontaneous abortion were associated with an altered vaginal microbial composition (55.5% with intermediate status and 22.2% with BV condition).

Nugent score category	**I trimester miscarriage (n=9)**	**Normal pregnancies (n=63)**			**Puerperium (n=30)**
		I trimester	**II trimester**	**III trimester**	
0-3 (H)	22.2% (2/9)	49.2% (31/63)	**74.6% (47/63)**	82.5% (52/63)	33.3% (10/30)
4-6 (I)	55.5% (5/9)	33.3% (21/63)	**15.9% (10/63)**	11.1% (7/63)	26.6% (8/30)
7-10 (BV)	22.2% (2/9)	17.5% (11/63)	**9.5% (6/63)**	6.4% (4/63)	40.0% (12/30)

Table 11. Characteristics of the vaginal environment (NS), stratified by the different gestation periods and conditions. H, lactobacilli-dominated microbiome; I, intermediate flora; BV, bacterial vaginosis. Statistical significance was reported in bold and as referred to the differences in microbial composition (NS) during the three trimesters of pregnancy, excluding women suffering a first trimester miscarriage.

3.2 Vaginal microbiota structure characterization

Overall, microbiota composition assessed through 16S rRNA sequencing was in accordance with what expected for the vaginal environment, with the *Lactobacillus* genus having an average relative abundance of 77.9%, followed by *Gardnerella* (8.9% on average), *Bifidobacterium* (3.5%), *Atopobium* (2.1%), *Prevotella* (1.8%), and *Megasphaera* (1.3%). Other genera, such as *Sneathia*, *Ureaplasma*, *Aerococcus*, and *Dialister* had <1% abundance. The first 12 genera accounted for 97.7% of the overall relative abundance, confirming the relatively low biodiversity of the vaginal samples (Fig. 23).

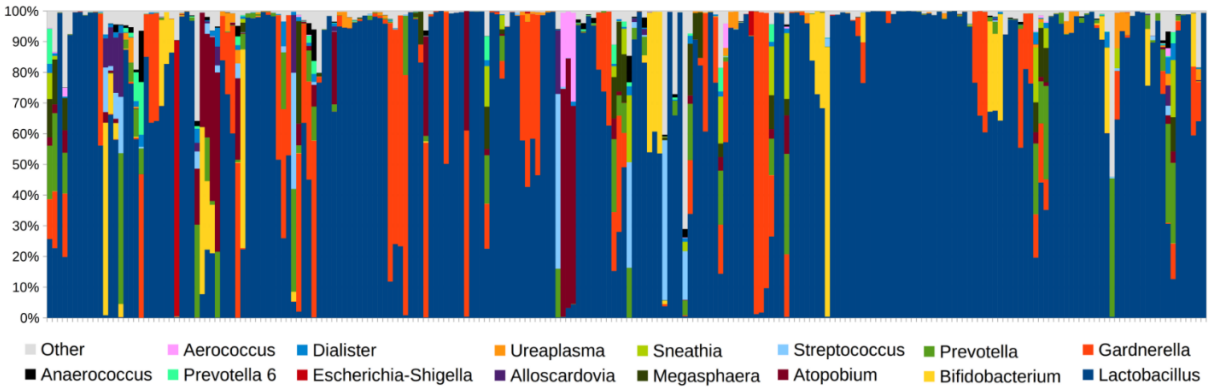

Figure 23. Overall microbiota composition. Barplots of relative abundances at genus level for the samples collected from 63 women during three trimesters of pregnancy (n=189). Genera with an average relative abundance <0.25% were grouped in "Other" category.

Microbiota structure was evaluated according to the vaginal status derived from the NS, for a total of 189 samples (63 women at 3 time points each), comparing H, I and BV status.

As expected, BV condition was characterized by a profound alteration of the microbiota, with a dramatic reduction of *Lactobacillus* spp. (83.8% vs 29.8% for H and BV, respectively) and an increase of opportunistic bacteria, (*i.e., Gardnerella, Atopobium, Prevotella, Megasphaera, Sneathia, Aerococcus*). The I condition seemed to be composed by nearly the same bacterial members of the H samples, with little exceptions in low-abundant members of the community (*i.e., Dietzia, Actinomyces, Enterococcus, Cutibacterium*, and "*Eubacterium eligens* group"), all contributing with <0.2% of average abundance (Fig. 24).

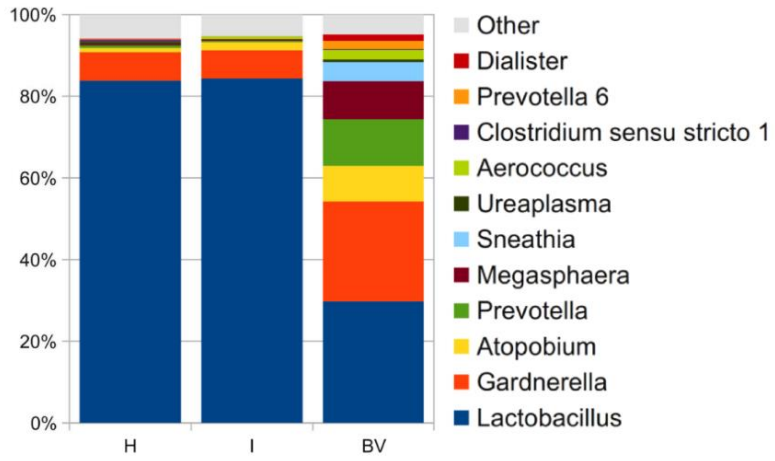

Figure 24. Microbiota characterization according to the vaginal status (H, I or BV). Barplot of average relative abundances at genus level. Genera with relative abundance (rel. ab.) ≤1% were grouped in "Others" category.

Among the lactobacilli species, a significant reduction of *L. crispatus* was highlighted in BV samples as compared to both H and I (average abundance (avg. ab.) 4.1% BV vs 32.7% H, 42.9% I); on the other hand, *L. iners* (avg. ab. 19.9% H, 17.4% I, 16.5% BV), as well as other *Lactobacillus* species, was not affected (Fig. 25A).

Differences in microbial composition were reflected in α-diversity analysis, which highlighted a significant increase in biodiversity in BV samples as compared to H and I ones (p=0.003 for all metrics) (Fig. 25B).

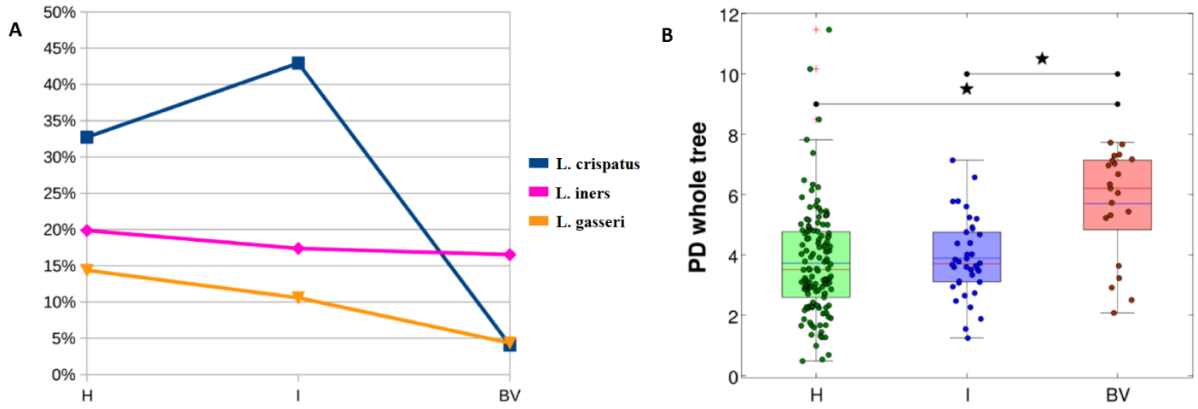

Figure 25. Microbiota characterization according to the vaginal status (H, I or BV). A) Line plot of average Lactobacillus species abundance per vaginal status; only the 3 most abundant species are represented; B) Boxplot of Faith's phylogenetic diversity of the samples (estimated at endpoint) for each vaginal status. Stars above the plots represent statistical significance ($p<0.05$).

Moreover, a significant separation of microbial profiles (β-diversity) among H, I and BV was recorded ($p<0.039$ for all pairwise group comparisons, unweighted Unifrac); at the same time, BV samples differed from the other two groups in major contributors of the microbiota ($p=0.001$ against both H and I conditions, weighted Unifrac) (Fig. 26).

Similarly, distances between H and BV samples were higher than H vs I ones and I vs BV distances were higher than H vs I (both for weighted and unweighted Unifrac); at the same time, weighted Unifrac distances among BV samples resulted higher than that among H or I samples, confirming that BV status was characterized by a deeper alteration of the microbial composition with respect to other conditions.

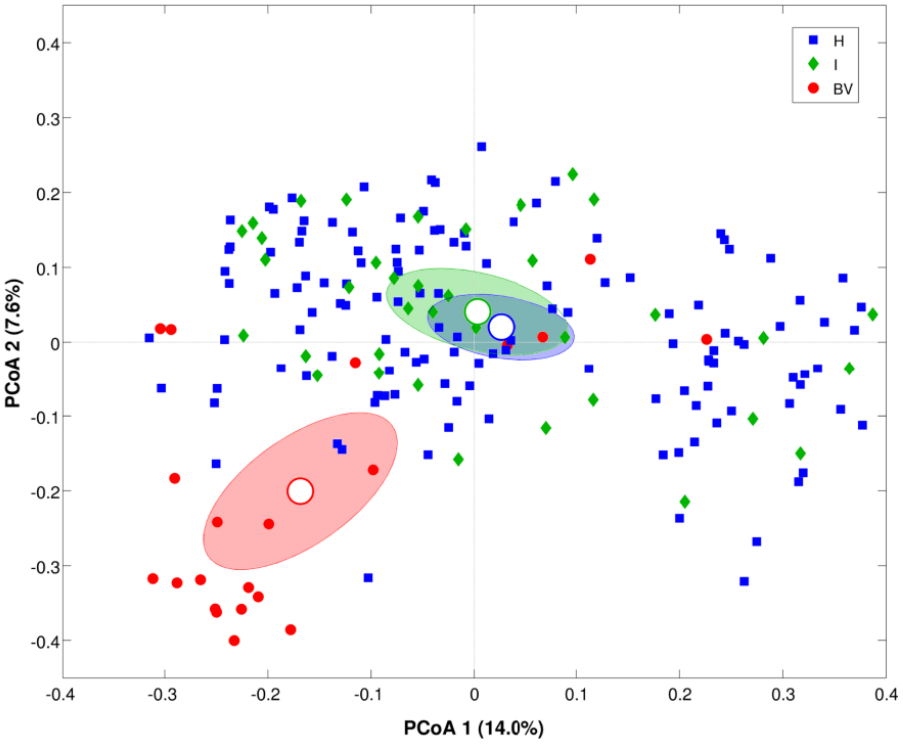

Figure 26. Microbiota characterization according to the vaginal status (H, I or BV). PCoA based on unweighted Unifrac distance among samples. Each point represents a sample; ellipses are 95% SEM-based confidence intervals; point and ellipses are grouped according to vaginal status; the first and the second coordinate are represented.

3.3 Taxonomic composition of the vaginal bacterial communities during pregnancy

The VMB dynamics along the three trimesters of pregnancy for a total of 63 women (189 total samples) were evaluated. The proportion of samples with the same vaginal status at each trimester was found to be statistically different for the H subjects ($p<0.001$, two-sided proportion test without continuity correction; increasing from 49.2% to 74.6% and 82.5% respectively at T1, T2, and T3) and the I subjects ($p=0.005$; decreasing from 33.3% at T1 to 15.9% at T2 and 11.1% at T3).

On the other hand, no differences were highlighted for BV status (proportion of 17.5%, 9.5%, and 6.3% respectively at T1, T2, and T3).

There were no significant or noticeable differences in biodiversity over time, other than T1 vs T2 in chao1 ($p=0.039$) and T1 vs T3 for the Faith's phylogenetic diversity metric ($p=0.015$). With regards to microbial composition, T3 points were statistically separated from T1 and T2 sets ($p\leq0.015$, unweighted Unifrac), which were indistinguishable from each other; no differences on the weighted Unifrac distance matrix were highlighted (Fig. 27).

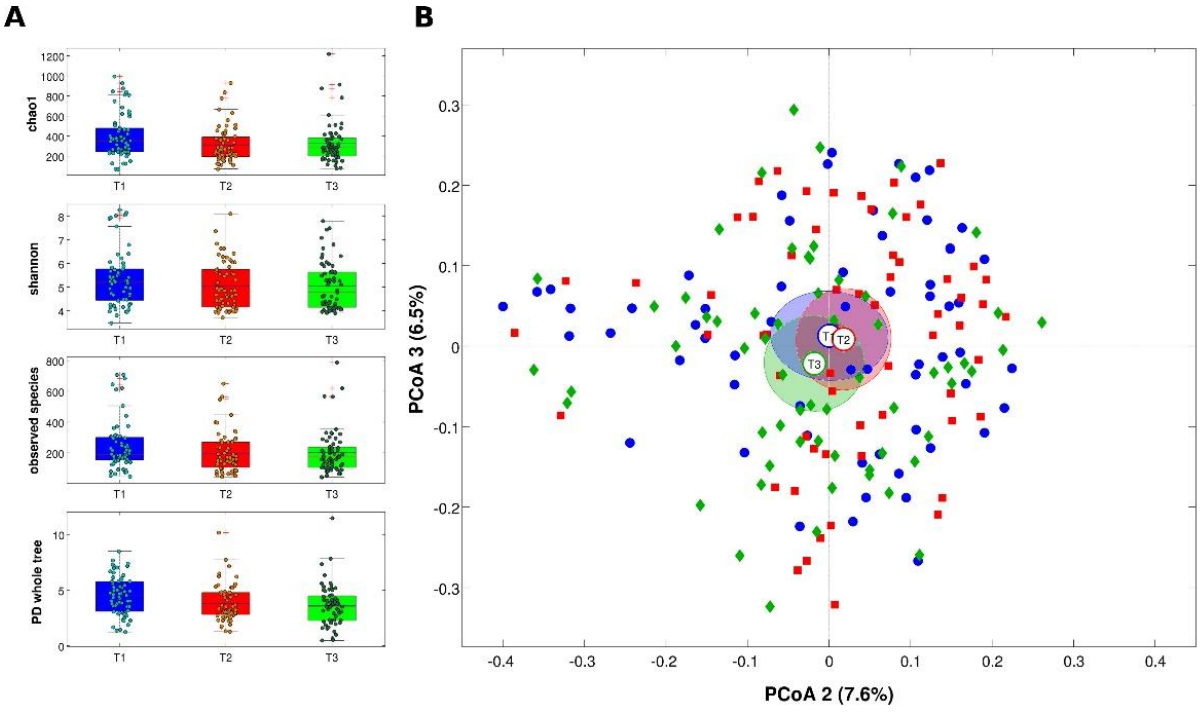

Figure 27. Microbiota characterization during pregnancy. (A) Boxplots of α-diversity values according to four different metrics over time; (B) PCoA based on unweighted Unifrac distance among samples. Each point represents a sample; ellipses are 95% SEM-based confidence intervals; point and ellipses are grouped according to time point; the second and third coordinate are represented.

Analyzing bacterial genera co-abundance patterns, four CAGs were identified: (i) *Ureaplasma* alone; (ii) *Lactobacillus* CAG (also including *Clostridium*); (iii) 'opportunistic' bacteria CAG (including *Bifidobacterium*, *Prevotella* and *Dialister*); (iv) BV-associated bacteria CAG (*i.e., Gardnerella, Atopobium, Megasphaera, Sneathia* and *Aerococcus*).

At all three time points, *Lactobacillus* CAG was inversely correlated to other CAGs, whereas opportunistic and BV-related CAGs were directly associated to one another, although with a different strength of correlation.

Many genera were statistically different over time, suggesting a deep reshaping of the microbiota between the first two trimesters: all groups were differential in both T1 vs T2 and T1 vs T3 comparisons, but not for T2 vs T3. In particular, analysis has revealed increased *Lactobacillus* abundances and reduced levels of opportunistic (such as *Bifidobacterium* and *Prevotella*) and BV-related bacteria (*Atopobium* and *Sneathia*) (Fig. 28).

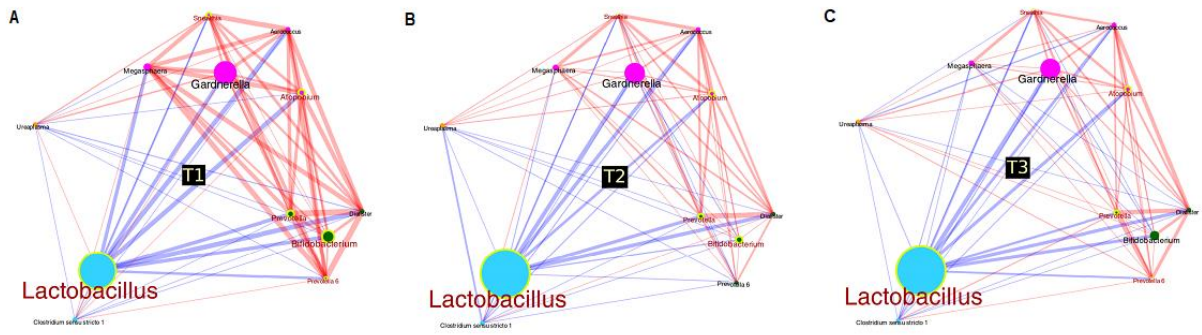

Figure 28. Microbiota evolution during three trimesters of pregnancy. Co-abundance networks of bacterial genera over time. Circle size is proportional to genus relative abundance for each time and colours are according to co-abundance groups; edge size is proportional to the strength of correlation; red lines mean positive correlation, while blue lines indicate negative correlation. Genera resulting statistically different over time points are highlighted with a yellow circle and a red label.

Stratification by the vaginal status allowed a deeper evaluation of changes over time: in BV samples, it has been highlighted a shift between T1 and T2 among the phyla *Actinobacteria* (increased) and *Fusobacteria* (decreased), while at genus level *Prevotella* (group 6) was found decreased.

For the I condition, no major members of the microbiota were statistically different among pregnancy trimesters (only a total of 6 differential genera over time were detected, all with average relative abundance <0.3%).

Finally, in the H samples, a reduction of the phylum Actinobacteria and of its related genus *Bifidobacterium* was noticed (average relative abundance (avg. rel. ab.) of 7.5%, 2.1%, and

3.2% respectively at T1, T2, T3); the *Bifidobacterium* reduction was also confirmed when limiting the analysis to the 22 women with a "healthy" microbiota (H group) at each pregnancy time-point (avg. rel. ab. 8.4%, 1.6%, and 1.0% respectively at T1, T2, T3).

The *Streptococcus* genus was also decreased in the H group (entirely taken), but with a consistently lower abundance (avg. rel. ab. 0.5%, 0.1%, 0.2% respectively for T1, T2, and T3) (Fig. 29).

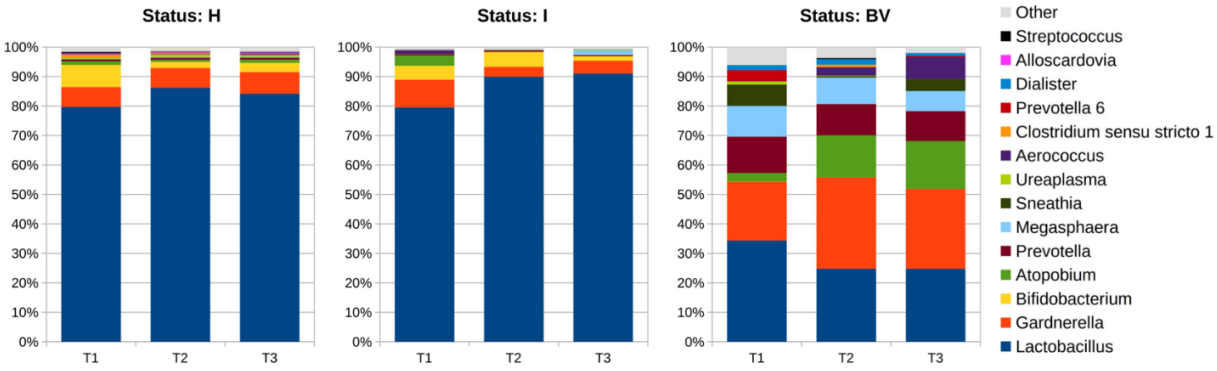

Figure 29. Barplots of average relative bacteria abundances at genus level during pregnancy, stratified according to the vaginal status. Only the 14 most abundant genera are represented.

As for evaluations within the *Lactobacillus* species, stratifying for the vaginal status, there were observed several variations. In the H group, a slight (non-significant) reduction of *L. iners* and the increase of *L. gasseri* in T1 vs T2 was highlighted, whereas abundances were nearly identical for T2 and T3; on the other hand, *L. crispatus* abundances were fundamentally unaltered.

Considering the I group, a significant reduction of proportion of *L. iners* and a significant increase of *L. crispatus* was observed between T1 and T2. BV samples had the opposite trend, with a reduction of *L. crispatus* and an increase of *L. iners* between T1 and T2 (Fig. 30).

