Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP
Faculdade de Medicina
Unidade de Pesquisa Experimental (Unipex)
Laboratório de Imunopatologia da Relação Materno-Fetal

LAURA EMI YONEZAWA DE SOUZA

Além da presença: contribuições da abundância absoluta de Gardnerella vaginalis e Fannyhessea vaginae para a resposta inflamatória vaginal

Dissertação apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu - Universidade Estadual Paulista “Júlio de Mesquita Filho” - para obtenção do título de Mestre em Ciências, Área de Patologia.

Orientadora: Profa. Associada Márcia Guimarães da Silva

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ATA DA DEFESA PÚBLICA DA DISSERTAÇÃO DE MESTRADO DE LAURA EMI YONEZAWA DE SOUZA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA, DA FACULDADE DE MEDICINA - CÂMPUS DE BOTUCATU.

Aos 23 de fevereiro de 2026, às 14h, por meio de Videoconferência, realizou-se a defesa de DISSERTAÇÃO DE MESTRADO de LAURA EMI YONEZAWA DE SOUZA, intitulada "Além da presença: contribuições da abundância absoluta de Gardnerella vaginalis e Fannyhessea vaginae para a resposta inflamatória vaginal". A Comissão Examinadora foi constituída pelos seguintes membros: Profa. Dra. MARCIA GUIMARÃES DA SILVA (Orientador(a) - Participação Presencial) do(a) Depto. de Patologia / FM/Botucatu - Unesp, Profa. Dra. ANDRÉA DA ROCHA TRISTÃO (Participação Presencial) do(a) Depto. de Ginecologia e Obstetrícia / FM/Botucatu - Unesp, Prof. Dr. JULIANO NOVAK (Participação Virtual) do(a) FM/Ribeirão Preto - USP, Após a exposição pela mestranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: Aprovada. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. MARCIA GUIMARÃES DA SILVA

Impacto potencial desta pesquisa

Impacto científico: Este estudo contribuiu para ampliar o conhecimento sobre como bactérias associadas à vaginose bacteriana, especialmente as espécies *Gardnerella vaginalis* e *Fannyhessea vaginae*, influenciam a resposta inflamatória no ambiente vaginal. Ao analisar diferentes padrões de microbiota vaginal, incluindo a microbiota intermediária, frequentemente pouco explorada em estudos científicos, o estudo demonstra que alterações na composição bacteriana estão relacionadas a mudanças mensuráveis na inflamação local. Esses achados contribuem para uma compreensão mais completa e contínua da saúde vaginal, fornecendo bases científicas para o desenvolvimento de novos estudos e estratégias de prevenção, diagnóstico e tratamento de disbioses vaginais.

Impacto social: A vaginose bacteriana e as alterações intermediárias da microbiota vaginal são condições comuns entre mulheres em idade reprodutiva, mas muitas vezes subdiagnosticadas ou tratadas de forma inadequada. Ao mostrar que a microbiota intermediária também apresenta sinais moleculares de inflamação, este estudo corrobora a importância do reconhecimento precoce dessas alterações, mesmo quando os sintomas são leves ou ausentes. Dessa forma, a pesquisa contribui para o fortalecimento da atenção à saúde da mulher, promovendo cuidados mais individualizados, prevenção de complicações ginecológicas e melhoria da qualidade de vida.

Impacto econômico: A identificação de padrões inflamatórios associados a diferentes perfis de microbiota vaginal pode auxiliar na criação de estratégias de diagnóstico mais precisas e intervenções mais eficazes. Isso pode reduzir o uso inadequado de antibióticos, a recorrência de infecções e os custos relacionados ao tratamento de complicações, como infecções ascendentes e problemas reprodutivos.

Potential impact of this research

Scientific impact: This study contributed to expanding knowledge on how bacteria associated with bacterial vaginosis, especially the species *Gardnerella vaginalis* and *Fannyhessea vaginae*, influence the inflammatory response in the vaginal environment. By analyzing different patterns of vaginal microbiota, including the so-called intermediate microbiota, which is often underexplored in scientific studies, the research demonstrates that changes in bacterial composition are associated with measurable alterations in local inflammation. These findings contribute to a more comprehensive and continuous understanding of vaginal health, providing a scientific basis for the development of new studies and strategies for the prevention, diagnosis, and treatment of vaginal dysbiosis.

Social impact: Bacterial vaginosis and intermediate alterations of the vaginal microbiota are common conditions among women of reproductive age, but they are often underdiagnosed or inadequately treated. By showing that the intermediate microbiota also presents molecular signs of inflammation, this study corroborates the importance of early recognition of these alterations, even when symptoms are mild or absent. In this way, the research contributes to strengthening women's health care by promoting more individualized care, preventing gynecological complications, and improving quality of life.

Economic impact: The identification of inflammatory patterns associated with different vaginal microbiota profiles may help in the development of more accurate diagnostic strategies and more effective interventions. This can reduce the inappropriate use of antibiotics, infection recurrence, and costs related to the treatment of complications, such as ascending infections and reproductive health problems.

Dedicatória

Dedico este trabalho à minha mãe e à minha avó, por todo amor incondicional e incentivo ao longo da minha trajetória. Tudo o que sou e conquistei carrega um pouco de vocês.

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Resumo

Introdução: A microbiota vaginal saudável, predominada por espécies lactobacilares, em mulheres na menacme, possui importante papel para o equilíbrio da saúde reprodutiva feminina, possibilitando um microambiente protetivo contra patógenos causadores de infecções sexualmente transmissíveis (ISTs) e hostil ao crescimento de outras espécies bacterianas. Por ser um ambiente dinâmico, a microbiota vaginal pode sofrer alterações. Desequilíbrios na microbiota vaginal podem levar à ocorrência da vaginose bacteriana (VB), uma disbiose clássica, comum em mulheres em idade reprodutiva, caracterizada pela redução ou ausência de *Lactobacillus* spp., juntamente com o aumento da abundância de espécies bacterianas anaeróbias facultativas, como *Gardnerella vaginalis*, *Fannyhessea vaginae*, *Mycoplasma hominis*, *Prevotella bivia*, *Mobiluncus* spp., entre outras. Essa condição, quando sintomática, pode causar corrimento e mau odor, além de estar associada à maior vulnerabilidade às ISTs e a complicações gestacionais. Uma das principais espécies bacterianas associadas à VB é a *G. vaginalis*, historicamente considerada a principal espécie envolvida na etiologia dessa disbiose. Trata-se de uma espécie produtora de sialidase A e vaginolisina como fatores de virulência, sendo que ambos permitem a formação do biofilme. *F. vaginae* é outra espécie bacteriana frequente no core patológico da VB e que geralmente está em combinação com *G. vaginalis*, possuindo importante papel na formação e no estabelecimento do biofilme. A microbiota vaginal atua na modulação imunológica do microambiente vaginal. A VB é clinicamente considerada uma condição não inflamatória; apesar disso, está associada a uma inflamação molecular, caracterizada pela produção de citocinas pró-inflamatórias. Comunidades dominadas por espécies anaeróbias estão associadas a uma resposta pró-inflamatória mais intensa em comparação àquelas dominadas por *Lactobacillus* spp. A coocorrência de *G. vaginalis* e *F. vaginae* no biofilme vaginal pode resultar em um efeito sinérgico sobre a resposta imune do hospedeiro. Diante desse cenário, estudos são necessários para determinar a coexistência de *G. vaginalis* e *F. vaginae* nas disbioses vaginais e na microbiota normal de mulheres em idade reprodutiva. **Metodologia:** Foi realizado um estudo prospectivo, transversal, que incluiu 152 mulheres atendidas no Ambulatório de Infecções Genitais Femininas do Complexo HCFMB, Unesp, nos anos de 2024 e 2025. O projeto foi aprovado pelo Comitê de Ética em Pesquisa local (CAAE 91235025.0.0000.5411). No momento da inclusão no estudo, foi realizada a coleta de conteúdo vaginal para posterior classificação da microbiota vaginal em exame microscópico corado pelo método de Gram, aplicando os critérios de Nugent et al. (1991). Foram incluídas mulheres com diagnóstico de Flora I (N = 68), Flora II (N = 24) e VB (N = 60). Foi realizada a coleta de material endocervical para pesquisa de *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis* e *M. genitalium*, bem como a coleta de lavado vaginal para a pesquisa de *G. vaginalis* e *F. vaginae* e a dosagem de citocinas. A extração do DNA das amostras endocervicais foi realizada com o kit QIAmp DNA Mini and Blood Mini Handbook (Qiagen, Hilden, NRW, Alemanha). Para a pesquisa de *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis* e *M. genitalium*, foi realizada PCR em tempo real multiplex utilizando o kit Allplex™ CT/NG/MG/TV Assay (Seegene). A extração do DNA dos lavados cervicovaginais foi realizada com o kit DNeasy PowerSoil Pro (Qiagen, Hilden, NRW, Alemanha). Para a obtenção da curva padrão de *G. vaginalis*, bactérias (ATCC #14018) adquiridas da American Type Culture Collection (ATCC) foram cultivadas em meio New

York City III e, em seguida, foi realizada uma etapa de clonagem. O DNA plasmidial obtido foi utilizado para a construção da curva padrão. A quantificação absoluta de *G. vaginalis* foi realizada por PCR quantitativo em tempo real (qPCR), utilizando os primers F-GV1 e RGV3. Para a obtenção da curva padrão de *F. vaginae*, foi utilizado um plasmídeo contendo o gene de interesse que codifica a região 16S rRNA de *F. vaginae* (Thermo Fisher Scientific GENEART). A quantificação absoluta de *F. vaginae* foi realizada por PCR quantitativo em tempo real (qPCR), utilizando os primers Fw e Rv. Para determinar a produção das citocinas inflamatórias IL-1β, IL-6, CXCL-8, IL-10 e TNF-α, foram coletados lavados cervicovaginais e utilizado o kit DuoSet, disponível comercialmente (R&D Systems, MN, EUA), seguindo as instruções do fabricante. **Resultados:** *G. vaginalis* e *F. vaginae* foram detectadas em 62,5% e 69,7% das amostras, respectivamente. As cargas bacterianas de *G. vaginalis* foram significativamente maiores no grupo VB [7,2 cópias/μL (6,1–8,7)] quando comparadas ao grupo Flora I [3,4 cópias/μL (1,8–6,7); $p < 0,001$]. Já as cargas bacterianas de *F. vaginae* foram estatisticamente maiores no grupo VB [6,5 cópias/μL (5,5–7,5)] quando comparadas aos grupos Flora I [4,4 cópias/μL (3,9–5,1); $p < 0,001$] e Flora II [4,5 cópias/μL (3,7–5,4); $p < 0,001$]. Os níveis de IL-1β foram significativamente maiores nos grupos Flora II [2,3 pg/mL (1,8–2,7); $p = 0,011$] e VB [2,1 pg/mL (1,6–2,5); $p = 0,024$] quando comparados ao grupo Flora I [1,7 pg/mL (1,3–2,3)], enquanto os níveis de CXCL-8 foram mais elevados no grupo Flora II [3,3 pg/mL (3,1–3,6)] quando comparado ao grupo Flora I [3,1 pg/mL (2,7–3,5); $p = 0,021$]. Não houve diferença significativa nos níveis das demais citocinas (IL-6, IL-10 e TNF-α) entre os diferentes padrões de microbiota. **Conclusão:** A VB está associada ao aumento das cargas de *G. vaginalis* e *F. vaginae* e ao aumento seletivo de IL-1β, evidenciando uma assinatura inflamatória distinta associada à disbiose vaginal.

Abstract

Introduction: A healthy vaginal microbiota, predominantly composed of lactobacilli species in women of reproductive age, plays an important role in maintaining female reproductive health by providing a protective microenvironment against pathogens that cause sexually transmitted infections (STIs) and by inhibiting the growth of other bacterial species. As a dynamic environment, the vaginal microbiota may undergo changes. Imbalances in the vaginal microbiota can lead to the development of bacterial vaginosis (BV), a classical dysbiosis common among women of reproductive age, characterized by the reduction or absence of *Lactobacillus* spp. together with an increased abundance of facultative anaerobic bacterial species such as *Gardnerella vaginalis*, *Fannyhessea vaginae*, *Mycoplasma hominis*, *Prevotella bivia*, *Mobiluncus* spp., among others. When symptomatic, this condition may cause vaginal discharge and unpleasant odor, and is also associated with increased susceptibility to STIs and pregnancy-related complications. One of the main bacterial species associated with BV is *G. vaginalis*, historically considered the primary species involved in the etiology of this dysbiosis. This species produces sialidase A and vaginolysin as virulence factors, both of which enable biofilm formation. F. vaginae is another bacterial species frequently found in the pathological core of BV and is usually present in combination with *G. vaginalis*, playing an important role in biofilm formation and establishment. The vaginal microbiota also plays a role in the immunological modulation of the vaginal microenvironment. Although BV is clinically considered a non-inflammatory condition, it is associated with molecular inflammation characterized by the production of pro-inflammatory cytokines. Communities dominated by anaerobic species are associated with a more intense pro-inflammatory response compared to those dominated by *Lactobacillus* spp. The co-occurrence of *G. vaginalis* and *F. vaginae* in the vaginal biofilm may result in a synergistic effect on the host immune response. In this context, further studies are needed to determine the coexistence of G. vaginalis and *F. vaginae* in vaginal dysbiosis and in the normal microbiota of women of reproductive age. **Methods:** A prospective, cross-sectional study was conducted including 152 women attended at the Female Genital Infections Outpatient Clinic of the HCFMB Complex, Unesp, between 2024 and 2025. The study was approved by the local Research Ethics Committee (CAAE 91235025.0.0000.5411). At enrollment, vaginal samples were collected for microbiota classification using Gram-stained microscopic examination according to the criteria of Nugent et al. (1991). Women were classified as Normal microbiota (N = 68), Intermediate microbiota (N = 24), or BV (N = 60). Endocervical samples were collected for the detection of *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, and *M. genitalium*, and vaginal lavage samples were collected for the detection of *G. vaginalis* and *F. vaginae* and for cytokine quantification. DNA extraction from endocervical samples was performed using the QIAmp DNA Mini and Blood Mini Handbook kit (Qiagen, Hilden, NRW, Germany). Multiplex real-time PCR was carried out using the Allplex™ CT/NG/MG/TV Assay (Seegene) for the detection of *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, and *M. genitalium*. DNA extraction from cervicovaginal lavage samples was performed using the DNeasy PowerSoil Pro kit (Qiagen, Hilden, NRW, Germany). For the construction of the standard curve for G. vaginalis, bacteria (ATCC #14018) obtained from the American Type Culture Collection (ATCC) were cultured in New York City III medium and subsequently subjected to a cloning step. The resulting plasmid DNA was used to generate the standard curve. Absolute quantification of G. vaginalis was performed by real-time quantitative PCR (qPCR) using the F-GV1 and

RGV3 primers. For the standard curve of *F. vaginae*, a plasmid containing the gene encoding the 16S rRNA region of *F. vaginae* (Thermo Fisher Scientific GENEART) was used. Absolute quantification of F. vaginae was carried out by real-time qPCR using the Fw and Rv primers. To measure the production of inflammatory cytokines (IL-1β, IL-6, CXCL-8, IL-10, and TNF-α), cervicovaginal lavage samples were collected and analyzed using the commercially available DuoSet kit (R&D Systems, MN, USA), following the manufacturer's instructions. **Results:** *G. vaginalis* and *F. vaginae* were detected in 62.5% and 69.7% of the samples, respectively. The bacterial loads of *G. vaginalis* were significantly higher in the BV group [7.2 copies/µL (6.1–8.7)] compared to the Flora I group [3.4 copies/µL (1.8–6.7); $p < 0.001$]. Similarly, *F. vaginae* loads were significantly higher in the BV group [6.5 copies/µL (5.5–7.5)] compared to both normal microbiota [4.4 copies/µL (3.9–5.1); $p < 0.001$] and intermediate microbiota [4.5 copies/µL (3.7–5.4); $p < 0.001$]. IL-1β levels were significantly higher in the intermediate microbiota [2.3 pg/mL (1.8–2.7); $p = 0.011$] and BV groups [2.1 pg/mL (1.6–2.5); $p = 0.024$] compared to the normal microbiota group [1.7 pg/mL (1.3–2.3)]. CXCL-8 levels were also higher in the intermediate microbiota group [3.3 pg/mL (3.1–3.6)] than in the normal microbiota group [3.1 pg/mL (2.7–3.5); $p = 0.021$]. No significant differences were observed in the levels of the other cytokines (IL-6, IL-10, and TNF-α) among the different microbiota patterns. **Conclusion:** BV is associated with increased loads of *G. vaginalis* and *F. vaginae* and with a selective increase in IL-1β, indicating a distinct inflammatory signature associated with vaginal dysbiosis.

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Lista de abreviaturas

ATCC	*American Type Culture Collection*
BVAB	Bactérias associadas à vaginose bacteriana
CD59	Glicoproteína CD59
CIM	Concentração inibitória mínima
Cpn60	Chaperonina 60
CSTs	*Community State Types*
CXCL-8	Ligante 8 da quimiocina CXC
EPS	Substâncias poliméricas extracelulares
FISH	Hibridização fluorescente *in situ*
GM-CSF	Fator estimulador de colônias de granulócitos e macrófagos
Gp130	Receptor de glicoproteína 30
H_2O_2	Peróxido de hidrogênio
hBD	Beta-defensina humana
HIV	Vírus da imunodeficiência humana
HPV	Papilomavírus humano
HSP60	*Heat shock protein 60*
IFN-γ	Interferon gama
IL	Interleucina
IL-1RA	Antagonista de receptor de interleucina 1
IP-10	*Interferon gamma induced protein 10*
ISTs	Infecções sexualmente transmissíveis
KOH	Hidróxido de potássio
MALDI-TOF	*Matrix-Assisted Laser Desorption/Ionization*
MAPK p38	Proteína quinase ativada por mitógeno p38
MCP-1	Proteína Quimiotática de Monócitos-1
mgCSTs	Metagenomic Community State Types
mgSs	Subespécies metagenômicas
mIL-6R	Receptor de interleucina 6 ligado à membrana
MIP-1β	Proteína inflamatória de macrófagos 1β
MIP-3α	Proteína inflamatória de macrófagos 3α
NF-kB	Fator nuclear kappa B
NLRP3	Proteína 3 contendo domínios NOD, LRR e pirina
NLRs	Receptores do tipo Nod
OMS	Organização Mundial da Saúde
PRR	Receptores de reconhecimento de padrões
RANTES	*Regulated on activation, normal T-cell expressed and secreted*
SLPI	Inibidor de protease leucocitária secretora

TLRs	Receptores do tipo Toll
TNF-α	Fator de necrose tumoral α
VB	Vaginose bacteriana

Sumário

Capítulo 1

Revisão de Literatura

1. Introdução

A saúde da mulher em idade reprodutiva é influenciada por uma complexa interação de fatores biológicos, ambientais e sociais. Entre os aspectos biológicos, a microbiota desempenha papel central na manutenção da homeostase, atuando como barreira contra microrganismos patogênicos, modulando a resposta imune e participando de processos metabólicos essenciais (1,2). Nas últimas décadas, o desenvolvimento e a consolidação de técnicas de biologia molecular têm permitido a análise detalhada da composição microbiana em distintos sítios do corpo humano, revelando sua relevância tanto para a homeostase quanto para o desenvolvimento de condições patológicas do sistema reprodutivo feminino. Nesse contexto, a microbiota vaginal emerge como um elemento-chave na proteção contra infecções e na manutenção da saúde ginecológica e reprodutiva. Alterações nesse ecossistema podem predispor ao estabelecimento de disbioses, com repercussões clínicas relevantes, incluindo maior suscetibilidade a aquisição de infecções sexualmente transmissíveis (ISTs), menor taxa de *clearance* da infecção pelo Papilomavírus Humano (HPV) e complicações gestacionais, com destaque para a Rotura Prematura de Membranas Pré-Termo e para o Parto Pré-Termo Espontâneo (2,3).

1.1. A microbiota vaginal

De forma geral, a microbiota estabelece uma relação mutualística com o corpo humano, desempenhando funções cruciais em diversos processos fisiológicos, modulação do sistema imunológico e na proteção contra ação dos microrganismos patogênicos. Entre os diferentes nichos microbianos dos nossos sistemas, a microbiota vaginal se destaca por representar cerca de 9% do total, exercendo importante papel para o equilíbrio da saúde reprodutiva feminina (1).

