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Composição taxonômica e potencial funcional do microbioma vaginal e relação com o papilomavírus humano em mulheres brasileiras

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Ciências – Área Patologia.

Orientadora: Profa. Dra. Camila Marcon
Coorientadora: Profa. Dra. Márcia Guimarães da Silva

Botucatu
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ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE RAFAEL BELLETI, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA, DA FACULDADE DE MEDICINA - CÂMPUS DE BOTUCATU.

Aos 23 dias do mês de agosto do ano de 2024, às 09:00 horas, por meio de Videoconferência, realizou-se a defesa de TESE DE DOUTORADO de RAFAEL BELLETI, intitulada **Análise da composição e função da microbiota vaginal e relação com o papilomavírus humano em mulheres brasileiras**. A Comissão Examinadora foi constituida pelos seguintes membros: Profa. Dra. CAMILA MARCON (Orientador(a) - Participação Virtual) do(a) Depto. de Patologia Básica / Universidade Federal do Paraná , Profa. Dra. RITA MAIRA ZANINE (Participação Virtual) do(a) Depto. de Tocoginecologia / Universidade Federal do Paraná , Profa. Dra. ALINE DO NASCIMENTO BOLPET (Participação Virtual) do(a) Universidade Nove de Julho (Uninove) / Bauru, Profa. Dra. MICHELLE GARCIA DISCACCIATI DE CARVALHO (Participação Virtual) do(a) Centro de Atenção Integral à Saúde da Mulher - CAISM/Campinas - Unicamp, Profa. Dra. ANA KATHERINE DA SILVEIRA GONÇALVES (Participação Virtual) do(a) Centro de Ciências da Saúde - Universidade Federal do Rio Grande do Norte. Após a exposição pelo doutorando e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, o discente recebeu o conceito final: APROVADO . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

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Resumo

Introdução: Estudos sobre o microbioma vaginal são fundamentais para entender como este microambiente contribui para a saúde das mulheres. Utilizando dados metagenômicos, como VALENCIA e o classificador de CST metagenômico (mgCST), as recentes classificações dos tipos de comunidade (CSTs) do microbioma vaginal têm fornecido importantes caracterizações do microbioma vaginal. No Brasil, os estudos de composição do microbioma são escassos, mas já foi apontado altas prevalências de CST IV, caracterizado pela depleção de *Lactobacillus* spp.. A CST IV engloba a maioria dos casos de vaginose bacteriana (VB), que é a principal disbiose encontrada na microbiota vaginal. Esta disbiose já foi previamente associada à diversas infecções sexualmente transmissíveis (ISTs), como a persistência do papilomavírus humano de alto risco (hrHPV). No entanto, o mecanismo envolvido nesta associação não está elucidado. *Gardnerella* spp., *Fannyhessea vaginae* e *Prevotella bivia* são apontados como espécies importantes na patogênese da VB. Por isso, o objetivo foi a caracterização taxonômica e potencial funciona do microbioma vaginal e relação com o papilomavírus humano em mulheres brasileiras.

Metodologia: De um total de 609 mulheres em idade reprodutiva incluídas em um estudo anterior que caracterizou o microbioma vaginal com base em sequências de 16S rRNA, 254 amostras vaginais foram selecionadas aleatoriamente para a análise metagenômica atual. Dados sociodemográficos, comportamentais e clínicos foram avaliados através de questionário e as amostras foram analisadas quanto ao pH vaginal, escores de Nugent do conteúdo vaginal e presença de infeccões sexualmente transmissíveis como *Chlamydia trachomatis*, *Neisseria gonorrhoeae* e *Trichomonas vaginalis*. Para a análise de cargas bacterianas envolvidas na VB, foram incluídas 216 mulheres positivas para hrHPV de um estudo anterior que incluía 1638 mulheres em idade reprodutiva. As cargas bacterianas de *Gardnerella* spp., *F. vaginae* e *P. bivia* basais foram realizadas através de *Real Time-PCR* e comparadas entre os grupos de 'persistência' e 'clearance', categorizados com base no status do HPV no recrutamento e no acompanhamento após 12 e 24 meses.

Resultados: As análises revelaram que a CST I, dominado por *L. crispatus*, representou 31.1% das participantes, a *CST III*, dominado por *L. iners*, 31.9%, e a CST IV, uma comunidade com uma diversidade de espécies, 33.8%. *L. crispatus* mgCST 1 foi a mais frequente (23.4%), seguido por *L. iners* mgCST 12 (15.0%) e *G. vaginalis* mgCST 20 (8.7%) e 24 (9.0%). As mgCSTs de *G. vaginalis* foram tiveram

maior frequência de maior número de parceiros sexuais/ano, aumento do pH vaginal e escores de Nugent mais elevados, sugerindo que fatores comportamentais e clínicos poderiam estar associados a essas mgCSTs.

Gardnerella spp. exibiu cargas significativamente maiores no grupo persistência (3.20E05 cópias/µL) em comparação ao grupo de eliminação (2.15E04 cópias/µL), indicando seu potencial efeito negativo na eliminação do hrHPV após 12 meses. No entanto, *F. vaginae* e *P. bivia* não mostraram associação significativa com a persistência do hrHPV.

Conclusão: O estudo fornece uma nova caracterização metagenômica do microbioma vaginal brasileiro e mostra que componentes microbianos e comportamentais podem influenciar tanto a manutenção do ambiente vaginal saudável, como também a persistência de infecções por HPV de alto risco.

Palavras-chave: Microbioma; microbiota vaginal; CST; metagenômica; papilomavírus humano; *Gardnerella*; *Fannyhessea vaginae*; *Prevotella bivia*.

Abstract

Introduction: Studies on the vaginal microbiome are essential for understanding how this microenvironment contributes to women's health. Utilizing metagenomic data, such as VALENCIA and the metagenomic CST classifier (mgCST), recent classifications of the community-state types (CSTs) of the vaginal microbiome have provided important characterizations of the vaginal microbiota. In Brazil, studies on microbiome composition are scarce, but high prevalence rates of CST IV, characterized by the depletion of *Lactobacillus* spp., have already been reported. CST IV encompasses the majority of bacterial vaginosis (BV) cases, which is the main dysbiosis found in the vaginal microbiota. This dysbiosis has been previously associated with several sexually transmitted infections (STIs), such as the persistence of high-risk human papillomavirus (hrHPV). However, the mechanism involved in this association is not yet elucidated. *Gardnerella* spp., *Fannyhessea vaginae*, and *Prevotella bivia* are pointed out as important species in the pathogenesis of BV. Therefore, the first objective was to characterize the vaginal microbiome with different metagenomic approaches (VALENCIA and the mgCST classifier) among Brazilian women and to investigate sociodemographic, behavioral, and clinical variables associated with communities dominated by *L. crispatus*, *L. iners*, and *Gardnerella vaginalis*. Secondly, to compare the cervicovaginal loads of *Gardnerella* spp., *F. vaginae*, and *P. bivia* between women with persistent hrHPV infection and those who cleared the infection after 12 and 24 months.

Methods: From a total of 609 reproductive-aged women included in a previous study that characterized the vaginal microbiome based on 16S rRNA sequences, 254 vaginal samples were randomly selected for the current metagenomic analysis. Sociodemographic, behavioral, and clinical data were evaluated through a questionnaire, and the samples were analyzed for vaginal pH, Nugent scores of vaginal content, and the presence of sexually transmitted infections such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*. For the analysis of bacterial loads involved in BV, 216 hrHPV-positive women from a previous study that included 1638 reproductive-aged women were included. Basal bacterial loads of *Gardnerella* spp., *F. vaginae*, and *P. bivia* were performed using Real Time-PCR and compared between the 'persistence' and 'clearance' groups, categorized based on HPV status at enrollment and follow-up after 12 and 24 months.

Results: The analyses revealed that CST I, dominated by *L. crispatus*, represented

31.1% of the participants, CST III, dominated by *L. iners*, 31.9%, and CST IV, a community with a diversity of species, 33.8%. *L. crispatus* mgCST 1 was the most frequent (23.4%), followed by *L. iners* mgCST 12 (15.0%) and *G. vaginalis* mgCST 20 (8.7%) and 24 (9.0%). *G. vaginalis* mgCSTs were associated with a higher frequency of multiple sexual partners per year, increased vaginal pH, and higher Nugent scores, suggesting that behavioral and clinical factors could be associated with these mgCSTs. *Gardnerella* spp. exhibited significantly higher loads in the persistence group (3.20E05 copies/μL) compared to the clearance group (2.15E04 copies/μL), indicating its potential negative effect on the clearance of hrHPV after 12 months. However, *F. vaginae* and *P. bivia* did not show a significant association with hrHPV persistence.

Conclusion: This study provides a new metagenomic characterization of the Brazilian vaginal microbiome and shows that microbial and behavioral components can influence both the maintenance of a healthy vaginal environment and the persistence of high-risk HPV infections.

Keywords: Microbiome; vaginal microbiota; CST; metagenomics; human papillomavirus; *Gardnerella*; *Fannyhessea vaginae*; *Prevotella bivia*.

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Capítulo I

Revisão de Literatura

1. Classificação do microbioma vaginal

O microbioma é uma comunidade microbiana em um ambiente com características físico-química, imunológicas, estresse oxidativo específicas, que considera não só a composição taxonômica como também seu potencial funcional (1). Nesse contexto, a saúde reprodutiva das mulheres é afetada pela composição e estabilidade do microbioma vaginal, influenciado por diversas variáveis como hormônios, gestação e fertilidade (2–5). A exemplo disso, estudos mostram que o microbioma vaginal muda significativamente ao longo da gravidez, e fatores hormonais, como o estrogênio, têm impacto na sua composição (3,6). Além disso, há evidências que associam o microbioma ao parto pré-termo, onde composições microbianas específicas foram associadas a um maior risco de parto pré-termo (6–8). Por isso, o equilíbrio do microambiente vaginal é importante para proteger contra aquisição de infecções e enfermidades, tornando-se um aspecto importante da saúde feminina.

Inicialmente, eram utilizadas culturas microbiológicas convencionais para analisar a microbiota vaginal, o que limitava o conhecimento de apenas algumas espécies bacterianas. A partir da evolução dos métodos moleculares, permitiu-se uma melhor determinação do microbioma local usando sequenciamento de nova geração, principalmente do gene codificante do RNA ribossômico 16S. Nesse sentido, Ravel et al.(9), em 2011, caracterizaram pela primeira vez o microbioma vaginal de mulheres em idade reprodutiva num estudo com mulheres americanas em idade reprodutiva de diferentes etnias. Tais autores verificaram que, apesar da grande diversidade microbiana presente nesse ambiente, o microbioma vaginal de todas as mulheres podem ser agrupados em 5 tipos de comunidades bacterianas vaginais (*community-state type*, CST), conforme predominância de determinadas espécies. Do total, quatro são caracterizadas pelo predomínio de *Lactobacillus* spp.: CST I (*Lactobacillus crispatus*), CST II (*L. gasseri*), CST III (*L. iners*) e CST V (*L. jensenii*). Na comunidade bacteriana vaginal remanescente, a CST IV, a predominância de espécies de *Lactobacillus* é substituída por uma maior diversidade de espécies bacterianas, onde se encontra a maioria dos casos de vaginose bacteriana (VB).

No Brasil, o microbioma vaginal foi caracterizado a partir do sequenciamento do de porções hipervariáveis do rRNA 16S em um estudo transversal com 609 mulheres das cinco macroregiões do Brasil. As comunidades foram alocadas em CST I (*L. crispatus*), CST II (*L. gasseri*), CST III-A (maior abundância de *L. iners*), CST III-B

(menor abundância de *L. iners*) e CST IV (diversas espécies bacterianas). Nessa população, a comunidade mais frequente encontrada foi a CST III (36,5%), seguida da CST I (30,5%) e CST IV (27,4%) (10).

Em 2020, uma nova classificação de de CSTs foi desenvolvida com base em centroides de referência que permitiram uma maior acurácia na classificação das comunidades bacterianas, usando o VALENCIA (*VA*gina*L* community state typ*E* *N*earest *C*entro*I*d cl*A*ssifier) (11). O centróide referência usado para isso foi baseado em 13.160 perfis de microbiota vaginal, que incluem representações de todas as CSTs previamente identificadas. Sete CSTs foram identificadas, sendo que quatro delas tinham uma alta abundância relativa de espécies de *Lactobacillus*. Essas sete CSTs foram subdivididos em treze sub-CSTs, com base em sua composição microbiana. As CSTs foram nomeadas de acordo com a classificação anterior, sendo CST I dominada por *L. crispatus*, CST II dominada por *L. gasseri*, CST III dominada por *L. iners*, e CST V dominada por *L. jensenii*. Além disso, foram identificadas três CSTs que não apresentavam uma alta abundância relativa de *Lactobacillus*, denominadas CST IV-A, IV-B e IV-C. A CST IV-A apresentava uma alta abundância relativa de *Candidatus* Lachnocurva vaginae (antes nomeada como BVAB1) (12) e uma abundância moderada de *G. vaginalis*, enquanto a IV-B tinha alta abundância relativa de *G. vaginalis* e baixa abundância relativa de *Ca.* L. vaginae. A CST IV-C, por sua vez, era caracterizado por uma baixa abundância relativa de *Lactobacillus* spp., *G. vaginalis*, a anteriormente conhecida *A. vaginae* e *Ca.* L. vaginae, sendo composta por uma variedade diversa de bactérias facultativas e estritamente anaeróbias. A CST IV-C foi ainda subdividida em cinco sub-CSTs, cada um dominado por diferentes tipos de bactérias: CST IV-C0 (*Prevotella* spp.), CST IV-C01 (*Streptococcus* spp.), CST IV-C02 (*Enterococcus* spp.), CST IV-C03 (*Bifidobacterium* spp.) e CST IV-C04 (*Staphylococcus* spp.). Até então, a distinção dessa nova classificação residia no fato de que a caracterização das CSTs se baseava na diversidade de espécies e na abundância bacteriana dentro de cada população estudada. Por isso, a comparação dos dados de sequenciamento usando os centroides possibilita uma avaliação mais precisa entre populações distintas.

Similar a CSTs, alguns autores recentemente descreveram o termo *vagitypes* na classificação do microbioma onde se consideram as espécies bacterianas com uma abundância relativa superior a 30%, podendo ter mais de duas espécies bacterianas dominantes em uma amostra. As *vagitypes* então são compostas pelas espécies

bacterianas mais dominantes e funcionais dentro do microbioma vaginal (13). Esta classificação tem sido aplicada tanto em mulheres não grávidas quanto grávidas, demonstrando sua relevância em diferentes estados fisiológicos (14,15). O uso de *vagitypes* propõe-se como uma caracterização dos microbiomas vaginais de diversos grupos raciais e étnicos, destacando a importância de considerar variações específicas da população na composição microbiana (6,16,17).

