Assessment of microbiota:host interactions at the vaginal mucosa interface

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***Abbreviations*:** BV, Bacterial vaginosis; CST, Community state type; DESI, Desorption electrospray ionisation; FWHM, full width at half maximum; GC, Gas chromatography; HIV, human immunodeficiency virus; HPLC, High performance liquid chromatography; HPV, human papilloma virus; MS, mass spectrometry; NMR, Nuclear magnetic resonance; PRR, pattern recognition receptors; PCR, Polymerase chain reaction; SIM, selected ion monitoring; SLPI, secretory leukocyte protease inhibitor; SRM, selected reaction monitoring

Abstract

There is increasing appreciation of the role that vaginal microbiota play in health and disease throughout a woman's lifespan. This has been driven partly by molecular techniques that enable detailed identification and characterisation of microbial community structures. However, these methods do not enable assessment of the biochemical and immunological interactions between host and vaginal microbiota involved in pathophysiology. This review examines our current knowledge of the relationships that exist between vaginal microbiota and the host at the level of the vaginal mucosal interface. We also consider methodological approaches to microbiomic, immunologic and metabolic profiling that permit assessment of these interactions. Integration of information derived from these platforms brings the potential for biomarker discovery, disease risk stratification and improved understanding of the mechanisms regulating vaginal microbial community dynamics in health and disease.

Highlights

- Vaginal microbiota-host interactions play a critical role in the dynamics of health and disease states during a woman's lifetime
- *Lactobacillus* species dominance of the vagina broadly indicates health, whereas *Lactobacillus* species depletion and increased diversity is more often associated with pathologies (e.g. bacterial vaginosis, poor pregnancy outcomes, sexual transmitted infections)
- Sampling of the vaginal mucosa and analysis by next-generation sequencing combined with immunological and metabolic profiling techniques can be used for detailed characterised of patient-level microbial composition and host response
- Optimisation of the methodological and analytical workflows for these multi-omic platforms is important for limiting technical and experimental biases that can hinder interpretation and reproducibility of data
- Ambient ionisation MS techniques such as DESI-MS permit rapid, direct analysis of vaginal swabs and the detection of metabolite changes associated with vaginal microbiota composition

1. Vaginal microbiota in health and disease

The human vagina hosts a complex microbial ecosystem that has long been recognised to influence health outcomes throughout a woman's lifetime [1]. As early as the late 19th century, bacteria and viruses present in the vagina began to be causally linked to lower reproductive tract diseases such as gonorrhoea and genital herpes [2]. Around the same time, Doderlein discovered a bacilli (later attributed to *Lactobacillus acidophilus* [3]) in vaginal secretions of pregnant women, which was described as having antagonistic effects towards pathogens [4]. It was also noted that vaginal secretions deplete of bacillus were often colonised instead by high numbers of morphologically distinct microorganisms with an elevated pH [5]. These clinical features today would be attributed to symptomatic bacterial vaginosis (BV) [6]. A century later, the concept of the relationship between reproductive health and lactobacillus dominance remains, yet through the application of modern culture-independent and molecular-based methods, is being redefined with increased appreciation of the complexity and dynamics of community structure within the vagina [7, 8].

1.1 The "healthy" and "unhealthy" vaginal microbiome

The vaginal microbiome describes the microorganisms (bacteria, fungi and viruses), their genomes and their surrounding biochemical environment in its entirety [9]. Establishment of the vaginal microbiome begins at the time of birth with delivery considered a key seeding event [10, 11]. In the immediate postpartum period, the residual effects of maternal oestrogen on the female neonate promotes thickening of the vaginal epithelium and deposition of glycogen, which is used as a primary carbon source by lactic acid-producing bacteria [12]. As estrogen levels fall these effects dissipate by around the fourth week of life when the vaginal epithelium becomes less stratified, glycogen content is reduced and vaginal pH increases [13]. This is accompanied by a shift toward a high-diversity community structure that persists throughout childhood [14] until just prior to the onset of menarche when it begins to resemble that of reproductive-aged women [15]. Cross-sectional studies of asymptomatic women have shown that vaginal microbiota profiles can be classified into at least five main groups often referred to as community state types (CST) [16]. Four of these groups are characterised by almost complete dominance of either *Lactobacillus crispatus* (CST I), *Lactobacillus gasseri* (CST II), *Lactobacillus iners* (CST III) or *Lactobacillus jensenii* (CST V), whereas CST IV is characterised by a low abundance of *Lactobacillus* species and increased diversity and richness of anaerobic bacteria often associated with BV. However, longitudinal studies reveal the dynamic nature of these vaginal microbial community compositions, which in some women flux and alter with hormonal changes during menstruation, while in other women it remains stable over long periods of time [17]. During pregnancy, where circulating concentration of oestrogen progressively rise [18], there is a reduction in vaginal bacterial diversity and a further shift towards *Lactobacillus* spp. dominance [19, 20]. Following delivery, when maternal oestrogen levels fall, vaginal *Lactobacillus* spp. dominance decreases and there is a shift towards a high-diversity microbiota community structure [19] that can persist in some women for up to one year postpartum [21, 22]. At menopause, reduced oestrogen levels are associated with decreased vaginal epithelial glycogen deposition, depletion of *Lactobacillus* spp. and increased colonisation of high-diversity microbial communities [23].

