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Aspectos da microbiota vaginal relacionados à resposta inflamatória local e infecção pelo papilomavírus humano

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

Orientadora: Profa. Dra. Camila Marconi
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Dedico este trabalho a minha mãe Elvira,
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RESUMO

A microbiota vaginal normal é composta por espécies de *Lactobacillus* que, principalmente devido a capacidade de produção de ácido lático, conferem um ambiente protetor contra as infecções sexualmente transmissíveis (ISTs), incluindo pelo papilomavírus humano (HPV), que é a IST mais prevalente mundialmente. Quando há substituição de *Lactobacillus* spp. por espécies anaeróbias e facultativas como *Gardnerella vaginalis*, *Fannyhessea vaginae*, *Prevotella* spp. entre outras, tem-se uma condição conhecida como vaginose bacteriana (VB). A microbiota vaginal pode ser classificada molecularmente, de acordo com o perfil dos genes bacterianos codificantes do RNA ribossômico 16S em cinco tipos de comunidades bacterinas (CSTs, do inglês *community-state types*) após sequenciamento. Em quatro CSTs, observa-se o predomínio de *Lactobacillus* spp., sendo: *L. crispatus* a espécie dominante na CST I, *L. gasseri* na CST II, *L. iners* na CST III e *L. jensenii* na CST V. Na CST IV, observa-se a depleção de *Lactobacillus*, acompanhada por uma microbiota diversa, com abundância de espécies anaeróbias e facultativas, compreendendo a maioria dos casos de VB. Na VB, observa-se o aumento dos níveis cérvico-vaginais de citocinas inflamatórias e de produtos bacterianos, como as sialidases. Tais alterações nos componentes cérvico-vaginais reduzem a capacidade de proteção da barreira física e imunológica local. Portanto, alinhado ao objetivo de correlacionar os parâmetros da microbiota vaginal e os níveis de citocinas no ambiente cérvico-vaginal nas diferentes CSTs vaginais, foi verificado que, em geral, a CST II apresenta um perfil distinto de correlações, principalmente entre carga bacteriana e citocinas, quando comparado às outras CSTs dominadas por *Lactobacillus* (CST I e CST III). Embora os mecanismos pelos quais a VB contribua para a aquisição e persistência de ISTs não sejam completamente elucidados, acredita-se que o aumento das sialidases bacterianas locais esteja envolvido. Neste sentido, objetivou-se comparar as cargas do gene nanH3 que codifica a sialidase

de *Gardnerella* spp. no fluido cérvico-vaginal de mulheres com persistência da infecção pelo HPV de alto risco oncogênico (hrHPV, do inglês high-oncogenic risk HPV) e aquelas que eliminaram a infecção (*clearance*) após 12 meses. Observou-se que, embora não haja diferença na carga de nanH3 entre os grupos com persistência e *clearance* de hrHPV, quando considerado apenas o genótipo HPV16, a carga de nanH3 é significativamente maior em mulheres com a persistência viral. Portanto, a classificação molecular da microbiota vaginal e a correlação dos parâmetros microbiológicos com citocinas permitem o melhor entendimento nos mecanismos envolvidos na relação entre a microbiota vaginal e a maior vulnerabilidade às ISTs. Além disso, a identificação de fatores de virulência bacterianos, como as sialidases, produzidos por componentes da microbiota vaginal alterada poderá contribuir para a identificação de preditores da microbiota associados à persistência de ISTs, como o HPV e as lesões HPV-induzidas.

Palavras-chave: Microbiota vaginal**;** citocinas; sialidases**,** infecções sexualmente transmissíveis**;** papilomavírus humano.

ABSTRACT

The normal vaginal microbiota is dominated by *Lactobacillus* spp. that, mainly due the capacity lactic acid production, provide an acidified and protective environment against changes in the microbiota and the acquisition of sexually transmitted infections (STIs). The shift of *Lactobacillus* spp. by anaerobic and facultative species such as *Gardnerella vaginalis*, *Fannyhessea vaginae*, *Prevotella* sp. among others, leads to a condition known as bacterial vaginosis (BV). Molecularly, the vaginal microbiota may be classified into five community-state type (CST) by sequencing the bacterial 16S rRNA gene. In four CSTs is observed predominance of *Lactobacillus* sp.: *L. crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III) and L. jensenii (CST V), while CST IV is classified as a diverse microbiota, with an abundance of anaerobic and facultative anaerobic species, comprising most cases of BV. In BV, there is an increase in cervicovaginal levels of inflammatory cytokines and bacterial products, such as sialidases. Such alterations in the cervicovaginal components reduce the protective capacity of the local physical and immunological barrier. Therefore, in line with the objective of correlating the parameters of the vaginal microbiota and the levels of cytokines in the cervicovaginal environment in the different CSTs, it was verified that, in general, CST II presents a distinct profile of correlations, mainly between bacterial load and cytokines, when compared to the CST I and III, both dominated by *Lactobacillus* spp. Although the mechanisms by which BV contributes to the acquisition and persistence of STIs are not fully elucidated, it is believed that the increase in sialidase production are involved. In this sense, it was aimed comparing the loads of the *Gardnerella* sialidase encoding-gene (nanH3) in the cervicovaginal fluid of women with persistent infection by high-risk oncogenic HPV (hrHPV) and those who cleared the infection after 12 months. It was observed no difference in the load of nanH3 between the clearance and persistence groups when considering infection by all hrHPV genotypes, however, the

nanH3 loads is significantly higher in women with HPV16 persistent infection. Therefore, the molecular classification of the vaginal microbiota and their correlation with cytokines allow a better understanding the mechanisms involved in the relationship between the vaginal microbiota and greater vulnerability to STIs. Furthermore, the identification of bacterial virulence factors, such as sialidases, produced by components of the disturbed vaginal microbiota may contribute to the identification of microbiota predictors associated with the persistence of STIs, such as HPV and HPV-induced lesions.

Keywords: Vaginal microbiota; cytokines; sialidase; sexually transmitted infections; human papillomavirus.

SUMÁRIO

CAPÍTULO I

REVISÃO DE LITERATURA

1. MICROBIOTA VAGINAL

A microbiota vaginal saudável apresenta predomínio de espécies do gênero *Lactobacillus* sp. [1] e a comunidade científica tem constantemente confirmado a importância da colonização por tais microrganismos [2] uma vez que estes são responsáveis pela manutenção do ambiente vaginal equilibrado além de conferir defesa contra patógenos [3]. Tal capacidade protetiva se deve à capacidade dos *Lactobacillus* sp. em produzir ácido lático utilizando o metabolismo do glicogênio armazenado nas células epiteliais vaginais, bem como produção de peróxido de hidrogênio e bacteriocinas [4-7]. Neste sentido, a literatura tem sido consistente ao demonstrar que mulheres com predomínio de *Lactobacillus* sp. apresentam menor risco de aquisição de infecções sexualmente transmissíveis (ISTs), sejam estas bacterianas, como *Chlamydia trachomatis* ou *Neisseria gonorrhoeae*, ou infecções virais, como pelo vírus da imunodeficiência humana (HIV) e papilomavírus humano (HPV) [3, 8, 9].

Diferentemente da microbiota vaginal saudável, na vaginose bacteriana (VB), principal disbiose da microbiota vaginal, há uma depleção parcial ou total dos *Lactobacillus* sp. Tais espécies são substituídas por outras como *Gardnerella vaginalis*, *Prevotella bivia*, *Atopobium vaginae* (atualmente renomeada para *Fannyhessea vaginae* [10]), entre outras [11]. Trata-se de uma condição que apresenta uma prevalência próxima a 30% nas mulheres em idade reprodutiva, sendo tal prevalência compatível com a encontrada no Brasil, embora possa variar de acordo com algumas características populacionais como idade, etnia e características comportamentais [11, 12]. Deste modo, os estudos já demonstraram que a VB está associada a fatores como comportamento sexual, incluindo histórico de mais de um parceiro sexual na vida, relato de novo parceiro sexual ou início de atividade sexual em idade mais jovem, além do uso de duchas vaginais e estresse crônico [12].

A VB está associada à diversas complicações obstétricas e ginecológicas, levando a efeitos deletérios para a saúde, visto que, mulheres que apresentam essa alteração de microbiota têm risco aumentado para aquisição e transmissão de ISTs, como por *C. trachomatis*, *N. gonorrhoeae*, *Trichomonas vaginalis*, HIV e HPV [13-17]. Além disso, no contexto da infecção pelo HPV, já foi demonstrado que a VB aumenta o tempo de *clearance* viral [18].

Para o diagnóstico da VB, a partir do estabelecido por Nugent et al. (1991), leva-se em conta a observação microscópica dos morfotipos bacterianos presentes no esfregaço do conteúdo vaginal após coloração pela técnica de Gram, sendo este o padrão-ouro para o diagnóstico [19]. Desta forma, baseando-se na semiquantificação dos morfotipos presentes, são atribuídos *escores* de 0 a 10, onde a microbiota normal recebe *escores* de 0 a 3, a intermediária de 4 a 6 e *escores* superiores são compatíveis com VB [19]. Clinicamente, as queixas mais relatadas são o aumento do conteúdo vaginal associado à um mau odor genital intenso, embora cerca de 50% dos casos sejam oligo- ou assintomáticos [12]. Deste modo, os critérios de Amsel são usados para diagnosticar a VB na maioria dos ambientes clínicos e levam em consideração a avaliação do pH vaginal, uma vez que na falta de *Lactobacillus*, este está aumentado (>4,5), a presença de corrimento vaginal, o aparecimento de *clue cells* (células epiteliais vaginais descamadas repletas de bactérias anaeróbias) e um *whiff-test* positivo, ou seja, percepção de um odor característico de “peixe” ao adicionar 10% de hidróxido de potássio à uma lâmina microscópica contendo o conteúdo vaginal [7].

Molecularmente, a vasta maioria dos casos de VB está inserida dentro da comunidade bacteriana que apresenta aumento da diversidade bacteriana conforme descrito por Ravel et al. (2011) [20]. Neste sentido, graças à evolução dos métodos moleculares para identificação bacteriana, como as técnicas de sequenciamento de nova geração, tais autores demonstraram

que, independentemente da grande diversidade do microbioma vaginal, ele pode ser classificado em cinco principais tipos de comunidades (*community-state type*, CST), conforme a predominância de determinadas espécies. Assim, quatro destas CSTs apresentam predomínio de *Lactobacillus*: CST I (*L. crispatus*), CST II (*L. gasseri*), CST III (*L. iners*) e CST V (*L. jensenii*). Já a CST IV apresenta grande diversidade bacteriana, sendo a *Gardnerella* sp. encontrada em abundância nesta CST, além de *F. vaginae*, *Prevotella* sp., entre outros [20]. No Brasil, nosso grupo de pesquisa caracterizou o microbioma vaginal de mulheres em idade reprodutiva das cinco regiões do país e revelou que 96% das mulheres brasileiras apresentam microbioma vaginal pertencente somente às CST I, CST III e CST IV, sendo que quase 30% das mulheres estão incluídas na CST IV [21].

Tais CSTs, mesmo que com predomínio lactobacilar, podem diferir em alguns aspectos, uma vez que cada espécie lactobacilar possui características particulares em relação a produção de fatores protetores, como por exemplo, a produção de ácido lático [22]. Em termos de estabilidade, autores apontam que a CST com predomínio de *L. iners* é mais instável e pode variar com relação à variedade de outras espécies coabitando o mesmo ambiente, enquanto que as comunidades com predomínio de *L. crispatus* e *L. gasseri* tendem a ser mais estáveis ao longo do tempo [9, 23]. Em relação às características da população associadas às CSTs, nosso grupo de estudo demonstrou recentemente que o hábito de fumar, o número de parceiros sexuais e ducha vaginal estão associados à CST IV [21], enquanto que a CST III foi independentemente associada às características da população anteriormente associadas à VB como baixa escolaridade, múltiplos parceiros sexuais e sexo desprotegido [24]. Deste modo, podemos observar que características sociodemográficas e comportamentais são associadas à composição da microbiota vaginal e tal composição parece

ter papel relevante na manutenção de um microambiente vaginal saudável, reduzindo inclusive a ocorrência de ISTs, embora os mecanismos pelos quais as alterações de microbiota, como a VB, favoreça a aquisição e persistência de ISTs não sejam totalmente compreendidos, acredita-se que a resposta inflamatória esteja envolvida [15].

2. MICROBIOTA VAGINAL E INFLAMAÇÃO

Tem sido descrito o desenvolvimento de uma resposta inflamatória na VB, com aumento dos níveis de diversas citocinas no microambiente vaginal resultando em lesão tecidual [15]. Contudo, os resultados mostrados são diversos, apesar da vasta maioria dos estudos mostrarem que os níveis de interleucina-1β (IL-1β) está aumentando no ambiente cérvico-vaginal de mulheres com VB [25]. Com relação a citocinas como IL-6, IL-8, IL-10 e fator de necrose tumoral (TNF-α) os resultados são mais variáveis [25]. Apesar dos resultados díspares, já foi demonstrado que níveis aumentados de citocinas inflamatórias como interleucina IL-1α, IL-1β, IL-6, IL-8 e TNF-α levam ao aumento da suscetibilidade à ISTs [26-28].

Em relação à associação entre espécies bacterianas presentes na microbiota vaginal e níveis de citocinas, as espécies de *Lactobacillus* são menos propensas a ocasionar aumento de citocinas inflamatórias no ambiente cérvico-vaginal, enquanto que as espécies associadas à VB são associadas ao aumento de tais citocinas. Mais recentemente, foi sugerido a participação de *Lactobacillus* sp. na modulação da inflamação local [29] uma vez que já foi demonstrado que o *L. crispatus* está associado à diminuição da contagem de células T CD4+ ativadas na mucosa vaginal, reduzindo assim o risco de aquisição do HIV [27, 28]. Além disso, isolados de *Lactobacillus* sp. foram capazes de suprimir a produção de IL-6 e IL-8 em estudos utilizando cultura de células epiteliais [30].

Como há alteração nos níveis cérvico-vaginais de citocinas na presença da microbiota vaginal alterada [27, 31, 32] e tal aumento pode ser dependente da carga bacteriana [33, 34] várias espécies bacterianas presentes na microbiota vaginal alterada já foram associadas ao aumento de tais citocinas. Por exemplo, nosso grupo de pesquisa já demonstrou que maiores cargas de *G. vaginalis* e *F. vaginae* são associadas a níveis mais elevados de IL-1β, enquanto níveis de *Megasphaera* sp. são inversamente associados com a quantidade de IL-8 [33]. Além disso, *G. vaginalis*, *F. vaginae* e *Prevotella sp.* já foram associadas com aumento dos níveis de IL-6 e IL-8 em diversos estudos [27, 35-37].

Apesar das associações encontradas entre VB e aumento dos níveis de citocinas inflamatórias bem como entre espécies bacterianas com tais citocinas, os estudos representam uma heterogeneidade nas medições que pode envolver vários fatores, tais como: variação na composição da comunidade microbiana, tamanho amostral e variabilidade dos métodos em cada estudo [25]. A avaliação transversal da microbiota e fatores imunes, ou seja, a avaliação em apenas um único momento, podem não retratar fidedignamente tal composição devido à natureza dinâmica da microbiota vaginal que algumas mulheres apresentam [38-40]. Além disso, a ampla maioria dos estudos está baseada na classificação microscópica da microbiota vaginal ou na identificação molecular de espécies particulares e a literatura carece de estudos baseados na classificação do microbioma vaginal em comunidades quando este é caracterizado pelo sequenciamento do gene rRNA 16S. Até o momento, De Seta et al. (2019) demonstrou que não há diferença nos níveis de citocinas como IL-1α, IL-1β, IL-1ra, IL-2 e TNF-α entre as CST I (dominada por *L. crispatus*) e CST III (dominada por *L. iners*) apesar da associação entre *L. iners* com algumas citocinas como a IL-2, IL-12 e TNF-α [29]. Portanto, se faz necessário o entendimento da relação entre fatores da microbiota vaginal e imunológicos para melhor compreensão do papel do microbioma vaginal

na manutenção de um microambiente saudável e na capacidade de proteção contra agentes infecciosos e sua persistência.

