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Carolina Sanitá Tafner Ferreira

Aspectos microbiológicos do fluído cérvico-vaginal da vaginose bacteriana em mulheres em idade reprodutiva

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

Orientadora: Profa. Dra. Camila Marconi

Coorientadora: Profa. Dra. Márcia Guimarães

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Palavras-chave: *Atopobium vaginae*; *Gardnerella vaginalis*; Vaginose bacteriana; metronidazol; sialidases bacterianas.

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1. Resumo

A microbiota vaginal normal é predominantemente composta por espécies de *Lactobacillus* spp., enquanto que o principal tipo de alteração de microbiota vaginal, a vaginose bacteriana (VB), é composta por uma diversidade de bactérias, não pela predominância de uma única espécie. Todavia, dentre as bactérias mais prevalentes nessa condição estão *Atopobium vaginae* e *Gardnerella vaginalis*, espécies conhecidamente produtoras de fatores de virulência, dentre eles a capacidade de formar biofilmes vaginais, dificultando a ação de drogas antimicrobianas, como o metronidazol, para o tratamento da VB. No caso particular da *G. vaginalis* deve-se destacar a produção de sialidases por algumas linhagens que conferem a capacidade de comprometer a barreira mucosa e degradar IgA vaginal, contribuindo para a adesão e proliferação de diversas espécies bacterianas que pertencem ao *core* patológico da VB. Entretanto, a ação das sialidases sobre a composição desse microbioma vaginal ainda não foi investigada. Dessa forma, os objetivos desse estudo foram: (1) determinar se as cargas cérvico-vaginais das espécies *A. vaginae* e *G. vaginalis* na VB estão associadas com o desfecho do tratamento, utilizando o tratamento convencional com metronidazol; (2) avaliar a influência da presença do gene da sialidase de *G. vaginalis* e da produção de sialidases sobre a composição do microbioma vaginal. Para tanto, foram incluídas 245 mulheres em idade reprodutiva no primeiro estudo, classificadas de acordo com o padrão de microbiota vaginal em normal, intermediária e VB. Daquelas com VB que realizaram o tratamento corretamente, foram classificadas como cura e falha no tratamento. As 114 mulheres incluídas no segundo estudo também foram separadas de acordo com o padrão de microbiota vaginal. Das 100 mulheres sequenciadas com sucesso, apenas aquelas com microbiota vaginal alterada foram avaliadas quanto à presença ou ausência do gene da sialidase de *G. vaginalis* e das sialidases cérvico-vaginais. A microbiota vaginal das participantes foi classificada por microscopia e o microbioma vaginal foi determinado pelo sequenciamento em equipamento 400PE MiSeq (Illumina Inc., San Diego, Califórnia). As quantificações de *A. vaginae* e *G. vaginalis*, bem como a detecção do gene da sialidase de *G. vaginalis* foram realizadas pela Reação em Cadeia da Polimerase (PCR) em tempo real e a atividade de sialidases foi detectada por meio de ensaio fluorogênico. Os resultados desses estudos demonstraram que: (1) não há diferença significativa com relação à carga bacteriana de *A. vaginae* no pré-tratamento entre as mulheres que curaram e aquelas que falharam no tratamento, enquanto que a carga de *G. vaginalis* foi significativamente maior naquelas que obtiveram cura; (2) a presença de linhagens de *G. vaginalis* produtoras de sialidases na microbiota vaginal anormal está associada a maior abundância de determinadas bactérias associadas à VB. Em conclusão: (1) as cargas bacterianas de *A. vaginae* e *G. vaginalis* não estão associadas à falha no tratamento, portanto devem ser considerados outros aspectos microbiológicos, bem como os aspectos imunológicos do hospedeiro; (2) Devem ser consideradas as peculiaridades referentes a determinadas linhagens bacterianas, como a produção de fatores de virulência no estabelecimento da microbiota vaginal anormal, uma vez que as sialidases são capazes de modular componentes do microbioma.