Adicionalmente, os avanços dos estudos com metagenômica, caracterizada pela análise genômica de uma população de microrganismos que permite identificar e avaliar comunidades microbianas em diferentes ecossistemas, permitiram entender não só a composição como também o potencial funcional das espécies bacterianas que integram as CSTs. Nesse contexto, foi desenvolvido o VIRGO (*non-redundant gene catalog for the microbial communities that inhabit the human vagina*) que é um catálogo de genes não redundantes para as comunidades microbianas do ambiente vaginal (18). Ele foi construído usando uma combinação de sequências metagenômicas e genomas isolados urogenitais. O VIRGO pode ser utilizado para caracterizar a composição taxonômica e/ou funcional de conjuntos de dados metagenômicos e metatranscriptômicos vaginais.

A partir disso, recentemente foram descritas 27 mgCSTs *(metagenomic community-state types*) com base no VIRGO onde cada uma é definida por uma subespécie bacteriana diferente (mgSs), sendo que essa subdivisão ocorre de acordo com a constituição genômica e potencial funcional de cada tipo. Nessa nova divisão, mgCSTs 1-6 são dominadas por *L. crispatus*, mgCSTs 7-9 por *L. gasseri*, mgCSTs 10-14 por *L. iners,* mgCSTs 15-16 por L. jensenii, mgCSTs 17-19 por *Ca.* La. vaginae, mgCSTs 20-25 por *G. vaginalis*, mgCST 26 por *Bifidobacterium breve* e mgCST 27 por outras espécies bacterianas (19).

O estudo do microbioma vaginal tem evoluído significativamente com o avanço das técnicas moleculares e desde a caracterização inicial das comunidades bacterianas vaginais até a recente classificação com base em centroides de referência, observamos uma diversidade notável na descrição das CSTs presentes. Além disso, o uso da metagenômica permitiu uma compreensão mais profunda não apenas da composição, mas também do potencial funcional das espécies bacterianas que compõem essas comunidades. O catálogo de genes VIRGO facilita a caracterização tanto taxonômica quanto funcional das comunidades microbianas vaginais. Os

avanços se mostram importantes para melhorar a compreensão da microbiota vaginal e seu papel na saúde da mulher.

2. Aspectos da disbiose vaginal

A principal disbiose vaginal, a VB é caracterizada à microscopia pela substituição da microbiota predominantemente lactobacilar por uma microbiota cocobacilar Gram mista (21). Sua prevalência é de cerca de 30% na população brasileira (22) e o padrão ouro clínico para seu diagnóstico é o descrito por Nugent (23). Este critério semi-quantifica os moforfotipos bacterianos, utilizando a coloração pelo método de Gram, em amostras de esfregaços vaginais de acordo com um escore, sendo de 0-3, microbiota normal, 4-6, microbiota intermediária e 7-10, VB.

Os estudos com base no sequenciamento de nova geração classificam a maioria dos casos de VB nas CSTs IV, onde existe a depleção lactobacilar e maior diversidade de espécies bacterianas (11,24). Embora essa condição esteja associada a uma grande diversidade bacteriana no ambiente vaginal, a espécie *Gardnerella vaginalis* está presente em quase a totalidade dos casos (25), sendo o primeiro microrganismo associado à essa alteração (26).

O gênero *Gardnerella* permaneceu, muito tempo, único e composto por como somente uma espécie. Em 2011, Santiago et al. (27), a partir de métodos de análise de restrição de DNA ribossomal (ARDRA), detectou a presença de três genótipos de *G. vaginalis* diferentes. Posteriormente, Ahmed et al.(28) realizou uma nova classificação, pelo pirossequenciamento, no qual foram identificados 4 subgrupos (1 a 4). Em 2016, mais um achado, usando sequenciamento de Sanger do gene CPN60, confirmou a existência dos 4 subgrupos, classificados como A, B, C e D, equivalente aos grupos 4, 2, 1 e 3, respectivamente(28,29). Adicionalmente, um outro estudo partir da hibridização de DNA-DNA (dDDH) e da identidade média de nucleotídeos (ANI), identificou, a partir de 10 linhagens de *G. vaginalis*, 3 novas espécies genômicas de *Gardnerella* spp., que com base na classificação de Ahmed et al.(28), o grupo 1 possui duas espécies, descrita como *G. vaginalis* (30) e o grupo 2 contém 3 espécies, descritas como *G. piotii.* Já o grupo 4 contém 2 espécies descritas como *G. leopoldii* e *G. swidsinskii*. Atualmente, existem outras espécies genômicas de *Gardnerella*

descritas que compõem 6 mgCSTs classificadas de acordo com seus diferentes genes (19).

A presença de *Gardnerella* spp. é encontrada em quase a totalidade dos casos de VB e os fatores virulência desta podem contribuir pra isso. Um estudo recente mostrou que a presença de atividade de sialidase desse gênero favorece o crescimento de outras espécies bacterianas associadas à VB, *como Leptotrichia amnionii*, *Megasphaera* spp. e *Mobiluncus curtisii* (31). As sialidases são enzimas produzidas por algumas linhagens de *Gardnerella* que hidrolisam o ácido siálico das células epiteliais do hospedeiro, expondo sítios de ligação que facilitam a adesão da *Gardnerella* spp. ao epitélio (32,33). A presença de biofilmes associados à VB propicia uma proteção dos microrganismos que os compõe da ação do peróxido de hidrogênio, ácido lático e bacteriocinas secretadas em condições normais pelos *Lactobacillus* spp. (34). Além disso, as moléculas de ácido siálico podem ser utilizadas no metabolismo energético da bactéria (35), ou ainda podem recobrir a superfície bacteriana, dificultando a fagocitose (33,36). Estudos já demonstraram que as sialidases são capazes de diminuir a viscosidade do conteúdo cérvico-vaginal, além de exercerem ação proteolítica nas imunoglobulinas A presentes na secreção cervical (37,38).

Trabalhos demonstraram que, embora a capacidade de produção de sialidases pela *G. vaginalis* esteja presente em apenas algumas linhagens, esta espécie contribui para a quase totalidade das sialidases detectadas no ambiente cérvico-vaginal (38). A presença de *G. vaginalis* sialidases-produtoras está associada tanto à VB como à produção de biofilmes vaginais (30,39). Adicionalmente, outros fatores de virulência são produzidos pelo gênero *Gardnerella*, a exemplo de espécies genômicas como *Gardnerella* spp. 11 e *Gardnerella* spp. 13 que produzem a vaginolisina, responsável pela lise das células epiteliais; hemolisina, responsável pela lise dos eritrócitos; e o precursor da enzima muralítica (RpF2) (19).

O modelo proposto da patogênese da VB descrito por Muzny et al. (40,41) discorre que inicialmente linhagens a presença de linhagens virulentas de *G. vaginalis* contribui para depleção lactobacilar e inicia a formação de biofilme pela produção de sialidase. Essas bactérias provocam a proteólise aumentando a quantidade de aminoácidos que promovem o crescimento de *Prevotella bivia*. Concomitantemente, a *P. bivia* produz amônia que induz o crescimento de *G. vaginalis*, o que estabelece uma relação de sinergia entre as duas espécies (42). O biofilme então cresce, e a sialidase produzida tanto pela *G. vaginalis* quanto pela *P. bivia* promove a quebra na camada protetora do

epitélio vaginal através da quebra do ácido siálico provocado pela imunoglobulina A.Isto, expõe sítios de ligação que permitem a ligação de outras espécies presentes na VB. Uma delas comumente encontrada é a *Fannyhessea vaginae* (antes nomeada *Atopobium vaginae*) que cresce no biofilme já maduro (**Fig. 1**).

A relação da *P. bivia* e da *F. vaginae* na composição do core bacteriano presente na VB pode ser explicada por um trabalho do mesmo grupo que descreveu o modelo onde foi mostrada a capacidade de *F. vaginae* (*F. vaginae*) e *P. bivia* (*P. bivia*) de se integrarem ao biofilme já existente de *G. vaginalis* (*G. vaginalis*), mostrado em biofilmes duplos (*G.vaginalis+F. vaginae e G. vaginalis+P. bivia*) e triplos (*G. vaginalis+F. vaginae+P. bivia*). No entanto, a habilidade de integração ao biofilme por parte de *F. vaginae* e *P. bivia* não necessariamente influencia a expressão dos fatores de virulência em *G. vaginalis.* Enquanto a sialidase, vaginolisina e os transcritos que codificam genes de resistência antimicrobiana específicos não apresentaram diferenças significativas na expressão entre o mono-biofilme de *Gardnerella* e os biofilmes duplos ou triplos, os genes de glicosiltransferases tipo II (envolvidos na manutenção do biofilme) e a Rib-proteína (que desencadeia imunidade protetora por meio da variação de tamanho entre as cepas de *G. vaginalis*) foram encontrados superexpressos nos biofilmes triplos em comparação com o mono-biofilme de *G. vaginalis* (43). Esses resultados enfatizam a complexidade da VB e sua relação com a microbiota vaginal e seus papéis na patogênese dessa condição.

3. Características associadas à microbiota vaginal

Considerando a diversidade da microbiota vaginal em relação a sua composição, existe uma relação entre sua variância e diversos fatores clínicos, comportamentais e sociodemográficos. A exemplo disso, o estilo de vida, como dieta, práticas de higiene e atividade sexual podem alterar a composição do microbioma (2,44). Ademais, o uso de contraceptivos de progesterona e a prática de exercícios intensos, por exemplo, foram associadas com a diminuição de espécies lactobacilares (2,45).

Etnicamente, as mulheres asiáticas e brancas têm mais probabilidade de possuir CSTs dominadas por Lactobacillus, como CST I, II, III e V, em comparação a mulheres negras ou hispânicas (45). Mulheres asiáticas também apresentaram, embora uma alta presença de CST III dominada *L. iners*, uma baixa presença de VB em relação a

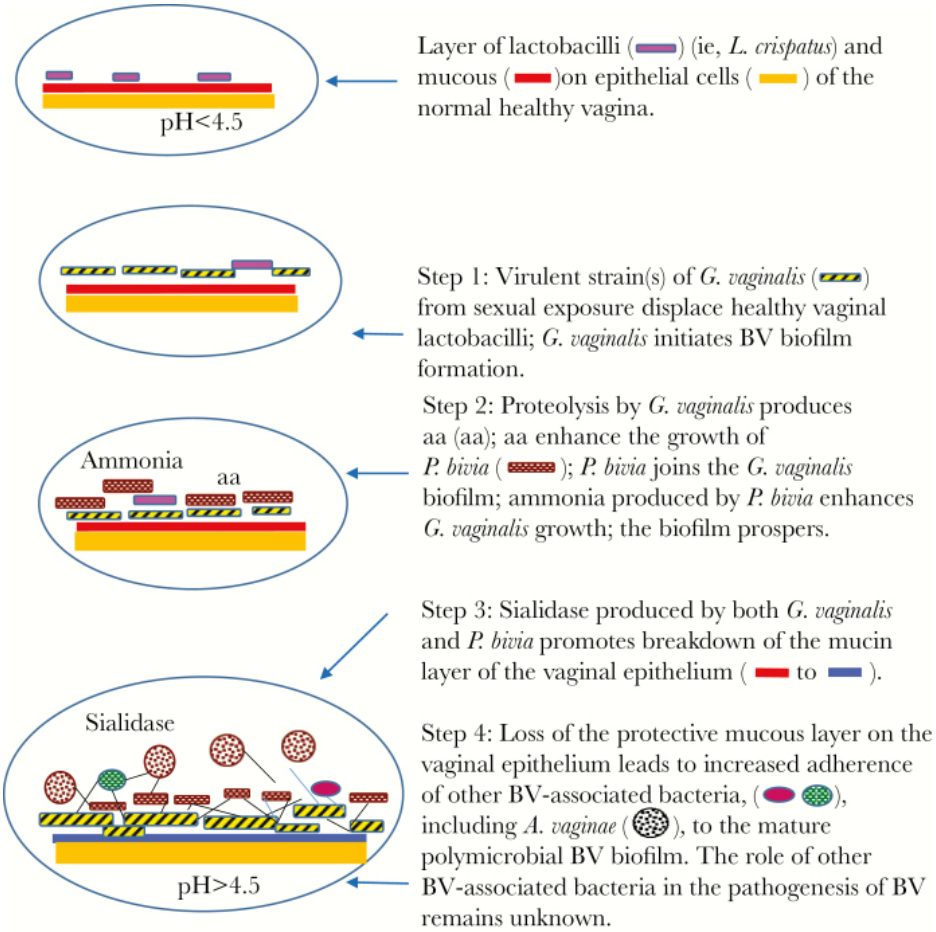

Figura 1. Modelo da patogênese da vaginose bacteriana. Linhagens virulentas de *G. vaginalis* inicia a formação do biolfime, seguida da *P. bivia* e seus fatores de virulência facilitam a adesão no biofilme maduro de outros microrganismos como *F. vaginae*. Figura retirada de Muzny et al. (40)

outras etnias (46). Isso pode se dar ao fato de que variáveis geográficas e culturais também podem impactar a composição microbiana da microbiota vaginal (16).

A produção de ácido láctico pelas espécies de Lactobacillus é essencial para manter um pH vaginal baixo, inibindo o crescimento de bactérias patogênicas (47). Alterações no microbioma, especialmente a redução de Lactobacillus, podem elevar o pH vaginal, tornando-o mais suscetível à invasão de patógenos (48). A fertilidade e os níveis hormonais e também podem influenciar a composição do microbioma vaginal, afetando o pH (49). Por isso, a disbiose provocada por essas alterações, como a VB, podem estar associadas a essa alteração de pH (50,51). Durante a menopausa, a redução nos níveis de estrogênio eleva o pH vaginal, o que provoca a diminuição do domínio de *Lactobacillus* spp. (52).

O comportamento sexual, como o aumento no número de parceiros sexuais pode também mudar a microbiota vaginal e alterar a alfa-diversidade microbiana (53). Outro

achado apontou que a relação sexual facilita a troca de componentes da microbiota entre parceiros, o que pode alterar as microbiotas urinária e vaginal, onde se observou um aumento de *G. vaginalis* após a relação sexual (54,55). Além disso, essa interação pode levar a uma maior diversidade bacteriana no sêmen e a uma menor abundância relativa de *L. crispatus* em amostras vaginais, resultando em maior concordância entre os microrganismos encontrados entre parceiros (56).