3. INFECÇÃO PELO PAPILOMAVIRUS HUMANO (HPV)

O HPV, responsável pela IST viral mais comum no mundo [41], possui mais de 170 genótipos caracterizados que são classificados em 3 gêneros: Alpha-papilomavirus (α-HPV), Beta-papilomavirus (β-HPV) e Gamma-papilomavirus (γ-HPV) [42]. Destes, os α-HPVs são mais bem caracterizados e apenas a infecção por HPV pertencente a este gênero foi claramente associada a todas ou quase todas as lesões intraepiteliais escamosas e cânceres anogenitais, que inclui o câncer cervical, anal, peniano, vulvar e vaginal, sendo então considerados de alto risco oncogênico [43]. Pertencentes ao gênero α-HPVs, os genótipos de alto risco oncogênico, HPV16 e HPV18, representam aproximadamente 70% dos cânceres cervicais na maioria dos estudos epidemiológicos[44]. Além disso, estima-se também que contribuam para 88% dos cânceres anais e mais de 50% dos cânceres da vulva, vagina e pênis [45].

Acredita-se que o HPV seja responsável por até 8% de todas os casos de câncer em humanos sendo associado a 36% dos casos de cânceres penianos, 51% dos cânceres vulvares, 63% de carcinomas orofaríngeos, 64% dos cânceres vaginais, 93% dos casos de cânceres anais e 96% dos cânceres cervicais. Deste modo, mais de 600.000 cânceres são atribuídos à infecção pelo HPV em todo o mundo [43].

Além da relação com os casos de câncer anogenital, infecção por HPV também está associada a cânceres não anogenitais, uma vez que o DNA do HPV é detectável em cerca de 60% dos cânceres de orofaringe, cuja incidência tem aumentado em todo o mundo, indo em sentido contrário à incidência de outros cânceres de cabeça e pescoço, que diminuiu nas últimas três décadas [46]. Tal fato pode estar associado ao aumento do

risco de aquisição oral da infecção por HPV por via sexual, uma vez que o parceiro sexual tem risco aumentado de ter HPV+ em câncer de orofaringe quando a mulher é diagnosticada com câncer cervical [47]. Além disso, o DNA do HPV foi detectado em outros cânceres do trato aerodigestivo superior, como na papilomatose laríngea, que é causada por HPV6 e HPV11, ambos de baixo risco [48], que quando recorrente, pode evoluir para carcinoma de células escamosas, provavelmente causado pela coinfecção com HPV16 e HPV18, ambos de alto risco que são comumente detectados nos casos de cânceres de laringe [49]. Além disso, apesar de frequência variável e merecendo uma investigação mais aprofundada, a infecção por HPV também foi detectada no câncer de esôfago e sua lesão pré-maligna, o esôfago de Barrett [50]. Uma estimativa demonstrou que a infecção pelo HPV está associada a 22,2% dos casos de carcinoma espinocelular de esôfago e a 35,0% dos casos de adenocarcinoma de esôfago [51].

Embora a maioria dos α-HPVs, incluindo o HPV16, infectem as mucosas e promovam o câncer, particularmente na junção entre os epitélios escamoso e glandular, os β-HPVs e γ-HPVs e um pequeno grupo de α-HPVs infectam o epitélio escamoso cutâneo [52]. Em indivíduos saudáveis, comumente é encontrado a presença de DNA de HPV na pele, sendo identificados mais de 40 tipos de β-HPV e 50 tipos de γ-HPV identificados [53], e geralmente são considerados como parte da microbiota na pele humana saudável [54]. Apesar da infecção por β- e γ-HPVs induzirem lesões cutâneas, sugere-se que apenas β-HPVs estejam associados à indução de câncer [55], atuando como um cofator para o surgimento do câncer de pele não melanoma, comum em caucasianos, que vem apresentando incidência crescente em todo o mundo, e tem como principal fator de risco a exposição à radiação ultravioleta [56-58]. Tem sido relatado que o DNA do β-HPV é mais comumente encontrado na pele exposta ao sol [59], resultando em aumento da

replicação do β-HPV com exposição à radiação ultravioleta, o que pode desencadear a transcrição do DNA do β-HPV [60, 61].

O mais comum dos cânceres relacionados ao HPV é o câncer cervical, sendo que a maioria dos casos é diagnosticada em países de baixa e média renda. A estimativa de casos em 2020 foi de 604.000 novos casos e mais de 340.000 mortes no mundo [62]. No Brasil, a incidência de casos novos em 2022 foi de 17.010, representando um risco considerado de 15,38 casos a cada 100 mil mulheres, sendo o terceiro mais comum na população feminina. Além disso, no Brasil, o câncer do colo do útero representa a quarta causa de morte por câncer na população feminina do país, com mais de 6.600 mortes no ano de 2022, representando 6,1% dos casos de morte por câncer em mulheres [63, 64]. As estimativas para o ano de 2023 não apresenta cenário favorável para a redução do câncer do colo do útero, sendo esperados 17 mil casos no Brasil, sendo o terceiro mais incidente quando excluído o câncer de pele não melanoma [63, 64]. Apesar dos esforços dos órgãos de saúde para redução da incidência de câncer do colo do útero, incluindo medidas de prevenção primária (vacinação contra o HPV) e secundárias (triagem), tais medidas não são implementadas de forma equitativa quando se compara países desenvolvidos e aqueles em desenvolvimento. Neste sentido, é de suma importância que medidas de prevenção sejam promovidas para transformar essa situação, uma vez que os programas de vacinação contra o HPV podem reduzir as taxas futuras do câncer cervical a longo prazo [65].

4. BIOLOGIA DO VÍRUS E PATOGÊNESE VIRAL

Pertencente à família *Papillomaviridae* [66], o HPV é um vírus de 50 a 55 nanômetros que apresenta DNA circular de fita dupla, não envelopado, com aproximadamente 8000 pares de base [67] sendo que genoma viral está dividido em regiões, de acordo com a sua localização e propriedades

funcionais que são designados como precoces (E) ou tardias (L). Na região precoce encontra-se até oito genes (E1-E8) que possuem múltiplos papéis na replicação do vírus (E1 e E2), transcrição do DNA (E2), maturação e liberação das partículas virais (E4), transformação celular (E5, E6, E7) e imortalização (E6 e E7). Além disso, E6 e E7, são genes que codificam proteínas associadas à malignização de lesões [67]. A expressão destes genes ocorre durante todo o ciclo de vida viral, mas diminui durante os estágios mais tardios da infecção diferindo dos dois genes virais de expressão tardia (L1 e L2), que codificam as proteínas do capsídeo viral [68]. A região L1 é a região mais conservada e seu produto, a proteína L1, representa 80% das proteínas do capsídeo viral, sendo a proteína mais abundante e de alta imunogenicidade. As proteínas L2, juntamente com L1, contribuem para a incorporação do DNA viral dentro do vírion [67]. Há ainda uma terceira região, LCR (*long control region*), que está envolvida com a expressão gênica e replicação viral que ocorre no núcleo da célula do hospedeiro. Esta região possui entre 500 e 1000 pares de bases e está localizada entre L1 e E6 (Figura 1) [67].

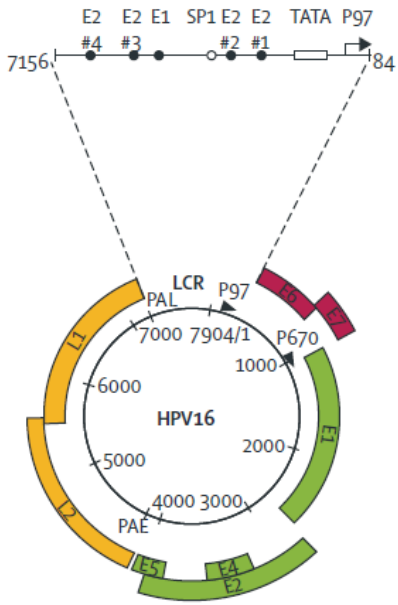

Figura 1: Representação esquemática do genoma do Papilomavírus Humano (HPV16). Adaptado de Schiffman et al. (2007) [69].

Por apresentar tropismo por células epiteliais o HPV causa infecções no tecido de revestimento, tais como a pele e mucosas, sugere-se que a infecção requer ferimento epitelial para permitir o acesso viral à lâmina basal, onde os queratinócitos basais estão localizados [68, 70]. A entrada do vírus em uma célula hospedeira se dá de forma lenta (até 12 h), provavelmente devido alterações conformacionais no capsídeo e nos receptores [71], e é iniciado pelas proteínas L1 e L2 [72-75], que após a ligação ao receptor, o vírus é internalizado na célula por endocitose [76]. A entrada do genoma viral ao núcleo ocorre devido quebra da membrana nuclear e então, o genoma viral é transportado para o núcleo e mantido como DNA episssomal [77], iniciando a fase não produtiva do ciclo de vida do vírus. Nesta fase, a expressão do gene viral é mínima, sendo que o DNA episssomal viral é amplificado para 50 a 100 cópias por célula no núcleo das células basais proliferativas [78]. Quando as células basais saem do ciclo celular e entram no processo de diferenciação, o HPV inicia a fase produtiva, aumentando significativamente a amplificação de DNA e atividade de expressão gênica [79].

Ao utilizar a maquinaria de replicação do DNA do hospedeiro, que é suprimida nas células em diferenciação, a proteína helicase E1 é expressa pelo HPV para facilitar o acesso ao DNA viral de fita simples para replicação enquanto que as oncoproteínas E6 e E7 são expressas para retardar a diferenciação celular. Há formação de um complexo de proteína E6 com a proteína supressora de tumor p53 recrutando enzimas de ubiquitinação para degradar a p53, para evitar a morte celular prematura. Por outro lado, a proteína E7 interrompe a ligação entre a proteína do retinoblastoma (Rb) e o fator de transcrição E2F, permitindo a liberação de E2F que atua ativando a transcrição de genes promotores da fase S nas células hospedeiras. Uma vez combinadas, a expressão das proteínas E6 e E7 substitui os pontos de controle do ciclo celular permitindo que o HPV se replique [80, 81]. Deste modo, o número de cópias do HPV aumenta

acentuadamente, chegando a milhares por célula nas camadas superiores do epitélio, ocorrendo a síntese de proteínas do capsídeo viral. Uma vez montadas, as proteínas do capsídeo viral formam um revestimento que encapsula o genoma viral, e os vírions são então liberados das células epiteliais infectadas diferenciadas [68, 82]. Uma vez que as proteínas virais imunogênicas são liberadas apenas por células epiteliais das camadas mais apicais, o HPV é capaz de evadir-se do sistema imunológico do hospedeiro, pois trata-se de um local menos acessível pela vigilância imunológica [83].

Considera-se que a maioria dos indivíduos com atividade sexual ativa será infectada por pelo menos um tipo de HPV genital durante a vida, onde cerca de 30 genótipos são conhecidos por causar infecção. No entanto, os HPVs podem ser divididos em genótipos de alto e baixo risco oncogênico [68]. No contexto da infecção de células do colo uterino, a ocorrência de replicação viral inicialmente causa lesões intraepiteliais escamosas de baixo grau (LSIL), também conhecidas como neoplasia intraepitelial cervical grau 1 (NIC 1), sendo lesões que apresentam apenas alterações displásicas leves, que podem ser eliminadas pelo sistema imunológico em torno de 1 ano. No entanto, se houver persistência das lesões, estas podem progredir para lesões intraepiteliais escamosas de alto grau (HSIL), também conhecidas como NIC 2 (displasia moderada) ou NIC 3 (displasia grave e carcinoma in situ) [84, 85]. Vale ressaltar que há aumento do risco de progressão para câncer cervical quando não há tratamento oferecido às mulheres que apresentam HSIL no colo do útero, uma vez que tais lesões podem durar por muitos anos. Assim, o risco de desenvolver câncer cervical invasivo em pacientes com HSIL é de aproximadamente 20% em 5 anos, aumentando para 50% em 30 anos [84, 86].

Embora os HPVs de baixo risco também produzam as proteínas E6 e E7, nos genótipos de alto risco a interação dessas proteínas com as proteínas

celulares ocorre de maneira diferente, assim a infecção persistente por HPVs de alto risco é o maior fator de risco para o desenvolvimento de câncer cervical [84, 87] devido à função complementar das oncoproteínas E6 e E7 de alto risco nas células infectadas. Por exemplo, tanto a proteína E6 do HPV de alto risco quanto a do HPV de baixo risco são capazes de se ligar a proteína p53, no entanto, apenas a E6 do genótipos de alto risco entram em contato com o domínio central da p53, sendo essencial para recrutar a ubiquitina ligase e marcar a p53 para degradação [88]. O mesmo ocorre com as proteínas E7, mas apesar de ambas interagirem com as proteínas Rb supressoras de tumor, somente as proteínas E7 de alto risco têm uma afinidade muito maior para Rb, sendo um fator essencial para interromper as interações entre Rb e E2F [89].

Outra diferença sugerida está na capacidade de integração dos genomas do HPV de alto risco no genoma do hospedeiro, sendo esta integração facilitada pelas proteínas E6 e E7 de alto risco, um evento chave para a carcinogênese associada ao HPV [90]. Ainda, a expressão dos genes E1 e E2 na fase inicial da infecção desempenha papéis essenciais na regulação negativa da expressão dos genes E6 e E7 e quando há interrupção dos genes E1 e E2 ocorre perda do controle da expressão de E6 e E7, promovendo ainda mais a progressão para o câncer. Além disso, tais proteínas (E6 e E7) podem ter maior potencial para interromper diretamente as vias normais de reparo do DNA [90]. Tal integração entre genoma do HPV de alto risco e do hospedeiro pode ocasionar tanto a deleção quanto a mutação dos genes hospedeiro e viral [91]. Diferente disso, as proteínas E6 e E7 do HPV de baixo risco desempenham papéis críticos durante o ciclo de vida viral, porém apresentam pouca atividade de transformação e não contribuem para a instabilidade genômica. Enquanto os genótipos de alto risco são capazes de manter baixo número de cópias nas células infectadas persistindo por décadas sem causar doença clínica, os genótipos de baixo risco apresentam um ciclo de vida caracterizado

pela rápida produção de prole viral e formação de grandes lesões produtivas para maximizar sua transmissão a um novo hospedeiro [92].

5. FATORES ASSOCIADOS À PERSISTÊNCIA OU *CLEARANCE* DO HPV

O desenvolvimento de câncer cervical tem como principal fator de risco a infecção persistente pelo HPV de alto risco oncogênico [84, 93]. Neste sentido, a comunidade científica tem, mais recentemente, estudado os fatores que contribuem para a persistência ou *clearance* da infecção em mulheres a fim de conhecer a população com maior vulnerabilidade de desfecho negativo, bem como possibilitar a elaboração de estratégias preventivas nas populações de risco aumentado.

Antes dos fatores associados a persistência ou *clearance* viral serem explorados, é necessário se conhecer quais destes já foi associado à infecção pelo HPV, uma vez que tais fatores podem ser distribuídos conforme os aspectos sociocomportamentais do hospedeiro, os aspectos inerentes ao vírus e fatores inerentes ao hospedeiro. Dentre os fatores sociocomportamentais a dieta pobre em nutrientes, o uso de contraceptivo hormonal, múltipla paridade, aumento de parceiros sexuais, iniciação sexual precoce, consumo de álcool e tabaco, e coinfecção por outras ISTs, como *Chlamydia trachomatis* e *Neisseria gonorrhoeae,* já foram associados a infecção por HPV. Quanto aos aspectos do vírus, como o genótipo, a presença de múltiplos genótipos e carga viral são os principais fatores associados a infecção. Dentre os fatores do hospedeiro que já foram associados à infecção pelo HPV, tem-se os hormônios endógenos, fatores genéticos, como polimorfismos de moléculas de histocompatibilidade, e fatores relacionados à resposta imunológica [94-98].

Estima-se que em cerca 70% das mulheres infectadas pelo HPV há o *clearance* viral, ou seja, eliminarão a infecção antes da integração viral ao

genoma do hospedeiro, evitando a carcinogênese, enquanto apenas uma menor porcentagem das mulheres apresenta a persistência da infecção. Neste sentido, a maioria das mulheres infectadas pelo HPV tende a eliminar o vírus dentro de 12 a 24 meses após a primeira detecção sendo que a taxa de eliminação pode variar entre 55% e 64% aos 6 meses e entre 67% e 80% aos 12 meses [99-102].