2. Revisão de literatura

O equilíbrio de interação entre o indivíduo e a microbiota residente é de fundamental importância para a manutenção de um organismo saudável, visto que esta exerce um papel protetor contra inúmeras doenças [1-3]. A microbiota intestinal e sua relação com condições patológicas têm sido a mais amplamente estudada e estudos já demonstraram que alterações na sua composição estão associadas a doenças crônicas como obesidade, doença inflamatória intestinal [2, 4]. Em vista da importância da relação –microbiota-hospedeiro, um grande número de estudos tem procurado determinar a composição exata do microbioma humano, viabilizado a partir dos avanços nas técnicas de sequenciamento de nova geração, incluindo as de metagenômica [5].

No contexto da microbiota vaginal, já foi demonstrado que ela exerce um papel importante para a saúde reprodutiva da mulher [6, 7]. A microbiota vaginal normal é composta por diferentes espécies do gênero *Lactobacillus* [8]. As espécies de *Lactobacillus* mais frequentemente encontradas no ambiente vaginal são *L. crispatus, L. iners, L. gasseri* e *L. jensenii* [8]. Inúmeros estudos demonstraram que tais espécies produzem substâncias que contribuem para a manutenção do ambiente vaginal saudável [6, 7, 9-13]. Dentre estas substâncias está o ácido lático, que mantém o pH vaginal baixo entre 3,8 e 4,5 [7]. Sua produção ocorre a partir da fermentação da glicose no metabolismo energético dos *Lactobacillus*. O glicogênio disponível nas células epiteliais é liberado a partir da citólise dessas células, sendo ele convertido em monossacarídeos pelas enzimas líticas da célula, os quais são convertidos em ácido lático pelas bactérias [7, 13]. Algumas espécies de *Lactobacillus* também são capazes de produzir peróxido de hidrogênio (H_2O_2), o qual apresenta potencial antimicrobiano, inibindo a proliferação de micro-organismos potencialmente patogênicos [6, 10]. Outras substâncias antimicrobianas, como as bacteriocinas também podem ser

produzidas por *Lactobacillus*, como a lactocina e a gassericina A [11, 12]. A maioria das bacteriocinas tem ação pela formação de poros na membrana celular e consequente efluxo de adenosina trifostato (ATP) [11]. Embora os lactobacilos sejam as espécies mais prevalentes na microbiota vaginal, inúmeras outras bactérias podem estar presentes mesmo em condições normais. Aproximadamente 30% das mulheres com escore de Nugent correspondente a microbiota vaginal normal apresentam *G. vaginalis* concomitantemente com *Lactobacillus* spp [14]. Portanto, apesar dos inúmeros produtos de lactobacilos benéficos ao ambiente vaginal, ainda é necessária melhor compreensão da interação entre a microbiota vaginal normal e seus componentes com o hospedeiro, bem como das alterações dessa microbiota e os produtos do metabolismo das espécies envolvidas.

Mediante a depleção total ou parcial dos *Lactobacillus* há o crescimento aumentado das outras espécies bacterianas que compõem o ambiente vaginal [15]. Tais espécies são em sua maioria anaeróbias facultativas ou estritas, dentre elas *Gardnerella vaginalis, Atopobium vaginae, Prevotella bivia, Mobiluncus curtisii,* entre outras [16, 17]. O primeiro micro-organismo identificado como associado à vaginose bacteriana foi *G. vaginalis* através dos métodos tradicionais de cultura [18]. Posteriormente, com o avanço das técnicas moleculares, foi possível a identificação de outras bactérias [17].