A microbiota vaginal também influencia a susceptibilidade a várias infecções sexualmente transmissíveis (ISTs). Pesquisas mostraram que alterações na composição da microbiota vaginal podem aumentar a vulnerabilidade a ISTs causadas por *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae* e o papilomavírus humano (HPV) (57–62). Particularmente, uma microbiota vaginal diversa, com baixa presença de espécies de *Lactobacillus*, como observado na vaginose VB, está ligada a uma maior prevalência de ISTs (59). Além disso, o metaboloma vaginal está associado a prevalência de ISTs, com mulheres com diagnótisco por ISTs exibindo maior prevalência de VB, sugerindo uma possível interação entre os metabólitos vaginais e os desfechos de aquisição de ISTs (63).

4. Papilomavírus Humano e microbiota vaginal

O papilomavírus humano (HPV) é um vírus da família *Papillomaviridae*, caracterizado por ser não envelopado, constituído por um capsídeo de simetria icosaédrica, que engloba uma molécula de DNA circular de dupla fita com aproximadamente 8000 bp (64). O genoma do HPV é dividido em três regiões. A primeira delas é uma região regulatória (LCR, *long control region*) que contém a origem de replicação do DNA e, portanto, responsável pelo início do ciclo viral. A próxima região é a precoce que codifica 6 proteínas (E1, E2, E4, E5, E6 e E7) que possuem ação enzimática ou reguladora no ciclo viral. Dentre essas, merecem destaque as proteínas E6 e E7, que inativam, respectivamente, os supressores tumorais p53 e pRB da célula infectada, estando envolvidas diretamente na imortalização dessas células e, portanto, no desenvolvimento de neoplasias (65–67). Finalmente, os genes da região tardia (*late*) que codificam as duas proteínas que compõe o capsídeo viral, a L1 e L2, altamente imunogênicas, utilizadas na produção de vacinas profiláticas (67). O HPV é um vírus epiteliotrópico, ou seja, sua infecção ocorre pela camada basal do epitélio a partir de microlesões, e após migrar para as

camadas intermediárias do epitélio, se replica, podendo levar ao aparecimento de lesões proliferativas (68).

Atualmente, são descritos mais de 100 genótipos de HPV que infectam o homem, dentre os quais 40 tem afinidade para o epitélio da região anogenital e alguns destes tipos estão envolvidos no aparecimento do fenótipo celular maligno (64). Dessa forma, a classificação dos HPV genitais é feita de acordo com seu potencial oncogênico, divididos em genótipos de baixo (lrHPV) ou intermediário risco e alto risco (hrHPV) oncogênico, sendo este responsável por 5% de todos os cânceres diagnosticados anualmente no mundo (69–72). Em relação aos lrHPV, destacam-se os HPV6 e HPV11, associados ao desenvolvimento de verrugas genitais. Já os HPV16 e HPV18 são associados ao maior período de persistência da infecção levando ao desenvolvimento das lesões intraepiteliais de alto grau e carcinomas invasivos do colo do útero, sendo responsáveis por 70% dos casos de carcinomas epidermóides e mais de 80% dos adenocarcinomas do colo do útero (73–76).

As infecções por HPV genitais são, em geral, de caráter transitório, visto que a maioria das infecções é eliminada pelo sistema imunológico do hospedeiro (*clearance* viral) num período entre 1 a 2 anos (77), entretanto, algumas infecções permanecem, pela presença da infecção por hrHPV (71). A literatura é consistente ao demonstrar que o HPV16 apresenta os períodos de infecção mais longos, mesmo quando comparado aos demais HPV de alto risco oncogênico (78,79). Além disso, também foi apontado que a presença de múltiplos genótipos de HPV atrasa o tempo de *clearance* (80).

Contudo, além do genótipo viral, o tempo de *clearance* do HPV pode ser influenciado por outros fatores como: dietas pobres em nutrientes, uso de absorventes internos, contraceptivos de progesterona, presença de outro vírus como o da imunodeficiência humana (HIV) (81,82) e presença de coinfecções, relacionadas ao ambiente vaginal, como as endocervicites causadas pela *Chlamydia trachomatis* e ectocervicite *Trichomonas vaginalis* (83–85).

Além das infecções sexualmente transmissíveis, diversos estudos com base no sequenciamento de nova geração foram realizados para determinar o papel da microbiota vaginal no contexto das infecções pelo HPV (86).

A microbiota lactobacilar é importante para a manutenção do ambiente vaginal saudável, sendo um gênero muito estudado na sua relação com HPV. Menores abundâncias de *Lactobacillus* e maior diversidade de outras espécies como *Prevotella*

e *Megasphaera* foram encontras em mulher com câncer cervical (87). Mais especificamente, o aumento de *L. crispatus* foi associado a ausência de hrHPV enquanto a diminuição foi associada a presença deste (88). Por outro, o *L. iners* foi negativamente associado ao HPV *clearance* (89,90) e também ao HPV positivo (91).

Alguns estudos mostram espécies relacionadas a VB com lesões cervicais. Zhai et al. (92) mostraram uma maior abundância do filo *Actinobacteria*, e dos gêneros *Gardnerella*, e *Prevotella* em mulheres com lesões ou hrHPV positivo. Outros autores avaliaram um aumento dos gêneros *Lactobacillus*, *Gardnerella*, *Prevotella* em mulheres com neoplasia intraepitelial cervical (NIC) e HPV (93) e em NIC2+ aumento de *L. crispatus*, *S. agalactiae* e *B. fragilis* (94).

Outros achados avaliaram a diversidade de espécies bacterianas em relação ao HPV. Hu et al. (95) mostrou que o microbioma com a presença de HPV exibe maior diversidade de espécies em relação as pacientes com HPV negativo. Adicionalmente, a transição de CST I para CST IV foi encontrada em mulheres com hrHPV persistentes (91) e maior diversidade de espécies no microbioma cervical em relação ao vaginal em mulheres HPV16 e 18 positivas também foi encontrada (90). Em relação as lesões cervicais, já foi apontado que mulheres com lesão intraepitelial de alto grau (HSIL) e carcinoma escamoso *in situ* (SCC) apresentaram maior diversidade em comparação a mulheres sem lesão (96).

Considerando as espécies bacterianas envolvidas no modelo da patogênese da VB proposto por Muzny et al. (*Gardnerella* spp., *F. vaginae* e *P. bivia*) (40,41), *Gardnerella* spp. foi associado previamente com a infecção pelo HPV. Maior abundância de *Gardnerella* foi encontrada em mulheres HPV positivas (89,97) e hrHPV persistentes (98), sendo também um potencial biomarcador para a progressão de hrHPV (99). Outros achados recentes do nosso grupo apontaram que a maior carga de *Gardnerella* spp. está associada a persistência do HPV16 e HPV18 após 11 meses (100), assim como o gene codificante da sialidase (NanH3), importante fator de virulência, também se encontrou aumentado em mulheres com HPV16 persistentes (101). Adicionalmente, um estudo baiano realizado por reação em cadeia polimerase (PCR) mostrou associação da presença de *Gardnerella* spp. no ambiente vaginal com a presença de lesões cervicais, enquanto outro, realizado no Paraná, mostrou que somente a bactéria *G. vaginalis* não estaria associada as lesões precursoras do câncer do colo de útero (102,103). O aumento na abundância de *F. vaginae* foi encontrado tanto em mulheres HPV positivas quanto em mulheres com hrHPV em

relação àquelas com lrHPV ou sem HPV (91,104). *Atopobium* e *Gardnerella* foram encontradas em maior quantidade em mulheres com HPV16 e 18 com câncer cervical (105) e em menor proporção em mulheres hrHPV negativas (88). O gênero *Prevotella* foi associada também a positividade do HPV (91) e seu aumento junto com *G. vaginalis* e *Atobobium* foram presentes em pacientes com lesões cervicais (106). Acerca disso, outro achado revelou que ausência de *L. crispatus* e presença de *L. iners*, *Atopobium* spp., *Prevotella* e *Gardnerella* podem indicar desenvolvimento de lesão cervical (96).

A formação de biofilme se mostra importante para o estabelecimento da VB (43,107), mas os estudos relacionando isso com o HPV são escassos. Um estudo recente mostrou que houve uma maior taxa de formação de biofilme em mulheres com HPV em comparação a ausência deste. Ademais, foi observado uma relação estatisticamente significativa entre a presença de um único genótipo de HPV único e os hrHPVs em relação a formação de biofilme. Além disso, verificou-se que a formação de biofilme ocorreu em 80% das mulheres com esfregaço com alguma alteração indicando a presença de células epiteliais atípicas (108).

A importante relação entre a infecção pelo HPV e a composição do microbioma vaginal, bem como as graves complicações associadas às duas condições, ressaltam a necessidade de se determinar qual parcela da população apresenta, em risco, para infecções persistentes de HPV. Dessa forma, os estudos de espécies bacterianas, como *Gardnerella* spp., *P. bivia e A. vaginae*, que estão envolvidas na etiologia e persistência da VB, permite a melhor compreensão da relação entre a microbiota vaginal e o desfecho da infecção pelo HPV.

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Capítulo II

Artigo científico I

Manuscrito apresentado de acordo com as normas para submissão no periódico *Scientific Reports* (Fator de impacto: 3.8)

Metagenomic characterization of the vaginal microbiome in Brazil

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Abstract

Vaginal microbiome studies are important for understanding how this microenvironment contributes to women's health. Recent dataset-based classifications of the community state types (CSTs) of the vaginal microbiota utilizing metagenomic data as VALENCIA and metagenomic CST (mgCST) classifier have added important insights on the knowledge of vaginal microbiota. Brazil is an ethnically diverse population with high prevalence rates of *Lactobacillus*-deprived CST IV and *L. iners*-dominated CST III. While CST IV is acknowledged dysbiotic, protective role of *L. iners* has been often considered sub-optimal for this environment. Thus, the aim was to characterize the vaginal microbiome with different metagenomic approaches (VALENCIA and mgCST classifier) among Brazilian women and look for sociodemographic, behavioral and clinical variables associated with *L. crispatus*, *L. iners* and *Gardnerella vaginalis* dominated communities. From a total of 609 reproductive-aged women Brazilian women included in a previous study that characterized the vaginal microbiome based on 16S rRNA sequences, 254 vaginal samples were randomly selected for the current metagenomic analysis. Data on population characteristics were available. Vaginal swabs samples were obtained from the mid-third of the vaginal wall. Vaginal pH and Nugent scores were also recorded. Participants were molecularly tested for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*. Illumina sequencing using random primers was performed for further and metagenomic analyses by VALENCIA and mgCST

classifier. χ2 and the nonparametric Kruskal-Wallis tests were utilized to compare distribution of categorical and continuous variables across group of participants accoding to the dominating species: *L. crispatus*, *L. iners* and *G. vaginalis*. VALENCIA showed that CST I, dominated by *L. crispatus*, accounted for 31.1% (n=79) of participants, CST III, dominated by *L. iners*, represented 31.9% (n=82), and CST IV, multispecies community, with 33.8%(n=86). Regarding mgCST classification, 20 (74.1%) out of the 27 mgCSTs described, were detected in this population. *L. crispatus* mgCST 1 were the most frequent (23.4%, n=59), followed by *L. iners* mgCST 12 (15.0%, n=38). *G. vaginalis* mgCST 20 (8.7%, n=22) and mgCST 24 (9.0%, n=23) had similar rates. *G. vaginalis* mgCSTs were associated with having more than one recent sex partners (p=0.02), increased vaginal pH (p<0.001) and higher Nugent scores (p<0.001). These findings provide the first brazilian metagenomic characterization of the vaginal environment and suggests that behavioral and clinical factors could be associated to *G. vaginalis* mgCSTs.

Keywords: Microbiome; vaginal microbiota; metagenomic; *Lactobacillus*; *Gardnerella*; CST.

1. Introduction

The microbiome refers to a specific microbial community that resides within a defined environment with unique physicochemical attributes. It includes not only the microorganisms present but also their activities, leading to the formation of distinct ecological niches (1). Molecular methods, such as next-generation sequencing techniques, have contributed to determining the local microbiome and find its association with different conditions like bacterial vaginosis (BV), cervical cancer and HIV (2–4). Ravel et al. (5) characterized 5 types of vaginal bacterial communities (community-state type, CST) in the vaginal microbiome of reproductive-aged women according to the predominance of certain species. Of these, 4 are characterized by the predominance of *Lactobacillus* spp.: CST I (*Lactobacillus crispatus*), CST II (*L. gasseri*), CST III (*L. iners*), and CST V (*L. jensenii*). In the remaining vaginal bacterial community, CST IV, the predominance of *Lactobacillus* species is replaced by a greater diversity of bacterial species, where the majority of cases of BV are found.

Additionally, metagenomic approaches have facilitated the identification of fine-scale variations within the vaginal microbiome, highlighting the diversity and complexity of microbial communities in this environment (6). Previous findings show that the vaginal microbiome typically exhibits lower community diversity compared to other anatomical sites, often being dominated by certain species (7).

Recently, a VAginaL community state typE Neare st CentroId clAssifier (VALENCIA) (8) reclassified the vaginal microbiome in 13 CSTs the vaginal microbiome. CST I-A and CST I-B dominated by L. crispatus according to its abundance, CST II dominated by L. gasseri, CST III-A and CST III-B dominated by L. iners according to its abundance, CST V dominated by L. jensenii and CST IV-A (more abundance of *Candidatus* Lachnocurva vaginae and *Gardnerella vaginalis*), IV-B (more abundance of *G. vaginalis* and *Fannyhessea vaginae*) and 5 more communities at CST IV-C (0-4) with lower abundance of *G. vaginalis*, Lactobacillus and *Ca.* L. vaginae and presence of other species.

Moreover, it was also described a new classification based on both composition and function of the vaginal microbiome in 27 metagenomic community-state types (mgCSTs) (9). The mgCSTs was constructed based on the human vaginal non-redundant gene catalog (VIRGO) (10), where each one is defined by different bacterial metagenomic subspecies (mgSs), and this subdivision occurs according to the

genes/functions of each one. The division consists in mgCSTs 1-6 dominated by *L. crispatus*, mgCSTs 7-9 by *L. gasseri*, mgCSTs 10-14, by *L. iners*, mgCSTs 15-16 by *L. jensenii*, mgCSTs 17-19 by *Canditatus* Lachnocurva vaginae, mgCSTs 20-25 by *Gardnerella vaginalis*, mgCST 26 by *Bifidobacterium breve*, and mgCST 27 by other bacterial species.

The mgCSTs showed to be more accurate to specify which mgSs could be harmful or protective to the vaginal environment. For example, *L. iners* mgSs 3 (mgCST 12) is associated to BV negative women, what could suggest a more protective potential compared to the other mgSs as described (11). In addition, it was possible to evaluate that *L. crispatus* mgCSTs variate according to their stability with more or less diversity for each mgSs (9).