While *Lactobacillus* species dominance of the lower genital tract is generally associated with states of health, a shift in microbial composition towards a high-diversity structure is often associated with disease risk and pathologies such as urogenital tract infections [24] and BV [25]. High diversity community structures and low relative abundance of *Lactobacillus* species are also associated with increased risk of sexually transmitted diseases such as human immunodeficiency virus (HIV) [26, 27], human papillomavirus infection and the progression of cervical intraepithelial neoplasia towards cervical cancer [28, 29]. Adverse pregnancy outcomes such as miscarriage and preterm birth have also been linked with vaginal pathogen infection or abnormal vaginal microbial composition [21, 30-34]. Although less studied, the fungal component of the microbiome, termed the “mycobiota”, may also be an important mediator of health and disease [35]. Many yeast species are vaginal commensals, however overgrowth of some species such as *Candida albicans* occurs commonly in the population with at least 75% of women experiencing symptomatic vaginal candidiasis at least once in their lifetime [36]. Common *Candida* species can cause urinary tract infections [37] and HIV viral load in vaginal fluid of HIV-positive women is significantly increased in the presence of *Candida* colonisation [38]. The precise mechanisms by which fungi act as modulators of microbiome structure in the context of health and disease states are poorly defined and warrant further investigation.

Lactobacillus species domination of the vaginal microbiome has often been described as a hallmark of vaginal health, however a number of recent studies have shown that this is not always the case. High diversity compositions deplete of *Lactobacillus* species are prevalent in around 40% of reproductive-aged women of Black and Hispanic ethnic background in contrast to around 10-20% of women from White or Asian ethnic backgrounds [39, 40]. It is also noteworthy that vaginal microbiota or community compositions associated with pathology or disease states can often still be observed in asymptomatic, healthy controls. This highlights the likelihood of individual-level microbiota:host interactions, mediated by factors such as genetic background and individual host immunity, as being key determinants of either normal or pathological responses to vaginal microbiota [1]. Methodologies that permit assessment of these interactions can broadly be divided into techniques that facilitate detection and identification of microbiota, and those that permit examination of the functional response of both microbiota and host.

2. Identification and characterisation of vaginal microbiota

In a clinical setting, analysis of vaginal microbes is performed almost exclusively using a combination of culture and microscopy based methods. This typically involves the analysis of material collected from vaginal swabs by routine aerobic, anaerobic and fungal culture using selective media combined with assessment of microscopic features of the samples (e.g. the presence or absence of clue cells and bacterial morphotypes) [41-44]. However, these methods are limited as many microbes cannot be cultured *in vitro* and information regarding relative abundance of specific species within a community cannot be obtained. As a result, culture-independent, molecular-based approaches are increasingly applied to assess vaginal microbiota composition and structure and will be primarily described in this review [45].

2.1 Sampling and isolation of microbial genetic material

Sampling of vaginal microbiota is most commonly performed using a rayon or cotton swab, however cytobrushes are also used where exfoliation of the top layer of epithelial cells is required (e.g. for cytological detection of dysplasia in vaginal intraepithelial neoplasia). Although vaginal microbiota composition and diversity measurements appear to be comparable between the main sampling devices [46, 47], DNA and protein yields can differ [47, 48] and this should be taken into account during study design.

Isolation and extraction of genomic DNA from vaginal microbiota requires cell lysis, which is generally performed using enzymatic, chemical and/or mechanical (bead beating) methods followed by the phenol-chloroform-isoamyl alcohol DNA precipitation or using more popular silica based columns. DNA extraction is a major source of bias in 16S rRNA gene sequencing analyses [49-51]. Several DNA extraction methods from vaginal swabs have been described yet there is no one approach regarded as the gold standard [19, 32, 52-59]. An enzyme cocktail consisting of mutanolysin and lysostaphin, in addition to lysozyme, is commonly used in order to lyse those bacterial species resistant to lysozyme digestion [60, 61]. For example, vaginal bacterial species such as *Neisseria gonorrhoeae*, *Proteus mirabilis* and *Staphylococcus aureus* are resistant to c-type lysozyme due to the modified peptidoglycan structure of their O-acetylated peptidoglycans [62]. Instead these bacteria are sensitive to mutanolysin, a N-acetylmuramidase that catalyses the cleavage of β-N-acetylmuramyl-(1 → 4)-N- acetylglucosamine linkage of the Gram-positive bacterial cell wall. Other bacteria commonly found in the human vagina such as *Streptococcus and Lactobacillus* species are also sensitive to mutanolysin [52, 63]. Although the use of mechanical disruption by bead beating following enzymatic lysis is widely used to increase DNA yields [64-66] and has been shown to improve overall representation of microbial diversity [61], excessive bead beating can lead to shearing of DNA and increased formation of chimeric products during polymerase chain reaction (PCR) amplification [67] therefore it is important to optimise speed and duration of mechanical disruption [68].

Extracted microbial DNA is then amplified by the PCR and analysed using whole genome-shotgun sequencing or targeted sequencing of bacterial gene amplicons derived from specific genomic regions (e.g. 16S rRNA and 18S rRNA genes) [24]. Resulting sequence data are then aligned and assigned using highly curated, broad coverage 16S rRNA sequence libraries [69] or more targeted reference databases enriched for taxa commonly isolated from the vagina [70]. Downstream of DNA extraction, various studies have highlighted additional sources of potential bias that can lead to erroneous findings and limit comparison of data across studies of vaginal microbiota. These include, but are not limited to, primer design and PCR amplification efficiency [71-73], sequencing depth [74] and data processing [75]. Selection of primers is particularly important when considering characterisation of vaginal microbiota communities by 16S rRNA gene sequencing. Bacterial 16S rRNA genes contain nine “hypervariable regions” (V1 – V9) that demonstrate considerable sequence diversity among different bacteria, thus permitting their use for species identification [76]. However, as current high-throughput sequencing platforms are limited to the analysis of short, partials sequence amplicons, this sequence diversity