Desde o primeiro estudo acerca da composição da microbiota vaginal realizado em 1892 por Doderlein [19], autores propuseram sistemas de classificação microscópica da microbiota. Os principais sistemas de classificação atualmente aceitos pelo Ministério da Saúde [20] são o método de Amsel [21] e o de Nugent [22], sendo este último considerado o método padrão-ouro para o diagnóstico da vaginose bacteriana. Ele se baseia na semi-quantificação dos morfotipos bacterianos observados microscopicamente no esfregaço vaginal em lâmina após a coloração de Gram e atribui escores a essa microbiota. De forma que escores de 0-3 correspondem a microbiota vaginal normal; de 4-6 como microbiota intermediária e de 7-10 como vaginose bacteriana, enquanto que o método de Amsel se

baseia na presença de três ou mais dos seguintes achados: corrimento vaginal branco, fino e homogêneo; aumento do pH vaginal (>4,5); *whiff test* positivo (presença de um odor fétido com a adição de hidróxido de potássio a 10% ao conteúdo vaginal) e/ou a presença de *clue cells,* que são células totalmente envoltas por bactérias, no esfregaço vaginal a fresco em lâmina avaliado por microscopia [21].

A vaginose bacteriana é o principal tipo alteração de microbiota vaginal em mulheres em idade reprodutiva. Sua prevalência é variável, mas assume-se que em média 30% da população feminina em idade reprodutiva no mundo apresentem tal alteração [23-26]. Mulheres com essa alteração de microbiota apresentam maior risco para a aquisição de infecções sexualmente transmissíveis (IST) como o vírus da imunodeficiência humana (HIV), *Chlamydia trachomatis* e *Neisseria gonorrhoeae* [27-29]. Além disso, a vaginose bacteriana está associada à infertilidade e ao mau desfecho gestacional, como parto prematuro e baixo peso do recém-nascido [30-32].

A composição microbiana vaginal pode variar de mulher pra mulher e ao longo do tempo, dependendo da população estudada, podendo ser relativo aos hábitos comportamentais, de higiene, histórico clínico ou ainda a características genéticas, inclusive na vaginose bacteriana, sendo considerada uma condição bastante heterogênea [33, 34]. Estudos iniciais para a identificação das bactérias associadas à vaginose bacteriana foram baseados em métodos de cultura, limitando o número de espécies identificadas [15, 18, 35]. Os métodos de cultura tradicionais possibilitam apenas a identificação daquelas espécies capazes de crescer nesses meios de cultivo. Com o desenvolvimento de técnicas moleculares como a Reação em Cadeia da Polimerase (PCR) e a clonagem do gene bacteriano do RNAr 16S foi possível a identificação de outras espécies bacterianas [17], mesmo embora estas técnicas ainda sejam limitadas pela escolha dos *primers* utilizados. Estudos mais recentes têm utilizado técnicas moleculares mais refinadas para a caracterização exata da microbiota, utilizando o sequenciamento de nova geração de parte do gene bacteriano

que codifica o RNA ribossômico 16S [8, 36]. Dessa forma, foi possível caracterizar o microbioma vaginal.

Estudo pioneiro envolvendo mulheres norte-americanas em idade reprodutiva verificou que a microbiota vaginal pode ser classificada em cinco tipos de comunidades (CST, do inglês *community-state type*), sendo quatro delas constituídas por espécies de *Lactobacillus* predominantes: *L. crispatus* (CSTI), *L. gasseri* (CSTII), *L. iners* (CSTIII) e *L. jensenii* (CSTV), representando 75% das mulheres e a CSTIV sem predominância de lactobacilos, na qual se enquadram a maioria dos casos de vaginose bacteriana, visto que nesta condição é observada maior alfa-diversidade bacteriana [8]. De fato, estudos de microbioma contribuíram para a melhor caracterização dos micro-organismos associados à vaginose bacteriana.

Tais espécies estabelecem relações simbióticas por meio dos metabólitos produzidos por elas. Por exemplo, foi demonstrado que *G. vaginalis* produz aminoácidos importantes para o metabolismo de *P. bivia*, enquanto que *P. bivia* produz amônia, que pode ser utilizada por *G. vaginalis*, favorecendo o seu crescimento [37]. Além disso, sugere-se que os fatores de virulência produzidos por algumas bactérias podem agir conjuntamente, de modo que a produção de um pode propiciar consequentemente o aumento de outro, uma vez que já foi demonstrado que a produção de sialidases por determinadas cepas de *G. vaginalis* aumentam a produção de biofilme devido a sua atividade como mucinase [38]. De fato, foi verificada uma forte associação entre *G. vaginalis* e *A. vaginae* na formação do biofilme bacteriano vaginal na vaginose bacteriana [39].