No differences in *Lactobacillus* species were highlighted considering all samples together, regardless of their vaginal status.

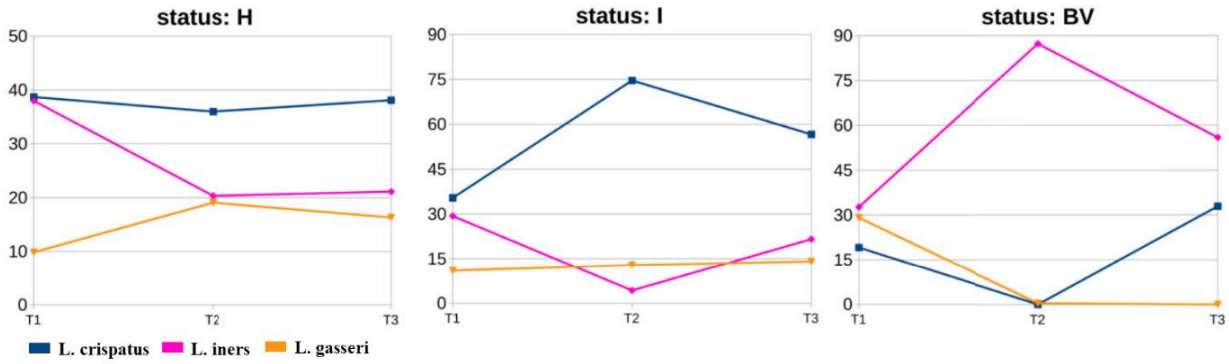

Figure 30. Microbiota evolution during three trimesters of pregnancy. *Lactobacillus* species abundance over time, stratified for vaginal status. Only the three most abundant species are represented.

Lastly, the Unifrac distances among samples was analyzed. The first interesting evidence was that the microbial profiles of all samples collected from one woman were more similar to each other than to those collected from the other women ($p<0.001$, intra- vs inter-distance for both weighted and unweighted Unifrac).When evaluating distances over time, it was recorded that T2 vs T3 distance (unweighted Unifrac) was significantly higher than both T1 vs T2 and T1 vs T3 (comparisons not significantly different), indicating that between the second and third trimester of pregnancy the microbiota develops in a more independent way (Fig. 31).

Considering the H samples alone, the evolution between T2 and T3 was confirmed; furthermore, our result suggests that T3 represents an evolution of the microbiota from T2 (as T1 had lower distance values to T2 than to T3), although average distances were very similar (T1-T2: 0.68, T1-T3: 0.71, T2-T3: 0.71).

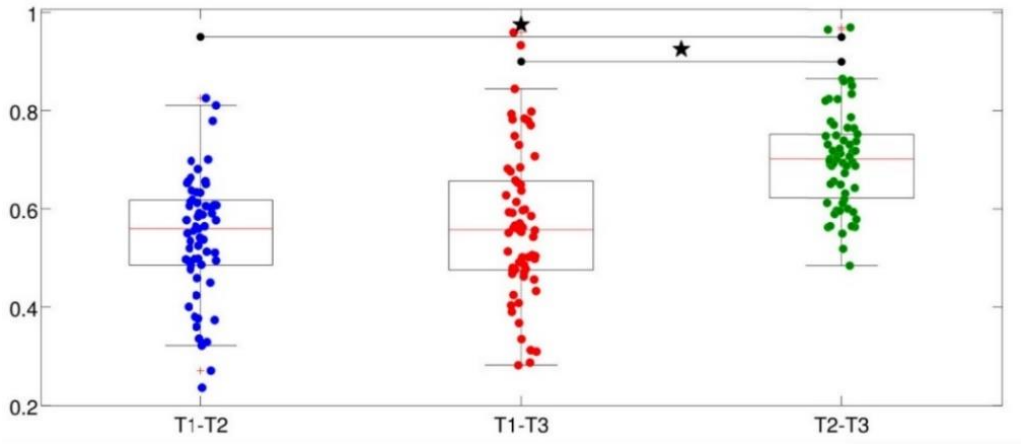

Figure 31. Microbiota evolution during three trimesters of pregnancy. The boxplot represents the unweighted Unifrac distances between samples over time. Distances were calculated for each pair of samples belonging to the same women, sampled at T1, T2 or T3; stars above the plots represent statistical significance ($p<0.05$).

3.4 Vaginal microbiota at the puerperium

In addition, the VMB characteristics during the puerperium period in a group of 30 women, sampled a fourth time during the study (total subset time-points: T1, T2, T3 during pregnancy; T4 at puerperium, 40-55 days after delivery) was evaluated. No differences in biodiversity over time were detected ($p>0.05$ for all α-diversity metrics tested).

On the other hand, there seemed to be some separation in microbial composition within the β-diversity analysis, as T4 points were statistically different from T1, T2, and T3 (both unweighted and weighted Unifrac) (Fig. 32A).

Over time, the analysis of microbial relative abundances at genus level suggested a composition variation at T4, with a lower content of *Lactobacillus* and a consistent presence of *Gardnerella, Prevotella, Atopobium,* and *Streptococcus*; those changes were significant ($p<0.05$) when compared to T1 (all except *Atopobium* and *Gardnerella*), to T2 (all except *Gardnerella*), and to T3 (all genera) (Fig. 32B).

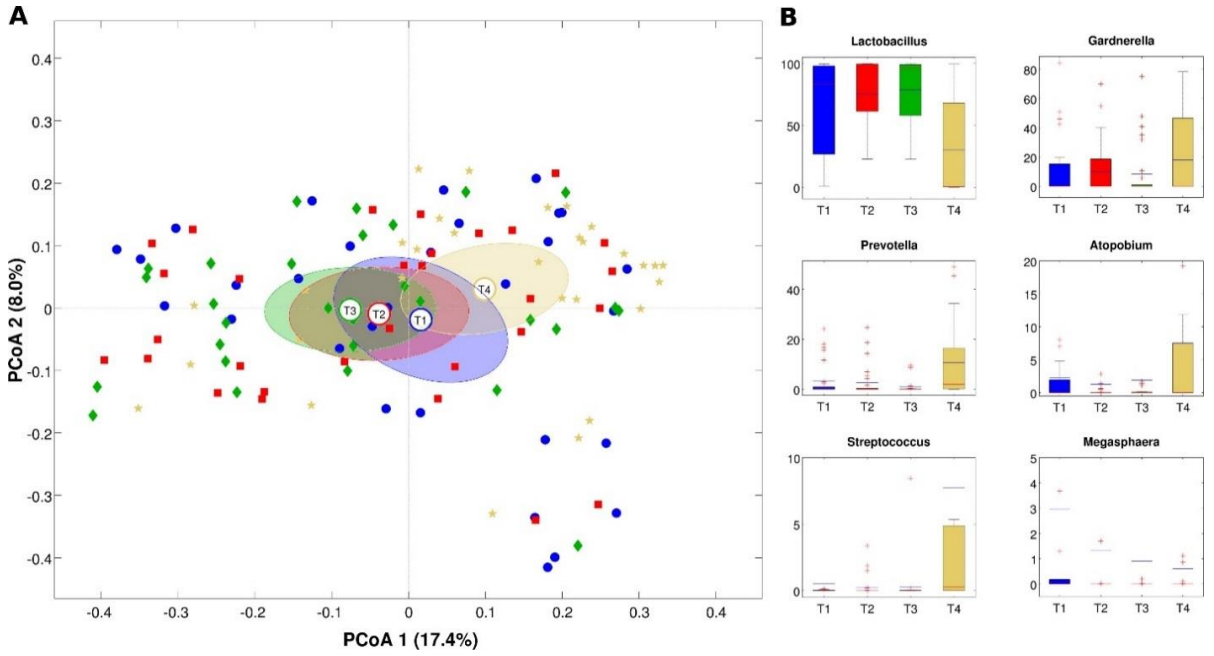

Figure 32. Microbiota characterization in puerperium. (A) PCoA based on unweighted Unifrac distance among samples. Each point represents a sample; ellipses are 95% SEM-based confidence intervals; point and ellipses are grouped according to time point; the first and second coordinate are represented; (B) Boxplots of relative abundances at genus level over time. The first six genera differential in at least one comparison are represented.

At species level, this was reflected in a significant decrease of all *Lactobacillus* species, *L. crispatus* and *L. jensenii* in particular ($p<0.05$), while *L. gasseri* was also found decreased but not significantly; contrariwise, *L. iners* was observed to be unchanged during the puerperium.

The stratification by vaginal status highlighted how these differences were mainly due to a change in bacterial members for the BV and I groups, whereas microbial profiles of H women resulted more stable, as evidenced by analyzing the correlation coefficients among average microbial profiles at genus level over time.

Within the H group, microbial composition did not vary consistently, with an average Pearson correlation between T4 and all of the other three time-points of r=0.977, similar to the average r=0.998 between the paired comparisons of T1, T2, and T3. On the other hand, correlation coefficients for BV women were lower and slightly different over time (r=0.659 between T4 and the other three time points; r=0.826 among T1, T2, and T3).

Finally, the most substantial differences were observed for the I group, as the correlation coefficient dropped from r=0.994 to r=0.340 when comparing T4 to the other three time points (Fig. 33).

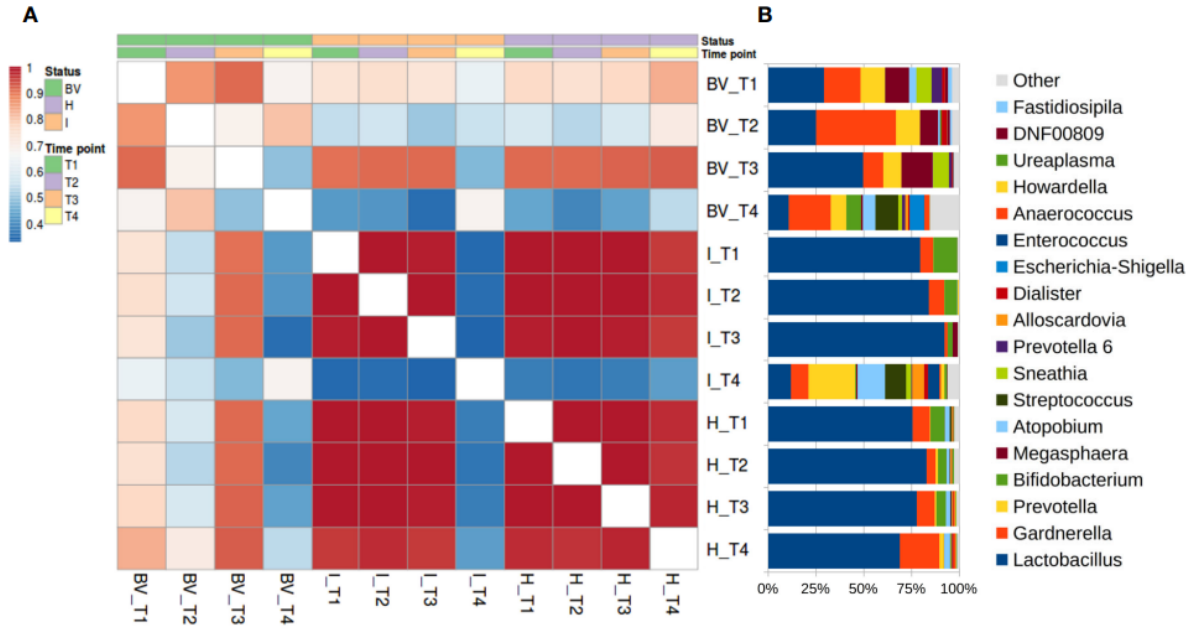

Figure 33. Microbiota evolution during puerperium (T4). (A) Heatmap of Pearson's correlation coefficients calculated between average relative abundances at genus level over time and stratified for vaginal status; (B) Barplots of average relative abundances at genus level over time and stratified for vaginal status; genera with rel. ab. ≤1% were grouped in "Others" category.

BV samples at T4 were characterized by a significant reduction of the genera *Lactobacillus*, *Megasphaera*, and *Prevotella*, and by an increase of *Streptococcus* and *Finegoldia* with respect to T1.

A significant reduction of *Lactobacillus* and an increase of *Prevotella*, *Streptococcus*, and *Dialister* for the I condition in the comparison of T4 to all other gestational time-points was observed; despite a slightly increase in the *Gardnerella* abundance, microbial profiles of the H group resulted very similar over all four time-points.

The *Lactobacillus* species analysis stratified by the vaginal status suggested that BV samples at puerperium had a switch compared to both the first and second trimester: *L. crispatus* showed a higher abundance (T4 7.6% vs T1 3.0% and T2<0.1%; $p<0.05$ for T2 vs T4) while *L. iners* a lowered one (T4 1.8% vs T1 12.0% and T2 11.5%; $p<0.05$ for T1 vs T4).

The BV composition during the third trimesters, T3, was not evaluated, since only one sample for this time-status combination was available.

In the I samples, T4 microbiota displayed a dramatic decrease of *L. crispatus* (0.1% vs 36.2% of the gestational time-points average; $p<0.05$ for T2 vs T4). A similar decrease of *L. crispatus* was observed for H women as well (0.4% vs 39.3% on average of the gestational time points), together with a somewhat higher abundance of *L. iners* (36.8% vs 19.0% of the gestational time-points average).

Due to the extreme variability among individuals, between T3 and T4 the sole *L. crispatus* reduction was statistically significant (Fig. 34).

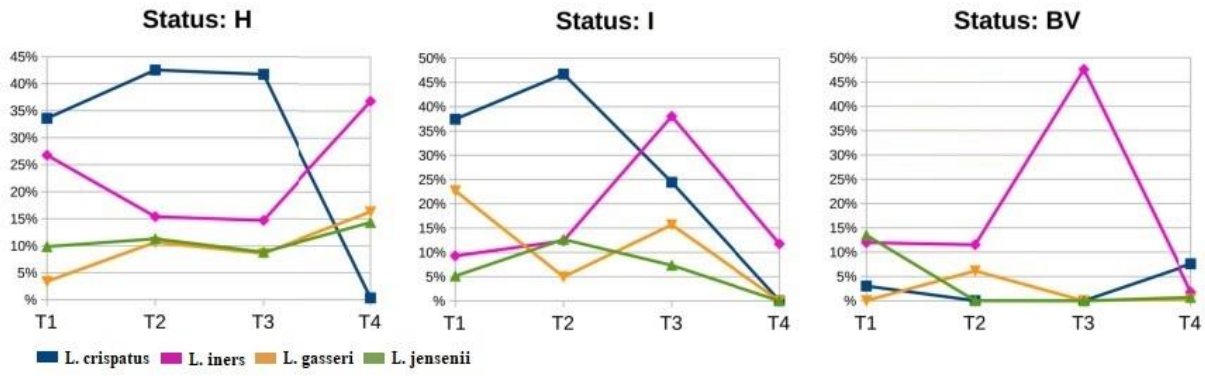

Figure 34. Microbiota evolution during three trimesters of pregnancy and puerperium. *Lactobacillus* species abundance over time, stratified for vaginal status. Only the four most abundant species are represented.

Among the women evaluated at T4, 10 out 30 (33.3%) received an IAP for GBS. Microbial profiles of these women did not result significantly different from the untreated group (n=20) neither by α- ($p>0.05$ for all metrics tested) nor β-diversity ($p=0.937$ and $p=0.112$ for unweighted and weighted Unifrac distances, respectively).

Only one taxon, the *Prevotella* genus, was significantly altered, showing an increase in antibiotic-treated women, when compared to the untreated ones (rel. ab. of 20.0% with antibiotics vs 6.0% without antibiotics) (Fig. 35).

At the same time, this difference was reflected in higher-level taxonomies as well (*Bacteroides, Bacteroidia, Bacteroidales*: 22.9% vs 8.0%; *Prevotellaceae*: 22.2% vs 7.3%, with vs without antibiotics).

No differences were highlighted by the species-level characterization of the *Lactobacillus* genus.

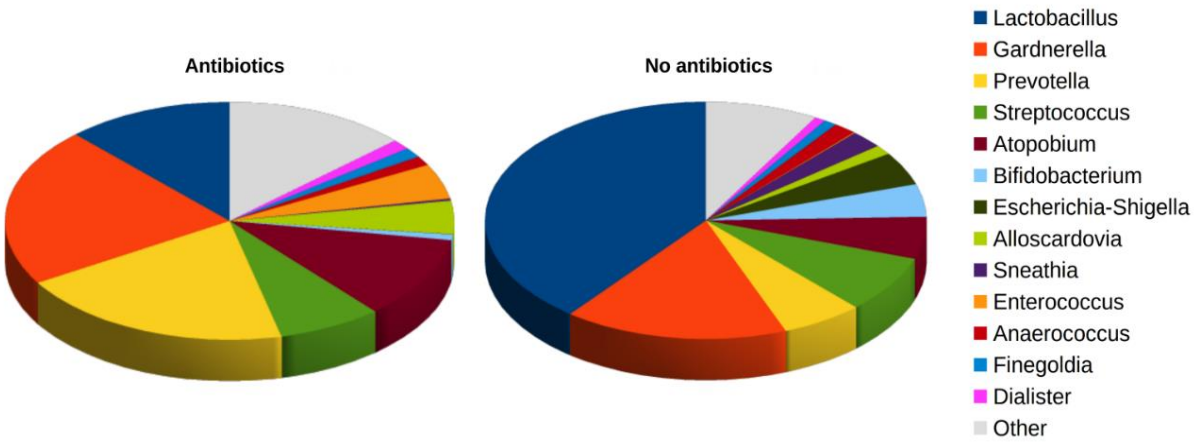

Figure 35. Effect of antibiotics treatment on microbiota. Pie charts of average microbial composition at genus level for samples taken from women treated with IAP to prevent GBS infection (n=10) and untreated ones (n=20). For graphical reasons, only the thirteen most abundant genera are represented.

3.5 Association between microbiome composition and first trimester miscarriage

The microbiome profiles at T1 of the 63 women with successful pregnancies to the ones of 9 women who suffered a first trimester miscarriage were compared. No significant differences were found on both α-diversity ($p>0.05$ for chao1, Shannon, Good's coverage, Observed species, Faith's phylogenetic diversity metrics) and β-diversity ($p=0.412$ and $p=0.110$ for unweighted and weighted Unifrac distances, respectively) analyses.

Nevertheless, an overgrowth of *Fusobacterium* (rel. ab. 1.1%, p=0.02) in the miscarriage group compared to successful pregnancies was observed (0.1%). No significant differences were highlighted for the *Lactobacillus* species.

3.6 Vaginal metabolites composition and metabolite-microbiome correlation

Among the 63 metabolites detected in the vaginal swab supernatant, molecules mainly belonged to the groups of SCFAs, organic acids, amino acids, and biogenic amines.

A correlation analysis for relating microbial composition to metabolite concentrations was assessed, using Spearman's rank correlation to determine monotonically increasing or decreasing relationships. All samples collected over the four time-points were considered (n=219); miscarriage samples were analyzed separately.

Results have shown three main clusters of correlations. Firstly, *Lactobacillus* stood by itself, separated from all other bacteria, strongly positively correlated to lactate and sarcosine (r=0.62 and r=0.61, respectively). Moreover, positive correlations were evidenced for many amino acids (*i.e.,* isoleucine, leucine, phenylalanine, aspartate, glutamate, valine, glycin, serine, threonine, tryptophan, with correlation values ranging from 0.26 to 0.65). Secondly, BV-associated genera, such as *Gardnerella, Prevotella, Atopobium, Dialister, Aerococcus,* and *Sneathia,* were positively correlated to putrescine, methylamine, tyramine, formate, trimethylamine (TMA), alcohols (*i.e.,* ethanol, isopropanol), and SCFAs (*i.e.,* acetate, butyrate, propionate). In conclusion, other lower-abundance bacteria, such as *Bifidobacterium, Streptococcus,* and *Alloscardovia* correlated with nucleotides (*i.e.,* adenine, glutamine, inosine, uracil), glucose, choline, benzoate, and fumarate (Fig. 36).