Apesar do caráter transitório da infecção, os genótipos de alto risco são aqueles que causam as infecções mais persistentes e apresentam aproximadamente o dobro do tempo de persistência quando comparado aos genótipos de baixo risco [102, 103]. Além disso, mais recentemente tem sido proposto que, além do genótipo viral, a persistência da infecção pelo HPV pode ser influenciada por outros fatores como dieta, tipos de absorventes utilizados, contraceptivos hormonais, presença de coinfecções cervicais e a presença de disbiose vaginal, como a VB [17, 104, 105], bem como ao microbioma vaginal que apresenta aumento da diversidade bacteriana, quando molecularmente categorizado pelas técnicas de sequenciamento [9, 106, 107].

6. MICROBIOTA VAGINAL E HPV

Os mecanismos envolvidos na associação entre a VB e as ISTs são parcialmente conhecidos. Sabe-se que na VB, como descrito anteriormente, há desenvolvimento de uma resposta inflamatória que aumenta os níveis de diversas citocinas no microambiente vaginal levando à lesão tecidual [15]. Além disso, enzimas bacterianas como as sialidases, são capazes de causar diversos efeitos deletérios ao microambiente vaginal causando degradação de diversos fatores protetores da mucosa vaginal e contribuindo para a esfoliação e descolamento das células epiteliais vaginais [108] facilitando a adesão bacteriana ao epitélio [109-111]. Por exemplo, tais enzimas podem clivar oligossacarídeos de mucina, resultando em redução da viscosidade do muco cérvico-vaginal,

comprometendo a barreira física e bioquímica contra patógenos [112]. Além disso, as sialidases comprometem as barreiras de proteção da imunidade inata devido sua capacidade de hidrolisar a Imunoglobulina-A [112-114]. As sialidases podem ainda hidrolisar o ácido siálico das células epiteliais do hospedeiro, fornecendo uma fonte de carbono para *Gardnerella* sp., permitindo sua captação e catabolismo. Além disso, ocorre a exposição de sítios de ligação, facilitando a adesão bacteriana ao epitélio e posterior formação de biofilmes, uma condição que contribui para a persistência da VB [109-111].

A produção de sialidase é um dos fatores de virulência mais importantes da *Gardnerella* sp. [115, 116], espécie bacteriana amplamente encontrada em casos de VB [19, 33]. Recentemente, novos estudos concluíram que nestas espécies, o genes responsáveis pela atividade da sialidase são os genes nanH2 e nanH3 [117], ao contrário do que se acreditava anteriormente, sendo tal atividade atribuída ao gene da sialidase A [118], atualmente renomeado para nanH1[117]. Vale ressaltar que apesar da atividade da sialidase ser encontrada na presença do gene nanH2, este quase não é encontrado sem a presença do nanH3 [116, 117].

Estudos já demonstraram que a VB é fator de risco para prevalência, incidência e persistência do HPV [18, 119], enquanto que estudos moleculares que classificam a microbiota vaginal em CST, demonstraram que a CST IV apresenta maior prevalência da infecção pelo HPV e mulheres incluídas dentro de CST com predomínio lactobacilar apresentam menor tempo de *clearance* da infecção [9, 119]. De fato, tanto a VB caracterizada microscopicamente, quanto a CST IV, apresentam em sua composição a presença de *G. vaginalis* [11, 19, 20, 120] e recentemente nosso grupo de estudo, ao quantificar a presença de *G. vaginalis* em mulheres infectadas por HPV, demonstrou que mulheres com infecção persistente por HPV16 e HPV18 apresentaram maiores cargas desta espécie comparadas àquelas que eliminaram a infecção [121].

Estudos sobre a microbiota vaginal demonstraram associação entre *Gardnerella* sp. e infecção persistente por HPV de alto risco, bem como progressão da lesão cervical [106, 107]. Recentemente, a partir dos resultados da associação entre *Gardnerella* sp. com persistência e progressão da infecção por HPV de alto risco, Usik et al. propõem que além da presença de *Gardnerella* sp. na persistência da infecção, esta espécie contribui com a alteração da composição da microbiota vaginal, levando para um estado polimicrobiano ao contribuir com o aumento da diversidade bacteriana, que por sua vez também contribui para a persistência e progressão da infecção pelo HPV [107].

Considerando os efeitos deletérios da sialidase para o ambiente cérvico-vaginal e as associações demonstradas entre a *Gardnerella* sp. e a infecção e persistência do HPV, é importante estudar a relação do gene nanH3, responsável pela atividade da sialidase, com a persistência ou *clearance* do HPV de alto risco, uma vez que a microbiota vaginal alterada tem sido associada à persistência da infecção pelo HPV de alto risco oncogênico.

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OBJETIVOS

8. OBJETIVO

I. Testar as correlações entre parâmetros da microbiota vaginal e os níveis de citocinas no ambiente cérvico-vaginal nas diferentes CSTs vaginais determinadas pelo sequenciamento do gene bacteriano codificante do RNA ribossômico 16S;

II. Comparar as cargas do gene nanH3, codificante da sialidase de *Gardnerella* sp., no fluido cérvico-vaginal de mulheres com infecção persistente por hrHPV com aquelas que eliminaram a infecção no período de 12 meses.

CAPÍTULO II

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Covariates of vaginal microbiota and pro-inflammatory cytokines levels in women of reproductive age

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ABSTRACT

This study aimed to assess the correlation between covariates of the vaginal microbiota and local levels of pro-inflammatory cytokines in women of reproductive age presenting four molecularly-defined bacterial community-state types (CSTs). We enrolled 133 non-pregnant women who attended primary care health clinics for routine Pap-testing. Molecular profiling of vaginal microbiota was performed by V3-V4 16S rRNA sequencing (Illumina). The covariates of vaginal microbiota included were: vaginal pH, total bacterial cell count, diversity (Shannon index), -richness and dominant taxa abundances. Levels of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF-α) were measured by enzyme-linked immunosorbent assays in supernatants of cervicovaginal fluids. Nonparametric Kruskal-Wallis test was used to compare microbiota covariates and cytokines among different CSTs. Spearman's tests were performed to assess correlations across the measured parameters. A total of 96 (72.2%) participants had CSTs dominated by *Lactobacillus* spp. (*L. crispatus* CST I, n=38; *L. gasseri* CST II, n=20; and *L. iners* CST III, n=38). A total of 37 (27.8%) presented the *Lactobacillus*-depleted CST IV. Total bacterial count was higher in CST II (1.29E+05, 3.40E+04-6.69E+05) compared to other *Lactobacillus*-dominated CSTs (p=0.0003). The highest values of microbiota diversity (1.85; 0.23-2.68) and richness (27.0; 5.0-37.0) were observed in CST IV (p<0.0001). Lower levels of IL-1β were observed in CST I (5.4; 0.0-3,256) when compared to CST III (51.7; 0.0-2,616) and to CST IV (56.2; 0.0-3,407) (p=0.008). Levels of IL-6 were higher in CST II (4.13; 0-131.4) than in CST IV (0.0-58.27) (p=0.02). Correlation tests showed an overall distinct profile of CST II when compared to other *Lactobacillus*-dominated CSTs, particularly regarding the correlation between total bacterial load and cytokines (r>0.39). In conclusion, this study provides evidence of a single pro-inflammatory signature of *L. gasseri*-dominated microbiota in response to bacterial load. Further studies evaluating a broader range of inflammation markers are warranted.

Keywords: *Lactobacillus* spp., bacterial vaginosis, bacterial count, bacterial diversity, 16s rRNA sequencing

1. INTRODUCTION

Species belonging to the *Lactobacillus* genus are the main components of the ´optimal´ vaginal microbiota, acting as first line of defense against pathogens. Bacteriocins, hydrogen peroxide and lactic acid are the main protective factors produced by *Lactobacillus* spp. (Lewis et al., 2017; O'Hanlon et al., 2013).

A recent report suggested the participation of *Lactobacillus sp.* in the modulation of local inflammation (De Seta et al., 2019). *Lactobacillus crispatus* already has been associated with decreased count of activated mucosal $CD4^+$ T cells, thus reducing risk of acquiring human immunodeficiency virus (HIV) (Gosmann et al., 2017; Masson et al., 2015). The overgrowth of a diverse array of non-*Lactobacillus* spp. in the vaginal microenvironment leads to changes in the cervicovaginal levels of cytokines (Gosmann et al., 2017; Hedges et al., 2006; Santos-Greatti et al., 2016) in a load-dependent manner (Marconi et al., 2013; Nikolaitchouk et al., 2008). Increased levels of inflammatory cytokines as interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor-alpha (TNF-α) lead to increased susceptibility to sexually transmitted infections (STIs), including HIV (Anahtar et al., 2015; Gosmann et al., 2017; Masson et al., 2015).

Culture- and molecular-based studies showed that total load of bacterial cells is associated with changes in the cervicovaginal cytokine levels. However, the accuracy of such approaches for determining the bacterial counts may not be sufficiently accurate (Marconi et al., 2013; Nikolaitchouk et al., 2008). Thus, flow cytometry has been employed as reliable tool for determining the bacterial loads in the cervicovaginal fluid samples (Luchiari et al., 2016; Schellenberg et al., 2008).

Using molecular techniques, the composition of vaginal microbiota can be assessed by sequencing of 16S ribossomal RNA (rRNA) hypervariable regions and further clustered into community-state types (CSTs). The pioneer study conducted by Ravel et al. (2011), described four CSTs dominated by certain *Lactobacillus* spp., as follows: *L. crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III) and *L. jensenii* (CST V). The *Lactobacillus*-depleted CST IV shows increased bacterial diversity when compared to other CSTs and comprise most of the cases of microscopic bacterial vaginosis (BV) (Ravel et al., 2011).

Although microbial and cytokine parameters of BV have been deeply investigated (Mitchell and Marrazzo, 2014; Marconi et al., 2013), only few molecular-based studies about cytokines in each CST assessed by sequencing were performed up to date (De Seta., 2019; Gosmann et al., 2017). Since the vaginal microbiota is highly diverse, especially considering the types of *Lactobacillus*-dominated CST, this study aimed (i) to determine the differences in microbiota covariates and cytokines across CSTs; (ii) test the correlations between parameters of vaginal microbiota with levels of cytokine in the cervicovaginal environment for each CST.

2. MATERIALS AND METHODS

2.1. Population and sampling procedures

This study included a subset of 133 out of 609 participants of a prior study that characterized the vaginal microbiota of Brazilian woman of reproductive age (Marconi et al., 2020). Women were enrolled when attending primary care health clinics for Pap-testing if aged between 18 and 50 years-old, not pregnant, not using intrauterine device, no reported urinary loss or infection and at least five days since the end of last menstrual period. Women were not included if they reported use of any antimicrobial drug within the 45 days prior to enrollment or had less than 72 hours of sexual abstinence.

All participants were informed about the purpose of the study and signed the informed consent form. The study was reviewed and approved by the Ethics Committee of Botucatu Medical School (Approval numbers: 306.547 and 2.929.149). The sociodemographic, behavioral and gynecological variables were assessed using a structured questionnaire.

Vaginal pH was measured by allowing the contact of a pH strip with mid-vaginal wall and compared with the color scale provided by the manufacturer (Merck, Darmstadt, Germany). A sterile swab was used for sampling the middle third of the vaginal wall and stored in Amies liquid transport medium (Copan, Brescia, Italy) at -80^{0}C. Using MoBio Powersoil Kit (MoBio Lab Inc, Carlsbad, CA) the total bacterial DNA was extracted, according to previous published and validated procedure (Fadrosh et al., 2014; Holm et al., 2019). The V3-V4 regions of the 16S rRNA gene was amplified and sequenced on an Illumina MiSeq platform (Illumina Inc.) according to the methodology developed by Fadrosh et al. (2014) and the sequence analysis details were previously reported by Marconi et al. (2020). Briefly, assembled reads were de-multiplexed by binning sequences with the same barcode and quality trimmed in QIIME (version 1.8.0) (Fadrosh et al., 2014). Samples with at least 1000 reads were used for subsequent microbiota analysis. Both *de novo* and reference-based chimera detection were conducted in UCHIME (v5.1) using Greengenes database of 16S rRNA sequences (Mcdonald et al., 2011). Study samples were then clustered into CSTs based on their taxonomic composition, taxa relative abundances and Jensen-Shannon divergence metrics, according to methods previously described by Ravel et al., (2011). Cervicovaginal fluid samples were obtained by inoculating 3 mL of saline solution with a sterile pipette into the posterior vaginal wall and ectocervix. After homogenizing with the local content, the fluid was recovered with the same pipette and stored at -80^{o}C until total bacterial count using flow cytometry and measurement of pro-inflammatory cytokine levels.

2.2. Study design and selection of participants

To perform the total bacterial count and cytokine levels, sample size was calculated based on estimates of mean total bacterial counts determined by flow cytometry in normal cervicovaginal fluid and with BV (Luchiari et al., 2016). Given the expected minimum difference of log 13.5 and standard deviation of log 2131.5 (Colony-forming unit) between the two groups and considering a type I error of 5% and a type II error of 10%, minimum sample size of 20 was obtained for each group. Thus, 133 were participants selected using random number tables

using Microsoft Excel 2016 (Microsoft Corporation, Redmond, Washington) out of the original study dataset of the of 609 women previously divided according to molecular CSTs in CST I (*L. crispatus*-dominant, n=186), CST II (*L. gasseri*-dominant, n=27), CST III (*L. iners*-dominant, n=222), CST IV (*Lactobacillus*-depleted, n=167), and CST V (*L. jensenii* -dominant, n=7) (Marconi et al., 2020). Samples that tested positive (n=40) for *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and/or *Trichomonas vaginalis* tested by molecular methods and *Candida* sp. morphotypes detected by microscopy (n=47) were excluded prior to selection. For details on the infections evaluated, please refer to the parent study (Marconi et al., 2020). The 133 cervicovaginal samples analyzed for the current study fitted into CST I (n=38), CST II (n=20), CST III (n=38) and CST IV (n=37). Since CST V is underrepresented in our population, it was not included in the analysis.

2.3. Microbiota diversity, richness and taxa relative abundances

A total of 122 taxa were identified through V3-V4 16S rRNA sequencing in this study population (Marconi et al., 2020). Bacterial diversity was calculated by the Shannon-Wiener index calculated using the *vegan* package in R (Oksanen et al., 2013). Richness was defined as the total number of taxa identified for each sample. Taxa relative abundances (RA) were obtained after Centered Log-Ratio (CLR) transformation using the MicrobiomeAnalyst (Chong et al., 2020).

2.4. Assessment of total bacterial load

Twenty-four hours before analysis, cervicovaginal fluid samples were thawed. Aliquots of 400 µL of each sample and diluted 1:10 in sterile saline solution and fixed with 200 µL of 4% paraformaldehyde for 16h at 4°C. Around one hour before reading on flow cytometer the samples diluted and fixed were stained with 8 µl of 0.002% propidium iodide (BD, San Jose, CA) and incubated at 37 °C for 15 minutes according to Schellenberg et al. (2008). The samples were then centrifuged at 800 rpm for 20 seconds to remove larger residues. A 500 uL volume of supernatant was divided in equal volumes of 250uL into two different tubes, first was used for cytometer calibration and the second for bacterial count after the addition of 100 µL of AccuCheck Counting Beads fluorescent microspheres with two populations of these microspheres (Invitrogen, Rockville, MD). These analyzes were performed on a FACSCalibur (BD, San Jose, CA) and the data were acquired and processed using the Cell Quest Pro (Version 5.2, San Jose, CA) with a double threshold of 280 in forward scatter (FSC) as a primary parameter and in side scatter (SSC) as a secondary parameter, both in logarithmic scale.

The pattern of sample readings showed that the bacterial and fluorescent microsphere populations can be differentially identified in the cervicovaginal fluid samples, first observed in the lower portion of the FSC x SSC graph and second represented in the upper portion (Figure 1A). For the determination of the total bacterial count, the volume of samples was considered equivalent to the reading of 5000 events of the fluorescent microspheres. As seen in Figure 1B, the bacterial cell count is determined at gate (R2) excluding the two types of fluorescent

structures. The final bacterial count was expressed in bacterial cell unit per µL of cervicovaginal fluid.