Atualmente, considera-se que a *G. vaginalis* seja o agente iniciador da formação do biofilme bacteriano vaginal, tornando um ambiente favorável para a proliferação de outras bactérias anaeróbias como *A. vaginae* e que estas espécies possam estar estabelecendo relações mutualísticas [39]. Esse biofilme pode estar associado à

transferência de carga bacteriana para o parceiro pela relação sexual desprotegida, o que pode estar associado à recorrência dessa condição quando não ocorre o tratamento do parceiro em conjunto [40]. Além disso, a produção de biofilme, subsequente a adesão inicial das bactérias, é de fundamental importância para a persistência dos micro-organismos no local, conferindo aumentada tolerância a antibióticos, o que contribui para a cronicidade das doenças e/ou recidivas [41].

O tratamento usual para a vaginose bacteriana recomendado pelo Centers for Disease and Control and Prevention (CDC) [42], que consiste na administração de 500 mg de metronidazol duas vezes ao dia durante 7 dias tem demonstrado baixa eficiência [43, 44]. Embora nenhuma evidência conclusiva tenha sido demonstrada, sugere-se que a falha no tratamento da vaginose bacteriana pode estar associada à formação de biofilmes e a heterogeneidade dessa condição [45]. A ausência de identificação de um único agente etiológico faz com que o tratamento seja baseado no empirismo, contribuindo assim para o aumento da resistência antimicrobiana [46].

A abundância de *A. vaginae* no ambiente vaginal já foi positivamente correlacionada com os valores de pH vaginal e com os escores segundo o sistema de Nugent de classificação microscópica da microbiota vaginal [47]. Além disso, já foi demonstrado que a *G. vaginalis* produz algumas enzimas que propiciam a invasão do epitélio vaginal, como a citotoxina vaginolisina, bem como prolidases e sialidases, capazes de degradar componentes da camada mucosa, a qual constitui uma barreira de proteção contra micro-organismos patogênicos [48]. De fato, a atividade de sialidases está aumentada em grande parte das mulheres com vaginose bacteriana. Tais enzimas contribuem para evasão dos micro-organismos associados à vaginose bacteriana da resposta imune do hospedeiro, uma vez que degradam importantes moléculas envolvidas na resposta imune, como a imunoglobulina A e interferon-gamma [48]. A presença das sialidases tem sido associada a maior suscetibilidade a aquisição de IST, como por *N. gonorroheae* [49] e HIV

[50] e também a complicações gestacionais como nascimento pré-termo [51].

Dada a grande diversidade de fatores de virulência produzidos por *G. vaginalis*, como de enzimas propiciadoras da adesão ao epitélio vaginal, formação de biofilme e citotoxicidade, têm se proposto que *G. vaginalis* possa ser o agente etiológico primário na patogênese da maioria dos casos de vaginose bacteriana. Por esse motivo linhagens de *G. vaginalis* associadas à vaginose bacteriana parecem ser potencialmente mais patogênicas que a maioria dos outros micro-organismos também associados a essa condição [52, 53]. Patterson et al. [53], demonstraram que dentre os micro-organismos associados a vaginose bacteriana, apenas a *G. vaginalis* apresenta todos os três fatores, evidenciando que *G. vaginalis* possui maior potencial de virulência em relação aos outros micro-organismos e que os outros microrganismos sejam colonizadores oportunistas secundários a invasão do epitélio vaginal por *G. vaginalis*. Contribuindo com a hipótese de a *G. vaginalis* ser a primeira colonizadora, Machado et al. [54] demonstraram que *G. vaginalis* possui maior capacidade de adesão às células epiteliais na presença de *L. crispatus*, que outras bactérias associadas a vaginose bacteriana. No entanto, não deve ser considerado que esta bactéria possua sozinha essa capacidade visto que Criswell et al. [55] já tinham demonstrado em 1969 que a inoculação apenas de *G. vaginalis* no fluído vaginal não era o suficiente para o desenvolvimento de VB, somente a transferência do corrimento vaginal de mulheres com vaginose bacteriana foi capaz de ocasionar a mesma condição. Portanto, há controvérsias com relação ao papel de *G. vaginalis* na patogênese da vaginose bacteriana, bem como dos outros micro-organismos anaeróbios associados a essa condição.