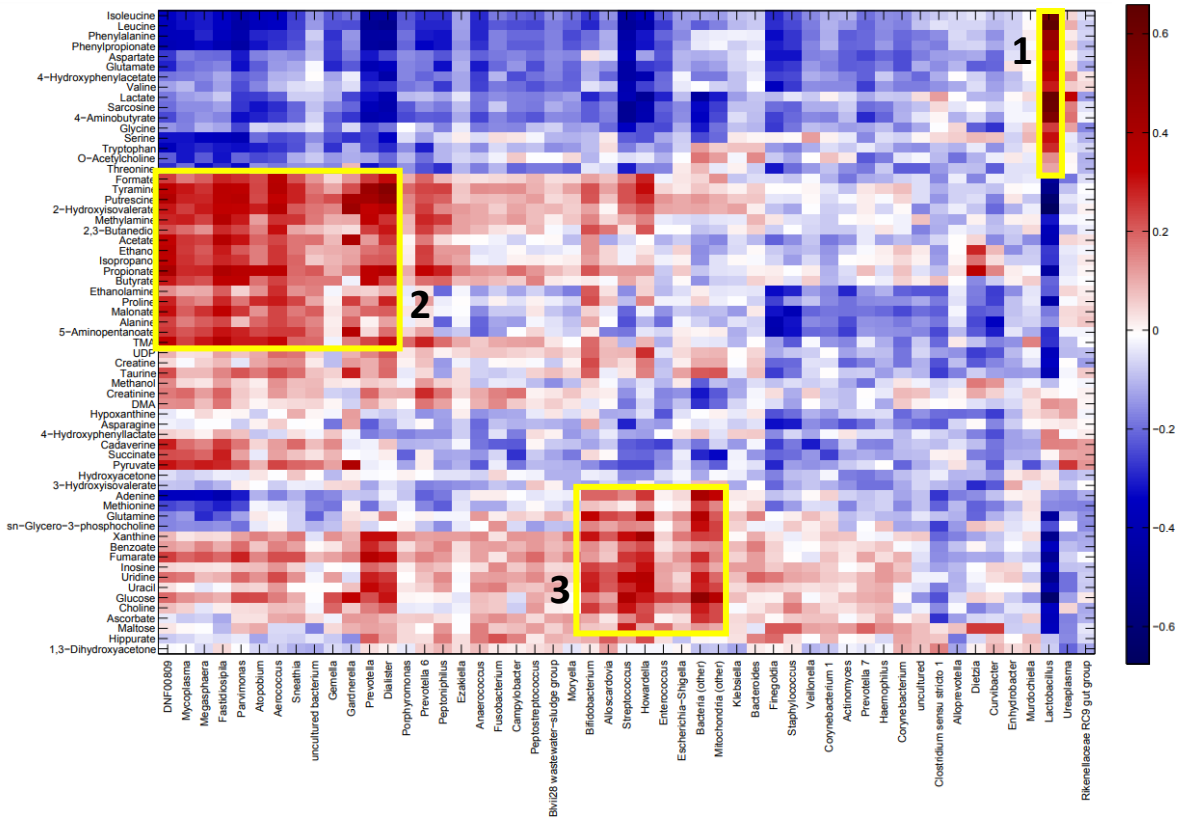

Figure 36. Correlation between metabolome and microbiota. Heatmap showing the Spearman's correlation coefficient between metabolites concentration and the relative abundances of the main bacterial genera over all samples collected, excluding miscarriages (n=219). Only groups present at >1% of relative abundance in at least one sample were considered. Metabolite and microbial data were clustered using Pearson's correlation metric and average linkage.
1. *Lactobacillus* stand by itself, separated from all other bacteria (as expected, in the vaginal environment), strongly positively correlated to lactate and sarcosine (ρ=0.62 and ρ=0.61, respectively). Moreover, positive correlations were evidenced for many aminoacids (*i.e.,* isoleucine, leucine, phenylalanine, aspartate, glutamate, valine, glycin, serine, threonine, tryptophan);
2. BV-associated genera, such as *Gardnerella, Atopobium* and *Sneathia* were correlated to putrescine, methylamine, formate, TMA, alcohols (*i.e.,* ethanol, isopropanol), acetate and short-chain fatty-acids (*i.e.,* buthyrate, propionate);
3. Other lower-abundance bacteria, such as *Bifidobacterium,* Streptococcus and *Alloscardovia* correlated with nucleotides (*i.e.,* adenine, glutamine, inosine, uracil), glucose, choline, benzoate and fumarate.

Microbiome-metabolites correlation patterns were further refined by looking at the possible relationships with spontaneous miscarriages (n=9).

Overall, only few correlations were significant (p-value<0.05). Correlation patterns for *Lactobacillus* and BV-associated genera were in accordance with those described above for the samples of women with a successful pregnancy. Interestingly, *Fusobacterium* was found positively correlated to the nucleotides and their components (*i.e.,* uracil, adenine, UDP, tyramine, r range 0.36-0.56), as well as to methionine (r=0.65), formate (r=0.54), choline, xanthine, and maltose (r=0.43-0.49), putrescine (r=0.38), and methylamine (r=0.31) (Fig. 37).

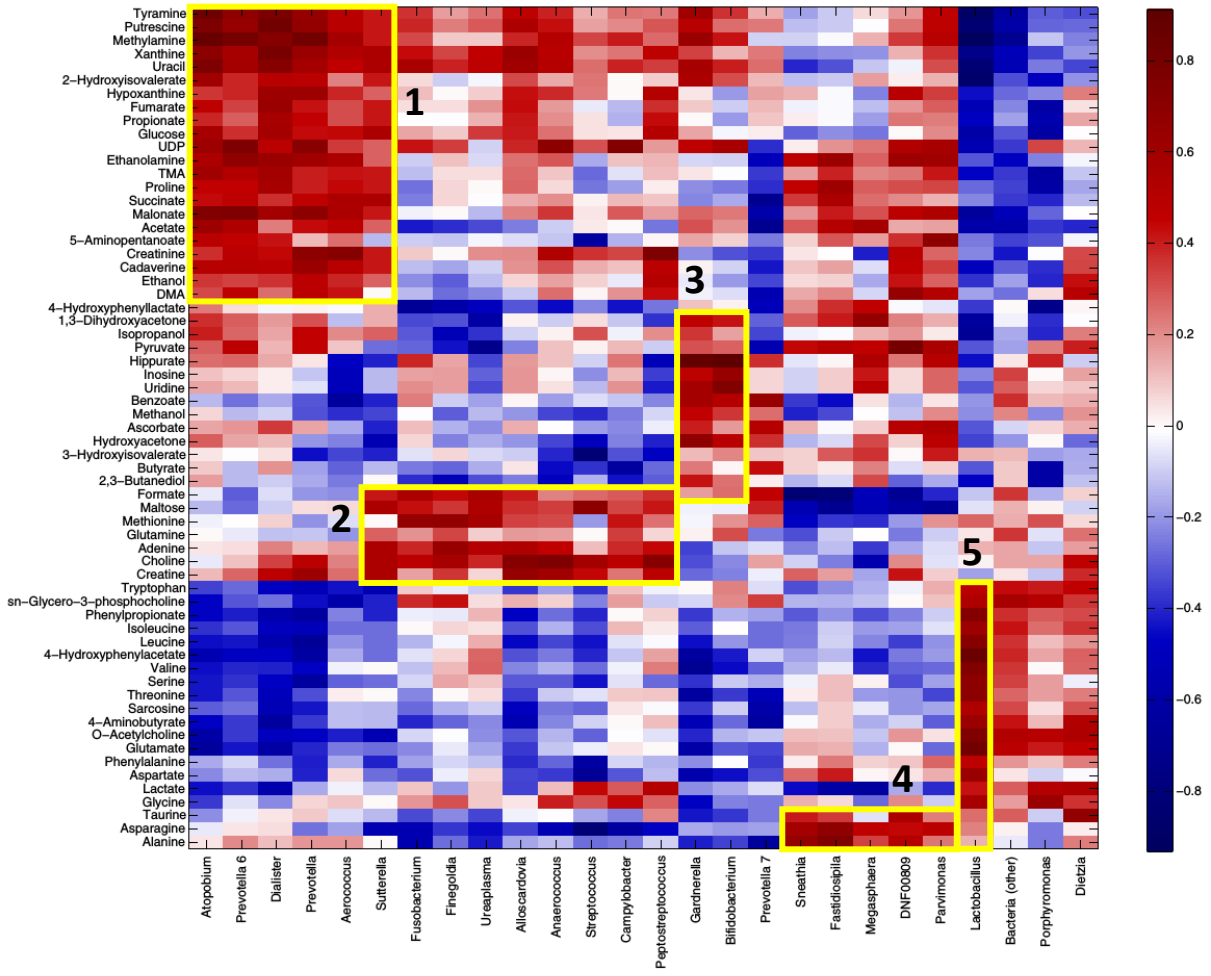

Figure 37. Correlation between metabolome and microbiota for miscarriages. Heatmap showing the Spearman's correlation coefficient between metabolites concentration and the relative abundances of the main bacterial genera over samples from women who underwent first-trimester miscarriage (n=9). Only groups present at >1% of relative abundance in at least one sample were considered. Metabolite and microbial data were clustered using Pearson's correlation metric and average linkage.

1. *Atopobium, Prevotella, Dialister, Aerococcus* and *Sutterella* were positively correlated to tyramine, putrscine, methylamine, propionate, acetate, succinate, malonate, creatinine, cadaverine, ethanol and DMA;
2. *Fusobacterium, Ureaplasma, Alloscardovia, Streptococcus* and *Anaeorcoccu*s were correlated to 2,3-butanediol, formate, maltose, methionine, glutamine, adenine, choline, creatine, tyramide, putrescine, methylamine;
3. *Gardnerella* and *Bifidobacterium* were correlated to 4−hydroxyphenyllactate, 1,3-dihydroxyacetone, isopropanol, pyruvate, hippurate, inosine, uridine, benzoate, methanol, ascorbate, hydroxyacetone, 3−hydroxyisovalerate, butyrate and 2,3-butanediol;
4. *Sneathia, Megasphaera* and *Parvimonas* showed a correlation to taurine, asparagine and alanine;
5. *Lactobacillus* were separated from other bacteria and showed nearly the same correlations as above: lactate, sarcosine, isoleucine, leucine, phenylalanine, aspartate, glutamate, valine, glycin, serine, threonine, and tryptophan.

4. Discussion

A deep comprehension of the vaginal ecosystem may hold promise for unravelling the pathophysiology of pregnancy and may provide novel markers to identify women at risk of complications, such as miscarriage and PTBs. Moreover, considering that microbial communities can be transferred from the mother's vaginal niche to the newborn gut, the study of the VMB during pregnancy and puerperium can open new perspectives for infant's microbiome development and future health (Dominguez-Bello *et al.,* 2010).

That is why in this study the vaginal environment in the situations of both a normal pregnancy, at the three gestational trimesters and the puerperium period, and a spontaneous first trimester miscarriage was characterized. In particular, the vaginal bacterial composition and the vaginal metabolic profiles were assessed.

At first, confirming results already report in the literature, irrespective of the period and type of pregnancy, BV cases were characterized by a dramatic reduction of *Lactobacillus* and an increase of anaerobic bacteria, such as *Gardnerella, Atopobium, Prevotella, Megasphaera, Sneathia* and *Aerococcus* (Nunn *et al.,* 2021).

In line with previous findings, the relative and absolute proportion of *L. crispatus*, a hallmark of vaginal eubiosis, inclined to decrease in the transition from H to BV conditions (Ceccarani *et al.,* 2019). As for *L. iners*, the abundance of this species did not differ between H and BV groups in our cohort, even though it has been considered a transitional species typically associated with dysbiotic conditions. On the other hand, *L. iners* has also been reported to be the dominating taxon in a large subset of women worldwide, being its presence associated with young age and unprotected sexual practices (Zheng *et al.,* 2021).

When considering changes in the VMB during the three trimesters of pregnancy, several bacterial genera showed statistically significant differences between the first and second trimesters. This suggests a significant reshaping of the microbiome profile toward a "healthier condition" as pregnancy progresses from the first to the third trimester. In line with the higher proportion of "H" cases, there was an increase in the *Lactobacillus* genus and a decrease in BV-related genera (*e.g., Prevotella, Atopobium, Sneathia*), with no differences in *Lactobacillus* species.

Taken together, these data confirmed that the VMB becomes more stable throughout the entire pregnancy, being less diverse and mainly dominated by lactobacilli (Li *et al.,* 2019).

It is worth noting that bifidobacteria, typical beneficial commensals inhabiting the human intestine, have tended to decrease their vaginal ecosystem abundances at the end of pregnancy.

It has been shown that *Bifidobacterium* is the dominant genus of some VMB and that overall bifidobacteria have the potential to be as protective as lactobacilli, according to the current understanding of a healthy VMB (Freitas and Hill, 2017). Nevertheless, Lee and colleagues recently observed that the relative abundance of *Bifidobacterium* spp. significantly increased during pregnancy in women with an intermediate and BV status compared to normal VMB, and that some dysbiotic conditions were dominated by *Bifidobacterium breve* (Lee *et al.,* 2020).

In line with these observations, the present work highlights a co-abundant vaginal pattern, characterized by several BV-associated genera, such as *Prevotella* and *Dialister*, and *Bifidobacterium* spp. Since the role of this vaginal microbial group is yet to be understood, further studies are needed to investigate the clinical significance of the bifidobacteria reduction at the end of pregnancy, as well as to assess the potential impact on newborn's health.

Other interesting data emerged when looking at the vaginal environment after delivery. In agreement with previous reports, during the puerperium, a significantly lower content of *Lactobacillus* and higher levels of *Gardnerella, Prevotella, Atopobium,* and *Streptococcus* were observed compared to the third trimester of pregnancy. These variations are consequences of after-delivery vaginal alterations that profoundly altered the host environment and, thus, led to changes in different bacterial species survival and proliferation capabilities (Nunn *et al.,* 2021).

Moreover, a significant increase in *Prevotella* abundance in women who received an IAP for GBS was observed compared to untreated ones. This aspect deserves attention considering that members of *Prevotella* genera have been associated with negative outcomes of the cervicovaginal environment, being responsible for strong inflammatory conditions, cytotoxicity, and alterations of the reproductive tract (Campisciano *et al.,* 2020). It is well known that IAP can negatively affect the gut microbiome of infants vaginally delivered, specifically in relation to microbial composition and occurrence of antibiotic resistance genes (Garcia, 2021).

However, the effect of antibiotic prophylaxis on the VMB after delivery is still little explored. Even if further studies are needed to clarify the reasons behind the increase in *Prevotella* levels in women receiving IAP, it is possible to speculate that β-lactam antibiotics could have selected this bacterial genus, as it is potentially able to produce β-lactamase enzymes (Toprak *et al.,* 2020).

Moving to the analysis of bacterial relative abundances in women suffering a first trimester miscarriage, present data have highlighted a significant vaginal overgrowth of *Fusobacterium* in abortions compared to successful pregnancies, at all taxonomic levels. This microbial genus has been strongly associated with genital inflammation and dysbiosis, being *Fusobacterium*

able to cooperate with other taxa to disrupt the normal vaginal bacterial composition, leading to microbial imbalance (Agarwal *et al.,* 2020). It has been shown that *Fusobacterium* has a mutualistic relationship with the BV-correlated bacteria: as they are major sialidase-producers, they enable *Fusobacterium* to consume sialic acids from the host-produced mucus. At the same time, *F. nucleatum* exposure to vaginal communities may encourage features of dysbiosis (*e.g.,* increased sialidase activity and *G. vaginalis* abundance) in susceptible vaginal communities (Agarwal *et al.,* 2020). In addition, *F. nucleatum* has been previously associated with PTB, since it was found in greater abundance in preterm placental membranes than at term (Ansari *et al.,* 2020).

The vaginal bacterial community profiles found during pregnancy were accompanied by peculiar fingerprints in the composition of the vaginal metabolites. In agreement with recent observations, *Lactobacillus* abundance was strongly correlated with higher levels of lactate, sarcosine, and many amino acids, whereas BV-associated genera, such as *Gardnerella, Atopobium, Sneathia*, were correlated to amines (putrescine, methylamine, TMA), formate, alcohols (ethanol, isopropanol), and short-chain fatty-acids (SCFAs, as butyrate, acetate, propionate) (Ceccarani *et al.,* 2019).

On the one hand, the lactate production by *Lactobacillus* species reduces the vaginal pH, contributing to the homeostasis against potentially endogenous or exogenous pathogens. These microorganisms are also known producers of branched-chain amino acids, thus the higher concentration of some of them, such as valine, leucine, and isoleucine, is another fingerprint of the prevalence of lactobacilli in 'healthy' women. Conversely, during dysbiotic conditions, the proliferation of diverse bacterial genera, some of which typical of the gut microbiota, and the imbalance between lactobacilli and BV-related bacteria lead to higher levels of amines, organic acids, and SCFAs (Vitali *et al.,* 2015).

In this context, higher levels of *Fusobacterium*, associated with the higher risk of spontaneous abortion, were positively correlated to several vaginal molecules, including methionine, formate, putrescine, and methylamine. Considering the low number of data points (n=9), the exact role of the vaginal metabolome in first trimester miscarriages, as well as the causative relationship between microbiota and immune responses, remain to be further elucidated, to enable the best possible diagnosis and therapeutics of early pregnancy loss.

In conclusion, the present study deepened the existing literature knowledge about the composition of the vaginal ecosystem during pregnancy and puerperium, highlighting peculiar microbial/ metabolic fingerprints.

III. Distribution of *ermB, ermF, tet(W),* and *tet(M)* resistance genes in the vaginal ecosystem of women during pregnancy and puerperium

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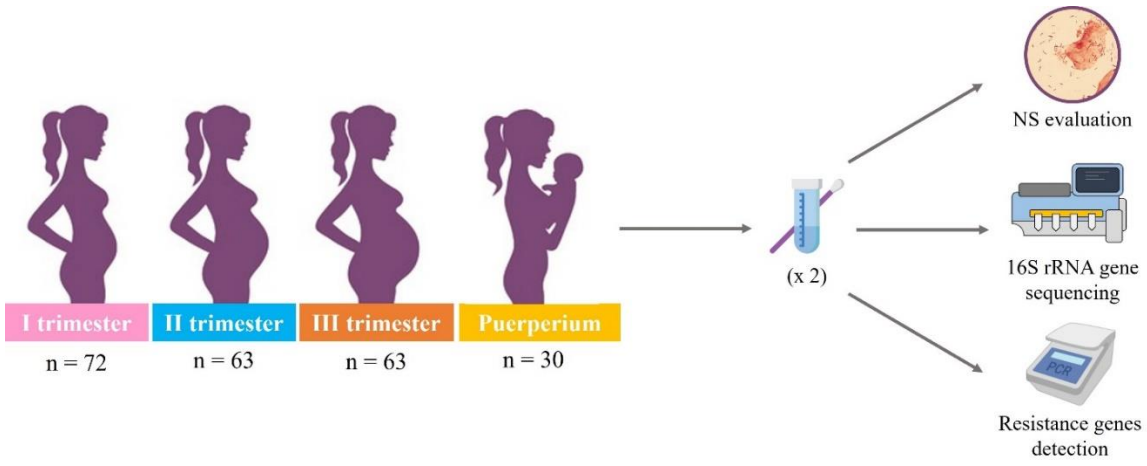

Figure 38. Graphical abstract.

1. Abstract

The inhabitants of vaginal ecosystem can harbour genetic determinants conferring antimicrobial resistance. However, detailed data about the distribution of resistance genes in the vaginal microbiome of pregnant women are still lacking. Therefore, the presence of macrolide (*i.e., erm* genes) and tetracycline (*i.e., tet* genes) resistance markers in the vaginal environment of Caucasian women at different gestational ages was tested. Furthermore, the detection of resistance genes was related to the composition of the VMB.

A total of 228 vaginal samples, collected at different trimesters of pregnancy or during the puerperium, were tested for the presence of *ermB*, *ermF, tet(W),* and *tet(M)* by in-house end-point Polymerase Chain Reaction (PCR) assays. The composition of the VMB was assessed through a microscopic evaluation (using NS system) and by means of sequencing V3-V4 hypervariable regions of the bacterial 16S rRNA gene. Overall, the most detected resistance gene was *tet(M)* (76.7%), followed by *ermB* (55.2%). In 17% of women, mainly with a 'normal' VMB, no resistance genes were found. Except for *tet(W),* a significant correlation between the positivity of resistance genes and a dysbiotic vaginal status (*i.e.,* BV) was noticed. Indeed, samples positive for at least one resistance determinant were characterized by a decrease in *Lactobacillus* spp. and an increase of BV-related genera (*Prevotella, Gardnerella, Atopobium, Sneathia*).

A high predominance of vaginal *Lactobacillus* spp. (>85%) was associated with a lower risk of *tet(W)* gene detection, whereas the presence of *Megasphaera* (>1%) increased the risk of positivity for all analyzed genes. Different types of VMB are associated with peculiar resistance profiles, being a lactobacilli-dominated ecosystem poor in or free of resistance genes.

These data could open new perspectives for promoting maternal and neonatal health.

2. Materials and methods

2.1 Study group and sample collection

From April 2019 to March 2021, all the Caucasian pregnant women presenting to the Family Advisory Health Centers of Ravenna (Italy) for prenatal care were considered eligible for the study. The following exclusion criteria were applied: (i) age <18 years; (ii) HIV positivity; (iii) BMI>33; (iv) medically assisted procreation; (v) use of any antibiotics in the month preceding the sampling; (vi) use of vaginal douches or topical agents in the two weeks before sampling; (vii) presence of uncontrolled chronic diseases; (viii) drug addiction or heavy smokers (>15 cigarettes/day).

Moreover, women with urogenital infections due to STIs, AV or VVC were excluded after the laboratory testing.

Women underwent a clinical visit at different gestational ages (*i.e.,* 9-13 weeks, first trimester; 20-24 weeks, second trimester; 32-34 weeks, third trimester) and during the puerperium (40-55 days after delivery). Demographic data and clinical information were recorded for each patient.

Two vaginal swabs were collected from each woman. The first one (E-swab, Copan, Brescia, Italy) was used for microbiological diagnostic tests and NS assessment. The second one (collected by a sterile cotton bud swab) was employed for microbiota analysis and for the detection of resistance genes.

Written informed consent was obtained from all subjects, and the study protocol was approved by CEROM (n◦ 2032 of 21 February 2018).