In Brazil, the vaginal microbiome was characterized through 16S rRNA sequencing in a cross-sectional study involving 609 women from the 5 regions of Brazil. In this population, the most frequent community found was CST III (36.5%), followed by CST I (30.5%) and CST IV (27.4%) (11). Brazil stands as one of the primary countries in Latin America, and understanding the microbiome within its ethnically heterogeneous population is important, particularly considering the microbiome variations among different ethnic groups (12). However, studies regarding the microbiome in this population remains scarce and predominantly focuses on compositional analysis (11). Based on this, there is an interest in characterize not only microbial composition but also the functional aspects of bacterial communities involved.

The advantage of these new metagenomic sequencing methods lies in both the improved accuracy of identifying present microorganisms and the fact that both analyses were based on reference databases (8–10). Thus, the prevalence of communities in the population can be more accurately estimated.

Thus, this study aimed to characterize with different approaches, VALENCIA and metagenomic community-state types (mgCSTs) classifier among Brazilian women from five geopolitical macroregions.

Furthermore, considering the high number of mgCSTs described, the analysis of mgCSTs grouped by each species allows a better evaluation of the population characteristics. Therefore, we aimed to characterize the sociodemographic, behavioral and clinical factors associated to metagenomic *L. crispatus*, *L. iners* and *G. vaginalis* dominated.

2. Material and Methods

Study design and population

This present transversal study obtained data from a subgroup of participants involved in a previous study that aimed to characterize the vaginal microbiota of 609 Brazilian women of reproductive age from 2012 to 2016 (11). A total of 254 participants were randomly selected across five geopolitical macroregions of Brazil (South, n=48; Southeast, n=49; Midwest, n=58; North, n=49; and Northeast, n=50). These women were recruited during visits to primary healthcare clinics, except in the South region, where recruitment occurred through a referral hospital. Only women attending routine cervical cancer screening (Pap testing) were considered eligible for enrollment. Participants were eligible if they were aged between 18 and 50 years, were not pregnant, and did not report using an intrauterine device, undergoing immunosuppressive therapy, or currently experiencing a urinary tract infection. Samples were collected at least 5 days after the end of the last menstrual period and at least 72 hours after the last sexual intercourse. Additionally, participants did not report using antimicrobial drugs in the 45 days preceding enrollment. For more details on participants enrollment, please refer to the parent study.

Data collection and sampling procedures

The procedures for data and biological sample collection were already detailed in the parent study (11). Briefly, participants underwent a structured face-to-face interview to obtain sociodemographic factors, behavioral characteristics, and medical history. Two sterile swabs were used to collect samples from the middle third of the vaginal wall. One swab was preserved in Amies liquid transport medium (Copan, Brescia, Italy) at -80°C for subsequent analysis of vaginal metagenomic composition, while the other swab was used for Nugent scoring by smearing it onto glass slides (13). KOH testing, vaginal pH measure and PCR testing of *C. trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* using cervical brush samples were perfomed (11).

Sequencing and metagenomic analysis

DNA extraction was performed using the MoBio PowerSoil kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. The final elution step was done by adding 100 µL of sterile nuclease-free warm water. After extraction, they were stored at -80^{o}C until the present analysis. The shotgun metagenomic sequence libraries were constructed from the extracted DNA using Illumina Nextera XT kits and sequenced on an Illumina Novaseq 6000 platform (150 bp paired end mode) at the Genomic Resource Center at the University of Maryland School of Medicine. All metagenomic sequencing data underwent host read removal using *BMTagger* and the *GRCh38* reference genome. Subsequently, the data underwent quality filtering using trimmomatic (v0.38), where parameters included a sliding window size of 4 bp, a quality threshold of Q15, and a minimum read length of 75 bp. Metagenomic sequence reads were mapped to VIRGO (10) using *bowtie*, producing a taxonomic and gene annotation for each read. The taxonomic composition table generated using VIRGO was run through the vaginal CST classifier VALENCIA (8) available in https://github.com/ravel-lab/VALENCIA#. A barplot was plotted to present the CSTs data using *matplotlib.pyplot*.

To run the mgCSTs classifier the required inputs are direct outputs from VIRGO (10) and include the taxonomic abundance table ("summary. Abundance.txt") and gene abundance table ("summary. NR.abundance.txt"). It is imperative that taxonomic and gene column headings match those output by VIRGO. A heatmap was produced showing taxon relative abundances in samples, where samples are labeled within the 27 assigned mgCSTs. The classifier is contained in an R script, which is available at https://github.com/ravel-lab/mgCST-classifer (9).

Statistical analysis

Descriptive statistics by frequency were calculated for VALENCIA and mgCSTs. According to the most frequent mgCSTs present we grouped them to facilitate the comparison in three groups: *L. crispatus* mgCSTs, *L. iners* mgCSTs and *G. vaginalis* mgCSTs. The sociodemographic, behavioral and clinical variables were compared groups using using $\chi 2$ categorical and Kruskal-Wallis non-parametric tests for continuous variables, with P-values <0.05 considered to be statistically significant. An additional analysis comparing the sociodemographic, behavioral and clinical variables

among subset of *L. crispatus* + *L. iners* mgCSTs and *G. vaginalis* + *L. iners* mgCSTs groups using χ2 or Fisher's Exact test and Mann-Whitney was performed for categorical and continuous variables, respectively.

3. Results

Table 1 displays the comparison of both VALENCIA and mgCST analyses showing the high concordance between the two approaches. The taxonomic composition and corresponding CST determined through VALENCIA are depicted in **Figure 1a**. CST I, dominated by *L. crispatus*, accounted for 31.1% (N=79) of the women included and CST III, dominated by *L. iners*, represented the most frequent *Lactobacillus*-dominated community (32.3%/N=82), with CST III-A as the most frequent one. CST II (1.6%/N=4) and CST V (1.2%/N=3), dominated by *L. gasseri* and *L. jensenii*, respectively, were the least frequent communities. Of the CST IV subcommunities, CST IV-B represented 26.1% (N=67) of all women, followed by CST IV-A, 7.0% (N=18) and CST IV-C was rare, found in only one sample (1.3%).

The results of the mgCSTs analysis indicate that 20 out of the 27 communities already described were present in our study population (**Figure 1b**). **Table 2** provides data on the distribution of the mgCSTs and their respective mgSs frequencies. *L. crispatus* mgCSTs were the most frequent, with subtype 1 (mgSs) being the most frequent (23.4%/N=59). mgSs 1 (mgCST 1) was found in all regions at similar rates, but the midwest showed the highest prevalence (25.4%/N=15). *L. iners* was also present in all regions, with mgSs 3 (mgCST 12) and 1 (mgCST 10) representing the most frequent ones, at 15.0% (N=39) and 10.6% (N=28) participants, respectively. mgCSTs dominated by *G. vaginalis* showed similar distribution across all regions, with mgSs 1, 3, 4, and 5 being identified, with a higher prevalence in the northeast (23.0%/N=17) and south (21.6%/N=16) regions. *L. gasseri* and *L. jensenii* were less common in our study population (2.7% combined/N=7) and were found only in selected regions. *Ca.* L. vaginae was not very prevalent in this population (2.4%/N=6)), with no presence in the northeast. The mgCST 27 (2.4%/N=6) was more prevalent in the northeast.

Table 3 presents the distribution of sociodemographic, behavioral and clinical variables of the study population across most prevalent mgCSTs, *L. crispatus* (mgCST1-mgCST6, *L. iners* 10-14 and *G. vaginalis* 20-25. Median age varied between

32 to 35 years across mgCSTs, with a higher representation of black women in *G. vaginalis* mgCSTs and white women in *L. crispatus* mgCSTs 10-14. Among the variables analyzed, the number of sexual partners (more than one partner/year) showed statistical significance between groups, with the *G. vaginalis* group displaying a higher prevalence compared to the other groups (p=0.02). Furthermore, a higher pH level was observed in the *G. vaginalis* group compared to both *L. crispatus* and *L. iners* mgCSTs groups (p<0.001). Significant disparities were also evident in Nugent scores across groups, with the *L. crispatus* group exhibiting a predominance of normal microbiota (93.6%), whereas the *G. vaginalis* group demonstrated a higher prevalence of BV (59.5%). Although not statistically significant (p=0.09), a trend was observed regarding the association with use of hormonal contraceptive and the presence of any STI across groups.

Considering the distinct characteristics of *L. iners* mgSs as described (11), 39 samples with mgCST 12 were grouped with *L. crispatus* mgCSTs group, while 44 samples with mgCST 10, 11 or 13 were assembled with *G. vaginalis* one. This classification allowed a comparative analysis of these two groups across the same set of variables as displayed in **Table 4**. Of the variables assessed, black women remained its higher frequency in *G. vaginalis* group (60.2%) and presented same proportion alongside white women in *L. crispatus* group (46.2%).

The pH maintained significantly higher in *G. vaginalis* with *L. iners* mgCSTs compared to *L. crispatus* + *L. iners* mgCST 12 (p<0.001). Similarly, Nugent scores exhibited a significant correlation, with combined *L. crispatus* + *L. iners* mgCSTs displaying a higher prevalence of normal microbiota (82.9%) and *G. vaginalis* + *L. iners* mgCSTs showing a higher incidence of bacterial vaginosis (41.5%) (p<0.001). The presence of any STI also showed a significant difference between groups (p=0.01) where *G. vaginalis* + *L. iners* (6.8%) had higher rate compared to *L. crispatus* + *L. iners* (1.7%). Although not statistically significant (p=0.06), a trend was observed regarding the association with skin color between groups.

4. Discussion

Agreeing with previous results on 16S sequencing (11), vaginal microbiome analyses with metagenomics showed higher prevalences of *L. crispatus*, *L. iners* and

Lactobacillus depleted communities in our population. The comparison between VALENCIA and mgCSTs showed a high similarity, which was expected since both methods were constructed based on the same gene catalog (10). This is the first metagenomic characterization of the brazilian vaginal microbiome, which contributes to a more complete understanding of this area in South America.

The metagenomic findings around the vaginal microbiome is important to understand the differences and stability changes even in communities dominated by the same species. The stability of *L. crispatus* communities within the vaginal microbiota plays an important role in maintaining vaginal health. CST I-A was majorly found within the CST I, characterized by a higher abundance of *L. crispatus*. mgSs 1 (mgCST 1) was the most common subspecies of *L. crispatus* identified in our population, which contain genes for both L- and two D-lactate dehydrogenase enzymes. The D-lactate dehydrogenase enzyme it is known for its important latic acid production, which characterizes the protective role in the vaginal environment.] (9,14,15).

CST III was the most frequent CST found in this population, which is particularly important as the protective role of *L. iners* in vaginal health remains controversial (11,16). *L. iners* has been associated with the regrowth of a *Lactobacillus*-dominant vaginal microbiota, suggesting a beneficial function in maintaining vaginal health (17,18). However, the presence of *L. iners* in the vaginal microbiota has also been linked to vaginal dysbiosis, potentially contributing to microbiota imbalance (19,20). The variability exhibited by different mgSs suggests that different subespecies may play distinct roles in microbiota health. mgSs 3, negatively associated to BV (9), was also found in Asian women at higher rates, albeit despite similarly high levels of *L. iners* compared to the Brazilian population (5,8,21), they exhibit lower incidences of BV compared to hispanic and black women (5,8,22–24). Despite the higher prevalence of mgSs 3 in the women included, the combined presence of other *L. iners* mgSs 1, 2 and 5 are majority and may contribute to higher BV rates.

CST IV has previously been linked to BV with the majority of cases exhibiting the non-lactobacilli community profile (25). This presence was notably associated with Gardnerella-dominated species (mgCST 20-25). Among these, mgCST 24, 20, and 23 were the most prevalent, respectively. MgCST 20 displayed greater genomic species variability compared to the other two. Gardnerella mgSs 4, the most predominant, primarily comprises genes from *G. swidsinkii* and *G. vaginalis* (9). Recent descriptions

of new *Gardnerella* species contribute to a better understanding of the variety and complexity of bacterial vaginal dysbiosis, particularly considering the virulence factors that may vary among them and within the context of symptomatic or asymptomatic BV (9,26). Regarding this, the presence of *Gardnerella* spp. in CST IV has been associated with sialidase activity in cervicovaginal fluid, suggesting its potential role in altering microbial components in the microbiota (27). Sialidases are important enzymes due to their potential to hydrolyze sialic acid in the host's cells, exposing binding sites that enable to form biofilm and are associated to the pathology of the BV (28).

Given the complexity of BV, it is noteworthy to highlight the presence of mgCSTs within CST IV, with different *Ca.* L. vaginae (before BVAB1) mgSs, which have been identified as biomarkers for this dysbiosis (29,30). The presence of *Ca.* L. vaginae has been linked to more diverse and heterogeneous microbial communities in symptomatic women with BV compared to asymptomatic individuals (31). In our studied population, we observed a prevalence of only 2.4%, which limits our ability to compare with other present communities, but larger sample sizes may provide a better understanding into their importance in the Latin American population.

The relationship between the number of sexual partners and the vaginal microbiome has been investigated in other studies (32,33). Previous findings have shown that sexual activity and the number of sexual partners can influence the composition of the vaginal microbiome on non-*Lactobacillus* dominant (34). In our population, a significant difference was found between groups, with a higher prevalence of participants that reported having more than one sex partner in the past year in the *G. vaginalis* mgCSTs group (33,34). Furthermore, evidence suggests the transmission of bacterial communities associated with bacterial vaginosis between sexual partners during intercourse (35). The pathogenesis model of bacterial vaginosis also suggests that virulent strains of G. vaginalis transmitted through sexual exposure are important for the establishment of BV, which reinforces the higher prevalence of higher sexual partners found in G. vaginalis mgCSTs group in our study (28).

In the study, higher pH values were consistently associated to *G. vaginalis* mgCSTs in contrast to both *L. crispatus* and *L. iners* communities. pH is one of the Amsel criteria for clinical BV diagnosis (36). mgCSTs 20 and 24, predominant in the study population, were previously associated with Amsel-BV diagnosis (9). Notably, *L. crispatus* mgCST 1, the most prevalent, earlier demonstrated greater stability and

maintained a lower vaginal pH compared to other *L. crispatus*-dominated mgSs, thus explaining the observed lower pH (8,9). While *L. iners* displayed similar vaginal pH values than *L. crispatus* mgCSTs, it is important to note the differences among their mgSs. When different *L. iners* subspecies were grouped with either *L. crispatus* or *G. vaginalis*, the predominant subtype 3 of *L. iners* (mgCST 12), along with *L. crispatus*, sustained a lower pH compared to the other group. This subspecies was previously linked to a less likelihood of Amsel-BV diagnosis by other study(9). On other side, the other subespecies of *L. iners* along with *G. vaginalis* mgCSTs maintained a higher pH presence which reinforces their association with Amsel-BV diagnosis (9).