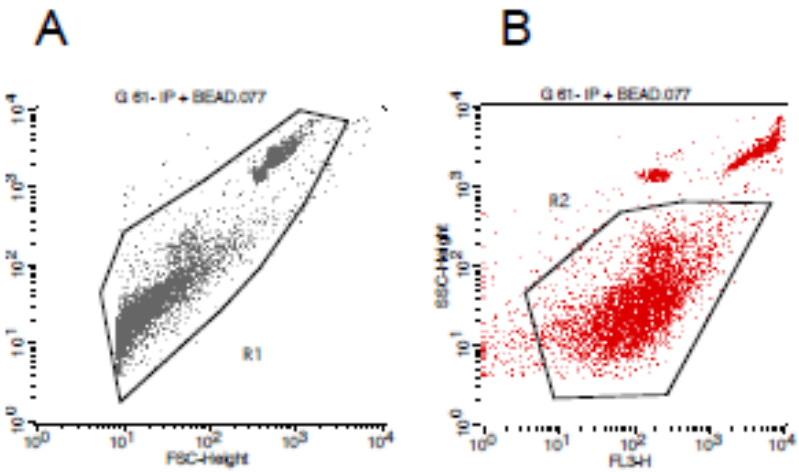

Figure 2: A) Scatterplot pattern of a cervicovaginal fluid sample. Bacterial populations are observed in the lower portion and fluorescent microparticles in the upper portion (delimited area - R1); B) Delimited area used for the total bacterial count (R2), after counting 5000 events in the two upper populations comprised by two types of fluorescent microparticles.

2.5. Cervicovaginal levels of IL-1β, IL-6, IL-8 and TNF-a

Interleukin-1β, IL-6, IL-8 and TNF-α levels were measured in the supernatants of cervicovaginal fluid sample by ELISA using Duo Set Kits (R&D Systems, Minneapolis, MN), according to manufacturer's instructions. Samples with cytokine values set above the standard curve range were diluted (1:2 and 1:4) and reassayed. The minimum detectable levels in the IL-1β, IL-6, IL-8 and TNF-α assays were, respectively, 0.34 pg/mL, 0.06 pg/mL, 1.20 pg/mL and 0.12 pg/mL. Intra and inter-assay variability were <10.0% for all cytokines.

2.6. Statistical Analysis

Sociodemographic, behavioral, and clinical characteristics were compared between women with *Lactobacillus*-dominated CSTs (CST I, CST II and CST III) and those with the *Lactobacillus*-depleted CST IV using the chi-square test or Fisher's exact test. Additionally, those variables were also compared across CSTs using the same approach. The Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test and P value adjustment method by Bonferroni was used to compare the microbiota covariates (vaginal pH, flow cytometry total bacterial cell count, molecular-diversity (Shannon index), -richness and -taxa abundances and cervicovaginal levels of cytokines). Spearman's correlation test was performed to test the correlation across microbiota and cytokine parameters in each CST as well as across all samples included in this study. Statistical analyzes were performed using the GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA) and Rstatix package for R. The correlation matrixes were built using Ggplot2 package for R (Wickham, 2016). Heatmap was built in the MicrobiomeAnalyst (Chong et al., 2020).

3. RESULTS

Sociodemographic, behavioral, and clinical characteristics of the 133 study participants are detailed in Table 1, according to the presence of a *Lactobacillus* dominant-CST, as well as across all the four CSTs included in the study (CSTs I to IV). Most of the participants were between 30 and 39 years-old (n=51, 31.3%). No association was observed for sociodemographic variables with vaginal microbiota. Regarding participants who reported genital symptoms, abnormal vaginal discharge (p=0.02) and odor (p=0.003) were more frequent among those with *Lactobacillus*-depleted CST IV. Abnormal vaginal discharge remained with a borderline significance when comparing across all CSTs (p=0.05). As expected, cases of microscopic bacterial vaginosis (i. e. Nugent score above 6) were more frequent among participants with CST IV. The heatmap in Figure supplementary 1 shows the relative abundance of the top 44 abundant taxa identified along each CST.

Table 2 shows differences in the median values of microbiota covariates and pro-inflammatory cytokine levels according to CSTs. Regarding the total bacterial count observed at each CST, the highest number of bacterial cells per volume of cervicovaginal fluid sample was showed by CST II when compared to other *Lactobacillus*-dominated CSTs (p<0.0003). The highest vaginal pH measurements were observed in CST IV (p=<0.0001).

In relation to cytokine levels, IL-1β concentrations were significantly higher in CST III and CST IV when compared with CST I (p=0.008). Levels of IL-6 differed statistically between CST II (highest) and CST IV (lowest) (p=0.02). No difference in IL-8 and TNF-α levels was observed among the CSTs (p=0.08 and p=0.09, respectively) (Table 2).

Figure 2 displays correlation matrices of each CST including all microbiota and cytokine parameters evaluated. For detailed Spearman correlation ranks, please refer to Table S1. For CST I, the stronger positive correlations were observed between IL-1β and IL-8 (r = 0.52; p <0.001) (Figure 3-A). Regarding CST II, positive correlations were observed between bacterial load and pH (r = 0.49; p <0.05), IL-1β (r = 0.56; p <0.01), IL-6 (r = 0.57; p <0.01), IL-8 (r = 0.49; p <0.05) and TNF-α (r = 0.48; p <0.05) (Figure 3-B). CST III showed strong positive correlation between vaginal pH and TNF-α (r = 0.43; p <0.01). (Figure 3-C). Finally, for CST IV, the strongest positive correlations were observed between IL-6 and IL-8 (r = 0.42; p <0.01) (Figure 3-D).

On the other hand, Figure 3 displays a matrix correlation of all samples included in this study. The main positive correlations were observed between vaginal pH and Shannon-index (r = 0.40; p<0.001), vaginal pH and IL-1β (r = 0.25; p<0.05), bacterial load correlated positively between RA of *G. vaginalis* and *L. gasseri* (r = 0.29 and 0.32, respectively; p<0.001), RA of *G. vaginalis* and Shannon-index (r = 0.46; p p<0.001). Negative correlations were observed between the RA of *L. iners* and *L. crispatus* and Shannon-index (r = -0.21 and -0.50; p<0.01 and p<0.001, respectively), RA of *L. crispatus* and *G. vaginalis* and vaginal pH (r = -0.56 and r = -0.42, respectively; p<0.001). For more details, please refer to Table S2.

Table 1: Sociodemographic, behavioral, and clinical data of the 133 women included in the study according to *Lactobacillus* spp. dominant CST (grouped CST I, II and III) and the *Lactobacillus*-depleted CST IV, as well as fully stratified by CST.

	***Lactobacillus* spp. dominant CST (n=96)**	***Lactobacillus* depleted (CST) (n=37)**	**P-value***	**CST I (n=38)**	**CST II (n=20)**	**CST III (n=38)**	**CST IV (n=37)**	**P-value**
Age (years)			0.75					0.42
18-29	28 (29.2)	13 (35.1)		9 (23.7)	7 (35.0)	12 (31.6)	13 (35.1)	
30-39	37 (38.5)	14 (37.8)		18 (47.4)	9 (45.0)	10 (26.3)	14 (37.8)	
>40	31 (32.3)	10 (27.0)		11 (28.9	4 (20.0)	16 (42.1)	10 (27.0)	
Marital status			0.10					0.33
Living with partner	67 (69.8)	20 (54.1)		25 (65.8)	14 (70.0)	28 (73.7)	20 (54.1)	
Single	29 (30.2)	17 (45.9)		13 (34.2)	6 (30.0)	10 (26.3)	17 (45.9)	
Ethnicity			0.28					0.49
Black/Brown	49 (51.0)	24 (64.9)		18 (47.4)	10 (50.0)	21 (55.3)	24 (64.9)	
White	43 (44.8)	11 (29.7)		17 (44.7)	9 (45.0)	17 (44.7)	11 (29.7)	
Other	4 (4.2)	2 (5.4)		3 (7.9)	1 (5.0)	0 (0)	2 (5.4)	
Education level[+]			0.67					0.57
≤ 8 years	30 (68.8)	10 (27.0)		12 (31.6)	4 (20.0)	14 (36.9)	10 (27.0)	
≥ 9 years	66 (31.3)	27 (72.9)		26 (68.4)	16 (80.0)	24 (63.1)	27 (72.9)	
Condom use			0.84					0.68
Yes	37 (38.5)	15 (40.5)		14 (36.8)	10 (50.0)	13 (34.2)	15 (40.5)	
No	59 (61.5)	22 (59.5)		24 (63.2)	10 (50.0)	25 (65.8)	22 (59.5)	
Hormonal contraceptive use			0.69					0.23
Yes	39 (40.6)	17 (45.9)		14 (36.8)	12 (60.0)	13 (34.2)	17 (45.9)	
No	57 (59.4)	20 (54.1)		24 (63.2)	8 (40.0)	25 (65.8)	20 (54.1)	
STI history			0.38					0.86
Yes	23 (23.9)	11 (29.7)		8 (21.1)	5 (25.0)	10 (26.3)	11 (29.7)	
No	73 (76.0)	26 (70.3)		30 (78.9)	15 (75.0)	28 (73.7)	26 (70.3)	
Referred abnormal vaginal discharge			0.02					0.05
Yes	35 (36.5)	22 (59.5)		12 (31.6)	10 (50.0)	13 (34.2)	22 (59.5)	

No	61 (63.5)	15 (40.5)		26 (68.4)	10 (50.0)	25 (65.8)	15 (40.5)	
Referred unpleasant genital odor			0.003					0.03
Yes	17 (17.7)	16 (43.2)		8 (21.1)	2 (10.0)	7 (18.4)	16 (43.2)	
No	79 (82.3)	21 (56.8)		30 (78.9)	18 (90.0)	31 (81.6)	21 (56.8)	
Nugent score			0.0001					0.0001
0-3	75 (78.1)	6 (16.2)		36 (94.8)	13 (65.0)	26 (68.4)	6 (16.2)	
4-6	10 (10.4)	2 (5.4)		1 (2.6)	4 (20.0)	5 (13.2)	2 (5.4)	
7-10	11 (11.5)	29 (78.4)		1 (2.6)	3 (15.0)	7 (18.4)	29 (78.4)	

*Qui-squared or Fisher exact test
Values represented in number of cases followed by percentages (%)
+ ≥ 9 years correspond to complete high school degree or higher
CST: community state type
STI: sexually transmitted infection

Table 2: Median values of microbiota covariates and pro-inflammatory cytokine levels and their respective range according to the four molecular community state type (CST) identified in the study population.

Variables	CST I (n=38)	CST II (n=20)	CST III (n=38)	CST IV (n=37)	P-value*
Bacterial load (cells/µL)	3.60E+04 (3.55E+03 – 4.12E+05)	1.29E+05 (3.40E+04 - 6.69E+05)	4.36E+04 (2.44E+03 - 5.07E+05)	5.40E+04 (2.75E+03 - 5.01E+05)	**0.0003[a]**
Bacterial diversity#	0.33 (0.13-1.07)	0.62 (0.08-1.57)	0.29 (0.00-2.26)	1.85 (0.23-2.68)	**<0.0001[b]**
Richness	16.00 (4.00-38.00)	23.50 (11.00-35.00)	16.50 (2.00-53.00)	27.00 (5.00-37.00)	**<0.0001[c]**
Vaginal pH	4.40 (4.00-5.00)	4.40 (4.00-5.00)	4.70 (4.00-6.00)	5.00 (4.00-5.80)	**<0.0001[d]**
IL-1β (pg/mL)	5.41 (0-3,256)	28.86 (0.35-746.5)	51.74 (0-2,616)	56.22 (0-3,407)	**0.008[e]**
IL-6 (pg/mL)	1.01 (0-49.62)	4.13 (0-131.4)	4.77 (0-316.6)	0.00 (0-58.27)	**0.02[f]**
IL-8 (pg/mL)	56.17 (0-3,205)	64.57 (10.92-5,582)	59.96 (0-4,604)	41.66 (1.20-220.7)	0.08
TNF-α (pg/mL)	0.00 (0-20.07)	2.86 (0-13.28)	0.00 (0-49.23)	0.00 (0-33.11)	0.07

*Kruskal Wallis test followed by Dunn's multiple comparison test (P< 0.05); P value adjustment method: Bonferroni
[a]CST II > CST I (Padj = 0.0001) and; CST II > CST III (Padj = 0.0051)
[b]CST IV > CST I (Padj = 0.000); CST IV > CST II (Padj = 0.0003) and; CST IV > CST III (Padj = 0.000)

[c]CST II > CST I (Padj = 0.0027); CST II > CST III (Padj= 0.0084); CST IV > CST I (Padj = 0.000) and; CST IV > CST III (Padj = 0.0002)
[d]CST III > CST I (Padj = 0.0146); CST IV > CST I (Padj = 0.000) ; CST IV > CST II (Padj = 0.0002) and; CST IV > CST III (Padj = 0.0092)
[e]CST III > CST I (Padj = 0.0209) and; CST IV > CST I (Padj = 0.0045)
[f]CST II > CST IV (Padj = 0.0139)
IL: Interleukin
TNF: Tumor Necrosis Factor

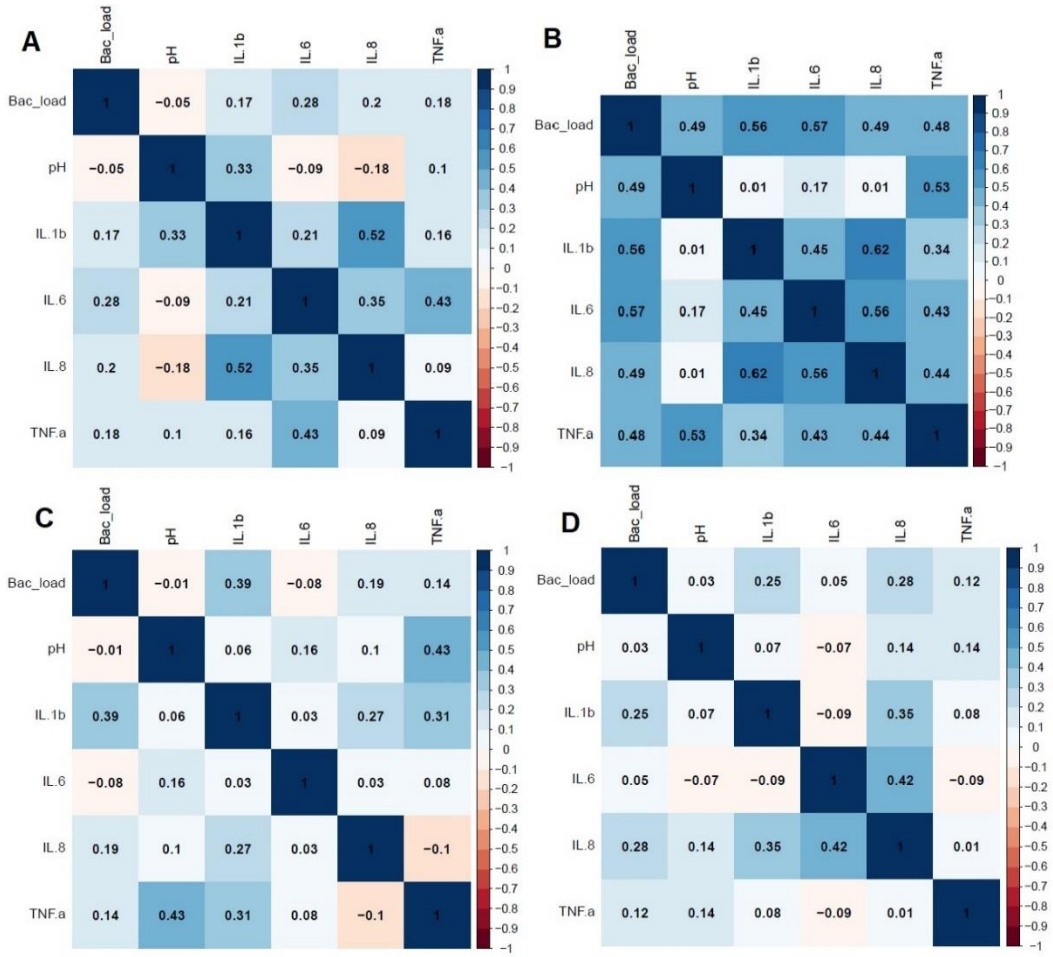

Figure 2: Correlation matrices of Spearman correlation rank between the microbiological and immunological parameters assessed in each community state type (CST). A) CST I, dominated by *L. crispatus*; B) CST II, dominated by *L. gasseri*; C) CST III, dominated by *L. iners*; CST IV, non-*Lactobacillus*-dominated. IL: Interleukin TNF: Tumor necrosis factor.