This study was supported by “Fondazione del Monte di Bologna e Ravenna” (Prot. N°329bis/2017).

2.2 Microbiological investigations

Nucleic acids were extracted from vaginal swabs by means of the Versant molecular system (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

Microscopic examination and cultures for AV and VVC, as well as STIs testing, were performed as elsewhere reported (Paper I, pages 56-58).

NS system was used for a preliminary assessment of the vaginal flora composition. Based on this score, women were grouped as follows: “H” group (NS≤3), “I” group (NS 4-6), “BV” group (NS≥7) (Nugent *et al.,* 1991).

2.3 Detection of resistance genes

Starting from the remained DNA eluate, each sample underwent both the detection of resistance genes and the analysis of the vaginal microbiome composition (see paragraph below).

The presence of tetracycline (*tet*) and macrolides (*erm*) resistance genes was assessed by means of in-house end-point PCR. *tet(W)* and *tet(M)* genes confer antimicrobial resistance to tetracyclines by encoding for ribosome protection types of tetracycline resistance proteins, whereas *ermB* and *ermF* are methylase-type erythromycin resistance genes, conferring resistance to macrolides (Chopra and Roberts, 2001; Liu *et al.,* 2009).

A PCR test targeting the conjugative transposon carrying the resistance gene *tet(M)*, namely *tet(M)-tn916*, was performed as well (Jeters *et al.,* 2009).

Each reaction consisted of 45 µL of PCR mix (GoTaq® G2 Master Mix, Promega, Milan, Italy) and 5 µL of target. For each gene, primer sequences, PCR conditions, and amplicon size are reported in detail in Table 12.

The amplification products were subjected to electrophoresis on a 1.5% agarose gel (1.5 g of agarose in 100 mL of 0.5X TAE buffer) added with ethidium bromide and visualized using a transilluminator. BenchTop 100 bp DNA Ladder (Promega) was used as a ladder for reference. A sample was considered positive if the PCR test, after 35 cycles, gave an amplicon of the expected size.

Associations between the presence of resistance genes and available variables (*e.g.,* NS, BMI, age) were searched by t-test or Chi-square test, where appropriate. A p-value<0.05 was considered as statistically significant.

Gene	Primers	PCR conditions	Amplicon Size	Reference
ermB	5'-GAAAAGGTACTCAACCAAATA-3' 5'-AGTAACGGTACTTAAATTGTTTAC-3'	95°C for 10 min; 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min; 72°C for 7 min	639 bp	Milanovic *et al.*, 2017
ermF	5'-CGGGTCAGCCTTTACTATTG-3' 5'-GGACCTACCTCATAGACAAG-3'	95°C for 10 min; 35 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min; 72°C for 7 min	466 bp	Sirichoat *et al.*, 2020
tet(M)	5'-ACCCGTATACTATTTCATGCACT-3' 5'-CCTTCCATAACCGCATTTTG-3'	95°C for 10 min; 35 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min; 72°C for 7 min	1115 bp	Milanovic *et al.*, 2017
tet(W)	5'-GAGAGCCTGCTATATGCCAGC-3' 5'-GGGCGTATCCACAATGTTAAC-3'	95°C for 10 min; 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min; 72°C for 7 min	168 bp	Milanovic *et al.*, 2017
tet(M)-tn916	5'-TACTACCGGTGAACCTGTTTGCCA-3' 5'-TTTAGCCAGCGGTATCAACGAAGC-3'	95°C for 10 min; 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; 72°C for 7 min	472 bp	Jeters *et al.*, 2009

Table 12. List of primers and PCR conditions used for the detection of resistance genes.

2.4 Microbiota analysis

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified and sequenced following the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). Quality filtering, taxonomy assignment, and sample diversity analysis were performed as previously reported (Pages 80-82).

2.5 Data analysis and statistics

Statistical evaluation of α-diversity, β-diversity, pairwise relative abundance and comparison between relative abundances across multiple categories were performed as previously described (Pages 82 and 83).

Correlation between microbial composition at the genus level and presence/absence of each resistance gene was calculated using the point biserial correlation whereas correlation between microbial profiles and BMI was performed using Spearman's rank-based correlation coefficient.

In both the cases, only coefficients showing a p-value of the linear model <0.05 were reported. Statistical evaluations were performed in Matlab (Software version 7.7.0, Natick, MA, USA).

Survival analysis was performed through the R Studio software (version 1.2.1335; R version 3.6.3) using a custom pipeline employing the packages "survival" (v 3.2-3) and "survminer" (v 0.4.9); statistical differences between Kaplan-Meyer curves were determined through a log-rank test.

A p-value<0.05 was considered as statistically significant for all analysis.

3. Results

3.1 Study population and samples

During the study period, a total of 228 vaginal samples were collected from 72 Caucasian women. In particular, 72 samples were collected during the first trimester of pregnancy, 63 during both the second and third trimester, and the remaining 30 during the puerperium.At the beginning of pregnancy, the mean age of women was 31.2±5.3 (range: 21-44 years), whereas the mean BMI was 23.6±3.6 (range: 16.3-32.5 kg/m).

Overall, based on NS, 142 vaginal samples (62.3%) were characterized by a lactobacilli-dominated bacterial composition (NS 0-3), 51 (22.4%) by an intermediate microbiota (NS 4-6), and the remaining 35 (15.3%) harbored a BV-associated microbial composition (NS 7-10). Cases of BV were mainly found during the first trimester of pregnancy (13/35; 37.1%) or during the puerperium (12/35; 34.3%) (Table 13).

	H (n= 142)	**I (n=51)**	**BV (n=35)**
I trimester	33 (23.2%)	26 (51.0%)	13 (37.1%)
II trimester	47 (33.1%)	10 (19.6%)	6 (17.1%)
III trimester	52 (36.6%)	7 (13.7%)	4 (11.4%)
Puerperium	10 (7.1%)	8 (15.7%)	12 (34.3%)

Table 13. Vaginal status, stratified by the gestational age.

3.2 Detection of resistance genes

The most detected resistance gene was *tet(M)*, with 175 cases (76.7%), followed by *ermB* (126 cases, 55.2%), *ermF* (58 cases, 25.4%), and *tet(W)* (33 cases, 14.4%). It is worth noting that in 39 women (17.1%), the majority of which belonged to the H group (34/39; 87.2%), no resistance genes were found. For all the four genes analyzed, the contemporary positivity of at least two of them in the same sample was more common than the detection of only one. Among the most common associations, *ermB* + *tet(M)* (55 cases) and *ermB* + *tet(M)* + *ermF* (31 cases) were found.

More than 80% of *tet(M)*-positive cases (147/175) were associated with the presence of the conjugative transposon *tet(M)-tn916.*

Interestingly, except for *tet(W)*, a significant correlation between the presence of resistance genes and a dysbiotic vaginal status was noticed (Table 14).

	H (n= 142)	I (n=51)	BV (n=35)	p-value
ermB	70 (49.2%)	30 (58.8%)	26 (74.2%)	**0.024**
ermF	27 (19.0%)	15 (29.4%)	16 (45.7%)	**0.003**
tet(M)	101 (71.1%)	42 (82.3%)	32 (91.4%)	**0.021**
tet(W)	20 (14.0%)	6 (11.7%)	7 (20.0%)	0.553

Table 14. Distribution of macrolide and tetracycline resistance genes, stratified for the vaginal status by NS. Statistical analysis was performed by Chi-square test. Statistical significance was reported in bold.

In particular, for *ermB, ermF,* and *tet(M)*, an increasing trend of positivity going from a normal vaginal flora to a condition of BV was noticed. Several cases of BV (10/35; 28.5%) were characterized by the contemporary positivity of *ermB* + *tet(M)* + *ermF* (Table 15).

Resistance genes	n° of cases (tot=228)	BV cases (n=35)
None	39 (17.1%)	1 (2.8%)
tet(M)	51 (22.3%)	6 (17.1%)
ermB	10 (4.4%)	1 (2.8%)
ermF	0 (0.0%)	0 (0.0%)
tet(W)	1 (0.4%)	0 (0.0%)
ermB + tet(M)	55 (24.1%)	7 (20.0%)
ermF + tet(M)	8 (3.5%)	2 (5.7%)
tet(M) + tet(W)	1 (0.4%)	0 (0.0%)
ermB + erm(F)	1 (0.4%)	1 (2.8%)
ermB + tet(W)	0 (0.0%)	0 (0.0%)
ermF + tet(W)	1 (0.4%)	0 (0.0%)
ermB + ermF + tet(M)	31 (13.6%)	10 (28.6%)
ermB + tet(M) + tet(W)	13 (5.7%)	4 (11.4%)
ermB + ermF + tet(W)	1 (0.4%)	0 (0.0%)
ermF + tet(M) + tet(W)	1 (0.4%)	1 (2.8%)
ermB + ermF + tet(M) + tet(W)	15 (6.6%)	2 (5.7%)

Table 15. Prevalence of resistance genes. Prevalence for all cases and those showing a BV vaginal status is shown.

Considering only the women enrolled at the first trimester of pregnancy, the presence of *ermB* was associated with a higher BMI (p=0.003; BMI= 24.6±3.9 *vs* 22.1±2.4). In this context, it is worth mentioning that no significant relationship was noticed between BMI and the vaginal status (H, I, or BV group; p=0.49). On the other hand, the detection of resistance genes was not related to the age of subjects. In agreement with the distribution of BV, it was noticed that most cases of positivity for *ermB, ermF,* and *tet(M)* were found in women at the first trimester of pregnancy (Table 16).

Time point	*ermB* (n=126)	*ermF* (n=58)	*tet(M)* (n=175)	*tet(W)* (n=33)
I trimester	44 (34.9%)	23 (39.6%)	55 (31.4%)	11 (33.4%)
II trimester	33 (26.2%)	11 (19.0%)	46 (26.3%)	8 (24.2%)
III trimester	27 (21.4%)	13 (22.4%)	47 (26.8%)	8 (24.2%)
Puerperium	22 (17.5%)	11 (19.0%)	27 (15.4%)	6 (18.2%)

Table 16. Distribution of macrolide and tetracycline resistance genes, stratified for the gestational age.

3.3 Correlation between resistance genes and vaginal microbiota

α-diversity evaluation showed a significant difference (p=0.001) in the biodiversity of vaginal samples, stratified for the positivity/negativity of resistance genes, for all the metrics (*i.e.,* chao1, observed species, phylogenetic diversity whole tree, Shannon, Good's coverage). Overall, for all the four genes tested, an increased biodiversity for samples positive to resistance determinants was observed (Fig. 39).

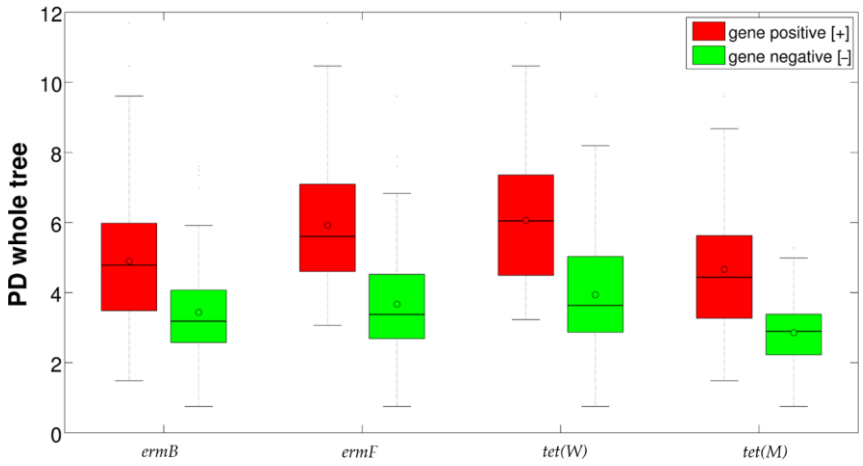

Figure 39. Boxplot of the α-diversities (Faith's phylogenetic diversity metric) of samples positive and negative for the four resistance genes tested. Black lines represent median values; circles represent means.

Stratifying for vaginal status, differences were statistically significant for H women for all resistance genes and metrics, whereas, when evaluating the combined presence of more resistance genes, a tendency (although not statistically significant) towards an increase of biodiversity following the number of resistance genes detected together was observed.

Evaluation of β-diversity confirmed the evidence suggested above, with samples displaying significant differences in composition based on the detection of resistance genes, for both unweighted and weighted Unifrac distances (Fig. 40A). When looking at the single or combined presence of more than one resistance gene per specimen (Fig. 40B), *ermF* and *tet(M)* seemed to be the determinants contributing most to the separation of the vaginal samples. All samples negative for *ermF* and *tet(M)* fell in the leftmost part of the PCoA, whereas *tet(M)*-positive samples were in the rightmost part. Interestingly, at the extreme left of the plot, samples negative to all four genes were found, whereas on the far-right samples positive for all the resistance determinants were positioned, in a sort of 'microbiota' trajectory, shifting from left to right with increasing number of resistance genes (Fig. 40C).

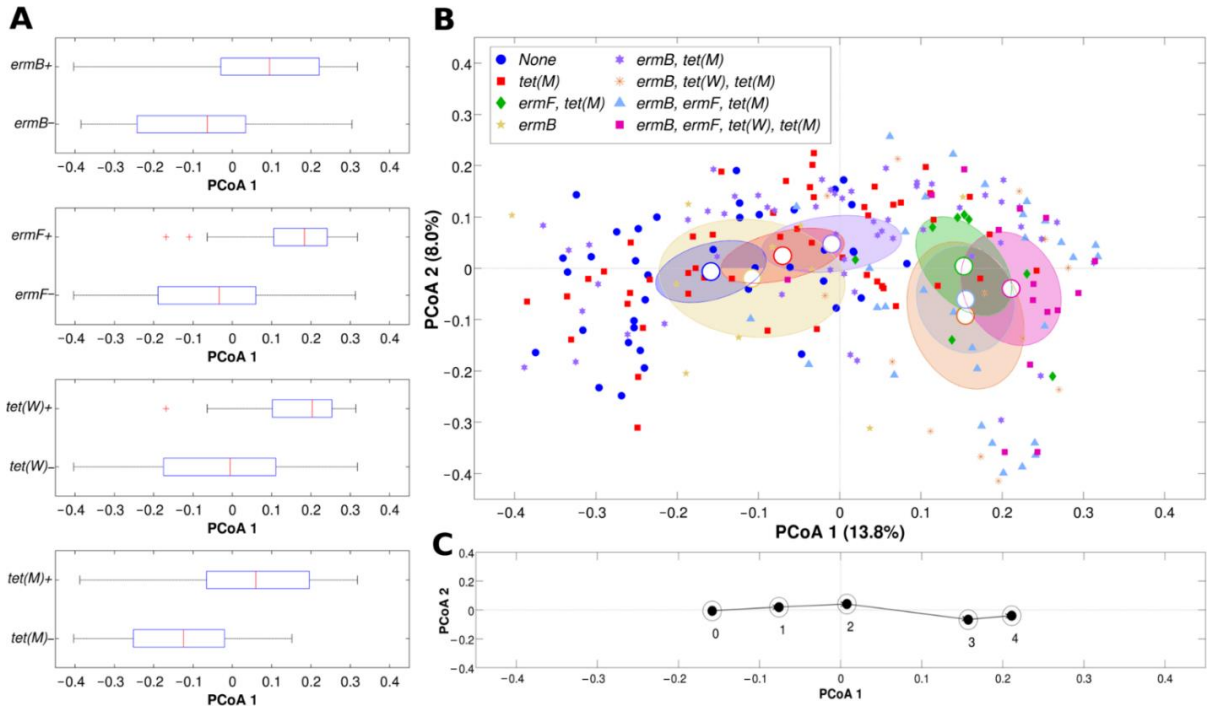

Figure 40. β-diversity analysis of microbial profiles according to the presence of the four resistance genes. (A) Horizontal boxplots representing the distribution of the first component deriving from PCoA, unweighted Unifrac distance, for samples positive or negative for each of the four resistance genes tested. (B) PCoA of unweighted Unifrac distances among samples; each point represents a sample, centroids are positioned at the average coordinate per group, ellipses are 95% confidence estimates of the standard error of the mean; colors indicate a different combination of the presence of the four resistance genes; only combinations with >1 sample per group were considered; the first and second principal coordinate are represented. (C) Plot representing the centroids of the PCoA (unweighted Unifrac) of the samples grouped according to the number of resistance genes; the first and second principal coordinates are represented.

Overall, resistance genes seemed to be associated with a status of dysbiosis, since in samples positive for at least one resistance determinant, a general decrease in *Lactobacillus* spp. and an increase of BV-related genera (*e.g., Prevotella, Gardnerella*) was noticed. Average relative abundances of each taxa showing a significant difference between samples negative or positive for resistance genes are shown in Table 17.

Gene	Phylogenetic name	Average relative abundance (%)		Significance
		Gene positive [+]	Gene negative [-]	
ermB	*Lactobacillus*	67.1	76.6	*
	Prevotella	5	0.8	***
	Atopobium	2.9	2.6	***
	Streptococcus	1.4	1.0	*
ermF	*Lactobacillus*	57.2	76.2	***
	Gardnerella	11.6	9.5	*
	Prevotella	7.3	1.8	***
	Atopobium	3.3	2.6	***
	Streptococcus	1.9	1.0	*
	Prevotella 6	1.3	0.1	***
	Anaerococcus	1.1	0.1	***
tet(W)	*Lactobacillus*	57.4	73.7	**
	Bifidobacterium	4.8	3.1	**
	Prevotella	7.0	2.5	***
	Atopobium	1.1	3.0	**
	Sneathia	1.5	0.6	**
	Prevotella 6	1.6	0.2	***
	Anaerococcus	1.5	0.2	**
tet(M)	*Lactobacillus*	67.8	83.0	***
	Bifidobacterium	4.2	0.8	**
	Prevotella	4.0	0.4	***
	Atopobium	3.4	0.7	***
	Streptococcus	1.6	0.0	***

Table 17. Bacterial genera statistically different between samples showing presence and absence of each resistance gene. "Gene positive [+]/negative [−]" refers to the average relative abundance of each genus on all samples positive/negative for the specified resistance gene. a p-value of Mann-Whitney U-test, with Benjamini-Hochberg FDR correction. *Prevotella* 6 group includes the following species: *P. bergensis, P. colorans, P. corporis,* and *P. salivae*, plus other non-species characterized strains and some uncultured bacteria. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

Samples negative for all the analyzed genes showed the highest average abundance of *Lactobacillus* spp. (86.4%) and the lowest of BV-associated taxa, such as *Prevotella, Atopobium,* and *Anaerococcus*. In contrast, the abundance of *Lactobacillus* spp. dropped drastically (about 50%) in samples positive for all the four genes. It is worth noting that the levels of *Prevotella, Anaerococcus, Streptococcus, Dialister, Sneathia, and Ureaplasma* tended to increase progressively as the number of positive genes per sample increased (Fig. 41).

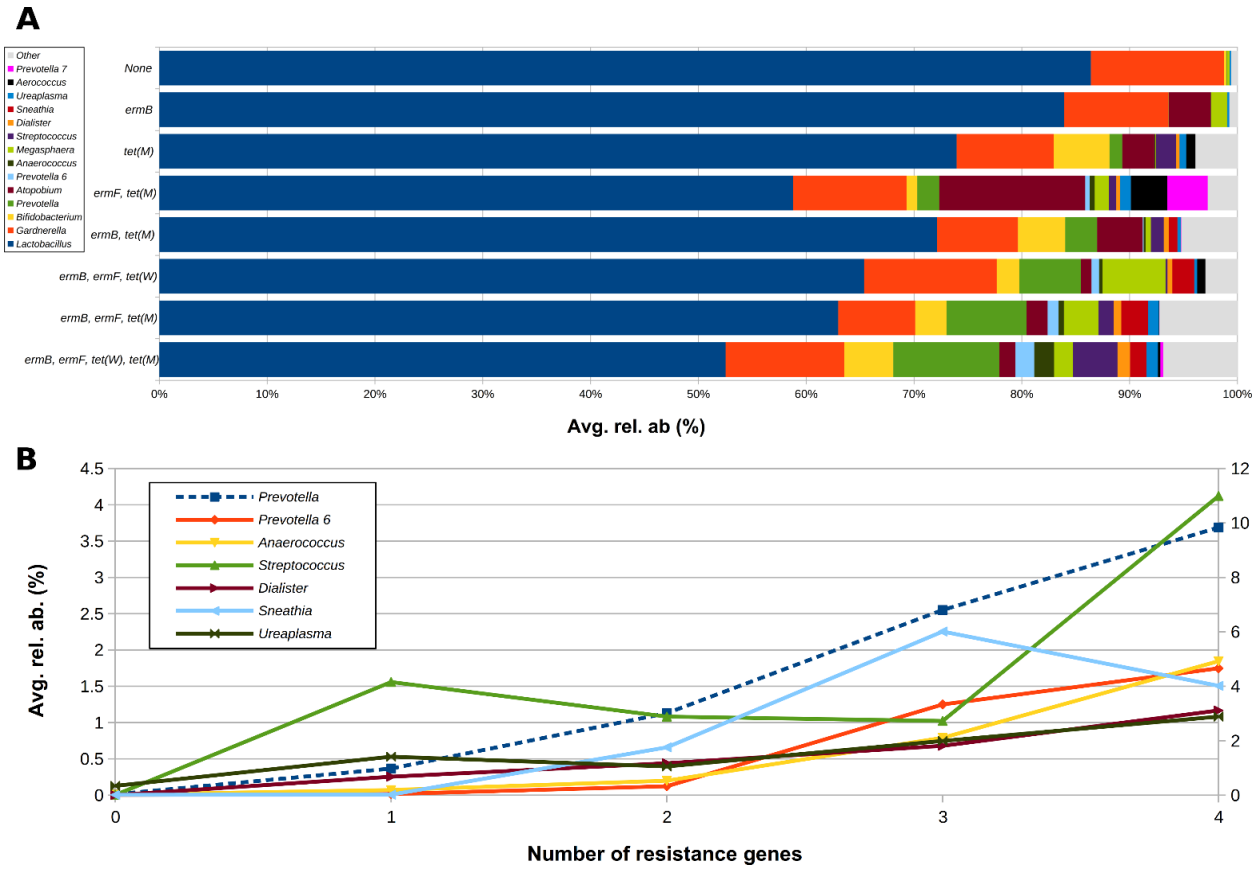

Figure 41. (A) Horizontal barplots of the average relative abundance of the main genera constituting the VMB of the tested women, grouped according to the combination of the different resistance genes; only combinations with >1 sample per group and only genera with avg. rel. ab. >1% in at least one combination were considered. (B) Line plot of the average relative abundance of a selection of genera from the VMB, showing an increasing trend with increasing number of resistance genes per sample; for graphical purposes, *Prevotella* abundance (dashed blue line) is represented on the secondary y-axis.