Nugent score was previously associated to CSTs in other studies, with *L. crispatus*-dominated CST more found in normal microbiota and non-*Lactobacillus* dominant CST found mostly in BV cases (11,23,24,37). Our findings contribute to this with high predominance of *L. crispatus* and G. vaginalis associated to normal microbiota and BV, respectively. *L. crispatus* mgCST 1 more prevalent in this population was previoulsy associated to lower Nugent score while G. vaginalis mgCST 20 and 24, more frequent, was associated to higher Nugent scores (9). The mgCST 23 had a certain presence in our population and this community was previously more found in intermediate or normal microbiota, therefore that could explain the variability of *G. vaginalis* group distribution about the Nugent scores. The association of the Nugent scores remained on the grouped *L. crispatus* + *L . iners* mgCSTs compared to *G. vaginalis* + *L. iners* mgCST, with similar distribution of *L. iners* intermediate and BV cases among groups.

Research also indicates that a vaginal microbiome with high species diversity, as seen in bacterial vaginosis, is associated with an increased risk of acquiring sexually transmitted infections (33). In our findings, it was observed a significant association between the presence of STIs (*C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis*) and the vaginal microbiota compositions by *L. crispatus* + *L. iners* mgCSTS compared to *G. vaginalis* + *L. iners*. mgCSTs. Previous studies have emphasized the important role of a healthy vaginal microbiome in reducing the risk of STIs, including HIV-1 (38), and have further identified specific associations with STIs such as *T. vaginalis* and *C. trachomatis*. For instance, *T. vaginalis* infections have been linked to vaginal microbiomes with low lactobacilli proportions and elevated levels of other anaerobes (39). Likewise, the presence of *N. gonorrhoeae* has been correlated with microbial communities typified by diverse bacterial taxa commonly observed in bacterial

vaginosis (40). Notably, our findings align with existing literature, as it is observed greater diversity in mgCSTs of *G. vaginalis* compared to those dominated by *L. crispatus* (9). Thus, the current finding contributes to the evidence on the interplay between vaginal microbiome composition and susceptibility to STIs.

The limitations of this study that may be pointed out include the small sample size (about 50 per region), which restricts the ability to compare communities with lower prevalence in our study population due to the presence of multiple community types. Despite that, our study brings a new view of the characterization of the Brazilian women vaginal microbiome profile in 5 different regions, with more specificity (20 of 27 mgCSTs identified) considering the subspecies by their genes and functions. The high presence of CSTs I, III, and IV found also reinforce the previous found in this population (11). Additionally, the presence of multiple different *L. iners* mgSs suggests its potential dual impact on vaginal health, with both beneficial and negative effects. Moreover, the higher pH associated to *G. vaginalis* mgCSTs emphasize its correlation with BV. Furthermore, the STI presence in *G. vaginalis* mgCSTs suggests that the vaginal environment could influence the risk of coinfections.

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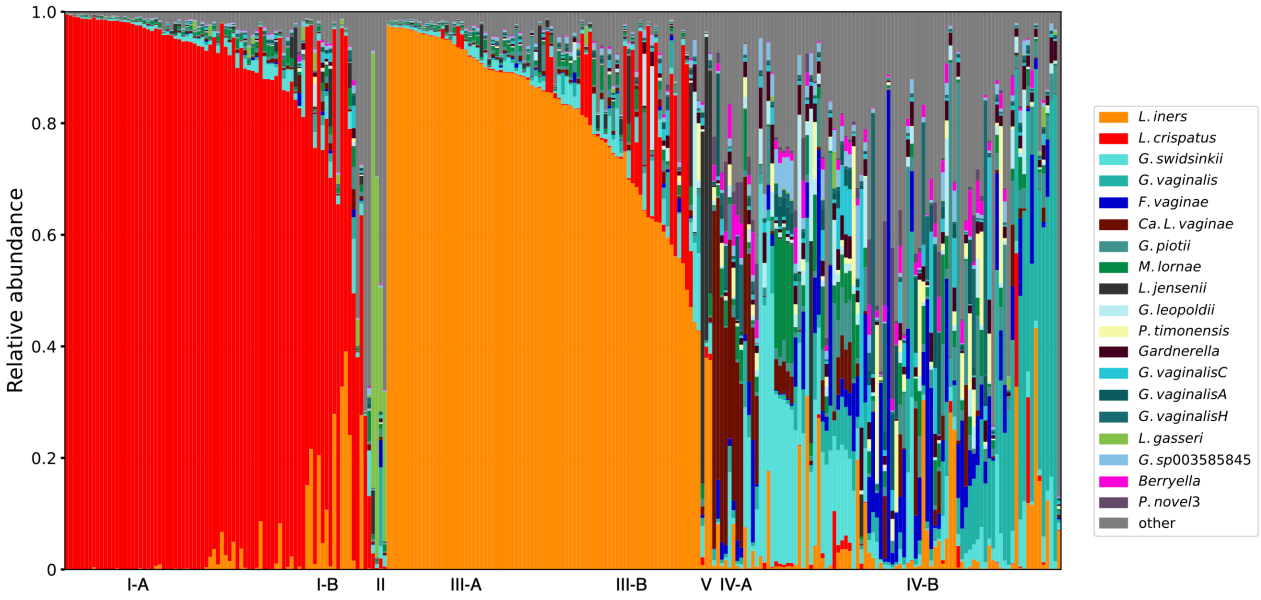
6. Tables and Figures

Table 1. Distribution of 254 samples in metagenomic CSTs analyses according to VALENCIA and mgCST classifier.

	mgCST dominant species and number						
	L. crispatus	*L. gasseri*	*L. iners*	*L. jensenii*	*Ca.* L. vaginae	*G. vaginalis*	Other
	1-6 (n=78)	7-9 (n=4)	10-14 (n=83)	15-16 (n=3)	17-19 (n=6)	20-25 (n=74)	27 (N=6)
CST I-A (N=63)	62 (79.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	0 (0.0%)	0 (0.0%)
CST I-B (N=16)	16 (20.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
CST II (N=4)	0 (0.0%)	4 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
CST III-A (N=64)	0 (0.0%)	0 (0.0%)	64 (77.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
CST III-B (N=18)	0 (0.0%)	0 (0.0%)	18 (21.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
CST IV-A (N=18)	0 (0.0%)	0 (0.0%)	1 (1.1%)	0 (0.0%)	4 (66.6%)	11 (14.9%)	1 (16.7%)
CST IV-B (N=67)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	62 (83.8%)	5 (83.3%)
CST IV-C (N=1)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.3%)	0 (0.0%)
CST V (N=3)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Figure 1. Taxonomic composition of all vaginal samples (n=254) taxa relative abundances are categorized by sub-CST assignment utilizing? VALENCIA (8) (**a**). Heatmap representing the Vaginal Metagenomic Community State Types (mgCSTs) (9) of 254 metagenomic samples, showing the 30 most abundant species (**b**).

a)



b)

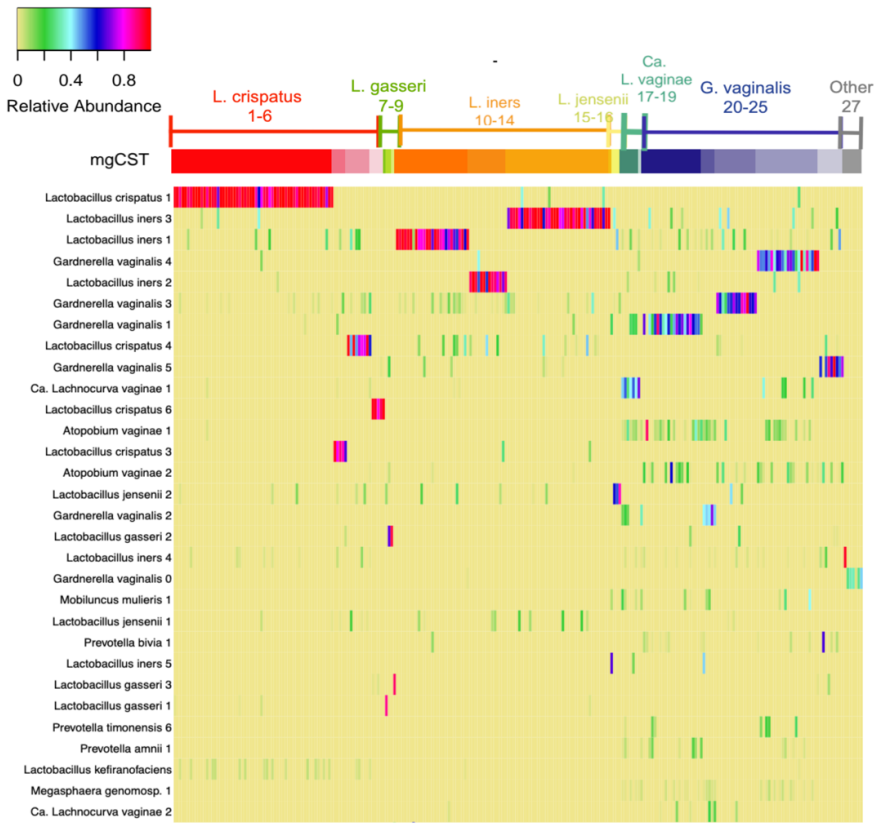


Table 2. Distribution of mgCSTs of the study population according to the 5 geographic regions of Brazil.

			Region				
mgCST	Most abundant mgSs	N	South	Southeast	Midwest	Northeast	North
L. crispatus 1	*L. crispatus* 1	59	11 (18.6%)	11 (18.6%)	15 (25.4%)	9 (15.2%)	13 (22.2%)
L. crispatus 3	*L. crispatus* 3	5	0 (0.0%)	1 (20.0%)	0 (0.0%)	2 (40.0%)	2 (40.0%)
L. crispatus 4	*L. crispatus* 4	9	2 (22.2%)	1 (11.1%)	1 (11.1%)	3 (33.3%)	3 (33.3%)
L. crispatus 6	*L. crispatus* 6	5	1 (20.0%)	0 (0.0%)	0 (0.0%)	3 (60.0%)	1 (20.0%)
L. gasseri 7	*L. gasseri* 1	1	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
L. gasseri 8	*L. gasseri* 2	2	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (100.0%)
L. gasseri 9	*L. gasseri* 3	1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (100.0%)
L. iners 10	*L. iners* 1	28	4 (14.8%)	6 (22.2%)	4 (14.8%)	8 (26.0%)	6 (22.2%)
L. iners 11	*L. iners* 2	15	2 (14.3%)	3 (21.4%)	6 (35.7%)	3 (21.4%)	1 (7.2%)
L. iners 12	*L. iners* 3	39	6 (15.8%)	10 (26.3%)	13 (31.6%)	4 (10.5%)	6 (15.8%)
L. iners 13	*L. iners* 5	1	0 (0.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)
L. jensenii 16	*L. jensenii* 2	3	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (100.0%)
Ca. L. vaginae 17	*Ca.* L. vaginae 1	5	2 (40.0%)	2 (40.0%)	1 (20.0%)	0 (0.0%)	0 (0.0%)
Ca. L. vaginae 19	*Ca.* L. vaginae 3	1	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
G. vaginalis 20	*G. vaginalis* 1	22	5 (22.7%)	2 (9.1%)	4 (18.2%)	5 (22.7%)	6 (27.3%)
G. vaginalis 22	*Prevotella amnii* 4	5	0 (0.0%)	0 (0.0%)	3 (60.0%)	2 (40.0%)	0 (0.0%)
G. vaginalis 23	*G. vaginalis* 3	15	3 (20.0%)	3 (20.0%)	1 (6.7%)	6 (40.0%)	2 (13.3%)
G. vaginalis 24	*G. vaginalis* 4	23	7 (30.4%)	7 (30.4%)	4 (17.4%)	3 (13.1%)	2 (8.7%)
G. vaginalis 25	*G. vaginalis* 5	9	1 (11.1%)	3 (33.3%)	2 (22.2%)	1 (11.1%)	2 (22.2%)
Other 27	*Enterococcus faecalis* 3	6	0 (0.0%)	0 (0.0%)	2 (33.3%)	4 (66.7%)	0 (0.0%)

Table 3. Comparison of sociodemographic, behavioral and clinical variables between the most frequent mgCSTs grouped in *L. crispatus* (1-6), *L. iners* (10-14) and *G. vaginalis* (20-25).

Variable	**mgCSTS**			
	***L. crispatus* 1-6 (n=78)**	***L. iners* 10-14 (n=83)**	***G. vaginalis* 20-25 (n=74)**	***P-value***
Region*				0.67
South	14 (17.9%)	12 (15.0%)	16 (21.6%)	
Southeast	13 (16.7%)	19 (23.7%)	15 (20.3%)	
Midwest	16 (20.5%)	25 (27.5%)	14 (18.9%)	
Northeast	17 (21.8%)	14 (17.5%)	17 (23.0%)	
North	19 (23.1%)	13 (16.3%)	12 (16.2%)	
Age†				0.29
median (range)	35 (19-50)	32 (18-50)	34 (18-48)	
Skin color*				0.26
Black	35 (44.9%)	44 (53.0%)	46 (62.2%)	
White	38 (48.7%)	35 (42.2%)	24 (32.4%)	
Other	5 (6.4%)	4 (4.8%)	4 (5.4%)	
Living with partner*				0.98
Yes	46 (59.0%)	50 (60.2%)	44 (59.4%)	
No	32 (41.0%)	33 (39.8%)	30 (40.6%)	
High school*				0.13
Yes	52 (66.7%)	44 (53.0%)	39 (52.7%)	
no	26 (33.3%)	39 (47.0%)	35 (47.3%)	
Smoking*				0.96
Yes	9 (11.5%)	9 (10.8%)	9 (12.2%)	
No	69 (88.5%)	74 (89.2%)	65 (87.8%)	
Douching*				0.42
Yes	8 (10.2%)	12 (14.4%)	13 (17.6%)	
No	70 (89.8%)	71 (85.6%)	61 (82.4%)	
Hormonal contraceptive*				0.09
Yes	27 (34.6%)	38 (45.8%)	22 (29.7%)	
No	51 (65.4%)	45 (54.2%)	52 (70.3%)	
Condom*				0.33
Yes	31 (39.7%)	24 (28.9%)	27 (36.5%)	
No	27 (60.3%)	59 (71.1%)	47 (63.5%)	
Number partner/year*				**0.02**
≤1	74 (94.9%)	72 (86.7%)	59 (79.7%)	
>1	4 (5.1%)	11 (13.3%)	15 (20.3%)	

*Chi-squared or Fisher's exact test. $P<0.05$ considered as significant.
†Mann–Whitney Test, expressed by median (minimum–maximum). $P<0.05$ considered as significant.
§Considered presence of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and/or *Trichomonas vaginalis*.