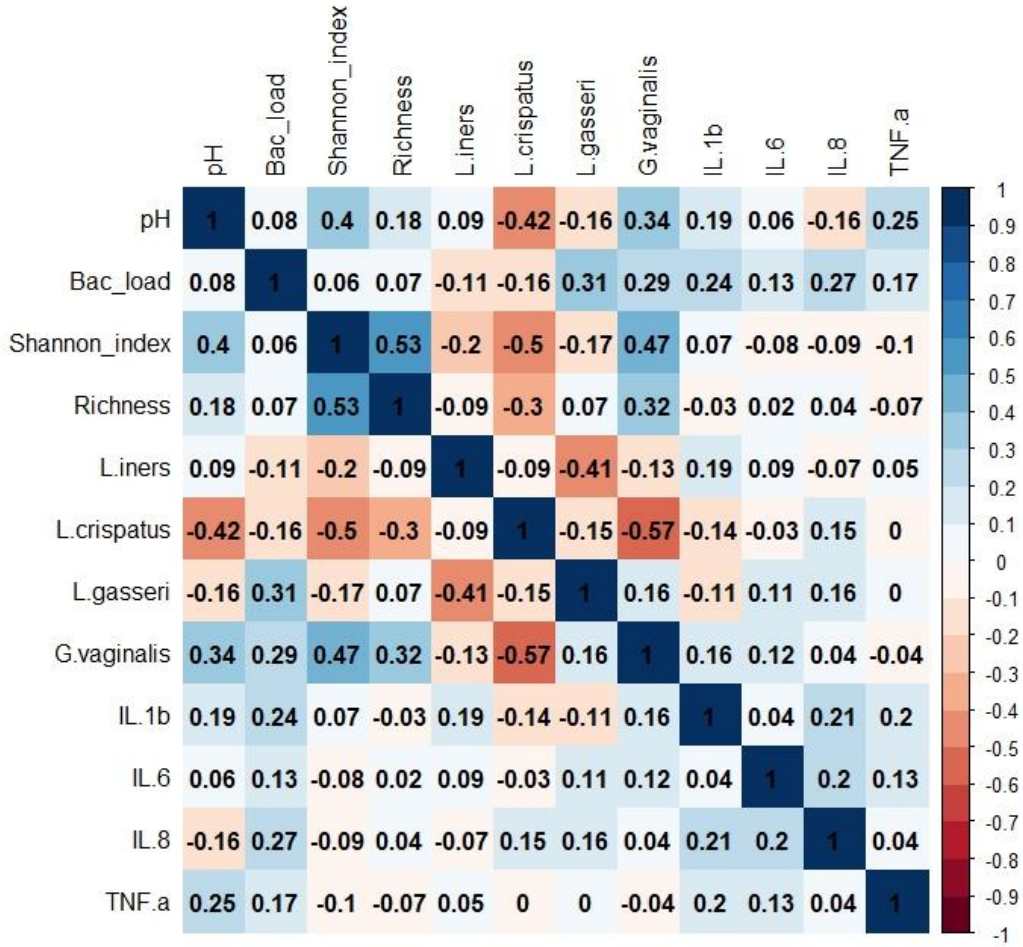

Figure 3: Correlation matrix of Spearman correlation rank between the microbiological and immunological parameters assessed in all samples. Data of relative abundance was considered for bacterial species. IL: Interleukin TNF: Tumor necrosis factor.

4. DISCUSSION

Despite the increasing knowledge on the relation between vaginal microbiota and the host´s immunity, there are still several gaps for the full understanding on how microbial components are related to a woman´s genital health. Literature lacks information on the number of bacterial cells are present on the different molecular-determined microbiota types, i.e., CSTs. Also, data from studies assessing pro-inflammatory cytokines associated with CSTs have presented conflicting results (De Seta et al., 2019; Gosmann et al., 2017). Thus, up to the best of our knowledge this is the first report of the total bacterial count using flow cytometric among the different molecular defined-CST and correlation with other microbiota covariates and pro-inflammatory cytokines. Previous study by our group showed that total bacterial counts assessed by flow cytometry did not differ between Nugent-defined BV and normal microbiota (Lucchiari et al., 2016). Current data allowed the stratification of ´optimal microbiota´ into three *Lactobacillus*-dominated CSTs and showed that number of bacterial cells is higher

in *L. gasseri*-dominated CST II. In addition, correlation analysis between covariates of vaginal microbiota and pro-inflammatory cytokines were assessed for each CST, showing that overall patterns of CST II have a difference from other *Lactobacillus*-dominated microbiota, especially regarding the correlation between bacterial load and cytokine levels.

The *L. crispatus*-dominated CST I is often considered as the ´optimal´ microbiota due to its high stability over time and protection against STIs, such as *Chlamydia trachomatis*, human papillomavirus (HPV) and HIV and the low vaginal pH is thought as the main source of protection in this environment (Lewis et al., 2017). Two isomers of lactic acid (D- and L-lactic acid) contributes to an *in vivo* acidic environment (O'Hanlon et al., 2013), while lowest *in vitro* pH levels are observed in the presence of the D-isomer (Witkin et al., 2013). While *L. crispatus* and *L. gasseri* can produce both, *L. iners* produce only L-isomer (Witkin et al., 2013). Thus, in this study vaginal pH was inferior in the CST I and correlated negatively with relative abundance of *L. crispatus*, reinforcing that *L. crispatus* may contribute efficiently for the maintenance of an acidic vaginal environment.

Besides that, lactic acid may act as an immunomodulator, creating an anti-inflammatory state through increased production of interleukin-1 receptor antagonist (IL-1RA) and inhibition the Toll-like receptor agonist-elicited production of inflammatory mediators, as IL-6, IL-8, and TNF-α (Hearps et al., 2017).

In fact, current data show that vaginal pH is positively correlated with pro-inflammatory cytokines (IL-1β and TNF-α) in *Lactobacillus*-dominated CSTs, indicating that an acidic vaginal microenvironment may contribute for local immune regulation. In addition, CST I showed lower vaginal pH and IL-1β level, reinforcing the protective role of *L. crispatus* in the reduced inflammation previously reported (Anahtar et al., 2015; Gosmann et al., 2017).

Previous studies showed that microbiota dominated by *L. crispatus* (CST I), has the lowest levels of IL-1β, when compared to those dominated by *L. iners* (CST III) or a *Lactobacillus*-deprived and highly diverse microbiota (CST IV), agreeing with current data (De Seta et al., 2019; Orfanelli et al., 2013). Recently, Manhanzva et al. (2020) showed that isolates *Lactobacillus* sp. suppressed the production of IL-6 and IL-8 by epithelial cells in cultures, despite the inverse correlation between IL-6 and D-lactate, which is not observed for L-lactate. In this sense, it may be suggested that bacterial species producing D-lactate could maintain a less inflammatory microenvironment (Hearps et al., 2017).

Vaginal microbiota dominated by *L. gasseri*, CST II, showed a different pattern when compared to other *Lactobacillus*-dominated CSTs. As overall the increased bacterial load assessed by flow cytometric in CST II. When analyzing the molecular bacterial composition, *G. vaginalis* is fairly abundant in CST II. In fact, the frequent co-occurrence between these two species was previously reported (De Backer et al., 2007; De Seta et al., 2019). The positive correlation between bacterial load with pH in CST II may be explained by fact that *L. gasseri* rely on a mechanism other than lowering pH by lactic acid production (i.e., hydrogen peroxide production and proteolytic enzyme-resistant compounds), to reduce the growth of *G. vaginalis* as demonstrated in *in vitro* analysis (Atassi et al. 2006).

Moreover, only small fraction of *L. gasseri* isolates present bactericidal activity independent of lactic acid (Atassi et al., 2019).

Anahtar et al. (2015) demonstrated that highly diverse bacterial communities and RA of some genital bacteria such as *G. vaginalis* could induce a local inflammatory response. Although CST II does not present as a diverse bacterial community, an increased RA of *G. vaginalis* was observed. Furthermore, CST II showed significant positive correlations between bacterial load and all cytokines assessed, which suggest a different relation between bacterial components and host´s immunity in the presence of *L. gasseri*-dominance when compared to *L. crispatus*- and *L. iners*-dominated microbiota. Levels of pro-inflammatory cytokines may be influenced not only by bacterial diversity but also by bacterial load of specific taxon. Corroborating with this hypothesis, IL-6 levels was increased in CST II when compared to the highly diverse CST IV. Since, increased IL-6 and IL-8 levels were already associated with presence of *G. vaginalis*, *A. vaginae* and *Prevotella spp.* (Eade et al., 2012; Fichorova et al., 2013; Gosmann et al., 2017; Libby et al., 2008).

Regarding the current findings on *L. iners*-dominated CST III, pH levels were significantly higher than in CST I which may be explained by the lack of capacity of *L. iners* to produce D-lactic acid (Witkin et al., 2013). This CST has been described as unstable in relation to their bacterial composition (Brotman et al., 2014; Gajer et al., 2012). It is worth mentioning the participation of a portion of RA of *G. vaginalis* and other bacterial species in this community. Previous findings already demonstrated the non *in vitro* inhibition between *L. iners* and *G. vaginalis*, even in the presence of *G. vaginalis*-derived vaginolysin (Castro et al., 2013; Nowak et al., 2018; Shipitsyna et al., 2013).

In relation to cytokine levels, CST III, showed higher levels of IL-1β when compared to CST I. Previously, Gosmann et al. (2017) showed that *L. crispatus* and, to a lesser extent, *L. iners* were associated with reduced inflammation evaluated measured through levels of IL-1β, IL-1α, IL-8 and IL-23. A study that aimed to assess which microbial factors that best predicted genital inflammation showed several *Lactobacillus spp*., including *L. iners*, are significantly lower abundant in the presence of genital inflammation (Lennard et al., 2017). Moreover, a study failed to show differences in cytokine levels (e.g., IL-1α, IL-1β, IL-1ra, IL-2, TNF-α, among others), between CST III (*L. iners*-dominated) and CST I (*L. crispatus*-dominated), despite demonstrating *L. iners* positively correlated with TNF-α, IL-12p70, IL-2 and MIF (macrophage migration inhibitory factor) (De Seta et al., 2019). In present study, bacterial load had positive correlation with IL-1β in CST III. Thus, it may be hypothesized the increase in IL-1β in this CST could be due other bacterial taxa than *L. iners.*

As expected, CST IV which accounts for most of microscopic BV and was recently named as molecular-BV, yielded the highest values of bacterial richness. Data on cytokine levels in BV are often conflicting, especially regarding IL-8 (Mitchell and Marrazzo, 2014). When looking at CST IV, IL-8 levels did not differ of those showed by other CSTs. Interestingly, although IL-8 levels were positively correlated with IL-1β and IL-6. Thus, the bacterial diversity could not be the only factor that contributes to disrupt the immune homeostasis, as the

presence of specific certain bacteria that could act as pro-inflammatory agents. Corroborating with this hypothesis, *G. vaginalis*, *A. vaginae*, BVAB 1 and *Fusobacterium*, that are detected in virtually all cases of CST IV, were already associated with inflammation on genital tract due increases on pro-inflammatory cytokines and chemokines levels (Anahtar et al., 2015; Lennard et al., 2017). In fact, our study group already demonstrated the inverse correlation between *Megasphaera sp.* with IL-8, while other taxa, as *G. vaginalis* and *A. vaginae* correlated positively with IL-1β (Marconi et al., 2013). Thus, it is suggested that not only greater bacterial diversity disturb the local immune response, but some specific bacterial taxon could be associated with pro-inflammatory cytokines and chemokines (Sabo et al., 2020).

Despite the positive correlation between IL-1β and IL-8 in the CST IV, bacterial diversity did not correlate with cytokines. It can be hypothesized that in an already disrupted microbiota, the increases in bacterial diversity/ richness would not reflect in additional antigenic stimulus. This could also be an explanation to the lack of neutrophils recruitment in BV, since in BV there IL-1β levels are increased (Mitchell and Marrazzo, 2014). Additionally, the blockages of the inflammatory cascade could be influenced by bacterial enzymes. For example, the inhibition the expected amplification of the proinflammatory IL-1β cascade was already demonstrated by the down-regulation of the IL-8/IL-1β ratio in the presence of hydrolytic enzymes sialidase and prolidase, produced by BV-associated bacteria (Cauci et al., 2008). Despite not showing relation between IL-8 cleaving activity with bacterial species present on genital tract, Zariffard et al., (2015) showed inhibitors of matrix metalloproteinases (MMPs) reduce IL-8 cleavage, suggesting that MMPs, specifically MMP-9, could mediate this activity. Active MMPs have multiple complex roles in inflammation, such as inhibition and potentiation of cytokines and chemokines (Nissinen, Kähäri, 2014) with its activity dependent by lactic acid on genital tract (Witkin et al., 2013), therefore active MMPs could potentially alter immune responses on the lower genital tract microbiota due to the local bacterial composition.

It is acknowledged that the current study has limitations, such as low representativeness of CST II. This occurs due in our population, CST dominated by *L. gasseri* presented inferior prevalence than another *Lactobacillus*-dominated microbiome (Marconi et al., 2020). Also, a limited number of cytokines was assessed, and does not provide the immune profile of each CST. Another limitation to mention is that this study did not aim to detect presence of HPV cervical infection. Despite this limitation, all samples had previously screened and tested negative for *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* by PCR and for *Candida* sp. assessed by microscopy. Other strength of this study, all samples were collected by previously trained clinical staff, allowing for better accuracy and reproducibility during sampling.

Taken together, current data point out to a distinct overall profile of CST II when compared to other CSTs, suggesting a single pro-inflammatory signature associated to bacterial load. The lack of correlation between bacterial diversity and cytokine levels, points out to the importance of future studies focusing on key-taxa and their relationship with inferior genital tract inflammation. Additionally, use of

more comprehensive panels for immune profiling could provide better understand the relation between vaginal microbiota and host´s immunity.

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AUTHOR CONTRIBUTIONS
C.M. and M.G.S. designed the study. C.S.T.F. and C.M. lead the clinical study and biological sample collection. J.N., C.M. and C.S.T.F. performed measurement of cervicovaginal cytokines and other laboratory analysis. J.N and M.A.G. performed flow cytometry analysis. J.N. and C.M. conducted the statistical analysis and interpreted the data. J.N. drafted the manuscript under the supervision of C.M. and M.G.S. All authors reviewed and approved the submitted version of the manuscript.