also means that no single hypervariable region can differentiate among all bacteria species [77, 78]. Uneven amplification of certain species can thus lead to either an under- or over-representation of specific species within a microbial community [79-81]. For example, the V1-V3 region of the 16S rRNA gene is often targeted for vaginal microbiota characterisation as it permits the most robust classification of the most common vaginal *Lactobacillus* species (*L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*)[82]. However, the commonly used universal forward (27F) primer has mismatches against the *G. vaginalis* 16S rRNA gene sequence, a key bacterium associated with high diversity communities [77, 83], potentially leading to lower representation of these sequences within samples [77, 84]. This can be overcome through the use of a mixed formulation of the 27F forward primer (27F-YM), which permits maintenance of the rRNA gene ratio of *Lactobacillus* spp. to *Gardnerella* spp. [77]. Primers targeting the V6 region have also been used to characterised vaginal microbiota however this region has known bias against *Sneathia*, *Leptotrichia*, *Ureaplasma* and *Mycoplasma* species [85]. Mixture experiments using mock communities composed of known quantities of bacterial isolates or PCR products have been proposed recently as an effective approach for identifying and quantifying such biases [49].

3. Functional assessment of vaginal microbiota:host interactions

Molecular-based, culture independent approaches for identifying and characterising vaginal microbiota have revolutionised our understanding of community composition. However, these approaches fail to capture the functional interactions occurring between microbiota and the host that ultimately dictate health and disease phenotypes in the reproductive tract. Microbial composition of the vagina can be rapidly altered through host behaviour (e.g. sexual activity, douching, antibiotics) but it is also shaped by intrinsic selection pressures including nutrient availability, the ability to adhere and attach to host epithelial surfaces or other microbiota, chemical composition of the local environment and the host innate and adaptive immune responses [8]. Assessment of the biochemical and immunological interplay between host and vaginal microbiota are therefore critical for understanding their functional implications in health and disease. As described below, this is increasingly being achieved through the integration of microbiomic, metabolomic and immunologic analyses of vaginal mucosa samples. Although beyond the scope of this review, readers are encouraged to examine strategies and approaches for integrating and fusing these forms of multi-omic datasets [86-88].

3.1 Vaginal microbiota and local immune response

As reviewed elsewhere [89], innate and adaptive mucosal immune responses in the vagina protect against colonisation by pathogens. This involves coordinated immune cell trafficking and activation regulated by endocrine signalling [90], tightly mediated expression of pattern recognition receptors (PRR) throughout the reproductive tract [91, 92] and secretion of antimicrobial peptides like beta-defensins, elafin and secretory leukocyte protease inhibitor (SLPI) [93-95]. Although the immune system appears to be largely tolerant of *Lactobacillus* colonisation, certain species can dampen or enhance local immune and inflammatory responses. For example,

Lactobacillus crispatus dominated vaginal communities are associated with lower levels of pro-inflammatory cytokines (e.g. IL-1α, IL-1β, and IL-8) compared to communities dominated by BV-associated bacteria or *Lactobacillus iners* [96-101]. Furthermore, BV is also linked to decreased levels of the anti-inflammatory, antimicrobial peptide SLPI [102, 103]. A detailed description of inflammatory and immune mediators associated with differing vaginal microbial communities and pathologies is provided in Table 1.

Table 1. Examples of cytokine responses at the vaginal mucosal interface in response to altered microbiota composition in pathology and infection.

Study cohort	Cytokines/Chemokines	References
BV	IL-1α ↑	[96],[99]
	IL-1β ↑	[96],[104],[105],[97],[106],[107]
	TNF-α ↑	[96]
	IFN-γ ↑	[96]
	IL-10 ↑	[96]
	IL-8 ↑	[96],[108],[107]
	IL-12p70 ↑	[96],[106]
	IL-4 ↑, IL-4 ↓	[96], [109]
	FLT-3L ↑	[96]
	Eotaxin ↓	[104]
	IL-2 ↑	[110]
	IL-12p70 ↑	[110]
	IL-6 ↑	[110],[111],[107],[108]
	G-CSF ↑	[111]
	IP-10 ↓	[106]
	Elafin ↓	[112]
	Mip-1β ↑	[111]
	RANTES ↑	[111]
	Gro-α ↑	[111]
	hBD2 ↑	[109]
	hD5 ↑	[109]
	TGF-β1 ↓	[107]
	β-defensin 4 ↑	[108]
	Endotoxin ↑	[99]
HPV	IL-2 ↑, IL-12 ↑, IFN-γ ↑	[110]
Cervicitis/Vaginitis	IL-1β ↑, TNF-α ↑, IL-6 ↑ Neutral sphingomelinase ↓	[105]

BV = Bacterial vaginosis; IFN = Interferon; FLT-3L = Fms-related tyrosine kinase 3 ligand; G-CSF = Granulocyte-colony stimulating factor; Gro = growth regulated oncogene; hBD = human beta defensin; hD = human defensin; IL = Interleukin; IP = interferon gamma-induced protein; Mip = Macrophage inflammatory protein; Normal T Cell Expressed and Secreted; RANTES = Regulated on activation; TGF = Transforming growth factor; TNF = Tumour necrosis factor;

In vitro studies using epithelial cell models have shown that immune and inflammatory responses from vaginal epithelial cells are species specific. Co-culture of *Gardnerella vaginalis* and *Atopobium vaginae* with vaginal epithelial cells leads to the upregulation of pro-inflammatory cytokines (e.g. IL-6, IL-1β, TNFα and IL-8) and chemokines (e.g. RANTES, MIP-1β) as well as antimicrobial peptides (e.g. hBD-2) [108, 111, 113, 114]. In contrast, other species often detected in BV cases such as *Prevotella bivia* do not elicit similar pro-inflammatory responses indicating differing pathogenicity of some BV-associated bacteria [114]. A summary of key *in vitro* inflammatory (cytokine and chemokine) responses by vaginal epithelial cells in co-culture with commensal or pathogenic vaginal microbes is provided in Table 2.