A primeira descrição científica da microbiota vaginal foi realizada por Albert Döderlein em 1892 (4), que a caracterizou como ambiente homogêneo, constituída por bacilos Gram-positivos, posteriormente denominados como bacilos de Döderlein. Atualmente, sabe-se que esses morfotipos bacterianos pertencem ao gênero *Lactobacillus,* e seu domínio está associada ao microambiente vaginal saudável (2), uma vez que os *Lactobacillus* spp. são produtores de ácido lático, peróxido de hidrogênio (H_2O_2) e bacteriocinas que, ao contribuírem para a manutenção de um pH ácido, inibem o crescimento de microrganismos patogênicos (2).

Durante a menacme, os níveis elevados de estrogênio promovem o acúmulo de glicogênio nas células epiteliais vaginais. Esse substrato é catabolizado pela α-amilase humana em açúcares simples, posteriormente metabolizados em ácido lático pelos lactobacilos, resultando na acidificação do microambiente vaginal (pH 3,8 – 4,5). Esse ambiente ácido favorece a lise de bactérias oportunistas, contribuindo para um estado anti-inflamatório, estimulando a produção de citocinas reguladoras, como o antagonista de receptor de interleucina 1 (IL-1RA), e inibindo a expressão de mediadores inflamatórios, como interleucina (IL)-6, fator de necrose tumoral alfa (TNF-α) e quimiocinas (CXCL-8, RANTES e MIP-3α) (2).

Além do ácido lático, os *Lactobacillus* spp. também são produtores de outros fatores antimicrobianos, como o H_2O_2, que inibe principalmente o crescimento de bactérias anaeróbias (2); a arginina deaminase, que metaboliza arginina em citrulina e amônia, reduzindo a disponibilidade desse aminoácido necessário para o crescimento de certos patógenos anaeróbios, além de promover resistência ao ambiente ácido (3,5); e as bacteriocinas e biossurfactantes, que promovem a autofagia de bactérias intracelulares, vírus e protozoários (2). As bacteriocinas são pequenos peptídeos antimicrobianos sintetizados por bactérias, especialmente aquelas que vivem em ambiente polimicrobiano competitivo, capazes de formar poros nas membranas celulares e inibir enzimas do metabolismo celular, promovendo citotoxicidade e impossibilitando o crescimento e formação do biofilme (6,7). Já os biossurfactantes são compostos anfifílicos que incluem glicolipídeos, lipopeptídeos, ácidos graxos, ramnolipídeos, fosfolipídeos e complexos proteína-polissacarídeo, que tem funções altamente seletivas e especializadas, sendo capazes de alterar a química de superfícies, reduzindo a tensão superficial e modulando a adesão de patógenos e a formação do biofilme, além de interagirem com as membranas celulares e alterarem sua permeabilidade (6, 8). Além disso, as espécies lactobacilares também são capazes de se aderir às células epiteliais vaginais, competindo com outros microrganismos pelos sítios de ligação (9).

Através desses mecanismos, os lactobacilos estabelecem um microambiente protetivo contra a infecção de patógenos, dentre eles, os causadores de cervicites clássicas como *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Mycoplasma genitalium e Trichomonas vaginalis*, além de infecções pelo HPV e pelo vírus da Imunodeficiência Humana (HIV) (2). Além disso, a manutenção de um ambiente vaginal eubiótico está diretamente associado a melhores desfechos reprodutivos, incluindo maior taxa de sucesso em técnicas de fertilização assistida (10) e menor risco de resultados adversos da gestação (3).

1.2. Classificação dos padrões de microbiota

Em 2011, Ravel et al. (11) clusterizaram genomicamente a microbiota de mulheres em idade reprodutiva em cinco diferentes comunidades bacterianas, denominadas de *Community State Types* (CSTs). As CSTs se diferenciam de acordo com a espécie de maior abundância, sendo as CSTs I, II, III e V dominadas, respectivamente, por *L. crispatus*, *L. gasseri*, *L. iners* e *L. jensenii*, enquanto a CST IV é composta por um grupo heterogêneo de espécies bacterianas, predominantemente anaeróbias, as quais estão frequentemente associadas ao *core* patológico da vaginose bacteriana (VB).

Com avanços na área da Biologia Molecular e Genômica, em 2020, France et al. (12) propuseram o modelo VALENCIA (*VAginaL community state typE Nearest CentroId clAssifier*) como método de classificação das CSTs. A partir da análise de mais de 13.000 perfis de microbiota vaginal, o VALENCIA permitiu o refinamento da classificação anterior, incorporando composições menos frequentes e distinguindo subtipos com base na abundância relativa das espécies dominantes. Esse método baseia-se no treinamento de um classificador que define um centroide para cada categoria de comunidade bacteriana, e o utiliza como referência para atribuir novas amostras aos tipos de CST mais semelhante. Com esse modelo, foram identificadas 13 diferentes CSTs. Seis delas são dominadas por espécies lactobacilares: CST I-A e CST I-B, ambas dominadas por *L. crispatus*, sendo que A possui maior abundância relativa dessa espécie do que B; CST II, dominada por *L. gasseri*; CST III-A e CST III-B, dominada por *L. iners*, com A apresentando maior abundância relativa de *L. iners* do que B; e CST V, dominada por *L. jensenii*.

Além das comunidade lactobacilares, o modelo identificou CSTs dominadas por espécies anaeróbias associadas à disbiose: CST IV-A, dominada por Candidatus *Lachnocurva vaginae* (anteriormente bactérias associadas à vaginose bacteriana - BVAB1) e com moderada abundância relativa de *Gardnerella vaginalis* e *Fannyhessea vaginae*; CST IV-B, dominada por *G. vaginalis*, com baixa abundância relativa de Ca. *L. vaginae* e moderada abundância relativa de *F. vaginae*; e CST IV-C, composta por uma comunidade diversificada de bactérias anaeróbias estritas ou facultativas, e baixa abundância de *Lactobacillus* spp., *G. vaginalis* e *F. vaginae*. Esta última foi subdividida em cinco subcategorias: CST IV-C0, mais homogênea e com moderada abundância de *Prevotella*; IV-C1, dominada por *Streptococcus*; IV-C2, dominada por *Enterococcus*; IV-C3, dominada por *Bifidobacterium*; e IV-C4, dominada por *Staphylococcus* (12).

Mais recentemente, em 2023, foi proposta uma nova classificação capaz de descrever o microbioma vaginal de maneira mais precisa e funcional. Através da análise de 1.890 metagenomas vaginais de mulheres em idade reprodutiva, foram definidos os *Metagenomic Community State Types* (mgCSTs) (13). Tal classificação leva em consideração agrupamentos de cepas da mesma espécie que coexistem na microbiota vaginal, denominados de subespécies metagenômicas (mgSs). Cada mgCSTs representa uma combinação única de mgSs, permitindo integrar informações taxogenômicas e de seus potenciais funcionais.

Ao todo, foram estabelecidas 27 mgCSTs, em sua maioria, dominadas por espécies comumente observadas no ambiente vaginal: mgCSTs 1 a 6 dominadas por *L. crispatus*, mgCSTs 7 a 9 por *L. gasseri*, mgCSTs 10 a 14 por *L. iners*, mgCSTs 15 e 16 por *L. jensenii*, mgCSTs 17 a 19 por Ca. *Lachnocurva vaginae*, mgCSTs 20 a 25 por *Gardnerella*, mgCST 26 por *Bifidobacterium breve*; e mgCST 27 contendo espécies menos comuns como *Streptococcus anginosus* ou não possuindo um táxon predominante (13).

Por ser um ambiente dinâmico, a microbiota vaginal pode sofrer alterações associadas a flutuações hormonais, gestação, ciclo menstrual e, ainda, a fatores externos como o estresse, métodos contraceptivos e práticas de higiene íntima e sexuais (9,14). Assim, enquanto algumas mulheres mantêm comunidades microbianas relativamente estáveis, outras podem apresentar mudanças mais drásticas na composição da sua microbiota (15). As diferentes classificações do microbioma vaginal, sintetizadas na Figura 1, ilustram como o conhecimento sobre esses perfis comunitários tem sido refinado ao longo dos anos, reconhecendo um espectro que inclui tantas comunidades dominadas por *Lactobacillus* spp. quanto perfis menos estáveis e associados à disbiose. Embora os perfis predominados por *Lactobacillus* spp. sejam geralmente associados a um ambiente estável e protetor, a redução dessas espécies e o consequente aumento de bactérias anaeróbias alteraram significativamente o equilíbrio vaginal. Essas mudanças na estrutura da comunidade microbiana caracterizam os estados de disbioses vaginais.

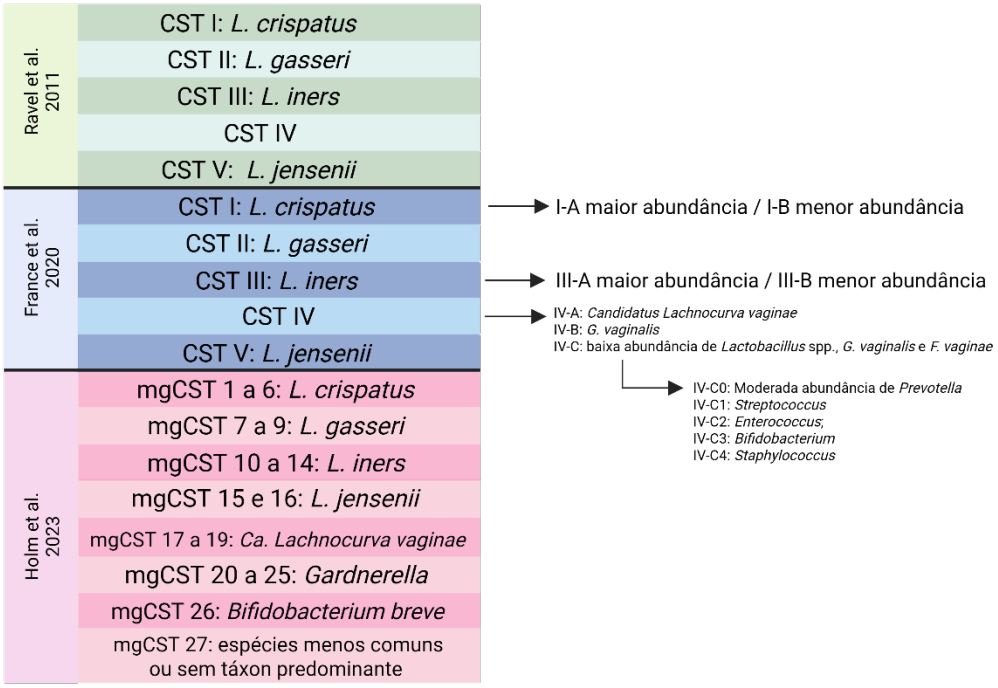

Figura 1. Evolução das classificações da microbiota vaginal ao longo do tempo. Representação comparativa dos modelos de classificação do microbioma vaginal propostos por Ravel et al. (2011), France et al. (2020) e Holm et al. (2023). O modelo inicial (7) descreveu cinco *Community State Types* (CSTs) baseados na espécie bacteriana dominante. O modelo VALENCIA (8) refinou essa categorização, reconhecendo subtipos e maior diversidade intraespécies. Posteriormente, o modelo metagenômico (9) introduziu os *Metagenomic Community State Types* (mgCSTs), que integram informações taxonômicas e funcionais, ampliando para 27 perfis distintos de microbiota vaginal. Imagem de autoria própria (BioRender).

1.3. Vaginose Bacteriana

Dentre as disbioses mais comuns em mulheres em idade reprodutiva, destaca-se a VB. Essa disbiose é caracterizada pela redução drástica ou ausência de espécies do gênero *Lactobacillus*, juntamente com o aumento da abundância relativa de espécies bacterianas anaeróbias estritas e facultativas (15), como *G. vaginalis*, *F. vaginae*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Prevotella bivia*, *Mobiluncus* spp., *Leptotrichia* spp., entre outras (16).

As taxas de VB podem variar de 5% a 70%, dependendo das condições socioeconômicas da população estudada, bem como de fatores comportamentais e clínicos, incluindo práticas sexuais e o hábito tabagista, além dos métodos diagnósticos utilizados (17). Mundialmente, a prevalência é mais alta em países do continente africano, com taxas que chegam a 51% no Lesoto e 44% no Quênia (17,18), enquanto na região ocidental africana os valores são menores, como 8% na Burkina Faso (19). Na Europa, as prevalências são geralmente mais baixas, embora altas prevalências são

observadas em países como a Polônia (19%), Turquia (23%) e Noruega (24%) (18). Taxas semelhantes são observadas em regiões do continente asiático e da Oceania, com prevalências que variam entre 30 e 32% (20,21).

No Brasil, a prevalência de VB apresenta ampla variação entre estudos, em razão de diferenças metodológicas e do constituinte populacional. Em inquérito populacional conduzido em área rural do nordeste, estimou-se prevalência aproximada de 20% (22), enquanto em estudo de triagem realizado no sudeste descreveu prevalência de 30,1% em mulheres de 15–64 anos (23). Em subgrupos de maior risco, como mulheres que fazem sexo com mulheres, as prevalências foram ainda mais elevadas em estudos clínicos. Uma revisão sistemática nacional reportou prevalência média de 25,4% (IC95% 24,0–26,8), com 18,1% em estudos populacionais e 27,2% em serviços de saúde (24).

1.3.1. Diagnóstico da vaginose bacteriana

As ferramentas mais utilizadas para o diagnóstico de VB são as de abordagens clínicas e as microscópicas, sendo os critérios de Amsel et al. (1983) (25) e o escore de Nugent et al. (1991) (26) os métodos mais difundidos mundialmente. O primeiro baseia-se predominantemente em sinais clínicos, e a subjetividade do teste das aminas, associada à suscetibilidade da variação do pH a interferências externas, comprometem a acurácia do diagnóstico. Para o diagnóstico clínico de VB é necessária a presença de pelo menos três dos quatro critérios, que incluem: presença de corrimento vaginal fluido, homogêneo, branco acinzentado; pH vaginal maior ou igual a 4,5; teste das aminas (ou *whiff test*) positivo, com presença de percepção odorífera desagradável do conteúdo vaginal após a adição de hidróxido de potássio (KOH) a 10%; e presença de *clue cells* em exame a fresco (25).

Já os critérios de Nugent et al. (26) representam uma avaliação microscópica do conteúdo vaginal de mulheres em idade reprodutiva, baseada na coloração do esfregaço vaginal pelo método de Gram. Nessa técnica, são avaliados e semi-quantificados diferentes morfotipos bacterianos, como os morfotipos lactobacilares, cocobacilares Gram variáveis e de bacilos curvilíneos Gram lábeis, gerando uma pontuação que varia de 0 a 10. Assim, os escores de 0 a 3, que possuem predomínio de *Lactobacillus* spp., são classificados como microbiota normal (Flora I), de 4 a 6 são classificados como microbiota intermediária (Flora II), e de 7 a 10 são classificados como VB (26). Por sua objetividade e reprodutibilidade, o uso do critério de Nugent et al. é considerado padrão-ouro para diagnóstico de VB, em mulheres na menacme, pela Organização Mundial da Saúde (OMS) (3).

Apesar da disponibilidade de métodos diagnósticos bem estabelecidos, a prática clínica ainda se baseia predominantemente na avaliação subjetiva dos sintomas e nos achados do exame ginecológico, com uso limitado da avaliação microscópica. Estudos comparativos demonstram que os critérios de Amsel apresentam sensibilidade inferior quando comparados ao escore de Nugent. Sha et al. (27) demonstraram sensibilidade de aproximadamente 37% dos critérios clínicos em relação ao escore microbiológico, evidenciando importante subdiagnóstico da VB quando apenas critérios clínicos são utilizados. Resultados semelhantes foram observados em estudo mais recente (28), no qual a sensibilidade de Amsel variou entre 37% e 70%, reforçando a maior acurácia do escore de Nugent, especialmente em mulheres assintomáticas ou com microbiota intermediária. Como consequência, avaliações baseadas exclusivamente no exame especular ou na anamnese mostram baixa concordância com métodos microbiológicos, favorecendo o uso inadequado de antifúngicos e antibióticos, o desequilíbrio adicional da microbiota vaginal e a seleção de microrganismos resistentes.

Além disso, alterações endócrinas, imunológicas e reações alérgicas podem modificar o volume e o aspecto do conteúdo vaginal, mimetizando quadros infecciosos e dificultando a distinção clínica entre conteúdo fisiológico e disbiose vaginal. Essa inespecificidade é particularmente relevante no contexto da VB, uma vez que a condição é frequentemente assintomática, o que contribui para seu subdiagnóstico e persistência na população feminina. Quando sintomática, a VB manifesta-se principalmente por corrimento vaginal homogêneo, decorrente da esfoliação de células epiteliais vaginais, e por odor genital característico, resultante da liberação de aminas voláteis, como putrescina, cadaverina e trimetilamina, que podem impactar negativamente a qualidade de vida das mulheres (3,9). Nessas situações, a adoção de tratamentos empíricos, sem confirmação microbiológica, não apenas pode falhar em aliviar os sintomas, como também intensificar o desconforto e perpetuar a disbiose vaginal.

1.3.2. Vaginose bacteriana e suas repercussões clínicas

Clinicamente, a VB está associada a uma série de repercussões ginecológicas e obstétricas. Essa associação decorre de alterações estruturais e imunológicas induzidas pela disbiose no microambiente vaginal. A VB promove a elevação do pH vaginal, redução da produção de metabólitos protetores por *Lactobacillus* spp. e comprometimento da integridade da barreira mucosa, tornando o epitélio vaginal mais suscetível à adesão de patógenos (29). Além disso, a atividade enzimática de bactérias associadas à VB contribui para a degradação do muco cervicovaginal e da matriz

extracelular, facilitando a exposição de receptores celulares envolvidos na infecção por agentes sexualmente transmissíveis (30).

Além do aumento do risco de ISTs, a vaginose bacteriana está associada ao desenvolvimento de infecções do trato genital superior, incluindo doença inflamatória pélvica, endometrite e salpingite (3,14). A disfunção da barreira do trato genital inferior favorece a ascensão bacteriana para a cavidade uterina e anexos, enquanto a inflamação subclínica persistente, caracterizada pelo aumento de citocinas pró-inflamatórias, pode resultar em dano tecidual progressivo (31). Esse processo inflamatório está relacionado à alteração da função ciliar tubária, à formação de aderências e ao aumento do risco de infertilidade, especialmente de origem tubária, além de elevar a probabilidade de gravidez ectópica (32). No contexto obstétrico, a VB também tem sido associada a complicações gestacionais, como corioamnionite, parto pré-termo, rotura prematura de membranas pré-termo, baixo peso ao nascer e infecções pós-parto (33).

Vale ressaltar que além das repercussões biológicas, a VB pode também acarretar impactos psicossociais relevantes. A presença persistente de sintomas como corrimento vaginal e odor desagradável pode gerar constrangimento, sofrimento emocional e prejuízos na autoestima e na vida sexual das mulheres, reforçando a importância de estratégias adequadas de diagnóstico, educação em saúde e tratamento efetivo (34).

1.4. Gardnerella vaginalis

Entre as espécies bacterianas mais frequentemente associadas à VB, destaca-se *G. vaginalis*, historicamente considerada a principal espécie envolvida na etiologia dessa disbiose. Trata-se de uma espécie cocobacilar Gram variável e anaeróbia facultativa, pertencente à família *Bifidobacteriaceae*, que tem fatores de virulência que permitem formação de biofilme (35,36). Foi isolada pela primeira vez em 1953 por Leopold (37) e, posteriormente, associada à “vaginite bacteriana não específica” por Gardner e Dukes (38), recebendo o nome de *Haemophilus vaginalis*. Mais tarde, foi reclassificada como parte do gênero *Corynebacterium* (39). Em 1980, estudos de Greenwood e Pickett propuseram o gênero *Gardnerella* e renomearam a bactéria como *G. vaginalis* (40).

Desde a sua primeira descrição, a *G. vaginalis* tem sido amplamente estudada devido ao seu papel central na patogênese da VB. Essa espécie apresenta um conjunto de fatores de virulência que favorecem sua colonização, evasão imunológica e formação do biofilme, destacando-se, entre eles, a sialidade e a vaginolisina (35, 41-43) Por muito tempo acreditou-se que a atividade da sialidase em *G. vaginalis* era codificada pelo gene da sialidase A (*nanH1*). Entretanto, estudo de Robinson et al. descreveu dois novos genes codificantes de sialidase (*nanH2* e *nanH3*), demonstrando que sua presença nos

genomas das cepas de *G. vaginalis* reflete a capacidade de produzir sialidase e que essas enzimas exibem ampla gama de atividades contra glicoproteínas da mucosa vaginal, sendo as principais fontes dessa neuraminidase em *G. vaginalis.* (44).