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6. SUPPLEMENTARY MATERIAL

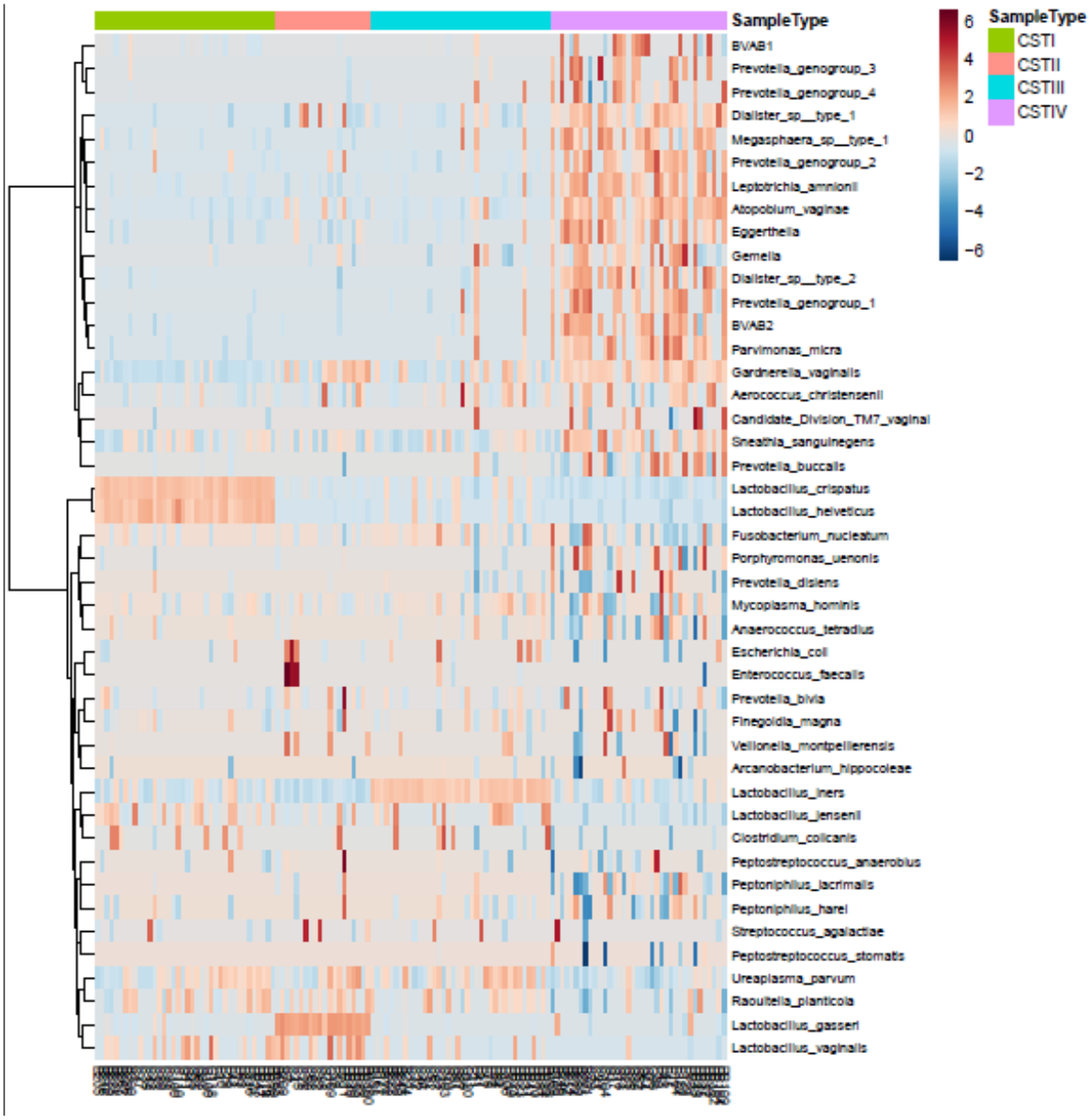

Figure Suppl 1: Heatmap of Centered Log-Ratio (CLR) transformation relative abundance of the top 44 abundant taxa identified, clustered by community-state type (CST).

Table S1: Spearman correlation rank between the microbiological and immunological features assessed in each CST

CST I	**Bac_load**	**pH**	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
Bac_load	1.00					
pH	-0.05	1.00				
IL-1β	0.17	**0.33***	1.00			
IL-6	0.28	-0.09	0.21	1.00		
IL-8	0.20	-0.18	**0.52*****	**0.35***	1.00	
TNF-α	0.18	0.10	0.16	**0.43****	0.09	1.00
CST II	**Bac_load**	**pH**	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
Bac_load	1.00					
pH	**0.49***	1.00				
IL-1β	**0.56****	0.007	1.00			
IL-6	**0.57****	0.17	**0.45***	1.00		
IL-8	**0.49***	0.007	**0.62****	**0.56****	1.00	
TNF-α	**0.48***	**0.53***	0.34	0.43	**0.44***	1.00
CST III	**Bac_load**	**pH**	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
Bac_load	1.00					
pH	-0.007	1.00				
IL-1β	**0.39****	0.06	1.00			
IL-6	-0.08	0.16	0.03	1.00		
IL-8	0.19	0.10	0.27	0.03	1.00	
TNF-α	0.14	**0.43****	0.31	0.08	-0.10	1.00
CST IV	**Bac_load**	**pH**	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
Bac_load	1.00					
pH	0.03	1.00				
IL-1β	0.25	0.07	1.00			
IL-6	0.05	-0.07	-0.09	1.00		
IL-8	0.28	0.14	**0.35***	**0.42****	1.00	
TNF-α	0.12	0.14	0.08	-0.09	0.01	1.00

*p<0.05; **p<0.01

[+]Five most relative abundant taxa

CST: Community state type

Bac_load: Bacterial load

IL: Interleukin TNF: Tumor necroses factor

Table S2: Spearman correlation rank between the microbiological and immunological features assessed in all samples included

	pH	**Load**	**Shannon-index**	**Richness**	***L. iners*[#]**	***L. crispatus*[#]**	***L. gasseri*[#]**	***G. vaginalis*[#]**	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
pH	1.00											
Load	0.08	1.00										
Shannon-index	**0.40*** **	0.06	1.00									
Richness	0.18	0.07	**0.53*** **	1.00								
L. iners	0.09	-0.11	**-0.21** **	-0.09	1.00							
L. crispatus	**-0.42*** **	-0.16	**-0.50*** **	**-0.30*** **	-0.09	1.00						
L. gasseri	-0.15	**0.32*** **	-0.17	0.07	**-0.41*** **	-0.16	1.00					
G. vaginalis	0.34	**0.29*** **	**0.46*** **	**0.32*** **	-0.13	**-0.56*** **	0.17	1.00				
IL-1β	**0.19***	0.24	0.07	0.03	**0.18***	-0.14	-0.11	0.16	1.00			
IL-6	0.06	0.13	-0.08	0.02	0.09	-0.02	0.10	0.16	0.04	1.00		
IL-8	-0.16	**0.27** **	-0.09	0.04	-0.07	0.15	0.16	0.21	**0.21***	**0.20***	1.00	
TNF-α	**0.25** **	0.17	-0.10	-0.07	0.05	0.00	0.00	0.20	**0.20***	0.13	0.04	1.00

*p<0.05; **p<0.01; ***p<0.001
[#]Relative abundance
Load: Bacterial load
IL: Interleukin
TNF: Tumor necroses factor

CAPÍTULO III

ARTIGO CIENTÍFICO II

ARTIGO SUBMETIDO PARA PUBLICAÇÃO NA REVISTA SCIENTIFIC REPORTS (ID E8F5FD80-A027-48FD-8027-0529E531DB1B)

Cervicovaginal *Gardnerella* sialidase-encoding gene in persistent human papillomavirus infection

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Abstract

Disturbed vaginal microbiota have a role in the persistence of high-oncogenic-risk human papillomavirus (hrHPV) and *Gardnerella* spp. is closely related with this condition. Such bacteria are the major source of cervicovaginal sialidases, important for microbiota alterations. The sialidase-encoding gene nanH3 is account for their sialidase activity. Thus, a subset of 212 women positive for hrHPV at the first visit were included in the analysis of the current study aiming to compare the loads of nanH3 in cervicovaginal fluid (CFV) of women with persistent hrHPV infection and with those cleared the infection after a year. Participants were assigned to two study groups named “persistence” (n=124, 53.22%) or “clearance” (n=88, 37.77%), according to the HPV status upon enrollment and follow-up. Absolute quantification of nanH3 gene was performed using quantitative real-time PCR (qPCR). Persistence and clearance group did not show statistical difference in the load of nanH3 gene (p=0.19). When considering the subset of women with HPV16, differences in number of copies of nanh3 gene was observed between the persistent (7.39E+08 copies/uL) and clearance group (2.85E+07 copies/uL) (p=0.007). Therefore, baseline loads of nanH3 gene is increased in women that persist with cervical HPV16 infection after 12 months.

Key-words: Sexually transmitted infection; urogenital cancer; persistent infection; bacterial vaginosis

1. INTRODUCTION

Human papillomavirus (HPV) cervical infection is the most frequent sexually transmitted infection (STI) worldwide[1-2] and the persistence of cervical infections by high-risk-HPV (hrHPV) for long periods of time cause virtually all precursor lesions and cervical cancers[3]. Despite that, the vast majority of the cases of cervical HPV infection are cleared within 2 years[4-5].

Factors associated with developing cervical lesion and cancer include smoking[6], hormonal contraceptive use[7-8], and parity[9], while immune responses are important for clearance[10]. In addition, the local cervical microenvironment, including the vaginal microbiota, may also influence the natural history of HPV infection[11]. Therefore, a *Lactobacillus*-deprived vaginal microbiota, as in bacterial vaginosis (BV), have been associated with persistent HPV infection and lesion progression[12-14].

Bacterial vaginosis is considered a polymicrobial dysbiosis, in which occurs the replacement of beneficial *Lactobacillus* and increases of anaerobic and facultative anaerobic bacteria, of which, *Gardnerella* spp. is found in nearly all cases of this condition[15-16]. Sialidase production is one of the most important virulence factor for *Gardnerella* spp.[17-18]. Among the deleterious effects of bacterial sialidases, it is the degradation of several protective factors of the vaginal mucosa and contribution to the exfoliation and detachment of vaginal epithelial cells[19] facilitating bacterial adhesion to the epithelium and biofilm formation[20-22], a condition already associated with persistence of BV[23].

At first, the putative sialidase gene, nanH1 (sialidase A gene), was identified in *Gardnerella* spp. and was thought to be the gene responsible for sialidase production[24]. However, the recent studies concluded that nanH2 and nanH3 account for the sialidase activity observed in cultured *G. vaginalis*[25]. Besides that, nanH3 gene is more commonly find and nanH2 has not being detected without nanH3[18, 25].

Considering the importance of better understanding the relationship between the bacterial components of the vaginal microbiota and the outcome of hrHPV infection, the aim of this study was to compare the loads of the sialidase-encoding gene nanH3 of *Gardnerella* spp. in the cervicovaginal fluid of women between persistent hrHPV infection and those who cleared the infection after a 12-month period.

2. RESULTS

Table 1 displays the sociodemographic, behavioral and clinical characteristics of the participants, according to the study groups. The groups showed differences in few variables such as age (p=0.04), and the number of sex (p<0.0001). When considering the number of hrHPV genotypes detected, persistent group had more mixed infection than clearance group (p=0.002). In relation to the HPV genotypes, HPV52 differed between the groups when considering single or mixed infection (p=0.01).

Table 1: Sociodemographic, behavioral and clinical characteristics of the participants, according to the study groups

	Outcome of hrHPV infection		
Variable	**Clearance (88)**	**Persistence (124)**	**P value**
Age[#]	29 (17-50)	26.5 (17-50)	**0.04**
Ethnicity*			0.52
Black	35 (39.77)	59 (47.58)	
White	51 (57.95)	62 (50.00)	
Other	2 (2.27)	3 (2.42)	
Living with partner[$]			0.89
No	41 (46.59)	60 (48.39)	
Yes	47 (53.41)	64 (51.61)	
Years at school[#]	11 (1-16)	11 (3-17)	0.14
Smoking[$]			0.66
No	57 (64.77)	85 (68.55)	
Yes	31 (35.23)	39 (31.45)	
Douching[$]			1.00
No	84 (95.45)	117 (94.35)	

Yes	4 (4.55)	7 (5.65)	
Hormonal contraceptive use[$]			0.14
No	33 (37.50)	34 (27.42)	
Yes	55 (62.50)	90 (72.58)	
Condom use[$]			0.88
No	63 (71.59)	87 (70.16)	
Yes	25 (28.41)	37 (29.84)	
Sex partners[#]	0 (1-15)	3 (1-100)	**<0.0001**
Sex partners (last year)[#]	1 (0-2)	1 (0-12)	0.26
Vaginal abnormal discharge[#]			0.25
No	27 (30.68)	48 (38.71)	
Yes	61 (69.32)	76 (61.29)	
Nugent*			0.59
Normal	50 (56.82)	68 (54.84)	
Intermediate	10 (11.36)	10 (8.06)	
BV	28 (31.82)	46 (37.10)	
Number of hrHPV genotypes[#]	1 (1-3)	1 (1-5)	**0.002**
HPV16[$]			1.00
Single	12 (13.64)	31 (25.00)	
Mixed	5 (5.68)	16 (12.90)	
HPV31[$]			0.43
Single	6 (6.82)	9 (7.26)	
Mixed	3 (6.41)	10 (8.06)	
HPV51[$]			0.12
Single	12 (13.64)	5 (4.03)	
Mixed	1 (1.14)	4 (3.23)	
HPV52[$]			**0.01**
Single	9 (10.23)	6 (4.84)	
Mixed	1 (1.14)	10 (8.06)	
HPV58[$]			1.00
Single	4 (4.55)	7 (5.65)	
Mixed	3 (3.41)	8 (6.45)	

[#]Mann–Whitney Test, expressed by median (minimum–maximum).
[*]Chi-square test.
[$]Fisher's exact test.
For all analysis P-value<0.05 was considered as significant

The most frequently genotype detected in the population (showed in supplementary material), was HPV16, followed by HPV31, HPV52, HPV51, HPV58 and HPV45, respectively. Of 233 women included in this

study, 21 cleared their HPV genotype detected at baseline and a new genotype was detected at follow-up, therefore these women were not included in the analysis. Thus, clearance group included women without detection of HPV at follow-up (n=88) and persistence group includes those women showing at least one genotype detected at baseline and follow-up (n=124). Thus, clearance and persistence rates were 41.50% (n=88) and 58.49% (n=124), respectively. For HPV16, rate of clearance was 26.56% (n=17) and persistence was 73.44% (n=47).

Regarding the presence of nanH3 gene and the outcome of cervical hrHPV infection displayed on the table 2, the groups did not show difference in relation to the positivity to the gene. When compared the absolute quantification of nanH3 gene, showed by median (minimum–maximum), in the presence of all hrHPV, no difference in the load was observed between the clearance (1.45E+08 copies/µL; 2.30E+04 – 6.15E+13) and persistence (9.16E+08 copies/µL; 3.02E+05 – 1.72E+13) groups (p=0.19). For 5 most prevalent genotypes (HPV16, 31, 52, 51 and 58) clearance group showed 1.02E+08 copies/µL (2.30E+04 – 6.15E+13) and persistence 1.82E+09 copies /µL (3.67E+05 – 1.72E+13) (p=0.02). When excluding HPV16, the 5 most prevalent genotypes (HPV31, 52, 51, 58 and 45) there was no difference, the clearance group showed 3.60E+08 copies/µL (2.30E+04 - 6.15E+13) and persistence 1.36E+09 (3,67E+05 - 1,72E+13) (p=0.42). Loads of nanH3 gene differed between the clearance (2.85E+07 ng/µL; 1.90E+05 – 4.23E+07) and persistence group (7.39E+08 copies/µL; 4.15E+06 – 4.09E+10) in the presence of HPV16 (p=0.007) (Table 3).

Table 2: Detection of nanH3 gene in the cervicovaginal fluid according to the study groups

	Detectable nanH3 gene		P value*
All hrHPV	**Yes**	**No**	0.77
Clearance	32 (36.36)	56 (63.64)	
Persistence	48 (38.71)	76 (61.29)	
5 most prevalent hrHPV genotypes[&]			0.60

	Clearance	20 (37.74)	33 (62.26)	
	Persistence	38 (42.70)	51 (57.30)	
5 most prevalent hrHPV genotypes, excluding HPV16[$]				0.70
	Clearance	19 (38.78)	30 (61.22)	
	Persistence	26 (44.07)	33 (55.93)	
HPV16				0.56
	Clearance	5 (29.41)	12 (70.59)	
	Persistence	19 (40.43)	28 (59.57)	

[&]Including HPV16, HPV31, HPV52, HPV51 and HPV58
[$]Including HPV31, HPV52, HPV51, HPV58 and HPV45
[*]Fisher's exact test
For all analysis P<0.05 was considered as significant

Analysis to test the association between vaginal microbiota and nanH3 gene were also performed. Of the 233 women, 127 (54.51%) had normal microbiota, 21 (9.01%) intermediate and 80 (34.33%) had BV, detected by Nugent scoring. Data of Nugent evaluation were not available for 5 participants. Among BV positive women, 52/80 (65%) were nanH3 positive, while 28/80 (35%) were nanH3 negative. Thus, sensitivity and specificity of the test was 65% (95% confidence interval, 54-75%) and 75% (66-82%), respectively. Figure 1A displays a bar graph illustrating the relationship of nanH3 gene status across normal and BV Nugent scoring, showing a significant difference between the categories (p<0.0001). In relation to the loads of nanH3 gene expressed by median (minimum–maximum) in the cervicovaginal fluid shown in figure 1B, there was difference between normal microbiota (2.06E+08 copies/µL; 2.30E+04 – 1.37E+13) and BV (2.91E+09 copies/µL; 8.17E+03 – 6.15E+13) (p=0.0041). Due the low representativeness, intermediate microbiota was not considered in this analysis.