Table 2. Summary of inflammatory response by vaginal epithelial cells following co-culture with commensal or pathogenic microbes.

Co-Culture	Upregulated Cytokines/Chemokines (unless indicated otherwise)	References
Atopobium vaginae	Membrane associated mucin, CCL20, hBD-2, IL-1β, IL-6, IL-8, TNF-α, hBD-3, G-CSF, IP-10, Mip Iβ, RANTES, Gro-α	[114], [111]
Candida albicans	TNF-α, Il-8, CXCL8, hBD2, IL-1α	[115], [116]
Gardnerella vaginalis	IL-6, IL-8, G-CSF, IP-10, Mip-Iβ, RANTES, Gro-α, IL-1β	[111]
Lactobacillus crispatus	AMP↓, defensins ↓	[114], [113]
Lactobacillus iners	IL-6 ↔, Il-8 ↔, PRR ↑, SLPI↑	[114]
Lactobacillus vaginalis	hBD2, IL-6, IL-8	[111]
Mobiluncus curtisii	IL-6, IL-8, G-CSF, IP-10, Mip-1β, RANTES, Gro-α, hBD2	[111]
Neisseria gonorrhoeae	IL-8, IL-6, CD-54, CD-66	[117]
Prevotella bivia	IL-6, IL-8, G-CSF, IP-10, Mip-Iβ, RANTES, Gro-α, hBD2, IL-1β	[111]
Streptococcus epidermidis	IL-1β, 1L-1RA, 1L-8, GCSF, TNF-α	[113]

CXCL = C-X-C motif ligand; G-CSF = Granulocyte-colony stimulating factor; Gro = growth regulated oncogene; hBD = human beta defensin; IP = interferon gamma-induced protein; IL = Interleukin; MDC = Macrophage-derived chemokine; Mip = Macrophage inflammatory protein; PRR = Pattern recognition receptor; RANTES = Regulated on activation; SLPI = secretory leukocyte peptidase inhibitor; TNF = Tumour necrosis factor.

3. Microbial and host metabolism in the vagina

3.1 Sugar compound metabolism

Mucus produced by the cervix contains a rich mixture of carbohydrates, fatty acids, and trace elements [118], which in combination with glycogen depositions found in the vaginal epithelia [119, 120], provides the majority of nutrients utilised by vaginal microbiota. *Lactobacillus* spp. have the capacity to hydrolyse glycogen into maltodextrins, maltobiose and maltose in vaginal fluid by host-encoded α-amylase [121, 122]. Consistent with this, women clinically diagnosed with BV have lower vaginal levels of α-amylase [121]. Breakdown products of glycogen are used as primary carbon sources by *Lactobacillus* species during anaerobic glycolysis, which results in the production of lactic acid [123, 124] and contributes to their capacity to dominate the niche. Metabolic profiling of vaginal mucosa comparing women with BV and healthy controls, revealed that relative concentrations of maltodextrins, maltotriose, maltopentose and maltose are positively correlated to relative abundances of *Lactobacillus crispatus* and *Lactobacillus jensenii* whereas levels of maltotriose, maltotetraose, maltopentaose and maltohexaose are decreased in BV cases [125]. In addition, BV is also characterised by comparatively low levels of simple sugars and sugar alcohols such as lactate, fructose and mannitol as well as metabolites involved in glycerol metabolism, namely glycerol and glycerol-3-phosphate. Further, it has been reported that N-acetylneuraminate, ethanolamine, and the lipid metabolites 4-trimethylaminobutanoate/gamma-butyrobetaine and 3-methyl-2-oxobutanoate are associated with colonisation by BV associated *Eggerthella* spp. and *Megasphaera* spp. [126].

Major functional metabolic differences also exist between closely related bacterial species. For example, a recent study comparing the genomes of the two most common inhabitants of the vagina, *Lactobacillus crispatus* and *Lactobacillus iners*, revealed the former has a genome size almost double that of the latter (4300 versus 2300 genes, respectively) [127]. A consequence of this is a reduced capacity for *Lactobacillus iners* to ferment as many carbon sources as *Lactobacillus crispatus*. Although both species have the capacity to metabolise glucose, mannose, maltose, and trehalose, only *Lactobacillus crispatus* has the genetic potential to ferment lactose, galactose, sucrose and fructose. Interestingly, Macklaim et al., reported that in BV cases *Lactobacillus iners* has the genetic and metabolic capacity to uptake and utilise glycerol as a carbon source for glycolysis or glycerophosholipid metabolism [128], which may provide a possible explanation for it's ability to co-colonise with BV-like community compositions.