A sialidase é uma glicosidase responsável pela degradação da barreira de células epiteliais vaginal, causando esfoliação e descamação do epitélio através da clivagem do ácido siálico dos sialoglicanos (mucinas) que compõem a mucosa vaginal. Essa clivagem permite a aderência bacteriana através da exposição dos sítios de ligação de glicanos, além de clivar o ácido siálico terminal da imunoglobulina A, aumentando sua suscetibilidade à degradação proteolítica e atuando como estratégia de evasão imunológica (35,41-43). As bactérias podem utilizar os ácidos siálicos liberados como fonte de carbono para sua nutrição, uma vez que ele pode ser transportado para o interior bacteriano por um sistema de alta afinidade, e posteriormente catabolizado pela reação intracelular de aldolase em N-acetilmanosamina (ManNAc) e piruvato (43,45). Ademais, sugere-se que pode haver a incorporação dos ácidos siálicos clivados à superfície da bactéria, o que permite que elas se camuflem e evadam-se da resposta imune. (41). Por fim, a sialidase está associada aos estágios iniciais da formação dos biofilmes, uma vez que permite a adesão da *G. vaginalis* às células epiteliais (46).

A vaginolisina é uma hemolisina dependente de colesterol, muito ativa em pH vaginal elevado (5.0–7.5) (43), responsável pela formação de poros nas células epiteliais vaginais através da interação com a molécula CD59. Essa interação desencadeia uma resposta epitelial que induz a fosforilação e ativação da MAPK p38 (quinase ativada por mitógeno p38), facilitando a lise celular, o que pode representar um mecanismo precoce de evasão imune, uma vez que esse fator é capaz de lisar neutrófilos e, possivelmente, outras células do sistema imunológico (35,36,41). Para além de sialidases, *G. vaginalis* secreta proteases, como a prolidase (43,45) que degradam componentes proteicos e glicoconjugados do ambiente vaginal, favorecendo a liberação de compostos voláteis derivados de aminas alifáticas (3). A superfície celular da *G. vaginalis* também apresenta fímbrias, que favorecem sua adesão às células epiteliais vaginais (3). Além disso, estudos genômicos indicam que a capacidade de produção de biofilme de *G. vaginalis* também está associada à presença de genes codificadores de glicosiltransferases, especialmente as do tipo II, que constituem compostos importantes na biossíntese de EPS (47). Em conjunto, esses fatores atuam de forma sinérgica para facilitar a colonização do epitélio vaginal, contribuindo para os estágios iniciais de formação do biofilme.

Os biofilmes são comunidades de bactérias que se aderem a uma superfície e crescem envoltas em uma matriz extracelular autoproduzida, que fornece proteção contra antimicrobianos, defesas do hospedeiro e outros estressores ambientais (48) (Figura 2). Sua formação ocorre de maneira progressiva, iniciando-se com uma fase de adesão reversível, mediada por interações físico-químicas transitórias entre a forma planctônica de um microrganismo e o epitélio vaginal. Posteriormente, há o estabelecimento da fase irreversível, caracterizada pela produção de substâncias poliméricas extracelulares (EPS), que incluem proteínas, glicoproteínas, glicolipídios e DNA extracelular, consolidando a fixação bacteriana, crescimento de microcolônias e formação da matriz responsável por sustentar as interações microbianas. Essa matriz é capaz de determinar porosidade, densidade, teor de água, carga, propriedades de adsorção, hidrofobicidade e estabilidade mecânica dos biofilmes, e sua composição depende das condições ambientais (49,50). Swidsinski et al. (51) analisaram amostras vaginais utilizando hibridização fluorescente *in situ* (FISH) e demonstraram que o gênero bacteriano predominante e componente essencial do biofilme da VB é *Gardnerella,* responsável por formar camadas aderidas firmemente à superfície do epitélio vaginal (51). Verstraelen & Swidsinski (52) demonstraram que *G. vaginalis* tem melhor capacidade de adesão às células epiteliais do que outras bactérias associadas à VB, mesmo na presença de espécies lactobacilares, o que sustenta a ideia de que ela é a colonizadora primária que permite a colonização por BVABs (53). Assim, os biofilmes vêm sendo considerados marca registrada da VB (54), contendo elevada abundância de *G. vaginalis* em associação com BVABs, *Prevotella* spp., *Snethia* spp., bem como *F. vaginae* (52).

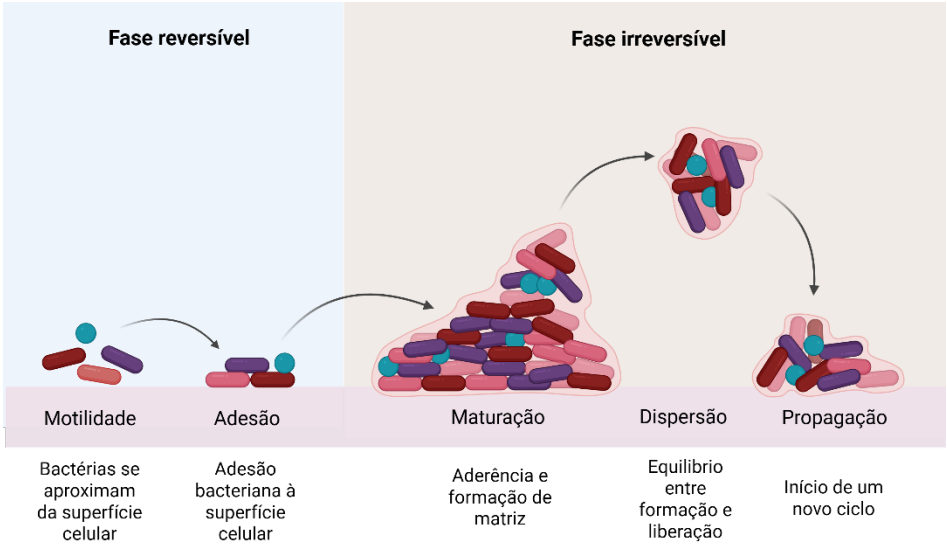

Figura 2. Representação esquemática das etapas de formação do biofilme bacteriano. Inicialmente, células bacterianas planctônicas interagem de forma transitória com a superfície (fase reversível). Na sequência, ocorre a adesão irreversível e a produção de substâncias poliméricas extracelulares (EPS), que promovem a maturação do biofilme e o desenvolvimento de microcolônias. Posteriormente, algumas células se dispersam, retornando à forma planctônica e permitindo a propagação e colonização de novas superfícies. Imagem de autoria própria (BioRender).

Devido à existência de várias camadas no biofilme, há a formação de gradientes de nutrientes, oxigênio e pH. O consumo de oxigênio pelas bactérias associadas ao biofilme aeróbio das camadas mais externas gera um gradiente de oxigênio que se desenvolve com condições anaeróbias crescentes em direção aos estratos internos. Conforme o biofilme cresce, as células internas ficam separadas da interface externa, onde a maioria das fontes essenciais de energia e nutrientes estão armazenadas. Além disso, produtos de resíduos e toxinas se acumulam no interior do biofilme. Esses gradientes desaceleram o crescimento e o metabolismo da subpopulação de bactérias que reside no núcleo, que é um grupo de bactérias dormentes influenciadas pelas condições de crescimento do biofilme. Quando expostas a antibióticos, algumas destas bactérias dormentes adquirem maior tolerância, sendo conhecidas como células persistentes (50).

As células persistentes são bactérias que crescem lentamente ou tem seu crescimento interrompido e que podem sobreviver sob condições de estresse, como antibióticos, espécies reativas de oxigênio, pH ácido ou condições inadequadas de nutrição, sendo frequentemente descritas como células dormentes. Após a remoção do estresse, as células persistentes podem continuar crescendo e permanecer sensíveis ao mesmo estressor. Elas exibem heterogeneidade fenotípica e são altamente dependentes de diferentes fatores, incluindo o tipo de cepa bacteriana, o antibiótico utilizado e as condições ambientais. A tolerância das células persistentes aos antibióticos é expressa apenas no nível fenotípico, e não há mutação nos genes de resistência. A persistência decorre da interação de diversos processos, que podem incluir a redução da energia celular, paralisação da replicação do DNA, bloqueio da tradução e redução da concentração intracelular de antibióticos. As células persistentes desempenham um papel significativo na recorrência de infecções, servem como um reservatório evolutivo a partir do qual bactérias resistentes podem surgir e aumentam a tolerância em infecções com biofilmes (55,56).

Portanto, os biofilmes representam uma das maiores barreiras à eficácia do tratamento da VB (57), aumentando a chance de resistência a antibióticos através de diversos mecanismos, que incluem: baixa penetração de células imunes e de compostos antimicrobianos na matriz extracelular, formando uma barreira que retarda ou diminui a difusão do fármaco; degradação dos compostos pelas bactérias ativas na subpopulação externa do biofilme; alterações fisiológicas no microambiente do biofilme devido ao seu crescimento lento causado pelo gradiente de nutrição e oxigênio, retardando o metabolismo das bactérias na camada interna e reduzindo a ativação do metronidazol de

pró-fármaco à forma ativa; alterações fenotípicas; comunicação celular por *quorum sensing* entre os microrganismos; expressão de bombas de efluxo e formação de células persistentes (42,50). Assim, esses fatores levam à uma taxa de recorrência em 20-30% das mulheres que passam pelo tratamento padrão da VB em até 3 meses, uma vez que o biofilme é apenas suprimido temporariamente pela ação do metronidazol e recupera sua atividade com o término do tratamento (58).

Durante décadas, a *G. vaginalis* foi a única espécie reconhecida em seu gênero, mas nos últimos anos sua descrição taxonômica foi alterada (59). Em 2012, Ahmed et al. (60) reconheceram a presença de quatro grupos (1, 2, 3 e 4) com base em análises de agrupamento de vizinhança. Em 2014, Balashov et al. (61) desenvolveram ensaios de PCR para identificar especificamente representantes desses quatro grupos utilizando a análise da sequência do gene chaperonina 60 (*cpn60*), que codifica a *heat shock protein* 60 (HSP60), uma proteína que atua como uma chaperona molecular envolvida na resposta ao estresse celular e é amplamente empregada como marcador filogenético devido ao seu elevado poder discriminatório entre espécies e subgrupos bacterianos. A análise de sequências de genomas completos diferenciou as cepas de *G. vaginalis* em quatro clados e demonstrou que os quatro subgrupos baseados em *cpn60* (A, B, C e D) correspondem aos clados 4, 2, 1 e 3, respectivamente. Além disso, encontraram correlação positiva entre VB e os clados 1 e 3, enquanto o clado 2 mostrou correlação positiva com a microbiota vaginal intermediária e o clado 4 não apresentou qualquer correlação (62). Em 2019, experimentos de sequenciamento do genoma completo e espectrometria de massa por *Matrix-Assisted Laser Desorption/Ionization* (MALDI-TOF) foram realizados em 81 cepas de *Gardnerella* por Vaneechoutte et al. (62), demonstrando a existência de 13 grupos suficientemente distintos para serem classificados como espécies distintas, o que resultou na inclusão de três espécies de *Gardnerella*, sendo elas *G. leopoldii*, *G. piotii* e *G. swidsinskii*, além de nove espécies genômicas. No mesmo estudo foi identificado que *G. vaginalis* e *G piotii* são sialidase-positivas enquanto *G. leopoldii* e *G. swidsinskii* são sialidase-negativas. Além disso, *G. vaginalis* é a única das quatro espécies que é β-galactosidase positiva (62).

Em estudo realizado com amostras vaginais de mulheres canadenses, várias espécies de *Gardnerella* foram detectadas na maioria das participantes do estudo, embora suas abundâncias variassem, coocorrências significativas foram observadas entre *G. vaginalis* e *G. swidsinskii* e entre *G. piotii* e a espécie genômica 3. Também foi identificada associação significativa entre a VB e maiores abundâncias relativas de *G. vaginalis*, *G. swidsinskii* e *G. piotii*, além de fortes relações entre os sintomas de odor

anormal e corrimento vaginal com maiores abundâncias relativas de *G. vaginalis* e *G. swidsinskii*, mas não com as demais espécies (63). Em relação aos fatores de virulência, existem diferenças na composição genética da vaginolisina entre as 13 genomospecies, sendo os tipos 1 e 2 de vaginolisina os mais comuns entre os isolados caracterizados (42), entretanto, não foi demonstrada diferença significativa na produção desta citolisina (63). Também não foram encontradas diferenças na capacidade de produção de biofilme entre as espécies (64,65). Quanto à resistência ao metronidazol, as cepas dos grupos 3 e 4 apresentaram 100% de resistência, os grupos 1 e 2 mostraram 35% e 7,1% de resistência, respectivamente (65).

Mais recentemente, em 2023, Sousa et al. (66) também utilizaram as técnicas de sequenciamento de genoma completo e espectrometria de massas por MALDI-TOF e descreveram duas novas espécies de *Gardnerella*: *G. pickettii* (anteriormente espécie genômica 3) e *G. greenwoodii* (anteriormente espécie genômica 8). O estudo identificou que ambas as espécies eram negativas para β-galactosidase. Em relação aos fatores de virulência, tanto *G.pickettii* quanto *G. greenwoodii* possuíam o gene *vly* codificante de vaginolisina tipo 1 e apenas *G. pickettii* foi positiva para atividade da sialidase. Além disso, foram identificados genes de resistência a antimicrobianos em ambas as cepas: *msr*(D) in tandem com a bomba de efluxo *mef*(A), que codifica resistência a macrolídeos, e *lsaC*, que codifica resistência cruzada com lincosamidas, estreptograminas A e pleuromutilinas, o que pode contribuir para uma baixa resposta ao tratamento com clindamicina. De forma complementar, Innamorati et al. (67) analisaram a resistência de espécies de *Gardnerella* ao metronidazol através do teste de concentração inibitória mínima (CIM) e observaram que as cepas testadas de *G. vaginalis*, *G pickettii* e *G. piotii* apresentaram respostas variadas, abrangendo CIMs que variavam entre baixa sensibilidade até alta resistência, enquanto as cepas de *G. swidsinskii* e *G. leopoldii* foram altamente resistentes a este nitroimidazol.

Buscando compreender a associação das diferentes espécies de *Gardnerella* com a VB, Munch et al. (68) desenvolveram quatro ensaios de PCR quantitativo utilizando o gene cpn60 como alvo e detectando quatro grupos de espécies divididos em *G. vaginalis*, *G.piotii*/*G.pickettii*, *G.swidsinskii*/*G. greenwoodii*, e *G.leopoldii*. Foi demonstrado que *G. vaginalis* foi a espécie mais frequente em pacientes com VB (69,7%), entretanto foi sempre encontrada em coocorrência com pelo menos uma outra espécie. Os quatro grupos de espécies foram detectados mais frequentemente em pacientes com VB, apresentando concentrações bacterianas significativamente mais elevadas. Além disso, as taxas de recorrência foram mais altas nos casos de presença de *G. vaginalis*,

chegando a 75%, seguida por 44,4% do grupo *G.piotii*/*G. pickettii* e 28,6% de *G. leopoldii*. Portanto, esses resultados sugerem que a presença de mais de uma espécie de *Gardnerella* pode ser um fator de risco para a VB.

Estudos apontam que a *G. vaginalis* faz parte do *core* patológico de grande parte das VB (35,69,70) e, na última década, vem sendo demonstrado que *G. vaginalis* possui maior potencial de virulência do que outras bactérias associadas à VB (71), além de ser capaz de produzir biofilme, permitindo sua evasão do sistema imune (72-74) e apresentando maior tolerância ao ácido lático e ao H_2O_2 (14). Além dessa, outras espécies bacterianas como *M. hominis*, *U. urealyticum*, *P. bivia* e *F. vaginae* também se apresentam em expressiva abundância relativa na VB (72).

1.5. Fannyhessea vaginae

Fannyhessea vaginae, anteriormente classificada como *Atopobium vaginae* (75), é outra espécie bacteriana frequente no core patológico da VB (76). Trata-se de um cocobacilo Gram positivo, anaeróbio estrito, que pode aparecer sozinho, em pares ou em pequenas cadeias (70). A presença de *F. vaginae* na microbiota vaginal dominada por espécies de lactobacilos ainda apresenta controvérsias, uma vez que alguns estudos demonstram sua presença em apenas 12-19% das amostras de pacientes que não possuem VB (77,78), enquanto outros estudos afirmam que sua presença é de cerca de 69% (79). Entretanto, sua quantificação parece ser um bom preditor de VB, uma vez que apresenta concentrações mais elevadas em amostras VB positivas. Dessa forma, *F. vaginae* vem sendo considerada como uma espécie indicadora da VB, uma vez que está presente em cerca de 50-95% dos casos (80) e geralmente em combinação com *G. vaginalis*, possuindo importante papel na formação e no estabelecimento do biofilme (81).

Nas análises de Swidsinski et al. (51), empregando a técnica de FISH, 86% dos biofilmes de *Gardnerella* spp. estavam associados a altas concentrações de *F. vaginae,* contribuindo com até 40% da massa total. Hardy et al. (81) também utilizaram a técnica de FISH para estudar o potencial de *F. vaginae* na VB e confirmaram que a espécie é importante constituintes do biofilme, uma vez que *F. vaginae* aderente foi visualizada em 54,1% das amostras de biofilme bacteriano. Além disso, apenas duas amostras continham *F. vaginae* na ausência de *G. vaginalis*, evidenciando a sinergia entre ambas as bactérias.

Os biofilmes de *G. vaginalis* criam um ambiente favorável para crescimento de bactérias anaeróbias pela presença de um gradiente de oxigênio em direção às camadas

internas, que se forma devido ao consumo de oxigênio pelas bactérias aeróbias nas camadas mais externas (50). Assim, *F. vaginae* consegue utilizar esse mecanismo para se proliferar, estabelecendo uma relação mutualística com *G. vaginalis* (59,71). A presença de *F. vaginae* no biofilme também pode ter impacto no tratamento, uma vez que estudos *in vitro* verificaram que a suscetibilidade ao metronidazol varia entre cepas de *F. vaginae*. (82). Bradshaw et al. (77) relataram taxas de recorrência da VB mais altas quando *F. vaginae* está associada à *G. vaginalis* e Ferris et al. (83) demonstraram que a presença de *F. vaginae* estava associada à falha parcial ou total do tratamento da VB com metronidazol. Adicionalmente, Luchiari et al. (84) mostraram que a falha terapêutica da VB não está relacionada à carga bacteriana total, mas possivelmente a características microbiológicas específicas, como a composição bacteriana e a organização em biofilme, reforçando o papel do consórcio microbiano *G. vaginalis–F. vaginae* na persistência da infecção (84).

1.6. Modulação imunológica no ambiente vaginal

A microbiota vaginal atua na modulação imunológica do microambiente vaginal. A vagina contém células imunes como neutrófilos, macrófagos, células T, células B e *natural killer*, e conta com receptores especiais como os *Toll-like* (TLRs) e *Nod-like* (NLRs), responsáveis pelo reconhecimento de patógenos. O estímulo microbiano leva à liberação de citocinas e quimiocinas, como IL-1β, IL-6, CXCL-8, e TNF-α, ativada e regulada pela via do Fator nuclear kappa B (NF-κB). A resposta inflamatória estimulada por patógenos geralmente controla a infecção, mas, em alguns casos, pode romper a barreira de células epiteliais e facilitar a aquisição de outras infecções (2).

A VB é clinicamente considerada uma condição não inflamatória, uma vez que não é associada a edema ou quimiotaxia neutrofílica (69). Uma possível explicação para isso é a natureza dos biofilmes em promover resposta mais silenciosa (42). Bertran et al. (85) demonstraram que *G. vaginalis* não induziu citotoxicidade nem aumento de citocinas pró-inflamatórias (TNF-α, IL-12p70, IFN-γ) por células dendríticas, sugerindo resposta quiescente. Outro estudo utilizando o mesmo isolado e outras bactérias associadas à VB mostrou aumento significativo na ativação de células dendríticas e maior expressão de IL-1β, IL-6, CXCL-8, IL-12A, IL-12B e RNA mensageiro de TNF-α em resposta ao estímulo de outras BVAB, mas, no caso da *Gardnerella*, apenas a secreção de CXCL-8 foi significativamente aumentada (42,86).