Stratifying for the vaginal status (H, I, or BV), this tendency was confirmed, particularly for the H group: *Lactobacillus* tended to decrease in samples positive for the resistance genes, whereas *Prevotella* and *Atopobium* tended to increase (Table 18).

Gene	Status	N+[a]	N-[b]	Phylogenetic name	Average relative abundance (%)		Direction
					Gene positive [+]	Gene negative [-]	
ermB	BV	26	9	*Prevotella*	13.0	2.6	↑
				Megasphaera	7.9	1.3	↑
				Sneathia	5.5	0.0	↑
				Prevotella 7	0.0	3.4	↓
				DNF00809	1.0	0.1	↑
	I	30	21	*Prevotella*	6.4	2.5	↑
	H	70	72	*Prevotella*	1.5	0.1	↑
				Atopobium	2.3	0.1	↑
				Streptococcus	0.1	0.3	↓
ermF	BV	16	19	*Sneathia*	6.2	2.3	↑
				Ureaplasma	1.0	0.1	↑
				Prevotella 6	3.3	0.6	↑
				DNF00809	1.3	0.3	↑
				Fastidiosipila	1.1	0.3	↑
	I	15	36	*Lactobacillus*	55.5	79.2	↓
				Prevotella	9.0	3.0	↑
				Atopobium	8.1	2.0	↑
				Streptococcus	4.9	0.6	↑
	H	27	115	*Lactobacillus*	74.3	84.8	↓
				Bifidobacterium	5.2	3.1	↑
				Prevotella	2.8	0.3	↑
				Atopobium	1.3	1.1	↑
tet(M)	BV	32	3	*Gardnerella*	18.13	76.18	↓
				Dialister	1.45	0.02	↑
				Prevotella 6	2.02	0.00	↑
	I	42	9	-	-	-	-
	H	101	41	*Lactobacillus*	79.87	90.06	↓
				Bifidobacterium	4.89	0.00	↑
				Prevotella	1.12	0.03	↑
				Atopobium	1.64	0.00	↑

Gene	Status	N+[a]	N- [b]	Phylogenetic name	Average relative abundance (%)		Direction
					Gene positive [+]	Gene negative [-]	
tet(W)	BV	7	28	*Megasphaera*	13.95	4.30	↑
				Dialister	1.99	1.17	↑
				Prevotella 6	4.51	1.18	↑
				Aerococcus	0.33	1.72	↓
				Fastidiosipila	1.29	0.48	↑
	I	6	45	*Lactobacillus*	51.74	74.93	↓
				Bifidobacterium	9.91	2.76	↑
	H	20	122	*Lactobacillus*	72.33	84.53	↓
				Bifidobacterium	4.96	3.24	↑

Table 18. Bacterial genera statistically different ($p<0.05$, Mann-Whitney U-test) between samples. Presence and absence of each resistance gene, stratified according to women's vaginal status, is shown. For each gene and category, the number of samples is indicated. N+[a], indicates the number of samples positive to each resistance gene per each vaginal status; N+[b], indicates the number of samples negative to each resistance gene per each vaginal status; "Direction", indicates an increase/decrease in gene positive samples with respect to gene negative ones.

The alterations suggested by analyzing the relative abundance of bacterial genera were confirmed by performing point-biserial correlation between genus-level relative abundances and the presence/absence of resistance genes. As shown in Table 19, the positivity to any resistance gene was negatively correlated to *Lactobacillus* spp. abundance and positively to *Prevotella, Dialister*, and *Anaerococcus*. Higher levels of *Atopobium* were associated with the positivity of all genes, except for *tet(W)*, whereas *Sneathia* was positively related to *ermB* and *tet(W)*.

A positive association between *Gardnerella*, *ermF*, and *tet(W)* was also found.

Interestingly, *Bifidobacterium* was positively associated with both the tetracycline resistance determinants.

Genera	Resistance gene			
	ermB	***ermF***	***tet(W)***	***tet(M)***
Lactobacillus	**-0.136**	**-0.239**	**-0.165**	**-0.185**
Gardnerella	-	**0.046**	0.074	-
Bifidobacterium	-	-	0.051	0.123
Prevotella	0.264	0.304	0.199	0.192
Atopobium	**0.016**	**0.029**	0.062	0.1
Streptococcus	**0.029**	0.066	-	0.104
Sneathia	0.205	-	0.093	-
Alloscardovia	-	-	-	0.105
Ureaplasma	-	0.175	-	-
Dialister	0.154	0.2	0.153	0.201
Prevotella 6	0.099	0.306	0.271	0.125
Aerococcus		**0.035**	**0.003**	0.076
Anaerococcus	0.067	0.28	0.3	0.125

Table 19. Correlation between the relative abundance of vaginal bacterial genera and presence of macrolide and tetracycline resistance genes. Only genera with an average relative abundance >0.4% were reported. "-" indicates that the p-value of the linear model for correlation calculation was >0.05. Significant values were reported in bold.

Since the positivity to a certain resistance gene could vary over time, a survival analysis was performed over the gestation and post-partum weeks to further investigate how the bacterial groups identified contributed to the positivity status of women during pregnancy.

Higher abundances of the *Lactobacillus* genus seem to have a protective role towards the incidence of resistance genes (for *tet(W)* in particular), which appeared less frequently when the vaginal microbiome was dominated by it. In contrast, higher abundances of BV-related bacteria (such as *Megasphaera, Prevotella,* and *Ureaplasma*) show an opposite trend: the higher the bacterial abundance, the higher the probability of manifesting resistance genes (Fig. 42).

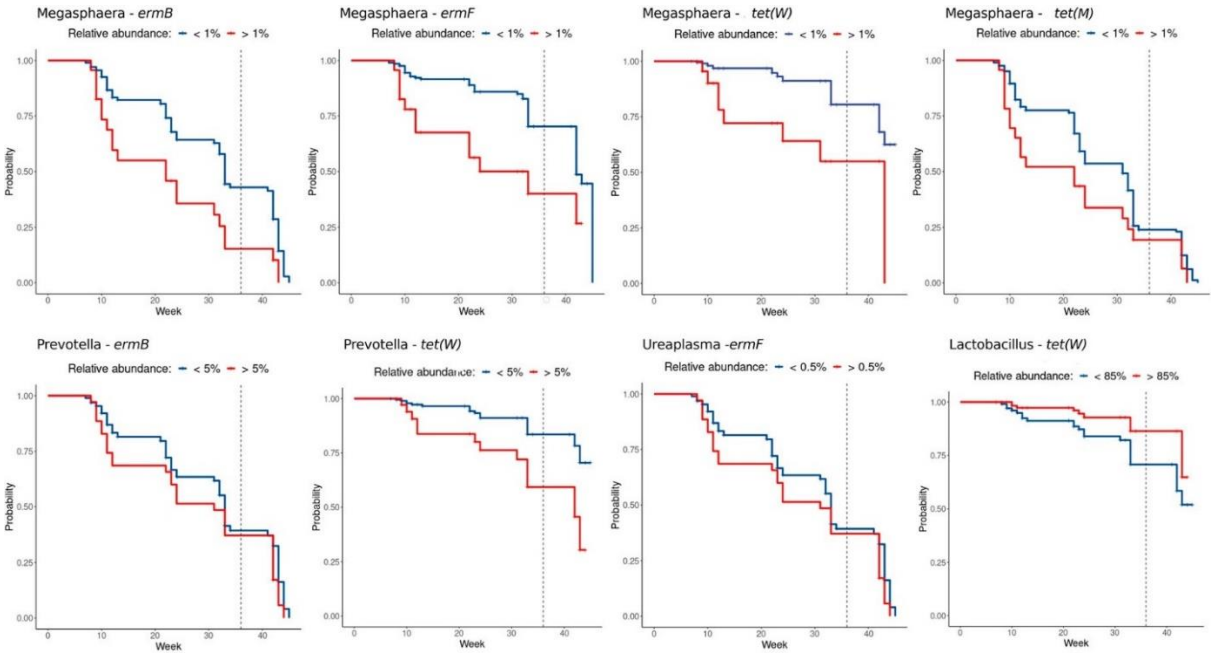

Figure 42. Kaplan-Meier curves of resistance gene positivity. Detection of a resistance gene was exploited as a survival event among samples above (red) or under (blue) bacterial relative abundance thresholds. Crosshairs represent censored observations. All curves reported have a significant log-rank separation (p-value<0.05).

3.4 Correlation between BMI and vaginal microbiota composition

In general, only a few significant correlations were observed between BMI (considered only for women during the first trimester of pregnancy) and the composition of the VMB. At the phylum level, there was a slightly negative correlation between BMI and *Tenericutes* abundance (r=-0.24). At the family and genus level, results have shown that BMI was negatively correlated with the *Leptotrichaceae* family (r=-0.28) and *Sneathia* genus (r=-0.31) and positively correlated with the *Prevotella* genus (r=0.24).

4. Discussion

In this study, the distribution of selected tetracycline and macrolide resistance genes in the VMB of pregnant women at different gestational ages was assessed. In particular, the presence of *ermB*, *ermF*, *tet(W)*, and *tet(M)* genes in the vaginal ecosystem of women during the three trimesters of pregnancy and puerperium was analyzed, deciphering the correlations between the presence of resistance determinants and the abundance of vaginal bacterial taxa.

At first, results have shown that some resistance genes were very common in the vaginal environment of pregnant women, with the prevalence of *tet(M)* and *ermB* exceeding 55%.

Other resistance determinants, namely *ermF* and *tet(W)*, were less common, showing 25% and 14% prevalence values, respectively. Recently, Roachford and colleagues assessed the cervicovaginal resistome in a cohort of Afro-Caribbean women by means of whole genome shotgun metagenomics. They confirmed that the most abundant resistance determinants are related to tetracyclines (*tet*; about 50%) and macrolides (*erm*; about 15%), with genes encoding for tetracycline-resistant ribosomal protection proteins being the most common (Roachford, *et al.*, 2021).

The distribution of resistance genes found in the present cohort was similar to the one described for the human gut microbiomes (*i.e.,* fecal samples), with a significantly high prevalence of *ermB* and *tet(M)* genes (Milanović *et al.,* 2017). Thus, it is possible to speculate that the gastro-intestinal tract could serve as a reservoir of bacteria-related resistance genes, able to easily reach the vaginal environment by means of microbial translocation (Petricevic *et al.,* 2014b).

This aspect can partly explain the occurrence of resistance genes during puerperium, since a significant vaginal 'contamination' from intestinal-derived microbes occurs during labor and delivery.

Significant data emerged when the presence of resistance genes was related to the vaginal status (*i.e.,* H, I, and BV groups based on NS) and to the bacterial composition of the VMB (*i.e.,* 16S rRNA gene sequencing).

It was noticed that, except for *tet(W)*, the detection of resistance determinants was significantly associated with BV status, with the prevalence of resistance genes increasing along with the worsening of the vaginal dysbiosis (*i.e.,* going from H to I to BV status).

Interestingly, a higher number of combined resistance genes (*i.e.,* more than one resistance gene in the same sample) was related to a greater distance from a normal microbiota. Thus, an increased polymicrobism, typical of severe BV conditions, could led to an easier occurrence of multiple resistance genes at the same moment.

In agreement with these results, the presence of resistance genes was more common when, during pregnancy, the conditions of vaginal dysbiosis are more frequent (*i.e.,* first trimester and puerperium).

As expected, the positivity of resistance genes was positively related with several BV-related taxa (*e.g., Prevotella, Dialister, Anaerococcus, Atopobium,* and *Gardnerella)* and negatively related to the abundance of vaginal *Lactobacillus* spp. In line with these findings, the present data demonstrated that a high predominance of *Lactobacillus* spp. in the vaginal environment (>85%) during pregnancy is associated with a lower risk of *tet(W)* gene detection, whereas the presence of several BV-associated bacteria significantly increase, in time, the chance of positivity of one or more resistance determinants (*e.g.,* the presence of *Megasphaera* >1% increases the risk of positivity for all analyzed genes, whereas *Prevotella* >5% significantly increases the risk for *ermB* and *tet(W)*).

Even though culture-based approaches will be needed to assess the exact distribution of resistance genes among bacterial genera, is possible to speculate that each genus is characterized by a different antimicrobial resistance pattern, linked to a different bacterial plasticity and different responses to antibiotic selective pressure.

All these data strengthen the idea that a lactobacilli-dominated microbiota is associated with vaginal eubiosis and wellbeing, whereas a BV condition can negatively affect the women's health, being a state that broadly correlates with increased risk of infection, disease, and poor reproductive and obstetric outcomes (Kroon *et al.,* 2018).

As indicated by the present results (*i.e.,* high association between *tet(M)* and the conjugative transposon *tet(M)-tn916*), macrolide and tetracycline resistance genes can be linked to mobile elements, thus favoring horizontal transfer of resistance determinants from commensal vaginal inhabitants to pathogens (Chopra and Roberts, 2001).

It was demonstrated that exposure to tetracycline results in an elevated *tn916* transposition level (Manganelli *et al.,* 1995). This phenomenon likely arises due to heightened expression of *tet(M)*, induced by the selective pressure exerted by the antibiotic. In the primate VMB, approximately 50% of *tet(M)* genes were observed in conjunction with *tn916*, while within the human VMB, this association was observed in *E. faecalis* strains isolated from vaginal samples (Jeters *et al.,* 2009; Sirichoat *et al.,* 2020b).

Moreover, during delivery, microbial communities can be transferred from the mother's vaginal niche to the newborn gut, thus affecting the infant's microbiome development and future health (Dominguez-Bello *et al.,* 2010).

Along with microbial transfer, newborns can acquire bacteria-associated resistance genes. As previously shown newborns acquire tetracycline antibiotic resistance genes from mothers at birth, especially *tet(M)* and *tet(O)* in case of vaginal delivery (Alicea-Serrano *et al.,* 2013).
Although the strongest correlations were found for BV-associated genera, it is worth mentioning that even 'health-promoting' microorganisms can harbor resistance determinants. In agreement with our results, it has been shown that resident *Bifidobacteria* can possess genes conferring resistance to tetracyclines. However, it should be remembered that *Bifidobacteria* are typical beneficial commensals inhabiting the human intestine and are only minority components of the vaginal consortium (Cao *et al.,* 2020).
It is worth noting that the presence of the *ermB* gene was associated with a higher BMI at the beginning of pregnancy. Moreover, *ermB* was found to be specially correlated with higher vaginal levels of *Prevotella* genus, in turn associated with higher BMI levels. In this context, it has been shown that host obesity significantly increased the diversity of the VMB in association with *Prevotella*, whose relative abundances are strongly associated with BV (Si *et al.,* 2016).
In conclusion, a sort of "vaginal fingerprint" was find, being different types of microbiota composition associated with peculiar resistance profiles. If a 'normal' vaginal ecosystem is poor in or free of resistance genes, a condition of dysbiosis (*i.e.,* BV) is strongly associated with the presence of more than one determinant of antimicrobial resistance. These data could open new perspectives for promoting vaginal health during pregnancy, with the aim of maintaining a lactobacilli-dominated vaginal ecosystem, in turn depleted of antimicrobial resistance genes.
Further studies are needed for a deeper comprehension of the potential origin and 'sources' of the antimicrobial resistance genes (*e.g.,* food, water, past use of antibiotics, or microbiome 'sharing' with partner) (Garofalo *et al.,* 2007).

IV. Torquetenovirus in pregnancy: correlation with vaginal microbiome, metabolome and pro-inflammatory cytokines

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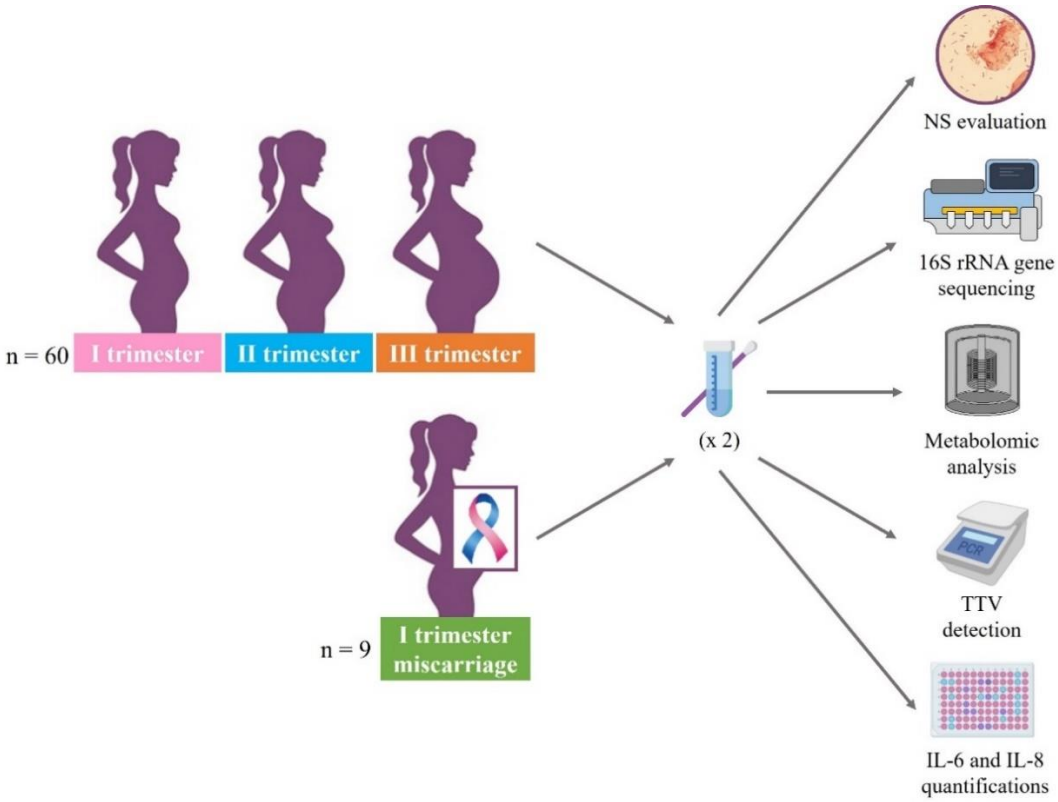

Figure 43. Graphical abstract.

1. Abstract

Torquetenovirus (TTV) is a negative sense, single-stranded DNA virus present in many body fluids of apparently healthy individuals. At present, it is considered a non-pathogenic endogenous virus. TTV can be detected in the vagina of pregnant women, its abundance being modulated with the extent of immune system activation. Until now, there is only scarce information regarding the association between TTV and the composition of the vaginal environment.

Therefore, this study aimed to assess the presence of TTV in the vaginal ecosystem of a cohort of white women with a normal pregnancy (n=60) at different gestational stages (first, second and third trimester) and in 9 subjects suffering a first trimester miscarriage.

For each woman, (i) the presence and titer of TTV, (ii) the vaginal bacterial composition by means of NS and 16S rRNA gene sequencing, (iii) the vaginal metabolic profiles through ^{1}H-NMR spectroscopy, and (iv) the vaginal concentration of two pro-inflammatory cytokines (IL-6 and IL-8) were determined.

More than one third of women were found negative for TTV at all gestational stages. Although not statistically significant, the positivity for TTV dropped from 53.3% in the first to 36.6% in the third trimester. TTV loads varied greatly among vaginal samples, ranging between 2×10 and 2×10^{5} copies/reaction. No difference in TTV prevalence and loads was observed between women with normal pregnancies and miscarriages. The presence of TTV was more common in women with a higher vaginal leucocyte count ($p=0.02$).

The levels of IL-6 ($p=0.02$), IL-8 ($p=0.03$), propionate ($p=0.001$) and cadaverine ($p=0.006$) were significantly higher in TTV-positive samples. TTV titer was positively correlated with the concentrations of 4-hydroxyphenyllactate ($p<0.0001$), isoleucine ($p=0.01$) and phenylalanine ($p=0.04$).