Table 3: Load of nanH3 gene in the cervicovaginal fluid according to the study groups

	NanH3 loads (copies/µL)[#]		
	Clearance	**Persistence**	**P value**
All hrHPV	1.45E+08 (2.30E+04 - 6.15E+13)	9.16E+08 (3.67E+05- 1.72E+13)	0.19
5 most prevalent hrHPV genotypes[&]	1.02E+08 (2.30E+04 – 4.34E+13)	1.82E+09 (3.67E+05 - 1.72E+13)	**0.02**
5 most prevalent hrHPV genotypes, excluding HPV16[$]	3.60E+08 (2.30E+04 - 6.15E+13)	1.36E+09 (3.67E+05 – 1.72E+13)	0.42
HPV16	2.85E+07 (1.90E+05 - 4.23E+07)	7.39E+08 (4.15E+06 - 4.09E+10)	**0.007**

[&]Including HPV16, HPV31, HPV52, HPV51 and HPV58
[$]Including HPV31, HPV52, HPV51, HPV58 and HPV45
[#]Mann–Whitney Test, expressed by median (minimum–maximum)
For all analysis P<0.05 was considered as significant

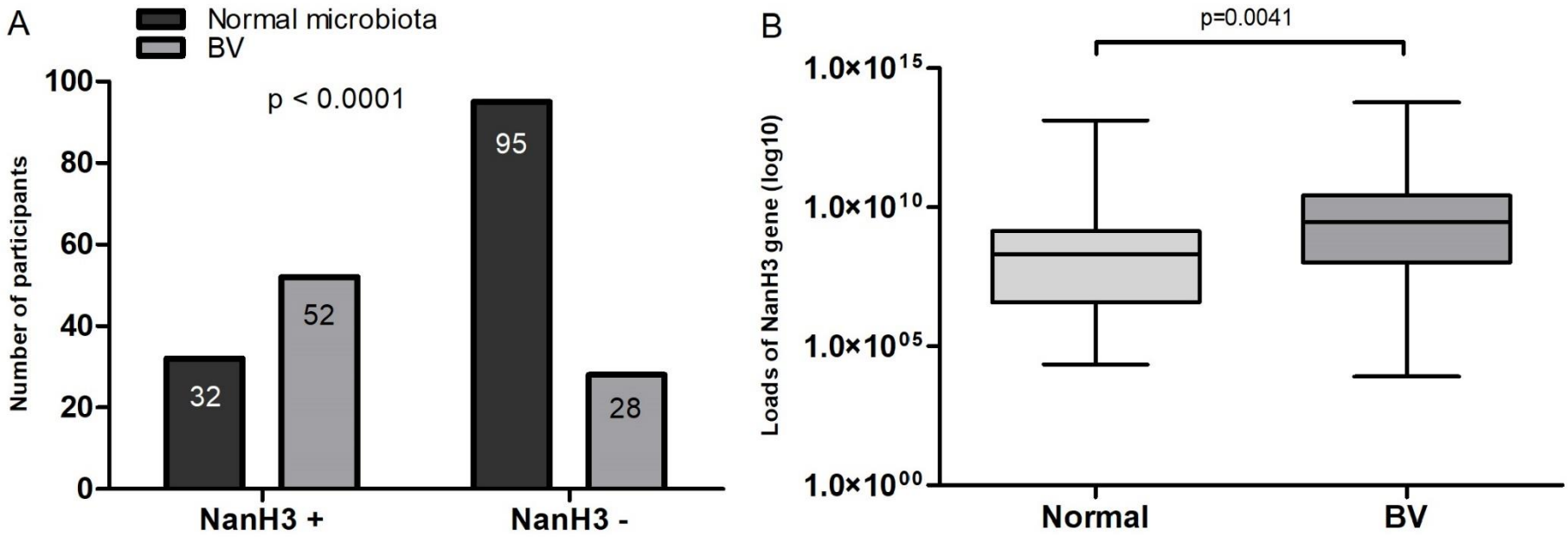

Figure 1: Characteristics of association between nanH3 gene and vaginal microbiota. A) A bar graph illustrating the relationship of nanH3 gene status across the two Nugent score categories (p<0.0001); B) Loads of nanh3 gene compared between normal microbiota and BV (p=0.0041).

3. DISCUSSION

Recently our study group showed that women with persistent HPV16 and HPV18 infection have increased baseline loads of *G. vaginalis*[26]. Thus, it was hypothesized that gene encoding sialidase, frequently found in *Gardnerella* spp.[18] could have a role in the outcome of HPV infection, being a useful marker for hrHPV persistence. Surprisingly, loads of nanH3 gene differed between clearance and persistence group in the presence of 5 most prevalent genotypes, however such difference remains only in the presence of HPV16.

This study showed high rates of HPV persistence, 58.49% for all hrHPV infection and 73.44% when considered only the HPV16. Such rates were similar to the rates showed in another study conducted in Brazil, with 61.8% of persistence rate[27]. Persistent rates in a large retrospective cohort study in China was 42.7% within 24 months[28], while a multicentric study showed 54.1% of persistence rate over a year in American, Canadian and Brazilian women[29]. In relation to HPV16, the persistence rate was 52.1% after 12 months in Dutch cohort of young women[30].

In relation to sociodemographic and behavioral characteristics of the study population, few variables differed between the groups. The median of age was higher in clearance group, agreeing with a study that showed that the highest HR-HPV persistence occurred in the 22-27 years old group, whereas clearance increased in women aged 28-33 years[31]. However, other Brazilian study did not show differences of age when evaluating factors associated with clearance and persistence after 24 months[27]. The number of sex partners and the number of detected genotypes differed between the clearance and persistence group. Furthermore, 1 of 5 most prevalent HPV genotypes differed among the groups when considering their detection in single or mixed infection. In fact, a previous study showed that HPV genotypes differ in terms of the period for clearance, as women with mixed infections had a longer time to clear their infections compared to those with a single infection[29]. The increasing in number of sex partners

could contribute to persistence since it increases the risk of acquiring new HPV genotypes.

Studies on vaginal microbiota, have been demonstrated association between *Gardnerella* spp. and persistent infection of hrHPV, as well as progression of the cervical lesion[32-33]. Of the results of association between *Gardnerella* spp. with persistence and progression of hrHPV, Usik et al. propose that beyond the influences of *Gardnerella* spp. in the persistence, such specie contributes with disruption on vaginal microbiota composition, increasing bacterial diversity, which in turn also contributes to the persistence and progression of HPV infection[33].

To the best of our knowledge, this is the first study to assess the relationship between hrHPV persistence infection and loads of nanH3 gene, responsible for sialidases production. It is known that sialidases hydrolyze the sialic acid of the host's epithelial cells, provides a carbon source to *Gardnerella* allowing their uptake and catabolism and expose binding sites, facilitating bacterial adhesion to the epithelium and further biofilms formation[20-22;34]. Sialidases can still cleave mucin oligosaccharides, that decreased the cervicovaginal mucus viscosity, compromising the physical and biochemical barrier against pathogens[35]. Besides that, sialidases compromise the immune barrier by hydrolyzing immunoglobulin A[34; 36]. In the present study, results on the quantification of nanH3 did not differ when included all hrHPV infection grouped, however loads of nanH3 were higher in the cervicovaginal fluid of women with HPV16 persistent.

Studies have been reporting the association of altered vaginal microbiota with HPV infection[12-14; 32-33; 37], despite the mechanisms by it can influence in HPV infection remain unknow. It is known that *Lactobacillus* spp. produce several microbicidal factors, including lactic acid[38], an important agent to acidify the vaginal environment and to control overgrowth of bacteria and preventing STIs[39-40]. The difference of nanh3 loads found in HPV16 infection is intriguing, are needed new approaches involving

vaginal microbiota and HPV16, since this genotype is responsible for the most cases of high-grade intraepithelial lesions and invasive cervical carcinomas[41].

Human papillomaviruses are able to modulate the host immune response by blocking immune-related gene expression and immune signaling pathways and different genotypes may act by different pathways[42]. In addition, cervical microbiome and cytokine profile showed notably different in all stages of the natural history of cervical cancer[43]. Thus, bacterial species may interact with immune signaling during HPV infection contributing to persistence and progression[43]. On the other hand, a consequence of HPV immune evasion is the imbalance in the vaginal microbiota due the reduced amino acid source sustaining the survival of *Lactobacillus* species[44]. Therefore, the association between BV and HPV seems to be bi-directional. In fact, a study showed that the stability of vaginal microbiome is necessary to maintenance of immune surveillance for HPV16 clearance[45]. In this sense, the differences in the load of nanH3 gene in the presence of HPV16 lead to hypothesize that the vaginal microenvironment would have a different modulation in the presence of different genotypes and the changes in the immune response might create a more permissive microenvironment for bacterial overgrowth.

Gardnerella spp. is present in virtually all cases of BV, the most frequent dysbiosis of the vaginal microbiota[46]. In this study, an association between the nanH3 gene and BV was observed. Robinson et al. suggested the potential use of nanH3 as a molecular diagnostic marker of BV, such PCR test showing 80.95% sensitivity and 78.26% specificity compared with the Nugent score for BV diagnosis[25]. Although the association, in this study values of sensitivity and specificity were lower than the mentioned study. It is worth mentioning that studies differ in terms of prevalence of BV and number of study participants.

Besides the association between nanH3 gene and BV, the load of the gene was significantly increased in BV compared to normal microbiota. As

previously discussed, sialidases have a deleterious effects in the vaginal microenvironment[20-22; 34-36]. Recently, our study group showed that sialidase activity in molecular-BV, assessed by V3-V4 16S rRNA sequencing, is associated with changes in bacterial components of the local microbiome as well as increased sialidase levels[47]. Indeed, sialidase activity in cervicovaginal fluid already was demonstrated in the presence of microscopy-detected BV[48-49]. In fact, sialidase activity could promote the growth or colonization of *Prevotella*, *Bacteroides* as well as *G. vaginalis*, vaginal bacterial sialidase producers[50-51], being the production of sialidase an important step in the biofilm formation[24]. Such mechanism reinforces the role of *Gardnerella* spp. as the scaffold on vaginal mucosa for attachment of other bacterial species, which can start to form biofilm [42; 52], a condition already associated with refractory treatment of BV and persistence of BV-associated bacteria after treatment[23; 53].

Although the amount of sialidase-encoding nanH3 gene did not differ between clearance and persistence in the presence of all hrHPV infection, the loads are high in the persistent group and there is a difference among the groups when considering only HPV16 infection. A limitation of this analysis is due the low sample size when stratifying each genotype. Furthermore, such results contribute to better understanding the role of *G. vaginalis* sialidase-encoding gene in BV, since this condition seems have an important role in the persistence and progression of HPV infection. Thus, microbial products are promising tools for identifying women with increased risk for HPV persistence.

4. METHODS

4.1. Data collection, sampling and laboratorial procedures

Between 2012 to 2014, 1635 reproductive-aged HIV-negative women screened for HPV infection in Botucatu, SP, Brazil. A sociodemographic, behavioral, gynecological history and clinical data were obtained during a face-to-face interview using a structured questionnaire and were entered

into a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA). HPV positive women were then invited to a follow-up within 12 months. All participants were informed about the procedures of study, signed an informed consent form. This current study was reviewed and approved by the Ethics Committee of Botucatu Medical School (Approval number: 5.660.472) and the samples included in the present study belongs to the biorepository of principal study (Biorepository approved number: 3.140.843).

Same sampling procedures during physical examination were conducted by nurses/physicians at enrolment and at follow-up visit. After speculum insertion, vaginal content was obtained with swabs for smearing into microscopic glass slides for Nugent scoring, after gram staining[15]. Mid vaginal wall was utilized for *Trichomonas vaginalis* culture in Diamond's medium[54]. Samples obtained with endocervical brushes were stored at -20 ºC until *Chlamydia trachomatis* and *Neisseria gonorrhoeae* detection, as described previously[46; 55] and for HPV detection and genotyping using the Linear Array HPV Genotyping kit (Roche Molecular Systems, Pleasanton, CA, USA). For cervicovaginal fluid sampling, 3 ml of sterile 0.9% NaCl was inserted into vaginal wall, homogenized with cervicovaginal secretion and recovered using a plastic sterile pipette. The cervicovaginal samples were stored at -20 ºC until genomic DNA extraction and *G. vaginalis* nanH3 gene absolute quantification.

Genomic DNA extraction of cervicovaginal fluid was performed using the UltraClean Soil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) as recommended by the manufacturer. Extracted DNA was quantified using an Epoch spectrophotometer (Biotek, Winooski, VT, USA) and the quality of the extraction was confirmed by the 260/280 nm absorbance ratio.

A cloning step was performed to obtain the plasmid DNA with *Gardnerella* nanH3 gene, through polymerase chain reaction (PCR) products from clinical samples using the primers *Gardnerella* nanH3 F (5′-

CAGTTCCAATGGAAGTGTGC-3') and *Gardnerella* nanH3 R (5′-AGCATCTGGGAATGCTCTTG-3') with 51ºC of annealing temperature. Expected amplicon size was 322 bp for nanH3 gene, confirmed in 1.5% agaroses gel band[25]. Positive samples had the amplicon purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Buckinghamshire, UK) and sequenced by Sanger method in ABI 3500 (Applied Biosystems, Foster City, CA, USA) and confirmed by blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_2235500528). The amplified products were used for the ligation step using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Carlsbad, CA, USA), cloned into *Escherichia coli* DH5-α (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was extracted using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Carlsbad, CA, USA).

The absolute quantification of nanH3 gene was performed by quantitative real-time PCR (qPCR) using 2x qPCRBIO SyGreen Blue Mix Hi-ROX (PCR Biosystems, London, UK), primers *Gardnerella* nanH3 F and *Gardnerella* nanH3 R under the following cycling conditions: 95 °C for 2 min, 25 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 25 s, and an extension at 95 °C for 15 s 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, performed in a StepOnePlus Real-Time System (Thermo Fisher Scientific, Waltham, MA, USA). Samples with a melting temperature value of 81 °C +/−0.5 °C were considered positive for nanH3 gene. For constructing the standard curve, three plasmid dilutions (3,05E+05 copies/µL, 3,05E+07 copies/µL and 3,05E+09 copies/µL) were utilized for sample cycle threshold interpolation. Loads of nanH3 gene were expressed as the number of copies per volume (µL) of cervicovaginal fluid.

4.2. Selection of participants and group assignments

In the first visit, 544 (33.27%) women were positive for any HPV infection, of which 413 (25.26%) tested positive for hrHPV genotypes and were

recruited for the follow-up visit after 12 months. Four hundred sixty-two (27.65%) women returned to the follow-up. For the present study, women positive for *Chlamydia trachomatis* (138, 8.44%), *Trichomonas vaginalis* (23, 1.41%), *Neisseria gonorrhoeae* (2, 0.12%) and only low-risk HPV (131, 8.01%) were not included in the analysis. Five women were excluded of this study due conflicting data in the spreadsheet database. From that, 245 (14.96%) women were hrHPV positive (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV68, HPV73, HPV82), however, 12 samples were excluded due insufficient volume of cervicovaginal fluid for laboratorial analysis of the current study. Therefore, 233 cervicovaginal fluid samples were included for this study analysis. Participants were assigned at two groups according to the status of hrHPV infection at enrolment and at follow-up: ‘clearance group’ comprised participants who tested negative for any hrHPV at follow-up and in ‘persistence group’ were assigned participants positive for hrHPVs genotype at first visit and follow-up, matched with those detected at follow-up. For clearance group, 21 women who showed clearance of baseline genotype and had a new genotype detected at follow-up were not included.