Table 3. Continued.

Variable	mgCSTS			
	***L. crispatus* 1-6**	***L. iners* 10-14**	***G. vaginalis* 20-25**	
	(n=78)	**(n=83)**	**(n=74)**	***P-value***
Abnormal discharge*				0.16
Yes	27 (34.6%)	41 (49.4%)	32 (43.2%)	
No	51 (65.4%)	42 (50.6%)	42 (56.8%)	
pH †				**< 0.001**
median (range)	4.4 (4.0-6.1)	4.4 (4.0-5.8)	4.7 (4.0-5.3)	
Nugent classification*				**< 0.001**
Normal	73 (93.6%)	57 (68.7%)	22 (29.7%)	
Intermediate	1 (1.3%)	14 (16.9%)	8 (10.8%)	
BV	4 (5.1%)	12 (14.4%)	44 (59.5%)	
Microscopic detection of *Candida* spp.*				0.71
Yes	5 (6.4%)	8 (9.6%)	7 (9.4%)	
No	73 (93.6%)	75 (90.4%)	67 (90.6%)	
Any STI*§				0.09
Yes	2 (2.6%)	4 (4.8%)	8 (10.8%)	
No	76 (97.4%)	79 (95.2%)	66 (89.2%)	

*Chi-squared or Fisher's exact test. $P<0.05$ considered as significant.
†Mann–Whitney Test, expressed by median (minimum–maximum). $P<0.05$ considered as significant.
§ Considered presence of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and/or *Trichomonas vaginalis*.

Table 4. Comparison of sociodemographic, behavioral and clinical variables between the most frequent mgCSTs grouped in *L. crispatus* (1-6) + *L. iners* (12) and *G. vaginalis* (20-25) + *L. iners* (10, 11, 13).

Variable			
	***L. crispatus* 1-6 + L. iners 12 (n=117)**	***G. vaginalis* 20-25 + L. iners 10, 11, 13 (n=118)**	***P-value***
Region*			0.39
South	20 (17.2%)	22 (19.0%)	
Southeast	23 (19.8%)	24 (20.7%)	
Midwest	31 (25.9%)	24 (20.7%)	
Northeast	18 (15.5%)	29 (24.1%)	
North	25 (21.6%)	19 (15.5%)	
Age†			0.38
median (min-max)	34 (18-50)	34 (18-48)	
Skin color*			0.06
Black	54 (46.2%)	71 (60.2%)	
White	54 (46.2%)	43 (36.4%)	
Other	9 (7.6%)	4 (3.4%)	
Living with partner*			0.79
Yes	71 (60.7%)	69 (58.5%)	
No	46 (39.3%)	49 (41.5%)	
High school*			0.44
Yes	61 (52.1%)	74 (62.7%)	
no	56 (47.9%)	54 (37.3%)	
Smoking*			0.54
Yes	15 (12.8%)	12 (10.2%)	
No	102 (87.2%)	106 (89.8%)	
Douching*			0.45
Yes	14 (12.0%)	19 (16.1%)	
No	103 (88.0%)	99 (83.9%)	
Hormonal contraceptive*			0.50
Yes	46 (39.3%)	41 (34.7%)	
No	71 (60.7%)	77 (65.3%)	
Condom*			0.58
Yes	43 (36.7%)	39 (33.0%)	
No	74 (63.3%)	79 (67.0%)	
Number partner/year*			0.07
≤1	107 (91.4%)	98 (83.0%)	
>1	10 (8.6%)	20 (17.0%)	

*Chi-squared or Fisher's exact test. $P<0.05$ considered as significant.
†Mann–Whitney Test, expressed by median (minimum–maximum). $P<0.05$ considered as significant.
§ Considered presence of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and/or *Trichomonas vaginalis*.

Table 4. Continued.

Variable			
	***L. crispatus* 1-6 + *L. iners* 12 (n=117)**	***G. vaginalis* 20-25 + *L. iners* 10, 11, 13 (n=118)**	***P-value***
Abnormal discharge*			0.99
Yes	47 (40.2%)	48 (40.7%)	
No	70 (59.8%)	70 (59.3%)	
pH†			**< 0.001**
median (min-max)	4.4 (4.0-6.1)	4.7 (4.0-6.1)	
Nugent*			**< 0.001**
Normal	97 (82.9%)	55 (46.6%)	
Intermediate	9 (7.7%)	14 (11.9%)	
BV	11 (9.9%)	49 (41.5%)	
***Candida* spp. ***			0.26
Yes	13 (11.1%)	8 (6.8%)	
No	104 (88.9%)	110 (93.2%)	
Any STI*§			**0.01**
Yes	2 (1.7%)	12 (6.8%)	
No	115 (98.3%)	106 (93.2%)	

*Chi-squared or Fisher's exact test. $P<0.05$ considered as significant.
†Mann–Whitney Test, expressed by median (minimum–maximum). $P<0.05$ considered as significant.
§ Considered presence of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and/or *Trichomonas vaginalis*.

Capítulo III

Artigo científico II

Manuscrito apresentado de acordo com as normas para submissão no periódico *Sexually Transmitted Infections* (Fator de impacto: 3.6)

Cervicovaginal loads of bacterial vaginosis-associated bacteria in immunocompetent women with persistent high-risk human papillomavirus infection

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Abstract

Human papillomavirus (HPV) is the most frequent sexually transmitted infection and persistent infections by high-risk HPV (hrHPV) are the cause of cervical cancer. Bacterial vaginosis (BV) was previously associated to hrHPV persistence, although the mechanism underlying this association remains unclear. *Gardnerella* spp., *Fannyhessea vaginae* and *Prevotella bivia* are key species in the pathogenesis of BV. Thus, the aim was to compare the cervicovaginal loads of these *Gardnerella* spp., *F. vaginae* and *P. bivia* between women with persistent hrHPV infection and those who cleared the infection after 12 and 24 months. In 216 hrHPV-positive women baseline bacterial loads were compared between 'persistence' and 'clearance' groups, categorized based on HPV status at enrollment and follow-up after 12 months. Absolute quantification of *Gardnerella* spp., *F. vaginae* and *P. bivia* were performed using quantitative real-time PCR (qPCR). Results indicated that *Gardnerella* spp. exhibited higher loads in the persistence group (3.20E05 copies/µL) compared to the clearance group (2.15E04 copies/µL) (p=0.04). However, no statistically significant differences were observed for *F. vaginae* (p=0.17) and *P. bivia* (p=0.75). Further analysis of loads, specifically for HPV16-positive women or those with other-hrHPV did not show difference between groups. 24 months analysis did not show difference between bacteria loads or presence of any species between groups. Therefore, these findings highlight the negative impact of *Gardnerella* spp. in the vaginal microbiota on

the clearance of hrHPV, while *F. vaginae* and *P. bivia* did not exhibit a similar association.

Keywords: human papillomavirus, persistence, high-risk HPV, bacterial vaginosis, *Gardnerella, Fannyhessea vaginae, Prevotella bivia*

1. Introduction

Human papillomavirus (HPV) urogenital infection is the most frequent sexually transmitted infection (STI) worldwide (1–3). HPV genotypes are categorized as of low (lrHPV) or high oncogenic risk (hrHPV) (4–6). The persistence of hrHPVs causes cervical lesions, the precursors of cervical cancer that is the fourth most prevalent cancer in women (7–12). The clearance rate for cervical HPV infections is nearly 80% within a span of 2 years (13,14). hrHPVs show to have longer infection periods than lrHPV, of which HPV16 seems to be the most persistent (15,16). Other factors can influence the HPV clearance, such as nutrient-poor diets, use of tampons, progesterone contraceptives, human immunodeficiency virus (HIV) infection and a *Lactobacillus*-deprived vaginal microbiota, as in bacterial vaginosis (17,18).

Bacterial vaginosis (BV) is the main type of dysbiosis of the vaginal microbiota and is characterized by the replacement of a dominant *Lactobacilli* gram-positive microbiota for a coccobacilli gram-variable microbiota (19). This condition has been associated with increased risk for acquisition of several STIs, including HPV (20,21). Despite its high microbial diversity, *Gardnerella* spp. was the first species associated to this condition and is present in almost all cases of BV (22,23). The association between *Gardnerella* and BV arises from the presence of virulence factors (24,25). For example, sialidase is responsible for hydrolyzing sialic acid in the host's cells, exposing binding sites that enable bacteria to adhere to the vaginal epithelium. This process facilitates the formation of a vaginal biofilm (26–28).

The description of five Community State Types (CSTs) of the vaginal microbiome showed four *Lactobacillus*-dominated CSTs and one gram-variable obligate or facultative anaerobic species or facultative *coccus* dominated (CST IV). (29). A recent molecular-based study (30) showed subdivisions on CST-IV, where the BV cases are usually designated, based on *Candidatus Lachnocurva vaginae* (formerly known as BVAB1 (31)), *G. vaginalis*, *F. vaginae*, *Prevotella* and others. Previous findings showed CST-IV associated with higher microbial diversity and HPV positivity (32,33).

A recent study demonstrated that cervicovaginal loads of *Gardnerella spp.* at baseline are significantly increased in women that persisted with HPV16 and

HPV18 infection for 11 months (34). Other data revealed that sialidase gene producer (NanH3) loads are increased in women with some persistent hrHPV showing that that presence of virulence factors encoding-gene can be associated with HPV persistence (35).

Current model of BV postulates that *Gardnerella* spp. serve as scaffold for other BV-associated bacteria as *Leptotrichia amnionii*, *Megasphaera* spp., *Mobiluncus curtisii* (36). Other microorganisms have been identified as contributors to the pathogenesis of BV with harmful effects on the vaginal environment, which include *Prevotella bivia* and *Fannyheassea vaginae* (before named as *Atopobium vaginae*). *P. bivia* demonstrates synergism with *Gardnerella vaginalis* (37)and also a producer of sialidases (38). Consequently, the presence of sialidase from *P. bivia* can intensify the impact of the enzyme produced by *Gardnerella* spp.. Regarding *F. vaginae*, it is noteworthy that this species, along with *G. vaginalis*, constitutes a primary component of vaginal biofilms and is closely associated with the development of BV (28).

Given the important relationship between HPV infection and the composition of the vaginal microbiota, as well as the serious complications associated with both conditions, a better understanding of the microbiologic factors associated with persistent HPV infections is needed. For this reason, the quantification of bacterial species, such as *Gardnerella* spp., *P. bivia and F. vaginae*, considered as part of the etiology BV (38,39), would allow for a better understanding of the relationship between the vaginal microbiota and the outcome of HPV infection. Therefore, the aim of the study is to compare the cervicovaginal loads of *Gardnerella* spp., *Prevotella bivia* and *Fannyhessea vaginae* in women with clearance and persistence of hrHPV cervical infection after 12 and 24 months follow-up.

2. Material and Methods

Population, Data and Samples collection

From 2012 to 2014, a total of 1638 reproductive-aged immunocompetent women attending all the 18 primary health care clinics for pap-testing constituted the cohort located in Botucatu-SP, Brazil. As part of the enrollment procedures,

data on sociodemographic, physical, behavioral and gynecological history, as well as clinical findings at the time of inclusion of women in the study were obtained during a face-to-face interview using a structured questionnaire. All data were entered into a Microsoft Excel spreadsheet (Microsoft Coporation, Redmond, WA).

Participants underwent same sampling procedures during physical exam at enrollment and at follow-up visit. After speculum insertion, nurses/physicians took mid-third vaginal swabs for smearing onto microscopic glass slides and further classification of the microbiota, according to Nugent et al. (19) We used vaginal vault samples for *Trichomonas vaginalis* culture in Diamond's medium (32). Women were also sampled for endocervical brushes by 3 rotations of 360◦. Brushes-containing samples were immersed in TET buffer (10mM Tris-HCl pH=8.0; 1 mM EDTA; 0.5% Tween 20) and kept at -20ºC until analysis. These samples were then used for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* genes detection, as described previously (40,41), and also for HPV detection and genotyping using the Linear Array HPV Genotyping kit (Roche Molecular Systems, Pleasanton, CA). Cervicovaginal fluid were sampled by inserting 3 ml of sterile 0.9% NaCl allowing the contact with vaginal wall, homogenized with cervicovaginal secretion and recovered using a plastic sterile pipette. Fluid samples were stored at -20ºC until processing *Gardnerella* spp. 23s rRNA gene, *F. vaginae* 16s rRNA gene and *P. bivia* 16s rRNA gene quantification.

This study was reviewed and approved by the Research Ethics Committee of the Botucatu Medical School/UNESP (approval numbers 3.140.843 and 5.660.472) and all participants signed a consent term consent after being explained about the study aims and procedures.

Study design

At the baseline 544 (33.2%) of 1638 women were positive for any HPV infection, and were recruited for a follow up visit after 12 and 24 months. 462 (28.2%) HPV-positive participants returned at the first follow-up, of which we excluded for the present study women who had only lrHPVs (131, 8.00%) and those with other infections, as *Chlamydia trachomatis* (138, 8.42%), *Trichomonas vaginalis* (23, 1.40%) and *Neisseria gonorrhoeae* (2, 0.12%). From the 250 (15.3%) a total of

14 participants were excluded because cervicovaginal samples were insufficient for carrying out all the analysis of the current study. In the remaining 236 women, 20 had cleared the hrHPV genotype detected at baseline, but had incident infection by other hrHPV genotypes at time of the first follow-up and were excluded. Therefore, we tested a total of 216 hrHPV positives cervicovaginal samples obtained at baseline. Participants were then assigned to the two study groups according to the hrHPV status (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) at enrollment and at 12 months follow up. For the 'persistence' group (N=135), was considered participants with at least one baseline hrHPVs genotype agreed with those detected at follow up. Participants were assigned to 'clearance' group (N=85) if they tested negative for all the baseline hrPHV at the follow-up. For the second follow-up analysis, we considered those who persisted with hrHPV at the first visit and attended the 24 months appointment (64/135). They were divided with the same criteria previously described in 'persistence' (N=43) and clearance' groups (N=21).

Cloning and absolute quantification of *Gardnerella* spp. 23s, *Fanyhessea vaginae* and *Prevotella bivia* 16s rRNA genes copies

DNA extraction was performed using the cervicovaginal fluid with the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) as recommended by the manufacturer. The samples were quantified in Epoch spectrophotometer (Biotek, Winooski, VT) to confirm the quality of the extraction by the absorbance ratio 260/280nm.