TTV-positive samples were characterized by a higher relative abundance of *Sneathia* ($p=0.04$) and *Shuttleworthia* ($p=0.0009$). In addition, a trend toward a decrease of *L. crispatus* and *L. jensenii*, and an increase of *L. iners* was observed for TTV-positive samples. In conclusion, results have shown that TTV is quite common in women with normal pregnancy outcomes, representing a possible predictor of local immune status.

2. Materials and methods

2.1 Study group and sample collection

From April 2019 to March 2021 all the Caucasian pregnant women attending the Family Advisory Health Centers of Ravenna (Italy) were considered eligible for the study.

Exclusion criteria included the following: (i) age<18 years; (ii) being positive for HIV infection; (iii) BMI>33; (iv) medically assisted procreation; (v) use of antimicrobials in the month prior the enrollment; (vi) use of vaginal topical agents in the 2 weeks before the enrollment; (vii) presence of chronic diseases; (viii) drug addiction or heavy smokers (>15 cigarettes/day).

Furthermore, women were excluded if a diagnosis of STIs, VVC and AV.

For each woman a clinical visit was performed at gestational stages 9-13 weeks (first trimester), 20-24 weeks (second trimester), and 32-34 weeks (third trimester).

At each time point, two vaginal swabs were collected: the first one (E-swab, Copan, Brescia, Italy) was used for microbiological tests. The second was collected with a sterile cotton bud, re-suspended in 1 ml of sterile saline, and the cell-free supernatants was used for metabolomic analysis and cytokine detection, whereas cell-pellets were employed both for TTV detection and vaginal microbiome profiling.

The study protocol was approved by the CEROM (no 2032 of 21st February 2018) and it was carried out in accordance with the Declaration of Helsinki. Each woman gave written informed consent to participate in the study.

2.2 Microbiological investigations

A commercial NAAT was used to exclude the presence of STIs, whereas VVC and AV diagnosis was performed by microscopic examination and microbial cultures, as elsewhere described (Pages 56-58).

Based on NS classification, women were categorized into 3 groups: “H” (NS≤3), “I” (NS 4-6), “BV” (NS≥7).

The presence of vaginal leukocytes (white blood cells: WBCs) was evaluated after visualization of a minimum of five fields under light microscopy at 400×. Samples were categorized as “minimal or no inflammation” in case of <5 WBCs in all visualized fields or as “significant inflammation” in presence of ≥5 WBCs in at least one field visualized (Geisler *et al.,* 2004).

2.3 Vaginal microbiota profiling

Nucleic acids were extracted from vaginal swabs by means of the Versant molecular system (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

The microbiota analysis was performed trough the sequencing of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene (Illumina, San Diego, CA, USA).

Quality filtering, taxonomy assignment, and sample diversity analysis were performed as previously reported (Pages 80-82).

2.4 Detection of Torquetenovirus in the vaginal ecosystem

Starting from the remaining DNA eluate, all the vaginal swabs were tested for the presence of TTV using a Quantitative Real-Time Polymerase Chain Reaction (qPCR) (Tozetto-Mendoza *et al.,* 2022).

The PCR reaction mixtures (final volume: 25 µL) included 12.5 µL of Platinum Quantitative PCR Supermix-UDG with ROX (Invitrogen, Waltham, MA, USA), 250 nM of primers, 62 nM of the probe, and 2.5 µL of template. All PCR reactions were performed with the following cycling conditions using a QuantStudio Real-Time PCR system (Applied Biosystems, Waltham, MA, USA): 2 min at 50°C, 15 s at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C.

A standard curve with known amounts of a synthetic oligonucleotide was used for TTV quantification. Results were expressed as $\log_{10}$ DNA copies/reaction.

2.5 Cytokine detection

The concentrations of IL-6 (pg/ml) and IL-8 (pg/ml) were determined on the cell-free supernatants of the vaginal swabs by means of commercial ELISA assays (Simple Plex Human IL- 6 and IL-8 Cartridges, R&D Systems, Minneapolis, MN, USA), following manufacturer's instructions.

2.6 Metabolome analysis

Metabolomic analysis was performed using ^{1}H-NMR spectroscopy starting from 700 µL of the cell-free supernatants of the second vaginal swabs, as reported in Paper I, pages 58 and 59.

2.7 Data analysis and statistics

Statistical analyses were conducted by using GraphPad Prism software (version 5.02; GraphPad Software, San Diego, CA, USA) and MATLAB (Software version 7.7.0, Natick, MA, USA). Fisher's exact test was used to compare categorical data (*i.e.,* presence of TTV stratified by the vaginal status), whereas ANOVA test, followed by Tukey's multiple comparisons test, was employed to compare TTV loads among the different categories. TTV loads were correlated to metabolite concentrations by calculating the Spearman correlation coefficient.

Statistical evaluation of α-diversity, β-diversity, pairwise relative abundance, and comparison between relative abundances across multiple categories were performed as previously described (Pages 82 and 83). Statistical significance was considered as p-value<0.05.

Correlation between microbial composition at the genus level and presence/absence of TTV was calculated using the point biserial correlation, whereas the correlation between microbial profiles and TTV loads (log-transformed TTV copy number) was performed using Spearman's rank-based correlation coefficient. Only coefficients showing a p-value of the linear model <0.05 were considered.

3. Results

3.1 Study population

A total of 60 pregnant women with a median age of 31 years (min-max: 21-44) completed the study. In addition, 9 women (median age: 35 years; min-max: 23-41) who had a spontaneous first trimester miscarriage (gestational age: 11-13 weeks) were also included.

Overall, excluding specimens from women with miscarriages, 118 vaginal samples (65.6%) were characterized by a lactobacilli-dominated flora (NS≤3), 43 (23.9%) by an intermediate microbiota (NS 4-6), and the remaining 19 (10.5%) had a BV-associated bacterial composition (NS≥7).

It is noteworthy that a significant reduction of dysbiotic cases was noticed (p=0.002) when moving from the first to the third trimester of pregnancy.

Finally, women who suffered a first trimester miscarriage (n=9) were mainly characterized by a condition of dysbiosis (*i.e.,* 6 with an intermediate microbiota, 2 with a BV condition and one with I condition).

3.2 Detection of Torquetenovirus

Overall, considering all the specimens belonging to the 60 women who completed the study, 42.7% (77/180) of the tested vaginal swabs were positive for TTV.

Stratifying the samples by the gestational age (n=60 per time point), there was a non-significant decrease in TTV positivity between samples obtained in the first, second and third trimester. In fact, 32 (53.3%) were TTV-positive in the first trimester, 23 (38.3%) in the second, and 22 (36.6%) in the third. No differences in TTV prevalence were found when comparing normal pregnancies with miscarriages: in fact, TTV was detected in about half (5/9; 55.5%) of the women who suffered a first trimester miscarriage.

Considering each subject throughout the pregnancy, more than one third of women were found negative for TTV at all three trimesters of pregnancy (25/60; 41.6%). Conversely, 26.6% of women (16/60) were positive for TTV at each trimester. Thirteen of the remaining cases were characterized by a TTV positivity in the first and/or second trimester, with a negativity at the end of pregnancy.

TTV loads (expressed as $\log_{10}$ DNA copies/reaction) varied greatly among vaginal samples, ranging between 1.4 (about 26 copies/reaction) and 5.3 (about 209,000 copies), with a mean (±

standard deviation, SD) of 3.06±0.96. No significant difference in TTV titer was found between women with a miscarriage and women with a normal pregnancy at the first trimester (2.8±0.8 vs. 2.1±0.5; p=0.06).

3.3 Correlations between Torquetenovirus and available variables

Considering only the first trimester of pregnancy, no difference in the median age among TTV-positive (30 years) and TTV-negative (32 years) women was found (p=0.36). Neither the presence of TTV (p=0.65) nor TTV loads were associated with a condition of BV (BV: 2.7±0.7; I: 2.8±0.9; H: 3.2±0.9; p=0.10). Conversely, the presence of TTV was significantly more common in women with a higher vaginal WBC count (37.7 vs. 57.7%; p=0.02).

In line with these findings, the levels of IL-6 (median, range: 0.81, 0.01-57.8 vs. 0.41, 0.0-31.2 pg/mL; p=0.02), as well as IL-8 (1,901, 34-35,783 vs. 652, 11.3-43,248; p=0.03) were significantly higher in TTV-positive vaginal samples.

Moreover, a trend in the correlation between TTV loads and IL-8 levels (R=0.19; p=0.09) was observed. The detection of vaginal *Candida* spp. was not significantly associated with the vaginal presence of the virus nor with higher TTV loads (p>0.5).

On the contrary, several correlations were observed between TTV presence/loads and the levels of some vaginal metabolites. In relation to this, it is worth mentioning that a total of 63 metabolites were detected in the vaginal cell-free supernatants, mainly belonging to the groups of SCFAs, organic acids, amino acids, and biogenic amines.

In particular, TTV-positive samples were characterized by higher levels of propionate (median, range: 0.01, 0.001-0.36 vs. 0.007, 0.001-0.23 mM; p=0.001) and cadaverine (0.008, 0.002-0.05 vs. 0.006, 0.001–0.06 mM; p=0.006), compared to TTV-negative ones.

Moreover, TTV titer was positively correlated with the levels of 4-hydroxyphenyllactate (p<0.0001), isoleucine (p=0.01) and phenylalanine (p=0.04). Vaginal molecules showing a negative correlation with TTV loads included benzoate (p=0.008), inosine (p=0.002), and creatine (p=0.004) (the full list is displayed in Table 20).

Metabolite	Sperman r	p-value
Xanthine	-0.276	0.01
Benzoate	-0.298	0.008
Phenylalanine	0.231	0.04
Tyramine	-0.259	0.02
4-Hydroxyphenyllactate	0.508	<0.0001
Inosine	-0.336	0.002
Uridine	-0.2285	0.04
Uracil	-0.265	0.02
Methanol	-0.256	0.02
Ethanolamine	-0.261	0.02
Creatinine	-0.2780	0.01
Creatine	-0.320	0.004
Asparagine	-0.288	0.01
TMA	-0.301	0.007
2,3-Butanediol	-0.285	0.01
Propionate	-0.366	0.001

Table 20. List of the vaginal molecules, whose concentration was found related to TTV loads.

3.4 Correlation between Torquetenovirus and vaginal microbiome profiling

For microbiota analysis, only samples with a number of reads >5000 (n=175) were considered, in order to have a reliable picture of the microbial composition. TTV-positive and negative samples showed no statistical difference ($p>0.05$) on both biodiversity (α-diversity) or microbial composition (β-diversity) for all the metrics considered (Fig. 44).

Moreover, the analysis of the bacterial relative abundances did not reveal any major changes in the bacterial groups between TTV+ and TTV- samples. Nevertheless, a significant difference in two low-abundant taxa (average relative abundance <1%), such as a higher abundance of *Sneathia* (0.92 vs. 0.29%; p=0.04) and *Shuttleworthia* (0.89 vs. <0.01%; p=0.0009) in TTV-positive samples was noticed.

The point-biserial correlation confirmed the significant positive correlations between TTV presence and the abundance of *Sneathia* (r=0.123) and *Shuttleworthia* (r=0.125). Spearman analysis showed a significant correlation between TTV loads and the abundance of *Sneathia* (r=0.166) and *Shuttleworthia* (r=0.313). Although not statistically significant, TTV-positive

samples were characterized by a decrease of *L. crispatus* (32 vs. 41%) and *L. jensenii* (7 vs. 10%), as well as by an increase of *L. iners* (25 vs. 15%), compared to TTV-negative ones.

In this context, it is worth mentioning that a negative correlation between the levels of pro-inflammatory cytokines and both *L. crispatus* (r=-0.354 and r=-0.277 for IL-6 and IL-8, respectively) and *L. jensenii* (r=-0.309 and r=-0.171 for IL-6 and IL-8, respectively) was found.

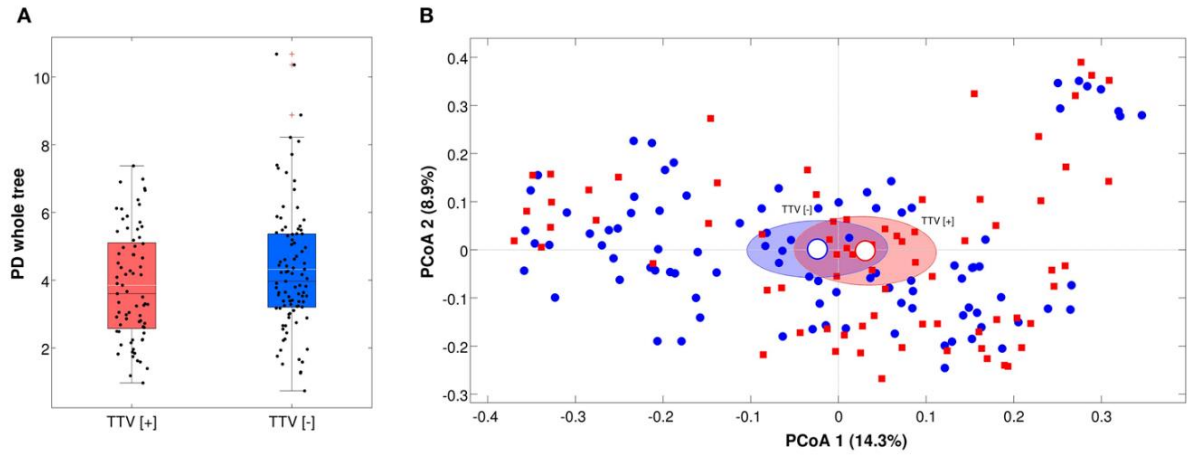

Figure 44. (A) Boxplot of the α-diversity according to Faith's phylogenetic diversity metric, grouped by TTV presence or absence. Each point represents a sample; median of the distributions are in black, whereas means are in white; (B) PCoA of the β-diversity values according to unweighted Unifrac distances. Each point represents a sample; data points are colored according to the presence or absence of TTV; ellipses represent the 95% SEM-based confidence intervals; the first and the second coordinates are represented.

4. Discussion

The presence and role of TTV in pregnant women is still only scarcely available, so this study aimed to provide new insights into the dynamics of TTV in the vaginal ecosystem during pregnancy. TTV presence and loads in a cohort of Caucasian pregnant women at different gestational stages were explored, and their correlation with the vaginal bacterial composition, with the vaginal metabolic profiles and with the vaginal concentration of two pro-inflammatory cytokines was assessed.

In line with previous findings (Tozetto-Mendoza *et al.,* 2022), TTV was quite common in women with normal pregnancy outcomes, with a prevalence ranging from 53% at the first trimester to 36% at the third. This is not surprising if its consider that TTV has been identified both in peripheral blood and in cervical/vaginal fluids (Maggi and Bendinelli, 2010; Chan *et al.,* 2001; Tozetto-Mendoza *et al.,* 2020).

No difference in TTV prevalence and loads was observed between women with normal pregnancies and miscarriages. Even though further studies including a larger cohort of women are needed for a better comprehension of TTV role during pregnancy, these results seem to indicate that TTV does not have clinical outcome consequences.

Interestingly, TTV presence was positively related to the number of vaginal WBC, as well as to higher concentrations of vaginal proinflammatory cytokines (*i.e.,* IL-6 and IL-8). This result is not surprising if its consider that TTV has been recognized as a predictor of local immune status (Focosi *et al.,* 2016). It has been speculated that, in the vaginal ecosystem, TTV loads are related to the presence of activated lymphoid cells, being the vaginal TTV an additional indicator of the local "immune" status in pregnant women (Brundin *et al.,* 2020; Tozetto-Mendoza *et al.,* 2022).

Other interesting data emerged when TTV presence and loads were related to the vaginal bacterial composition. The most significant results included (i) the association between TTV and higher levels of *Sneathia* and *Shuttleworthia*, (ii) a trend toward a decrease of *L. crispatus* and *L. jensenii*, as well as an increase of *L. iners* in TTV-positive samples.

In this context, it is worth underlining that a significant negative correlation between *L. crispatus* and *L. jensenii* and both IL-6 and IL-8 was observed.

Since TTV replication preferentially occurs in activated lymphoid cells, and immune system activation is at its lowest level in case of a *L. crispatus*-dominated vaginal microbiome, is possible to speculate that the absence/decrease of TTV is linked to the reduction of lymphoid cells or their pro-inflammatory molecules when *L. crispatus* is predominant (Brundin *et al.,*

2020). On the contrary, the presence of *L. iners* is associated with a higher expression of genes involved in leukocyte mediated immunity and activation, being potentially associated with higher levels of TTV in the vaginal environment (Mohd Zaki *et al.,* 2022).

The association between TTV and higher levels of *Sneathia* and *Shuttleworthia* probably goes in the same direction. In fact, genital inflammation can be linked to specific BV-related microorganisms, including *Sneathia* (Dabee *et al.,* 2021). In this context, the association between TTV-positive samples and higher concentrations of propionate and cadaverine could reflect these findings. In fact, these two molecules (belonging respectively to SCFAs and biogenic amines) are common markers of vaginal dysbiosis, typically produced by BV-related anaerobes, when a reduction of lactobacilli is present.

In addition, a highly significant correlation between TTV loads and the levels of 4-hydroxyphenyllactate was observed. This metabolite is produced by lactic acid bacteria and exerts both antifungal properties and radical scavenging activities (O'Hanlon *et al.,* 2013).

Further studies are needed to understand the exact role and origin of 4-hydroxyphenyllactate and if this molecule can possess antiviral activities against TTV.

The present study has some limitations: (i) for TTV detection, cell pellets after a low centrifugation step was tested instead of supernatants; thus, belonged to mainly detected the presence of the virus inside the host cells, (ii) the association between TTV and specific microbes of the vaginal ecosystem (*i.e.*, *Sneathia* and *Shuttleworthia*) could be a coincidental finding. Additional studies are needed to understand if there is a real biological cooperation or if these microbes are simple bystanders.

In conclusion, in agreement with previous reports, TTV was commonly found in the vaginal ecosystem of pregnant women, representing a possible predictor of local immune status (Focosi *et al.,* 2016; Tozetto-Mendoza *et al.,* 2020). In fact, its detection and loads vary with local vaginal conditions, being more common in presence of higher levels of leukocytes, higher levels of BV-related microbes, and lack of *L. crispatus* dominance.

V. *Gardnerella vaginalis* clades in pregnancy: new insights into the interactions with the vaginal microbiome

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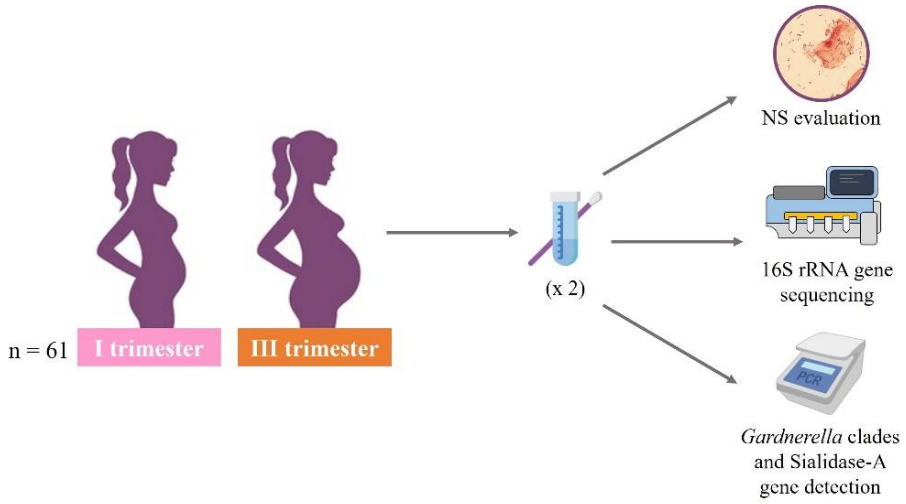

Figure 45. Graphical abstract.

1. Abstract

Gardnerella vaginalis (GV) is an anaerobic bacterial species involved in the pathogenesis of BV, a condition of vaginal dysbiosis associated with adverse pregnancy outcomes. GV strains are categorized into four clades, characterized by a different ability to produce virulence factors, such as sialidase.

The distribution of GV clades and sialidase genes in the vaginal ecosystem of a cohort of pregnant women were investigated, assessing the correlations between GV clades and the whole VMB.

A total of 61 Caucasian pregnant women were enrolled. Their vaginal swabs, collected both at the first and third trimester of pregnancy, were used for (i) evaluation of the vaginal status by NS, (ii) vaginal microbiome profiling by 16S rRNA sequencing, (iii) detection and quantification of GV clades and sialidase A gene by qPCR assays.

DNA of at least one GV clade was detected in most vaginal swabs, with clade 4 being the most common one. GV clade 2, together with the presence of multiple clades (>2 simultaneously), were significantly associated with a BV condition.

Significantly higher GV loads and sialidase gene levels were found in BV cases, compared to the healthy status. Clade 2 was related to the major shifts in the vaginal microbial composition, with a decrease in *Lactobacillus* and an increase in several BV-related taxa. As the number of GV clades detected simultaneously increased, a group of BV-associated bacteria tended to increase as well, while *Bifidobacterium* tended to decrease. A negative correlation between sialidase gene levels and *Lactobacillus*, and a positive correlation with *Gardnerella, Atopobium, Prevotella, Megasphaera*, and *Sneathia* were observed.