Apesar disso, a VB está associada a uma inflamação molecular, caracterizada geralmente por produção de citocinas pró-inflamatórias e níveis reduzidos de algumas quimiocinas (42). Comunidades dominadas por espécies anaeróbias estão associadas a resposta pró-inflamatória mais intensa em comparação àquelas dominadas por *Lactobacillus* spp.. Estes últimos exercem efeito imunomodulador por meio da forma protonada do ácido lático, que reforça a defesa antimicrobiana sem induzir mediadores inflamatórios. Esse mecanismo cria um ambiente anti-inflamatório, marcado pelo aumento de citocinas reguladoras, como IL-1RA, e pela redução da produção de citocinas pró-inflamatórias, como IL-6 e TNF-α, além da diminuição de quimiocinas, como CXCL-8, RANTES e MIP-3α (2).

Estudos demonstram que a colonização por BVAB em células epiteliais cervicais induz resposta pró-inflamatória medida pelo aumento de IL-6, CXCL-8, *Interferon gamma induced protein* 10 (IP-10), MCP-1, MIP-1β, MIP-3α e IL-1β (87). Em macrófagos, a cascata de sinalização resultante do estímulo com lavados de pacientes com VB leva à ativação de NF-κB, que ativa o inflamassoma *NLRP3* e leva à liberação aumentada de IL-1β e IL-18 (42). Eade et al. (88) utilizando *G. vaginalis* do *American Type Culture Colection* (ATCC 49145) demonstraram aumento da expressão de CXCL-8. Fichorova et al. (89) utilizando isolado primário de *G. vaginalis* obtido de uma amostra de VB, demonstraram aumento de CXCL-8, RANTES e inibidor de protease leucocitária secretora (SLPI). Anderson et al. (90), utilizando a técnica de cultura bacteriana, associaram a presença de *G. vaginalis* em conteúdos vaginais de gestantes com níveis cervicais mais altos de IL-1, IFN-γ, TNF-α e fator estimulador de colônias de granulócitos e macrófagos (GM-CSF). Similarmente, detecção genômica de *G. vaginalis* no conteúdo vaginal de pacientes com VB, associou sua presença à níveis aumentados de IL-1, IL-6, CXCL-8 e IL-12p70, embora níveis reduzidos de IP-10 e SLPI também tenham sido observados. *G. vaginalis* também foi uma das seis espécies dominantes identificadas pelo sequenciamento do RNA ribossômico16S de amostras caracterizadas por níveis cervicovaginais elevados de IL-1, IL-6, CXCL-8, TNF-α, IFN-γ e IL-10 em jovens sul-africanas (70,91).

Os níveis de citocinas específicas e de peptídeos antimicrobianos também podem variar de acordo com assinaturas imunes espécie-específicas. Colonizadores primários, como *G. vaginalis*, são capazes de inibir ativamente a resposta inflamatória do hospedeiro no epitélio vaginal, favorecendo o estabelecimento e a manutenção do biofilme característico da VB. Em contraste, *F. vaginae* pode induzir a ativação da resposta imune do hospedeiro, contribuindo para o desenvolvimento dos sinais e

sintomas da VB, incluindo a liberação de metabólitos, como aminas alifáticas (92). Corroborando esses achados, estudo conduzido utilizando o modelo de tecido EpiVaginal demonstrou que a infecção por *G. vaginalis* não promove alterações significativas nos níveis de mediadores pró-inflamatórios (93). De forma consistente, estudo anterior evidenciou que *G. vaginalis* não promove a indução de IL-1β, IL-6, MIP-3α ou TNF-α em um modelo humano tridimensional (3D) de células epiteliais endometriais. Esses achados sugerem que *G. vaginalis* atua predominantemente como um colonizador inicial, capaz de modular ou atenuar a resposta inflamatória do hospedeiro, favorecendo a adesão epitelial e o estabelecimento do biofilme polimicrobiano característico da VB. Em contraste, outras bactérias associadas à VB, como *F. vaginae*, parecem desempenhar papel central na amplificação da resposta inflamatória local. Nesse contexto, Doerflinger et al. (94) demonstraram, em modelo humano 3D de células epiteliais vaginais, que *F. vaginae* induz uma ampla gama de citocinas pró-inflamatórias, quimiocinas e peptídeos antimicrobianos, incluindo IL-1β, IL-6, IL-8, MIP-3α, TNF-α e β-defensina humana-2 (HBD-2) (92).

Evidências adicionais indicam que a coocorrência de *G. vaginalis* e *F. vaginae* no biofilme vaginal resulta em um efeito sinérgico sobre a resposta imune do hospedeiro. Quando cultivadas conjuntamente em modelos de células epiteliais vaginais, essas espécies desencadeiam resposta pró-inflamatória mais robusta, caracterizada pelo aumento da produção de citocinas (IL-6, IL-1β, TNF-α, IL-8) e quimiocinas (RANTES, MIP-1β), refletindo de forma mais fidedigna o perfil inflamatório observado em mulheres com VB (15). Esses dados reforçam a noção de que a patogênese da VB não depende de uma única espécie bacteriana, mas sim da interação funcional entre diferentes microrganismos organizados em biofilme, capaz de modular simultaneamente a colonização, a evasão imune e a inflamação local.

Além disso, as respostas de citocinas e quimiocinas podem variar dependendo do modelo celular e da espécie de *Gardnerella*. O epitélio vaginal possui um perfil diferente de receptores de reconhecimento de padrões (PRR) em comparação com as células endocervicais e da membrana basal, o que pode explicar as diferenças nas respostas ao mesmo patógeno. Aumentos na produção de IP-10 foram observados após o desafio endocervical com *G. vaginalis*, mas não com o desafio vaginal ou ectocervical. Em contraste, em resposta ao tratamento com o isolado *G. leopoldii*, houve redução do IP-10 secretado tanto na ectocérvice quanto na vagina (42).

A alteração da microbiota na VB e a inflamação subsequente podem levar a desfechos ginecológicos e reprodutivos indesejáveis. Noda-Nicolau et al. (95)

demonstraram que o estímulo de membranas fetais com *G. vaginalis*, sozinha ou em combinação com micoplasmas genitais, induziu aumento em citocinas inflamatórias (IL-1β, CXCL-8 e TNF-α) e anti-inflamatórias (IL-4, IL-10 e IL-13) e que combinações polimicrobianas com maiores cargas de *G. vaginalis* geraram forte resposta pró-inflamatória. Além disso, os tratamentos com *G. vaginalis* também aumentaram a produção de IL-6 e a expressão do receptor de interleucina 6 ligado à membrana (mIL-6R) nas células amnióticas e do receptor de glicoproteína 130 (gp130) em células amnióticas e coriônicas, indicando que essa bactéria ativa a via clássica de sinalização da IL-6 (96).

Análises moleculares de amostras de lavado cervicovaginal realizadas por Belleti et al. (97) identificaram que a persistência dos HPVs de alto risco 16 e 18 está associada não só com a presença, mas também com maiores cargas de *G. vaginalis*, o que suporta evidências de que essa bactéria possui importante papel no desfecho da infecção pelo HPV. De maneira complementar, Novak et al. (98) analisou a presença e a carga do gene codificador de sialidase *nanH3* de *G. vaginalis* em amostras HPV positivas demonstrando que, na presença de HPV16, o grupo que apresentou *clearance* viral possuía menores cargas de *nanH3* do que o grupo que apresentou persistência da infecção.

Mais recentemente, dados do nosso grupo demonstraram, utilizando um modelo *organ-on-chip* da interface materno-fetal, a capacidade de *Gardnerella* spp. de ascender até as membranas fetais, apresentando cargas significativamente mais altas nessas camadas em um período de 24 horas (dados não publicados). Assim, os mecanismos de ativação imunológica e inflamação que atuam durante a VB fornecem suporte para o papel desta disbiose em outras complicações (99).

Considerando o predomínio de *Gardnerella* spp. nos biofilmes da VB, as alterações na imunidade da mucosa mediadas por esse gênero bacteriano podem ajudar a explicar a persistência da VB e como ela aumenta o risco de aquisição de ISTs e resultados adversos da gestação (42). Diante desse cenário, estudos são necessários para determinar a coexistência de *Gardnerella* spp. e de *F. vaginae* nas disbioses vaginais e na microbiota lactobacilar de mulheres em idade reprodutiva.

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Capítulo 2
Artigo Científico I

São Paulo Medical Journal. Fator de Impacto: 1.6

Bacterial Vaginosis: vaginal microbiota composition, immune modulation, and the role of *Gardnerella vaginalis* and *Fannyhessea vaginae*.

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Abstract: Women's health during the reproductive age is influenced by a complex interaction of biological, environmental, and social factors. Among the biological determinants, vaginal microbiota plays a central role in maintaining homeostasis by acting as a barrier against pathogenic microorganisms, modulating immune response, and participating in essential metabolic processes. *Lactobacillus* spp. produce lactic acid and other antimicrobial compounds, thereby establishing a protective microenvironment against pathogenic infections. Disruptions in this ecosystem may predispose individuals to dysbiosis, with clinically relevant consequences, including increased susceptibility to sexually transmitted infections (STIs) and adverse pregnancy outcomes. Bacterial vaginosis (BV) is the most prevalent dysbiotic condition among women of reproductive age and is characterized by a marked reduction or absence of *Lactobacillus* species, accompanied by an increased relative abundance of facultative anaerobic bacteria. Among the species most frequently associated with BV, *Gardnerella vaginalis* harbors a repertoire of virulence factors that promote colonization, immune evasion, and biofilm formation, notably sialidase A and vaginolysin. *Fannyhessea vaginae* is another key species commonly identified within the pathological core of BV, typically detected in association with *G. vaginalis*, and plays a critical role in biofilm establishment. Although BV is clinically considered a non-inflammatory condition, it is associated with molecular inflammatory responses. The co-occurrence of *G. vaginalis* and *F. vaginae* within the vaginal biofilm exerts a synergistic effect on the host immune response, reinforcing the concept that BV pathogenesis depends on functional interactions among multiple microorganisms organized within a biofilm.

Keywords: *Gardnerella vaginalis*; *Fannyhessea vaginae*; vaginal microbiota; bacterial vaginosis; inflammatory cytokines

1. Introduction

Women's health during reproductive age is influenced by a complex interaction of biological, environmental, and social factors. Among biological factors, the vaginal microbiota plays a central role in maintaining homeostasis by serving as a barrier against pathogenic microorganisms, modulating the immune response, and participating in essential metabolic processes.[1,2] In recent decades, the development and consolidation of molecular biology techniques have enabled detailed characterization of microbial composition across different body sites, revealing its relevance both to homeostasis and to the development of pathological conditions of the female reproductive tract. In this context, the vaginal microbiota emerges as a key element in protection against infections and in the maintenance of gynecological and reproductive health. Alterations in this ecosystem may predispose to dysbiosis, with clinically relevant consequences, including increased susceptibility to sexually transmitted infections (STIs), reduced clearance rates of Human Papillomavirus (HPV) infection, and gestational complications, particularly preterm premature rupture of membranes and spontaneous preterm birth.[2,3]

1.1. Vaginal microbiota

In general, the microbiota establishes a mutualistic relationship with the human body, performing crucial functions in multiple physiological processes, including immune system modulation and protection against pathogenic microorganisms. Among the different microbial niches within human body sites, the vaginal microbiota stands out, accounting for approximately 9% of the total, playing an important role in maintaining female reproductive health.[1]

The first scientific description of the vaginal microbiota was provided by Albert Döderlein in 1892,[4] who characterized it as a homogeneous environment composed of Gram-positive bacilli, later referred to as Döderlein bacilli. It is now known that these bacterial morphotypes belong to the genus *Lactobacillus*, and their dominance is associated with a healthy vaginal microenvironment, since *Lactobacillus* spp. produce lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins, which contribute to the maintenance of an acidic vaginal pH and inhibit the growth of pathogenic microorganisms.[2]

During reproductive age, elevated estrogen levels promote glycogen accumulation in vaginal epithelial cells. This substrate is hydrolyzed by human α-amylase into simple sugars, which are subsequently metabolized into lactic acid by lactobacilli, leading to

acidification of the vaginal microenvironment (pH 3.8–4.5). This acidic environment inhibits the growth of opportunistic bacteria, contributing to an anti-inflammatory state by promoting the production of regulatory cytokines, such as interleukin-1 receptor antagonist (IL-1RA), and inhibiting the expression of inflammatory mediators, including interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), and chemokines (CXCL-8, RANTES, and MIP-3α).[2]

Additionally to lactic acid, Lactobacillus spp. also produce other antimicrobial factors, such as hydrogen peroxide (H_2O_2), which primarily inhibits the growth of anaerobic bacteria;[2] arginine deaminase, which metabolizes arginine into citrulline and ammonia, reduces the availability of this amino acid required for the growth of certain anaerobic pathogens, in addition to promoting resistance to acidic environments;[3,5] and bacteriocins and biosurfactants, which promote the autophagy of intracellular bacteria, viruses, and protozoa.[2] Bacteriocins are small antimicrobial peptides synthesized by bacteria, especially those that inhabit competitive polymicrobial environments, capable of forming pores in cellular membranes and inhibiting enzymes involved in cellular metabolism, thereby promoting cytotoxicity and preventing growth and biofilm formation.[6,7] Biosurfactants, on the other hand, are amphiphilic compounds that include glycolipids, lipopeptides, fatty acids, rhamnolipids, phospholipids, and protein–polysaccharide complexes. They have highly selective and specialized functions, being able to alter surface chemistry, reduce surface tension, and modulate pathogen adhesion and biofilm formation, in addition to interacting with cell membranes and altering their permeability.[6,8] Furthermore, lactobacilli species are also capable of adhering to vaginal epithelial cells, competing with other microorganisms for binding sites.[9]

Through these mechanisms, lactobacilli establish a protective microenvironment against infection with pathogens, including those responsible for classical cervicitis, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and *Trichomonas vaginalis*, as well as infections caused by Human Papillomavirus (HPV) and Human Immunodeficiency Virus (HIV).[2] Additionally, the maintenance of a eubiotic vaginal environment is directly associated with improved reproductive outcomes, including higher success rates in assisted reproductive technologies and a lower risk of adverse pregnancy outcomes.[3,10]

1.2. Classification of vaginal microbiota patterns

In 2011, Ravel et al. genomically clustered the vaginal microbiota of women of reproductive age into five distinct bacterial communities, termed Community State Types

(CSTs). The CSTs differ according to the most abundant species, with CSTs I, II, III, and V being dominated, respectively, by *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, and *Lactobacillus jensenii*, whereas CST IV comprises a heterogeneous group of predominantly anaerobic bacterial species, which are frequently associated with the pathological core of bacterial vaginosis (BV).[11]

With advances in the fields of Molecular Biology and Genomics, in 2020, France et al. proposed the VALENCIA model (VAginaL community state typE Nearest CentroId clAssifier) as a method for classifying CSTs. Based on the analysis of more than 13,000 vaginal microbiota profiles, VALENCIA enabled refinement of the previous classification by incorporating less frequent community compositions and distinguishing subtypes based on the relative abundance of dominant species. This method is based on training a classifier that defines a centroid for each bacterial community category and uses it as a reference to assign new samples to the most similar CST type. Using this model, 13 distinct CSTs were identified. Six of these are dominated by *Lactobacillus* species: CST I-A and CST I-B, both dominated by *L. crispatus*, with subtype A exhibiting a higher relative abundance of this species than subtype B; CST II, dominated by *L. gasseri*; CST III-A and CST III-B, dominated by *L. iners*, with subtype A showing a higher relative abundance of *L. iners* than subtype B; and CST V, dominated by *L. jensenii*. [12]

Beyond lactobacilli-dominated communities, the model identified CSTs dominated by anaerobic species associated with dysbiosis: CST IV-A, dominated by Candidatus *Lachnocurva vaginae* (formerly referred to as bacterial vaginosis associated bacteria — BVAB1) and characterized by a moderate relative abundance of *Gardnerella vaginalis* and *Fannyhessea vaginae*; CST IV-B, dominated by *G. vaginalis*, with a low relative abundance of *Ca. L. vaginae* and a moderate relative abundance of *F. vaginae*; and CST IV-C, composed of a diverse community of strict or facultative anaerobic bacteria and a low relative abundance of *Lactobacillus* spp., *G. vaginalis*, and *F. vaginae*. The latter was further subdivided into five subcategories: CST IV-C0, which is more homogeneous and characterized by a moderate abundance of *Prevotella*; CST IV-C1, dominated by *Streptococcus*; CST IV-C2, dominated by *Enterococcus*; CST IV-C3, dominated by *Bifidobacterium*; and CST IV-C4, dominated by *Staphylococcus*.[12]

More recently, in 2023, a new classification capable of describing the vaginal microbiome in a more precise and functional manner was proposed. Through the analysis of 1,890 vaginal metagenomes from women of reproductive age, Metagenomic Community State Types (mgCSTs) were defined. This classification takes into account clusters of strains from the same species that coexist within the vaginal microbiota,

referred to as metagenomic subspecies (mgSs). Each mgCST represents a unique combination of mgSs, allowing the integration of taxogenomic information and functional potential. [13]

In total, 27 mgCSTs were established, the majority of which are dominated by species commonly observed in the vaginal environment: mgCSTs 1–6 are dominated by *L. crispatus*; mgCSTs 7–9 by *L. gasseri*; mgCSTs 10–14 by *L. iners*; mgCSTs 15 and 16 by *L. jensenii*; mgCSTs 17–19 by Ca. *Lachnocurva vaginae*; mgCSTs 20–25 by *Gardnerella*; mgCST 26 by *Bifidobacterium breve*; and mgCST 27 containing less common species, such as *Streptococcus anginosus*, or lacking a predominant taxon.[13]

As a dynamic environment, the vaginal microbiota is subject to changes associated with hormonal fluctuations, pregnancy, and the menstrual cycle, as well as external factors such as stress, contraceptive methods, and intimate and sexual hygiene practices.[9,14] Thus, while some women maintain relatively stable microbial communities, others may exhibit more pronounced changes in the composition of their microbiota.[15] The different classifications of the vaginal microbiome, summarized in figure 1, illustrate how knowledge of these community profiles has been refined over time, recognizing a spectrum that includes both *Lactobacillus*–dominated communities and less stable profiles associated with dysbiosis. Although profiles dominated by *Lactobacillus* spp. are generally associated with a stable and protective environment, a reduction in these species, together with a concomitant increase in anaerobic bacteria, significantly disrupts vaginal homeostasis. These changes in microbial community structure characterize states of vaginal dysbiosis.

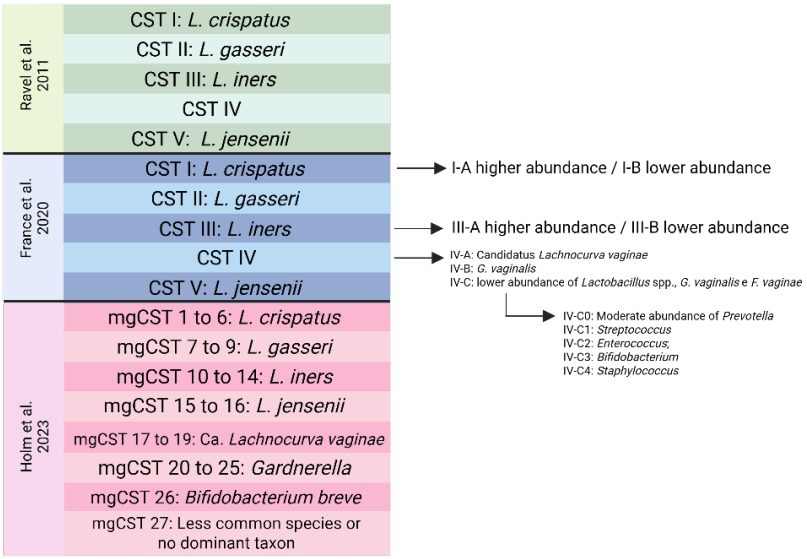

Figure 1. Evolution of vaginal microbiota classifications over time. Comparative representation of vaginal microbiome classification models proposed by Ravel et al. (2011), France et al. (2020), and Holm et al. (2023). The initial model described five Community State Types (CSTs) based on the dominant bacterial species.[11] The VALENCIA model refined this categorization by recognizing subtypes and greater intraspecies diversity.[12] Subsequently, the metagenomic model introduced Metagenomic Community State Types (mgCSTs), which integrate taxonomic and functional information, expanding the classification to 27 distinct vaginal microbiota profiles.[13] Figure created by the authors (BioRender).