Além dos fatores microbiológicos, os aspectos imunológicos do hospedeiro também estão envolvidos na proteção contra infecções vaginais, como candidíase ou por HPV [56, 57]. *Lactobacillus* spp. induzem a expressão de moléculas de defesa para a proteção contra infecções e manutenção de um ambiente vaginal saudável [58]. No entanto, até mesmo discretas alterações na microbiota são capazes de causar o

desequilíbrio em moléculas do sistema imune [59].

A vaginose bacteriana já foi associada a alterações nos níveis cérvico- vaginais de diversos mediadores da resposta imune, dentre eles interleucinas (IL)-1beta, IL-4, IL-6, IL-10, IL-8, IL-3, IL-7 e IL-12, além de beta-defensinas humanas, fator estimulador de colônias de granulócitos e macrófagos e lactoferrina [60-62]. Além disso, os níveis de sialidases bacterianas já foram correlacionados com o aumento nos níveis de citocinas pró-inflamatórias como a IL-1beta e IL-8 [63]. Esse desequilíbrio também pode contribuir para o aumento do risco de IST [64].

Levando em consideração as sérias consequências das alterações de microbiota vaginal para a saúde reprodutiva da mulher, é importante obter conhecimento do papel que desempenham os micro-organismos associados a essa condição, se sua abundância está associada à falha no tratamento, bem como o impacto da produção de fatores de virulência, como as sialidases, na composição da microbiota local.

3. Referências

1. Ohashi Y, Nakai S, Tsukamoto T, Masumori N, Akaza H, Miyanaga N, et al. Habitual intake of lactic acid bacteria and risk reduction of bladder cancer. Urol Int 2002;68(4):273-80.

2. Rayes N, Hansen S, Seehofer D, Muller AR, Serke S, Bengmark S, et al. Early enteral supply of fiber and lactobacilli versus conventional nutrition: a controlled trial in patients with major abdominal surgery. Nutrition 2002 Jul-Aug;18(7-8):609-15.

3. Naruszewicz M, Johansson ML, Zapolska-Downar D, Bukowska H. Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. Am J Clin Nutr 2002 Dec;76(6):1249-55.

4. Yang L, Lu X, Nossa CW, Francois F, Peek RM, Pei Z. Inflammation and intestinal metaplasia of the distal esophagus are associated with alterations in the microbiome. Gastroenterology 2009 Aug;137(2):588-97.

5. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, et al. The NIH Human Microbiome Project. Genome Res. 2009 Dec;19(12):2317-23.

6. Hillier SL, Krohn MA, Rabe LK, Klebanoff SJ, Eschenbach DA. The normal vaginal flora, H2O2- producing lactobacilli, and bacterial vaginosis in pregnant women. Clin Infect Dis 1993 Jun;16(4):S273-81.

7. Graver MA, Wade JJ. The role of acidification in the inhibition of *Neisseria gonorrhoeae* by vaginal lactobacilli during anaerobic growth. Ann Clin Microbiol Antimicrob 2011 Feb;10:8.

8. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci USA 2011 Mar;108(1):4680-7.

9. Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA. Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. Infect Immun. 1999 Oct;67(10):5170-5.

10. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, et al. Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J Clin Microbiol. 1989 Feb;27(2):251-6.

11. Kawai Y, Salto T, Toba T, Samant SK, Itoh T. Isolation and characterization of a highly hydrophobic new bacteriocin (gassericin A) from *Lactobacillus gasseri* LA39. Biosci Biotechnol Biochem 1994 Jul;58(7):1218-21.

12. Li J, Aroutcheva AA, Faro S, Chikindas ML. Mode of action of lactocin 160, a bacteriocin from vaginal *Lactobacillus rhamnosus.* Infect Dis Obstet Gynecol 2005 Sep;13(3):135-40.