The present results added knowledge about the interactions of GV clades with the inhabitants of the VMB, possibly helping to predict the severity of BV and opening new perspectives for the prevention of pregnancy-related complications.

2. Materials and methods

2.1 Study group and sample collection

From April 2019 to March 2021, a total of 61 Caucasian pregnant women attending Family Advisory Health Centers of Ravenna (Italy) for prenatal care were enrolled for the study.
The exclusion criteria were: (i) age <18 years; (ii) HIV positivity; (iii) BMI>33; (iv) medically assisted procreation; (v) use of antibiotics in the last month; (vi) use of vaginal douches or topical agents in the last two weeks; (vii) presence of uncontrolled chronic diseases; (viii) drug addiction or heavy smokers (>15 cigarettes/day).
Moreover, women with STIs, VVC and AV were excluded after the laboratory testing.
A vaginal swab (E-swab, Copan, Brescia, Italy) from each woman was collected at gestational stages between 9-12 weeks (first trimester) and between 31-34 weeks (third trimester).
For all patients, demographic data and information about urogenital symptoms were recorded.
A written informed consent was obtained from all subjects and the study protocol was approved by CEROM (n° 2032 of 21st February 2018).

2.2 Microbiological investigations

Nucleic acids were extracted from vaginal swabs by means of the Versant molecular system (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).
STIs analysis, microscopic examination and semi-quantitative cultures were performed as previously reported (Pages 56-58).
The composition of the VMB was evaluated by NS, and women were divided into three groups: “H” (NS≤3), “I” (NS 4–6), “BV” (NS≥7) (Zozaya-Hinchliffe *et al.,* 2010).

2.3 Vaginal microbiota profiling

Starting from the remaining eluate of the Versant PCR plate, the microbiota analysis was performed trough the sequencing of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene (Illumina, San Diego, CA, USA). Quality filtering, taxonomy assignment, and sample diversity analysis were performed as previously reported (Pages 80-82).

2.4 Detection of *G. vaginalis* clades

The residual DNA of each vaginal sample was tested against the four different GV clades using qPCR assays (Balashov *et al.,* 2014; Schuyler *et al.,* 2016). The list of primers and probes used are reported in Table 21.

After the production of a standard curve, a singleplex TaqMan real-time qPCR assay was used for each clade. The PCR reaction mixtures (final volume: 25 µL) included 12.5 µL of Platinum qPCR Supermix-UDG with ROX (Invitrogen, Thermo Fisher, Waltham, MA, USA), 400 nM of each DNA primer, 100 nM of the specific probe, and 2.5 µL of the template.

All PCR reactions were performed with the following cycling conditions using a QuantStudio qPCR system (Applied Biosystems, Thermo Fisher): 45°C for 3 minutes, 95°C for 3 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds.

Any reaction which failed to produce a cycling threshold value after 40 cycles was recorded as negative. Results were expressed as $\log_{10}$ DNA copies/reaction.

2.5 Detection of the putative *G. vaginalis* sialidase A gene

The residual DNA of each vaginal sample was used for molecular detection and quantification of the GV sialidase A gene, using the primers and probes described by Santiago and colleagues (Santiago *et al.,* 2011). The list of primers and probes used are reported in Table 21.

qPCR amplifications were performed in reactions (total volume 25 µL) containing 12.5 µL of Platinum qPCR Supermix-UDG with ROX (Invitrogen), 10 µM of each DNA primer, 5 µM of the specific probe, and 5 µL of the template.

All PCR reactions were performed with the following cycling conditions using a QuantStudio qPCR system (Applied Biosystems): 45°C for 3 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 5 seconds and 58°C for 10 seconds.

Starting from a quantitative standard curve, the results for each sample were expressed as $\log_{10}$ DNA copies/reaction.

Target	Primers/Probes
GV clade 1 (Putative α-L-fucosidase)	5'-CCAGTCATAAGTTTGCGTTTTACC-3' 5'-TGGCACTGGCAAAGTTTACAAC-3' 5'-FAM-CTCGCCGCAAGCACCATCAAGCCA-3'
GV clade 2 (Hypothetical protein)	5'-GCAAAGCAGACTGAGCGTATTAG-3' 5'-GTAATAATCAGGCTCCTCATCGC-3' 5'-FAM-CGCAGGCGCTCGCATAACAGTGCA-3'
GV clade 3 (Thioredoxin)	5'-TTCTGCTTCTTCTGCTATTTGCTG-3' 5'-TTCGTTGACTTTTGGGCAACATG-3' 5'-FAM-CGGTCCGTGCCGTTCATTTGGTCC-3'
GV clade 4 (Chloride transporter, CIC family)	5'-CCTACGCAAGCTCCAGACGAC-3' 5'-ACAAGTTGCACTCTTCGAGCTGG-3' 5'-FAM-ACTCGGCTGAAGCACACCACCACT-3'
Sialidase A	5'-GACGACGGCGAATGGCACGA-3' 5'-TACAAGCGGCTTTACTCTTG- 3' 5'-/56-FAM/CTCCGCGAT/ZEN/TTGCGCGAATAATC/3IABkFQ/-3'

Table 21. List of primers and probes used for the detection of GV clades and Sialidase A gene.

2.6 Data analysis and statistics

The software GraphPad Prism was used to perform statistical analyses (version 5.02; Graph-Pad Software, San Diego, CA, USA).

Categorical data were compared using Fisher's exact test (*i.e.,* presence of GV clades stratified by the vaginal status by NS), while ANOVA test, followed by Tukey's multiple comparisons test was used to compare GV or sialidase loads among the different categories.

Statistical evaluation of α-diversity, β-diversity, pairwise relative abundance, and comparison between relative abundances across multiple categories were performed as previously described (Pages 82 and 83). Statistical significance was determined at a p-value<0.05.

Statistical evaluations were performed in Matlab (Software version 7.7.0, Natick, MA, USA). Correlations between the presence/absence of each GV and microbial composition was evaluated using point biserial correlation. Instead, correlation between the quantity of sialidase A and microbiota was performed using Spearman's rank-based correlation coefficient on the $\log_2$-transformed sialidase copy number.

3. Results

3.1 Study population

A total of 61 Caucasian pregnant women with a median age of 31.0 years (min-max: 21-44) were enrolled, therefore 122 vaginal samples (61 at the first and 61 at the third trimester) were available for the analyses.

Going from the first to the third trimester of pregnancy, a significant decrease of cases of BV, together with an increase of cases characterized by a normal microbiota ($p<0.001$), was noticed. At the first trimester, 31 (50.8%) women showed a lactobacilli-dominated vaginal flora (NS≤3), 19 (31.1%) were characterized by an intermediate microbiota (NS 4-6), whereas the remaining 11 (18.1%) harbored a BV-associated microbial composition (NS≥7). Conversely, at the third trimester, most women (54; 88.5%) were characterized by a normal microbiota, with only 4 cases of BV (6.6%).

When looking to paired vaginal samples from the same women, it was found that all the subjects with a normal microbiota at the first trimester (n=31) maintained the same status at the third trimester, except in three cases where an intermediate microbiota was detected at the end of the pregnancy. All the women with a BV condition at the first trimester (n=11) showed a normal microbiota at the third trimester, except for 2 cases who continued to have BV-like status by NS. Only two new cases of BV, not present at the beginning of the pregnancy, was observed at the third trimester.

Overall, 85 cases of normal vaginal flora, 15 BV, and 22 cases of intermediate microbiota (I group) were considered for the analysis.

3.2 Detection and quantification of *G. vaginalis* clades

DNA of at least one GV clade was detected in all the vaginal swabs except in four samples (4/122, 3.2%). Two of them belonged to the same woman, negative both at the first (characterized by an intermediate flora) and third trimester (BV condition) of pregnancy. The remaining two cases belonged to two women at the first trimester, with a normal vaginal flora by NS.

Of the 122 vaginal swabs, 23 samples were positive for only one clade (18.9%), 47 for two clades (38.5%), and 40 for three clades (32.8%). Eight samples showed the contemporary presence of all four clades (6.6%), while four samples had no clades detected.

The distribution of clades was reported in Figure 46.

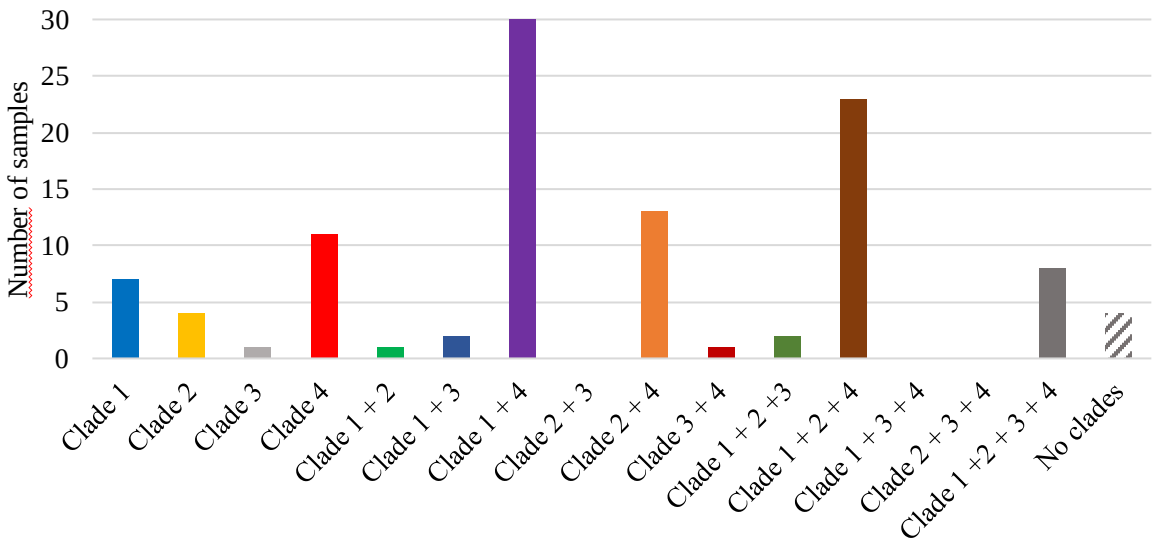

Figure 46. Single clades and clade association distribution in studied samples.

The most common GV clade was represented by clade 4 (101/122; 82.8%), followed by clade 1 (88/122; 72.1%), clade 2 (51/122; 41.8%), and clade 3 (19/122; 23.8%). Overall, the distribution of GV clades in the first trimester of pregnancy was like that of the third trimester. Considering the distribution of GV clades stratified by the vaginal status (*i.e.,* H vs I vs BV), a significant association between BV status and clade 2 was found (p=0.01) (Table 22).

	Clade 1		**Clade 2**		**Clade 3**		**Clade 4**	
	+	-	+	-	+	-	+	-
BV	15.9%	2.9%	21.6%	5.6%	20.7%	9.7%	11.9%	14.3%
H	65.9%	79.4%	66.7%	71.8%	65.5%	71.0%	72.3%	57.1%
I	18.2%	17.7%	11.8%	22.5%	13.8%	19.4%	15.8%	28.6%

Table 22. Distribution of GV clades, stratified by the vaginal status (H vs I vs BV, by the NS).

Interestingly, multiple GV clades (*i.e.,* more than two different clades at the same time) were more often detected in BV conditions than in intermediate or healthy VMB (p=0.035) (Table 23).

Number of clades	H (n=83)	I (n=21)	BV (n=14)
1	21.6%	23.8%	0%
2	42.3%	52.4%	7%
>2	36.1%	23.8%	93%

Table 23. Presence of GV clades (single clade, dual clade, multi-clade) stratified by vaginal status (H vs I vs BV, by the NS).

In terms of GV loads (expressed as log10 GV copies/reaction ± SD), clade 4 showed the highest mean levels (1.6±1.9), followed by clade 1 (1.5±1.9), clade 3 (1.0±1.9) and clade 2 (0.9±1.5). As shown in Table 24, significantly higher GV loads were found in BV cases compared to healthy conditions, especially for clades 1, 3, and 4. No differences in bacterial loads for any of the clades were found between H and I groups.

Considering paired vaginal samples, women who cleared their BV status at the third trimester of pregnancy were characterized by a significant reduction of GV clade 1 (4.1±1.9 vs 2.5±2.0; p=0.01) and clade 4 loads (4.3±1.9 vs 2.7±2.0; p=0.001). Moreover, in this group, the positivity for clade 2 decreased from 77.7% (7/9) to 22.2% (2/9). The two cases of BV observed at the third trimester, not present at the beginning of the pregnancy, showed a pattern of GV clades/loads similar to the third trimester.

Clade	H	I	BV	H vs BV (p-value)	I vs BV (p-value)	H vs I (p-value)
1	1.2 ±1.6	0.6 ± 1.3	3.9 ± 1.9	<0.0001	<0.0001	0.37
2	0.8 ± 1.5	0.3 ± 0.8	1.9 ± 1.1	0.06	0.08	0.78
3	0.5 ± 0.9	0.6 ± 0.3	3.0 ± 2.4	0.007	0.06	0.99
4	1.3 ± 0.7	1.4 ± 1.0	3.8 ± 1.3	0.0001	0.003	0.96

Table 24. GV bacterial loads stratified by the vaginal status (NS). GV loads are expressed as $\log_{10}$ DNA copies/reaction. Results are expressed as mean ± standard deviation. Statistical significance was deemed for $p<0.05$.

3.3 Presence of the putative *G. vaginalis* sialidase A gene

GV sialidase A gene was detected in 89.3% of the samples (109/122). All the negative samples belonged to H (n=10) or I women (n=3). When considering quantitative results (expressed as $\log_{10}$ DNA copies/reaction), higher sialidase levels in BV group (3.9±2.4) compared to both healthy (1.3±1.7; $p<0.0001$) and intermediate conditions (1.1±1.1; $p<0.0001$) was found. No significant difference was found between H and I groups ($p=0.9$).

Overall, considering paired vaginal samples from the same women, a significant reduction of sialidase gene count going from the first to the third trimester of pregnancy (2.0±2.2 vs 1.5±1.8, $p=0.02$) was noticed. These data reflected the increase of cases characterized by a normal microbiota at the end of the pregnancy. Women who cleared their BV status at the third trimester of pregnancy showed a significant decrease of sialidase levels (4.3±1.9 vs 2.6±1.3; $p=0.04$). The two cases of BV observed at the third trimester, not present at the beginning of the pregnancy were characterized by low levels of sialidase (0.09±0.09).

3.4 Correlation between *G. vaginalis* clades and vaginal microbiome

A correlation between the presence of the different GV clades and the vaginal microbiome profiles was performed on the whole dataset (122 vaginal samples), not dividing the two different sampling timepoint (first vs third trimester of pregnancy).

The evaluation of α-diversity showed a significant ($p<0.002$) difference in the biodiversity of samples positive or negative for GV clade 2 for all the metrics (*i.e.,* Chao1, Observed Species, Faith's Phylogenetic tree, Shannon index, Good's coverage), and for clade 1 ($p<0.035$, Observed Species and Good's coverage only), with an increase in biodiversity in positive samples. Moreover, it was detected a somewhat significant difference in biodiversity according to the number of clades contemporary found in the vaginal ecosystem, with increased diversity for samples positive for 3 clades compared to samples positive for 1 or 2 clades ($p<0.02$ for all metrics).

The evaluation of the microbial composition of the samples (β-diversity; Fig. 47A) confirmed that the most evident differences were associated with clade 2 (both unweighted and weighted Unifrac distances: $p=0.001$ and $p=0.002$, respectively).

On the other hand, no significant difference in the microbial composition was noticed between positive and negative samples for clades 1, 3, and 4 (with the only exception of weighted Unifrac distance for clade 1, $p=0.044$).

Stratification of the samples according to the vaginal status (by NS) highlighted the peculiar behavior of the BV samples for clade 2, the only combination that presented a statistically significant difference (p=0.003) (Fig. 47B).

Considering the number of clades simultaneously detected in the same sample, the unweighted Unifrac distances depicted a certain separation between samples negative to all clades and the other samples (although non-significant). On the other hand, samples positive to 3 clades were significantly different (p<0.03, unweighted Unifrac) to those positive to none, 1 or 2 clades; no differences were found between samples with 3 or 4 positive clades (Fig. 47C).

Moreover, the plot of the weighted Unifrac centroids for each group seemed to depict a sort of trajectory along PCoA first component axis (Fig. 47D).

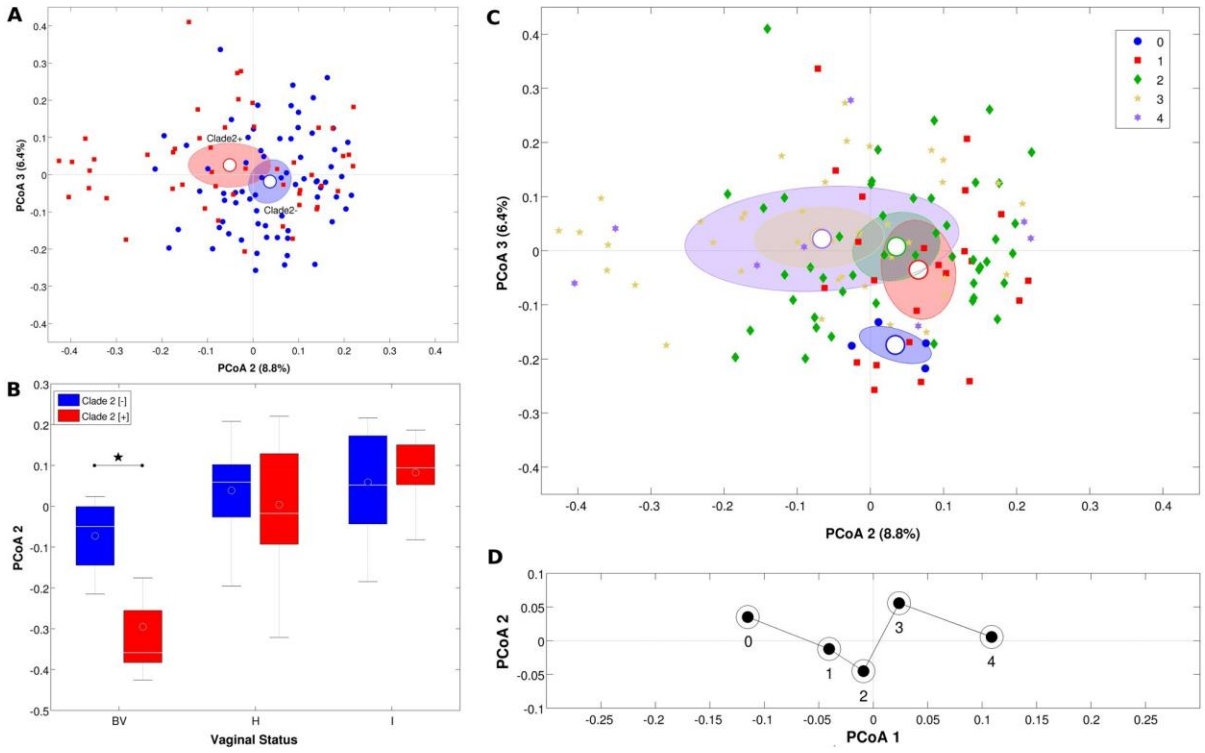

Figure 47. A) PCoA plots based on the unweighted Unifrac distance among samples clustered on the presence of GV clade 2. Each point represents a sample, colored according to the experimental category (blue=negative, red=positive). Ellipses are 95% SEM-based confidence intervals, and centroids represent the average coordinate per category. The second and the third coordinates are represented. B) Boxplots of the distributions of PCoA coordinate 2 on the unweighted Unifrac distances for presence/absence of GV clade 2, with samples divided according to their vaginal status (by NS). Asterisk indicates statistical significance (p<0.05, adonis test) C) PCoA plot based on the unweighted Unifrac distance among samples clustered according to the number of GV clades present in each sample. Each point represents a sample, which is colored according to the experimental category. Ellipses are 95% SEM-based confidence intervals, and centroids represent the average coordinate per each category. The second and the third coordinates are represented. D) Trajectory plot of the PCoA centroids obtained from the first and second coordinate of the PCoA analysis of the weighted Unifrac distances.

The analysis of bacterial relative abundances at genus level provided further evidence that GV clade 2 could be the one associated with the major shifts in the microbial composition. Major components of the VMB were found altered in samples positive for this clade, compared to clade 2-negative samples: *Lactobacillus* was observed to be decreased (-7.3% in clade 2-positive samples), while *Prevotella, Megasphaera, Sneathia*, and *Dialister* were all increased in relative abundance. In particular, both *Megasphaera* and *Sneathia* had a 14-fold increase, and *Prevotella* in samples positive for clade 2 was 8 times higher than in negative ones. On the other hand, clade 1 showed only an alteration of *Prevotella* (avg. rel. ab: 0.7% vs. <0.1% in clade positive and negative, respectively) plus some minor components, such as *Fastidiosipila* and *Parvimonas* (rel. ab.<0.2%). Detailed results are shown in Table 25.