1.3. Bacterial vaginosis

Among the most common dysbiotic conditions in women of reproductive age, bacterial vaginosis (BV) stands out. This dysbiosis is characterized by a marked reduction or absence of *Lactobacillus* species, concomitant with an increased relative abundance of obligate and facultative anaerobic bacterial species, such as *G. vaginalis*, *F. vaginae*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Prevotella bivia*, *Mobiluncus* spp., *Leptotrichia* spp., among others.[15,16]

BV prevalence rates may range from 5% to 70%, depending on the socioeconomic conditions of the population studied, as well as behavioral and clinical factors, including sexual practices and smoking habits, in addition to the diagnostic methods used.[17] Globally, prevalence is highest in countries on the African continent, reaching rates of up to 51% in Lesotho and 44% in Kenya,[17,18] whereas lower values are reported in West Africa, such as 8% in Burkina Faso.[19] In Europe, prevalence rates are generally lower; however, relatively high prevalences have been reported in countries such as Poland (19%), Turkey (23%), and Norway (24%).[18] Similar rates are observed in regions of the Asian continent and Oceania, with prevalence estimates ranging between 30% and 32%.[20,21]

In Brazil, BV prevalence shows wide variability across studies, largely due to methodological differences and population characteristics. In a population-based survey conducted in a rural area of the Northeast region, an approximate prevalence of 20% was estimated,[22] whereas a screening study carried out in the Southeast region reported a prevalence of 30.1% among women aged 15–64 years.[23] In higher-risk subgroups, such as women who have sex with women, prevalence rates were even higher in clinical studies. A national systematic review reported a mean prevalence of 25.4% (95% CI: 24.0–26.8), with 18.1% in population-based studies and 27.2% in healthcare settings.[24]

1.3.1. Diagnosis of bacterial vaginosis

The most commonly used tools for the diagnosis of BV include clinical and microscopic approaches, with the criteria proposed by Amsel et al. (1983) and the Nugent score described by Nugent et al. (1991) being the most widely applied methods worldwide.[25,26] The former is primarily based on clinical signs, and the subjectivity of the amine test, together with the susceptibility of vaginal pH to external interference, compromises diagnostic accuracy. For the clinical diagnosis of BV, the presence of at least three of the four criteria is required, including: the presence of thin, homogeneous, grayish-white vaginal discharge; vaginal pH ≥ 4.5; a positive amine (whiff) test,

characterized by a fishy odor of the vaginal contents after the addition of 10% potassium hydroxide (KOH); and the presence of clue cells on wet mount microscopy.[25]

The Nugent criteria, in turn, consist of a microscopic evaluation of vaginal contents from women of reproductive age, based on Gram staining of vaginal smears. In this technique, different bacterial morphotypes are assessed and semi-quantified, including lactobacillary morphotypes, Gram-variable cocobacilli, and curved, Gram-labile bacilli, generating a score ranging from 0 to 10. Accordingly, scores from 0 to 3, characterized by a predominance of *Lactobacillus* spp., are classified as normal microbiota; scores from 4 to 6 are classified as intermediate microbiota; and scores from 7 to 10 are classified as BV.[26] Due to its objectivity and reproducibility, the Nugent criteria are considered the gold standard for the diagnosis of BV in women of reproductive age by the World Health Organization (WHO).[3]

Despite the availability of well-established diagnostic methods, clinical practice still relies predominantly on subjective symptom assessment and gynecological examination findings, with limited use of microscopic evaluation. Comparative studies have shown that the Amsel criteria exhibit lower sensitivity when compared with the Nugent score. Sha et al. reported a sensitivity of approximately 37% for clinical criteria relative to the microbiological score, highlighting substantial underdiagnosis of BV when clinical criteria alone are used.[27] Similar findings were observed in a more recent study, in which the sensitivity of the Amsel criteria ranged from 37% to 70%, reinforcing the superior accuracy of the Nugent score, particularly among asymptomatic women or those with intermediate microbiota.[28] Consequently, assessments based exclusively on speculum examination or medical history show low concordance with microbiological methods, favoring inappropriate use of antifungals and antibiotics, further disruption of the vaginal microbiota, and the selection of resistant microorganisms.

Concurrently, endocrine and immunological alterations, as well as allergic reactions, may modify the volume and appearance of vaginal secretions, mimicking infectious conditions and hindering the clinical distinction between physiological discharge and vaginal dysbiosis. This nonspecificity is particularly relevant in the context of BV, as the condition is frequently asymptomatic, contributing to its underdiagnosis and persistence in the female population. When symptomatic, BV manifests mainly as homogeneous vaginal discharge, resulting from exfoliation of vaginal epithelial cells, and as a characteristic genital odor caused by the release of volatile amines such as putrescine, cadaverine, and trimethylamine, which may negatively impact women’s quality of life.[3,9] In such situations, the adoption of empirical treatments without microbiological

confirmation may not only fail to alleviate symptoms but also intensify discomfort and perpetuate vaginal dysbiosis.

1.3.2. Bacterial vaginosis and clinical implications

Clinically, BV is associated with a wide range of gynecological and obstetric implications. This association arises from structural and immunological alterations induced by dysbiosis within the vaginal microenvironment. BV promotes an elevation of vaginal pH, a reduction in the production of protective metabolites by *Lactobacillus* spp., and impairment of mucosal barrier integrity, rendering the vaginal epithelium more susceptible to pathogen adhesion.[29] Additionally, the enzymatic activity of BV-associated bacteria contributes to the degradation of cervicovaginal mucus and the extracellular matrix, facilitating exposure of cellular receptors involved in infection by sexually transmitted agents.[30]

Beyond the increased risk of STIs, bacterial vaginosis is also associated with the development of upper genital tract infections, including pelvic inflammatory disease, endometritis, and salpingitis.[3,14] Dysfunction of the lower genital tract barrier favors bacterial ascent into the uterine cavity and adnexa, while persistent subclinical inflammation—characterized by increased levels of proinflammatory cytokines—may result in progressive tissue damage.[31] This inflammatory process is linked to altered tubal ciliary function, adhesion formation, and an increased risk of infertility, particularly tubal-factor infertility, as well as a higher likelihood of ectopic pregnancy.[32] In the obstetric context, bacterial vaginosis (BV) has also been associated with gestational complications, including chorioamnionitis, preterm birth, preterm premature rupture of membranes, low birth weight, and postpartum infections.[33]

It is noteworthy that, beyond its biological repercussions, BV may also entail significant psychosocial impacts. The persistent presence of symptoms such, as vaginal discharge and unpleasant odor can lead to embarrassment, emotional distress, and impairments in self-esteem and sexual life, underscoring the importance of appropriate diagnostic strategies, health education, and effective treatment.[34]

1.4. Gardnerella vaginalis

Among the bacterial species most frequently associated with BV, *Gardnerella vaginalis* stands out, having historically been considered the primary species involved in the etiology of this dysbiosis. It is a Gram-variable, facultative anaerobic coccobacillary species belonging to the family *Bifidobacteriaceae*, and it possesses virulence factors that

enable biofilm formation.[35,36] *G. vaginalis* was first isolated in 1953 by Leopold and was subsequently associated with “nonspecific bacterial vaginitis” by Gardner and Dukes, at which time it was named *Haemophilus vaginalis*.[37,38] It was later reclassified as part of the genus *Corynebacterium*.[39] In 1980, studies by Greenwood and Pickett proposed the genus *Gardnerella* and renamed the bacterium *G. vaginalis*.[40]

Since its first description, *G. vaginalis* has been extensively studied due to its central role in the pathogenesis of BV. This species harbors a set of virulence factors that promote colonization, immune evasion, and biofilm formation, among which sialidase and vaginolysin are particularly notable.[35,41-43] For a long time, sialidase activity in *G. vaginalis* was thought to be encoded by the sialidase A gene (*nanH1*). However, a study by Robinson et al. described two additional sialidase-encoding genes (*nanH2* and *nanH3*), demonstrating that their presence in *G. vaginalis* genomes reflects the capacity to produce sialidase, and that these enzymes exhibit a broad range of activity against glycoproteins of the vaginal mucosa, representing the main sources of neuraminidase activity in this species.[44]

Sialidase is a glycosidase responsible for degrading the vaginal epithelial barrier, leading to epithelial exfoliation and desquamation through the cleavage of sialic acid residues from sialoglycans (mucins) that compose the vaginal mucosa. This cleavage facilitates bacterial adhesion by exposing glycan binding sites, in addition to removing terminal sialic acid from immunoglobulin A, thereby increasing its susceptibility to proteolytic degradation and contributing to immune evasion.[35,41-43] The released sialic acids can be utilized by bacteria as a carbon source, as they can be transported into the bacterial cell via a high-affinity system and subsequently catabolized intracellularly by aldolase into N-acetylmannosamine (ManNAc) and pyruvate.[43,45] Furthermore, it has been suggested that cleaved sialic acids may be incorporated into the bacterial surface, allowing bacteria to mimic host structures and evade immune recognition.[41] Finally, sialidase is associated with the early stages of biofilm formation, as it facilitates the adhesion of *G. vaginalis* to epithelial cells.[46]

Vaginolysin is a cholesterol-dependent hemolysin that is highly active at elevated vaginal pH (5.0–7.5) and is responsible for pore formation in vaginal epithelial cells through interaction with the CD59 molecule.[43] This interaction triggers an epithelial response that induces the phosphorylation and activation of p38 MAPK (mitogen-activated protein kinase p38), facilitating cell lysis, which may represent an early mechanism of immune evasion, as this factor is capable of lysing neutrophils and possibly other immune system cells.[35,36,41] In addition to sialidases, *G. vaginalis* secretes proteases, such as

prolidase,[43,45] which degrade protein components and glycoconjugates of the vaginal environment, promoting the release of volatile compounds derived from aliphatic amines.[3] The cell surface of *G. vaginalis* also features fimbriae, which enhance its adhesion to vaginal epithelial cells.[3] Furthermore, genomic studies indicate that the biofilm-forming capacity of *G. vaginalis* is also associated with the presence of genes encoding glycosyltransferases, particularly type II enzymes, which are important components in the biosynthesis of extracellular polymeric substances (EPS).[47] Together, these factors act synergistically to facilitate colonization of the vaginal epithelium, contributing to the early stages of biofilm formation.

Biofilms are communities of bacteria that adhere to a surface and grow embedded within a self-produced extracellular matrix, which provides protection against antimicrobials, host defenses, and other environmental stressors (Figure 2).[48] Biofilm formation occurs in a progressive manner, beginning with a reversible adhesion phase mediated by transient physicochemical interactions between the planktonic form of a microorganism and the vaginal epithelium. This is followed by the establishment of an irreversible phase, characterized by the production of extracellular polymeric substances (EPS), including proteins, glycoproteins, glycolipids, and extracellular DNA, thereby consolidating bacterial attachment, microcolony growth, and formation of the matrix that sustains microbial interactions. This matrix determines biofilm porosity, density, water content, charge, adsorption properties, hydrophobicity, and mechanical stability, and its composition depends on environmental conditions.[49,50] Swidsinski et al. analyzed vaginal samples using fluorescence in situ hybridization (FISH) and demonstrated that *Gardnerella* is the predominant bacterial genus and an essential component of BV biofilms, forming layers that are firmly adherent to the surface of the vaginal epithelium.[51] Verstraelen and Swidsinski further showed that *G. vaginalis* has a greater capacity to adhere to epithelial cells than other BV-associated bacteria, even in the presence of lactobacilli,[52] supporting the concept that it acts as a primary colonizer enabling subsequent colonization by BV-associated bacteria (BVABs).[53] Accordingly, biofilms have been considered a hallmark of BV,[54] containing high abundances of *G. vaginalis* in association with BVABs, *Prevotella* spp., *Sneathia* spp., as well as *F. vaginae*.[52]

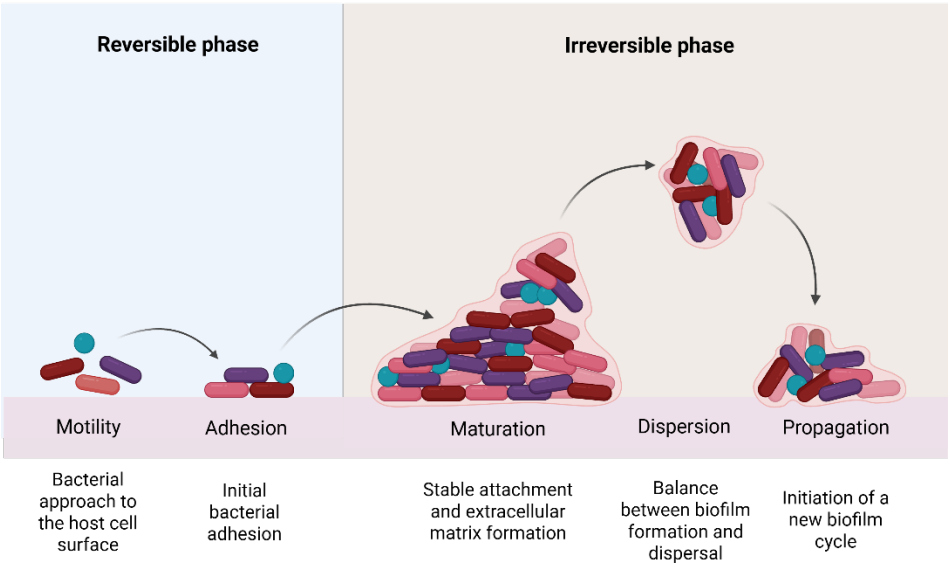

Figure 2. Schematic representation of the stages of bacterial biofilm formation. Initially, planktonic bacterial cells transiently interact with a surface (reversible phase). Subsequently, irreversible adhesion and the production of extracellular polymeric substances (EPS) occur, promoting biofilm maturation and microcolony development. Thereafter, some cells disperse, returning to the planktonic state and enabling propagation and colonization of new surfaces. Figure created by the authors (BioRender).

Due to the presence of multiple layers within the biofilm, gradients of nutrients, oxygen, and pH are established. Oxygen consumption by aerobic bacteria located in the outer layers of the biofilm generates an oxygen gradient, resulting in increasingly anaerobic conditions toward the inner strata. As the biofilm grows, inner cells become separated from the external interface, where most essential energy sources and nutrients are available. In addition, waste products and toxins accumulate within the biofilm. These gradients slow the growth and metabolism of the bacterial subpopulation residing in the core, which consists of dormant cells influenced by the biofilm's growth conditions. When exposed to antibiotics, some of these dormant bacteria exhibit increased tolerance and are known as persister cells.[50]

Persister cells are bacterial subpopulations characterized by reduced growth rates or growth arrest, enabling survival under stress conditions, such as exposure to antibiotics, reactive oxygen species, acidic pH, or nutrient limitation, and are often described as dormant cells. After the removal of the stressor, persister cells can resume growth and remain susceptible to the same stressor. They exhibit phenotypic heterogeneity and are highly dependent on various factors, including the bacterial strain, the antibiotic used, and environmental conditions. The tolerance of persister cells to antibiotics is expressed only at the phenotypic level, with no underlying mutations in resistance genes. Persistence arises from the interplay of multiple processes, which may include reduced cellular energy levels, arrest of DNA replication, inhibition of translation, and decreased intracellular antibiotic concentrations. Persister cells play a significant role

in the recurrence of infections, act as an evolutionary reservoir from which resistant bacteria may emerge, and increase tolerance in biofilm-associated infections.[55,56]

Therefore, biofilms represent one of the major barriers to the effectiveness of BV treatment,[57] increasing the likelihood of antibiotic resistance through multiple mechanisms, including: limited penetration of immune cells and antimicrobial compounds into the extracellular matrix, forming a barrier that delays or reduces drug diffusion; degradation of compounds by metabolically active bacteria in the outer subpopulation of the biofilm; physiological changes in the biofilm microenvironment due to slow growth driven by nutrient and oxygen gradients, which reduces the metabolic activity of bacteria in the inner layers and limits the activation of metronidazole from its prodrug to its active form; phenotypic alterations; cell-to-cell communication via quorum sensing among microorganisms; expression of efflux pumps; and the formation of persister cells.[42,50] As a result, these factors contribute to a recurrence rate of 20–30% among women undergoing standard BV treatment within up to three months, as the biofilm is only temporarily suppressed by metronidazole and regains activity after treatment cessation.[58]

For decades, *G. vaginalis* was the only species recognized within its genus; however, in recent years, its taxonomic classification has been revised.[59] In 2012, Ahmed et al. identified four groups (1, 2, 3, and 4) based on neighborhood clustering analyses.[60] In 2014, Balashov et al. developed PCR assays to specifically identify representatives of these four groups using sequence analysis of the chaperonin 60 (*cpn60*) gene, which encodes heat shock protein 60 (HSP60).[61] This protein functions as a molecular chaperone involved in the cellular stress response and is widely used as a phylogenetic marker due to its high discriminatory power among bacterial species and subgroups. Whole-genome sequence analysis further differentiated *G. vaginalis* strains into four clades and demonstrated that the four *cpn60*-based subgroups (A, B, C, and D) correspond to clades 4, 2, 1, and 3, respectively. Moreover, a positive correlation was observed between BV and clades 1 and 3, whereas clade 2 showed a positive correlation with intermediate vaginal microbiota, and clade 4 showed no correlation.[62] In 2019, whole-genome sequencing and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF) analyses were performed on 81 *Gardnerella* strains by Vaneechoutte et al., revealing the existence of 13 sufficiently distinct groups to be classified as separate species. This led to the formal inclusion of three additional *Gardnerella* species—*G. leopoldii*, *G. piotii*, and *G. swidsinskii*—along with nine genomic species. In the same study, *G. vaginalis* and *G. piotii* were identified as sialidase-positive,

whereas *G. leopoldii* and *G. swidsinskii* were sialidase-negative. Furthermore, *G. vaginalis* was the only species among the four that was β-galactosidase positive.[62]

In a study conducted using vaginal samples from Canadian women, multiple *Gardnerella* species were detected in most participants. Although their relative abundances varied, significant co-occurrence was observed between *G. vaginalis* and *G. swidsinskii*, as well as between *G. piotii* and genomic species 3. A significant association was also identified between BV and higher relative abundances of *G. vaginalis*, *G. swidsinskii* e *G. piotii*, together with strong associations between symptoms of abnormal odor and vaginal discharge and higher relative abundances of *G. vaginalis* and *G. swidsinskii*, but not with the other species.[63] With respect to virulence factors, differences in the genetic composition of vaginolysin have been identified among the 13 genomospecies, with vaginolysin types 1 and 2 being the most common among the characterized isolates[42] however, no significant differences in the production of this hemolysin have been demonstrated.[63] Likewise, no differences in biofilm-forming capacity were observed among the species.[64,65] Regarding metronidazole resistance, strains belonging to groups 3 and 4 exhibited 100% resistance, whereas groups 1 and 2 showed resistance rates of 35% and 7.1%, respectively.[65]

More recently, in 2023, Sousa et al. also employed whole-genome sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and described two novel *Gardnerella* species: *G. pickettii* (formerly genomic species 3) and *G. greenwoodii* (formerly genomic species 8). The study identified that both species were β-galactosidase-negative. With regard to virulence factors, both *G. pickettii* and *G. greenwoodii* harbored the *vly* gene encoding type 1 vaginolysin, whereas only *G. pickettii* exhibited sialidase activity. Furthermore, antimicrobial resistance genes were identified in both species, including *msr(D)* in tandem with the *mef(A)* efflux pump, which confers resistance to macrolides, and *lsaC*, which mediates cross-resistance to lincosamides, streptogramin A, and pleuromutilins, potentially contributing to a reduced response to clindamycin therapy.[66] Additionally, Innamorati et al. evaluated metronidazole resistance among *Gardnerella* species using minimum inhibitory concentration (MIC) testing and observed that tested strains of *G. vaginalis*, *G. pickettii*, and *G. piotii* displayed heterogeneous responses, with MICs ranging from low susceptibility to high resistance, whereas strains of *G. swidsinskii* and *G. leopoldii* were highly resistant to this nitroimidazole.[67]

Seeking to elucidate the association between different *Gardnerella* species and bacterial vaginosis (BV), Munch et al. developed four quantitative PCR assays targeting the *cpn60* gene, enabling the detection of four species groups: *G. vaginalis*; *G. piotii/G. pickettii*; *G. swidsinskii/G. greenwoodii*; and *G. leopoldii*. *G. vaginalis* was shown to be the most frequent species among patients with BV (69.7%); however, it was consistently detected in co-occurrence with at least one additional *Gardnerella* species. All four species groups were identified more frequently in women with BV and exhibited significantly higher bacterial loads. Moreover, recurrence rates were highest in cases involving *G. vaginalis*, reaching 75%, followed by 44.4% for the *G. piotii/G. pickettii* group and 28.6% for *G. leopoldii*. Collectively, these findings suggest that the presence of multiple *Gardnerella* species may constitute a risk factor for BV.[68]