13. Soares Ricardo, Vieira-Baptista Pedro, Tavares Sara. Vaginose citolítica: uma entidade subdiagnosticada que mimetiza a candidíase vaginal. Acta Obstet Ginecol Port [Internet]. 2017 Jun [citado 2019 Ago 22];11(2):106-112. Disponível em: http://www.scielo.mec.pt/scielo.php?script=sci_arttext&pid=S164658302017000200007&lng=pt.

14. Marconi C, Donders GG, Parada CM, Giraldo PC, da Silva MG. Do *Atopobium vaginae*, *Megasphaera sp*. and *Leptotrichia sp*. change the local innate immune response and sialidase activity in bacterial vaginosis? Sex Transm Infect 2013 Mar;89(2):167-73.

15. Larsen B, Monif GR. Understanding the bacterial flora of the female genital tract. Clin Infect Dis 2001 Feb;32(4):e69-77.

16. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Simaey LV, et al. Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae, Gardnerella vaginalis* and bacterial vaginosis. BMC Microbiol 2004 Apr;4:16.

17. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med. 2005 Nov;353(18):1899-911.

18. Gardner HL, Dukes C. *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. Am J Obstet Gynecol 1955 May;69(5):962-76.

19. Döderlein A. Das Scheidensekret und seine Bedeutung fuerdas Puerperalfieber. Die Arten des Scheidensekretes 1892;11:699.

20. Gestação de Alto Risco / Secretaria de Políticas, Área Técnica da Saúde da Mulher. Brasília: Ministério da Saúde, 2000.

21. Amsel R, Totten PA, Spiegel CA, Chen KCS, Eschenbach DA, Holmes KK. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. Am J Med 1983 Jan;74(1):14-22.

22. Nugent RP, Krohn MA, Hillier SL. Reability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. J Clin Microbiol 1991 Feb;29(2):297-301.

23. Marconi C, Duarte MT, Silva DC, Silva MG. Prevalence of and risk factors for bacterial vaginosis among women of reproductive age attending cervical screening in southeastern Brazil. Int J Gynaecol Obstet 2015 Nov;131(2):137-41.

24. Goldenberg RL, Culhane JF, Johnson DC. Maternal infection and adverse fetal and neonatal outcomes. Clin Perinatol 2005 Sep;32(3):523-59.

25. McGregor JA, French JI. Bacterial vaginosis in pregnancy. Obstet Gynecol Surv 2000 May;55(5 Suppl 1):S1-19.

26. Tolosa JE, Chaithongwongwatthana S, Daly S, Maw WW, Gaitan H, Lumbiganon P, et al. The International Infections in Pregnancy (IIP) study: variations in the prevalence of bacterial vaginosis and distribution of morphotypes invaginal smears among pregnant women. Am J Obstet Gynecol 2006 Nov;195(5):1198-204.

27. Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL. Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. Clin Infect Dis 2003 Mar;36(5):663-8.

28. Myer L, Denny L, Telerant R, Souza M, Wright TC Jr, Kuhn L. Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. J Infect Dis 2005 Oct;192(8):1372-80.

29. Sewankambo N, Gray RH, Wawer MJ, Paxton L, McNaim D, Wabwire-Mangen F, et al. HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. Lancet 1997 Aug;350(9077):546-50.

30. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and *Chlamydia trachomatis* infection with adverse pregnancy outcome. JAMA 1986 Oct;256(14):1899-903.

31. Gravett MG, Hummel D, Eschenbach DA, Holmes KK. Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. Obstet Gynecol 1986 Feb;67(2):229-37.

32. McGregor JA, French JI, Jones W, Milligan K, McKinney PJ, Patterson E, et al. Bacterial vaginosis is associated with prematurity and vaginal fluid mucinase and sialidase: results of a controlled trial of topical clindamycin cream. Am J Obstet Gynecol 1994 Apr;170(4):1048-59.