As mentioned previously, the end product of anaerobic glycolysis in *Lactobacillus* spp. is lactic acid, which is widely considered a hallmark of the species and therefore a proxy for good vaginal health. Under anaerobic growth conditions, physiological concentrations of lactic acid (55-111 mM) inactivate various BV associated bacteria [129] and viruses [130]. However, other bacteria often associated with BV also have the capacity to produce lactic acid including species of *Atopobium*, *Streptococcus*, *Staphylococcus*, *Megasphaera* and *Leptotrichia*, which are capable of both homolactic or heterolactic acid fermentation [131]. Vaginal epithelial cells produce almost uniquely the L-lactic acid enoantiomer, whereas major *Lactobacillus* spp. can encode genes for the production of both L- and D- enantiomers [132]. This appears to be of functional importance as women exhibiting vaginal microbiota dominated by

Lactobacillus iners have an increased ratio of L to D-lactic acid compared to women with *Lactobacillus crispatus* dominance, which is associated with increased expression of vaginal extracellular matrix metalloproteinase inducer (EMMPRIN) and activation of matrix metalloproteinase-8 (MMP8), an enzyme involved in remodelling of the fetal membranes and the cervix prior to the onset of labour [80].

3.2 Short chain fatty acids (SCFAs)

In contrast to lactic acid, SCFAs associated with high diversity vaginal microbial compositions have markedly less antimicrobial activity and appear to potentiate a pro-inflammatory vaginal environment [133-136] that may ultimately lead to reduced barrier integrity of the vaginal epithelium and increased risk of infection [137]. Elevated levels of SCFAs are readily detectable in vaginal mucosa collected from BV patients and include acetate, propionate, butyrate, succinate, formate, valerate and caproate [138-145]. Many BV associated bacteria are known SCFAs producers including *Bacteroides* (succinate) *Peptococcus* (butyrate and acetate), *Clostridium* and *Dialister* (propionate) species [139, 144, 146, 147]. *Gardnerella vaginalis* has also been shown to produce acetate and succinate [148]. Despite being historically associated with BV, it has recently been shown that succinate is also elevated in microbial communities dominated by *Lactobacillus crispatus* [149, 150]. Correlation analyses of matched vaginal metabolomic and microbiomic profiles has recently identified *Gardnerella vaginalis* as a major producer of γ-hydroxybutyrate (GHB) in vaginal secretions [149], however the role of GHB in the pathology of BV remains to be determined.

3.3 Lipid metabolism

Significant alterations in lipid metabolism can be observed in BV and appears to mainly involve eicosanoid and carnitine synthesis pathways. For example, BV has been associated with higher levels of the signalling eicosanoids, 12-HETE, a biomarker for inflammation, and lower levels of its precursor, arachidonate suggesting conversion of arachidonate to 12-HETE by BV-associated bacteria [125]. In contrast, carnitine, a product of lysine or methionine degradation involved in transport of long-chain fatty acids, is lower in vaginal mucosal samples isolated from BV patients while levels of the precursor, deoxycarnitine and ascorbic acid are higher [125]. Acyl-carnitines such as acetylcarnitine, propionylcarnitine, and butyrylcarnitine have also been previously reported to be lower in BV [22]. One proposed mechanism for lower carnitine levels in BV is through their conversion to trimethlyamine by BV associated bacteria [125].

3.4 Bioamines

Host cells are capable of producing putrescine, spermine and spermidine, which play important roles in immune regulation, lipid metabolism, nucleic acid stabilisation and cell division, however, certain bioamines are exclusively of microbial origin [151]**.** Most bioamines, with the exception of spermine and spermidine, are produced via specific amino acid decarboxylation reactions and modify redox status through consumption of hydrogen ions and the subsequent reduction of intracellular and extracellular acidity [152, 153]. Metabolic profiling studies of the *Lactobacillus* spp.

deplete, high-diversity communities as seen in BV have identified elevated levels of several bioamines including cadaverine, putrescine, agmatine and tyramine, which contribute to malodour [125, 154, 155].

3.5 Amino acids

Vaginal mucosa isolated from patients with BV contains numerous amino acid catabolites, whereas *Lactobacillus crispatus* and *Lactobacillus jensenii* dominance of the vaginal microbiota are associated with the presence of intact amino acids and dipeptides suggesting increased utilisation of amino acids in BV as a carbon and nitrogen source [125]. The amino acid arginine typically can act as a precursor for polyamines putrescine, spermidine and spermine and consistent with this, BV is associated with reduced arginine and increased putrescine [125]. Other amino acids depleted in vaginal secretions in BV include leucine, isoleucine, alanine, valine tyrosine and tryptophan [156].

4. Metabolic profiling approaches for characterisation of host-microbiota interactions

4.1 Sampling and metabolite extraction

Vaginal swab sampling [19, 33, 34, 149, 157, 158] or the collection of cervicovaginal lavage (CVL) [125, 156, 159] represent the two most common sampling techniques for metabolic profiling studies of the vaginal mucosa. To ensure adequate amount of vaginal mucosa is collected, the swab is rolled in a clockwise direction and moved across all four quadrants of the posterior fornix. The wet weight of the sample should be recorded to permit normalisation of metabolite concentrations and facilitate sample-to-sample comparison of data. For metabolic profiling studies it is recommended that swabs be stored directly in a sterile 1.5 ml microfuge tube without storage media to avoid contamination and matrix suppression when using MS based techniques. Collected swabs should be immediately processed or stored at -80°C.