Accumulating evidence indicates that *G. vaginalis* is part of the pathological core of most BV cases,[35,69,70] and over the past decade it has been increasingly demonstrated that *G. vaginalis* exhibits greater virulence potential than other BV-associated bacteria.[71] This species is capable of biofilm formation, facilitating immune evasion (62–64), and displays increased tolerance to lactic acid and hydrogen peroxide (H_2O_2).[14] In addition, other bacterial species, including *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Prevotella bivia*, and *Fannyhessea vaginae*, are also present at high relative abundances in BV.[72]

1.5. Fannyhessea vaginae

Fannyhessea vaginae, previously classified as *Atopobium vaginae*,[75] is another bacterial species frequently identified within the pathological core of bacterial vaginosis (BV).[76] It is a Gram-positive coccoid bacillus, a strict anaerobe, which may appear as single cells, in pairs, or in short chains.[70] The presence of *F. vaginae* in vaginal microbiota dominated by *Lactobacillus* species remains controversial, as some studies report its detection in only 12–19% of samples from women without BV,[77,78] whereas others have described prevalence rates as high as 69%.[79] Nevertheless, its quantitative abundance appears to be a reliable predictor of BV, as *F. vaginae* is found at significantly higher concentrations in BV-positive samples. Accordingly, *F. vaginae* has been considered an indicator species of BV, being detected in approximately 50–95% of cases,[80] typically in combination with *G. vaginalis*, and playing a critical role in biofilm formation and establishment.[81]

In analyses conducted by Swidsinski et al. using fluorescence *in situ* hybridization (FISH), 86% of *Gardnerella* spp. biofilms were associated with high concentrations of *F.*

vaginae, contributing up to 40% of the total biofilm mass.[51] Hardy et al. also employed FISH to investigate the role of *F. vaginae* in BV and confirmed that this species is a key biofilm constituent, as adherent *F. vaginae* was visualized in 54.1% of bacterial biofilm samples.[81] Moreover, only two samples contained *F. vaginae* in the absence of *G. vaginalis*, further highlighting the synergistic relationship between these two bacteria.[81]

Biofilms formed by *G. vaginalis* create a favorable environment for the growth of anaerobic bacteria due to the establishment of an oxygen gradient toward the inner layers, which arises from oxygen consumption by aerobic bacteria in the outer layers.[50] Thus, *F. vaginae* is able to exploit this mechanism to proliferate, establishing a mutualistic relationship with *G. vaginalis*.[59,71] The presence of *F. vaginae* within the biofilm may also impact treatment outcomes, as in vitro studies have shown that susceptibility to metronidazole varies among *F. vaginae* strains.[82] Bradshaw et al. reported higher rates of BV recurrence when *F. vaginae* was associated with *G. vaginalis,*[77] and Ferris et al. demonstrated that the presence of *F. vaginae* was associated with partial or complete failure of BV treatment with metronidazole.[83] Additionally, Luchiari et al. showed that therapeutic failure in BV is not related to total bacterial load but is instead likely associated with specific microbiological characteristics, such as bacterial composition and biofilm organization, further supporting the role of the *G. vaginalis–F. vaginae* microbial consortium in infection persistence.[84]

1.6. Immune modulation in the vaginal microenvironment

The vaginal microbiota plays a key role in immune modulation of the vaginal microenvironment. The vagina contains immune cells such as neutrophils, macrophages, T cells, B cells, and natural killer cells, and expresses specialized receptors, including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), which are responsible for pathogen recognition. Microbial stimulation leads to the release of cytokines and chemokines, such as IL-1β, IL-6, CXCL-8, and TNF-α, which are activated and regulated through the nuclear factor kappa B (NF-κB) signaling pathway. The inflammatory response elicited by pathogens generally contributes to infection control; however, in some cases, it may disrupt the epithelial cell barrier and facilitate the acquisition of additional infections.[2]

Clinically, BV is considered a non-inflammatory condition, as it is not associated with edema or neutrophilic chemotaxis.[69] One possible explanation for this phenomenon is the ability of biofilms to promote a more immunologically silent response.[42] Bertran et al. demonstrated that *G. vaginalis* did not induce cytotoxicity or increase the production of pro-inflammatory cytokines (TNF-α, IL-12p70, IFN-γ) by dendritic cells, suggesting a

quiescent immune response.[85] Another study using the same isolate and other BV-associated bacteria showed a significant increase in dendritic cell activation and higher expression of IL-1β, IL-6, CXCL-8, IL-12A, IL-12B, and TNF-α messenger RNA in response to stimulation by other BVAB; however, in the case of *Gardnerella*, only CXCL-8 secretion was significantly increased.[42,86]

Despite this, BV is associated with molecular inflammation, generally characterized by the production of pro-inflammatory cytokines and reduced levels of certain chemokines.[42] Communities dominated by anaerobic species are associated with a more intense pro-inflammatory response compared with those dominated by *Lactobacillus* spp., in contrast, exert immunomodulatory effects through the protonated form of lactic acid, which enhances antimicrobial defense without inducing inflammatory mediators. This mechanism establishes an anti-inflammatory environment, characterized by increased levels of regulatory cytokines, such as IL-1 receptor antagonist (IL-1RA), and reduced production of pro-inflammatory cytokines, including IL-6 and TNF-α, as well as decreased levels of chemokines such as CXCL-8, RANTES, and MIP-3α.[2]

Studies have shown that colonization by BV-associated bacteria (BVAB) of cervical epithelial cells induces a pro-inflammatory response, as evidenced by increased levels of IL-6, CXCL-8, interferon gamma–induced protein 10 (IP-10), MCP-1, MIP-1β, MIP-3α, and IL-1β.[87] In macrophages, the signaling cascade triggered by stimulation with vaginal lavages from patients with BV leads to activation of NF-κB, which in turn activates the NLRP3 inflammasome and results in increased release of IL-1β and IL-18.[42] Eade et al., using *G. vaginalis* from the American Type Culture Collection (ATCC 49145), demonstrated increased CXCL-8 expression.[88] Fichorova et al., using a primary isolate of *G. vaginalis* obtained from a BV sample, reported increased levels of CXCL-8, RANTES, and secretory leukocyte protease inhibitor (SLPI).[89] Anderson et al., employing bacterial culture techniques, associated the presence of *G. vaginalis* in vaginal specimens from pregnant women with higher cervical levels of IL-1, IFN-γ, TNF-α, and granulocyte–macrophage colony-stimulating factor (GM-CSF).[90] Similarly, genomic detection of *G. vaginalis* in vaginal samples from patients with BV was associated with increased levels of IL-1, IL-6, CXCL-8, and IL-12p70, although reduced levels of IP-10 and SLPI were also observed. *G. vaginalis* was also among the six dominant species identified by 16S rRNA sequencing in samples characterized by elevated cervicovaginal levels of IL-1, IL-6, CXCL-8, TNF-α, IFN-γ, and IL-10 in young South African women.[70,91]

Levels of specific cytokines and antimicrobial peptides may also vary according to species-specific immune signatures. Primary colonizers, such as *G. vaginalis*, are capable of actively inhibiting the host inflammatory response in the vaginal epithelium, thereby favoring the establishment and maintenance of the characteristic BV-associated biofilm. In contrast, *F. vaginae* can induce activation of the host immune response, contributing to the development of BV signs and symptoms, including the release of metabolites such as aliphatic amines.[92] Corroborating these findings, a study using the EpiVaginal tissue model demonstrated that infection with *G. vaginalis* does not promote significant changes in the levels of pro-inflammatory mediators.[93] Consistently, a previous study showed that *G. vaginalis* does not promote the induction of IL-1β, IL-6, MIP-3α, or TNF-α in a three-dimensional (3D) human model of endometrial epithelial cells. These findings suggest that *G. vaginalis* acts predominantly as an initial colonizer, capable of modulating or attenuating the host inflammatory response, favoring epithelial adhesion and the establishment of the polymicrobial biofilm characteristic of BV. In contrast, other bacteria associated with BV, such as *F. vaginae*, appear to play a central role in amplifying the local inflammatory response. In this context, Doerflinger et al. demonstrated in a 3D human model of vaginal epithelial cells that *F. vaginae* induces a wide range of proinflammatory cytokines, chemokines, and antimicrobial peptides, including IL-1β, IL-6, CXCL-8, MIP-3α, TNF-α, and human β-defensin-2 (HBD-2).[92,94]

Additional evidence indicates that the co-occurrence of *G. vaginalis* and *F. vaginae* within the vaginal biofilm results in a synergistic effect on the host immune response. When co-cultured in vaginal epithelial cell models, these species elicit a more robust pro-inflammatory response, characterized by increased production of cytokines (IL-6, IL-1β, TNF-α, CXCL-8) and chemokines (RANTES, MIP-1β), more accurately reflecting the inflammatory profile observed in women with BV.[15] These findings reinforce the notion that BV pathogenesis does not depend on a single bacterial species, but rather on the functional interactions among multiple microorganisms organized within a biofilm, which are capable of simultaneously modulating colonization, immune evasion, and local inflammation.

In addition, cytokine and chemokine responses may vary depending on the cellular model and the *Gardnerella* species involved. The vaginal epithelium exhibits a distinct pattern of pattern recognition receptors (PRRs) compared with endocervical cells and the basement membrane, which may account for differences in responses to the same pathogen. Increased IP-10 production has been observed following endocervical challenge with *G. vaginalis*, but not after vaginal or ectocervical challenge. In contrast,

exposure to the *G. leopoldii* isolate resulted in reduced IP-10 secretion in both the ectocervix and the vagina.[42]

Alterations in the vaginal microbiota associated with BV and the subsequent inflammatory response may lead to adverse gynecological and reproductive outcomes. Noda-Nicolau et al. demonstrated that stimulation of fetal membranes with *G. vaginalis*, either alone or in combination with genital mycoplasmas, induced increased levels of inflammatory cytokines (IL-1β, CXCL-8, and TNF-α) as well as anti-inflammatory cytokines (IL-4, IL-10, and IL-13), and that polymicrobial combinations with higher *G. vaginalis* loads elicited a strong pro-inflammatory response.[95] Notably, treatment with *G. vaginalis* also increased IL-6 production and the expression of membrane-bound interleukin-6 receptor (mIL-6R) in amniotic cells, as well as the expression of glycoprotein 130 (gp130) in both amniotic and chorionic cells, indicating that this bacterium activates the classical IL-6 signaling pathway.[96]

Molecular analyses of cervicovaginal lavage samples conducted by Belleti et al. identified that persistence of high-risk HPV types 16 and 18 is associated not only with the presence, but also with higher loads of *G. vaginalis*, supporting evidence that this bacterium plays an important role in the outcome of HPV infection.[97] Complementarily, Novak et al. analyzed the presence and load of the *G. vaginalis* nanH3 gene in HPV-positive samples and demonstrated that, in the presence of HPV16, the group that achieved viral clearance exhibited lower nanH3 loads than the group that showed persistent infection.[98]

More recently, data from our group demonstrated, using an organ-on-chip model of maternal–fetal interface, the ability of *Gardnerella* spp. to ascend to the fetal membranes, exhibiting significantly higher loads in these layers within a 24-hour period (unpublished data). Thus, the mechanisms of immune activation and inflammation operating during BV provide support for the role of this dysbiosis in other clinical complications.[99]

Considering the predominance of *Gardnerella* spp. in BV-associated biofilms, alterations in mucosal immunity mediated by this bacterial genus may help explain the persistence of BV and how it increases the risk of sexually transmitted infection acquisition and adverse pregnancy outcomes.[42] In this context, further studies are needed to determine the coexistence of *Gardnerella* spp. and *F. vaginae* in vaginal dysbiosis and within the lactobacilli-dominated microbiota of women of reproductive age.

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Capítulo 3
Artigo Científico II

Microorganisms. Fator de Impacto: 4.2

Bacterial load of *Gardnerella* spp. and *Fannyhessea vaginae* and its association with cervicovaginal inflammatory cytokine responses across vaginal microbiota patterns.

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Abstract: Bacterial vaginosis (BV) is a common vaginal dysbiosis characterized by the depletion of *Lactobacillus* species and the overgrowth of facultative anaerobic bacteria, particularly *Gardnerella* spp. and *Fannyhessea vaginae*. The vaginal microbiota plays a key role in local immune modulation, and BV has been associated with a molecular pro-inflammatory profile. This study included 152 women with normal microbiota (n = 68), intermediate microbiota (n = 24), or BV (n = 60). Vaginal lavage samples were used to quantify *Gardnerella* spp. and *F. vaginae* and to measure IL-1β, IL-6, CXCL-8, IL-10, and TNF-α levels. Bacterial loads of *Gardnerella* spp. were significantly higher in the BV group than in normal microbiota ($p < 0.001$). *F. vaginae* loads were higher in BV than in both normal and intermediate microbiota ($p < 0.001$). IL-1β levels were increased in intermediate microbiota ($p = 0.011$) and BV ($p = 0.024$) compared with normal microbiota, while CXCL-8 levels were higher in intermediate microbiota ($p = 0.021$). No differences were observed for IL-6, IL-10, or TNF-α. BV is associated with increased *Gardnerella* spp. and *F. vaginae* loads and a selective increase in IL-1β, supporting a distinct inflammatory signature linked to vaginal dysbiosis.

Keywords: *Gardnerella* spp.; *Fannyhessea vaginae*; vaginal microbiota; bacterial vaginosis; inflammatory cytokines

1. Introduction

Human vaginal microbiota establishes a mutualistic relationship with the host, playing a critical role in immune modulation and protection against pathogenic microorganisms. A healthy vaginal microenvironment is typically dominated by *Lactobacillus* species, which produce lactic acid, hydrogen peroxide, and bacteriocins, contributing to the maintenance of an acidic vaginal pH (3.8–4.5) and inhibiting the growth of bacterial and viral pathogens, including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, human papillomavirus (HPV), and human immunodeficiency virus (HIV) [1,2]. The maintenance of a eubiotic vaginal microbiota has also been consistently associated with favorable reproductive outcomes, including improved success rates in assisted reproductive technologies and reduced risk of adverse pregnancy outcomes [3,4].

As a dynamic ecosystem, vaginal microbiota is influenced by hormonal fluctuations, pregnancy, menstrual cycle, and external factors such as contraceptive use, stress, and sexual and hygiene practices [5,6]. Disruption of *Lactobacillus*-dominated communities and overgrowth of anaerobic bacteria lead to vaginal dysbiosis, most notably bacterial vaginosis (BV). BV is characterized by a marked reduction or absence of *Lactobacillus* species and increased abundance of facultative anaerobic bacteria, including *Gardnerella vaginalis*, *Fannyhessea vaginae*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Prevotella bivia*, *Mobiluncus* spp., and *Leptotrichia* spp. [7,8]. BV prevalence ranges from 5% to 70% worldwide, varies according to population characteristics and diagnostic criteria, and is frequently asymptomatic, contributing to underdiagnosis and persistence [9–12].

Among BV-associated bacteria, *G. vaginalis* has historically been considered a key species in BV pathogenesis due to its ability to colonize the vaginal epithelium, evade host immune responses, and form structured biofilms through virulence factors such as sialidase A and vaginolysin [13–17]. *F. vaginae*, formerly *Atopobium vaginae*, is also frequently detected within the pathogenic core of BV and has been proposed as an indicator species, being present in up to 95% of BV cases, most often in co-occurrence with *G. vaginalis* [18–21]. This synergistic interaction is thought to contribute to biofilm stability and BV persistence.

Vaginal microbiota plays a central role in mucosal immune regulation. Although BV has traditionally been considered a non-inflammatory condition, it is increasingly recognized as a state of molecular inflammation characterized by altered cytokine and chemokine profiles. Anaerobe-dominated communities are associated with enhanced pro-inflammatory responses, whereas *Lactobacillus*-dominated microbiota promote

antimicrobial defense without excessive inflammation. Notably, *G. vaginalis* and *F. vaginae* appear to exert distinct, species-specific effects on host immune responses, potentially influencing BV persistence and its association with sexually transmitted infections and adverse reproductive outcomes [22–24]. Therefore, this study aimed to quantify *Gardnerella* spp. and *F. vaginae* across different vaginal microbiota patterns and to evaluate their association with cervicovaginal cytokine responses in women of reproductive age.

2. Materials and Methods

2.1. Study design and population

This prospective, cross-sectional study included 152 women attending the Female Genital Infections Outpatient Clinic at Botucatu Medical School University Hospital Complex (HCFMB–UNESP), Brazil, between 2024 and 2025. At enrollment, participants completed a structured questionnaire to collect sociodemographic information, sexual and behavioral habits, and gynecological history. All participants provided written informed consent prior to inclusion in the study. The study protocol was approved by the local Research Ethics Committee (CAAE: 91235025.0.0000.5411).

Eligible participants were women aged 18 years or older who were of reproductive age, non-pregnant, had not received treatment for genital tract infections within the preceding 30 days, reported sexual abstinence for at least 48 h prior to sample collection, and were at least five days beyond the end of menstruation. Three distinct biological samples were collected from each participant. First, a vaginal swab was obtained from the middle third of the lateral vaginal wall and used for microscopic evaluation of the vaginal microbiota, including Gram staining and microbiota classification. Second, an endocervical sample was collected using a cytobrush and used exclusively for molecular screening of sexually transmitted infections (*N. gonorrhoeae*, *C. trachomatis*, *Trichomonas vaginalis*, and *M. genitalium*). Third, a cervicovaginal lavage sample was obtained by instilling 3 mL of sterile 0.9% saline solution onto the vaginal walls, gently mixing with cervicovaginal secretions, and recovering the fluid using a sterile plastic pipette. This lavage sample was used for the quantification of *Gardnerella* spp. and *F. vaginae* by quantitative PCR and for ELISA to assess cytokine levels.

Exclusion criteria included immunosuppression, history of solid organ transplantation, seropositivity for HIV or syphilis, and a positive endocervical molecular test for *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, or *M. genitalium*.

2.2. Microscopic diagnosis and vaginal sample collection

At study enrollment, a sterile, lubricant-free bivalve vaginal speculum was used for examination. Vaginal samples were collected using a sterile swab from the middle third of the lateral vaginal wall and used to prepare Gram-stained smears for microscopic evaluation of the vaginal microbiota. Microscopic classification of the vaginal microbiota was performed according to the Nugent scoring system, a standardized and widely accepted method based on the semi-quantitative assessment of bacterial morphotypes observed on Gram-stained smears. Specifically, three bacterial morphotypes are evaluated: (i) large Gram-positive rods, predominantly *Lactobacillus* species; (ii) small Gram-variable rods, consistent with *Gardnerella* and *Bacteroides* morphotypes; and (iii) curved Gram-variable rods, consistent with *Mobiluncus* species. Each morphotype is assigned a score ranging from 0 to 4 according to its relative abundance, and the total Nugent score is calculated by summing the individual scores. According to the original criteria described by Nugent et al., total scores of 0–3, 4–6, and 7–10 correspond to normal microbiota, intermediate microbiota, and bacterial vaginosis (BV), respectively. Throughout the manuscript, vaginal microbiota status refers to ecological states inferred from Nugent morphotype scoring of Gram-stained vaginal smears.

Additional microscopic criteria described in the literature were applied exclusively to identify conditions in which Nugent scoring is not applicable, such as cytolytic vaginosis (Cibley and Cibley, 1991) [25] and aerobic vaginitis (Dong et al., 2022) [26]. Participants diagnosed with these conditions were excluded from the study and were referred for clinical management in accordance with applicable clinical guidelines. Based on Nugent score classification, participants included in the analysis were categorized as having normal microbiota (n = 68), intermediate microbiota (n = 24), or bacterial vaginosis (n = 60).

Endocervical samples were collected for molecular screening of *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, and *M. genitalium* by real-time PCR. Cervical specimens were placed in 15-mL Falcon tubes containing 1000 µL of Tris–HCl buffer (50 mM, pH 8.5) supplemented with EDTA (1 mM, pH 8.0) and stored at −80 °C until processing. Vaginal lavage samples were also obtained for the detection and quantification of *Gardnerella* spp. and *F. vaginae*, as well as for cytokine measurements. Briefly, 3 mL of sterile 0.9% saline solution was instilled onto the vaginal wall, gently mixed with cervicovaginal secretions, and recovered using a sterile plastic pipette. Lavage samples were stored at −80 °C until further analysis.

2.3. Molecular screening for sexually transmitted infections

DNA was extracted from endocervical samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA integrity and amplifiability were verified by amplification of the constitutive β-globin gene using the PCO4 and GH20 primers, as previously described [27]. Molecular screening for *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, and *M. genitalium* was performed using a multiplex real-time PCR assay (Allplex™ CT/NG/MG/TV Assay; Seegene, Seoul, Republic of Korea), which employs multiple targets and pathogen-specific probes for simultaneous detection of the four microorganisms. PCR reactions were carried out on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's recommendations.