For the cloning step, to obtain the plasmid DNA, the DNA extracted of *G. vaginalis* ATCC 14018 standard strain were used for amplification by PCR, on the 23s rRNA region of *Gardnerella* spp., using the primers F-GV1 (5'-TTACTGGTGTATCACTGTAAGG-3') and R-GV3 (5'-CCGTCACAGGCTGAACAGT-3') (19). The amplificated products were used for the ligation step using pGEM T Easy Vector System Kit (Promega, Madison, WI), cloned into *Escherichia coli* DH5-α (Invitrogen, Carlsbad, CA) and sequenced using 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA), confirmed by BLAST (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). Same procedure was performed to obtain plasmid DNA from the 16s rRNA gene region of *F. vaginae*, primers Atop582F (5'-TAGGCGGTYTGTTAGGTCAGGA-3') and Atop665R (5'-

CCTACCAGACTCAAGCCTGC-3'), and from the 16s rRNA gene region of *P. bivia*, primers F-PB (5'-CGCACAGTAAACGATGGATG-3') and R-PB (5'-ATGCAGCACCTTCACAGATG-3'), both from a sequenced positive clinical sample.

The absolute quantification of *Gardnerella* spp. 23s rRNA, *F. vaginae* and *P. bivia* 16s rRNA genes were performed by quantitative real-time polymerase chain reaction (qPCR) using 2×qPCRBIO SyGreen Blue Mix Hi-ROX (PCR Biosystems, London, UK), with each targeting primers, in a StepOnePlus Real-Time System (Termo Fisher Scientifc, Waltham, MA, USA), under the following cycling conditions: 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 25 seconds, followed by a dissociation stage: 95 °C for 15 s; 60 °C for 1 min and 95 °C for 15 min, according to the manufacturer's recommendations. Samples with a melting temperature value of 87ºC +/- 0.5ºC, 83ºC +/- 0.5ºC and 86ºC +/- 0.5ºC were considered positive for *Gardnerella* spp, *F. vaginae* and *P. bivia*, respectively. For the sample cycle threshold interpolation, we used a standard curve with four plasmid dilutions (2.45E+08, 2.45E+07, 2.45E+06 and 2.45E+05 copies/µL) for *Gardnerella* spp., four (1.08E+10, 1.08E+09, 1.08E+08 and 1.08E+07 copies/µL) for *F. vaginae* and five (1.47E+10, 1.47E+09, 1.47E+08, 1.47E+07, 1.47E+06 copies/µL) for *P. bivia.* Samples were tested in duplicates and the loads of the genes were expressed as the number of copies per volume (µL) of cervicovaginal fluid.

Statistical analysis

Comparisons between the 'persistence' and 'clearance' groups regarding sociodemographic, behavioral, and clinical variables were conducted using Chi-squared, Fisher's Exact, or Mann-Whitney tests, as appropriate. Mann-Whitney tests were performed to assess the loads of Gardnerella spp., *F. vaginae*, and *P. bivia* genes between groups, considering all-hrHPV and subsets with only HPV16 and other-hrHPVs (HPV16 excluded), expressed as median (minimum-maximum). The presence/absence of *Gardnerella* spp., *F. vaginae*, and *P. bivia* genes and their combinations were compared between the two study groups using Chi-squared or Fisher's Exact tests.

Finally, we defined 'high *Gardnerella* spp., *F. vaginae*, and *P. bivia* loads' as those samples with 1.22E03, 1.15E05, and 1.12E06, respectively, or more

copies of the genes/µL (equivalent to the 40th percentile or higher, corresponding to the nearest inferior value of the median of each bacterium detected in the 'persistence group'). All analyses were performed using GraphPad Prism software (version 9.0, GraphPad, CA), with a p-value<0.05 considered statistically significant.

3. Results

The HPV frequency is presented in Table 1, with HPV16 as the most prevalent genotype found in the population, exhibiting a persistence rate of 74.2% (49/66) after 12 months. The second most prevalent was HPV31, followed by HPV52, HPV58, HPV51, HPV45, and HPV18. Out of the initial 236 women included, 20 were excluded due to the incidence of any hrHPV genotype at follow-up after clearing the initial hrHPV genotype(s) detected at enrollment. The persistence and clearance rates of hrHPV were 60.6% (131/216) and 39.4% (85/216), respectively, constituting the study cohort.

Table 2 displays the sociodemographic, behavioral, and clinical variables according to the study groups. The population was homogeneous for most of the variables evaluated, except for the presence of abnormal discharge, which was statistically associated with the persistence group. The women included are mostly less than 35 years old, white, living with a partner, having completed high school, and employed.

Loads of *Gardnerella* spp., *F. vaginae*, and *P. bivia* were measured and compared between the two groups (Figure 1). *Gardnerella* spp. exhibited significantly higher loads in the hrHPV persistence (3.2E05 (0-3.36E10) copies/uL) group compared to the clearance (2.1E4 (0-3.49E10 copies/uL) (P=0.04). However, this pattern was not observed when comparing loads of *F. vaginae* (P=0.17) and *P. bivia* (P=0.75). Additionally, we conducted comparisons of gene loads in two subsets, HPV16 only and other-hrHPV (HPV16 excluded), but no statistical differences were found based on the HPV status (Table S1).

Table 3 presents the Spearman correlation among the loads of the three bacteria. A positive correlation was identified in all pairwise evaluations, though none exhibited a strong correlation. *Gardnerella spp.* + *F. vaginae* displayed a higher correlation compared to the other combinations.

We also evaluated whether the presence of bacteria could be associated with outcome of hrHPV infection (Table 4). No association was found between the presence of *Gardnerella* spp., *F. vaginae*, and *P. bivia*, either alone or in combination, and the groups. Despite no statistical differences, we observed a higher frequency of *Gardnerella* spp. (P=0.09), *F. vaginae* (P=0.31), *P. bivia* (P=0.20), *Gardnerella* spp.+*P. bivia* (P=0.07), and all three together (P=0.41) in the persistence group. Additionally, we performed the same analysis, but considered only the samples with a high load of each individual species. There was no statistically significant difference between groups regarding the high load presence of the three bacteria analyzed, either individually or in mixed presence. However, we observed a higher frequency in the persistence group for *Gardnerella* spp. (87.0%) and *F. vaginae* (66.7%) and in pairwise combinations of *Gardnerella* spp. + *F. vaginae* (41.2%) and *Gardnerella* spp. + *P. bivia* (73.7%) (Table 5).

Additionally, a sub-analysis considering the 24-month follow-up was performed, including the women who persisted with hrHPV after 12 months and attended this visit, totaling 64 out of 131 participants. The bacterial loads (Table S2) and the presence/absence of species (Table S3) were compared between the persistence and clearance groups. No significant differences were found in the comparisons of load and presence for any species evaluated between the groups.

4. Discussion

Studies showing the association between HPV and biofilm are scarce, but a recent study showed that cervicovaginal biofilm formation is associated HPV and hrHPV positivity (42). Therefore, we hypothesized that high loads of *Gardnerella spp.*, *P. bivia*, and *F. vaginae*, BV-associated bacteria, could be observed in women with hrHPV persistence once they are the major components of the vaginal biofilm during BV (38,39). Of the three targeted species, only *Gardnerella* spp. showed higher loads associated to the persistence of hrHPV with higher loads compared to those women who cleared the infection within a year. Baseline increased *Gardnerella* spp. loads have previously been associated with the persistence of HPV16 and HPV18 in this same population

after 11 months (34). Unexpectedly, the similar findings were not observed for *F. vaginae* and *P. bivia* loads groups.

The present study observed a hrHPV persistence rate of 70.2% (131 out of 216 participants), which surpasses findings in American, Canadian, and Brazilian centers that screened 553 young women, revealing a 54.1% persistence rate for hrHPVs over a year (43). Another study with over 3000 young Dutch women reported a persistence rate of 52.1% (176 out of 338) after 12 months (44). Importantly, the microbiota in the aforementioned studies were not assessed, potentially allowing for the consideration that the prevalence of BV, already linked to HPV persistence, might be lower in those cohorts compared to the current study. These variations in rates de BV could be influenced by the economic and racial disparities specific to each country (45–48).

This cohort showed to be homogenous between groups in terms of all sociodemographic, behavioral and clinical variables, except for abnormal discharge that were more frequent in women with persistent hrHPV. This symptom is part of the Amsel criteria for the BV clinical diagnosis (49). Although the complaints show lower accuracy compared to Nugent microscopic method for BV diagnosis (50,51), Nugent classification did not change between the study groups. It could be hypothesized that immune response against HPV could potentialize the inflammation in response to BV.

The recent metagenomic subdivisions of communities (mgCSTs) have categorized CST IV into 11 mgCSTs, six of which are based on the abundance of various *Gardnerella* genomospecies. *Gardnerella* sp. 11 and *Gardnerella* sp. 13, among them, exhibit several virulence factors, such as vaginolysin, which is responsible for the lysis of epithelial cells; hemolysin, responsible for the lysis of erythrocytes; and the muralytic enzyme precursor (RpF2) that enhances the culturability of dormant bacteria (52). Although the associations between virulence factors and HPV have not been fully investigated, it has been found that the sialidase encoding gene (NanH3), another virulence factor in *Gardnerella* spp., is present in higher loads in women who are BV-positive and have HPV16-persistence after 12 months (35). Additionally, *Gardnerella* has been identified as a microbial indicator of high grade cervical intraepithelial neoplasia (CIN3) and as a biomarker for hrHPV, which reinforces its association with HPV persistence. (53,54).

Our analysis did not confirm any association between *F. vaginae* and *P. bivia* with hrHPV persistence, even when considering subsets with only HPV16 (most frequent) and other hrHPVs (excluding HPV16). Studies demonstrating an association between these BV-associated bacteria and HPV are mostly based on sequencing methods. The observed high abundance of *F. vaginae* has been previously linked to hrHPV persistence and HPV positivity, aspects that we were unable to verify with the results from our study (55,56). However, these studies included smaller sampling and postmenopausal women that influence the vaginal microbiota environment due to decreased estrogen levels. In addition, another study suggested that the dominant presence of *Atopobium* and *Prevotella* may contribute to the potential development of CIN (57).

Alongside the loads, we assessed whether the individual or combined presence of targeted genes was associated with hrHPV clearance or persistence. No association was observed, even when considering samples with high bacterial loads within an year. In contrast, other authors pointed that the combined presence of *G. vaginalis*, *F. vaginae*, and *Lactobacillus iners* a high risk for cervicovaginal cancer, but the study population age mean was over 40 years old, which is older than our study (median 28) and could influence the outcome (58). In addition, a recent study by Stoian et al. (59) demonstrated that the presence of *G. vaginalis*, *F. vaginae*, *P. bivia*, and *L. iners*, coupled with the lack of *L. crispatus*, may serve as an indicator for severe cervical lesions. However, we did not access the lactobacillus load not allowing the comparison. In fact, it is expected that Lactobacillus is diminished in the presence of high loads of Gardnerella. It is a limitation of this study since we cannot rule out the presence of high loads of the less protective *L. iners*. Additionally, the assessment through qPCR focused solely on target genes of three BV-associated bacteria, overlooking the complexity and involvement of multiple species in this condition. Additionally, with regard to the bacteria examined, the use of the 23s rRNA *Gardnerella* spp. gene allows us to identify only the *Gardnerella* genus without specifying the particular species associated with hrHPV.

Although there is no correlation between bacterial combinations and HPV, the loads of the evaluated bacteria were positively correlated with each other. This observation can be explained due to the ability of *F. Vaginae* (FV) and *P. bivia* (PB) to incorporate into the pre-existing biofilm of *G. vaginalis* (GV), as

evaluated in dual (GV+FV and GV+PB) and triple-species (GV+FV+PB) biofilm. However, the biofilm incorporation capacity of *F. Vaginae* and *P. Bivia* does not necessarily influence the expression of virulence factors in *G. vaginalis*, what could show its independent role. Sialidase, vagynolysin, and transcripts encoding antimicrobial-specific resistance proteins genes showed no significant differences in expression between Gardnerella mono-biofilm and dual or triple-species biofilms. In contrast, glycosyltransferases type II (involved in biofilm maintenance) and Rib-protein (eliciting protective immunity through *G. vaginalis* inter-strain size variability) genes were found to be overexpressed in triple-species biofilms compared to *G. vaginalis* mono-biofilm (60). Thus, it would be important to evaluate more specifically how these bacteria interactions could be associated to HPV.

Regarding the 24-month follow-up analysis, no association was found between the persistence and clearance groups concerning the loads of the three species or their presence/absence. These results suggest that for *Gardnerella* spp., a lower load may be sufficient to clear hrHPV within a year and reinforce the non-effect of *P. bivia* and *F. vaginae* on this condition, even with longer infection duration. The presence/absence of these species also did not appear to influence hrHPV outcomes. However, it is important to note that one of the study's limitations is that half of the women who persisted with hrHPV at 12 months did not attend the next follow-up, which could influence the results obtained. Another limitation is that we defined the start of HPV infection as the baseline for determining HPV clearance time, preventing us from knowing the duration of the virus's presence in the host before the baseline.

In conclusion, we suggest that *Gardnerella* spp. impact negatively the vaginal environment, independently of other BV-associated bacteria which could lead to HPV persistence after 12 months, as neither their presence nor bacterial loads differed according to the outcome of hrHPV infection. Therefore, such results contribute to better understanding the role of *Gardnerella spp.* in the HPV persistence, although additional studies investigating BV-associated bacteria and their virulence factors are essential to more accurately determine their influence on hrHPV persistence.

5. References

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6. Tables and Figures

Table 1. Distribution of high-risk HPV genotypes in the cohort study.

Baseline hrHPV genotypes detected	Frequency in single infection (n=168)	%	Frequency in mixed infection (n=48)	%	Total of infection for each hrHPV (216)	%
HPV16	45	26,79	21	43,75	66	30,56
HPV31	14	8,33	13	27,08	27	12,50
HPV52	16	9,52	11	22,92	26	12,04
HPV58	12	7,14	11	22,92	23	10,65
HPV51	15	8,93	7	14,58	22	10,19
HPV45	15	8,93	7	14,58	22	10,19
HPV18	13	7,74	8	16,67	21	9,72
HPV56	8	4,76	8	16,67	16	7,41
HPV35	6	3,57	7	14,58	13	6,02
HPV59	6	3,57	7	14,58	13	6,02
HPV73	8	4,76	5	10,42	13	6,02
HPV68	5	2,98	6	12,50	11	5,09
HPV82	3	1,79	6	12,50	9	4,17
HPV39	1	0,60	6	12,50	7	3,24
HPV33	1	0,60	2	4,17	3	1,39

Table 2. Sociodemographic, behavioral and clinical variables grouped into clearance and persistence for any-hrHPV* after 12 months follow-up.