Clade	N° [+]	N° [-]	Genus	Avg.clade [+] (%)	Avg.clade [-] %	p-value	Significance
Clade 1	88	34	*Prevotella 6*	0.65	0.01	0.0053	**
			DNF00809	0.19	0	0.0187	*
			Fastidiosipila	0.17	0	0.0143	*
			Parvimonas	0.09	0	0.0143	*
Clade 2	51	71	*Lactobacillus*	73.07	80.34	0.0191	*
			Prevotella	3.92	0.48	0.0189	*
			Megasphaera	3.10	0.21	0.0350	*
			Sneathia	1.74	0.12	0.0030	**
			Ureaplasma	0.33	0.45	0.0419	*
			Dialister	0.47	0.15	0.0244	*
			DNF00809	0.32	0	0.0049	**
			Fastidiosipila	0.28	0	0.0022	**
			Peptoniphilus	0.09	0.10	0.0008	***
			Dietzia	0.07	0.11	0.0463	*
			Porphyromonas	0.16	0.03	0.0223	*
			Parvimonas	0.16	0	0.0021	**
Clade 3	29	93	-	-	-	-	-
Clade 4	101	21	-	-	-	-	-

Table 25. Average relative abundances for samples positive and negative to each of the four GV clades. Number of samples per group, as well as the p-value of the Mann-Whitney U test is reported. Significance was assessed using a Benjamini-Hochberg FDR correction on the 25 most abundant genera (using an FDR of 0.15 as a threshold). For better clarity, in the table, raw unadjusted p-values were provided. Asterisks indicate graphically the statistical significance: ***: $p<0.001$, **: $p<0.01$; *: $p<0.05$.

When evaluating relative abundances according to the number of GV clades found simultaneously in the same sample, interesting trends were found (Fig. 48).

In particular, *Bifidobacterium* tended to decrease with the increasing number of positive clades (r=-0.994, Pearson's correlation coefficient, considering only samples positive to 1, 2 or 3 clades), whereas a group of BV-related bacteria (*i.e., Prevotella, Megasphaera, Sneathia, Prevotella 6, Ureaplasma,* and *Dialister*) tended to increase (Pearson's coefficients between 0.833 and 0.916). No significant trend was found between *Lactobacillus* spp. abundance and GV multi-clade colonization. In vaginal samples negative for GV (n=4), a significant proportion of *Atopobium* was found (average relative abundance about 30%).

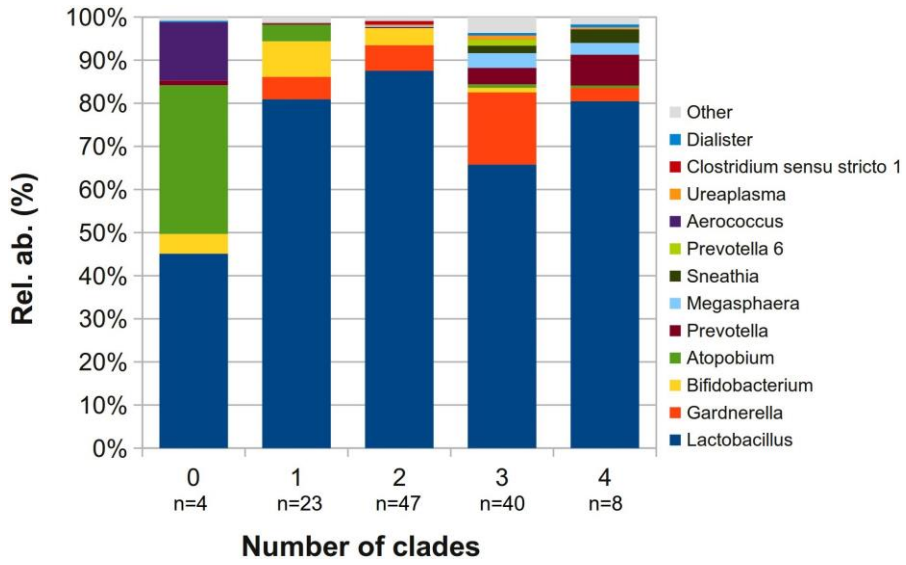

Figure 48. Barplot of the average relative abundance of the main genera, over the number of clades found at the same time in each sample. Only the first 12 most abundant genera are plotted, whereas the remaining genera are grouped in the 'Others' category.

The alterations suggested by analyzing the relative abundance of bacterial genera were confirmed with the point-biserial correlation between genus level relative abundances and the presence/absence of each of the four GV clades (Fig. 49). Clade 2 was the one displaying most of the correlations: indeed, 13 genera among the first 16 most abundant in the vaginal ecosystem showed a significant correlation. For example, clade 2 was negatively correlated with *Lactobacillus* (r=-0.122) and positively with several BV-associated bacteria (*i.e., Prevotella, Megasphaera, Sneathia, Dialister, Coriobacteriales DNF00809, Fastidiosipila*, with coefficients in the range 0.201–0.324). On the other hand, all the other GV clades displayed fewer and weaker correlations (*i.e.,* all significant correlations had r<0.159, Fig. 49).

Among them, a positive correlation between GV clade 1 and *Parvimomas, Prevotella, Coriobacteriales DNF00809,* and *Fastidiosipila*, as well as a positive correlation between clade 4 and *Peptoniphilus* and *Ureaplasma* was observed. Notably, all the significant correlations corresponded to the taxa found as differentially abundant in samples positive or negative to each clade.

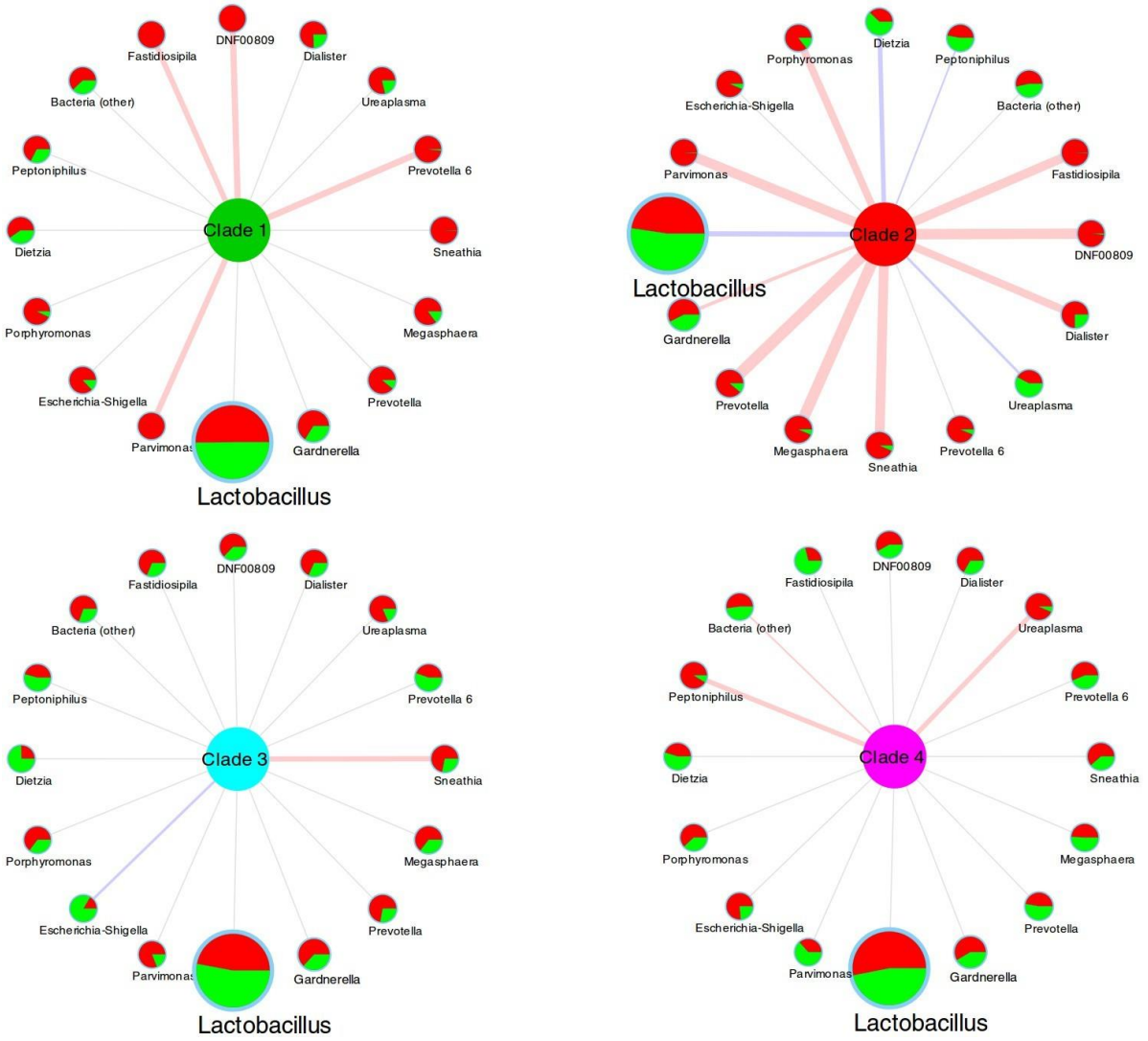

Figure 49. Diagram representing the correlations between bacterial genera and presence/absence of each of the four GV clades. Edge color represents the sign of the correlation (blue=negative, red=positive, gray=not significant) and edge thickness represents the strength of correlation. Node and label size of the bacterial genera is proportional to the average abundance over the whole set of samples. Piecharts in each node represent the skewness of the average relative abundance of the genera in samples not showing (green) or showing (red) the specific clade. Thus, for example, genera with a piechart nearly completely red are those whose abundance in the samples is more associated with positivity for the specific clade.

3.5 Correlation between sialidase A gene levels and vaginal microbiome

A correlation between sialidase gene levels and the vaginal microbiome profiles was performed on the whole dataset (122 vaginal samples).

When evaluating Spearman's correlation between the sialidase A gene count and the relative abundance of the main bacterial genera, a negative correlation with *Lactobacillus* (r=-0.27) while positive correlations were observed with *Gardnerella* (r=0.52), *Atopobium* (r=0.26), *Prevotella* (r=0.21), *Megasphaera* (r=0.30), *Sneathia* (r=0.28), and *Prevotella 6* (r=0.21) were observed (Fig. 50). Many of them were also the bacterial genera found as differentially abundant in the comparison between samples positive or negative for each clade.

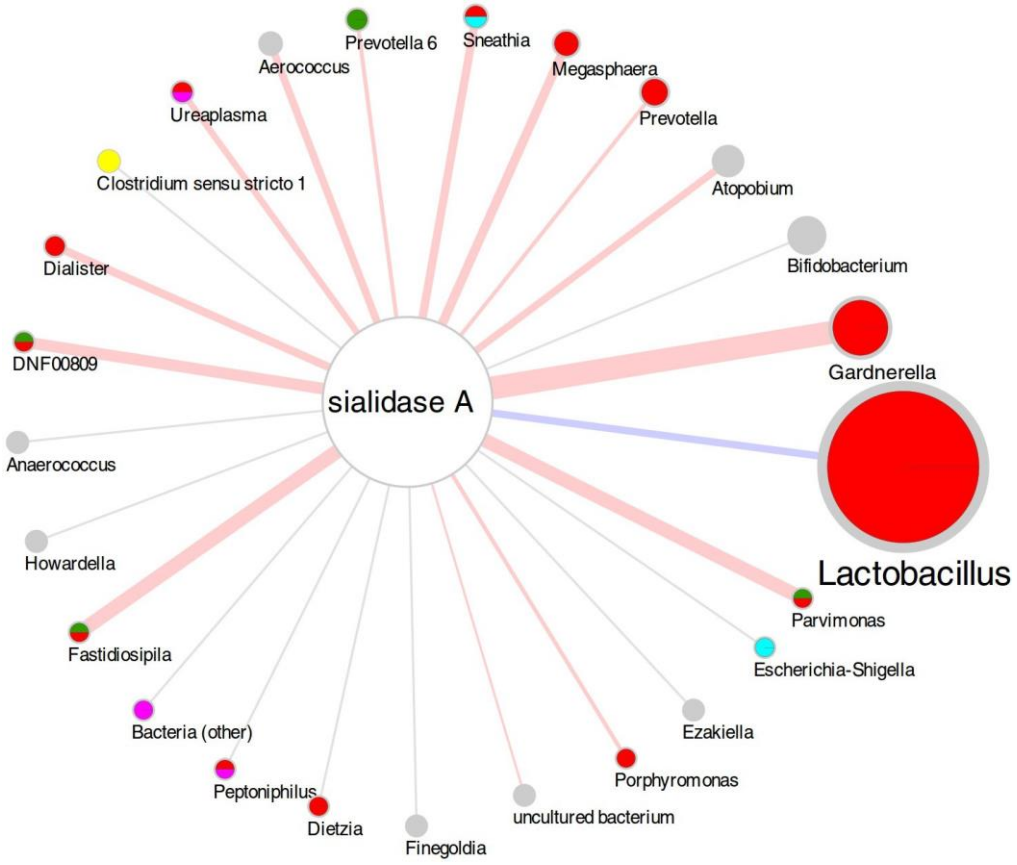

Figure 50. Diagram representing the correlations between bacterial genera and the number of copies of the sialidase gene, on a log2 scale. Edge color represents the sign of the correlation (blue=negative, red=positive, gray=not significant) and edge thickness represents the strength of correlation. Node and label size of the bacterial genera is proportional to the average abundance over the whole set of samples. Piecharts in each node indicate whether the genera were differentially abundant in samples positive or negative for each GV clade (green=clade 1; red=clade 2; cyan=clade 3; magenta=clade 4). Colors are the same as the clades in Fig. 49.

4. Discussion

In this work it was explored the distribution and loads of the different GV clades in the vaginal environment of a cohort of Caucasian pregnant women, deciphering the correlations of the different clades with the inhabitants of the vaginal microbiome. Moreover, considering the key role of putative GV sialidase A in the ability to form biofilm and BV pathogenesis, sialidase gene count in the vaginal ecosystem of each woman was assessed (Hardy *et al.,* 2017).

At first, in agreement with previous observations, the present results confirmed that GV is extremely common even in women harboring a lactobacilli-dominated VMB, thus confirming its commensal role in many healthy women (Shipitsyna *et al.,* 2019).

Moreover, the distribution of GV clades found was in line with previously published literature, with clade 4 being the most common one, followed by clade 1, clade 2, and clade 3 (Balashov *et al.,* 2014). When analyzing the distribution of GV stratified by the vaginal status (NS), a significant association between clade 2 and BV condition was found. In this context, it should be noted that the correlation between single GV clade and BV status is still under debate. Even though most studies indicate clade 1 as the one most associated with BV, even clades 2 and 3 were found related to an abnormal VMB, in conjunction with higher NS (Balashov *et al.,* 2014). In this dataset, the relationship between BV status and GV clade 2 was strengthened by the association of this clade to a higher vaginal microbial biodiversity, as well as to several BV-associated bacteria. Indeed, women positive for clade 2 were more likely to harbor a plethora of anaerobic bacteria in the vaginal ecosystem, typically found during dysbiotic conditions (*e.g., Gardnerella, Prevotella, Megasphaera, Sneathia, Dialister*).

Although some studies found a relationship between clade 4 and dysbiotic conditions, in the present study it was not observe this association, being clade 4 very frequently detected also in women with low NS (*i.e.,* normal lactobacilli-dominated flora) (Vodstrcil *et al.,* 2017).

The results, on the other hand, agree with several other observations suggesting that clade 4 is not associated with BV or altered VMB, presumably for its reduced pathogenicity. Indeed clade 4 strains are characterized by low or no production of virulence factors, including sialidase A enzyme, resulting in a reduced ability to mucin degradation and to form biofilm (Balashov *et al.,* 2014).

The discrepancies between studies in defining the specific GV clades associated with BV or healthy vaginal status could be due to population differences, including ethnicity, sexual activities and networks, pregnancy status, and behavioral practices such as smoking, diet, and hygiene.

Other interesting data emerged when considering the contemporary presence of more than one GV clade. Indeed, in our study, according to previous observations, BV-women were more likely (p=0.003) to have multiple clades of GV (*i.e.,* co-colonization with more than two different GV clades) compared to single or dual clades (Shipitsyna *et al.,* 2019; Vodstrcil *et al.,* 2017).

As previously hypothesized, multiple clades may act synergistically to suppress *Lactobacillus* spp. or form biofilms. During BV, biofilm can incorporate various bacteria, including different GV strains/clades, over time, so it can be hypothesized that in BV status, GV is commonly found as multi-clade, in contrast with healthy women characterized by dispersed forms of the microorganism (*i.e.,* single clade) (Plummer *et al.,* 2019).

In line with these findings, as the number of GV clades increased, an increase in the severity (*i.e.,* higher microbial diversity with an abundance of BV-associated taxa) of vaginal dysbiosis was observed as well. Indeed, women co-colonized by multi-clade of GV were characterized by lower levels of Bifidobacterium, and higher levels of BV-related bacteria, such as *Prevotella, Megasphaera, Sneathia, Ureaplasma,* and *Dialister*.

In this context, it should be remembered that, despite being typically associated with the gut microbiota, some *Bifidobacterium* are frequent and abundant colonizers of the 'healthy' VMB (Bradshaw *et al.,* 2006). Bifidobacteria can produce L-LA, which may contribute to lowering vaginal pH values, thus inhibiting the growth of dysbiosis-associated bacteria (Bradshaw *et al.,* 2006).

Overall, no significant trend was found between lactobacilli abundance and GV multi-clade colonization. However, additional studies based on more in-depth 16s rRNA gene analyses might possibly reveal associations between GV multi-clade colonization and specific *Lactobacillus* species (*e.g., L. crispatus, L. iners*).

During dysbiotic conditions, *Gardnerella* spp. can adhere to glycan-binding sites, and biofilm-forming *Gardnerella* spp. can, then, act as a scaffold for the attachment of other BV-associated species, such as *A. vaginae* and *Prevotella* spp. (Hardy *et al.,* 2017).

Few cases characterized by a high proportion of *Atopobium* spp. in absence of GV were found. Even though *A. vaginae* is considered a highly specific marker for BV, especially when combined with GV, it should be remembered that this microorganism can also be found in the VMB of healthy women and detected without GV (Bradshaw *et al.,* 2006). Considering the low number of data points (n=4), the exact role of *Atopobium* in GV-negative samples remains to be further elucidated.

BV conditions were characterized by higher GV bacterial loads for all the four clades, in agreement with previous observations demonstrating that the quantification of GV clades is a good and accurate predictor of an abnormal vaginal microbiome, associated with BV condition (Shipitsyna *et al.,* 2019).

Similarly, sialidase A gene levels were significantly higher in women with a BV status compared to H and I groups. In agreement with this observation, sialidase gene count was positively correlated with several BV-associated bacteria, such as *Gardnerella, Atopobium, Prevotella, Megasphaera,* and *Sneathia,* and negatively with *Lactobacillus* genera.

Recently, Ferreira and colleagues observed that many BV-related taxa (especially belonging to *Prevotella* genera) were enriched in sialidase-positive vaginal samples and that only two taxa, including *Lactobacillus helveticus*, were associated with sialidase-negative samples (Ferreira *et al.,* 2022).

The negative correlation between sialidase gene levels and *Lactobacillus* is not surprising if it is consider that these microorganisms are the hallmark of vaginal health and eubiosis, being detected only in low abundances in BV/dysbiotic bacterial communities (Ceccarani *et al.,* 2019).

Considering that sialidase A gene is not the best marker of functional sialidase in vivo, further studies assessing the presence of additional GV sialidase genes (namely, NanH2 and NanH3) are needed to better understand the correlation between sialidase gene count and BV presence/severity (Hardy *et al.,* 2017).

Taken together, our data highlight that BV status is associated with the presence of clade 2, the contemporary presence of multi-clades of GV (*i.e.,* >2 different clades), higher GV bacterial loads, and higher sialidase gene count.

However, this study had some limitations. First, the limited number of BV-affected women may have led to missing significant associations between specific GV clade and loads, sialidase gene count, and microbial signatures during vaginal dysbiosis. Second, the use of culture-based approaches will be needed to better understand the role of sialidase enzymes in BV pathogenesis and severity. Finally, detailed information about the clinical manifestations of BV, as well as an evaluation of Amsel criteria of each woman will help to better define the role of GV clades and sialidase activity in the conditions of vaginal dysbiosis.

In conclusion, the present study added knowledge on the distribution of GV clades in the vaginal ecosystem of a cohort of pregnant women, assessing the potential role of specific GV clades, bacterial loads, and sialidase A gene count in BV pathogenesis. Specific microbial fingerprints related to the different GV clades were identified, thus underlining the ability of

each clade to interact and cooperate in a different way with the inhabitants of the vaginal environment.

Further studies will be needed to understand if GV clades have a different clinical and prognostic impact on pregnancy status, as well as if one or more combined parameters (*e.g.,* number of GV clades, type of clades, GV loads) can predict the severity of BV in pregnant women.

Since a vaginal microbiome enriched in BV-related taxa, is associated with adverse pregnancy outcomes, a detailed characterization of BV conditions in pregnant women could open new perspectives for the prevention of pregnancy-related complications, such as first trimester miscarriage and PTB (Di Simone *et al.,* 2020).

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