Each run included an internal control to monitor PCR inhibition, as well as positive and negative controls. Target detection was based on automatically generated cycle threshold (Ct) values, and result interpretation (positive, negative, or invalid) was performed using Seegene Viewer software (V1.00.001), following the criteria established by the manufacturer.

2.4. Quantification of Gardnerella vaginalis by quantitative PCR

DNA was extracted from cervicovaginal lavage samples using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. To generate a standard curve, *G. vaginalis* (ATCC® 14018™) obtained from the American Type Culture Collection (ATCC) was cultured in New York City III (NYC III) medium containing 0.4% HEPES, 1.5% peptone, 0.5% sodium chloride, 0.4% resazurin, 0.5% glucose, 0.375% yeast extract, 10% horse serum, and 85.1% deionized water, with 1.5% agar added when required. Cultures were incubated under anaerobic conditions using an anaerobiosis generator (Anaerobac; Probac, #ANA16T, São Paulo, Brazil) for 24–48 h.

For cloning, bacterial cultures were used to amplify a 332 bp fragment of the 16S rRNA gene by conventional PCR using the primers F-GV1 (5′-TTACTGGTGTATCACTGTAAGG- 3′) and R-GV3 (5′-CCGTCACAGGCTGAACAGT-3′) [28]. Following confirmation of the expected amplicon, PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (Cytiva, Bangalore, India) and ligated into the pJET1.2/blunt cloning vector using T4 DNA ligase, according to the manufacturer's instructions. The ligation mixture was cloned into *Escherichia coli* DH5-α cells (Invitrogen, Carlsbad, CA, USA) by electroporation and heat shock. Transformed cells were cultured on Luria–Bertani (LB) medium supplemented with ampicillin as a

selective antibiotic. Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Vilnius, Lithuania), according to the manufacturer's instructions. Recombinant colonies were screened by PCR using internal primers specific to the cloned fragment. Plasmid DNA concentration was determined using the Qubit™ dsDNA Broad Range (BR) Assay, and the insert sequence was validated by Sanger sequencing on a 500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and confirmed by BLAST+ 2.17.0 analysis.

Absolute quantification of *Gardnerella* spp. was performed by quantitative real-time PCR (qPCR) using 2x qPCRBIO SyGreen Hi-ROX Master Mix (PCR Biosystems, London, UK) and the primers F-GV1 and R-GV3. Reactions were carried out in a final volume of 20 µL, containing 4 µL of extracted DNA (20 ng/µL), under the following cycling conditions: initial denaturation at 95 °C for 2 min; followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 25 s, and extension at 72 °C for 15 s. A melting curve analysis was subsequently performed (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s) using a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Samples presenting a melting temperature of 87 ± 1 °C were considered positive. Standard curves were generated using three plasmid dilutions (10^5, 10^6, and 10^7 copies), allowing the establishment of a linear relationship between cycle threshold (Ct) values and *Gardnerella* spp. copy number. All reactions were performed in triplicate.

2.5. Quantification of Fannyhessea vaginae by quantitative PCR

For construction of the standard curve, a synthetic plasmid containing a 301 bp fragment of the 16S rRNA gene of *F. vaginae* was designed and synthesized (GENEART, Thermo Fisher Scientific). The plasmid was linearized using NotI FastDigest (Thermo Fisher Scientific), quantified using the Qubit™ dsDNA High Sensitivity (HS) Assay, and purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (Cytiva, Bangalore, India).

Absolute quantification of *F. vaginae* was performed by quantitative real-time PCR (qPCR) using 2× qPCRBIO SyGreen Hi-ROX Master Mix (PCR Biosystems, London, UK) and the primers Fw (5′-TAGGCGGTYTGTTAGGTCAGGA-3′) and Rv (5′-CCTACCAGACTCAAGCCTGC-3′) [28]. Reactions were carried out in a final volume of 20 µL, containing 4 µL of extracted DNA (20 ng/µL), under the following cycling conditions: initial denaturation at 95 ◦C for 2 min, followed by 40 cycles of denaturation at 95 ◦C for 5 s, annealing at 60 ◦C for 25 s, and extension at 72 ◦C for 15 s. A melting curve analysis was then performed (95 ◦C for 15 s, 60 ◦C for 1 min, and 95 ◦C for 15 s) using a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA).

Samples presenting a melting temperature of 83.2 ± 0.5 ◦C were considered positive for *F. vaginae*. Standard curves were generated using five serial plasmid dilutions (5×10^8, 5×10^7, 5×10^6, 5×10^5, and 5×10^4 copies/µL), enabling the establishment of a linear relationship between Ct values and *F. vaginae* copy number. All samples were analyzed in triplicate.

2.6. Cytokine quantification

To assess inflammatory cytokine production, cervicovaginal lavage samples collected from the middle third of the vaginal wall were used for the quantification of IL-1β, IL-6, CXCL-8, IL-10, and TNF-α. These cytokines were selected based on their well-established roles in vaginal innate immune responses and bacterial vaginosis-associated inflammation, encompassing key pro-inflammatory mediators involved in epithelial activation and immune cell recruitment (IL-1β, IL-6, CXCL-8, and TNF-α), as well as the anti- inflammatory/regulatory cytokine IL-10, which contributes to immune homeostasis in the vaginal microenvironment.

Prior to analysis, samples were centrifuged to remove cellular debris, and supernatants were aliquoted and stored at −80 ◦C until use. Cytokine concentrations were measured using commercially available DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's protocols. Briefly, 96-well microplates were coated overnight at room temperature with capture antibodies diluted in PBS, washed, and blocked for 1 h. Subsequently, 100 µL of samples or standards was added to each well and incubated for 4 h at room temperature. After washing, detection antibodies were added and incubated for 2 h, followed by incubation with streptavidin–HRP for 20 min. Color development was achieved using substrate solution, and the reaction was stopped after 20 min. Absorbance values were measured at 492 nm using an automated ELISA plate reader (BioTek Instruments Inc., Winooski, VT, USA). The limits of detection for IL-1β, IL-6, CXCL-8, IL-10, and TNF-α were 3.9 pg/mL, 9.4 pg/mL, 15.6 pg/mL, 15.6 pg/mL, and 15.6 pg/mL, respectively.

2.7. Statistical analysis

Statistical analyses were performed using R software (version 2025.09.0-387). Bacterial loads of *Gardnerella* spp., *F. vaginae*, and the combined bacterial burden (*Gardnerella* spp. + *F. vaginae*) were log-transformed due to data skewness and differences in orders of magnitude across measurements. For descriptive analyses of vaginal microbiota groups (normal microbiota, intermediate microbiota and BV), median

values and interquartile ranges (25th–75th percentiles) were calculated. Zero values were excluded from log-transformed analyses, as logarithmic transformation is not applicable to zero. Comparisons among microbiota groups were performed using the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction. A significance level of $p < 0.05$ was adopted.

Correlations between cytokine levels (IL-1β, IL-6, CXCL-8, IL-10, and TNF-α) and bacterial variables (*Gardnerella* spp., *F. vaginae*, and *Gardnerella* spp. + *F. vaginae*) were assessed using Spearman's rank correlation coefficient (ρ), given the non-parametric distribution of the data. Scatter plots were generated to illustrate the relationships between bacterial load and cervicovaginal cytokine levels, with each point representing an individual sample. Bacterial loads are expressed as $\log_{10}$-transformed DNA copy numbers, while cytokine concentrations are also presented on a $\log_{10}$ scale to account for non-normal data distribution. Samples are color-coded according to vaginal microbiota classification (normal microbiota, intermediate microbiota, and bacterial vaginosis), allowing visualization of microbiota-specific patterns.

Associations between bacterial variables and cytokine levels were evaluated using Spearman's rank correlation coefficient, which was selected due to the non-parametric distribution of both bacterial load and cytokine data. Correlation strength was interpreted based on Spearman's ρ values, and statistical significance was defined as $p < 0.05$.

3. Results

3.1. Sociodemographic characteristics

The sociodemographic characteristics of the 152 study participants are presented in Table 1. The median ages were 37 years (22–54) in women with normal microbiota, 39 years (19–55) with intermediate microbiota, and 41 years (20–58) with bacterial vaginosis. Most participants self-identified as white, regardless of the microbiota profile. Among intermediate microbiota cases, married women predominated, whereas a higher proportion of single women was observed in the BV group. Most patients did not report vaginal discharge, odor, or pruritus, irrespective of the microscopic diagnosis of normal, intermediate microbiota or BV. A subset of participants did not provide information on symptoms, corresponding to 24.3% for vaginal discharge, 40.8% for odor, and 33.5% for pruritus.

Tabela 1. Sociodemographic and clinical characteristics of the study participants. Distribution of participants according to age, ethnicity, marital status, and symptoms, based on the microscopic classification of the vaginal microbiota (normal microbiota, intermediate microbiota, and bacterial vaginosis).

Variables	Normal microbiota (n=68)	Intermediate microbiota (n=24)	Bacterial vaginosis (n=60)
Age	37 (22-54)	39 (19-55)	41 (20-58)
Ethnicity *			
White	44 (63.2%)	12 (50.0%)	32 (53.3%)
Black	2 (2.9%)	1 (4.2%)	7 (11.7%)
Others	5 (7.4%)	4 (16.7%)	2 (3.3%)
Marital status**			
Single	25 (36.8%)	5 (20.8%)	23 (38.3%)
Married	25 (36.8%)	12 (50.0%)	18 (30.0%)
Vaginal discharge			
Yes	11 (16.2%)	9 (37.5%)	9 (15.0%)
No	40 (62.5%)	9 (37.5%)	37 (61.7%)
Vaginal odor			
Yes	4 (6.3%)	2 (8.3%)	6 (10.0%)
No	35 (51.5%)	13 (54.2%)	30 (50.0%)
Vaginal pruritus			
Yes	3 (4.4%)	2 (8.3%)	4 (6.7%)
No	40 (62.5%)	15 (62.5%)	37 (61.7%)

** Data available for 109 patients; ** Data available for 108 patients. Age values are presented as median (range).

3.2. Quantitative profile of bacterial species associated with BV.

Among the 152 participants included in the study, 95 (62.5%) tested positive for *Gardnerella* spp. Of these, 35 were classified as normal microbiota (36.8%), 14 as intermediate microbiota (14.7%), and 46 as BV (48.4%). In normal microbiota cases, the median *Gardnerella* spp. bacterial load was approximately 3.4 copies/µL (1.8–6.7), whereas in BV cases, the median was significantly higher at 7.2 copies/µL (6.1–8.7) ($p < 0.001$). In intermediate microbiota, the median load was 6.2 copies/µL (3.4–8.6), with no statistically significant difference compared to normal microbiota [3.4 copies/µL (1.8–6.7)] or BV [7.2 copies/µL (6.1–8.7)] ($p > 0.05$) (Figure 1).

Regarding *F. vaginae*, the species was detected in 106 (69.7%) vaginal samples. Of these, 51 (48.1%) belonged to normal microbiota, 15 (14.2%) to intermediate microbiota, and 40 (37.7%) to BV. Considering the vaginal microbiota profiles, 75.0% (51/68) of normal microbiota cases were positive for *F. vaginae*, compared with 63.0% (15/24) of intermediate microbiota cases and 66.7% (40/60) of BV cases. In normal microbiota, the median bacterial load was 4.4 copies/µL (3.9–5.1), which was significantly lower than in BV [6.5 copies/µL (5.5–7.5); $p < 0.001$]. Intermediate microbiota cases showed a median of 4.5 copies/µL (3.7–5.4), with no significant difference compared to

eubiosis, but with significantly lower values than those observed in BV ($p < 0.001$) (Figure 1).

Considering the synergistic interaction between *Gardnerella* spp. and *F. vaginae* in the formation of the characteristic BV biofilm, the combined bacterial loads of both species were also evaluated. A total of 74 (48.7%) women showed simultaneous positivity, of whom 28 (37.8%) were classified as normal microbiota, 10 (13.5%) as intermediate microbiota, and 36 (48.7%) as BV. The median combined loads were 4.6 copies/µL (4.2–6.5) for normal microbiota, 5.2 copies/µL (4.1–6.4) for intermediate microbiota, and 7.5 copies/µL (6.5–8.8) for BV. The combined bacterial loads followed the same trend observed for *F. vaginae* alone, with significantly higher values associated with dysbiosis compared to eubiosis ($p < 0.001$) and intermediate microbiota ($p = 0.006$). No statistically significant difference was observed between normal and intermediate microbiota (Figure 1).

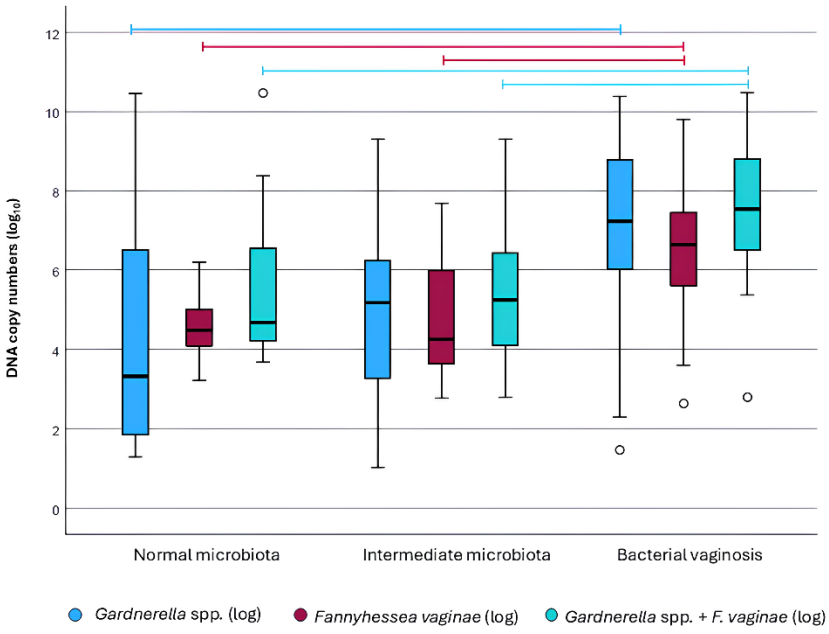

Figure 1. Bacterial load of *Gardnerella* spp., *Fannyhessea vaginae*, and their combined abundance across normal microbiota, intermediate microbiota, and bacterial vaginosis (BV) groups. Bacterial loads were quantified by absolute qPCR and are expressed as DNA copy numbers (log_{10}). Boxplots represent the median and interquartile range. Individual data points falling outside the whisker range are shown as small white circles and represent statistical outliers. Comparisons between microbiota groups were performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Horizontal bars indicate statistically significant differences between groups ($p < 0.05$). Blue bars indicate statistical differences in *Gardnerella* spp. load, burgundy bars indicate differences in *F. vaginae* load, and turquoise bars represent combined *Gardnerella* spp. + *F. vaginae* bacterial load.

3.3. Inflammatory cytokines associated with the vaginal microbiota profile

To investigate whether variations in bacterial load are associated with modulation of the local immune response, we quantified a panel of cytokines representative of key inflammatory pathways in the vaginal microenvironment, including pro-inflammatory mediators involved in epithelial activation and immune cell recruitment (IL-1β, IL-6, CXCL-8, and TNF-α), as well as the regulatory cytokine IL-10, which contributes to immune homeostasis. IL-1β levels were significantly higher in samples from patients diagnosed with intermediate microbiota [2.3 pg/mL (1.8–2.7); p = 0.011] and BV [2.1 pg/mL (1.6–2.5); p = 0.024] compared with women classified as normal microbiota [1.7 pg/mL (1.3–2.3)]. No statistically significant differences were observed between the intermediate microbiota [2.3 pg/mL (1.8–2.7)] and BV [2.1 pg/mL (1.6–2.5)] groups (p = 0.408) (Figure 2A).

CXCL-8 levels also differed significantly, being higher in the intermediate microbiota group [3.3 pg/mL (3.1–3.6)] compared with the normal microbiota group [3.1 pg/mL (2.7–3.5)] (p = 0.021). However, no significant differences were observed between normal microbiota [3.1 pg/mL (2.7–3.5)] and BV [3.2 pg/mL (2.8–3.4)] (p = 0.442), nor between intermediate microbiota [3.3 pg/mL (3.1–3.6)] and BV [3.2 pg/mL (2.8–3.4)] (p = 0.075) (Figure 2B). The other cytokines analyzed—IL-6, IL-10, and TNF-α—did not show significant differences among the vaginal microbiota patterns defined according to the criteria of Nugent [11] (Figure 2C–E).

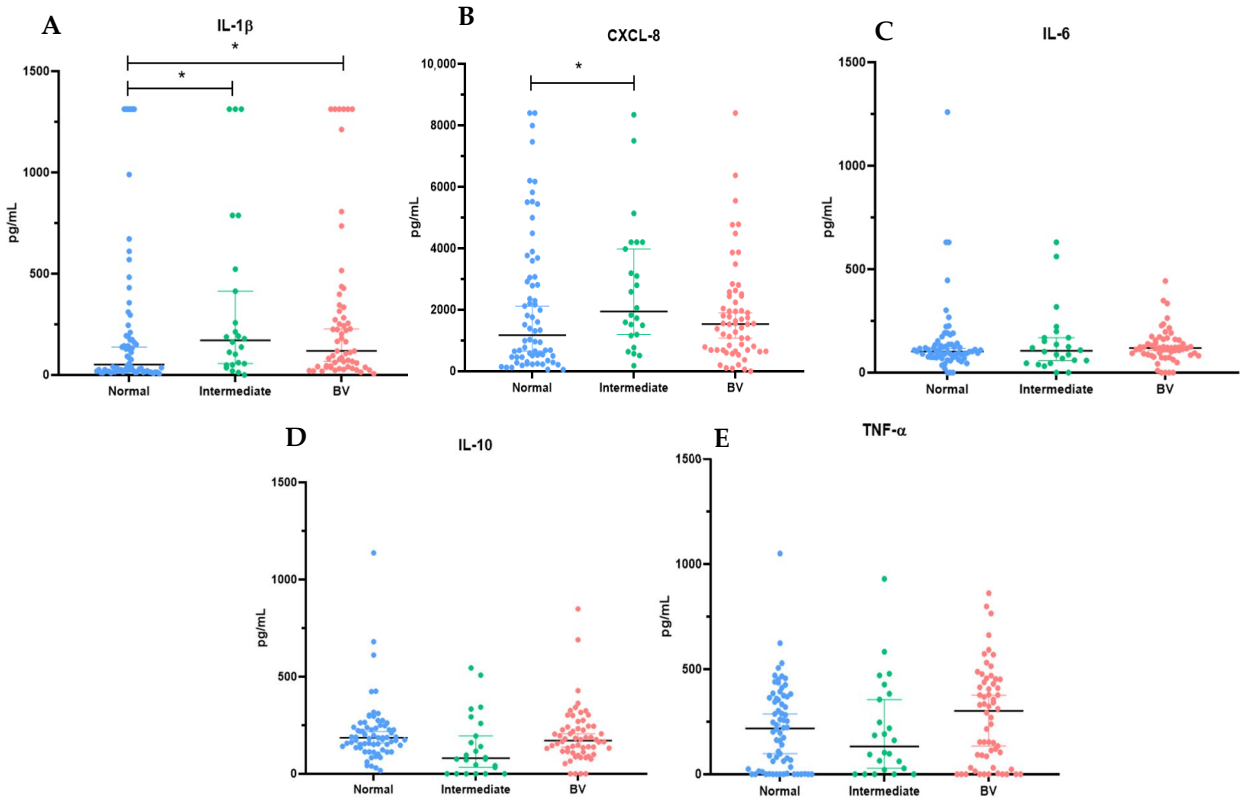

Figure 2. Quantitative representation of cytokine levels in cervicovaginal lavages from women according to the vaginal microbiota profile classified as Normal microbiota, Intermediate microbiota, and bacterial vaginosis (BV). Concentrations of **(A)** IL-1β (*p=0,011; p=0,024), **(B)** IL-6, **(C)** CXCL-8 (*p=0,021), **(D)** IL-10, and **(E)** TNF-α (pg/mL). Bars represent the median with dispersion of individual absolute values. * indicates statistical significance

After the individual evaluation of bacterial and immunological variables, multivariate analyses were performed to investigate the association between *Gardnerella* spp. and *F. vaginae* loads and inflammatory cytokine levels, while simultaneously considering the clinical classification of the vaginal microbiota (normal, intermediate and BV). In the adjusted model, both *F. vaginae* load ($p = 0.007$) and clinical microbiota classification ($p = 0.001$) remained associated with IL-1β levels, indicating that progressively more dysbiotic profiles exhibited higher *F. vaginae* loads and increased levels of this cytokine. In contrast, *Gardnerella* spp. load lost statistical significance in the adjusted model ($p = 0.079$) (Figure 3A–C). These findings suggest that the progression of vaginal dysbiosis—from normal microbiota to intermediate microbiota and to bacterial vaginosis—is associated with a concomitant increase in *F. vaginae* load and in the molecular inflammatory response, as reflected by higher IL-1β levels. Regarding CXCL-8, only the clinical microbiota classification remained associated with the levels of this chemokine ($p = 0.004$), whereas *Gardnerella* spp. and *F. vaginae* loads did not show independent associations ($p = 0.058$ and $p = 0.516$, respectively) (Figure 4A–C).