CHARACTERISTICS	Clearance (n=85) (Any-hrHPV)	Persistence (n=131) (Any-hrHPV)	p-value**
Age, median (range)[a]	28 (17-50)	28 (17-50)	0.13
Age[b]			0.19
<35 years	61 (71.75%)	105 (80.1%)	
≥35 years	24 (28.25%)	26 (19.8%)	
Skin color[b]			0.33
Black and other[c]	35 (41.2%)	64 (48.2%)	
White	50 (58.2%)	67 (51.8%)	
Living with partner[b]			0.89
No	39 (47.0%)	63 (48.1%)	
Yes	44 (53.0%)	68 (51.9%)	
Completed high school[b]			0.67
No	34 (41.0%)	50 (38.2%)	
Yes	49 (59.0%)	81 (61.8%)	
Employment[b]			0.99
No	24 (28.2%)	36 (27.5%)	
Yes	61 (71.8%)	95 (72.5%)	
Smoking[b]			0.15
No	50 (58.8%)	90 (68.7%)	
Yes	35 (41.2%)	41 (31.3%)	
Alcohol[b]			0.40
No	43 (50.6%)	75 (57.2%)	
Yes	42 (49.4%)	56 (42.8%)	
Douching[b]			0.99
No	80 (94.1%)	124 (94.7%)	
Yes	5 (5.9%)	7 (5.3%)	
Number of sexual partners[b]	3 (1-18)	3 (1-100)	0.60
Number of sexual partners/year	1 (0-15)	1 (0-12)	0.33
Hormonal contraceptive[b]			0.19
No use	34 (40.0%)	40 (30.5%)	
Oral/injectable	51 (60.0%)	91 (69.5%)	
Condom use[b]			0.72
No	61 (71.8%)	91 (69.5%)	
Yes	24 (28.2%)	40 (30.5%)	
Abnormal discharge[b]			<0.0001
No	58 (68.2%)	52 (39.7%)	
Yes	27 (32.8%)	79 (60.3%)	
Nugent microscopic categories[b]			0.77
Normal (scores 0-3)	48 (56.5%)	74 (56.5%)	
Intermediate (scores 4-6)	10 (11.8%)	11 (8.4%)	
BV (scores 7-10)	27 (31.7%)	46 (35.1%)	
Citology altered ever[b]			0.41
No	68 (80.0%)	98 (74.8%)	
Yes	17 (20.0%)	33 (25.2%)	

*hrHPVs genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82

**P<0.05 considered as statistically significant.

[a]Mann–Whitney Test, expressed by median (minimum–maximum).

[b]Chi-squared or Fisher's exact test.

[b]Another self-reported skin color (i. e. brown ´pardo´).

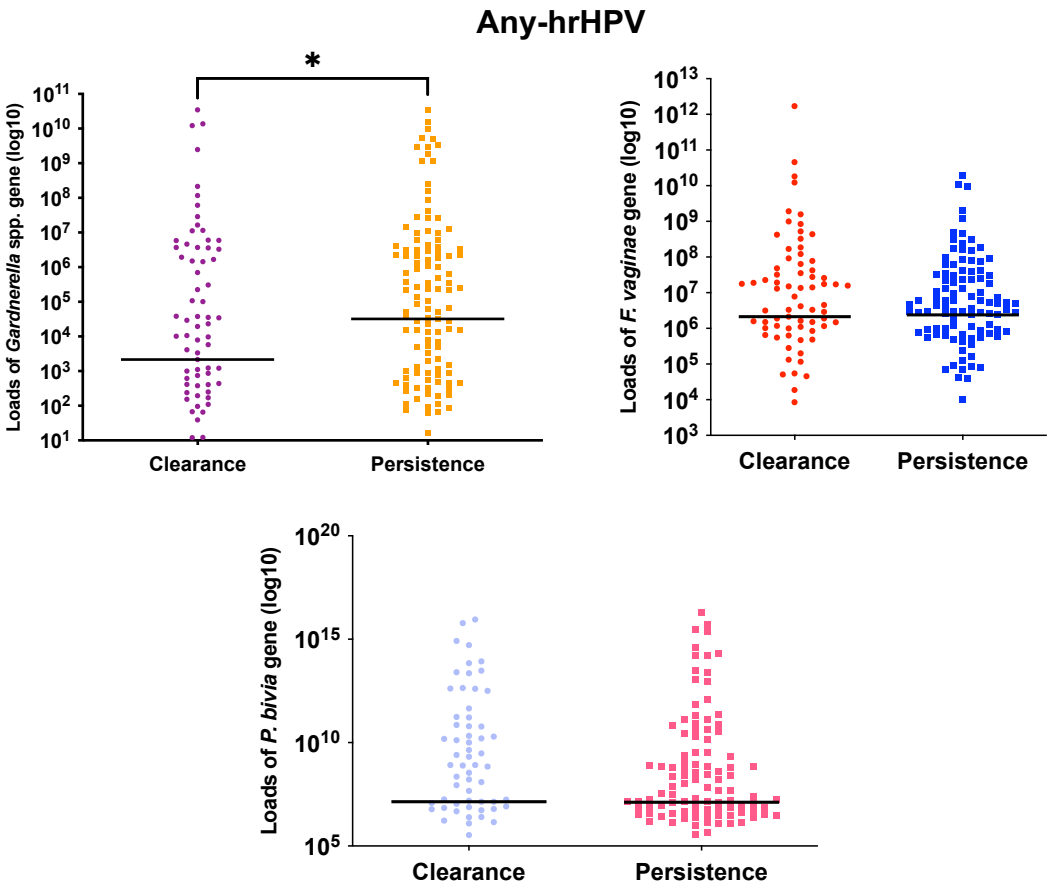

Figure 1. Scatter plot with loads of *Gardnerella* spp., *F. vaginae* and *P. bivia* between any-HPV clearance and persistence groups after 12 months follow-up. *p<0.05.

Table S1. Comparison of *Gardnerella* spp. 23s rRNA, *Fannyhessea vaginae* and *Prevotella bivia* 16s rRNA genes (copies/µl) loads considering HPV16-only or Other-hrHPV (HPV16 excluded) between 'clearance' and 'persistence' groups after 12 months follow-up.

Gene loads*	Clearance (n=17) (HPV16 only)	Persistence (n=49) (HPV16 only)	p-value**
***Gardnerella* spp. 23s rRNA gene**	1.94E05 (0-1.65E07)	1.04E06 (2.89E10)	0.52
***Fannyhessea vaginae* 16s rRNA gene**	5.52E06 (0-1.59E10)	2.65E07 (0-1.20E10)	0.80
***Prevotella bivia* 16s rRNA gene**	1.25E09 (0-4.53E13)	9.93E07 (0-2.05E14)	0.59
	Clearance (n=68) (Other hrHPV)	**Persistence (n=82) (Other hrHPV)**	
***Gardnerella* spp. 23s rRNA gene**	9.36E02 (0-3.49E10)	1.52E05 (0-3.36E10)	0.10
***Fannyhessea vaginae* 16s rRNA gene**	3.25E06 (0-1.70E10)	1.56E06 (0-1.80E10)	0.15
***Prevotella bivia* 16s rRNA gene**	1.29E07 (0-9.30E15)	1.37E07 (0-2.05E16)	0.99

*Mann-whitney test.

**P<0.05 considered as statistically significant.

Table 3. Spearman correlation rank between the bacteria loads measured in women with hrHPV after 12 months follow-up.

	Gardnerella spp.	*F. vaginae*	*P. bivia*
***Gardnerella* spp.**	1.00		
F. vaginae	0.44 (0.33-0.55)*	1.00	
P. bivia	0.36 (0.23-0.47)*	0.34 (0.21-0.45)*	1.00

*p<0.0001 considered as statistically significant. 95% confidence interval.

Table 4. Presence/absence distribution of *Gardnerella* spp., *Fannyhessea vaginae* and *Prevotella bivia* between groups after 12 months follow-up.

Bacteria presence/absence	Clearance (n=85) (Any-hrHPV)	Persistence (n=131) (Any-hrHPV)	p-value**
***Gardnerella* spp.**			0.09
Presence	66 (77.6%)	114 (87.0%)	
Absence	19 (22.4%)	17 (13.0%)	
Fannyhessea vaginae			0.31
Presence	70 (82.4%)	99 (75.6%)	
Absence	15 (17.6%)	32 (24.4%)	
Prevotella bivia			0.20
Presence	59 (69.4%)	102 (77.8%)	
Absence	26 (30.6%)	29 (22.2%)	
G.* spp + *F. vaginae			0.18
Presence	17 (20.0%)	17 (13.0%)	
Absence	68 (80.0%)	114 (87.0%)	
G.* spp + *P. bivia			0.07
Presence	5 (5.9%)	19 (14.5%)	
Absence	80 (94.1%)	112 (85.5%)	
P. bivia* + *F. vaginae			0.77
Presence	6 (7.1%)	7 (5.3%)	
Absence	79 (92.9%)	124 (94.7%)	
G.* spp + *F. vaginae* + *P. bivia			0.40
Presence	41 (48.2%)	71 (54.2%)	
Absence	44 (51.8%)	60 (45.8%)	

** P<0.05 considered as statistically significant. All variables

[a]Chi-squared or fisher's exact test.

Table 5. Distribution of high loads* of *Gardnerella* spp., *Fannyhessea vaginae* and *Prevotella bivia* between groups after 12 months follow-up.

High load	Clearance (Any-hrHPV)	Persistence (Any-hrHPV)	p-value**
***Gardnerella* spp.[a] (n=180)**	n=66	n=114	0.15
Yes	35 (53.0%)	73 (64.0%)	
No	31 (47.0%)	41 (36.0%)	
***Fannyhessea vaginae*[a] (n=169)**	n=70	n=99	0.42
Yes	42 (60.0%)	66 (66.7%)	
No	28 (40.0%)	33 (33.3%)	
***Prevotella bivia*[a] (n=161)**	n=59	n=102	0.16
Yes	44 (74.6%)	64 (62.7%)	
No	15 (25.4%)	38 (37.3%)	
***G.* spp + *F. vaginae*[a](n=34)**	n=17	n=17	0.72
Yes	5 (29.4%)	7 (41.2%)	
No	12 (70.6%)	10 (58.2%)	
***G.* spp + *P. bivia*[a] (n=24)**	n=5	n=19	0.60
Yes	3 (60.0%)	14 (73.7%)	
No	2 (40.0%)	5 (26.3%)	
***P. bivia* + *F. vaginae*[a] (n=13)**	n=6	n=7	0.59
Yes	3 (50.0%)	2 (28.6%)	
No	3 (50.0%)	5 (71.4%)	
***G.* spp + *F. vaginae* + *P. bivia*[a] (n= 112)**	n=41	n=71	0.84
Yes	20 (48.8%)	32 (45.1%)	
No	21 (51.2%)	39 (54.9%)	

*High load defined as >1,22E03 copies for *Gardnerella* spp., >1,15E05 copies for *Fannyhessea vaginae* and >1,12E06 copies for *Prevotella bivia*.

** P<0.05 considered as statistically significant.

[a]Chi-squared or fisher's exact test.

Table S2. Comparison of *Gardnerella* spp. 23s rRNA, *Fannyhessea vaginae* and *Prevotella bivia* 16s rRNA genes (copies/µl) loads between 'clearance' and 'persistence' groups after 24 months follow-up.

Gene loads*	Clearance (n=21) (Any-hrHPV)	Persistence (n=43) (Any-hrHPV)	p-value**
***Gardnerella* spp. 23s rRNA gene**	4.92E04 (0-1.57E10)	6.37E05 (2.89E11)	0.53
***Fannyhessea vaginae* 16s rRNA gene**	2.81E06 (0-1.59E10)	1.63E07 (0-1.20E10)	0.59
***Prevotella bivia* 16s rRNA gene**	4.95E06 (0-1.59E13)	1.39E07 (0-1.20E14)	0.61

*Mann-whitney test.

**P<0.05 considered as statistically significant.

Table S3. Presence/absence distribution of *Gardnerella* spp., *Fannyhessea vaginae* and *Prevotella bivia* between groups after 24 months follow-up.

Bacteria presence/absence	Clearance (n=21) (Any-hrHPV)	Persistence (n=43) (Any-hrHPV)	p-value**
***Gardnerella* spp.**			0.67
Presence	18 (85.7%)	39 (90.7%)	
Absence	3 (14.3%)	4 (9.3%)	
Fannyhessea vaginae			0.54
Presence	15 (71.4%)	34 (79.1%)	
Absence	6 (28.6%)	9 (20.9%)	
Prevotella bivia			0.54
Presence	15 (71.4%)	34 (79.1%)	
Absence	6 (28.6%)	9 (20.9%)	
G.* spp + *F. vaginae			0.27
Presence	5 (23.8%)	4 (9.3%)	
Absence	16 (76.2%)	39 (90.7%)	
G.* spp + *P. bivia			0.45
Presence	4 (19.0%)	4 (9.3%)	
Absence	17 (81.0%)	39 (90.7%)	
P. bivia* + *F. vaginae			0.10
Presence	2 (9.5%)	0 (0.0%)	
Absence	19 (90.5%)	43 (100.0%)	
G.* spp + *F. vaginae* + *P. bivia			0.29
Presence	10 (47.6%)	27 (62.8%)	
Absence	11 (52.4%)	16 (37.2%)	

** P<0.05 considered as statistically significant. All variables

[a]Chi-squared or fisher's exact test.

Capítulo IV

Conclusão final

Este trabalho apresentou a primeira caracterização metagenômica do microbioma brasileiro. A alta presença dos CSTs I, III e IV encontrados também reforça os achados anteriores nesta população. Além disso, a presença de diferentes mgSs de L. iners sugere seus potenciais efeitos positivos ou negativos para o ambiente vaginal. Ademais, o pH mais elevado associado aos mgCSTs de *G. vaginalis* enfatiza sua correlação com a VB. Por fim, a presença de ISTs nos mgCSTs de *G. vaginalis* também sugere que o ambiente vaginal pode influenciar o risco de coinfecções.

Em relação às espécies envolvidas na patogênese da VB e sua associação com o HPV de alto risco, sugere-se que as *Gardnerella* spp. afetam negativamente o ambiente vaginal, independentemente de outras bactérias associadas à VB, o que pode levar à persistência do HPV após 12 meses.