A schematic workflow for metabolic profiling assays from vaginal swab extractions and assessment of microbial composition and host immune response is presented in Figure 1. There exists a vast array of extraction procedures that can be used for isolating a broad range of metabolites from vaginal swabs. Ideally the method should permit robust and reproducible capture of metabolites with different chemical properties (e.g. polarity, solubility) and across a wide concentration range. Most commonly, a biphasic, liquid-liquid extraction is performed directly on swab tips but care should be taken to optimise the volumes, ratios and pH of organic and aqueous solvents used for the procedure. It is also worth noting that particular solvents can degrade swab material and generate contaminants that overlap with genuine metabolite signals. In the example presented in Figure 1, a 1:1 methanol:water mixture is added to a swab in a centrifuge tube to reach a suggested final concentration of 50 mg vaginal fluid/ml. The swab is then vortexed and sonicated to enhance release of metabolites from the swab. For entire recovery of extracted material, the swab can be inverted and transferred to a new microfuge tube, before being centrifuged with 8000 x *g* for 2 min. The swab tip is then removed using sterile forceps and the supernatants pooled before centrifugation at 16 000 x *g* for 10 min to

remove insoluble material. The metabolite extracts can then be further cleaned up and pre-concentrated with the use of solid phase microextraction cartridges. Extracts are then evaporated using a SpeedVac with the heating function turned off to avoid metabolite degradation.

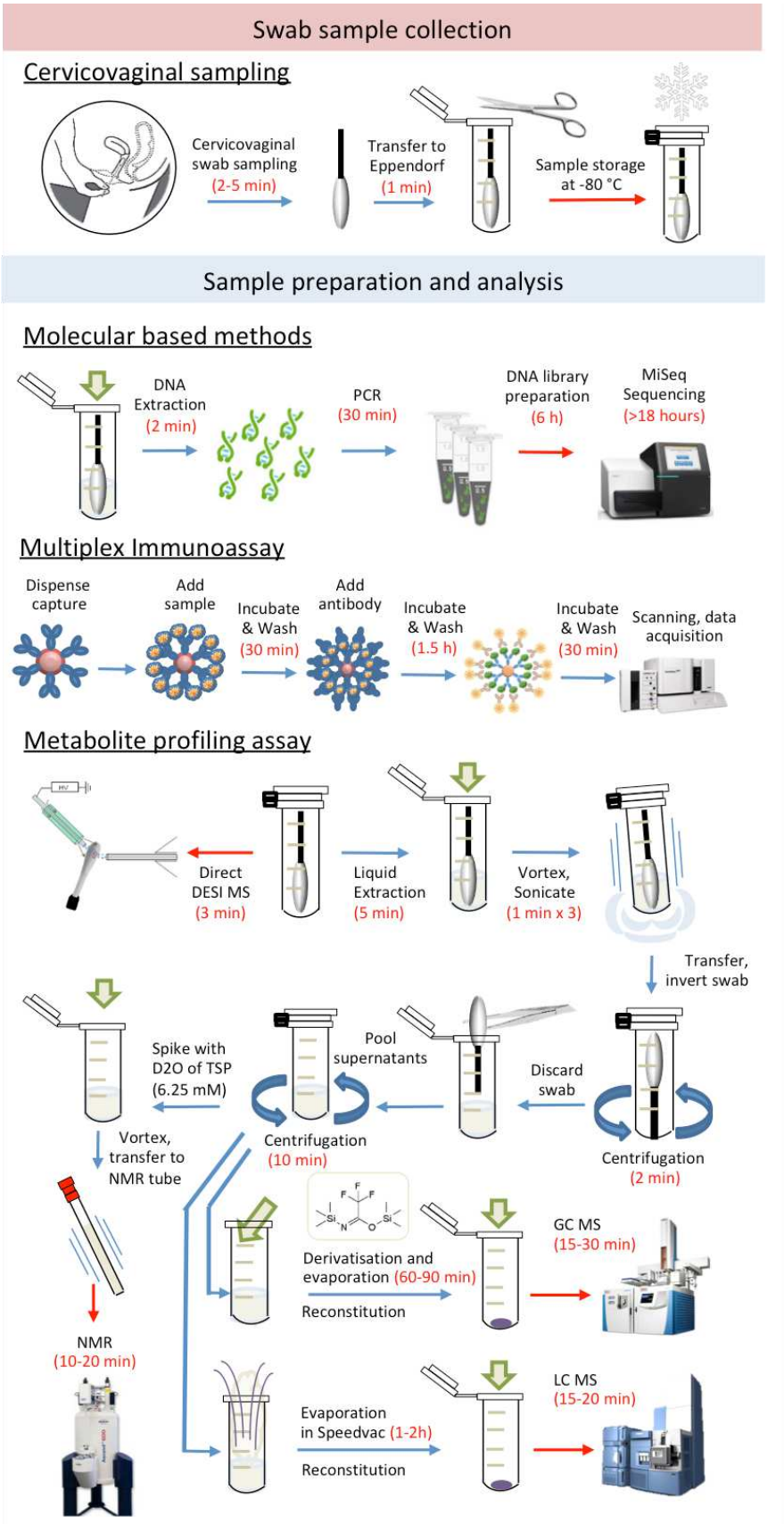

Figure 1. Example workflow for multi-omic microbiomic, immunologic and metabolome studies of the vaginal mucosal interface. Step wise workflow and time scheme illustrated for vaginal mucosal sampling, sample preparation and analysis for next generation sequencing, multiplexed immunoassay and metabolite profiling assays using NMR, GC-MS and/or LC-MS.