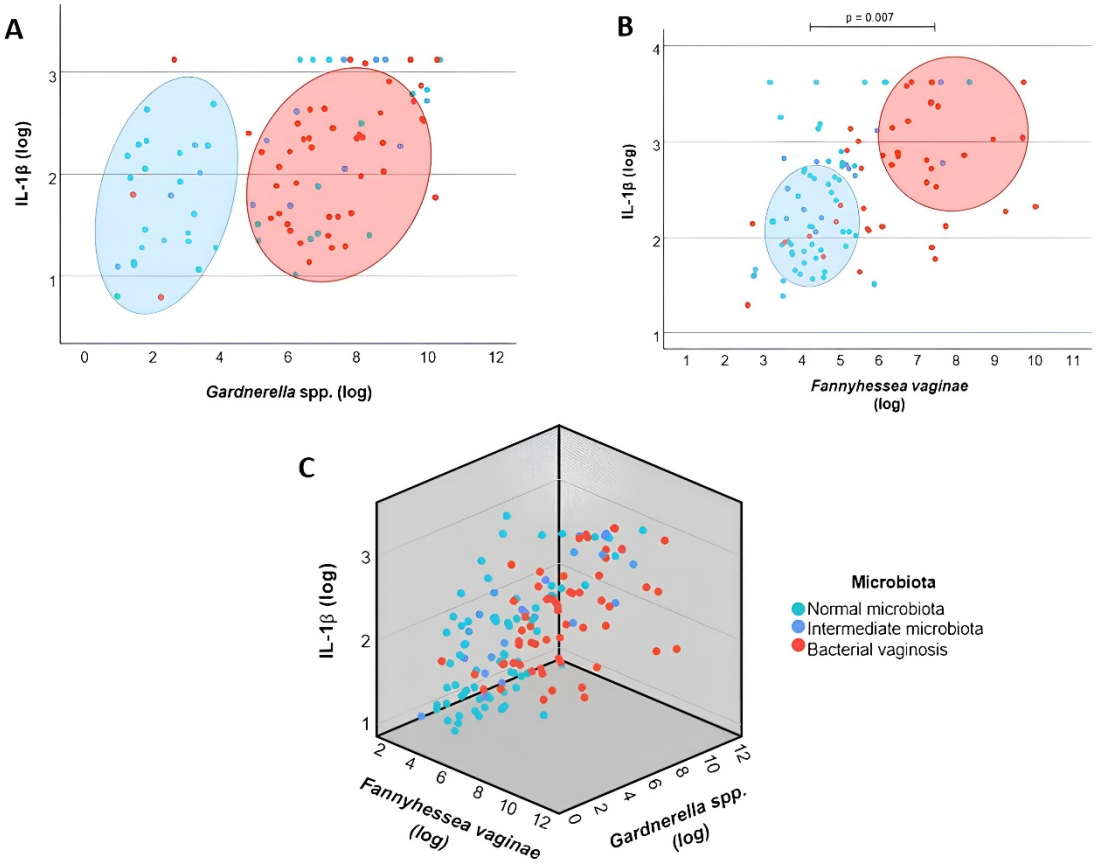

Figure 3. Two-dimensional (2D) and three-dimensional (3D) scatter plot representations showing the relationship between bacterial loads and IL-1β levels in cervicovaginal lavages. (**A**) Association between *Gardnerella* spp. bacterial load and IL-1β levels. (**B**) Association between *Fannyhessea vaginae* bacterial load and IL-1β levels. (**C**) Three-dimensional scatter plot illustrating the combined relationship between *Gardnerella* spp. and *F. vaginae* loads and IL-1β levels. Bacterial loads and cytokine concentrations are expressed as $\log_{10}$-transformed values. Green dots represent women with normal microbiota, blue dots represent intermediate microbiota, and red dots represent bacterial vaginosis.

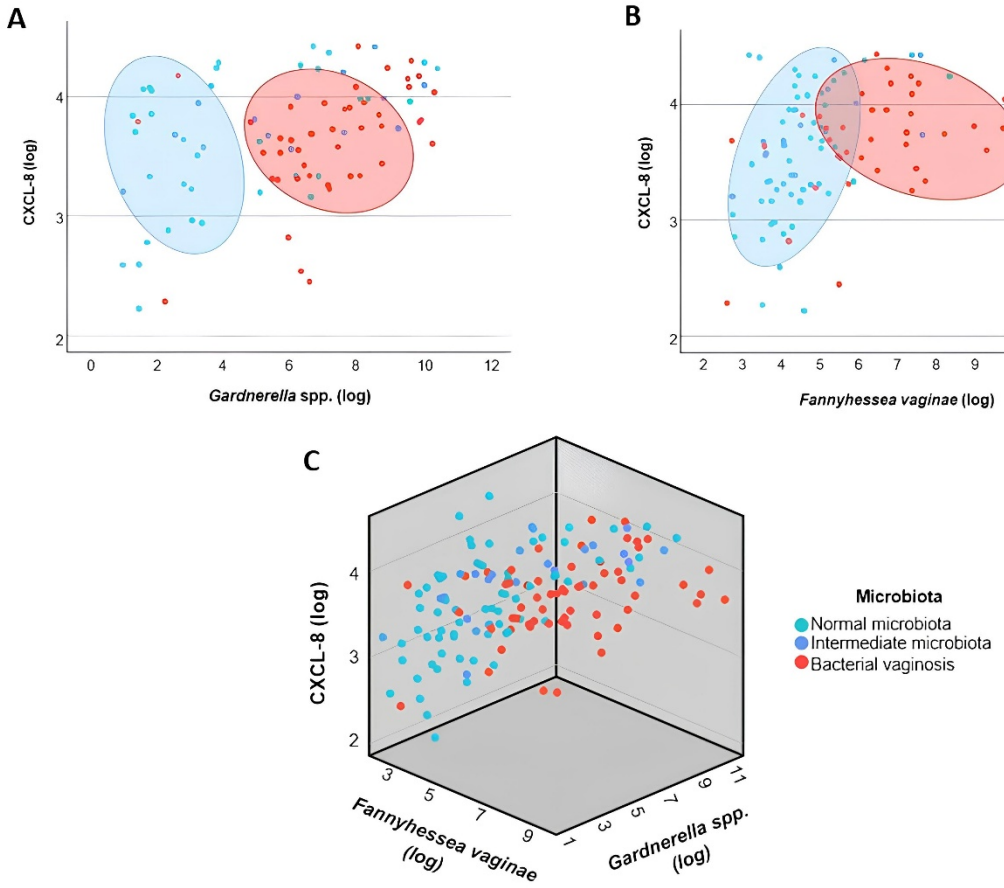

Figure 4. Two-dimensional (2D) and three-dimensional (3D) scatter plot representations showing the relationship between bacterial loads and CXCL-8 levels in cervicovaginal lavages. (**A**) Association between *Gardnerella* spp. bacterial load and CXCL-8 levels. (**B**) Association between *Fannyhessea vaginae* bacterial load and CXCL-8 levels. (**C**) Three-dimensional scatter plot illustrating the combined relationship between *Gardnerella* spp. and *F. vaginae* loads and CXCL-8 levels. Bacterial loads and cytokine concentrations are expressed as $\log_{10}$-transformed values. Green dots represent women with normal microbiota, blue dots represent intermediate microbiota, and red dots represent bacterial vaginosis.

4. Discussion

Bacterial vaginosis (BV), the main dysbiotic condition of the female lower genital tract, is characterized not only by a reduction in *Lactobacillus* spp. but also by an increase in anaerobic bacterial species, particularly *G. vaginalis* and *F. vaginae*. Although the presence of these species in BV is well documented, gaps remain regarding the impact of their bacterial loads and their relationship with modulation of the inflammatory response in the vaginal microenvironment. The present study aimed to address this gap by evaluating the quantitative profiles of *Gardnerella* spp. and *F. vaginae* in women with different vaginal microbiota patterns, as well as their association with inflammatory mediators.

Our main findings reaffirm that both species are present across different vaginal microbiota profiles, indicating that their presence alone does not define a dysbiotic state, but rather their abundance. In this context, bacterial loads—particularly of *F. vaginae*—

were significantly higher in the BV group, as was the combined load of both species, suggesting that the quantitative increase in these microorganisms is associated with dysbiosis progression. From an inflammatory perspective, a selective increase in IL-1β was observed in the intermediate microbiota and BV groups, and *F. vaginae* load remained associated with this elevation.

The detection of *Gardnerella* spp. in *Lactobacillus*-dominated microbiota is not unexpected and has been widely reported in the literature, reinforcing that the presence of this species alone is insufficient to characterize vaginal dysbiosis [29,30]. Microbiota classification-based studies, such as the VALENCIA model, have demonstrated that *Gardnerella* spp. can be detected across different CSTs, with varying abundance even in profiles considered eubiotic [31]. In that study, *Gardnerella* spp. were present at moderate abundance in CST IV-A and at high abundance in CST IV-B. In the present study, although *Gardnerella* spp. was detected across all vaginal microbiota profiles, its absolute bacterial load was significantly higher in BV compared with normal microbiota, supporting its role as a central component of dysbiosis. This finding is consistent with previous quantitative studies reporting elevated *Gardnerella* spp. loads in BV and low bacterial burdens in *Lactobacillus*-dominated microbiota [32–34]. In contrast, data regarding absolute *Gardnerella* spp. load in intermediate microbiota remain limited in the literature, as most studies focus primarily on binary comparisons between normal microbiota and BV.

However, no statistically significant difference was observed between BV and intermediate microbiota. This finding may reflect the heterogeneous nature of intermediate microbiota, which is often considered a transitional state between eubiosis and dysbiosis. A systematic review analyzing molecular characterization studies of the vaginal microbiota reported similar results, showing no significant difference in *G. vaginalis* bacterial load between intermediate microbiota and BV in most studies [34]. Another possible explanation for this finding relates to a limitation of the present study, namely the smaller sample size of the intermediate microbiota group in our cohort, as the number of intermediate microbiota cases was less than half that of normal microbiota and BV cases.

Regarding *F. vaginae*, our results showed that its bacterial load was similar between normal and intermediate microbiota, but significantly different from both when compared with BV. The higher load observed in BV suggests that this species is more strongly associated with established vaginal dysbiosis than with intermediate stages of microbial transition. In the literature, findings regarding the presence of *F. vaginae* in women without BV are heterogeneous [20,35,36]. While some studies report low prevalence of this species in *Lactobacillus*-dominated microbiota [35,36], others have

identified it at higher proportions [20]. This variability has been attributed to methodological differences, population characteristics, and analytical sensitivity of the tests employed. Nevertheless, the consistent increase in its load in BV cases reinforces its role as an important microbial marker of vaginal dysbiosis.

Our results are also consistent with previous studies showing substantially higher loads of *Gardnerella* spp. in association with *F. vaginae* in dysbiotic microbiota, as well as demonstrating that the coexistence of these species at high concentrations increases the predictive value for BV diagnosis [17,29,31,36–38]. Santos-Greatti et al. [39] and Marconi et al. [28] reported similar findings, observing higher bacterial loads in women with BV and reinforcing the role of these species as central components of the polymicrobial core associated with dysbiosis.

Importantly, unlike most studies available in the literature, the present work included the evaluation of intermediate microbiota, which is frequently excluded from analyses. Our findings indicate that *Gardnerella* spp. does not reach significantly elevated levels during transitional stages of the microbiota, whereas *F. vaginae* appears to be more closely associated with established dysbiosis, although its load begins to increase already in intermediate microbiota. This pattern suggests that intermediate microbiota presents intermediate bacterial loads of core BV-associated species, reinforcing the hypothesis that it represents a transitional state between eubiosis and dysbiosis and highlighting the need for greater attention to this profile in future studies.

In addition, the composition of the vaginal microbiota plays a decisive role in maintaining ecological stability and the physiological balance of the vaginal microenvironment [1,2]. This ecosystem directly influences epithelial barrier integrity and the modulation of the local immune response. Disruptions in this balance may favor the proliferation of dysbiosis-associated species, including those of interest in the present study. Accumulating evidence indicates that *G. vaginalis* and *F. vaginae* contribute to the organization of structured biofilms and to the modulation of the vaginal immune response, playing a central role in the establishment and persistence of BV [7,8].

G. vaginalis exhibits a range of virulence factors that promote colonization, immune evasion, and biofilm formation, notably sialidase A—which degrades components of the vaginal epithelial barrier—and vaginolysin, a cholesterol-dependent cytolysin responsible for pore formation in epithelial cells, facilitating cell lysis [14,22]. These mechanisms compromise epithelial integrity and create a microenvironment favorable to bacterial adhesion and colonization by other BV-associated species. *F. vaginae*, in turn, typically occurs in association with *G. vaginalis* and shows a high capacity for biofilm integration, contributing to its structural stability and resistance to antimicrobial treatment

[21]. The synergistic interaction between these species promotes the persistence of dysbiosis and is associated with the maintenance of a non-quiescent environment characteristic of BV. Thus, bacterial virulence factors, combined with biofilm organization, enable both modulation and evasion of the local immune response, sustaining persistent colonization.

Although BV is classically described as a clinically non-inflammatory condition, consistent evidence demonstrates that it is associated with a state of molecular inflammation, primarily characterized by increased levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and the pro-inflammatory CXC chemokine CXCL-8, a key mediator of neutrophil recruitment [16,22]. In the present study, IL-1β levels were significantly higher in intermediate microbiota and BV compared with normal microbiota, indicating that inflammatory activation associated with this cytokine begins in the transitional microbiota and persists in established dysbiosis.

Similar findings were reported by Santos-Greatti et al. [39], who also observed increased IL-1β levels in women with intermediate microbiota compared with those with *Lactobacillus*-dominated microbiota. These results suggest that intermediate microbiota represents a heterogeneous and transitional state, rather than merely an undefined profile, and highlight the importance of integrating quantitative bacterial measures with immuno- logical parameters to better understand BV pathogenesis. Longitudinal studies have shown that alterations in cervical innate immune mechanisms may precede the establishment of vaginal dysbiosis, suggesting that the immune response actively participates in the microbial transition process [40]. This provides biological support for the elevated IL-1β levels observed in intermediate microbiota in our study, indicating that inflammatory activation may occur before clinically established BV and that immunological changes may accompany—or even precede—the microbial shifts observed during progression to BV.

When multivariate analyses were performed considering both the microscopic classification of the vaginal microbiota and the bacterial loads of the species evaluated, IL-1β levels remained associated with the presence of *F. vaginae*, independently of the microbiota profile. This finding suggests that, among the species assessed, *F. vaginae* has a closer relationship with the activation of the local IL-1β inflammatory response than *Gardnerella* spp., reinforcing its potential role as a modulator of vaginal inflammation during dysbiosis progression.

From a mechanistic perspective, experimental studies have demonstrated that *F. vaginae* is capable of inducing innate immune responses in vaginal epithelial cells, promoting the production of pro-inflammatory cytokines and antimicrobial peptides [41,42].

Moreover, this species shows a high capacity for integration into the BV-associated biofilm, contributing to its structural stability and persistence [21,43]. These factors help explain why the presence—and especially the increased abundance—of *F. vaginae* is associated with elevated IL-1β levels.

With respect to inflammatory chemokines, CXCL-8 showed significant variation among the vaginal microbiota patterns analyzed. Its levels were higher in intermediate microbiota compared with normal microbiota, while no statistically significant differences were observed between BV and normal microbiota or intermediate microbiota. This pattern suggests that increased CXCL-8 may be associated with a transient state of vaginal microbiota imbalance, characteristic of intermediate microbiota, rather than with fully established dysbiosis. The absence of CXCL-8 elevation in BV may reflect immunomodulatory mechanisms associated with biofilm organization and inflammatory evasion by bacteria predominant in this fully dysbiotic state. Given that CXCL-8 is a potent neutrophil chemoattractant and that BV is classically not associated with neutrophilic chemotaxis, unchanged levels of this chemokine in BV are expected.

In contrast, intermediate microbiota states may be associated with a distinct inflammatory profile characterized by greater activation of the innate immune response. Previous studies indicate that CXCL-8 production varies according to vaginal bacterial composition, rather than solely based on the clinical diagnosis of BV [44,45]. Moreover, different bacterial species have been shown to correlate differently with local CXCL-8 levels [44]. As CXCL-8 exhibits a negative correlation with *Lactobacillus* abundance [46], the increase observed in intermediate microbiota may reflect a partial reduction in these protective bacteria, without yet reaching the more immunologically silent profile typical of BV.

When CXCL-8 was analyzed using multivariate models that simultaneously considered vaginal microbiota diagnosis and bacterial loads, only the microbiota profile remained associated with its levels, while the presence of *Gardnerella* spp. or *F. vaginae* showed no independent association. This suggests that CXCL-8 production is linked to global characteristics of the microbial community rather than to these two individual taxa specifically. Furthermore, other conditions associated with bacterial compositions distinct from BV, such as aerobic vaginitis, have also been linked to increased CXCL-8 levels [45], reinforcing that this chemokine reflects an inflammation profile dependent on the type of microbiota present.

The other cytokines evaluated in this study did not differ among vaginal microbiota patterns, a result that may be related to the immunomodulatory behavior of biofilm formation. Evidence suggests that *G. vaginalis*, as a primary colonizer, can attenuate

epithelial inflammatory responses during biofilm establishment [23], reducing activation of pathways such as CXCL-8, IL-6, and TNF-α. Studies using three-dimensional epithelial models also show that *G. vaginalis* alone does not robustly induce IL-6, CXCL-8, or TNF-α [47]. Nevertheless, other studies report broader inflammatory responses, including increased IL-1β, IL-6, CXCL-8, TNF-α, RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted), and MIP-1β (Macrophage Inflammatory Protein-1β) in cell cultures exposed to *G. vaginalis* or *F. vaginae* [48,49]. Similarly, clinical studies, such as one conducted in South African women, described elevations in several cytokines (IL-1, IL-6, CXCL-8, TNF-α, and IL-10) in association with BV [50]. These discrepancies may reflect methodological differences, population variability, the influence of co-infections, intra-species diversity (e.g., *Gardnerella* species), or temporal differences between dysbiosis stages.

Finally, this study has some limitations that should be considered when interpreting the results. The sample size of intermediate microbiota cases was numerically smaller than that of normal microbiota and BV cases, which may have reduced the statistical power to detect differences among these profiles. In addition, *Gardnerella* quantification in this study was performed using F-GV1/R-GV3 primer set targeting a region spanning the 16S-23S rRNA intergenic spacer. Although this assay has been widely applied in clinical investigations, it was developed prior to the genome-based taxonomic revision of the genus *Gardenerella*. As ITS-based assays do not reliably discriminate among all currently recognized species within the genus, species-level identification cannot be fully assured. Therefore, the bacterial loads reported herein should be interpreted as representing *Gardnerella* spp. detected by this molecular target, rather than confirmed identification of a specific *Gardnerella* species under current taxonomy. Furthermore, bacterial analysis was restricted to selected taxa and did not include comprehensive characterization of vaginal microbial diversity, limiting the ability to assess the potential contribution of other microbial species to the observed inflammatory responses.

5. Conclusions

Taken together, the findings of this study indicate that progression to the dysbiotic state of BV does not depend solely on the presence of dysbiosis-associated microorganisms, but rather on the quantitative increase in key species—particularly *F. vaginae*—and their interaction with the vaginal inflammatory microenvironment. The early detection of *F. vaginae* in intermediate microbiota, together with elevated IL-1β levels, suggests that inflammatory alterations may begin before the establishment of full-blown BV, reflecting a transitional ecological and inflammatory state. These results reinforce the

role of intermediate microbiota as a biologically active stage rather than merely an undefined profile and highlight the importance of quantitative and immunological approaches for understanding BV pathogenesis. From a clinical perspective, the identification of bacterial and inflammatory markers associated with dysbiosis progression may contribute to more accurate diagnostic strategies and to the development of targeted interventions aimed at preventing gynecological and obstetric complications associated with BV.

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