4.3. Statistical analysis

For sociodemographic, behavioral and clinical variables comparisons between the clearance and persistence groups, chi-squared or Fisher’s exact were utilized for categorical variables and Mann–Whitney tests was utilized for continuous variables. The loads and presence of nanH3 gene were compared between the study groups using, respectively, Mann–Whitney and chi-squared tests or Fisher’s exact test. All analyses considered a P-value <0.05 to be statistically significant and were performed using GraphPad Prism software (version 6.0, GraphPad, CA, USA).

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Author Contributions
CM and MG conceived and supervised the study. ANB and GVSP recruited volunteers. JN, RF, ANB and GVSP performed the experiments. JN performed the statistical analysis and interpreted the data. JN wrote the manuscript under supervision of CM. All authors read, reviewed and approved the final manuscript.

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6. SUPPLEMENTARY MATERIAL

Table Suppl 2: Frequency of each hrHPV detected as single or mixed infection

Baseline hrHPV genotypes detected	Frequency in single infection (n=164)	Frequency in mixed infection (n=48)	Total of infection for each hrHPV (212)
HPV16	43 (26.22)	21 (43.75)	64 (30.19)
HPV31	15 (9.15)	13 (27.08)	28 (13.21)
HPV52	15 (9.15)	11 (22.92)	26 (12.86)
HPV51	17 (10.37)	5 (10.42)	22 (10.38)
HPV58	11 (6.71)	11 (22.92)	22 (10.38)
HPV45	14 (8.54)	7 (14.58)	21 (9.91)
HPV18	12 (7.32)	8 (16.67)	20 (9.43)
HPV56	8 (4.88)	8 (16.67)	16 (7.55)
HPV35	6 (3.66)	7 (14.58)	13 (6.13)
HPV59	6 (3.66)	7 (14.58)	13 (6.13)
HPV73	7 (4.27)	5 (10.42)	12 (5.66)
HPV68	5 (3.05)	6 (12.50)	11 (5.19)
HPV82	3 (1.83)	6 (12.50)	9 (4.25)
HPV39	1 (0.61)	6 (12.50)	7 (3.30)
HPV33	1 (0.61)	2 (4.17)	3 (1.42)

CAPÍTULO IV

CONCLUSÃO

CONCLUSÕES

Embora a microbiota vaginal desempenhe um importante papel na manutenção de um microambiente vaginal saudável, quando esta é predominada por *L. gasseri* parece demonstrar um perfil de carga bacteriana e correlação entre fatores microbiológicos e citocinas distinto das demais comunidades com predomínio de outras espécies de Lactobacillus. Portanto, é de suma importância considerar que a interação e carga bacteriana são fatores importantes, uma vez que até mesmo uma microbiota com predomínio lactobacilar pode apresentar um perfil imunológico distinto. Neste sentido, destaca-se a importância em estudar as interações entre espécies bacterianas consideradas como fator chave no desencadeamento de resposta inflamatória, uma vez que as respostas inflamatórias podem contribuir para a aquisição de ISTs.

As cargas do gene nanH3 codificante da sialidase de *Gardnerella* sp. no lavado cérvico-vaginal não apresentaram diferença entre mulheres com infecção persistente por hrHPV e aquelas que tiveram *clearance* após 12 meses. No entanto, ao considerar apenas o genótipo do HPV16, as cargas de nanH3 foram maiores no grupo com persistência, sendo importante a elucidação da interação entre os diferentes genótipos de HPV e componentes da microbiota vaginal.

ANEXOS

Anexo I – Informe de aceite do artigo científico I

unesp

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Beneficial Microbes - Decision on Manuscript ID BM-2022-06-0060.R1

Beneficial Microbes <onbehalfof@manuscriptcentral.com> 18 de janeiro de 2023 às 06:08
Responder a: editor-in-chief@beneficialmicrobes.org
Para: juliano.novak@unesp.br, catafner@hotmail.com, marjorie.golim@unesp.br, marcia.guimaraes@unesp.br, marconi.cml@gmail.com

18-Jan-2023

Dear Dr. Novak,

I am pleased to inform you that your manuscript entitled "Covariates of Vaginal Microbiota and Pro-inflammatory Cytokines Levels in Women of Reproductive Age" is accepted for publication in Beneficial Microbes, under condition that no problems arise during the editing stage at the publisher. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

I also would like to draw your attention to the possibility of publishing your manuscript as 'open access' for 1950 Euro in Beneficial Microbes. With open access, your article will be free available online for everyone. Open access publishing significantly increases the exposure and citation of your work. Often research grants have funding available for dissemination of the results of the research.

Note that that the publisher needs a signed Copyright Transfer Agreement (CTA) from the first or the corresponding author before the manuscript can be published. Please fax (+31 317 453417) or send the CTA by email to the publisher (bm@wageningenacademic.com) as soon as possible if you have not done already. For your convenience a blank CTA is attached to this email.

Thank you for your fine contribution. On behalf of the Editors of the Beneficial Microbes, we look forward to your continued contributions to the Journal. Beneficial Microbes has an Impact Factor of 5.050 (Source: Clarivate 2022) and a 2021 CiteScore of 8.1 (81% percentile; calculated by Scopus, Elsevier 2022)

Sincerely,

Dr. Koen Venema
Editor in Chief, Beneficial Microbes
editor-in-chief@beneficialmicrobes.org

Anexo II – Informe de submissão do artigo científico II

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Scientific Reports - Receipt of Manuscript 'Cervicovaginal Gardnerella sialidase-encoding...'
1 mensagem

Scientific Reports <srep@nature.com> 4 de março de 2023 às 20:19
Para: juliano.novak@unesp.br

Ref: Submission ID e8f5fd80-a027-48fd-8027-0529e531db1b

Dear Dr Novak,

Thank you for submitting your manuscript to Scientific Reports.

Your manuscript is now at our initial Technical Check stage, where we look for adherence to the journal's submission guidelines, including any relevant editorial and publishing policies. If there are any points that need to be addressed prior to progressing we will send you a detailed email. Otherwise, your manuscript will proceed into peer review.

You can check on the status of your submission at any time by using the link below and logging in with the account you created for this submission:

https://researcher.nature.com/your-submissions?utm_source=submissions&utm_medium=email&utm_campaign=confirmation-email&journal_id=41598

Kind regards,

Peer Review Advisors
Scientific Reports

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Anexo III – Parecer do Comite de Ética em Pesquisa para o objetivo I

FACULDADE DE MEDICINA DE BOTUCATU -UNESP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Caracterização do microbioma vaginal de mulheres brasileiras em idade reprodutiva.

Pesquisador: Márcia Guimarães da Silva
Área Temática: Área 8. Pesquisa com cooperação estrangeira.
Versão: 3
CAAE: 02381512.5.1001.5411
Instituição Proponente: Hospital das Clínicas da Faculdade de Medicina de Botucatu
Patrocinador Principal: Fundação de Amparo a Pesquisa de São Paulo ((FAPESP))

DADOS DO PARECER

Número do Parecer: 306.547
Data da Relatoria: 17/06/2013

Apresentação do Projeto:
Sabe-se que a microbiota residente exerce papel fundamental na prevenção de inúmeras doenças humanas. Em especial, as modificações na microbiota vaginal (i.e.,alterações na composição bacteriana local)interferem na saúde da mulher, aumentando o risco de aquisição de doenças sexualmente transmissíveis (DST),vaginose bacteriana (VB) entre outros problemas de saúde. Portanto, a caracterização da microbioma vaginal de mulheres brasileiras em idade reprodutiva, provenientes de diferentes regiões do país, trará contribuições para o conhecimento da flora predominante. Assim, a partir dessa descrição será possível desenvolver estratégias futuras no país para manutenção de um ambiente vaginal saudável com a redução de importantes problemas na saúde reprodutiva da mulher.

Objetivo da Pesquisa:
Caracterizar a microbioma vaginal de mulheres brasileiras em idade reprodutiva, de acordo com: 1) Abundância das diferentes espécies presentes e padrões de classificação da microbiota vaginal; 2) Normal, intermediária e vaginose bacteriana; 3) Contagem total e caracterização das sub-comunidades bacterianas presentes na VB por clusters das espécies prevalentes; 3) Residência nas diferentes regiões brasileiras.

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Bairro: Rubião Junior **CEP:** 18.618-970
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FACULDADE DE MEDICINA DE BOTUCATU -UNESP

Avaliação dos Riscos e Beneficios:

Este estudo oferece riscos mínimos à saúde das mulheres participantes que incluem o desconforto durante o exame especular e a surpresa ou perturbação emocional após a divulgação do diagnóstico. As participantes incluídas neste estudo serão submetidas à pesquisa de tricomoníase, gonorréia e infecção por Chlamydia trachomatis, além da avaliação da microbiota vaginal por microscopia. As participantes que apresentarem sinais de vaginose bacteriana e/ou de DST serão tratadas conforme a necessidade e segundo o protocolo de cada serviço.

Comentários e Considerações sobre a Pesquisa:

A pesquisa trará informações importantes para o conhecimento da flora vaginal predominante da mulher brasileira. Todas as autorizações pertinentes foram incluídas no processo e os custos da pesquisa serão bancados por agência de fomento a pesquisa(FAPESP).

Considerações sobre os Termos de apresentação obrigatória:

O TCLE foi descrito na forma de convite, com linguagem acessível, com esclarecimentos sobre os objetivos do estudo, sigilo e participação voluntária dos envolvidos na pesquisa.

Foi incluído esclarecimentos as paciente que haverá uma equipe de saúde disponível para orientar e realizar o tratamento físico e emocional das pacientes diagnosticadas com doenças vaginais.

Recomendações:

Nada a acrescentar.

Conclusões ou Pendências e Lista de Inadequações:

Após atendimento aos questionamentos feitos pela CEP local e CONEP, sou de parecer favorável à aprovação da execução desse projeto de pesquisa.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Haja Vista que todas as recomendações feitas pela CONEP foram atendidadas de forma satisfatória,o projeto está em condições de ser iniciado. Deliberado em reunião EXTRAORDINÁRIA DO CEP de 17/06/2013.

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Anexo IV – Parecer do Comite de Ética em Pesquisa para o objetivo II

UNESP -FACULDADE DE MEDICINA DE BOTUCATU

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Relação entre espécies bacterianas associadas à vaginose bacteriana com a persistência da infecção pelo Papilomavírus Humano em mulheres imunocompetentes.

Pesquisador: RAFAEL BELLETI

Área Temática:

Versão: 1

CAAE: 61691222.3.0000.5411

Instituição Proponente: Departamento de Patologia

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.660.472

Apresentação do Projeto:

O projeto tem como objetivo comparar as cargas cérvico-vaginais de Gardnerella vaginalis, Prevotella bivia e Atopobium vaginae em mulheres com clearance e com persistência da infecção cervical por HPV de alto risco-oncogênico. Além disso, determinar se a presença do gene codificante da sialidase de G. vaginalis e P. bivia está associada com o desfecho da infecção no período de 12 meses em mulheres imunocompetentes em idade reprodutiva.

A população do estudo proposto envolve a coorte de 323 de 1638 participantes do projeto intitulado "Prevalência de coinfecção e fatores associados ao Papilomavírus Humano (HPV) e à Chlamydia trachomatis em mulheres em idade reprodutiva atendidas em Programa de Prevenção de Câncer do Colo Uterino na região sudeste do Brasil". As 323 participantes selecionadas são aquelas que apresentaram positividade para a infecção cervical pelo HPV e foram acompanhadas no follow-up de 12 meses nesse projeto. As participantes serão divididas em dois grupos de estudo de acordo com o desfecho da infecção pelo HPV após 12 meses. De forma que o grupo (1) de mulheres com persistência de HPV será constituído por aquelas que apresentaram positividade para o mesmo genótipo de HPV no momento da inclusão do estudo e na visita de follow up após 12 meses (controle positivo). O grupo (2) de mulheres nas quais houve o clearance viral será composto por aquelas que apresentaram, após 12 meses, resultado negativo para a infecção pelo HPV detectado no momento da inclusão no estudo

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útil para estabelecer se há associação deste fator de virulência com a persistência da infecção pelo HPV. Portanto, esse estudo poderá nortear o desenvolvimento de novas estratégias diagnósticas, permitindo rastrear, dentre a população de mulheres brasileiras em idade reprodutiva, a parcela com risco aumentado para a infecção persistente pelo HPV e as lesões HPV-induzidas.

Objetivo da Pesquisa:

Comparar as cargas cérvico-vaginais de Gardnerella vaginalis, Prevotella bivia e Atopobium vaginae em mulheres com clearance e com persistência da infecção cervical por HPV de alto risco-oncogênico. Além disso, determinar se a presença do gene codificante da sialidase de G. vaginalis e P. bivia está associada com o desfecho da infecção no período de 12 meses em mulheres imunocompetentes em idade reprodutiva.

Objetivo Secundário:

Selecionar os casos de infecção persistente (12 meses) por genótipos de HPV de alto risco oncogênico nas mulheres pertencentes à coorte do projeto "Prevalência de coinfecção e fatores associados ao Papilomavírus Humano (HPV) e à Chlamydia trachomatis em mulheres em idade reprodutiva atendidas em Programa de Prevenção de Câncer do Colo Uterino na região sudeste do Brasil" - HPV-CT;

Selecionar os casos de clearance (12 meses) da infecção cervical por genótipos de HPV de alto risco oncogênico nas mulheres pertencentes à coorte do projeto 7 "Prevalência de coinfecção e fatores associados ao Papilomavírus Humano (HPV) e à Chlamydia trachomatis em mulheres em idade reprodutiva atendidas em Programa de Prevenção de Câncer do Colo Uterino na região sudeste do Brasil" - HPV-CT;

Realizar a quantificação absoluta dos genes codificantes do RNAr 16S de G. vaginalis, P. bivia e A. vaginae no conteúdo cérvico-vaginal das mulheres selecionadas a partir da coorte "HPV-CT";

Comparar o número de cópias dos genes codificantes do RNAr 16S de G. vaginalis, P. bivia e A. vaginae entre as mulheres da coorte "HPV-CT" que tiveram o clearance do HPV com aquelas que tiveram persistência da infecção no período de 12 meses;

Detectar a presença dos genes codificantes de sialidade de G. vaginalis e P. bivia no conteúdo cérvico-vaginal das mulheres selecionadas a partir da coorte "HPV-CT";

Comparar a positividade dos genes codificantes de sialidade de G. vaginalis e P. bivia entre as mulheres da coorte "HPV-CT" que tiveram o clearance do HPV com aquelas que tiveram persistência da infecção no período de 12 meses;

Metodologia

Comitê de Ética em Pesquisa FMB/UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1986225.pdf	04/08/2022 11:21:06		Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoArmazenamentoNaSalaDeCriogeniaUnipex.pdf	04/08/2022 11:16:41	RAFAEL BELLETI	Aceito
Declaração de Instituição e Infraestrutura	AceiteUnipex.pdf	04/08/2022 11:16:16	RAFAEL BELLETI	Aceito
Declaração de Instituição e Infraestrutura	TermoDeAnuenciaInstitucional.pdf	04/08/2022 11:16:07	RAFAEL BELLETI	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoDePesquisa.pdf	04/08/2022 11:15:52	RAFAEL BELLETI	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Dispensatcle.pdf	04/08/2022 11:15:29	RAFAEL BELLETI	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Biorrep_HPV.pdf	04/08/2022 11:14:34	RAFAEL BELLETI	Aceito
Declaração de Pesquisadores	Declaracao.pdf	04/08/2022 11:14:03	RAFAEL BELLETI	Aceito
Parecer Anterior	CEP_HPV.pdf	04/08/2022 11:13:50	RAFAEL BELLETI	Aceito
Folha de Rosto	FolhaDeRostoAssinada.pdf	04/08/2022 11:13:39	RAFAEL BELLETI	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não