4.2 Comparison of different platforms for metabolomic assays

No single analytical instrument or instrumental method can detect all metabolites present in a given metabolome, thus numerous platforms are often used for metabolomic profiling including nuclear magnetic resonance (NMR) spectroscopy, Fourier transformed-infrared spectroscopy (FT-IR), and mass spectrometry (MS) coupled to separation techniques including gas chromatography-MS (GC-MS), or liquid chromatography-MS (LC-MS) or with direct flow injection methods [160-164].

NMR offers high throughput, high reproducibility metabolic profiling, minimal sample preparation and is a non-discriminatory and non-destructive technique. Unlike mass spectrometry, it is inherently quantitative thus permitting absolute concentration of detected metabolites. However, only medium to high abundance (μM) metabolites can be detected with NMR and the identification of individual metabolites based on chemical shift signals that cause sample clustering in multivariate analysis is challenging in complex mixtures [165].

Mass spectrometry (MS) offers high sensitivity detection of a wide variety of compounds including fragmentation patterns which contribute to the identification of the metabolites [165]. Both targeted profiling (in which metabolites are known *a priori*) and fingerprinting can be carried out using MS-based metabolomics approaches. Further, the use of separation techniques prior to MS reduces the complexity of the mass spectra due to metabolite separation in a time dimension, provides isobar separation, and delivers additional information on the physicochemical properties of the metabolites. However, such methods generally require a sample preparation step, which can cause metabolite losses, and based on the sample introduction system and the ionisation technique used, specific metabolite classes may be biased. Therefore, parallel application of several techniques, for example, GC-MS and LC-MS is desirable for comprehensive analysis of the metabolome [166-169]. This results in increasing time demand for analysis [170].

4.3 Direct infusion and ambient ionization techniques for rapid and direct sample analysis

Direct infusion measurements are most effective in combination with high-resolution mass spectrometers. This allows the determination of thousands of compounds based on their accurate masses in a single experiment. However, the simultaneous detection of so many compounds can in turn lead to sensitivity loss due to ion suppression effects. Ambient ionisation techniques are particularly advantageous compared to direct infusion methods as samples can be directly analysed in their native state [171, 172]. For routine clinical applications, faster and simpler methodologies are desirable for enabling the analysis of biomolecules in real time and without sample preparation to ensure effective, reproducible and immediate results. A promising technique for direct, on-sample analysis is desorption electrospray ionisation (DESI) MS [112]. DESI is an atmospheric pressure desorption ionisation method developed for the direct and rapid analysis of samples on arbitrary solid phase samples [173, 174]. Sampling and ionisation for DESI can be performed directly on various solid/gas interfaces for the ionisation of a variety of compounds ranging from lipids, peptides, proteins, drug molecules and their metabolites to secondary metabolites such as organic acids or amino acids.

Recently, we developed an analytical approach for the direct analysis of standard medical rayon swabs for rapid (<3 minutes) mucosal diagnostic point of care applications using DESI-MS [175]. In this study, we showed that DESI-MS applied to vaginal swabs permitted the detection of metabolite changes associated with BV including altered levels of lactate, short chain fatty acids and bioamines. Further it was demonstrated that DESI-MS offers the potential for culture-free microbial identification by permitting the rapid detection of microbiota-specific and associated metabolites.

4.4 Mass analysers used for targeted and untargeted metabolomics

Single quadrupol (Q), triple quadrupol (QqQ), quadrupol ion trap (QIT) and Orbitrap and Time of Flight (TOF) are the most frequently used mass analysers [176-178]. Single quadrupole is the simplest mass analyser, and can operate in the selected ion monitoring (SIM) mode which is preferred for targeted metabolomics due to its significantly higher sensitivity [179]). Triple quadrupole (QqQ) instruments have the advantage over single quadrupole in being able to perform selected reaction monitoring (SRM) experiments [180]. In SRM a specific precursor ion is selected in the first quadrupole, followed by fragmentation in the second quadrupole acting as a collision cell and finally, specific fragment ions are monitored in the third quadrupol to increase both sensitivity and selectivity [181]. The Orbitrap is one of the most recently developed mass analysers, and provides high mass resolution (>100.000 full-width at half maximum), high mass accuracy (2-5 ppm), high acquisition rates (12 Hz) and a wide dynamic range [182].

While mass accuracy describes the precision of the measured *m/z*, the mass resolving power describes the ability of a mass spectrometer to provide a specified value of mass resolution, i.e. generate distinct signals for two ions with a small *m/z* difference [183]. Untargeted metabolomics allows exploratory analyses of the majority of metabolites contained in a biological sample. The main features for mass analysers used frequently in untargeted metabolomics are mass resolution power, mass range and mass accuracy, sensitivity and linear dynamic range. In order to distinguish between isomeric and isobaric analytes, as is often the case for untargeted metabolomics, high-resolution mass spectrometers are typically the instrument of choice. High resolution also allows the calculation of empirical formulas, which enables a better confidence for compound identification [183].

The choice of mass analyser depends on the characteristics of the untargeted profiling technique to be deployed. For DESI-MS and other techniques that generate mass spectra without another dimension for feature deconvolution, mass resolution and mass accuracy are very important factors to consider. This makes Fourier Transform instruments such as the Orbitrap a natural choice for these methods. The impact of mass analysers on vaginal mucosal metabolite profiles captured using DESI-MS is presented in Figure 2. Comparatively, the Orbitrap instrument provides the best-resolved and most informative mass spectra with the highest spectral richness, mass resolution and mass accuracy. TOF-MS results in a similar number of detected spectral features as the Orbitrap, however the quality of mass accuracy is poorer and harder to stabilise over longer measurement times. The mass spectrum acquired with ion-trap provides comparatively lower mass accuracy (>20 ppm), weaker sensitivity, a smaller number of detected spectral features with reduced signal intensities and elevated baseline with lower signal/noise ratio.

Nevertheless, the reduced cost of ion trap instruments and their ability to consistently detect common, abundant spectral features including lipid molecules makes them valuable platforms for point of care diagnostics and biomarker measurements in clinical environments.

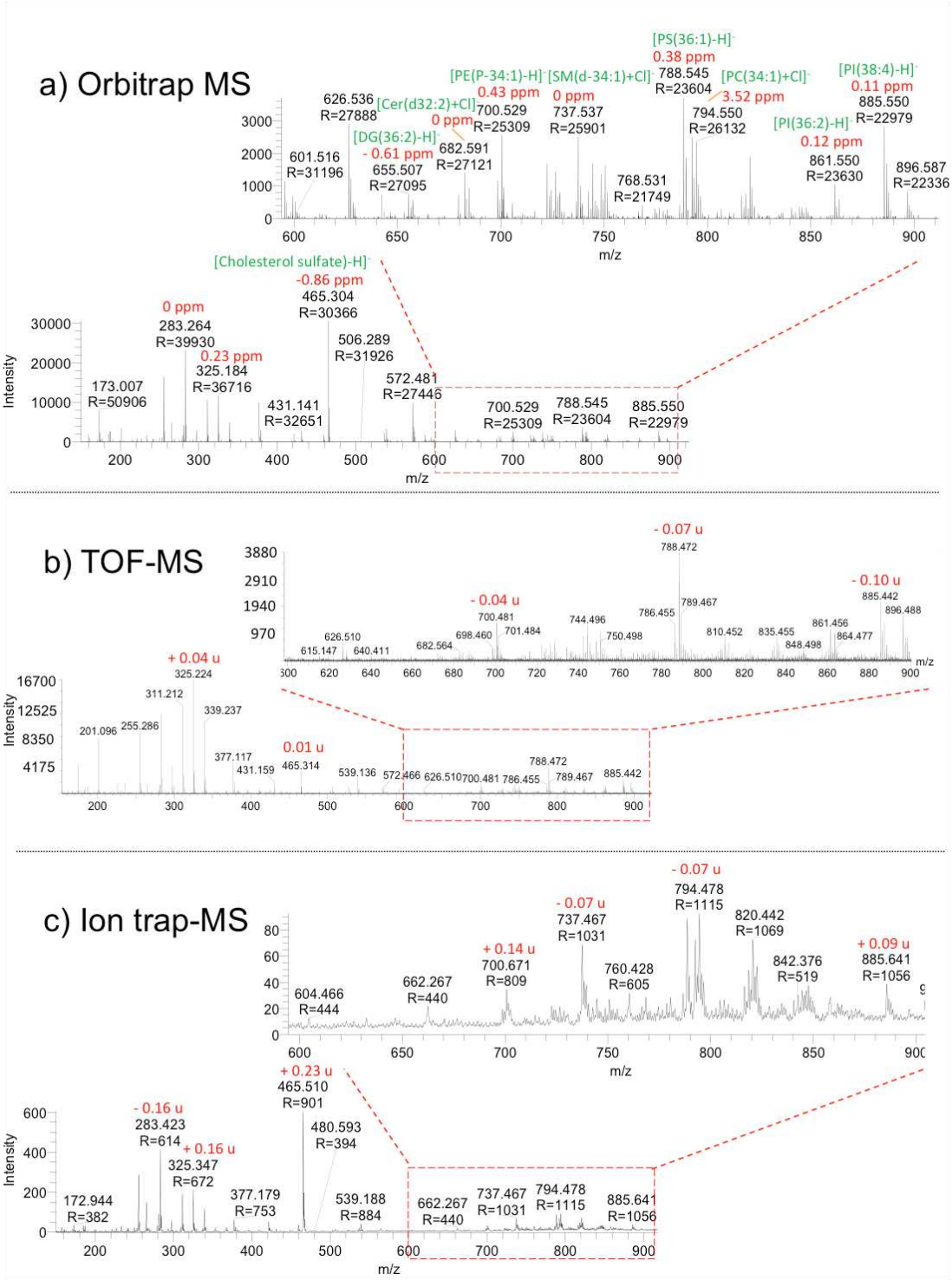

Figure 2. Comparison of MS spectra of matched cervicovaginal fluid samples obtained using a) Orbitrap MS (LTQ-Orbitrap Discovery, Thermo Scientific), b) TOF-MS (Xevo G2-S, Waters Coorporation) and c) Ion trap MS (LTQ-Orbitrap Discovery, Thermo Scientific) mass analysers combined with DESI as a metabolite ionisation source in the mass range from m/z 150-900 and m/z 600-900.

5. Conclusions

Detailed examination of host:microbiota interactions at the vaginal mucosal interface can be achieved throughout the application of multi-omic approaches including molecular, immunological and metabolomic profiling techniques and their integration via data fusion strategies. These profiling techniques are in a continual flux of development and evolution thus optimisation of the methodological and analytical workflow is critical for limiting the introduction of technical and experimental biases that can hinder interpretation and reproducibility of data across different studies. Despite these challenges, clear relationships exist between vaginal microbiota composition and disease-specific host responses that can be examined using immunological and metabolic profiling approaches. While validation and confirmation of purported mucosal biomarkers is still required, there exists strong potential for the development of rapid diagnostic and prognostic applications in women's health and the identification of novel targets for future therapeutic interventions.

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