33. Josey WE, Schwebke JR. The polymicrobial hypothesis of bacterial vaginosis causation: a reassessment. Int J STD AIDS 2008 Mar;19(3):152-4.

34. Kim TK, Thomas SM, Ho M, Sharma S Reich, Frank CI, et al. Heterogeneity of vaginal microbial communities within individuals. J Clin Microbiol 2009 Apr;47(4):1181-9.

35. Spiegel CA, Eschenbach DA, Amsel R, Holmes KK. Curved anaerobic bacteria in bacterial (nonspecific) vaginosis and their response to antimicrobial therapy. J Infect Dis 1983 Nov;148(5):817-22.

36. Fadrosh DW, Ma B, Gajer P, Ott S, Sengamalay N, Brotman RM, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome 2014 Feb;2(1):6.

37. Pybus V, Onderdonk AB. Evidence for a commensal, symbiotic relationship between *Gardnerella vaginalis* and *Prevotella bivia* involving ammonia: potential significance for bacterial vaginosis. J Infect Dis 1997 Feb;175(2):406-13.

38. Santiago GL, Deschaght P, El Aila N, Kiama TN, Verstraelen H, Jefferson KK, et al. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. Am J Obstet Gynecol 2011 May;204(5):450.e1-7.

39. Swidsinski A, Mendling W, Loening-Baucke V, Ladhoff A, Swidsinski S, Hale LP, et al. Adherent biofilms in bacterial vaginosis. Obstet Gynecol 2005 Nov;106(5 Pt 1):1013-23.

40. Danielsson D, Teigen PK, Moi H. The genital econiche: focus on microbiota and bacterial vaginosis. Ann N Y Acad Sci 2011 Aug;1230:48-58.

41. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, et al. Bacterial biofilms in nature and disease. Annu Rev Microbiol 1987;41:435–64.

42. Workowski KA, Berman S, Centers for Disease Control and Prevention (CDC). Sexually transmitted diseases treatment guidelines, 2010. MMWR Recomm Rep 2010 Dec;59(RR-12):1-110.

43. Bradshaw CS, Morton AN, Hocking J, Garland SM, Morris MB, Moss LM, et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. J Infect Dis 2006 Jun;193(11):1478-86.

44. Larsson PG, Forsum U. Bacterial vaginosis–a disturbed bacterial flora and treatment enigma. APMIS 2005 May;113(5):305-16.

45. Swidsinski A, Loening-Baucke V, Swidsinski S, Verstraelen H. Polymicrobial *Gardnerella* biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. Arch Gynecol Obstet 2015 Mar;291(3):605-9.

46. Stanley NR, Lazazzera BA. Environmental signals and regulatory pathways that influence biofilm formation. Mol Microbiol 2004 May;52(4):917-24.

47. Marconi C, Cruciani F, Vitali B, Donders GG. Correlation of *Atopobium vaginae* amount with bacterial vaginosis markers. J Low Genit Tract Dis 2012 Apr;16(2):127-32.

48. Harwich MD Jr, Alves JM, Buck GA, Strauss JF, Patterson JL, Oki AT, et al. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. BMC Genomics 2010 Jun;11:375.

49. Ketterer MR, Rice PA, Gulati S, Kiel S, Byerly L, Fortenberry JD, et al,. Desialylation of *Neisseria gonorrhoeae* lipooligosaccharide by cervicovaginal microbiome sialidases: The potential for enhancing infectivity in men. J Infect Dis 2016 Dec;214(11):1621-28.

50. Hu H, Shioda T, Moriya C, Xin X, Hasan MK, Miyake K, et al. Infectivities of human and other primate lentiviruses are activated by desialylation of the virion surface. J Virol 1996 Nov; 70(11): 7462–7470.

51. Olmsted SS, Meyn LA, Rohan LC, Hillier SL. Glycosidase and proteinase activity of anaerobic gram-negative bacteria isolated from women with bacterial vaginosis. Sex Transm Dis 2003 Mar;30(3):257-61.

52. Alves P, Castro J, Sousa C, Cereija TB, Cerca N. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in na in vitro biofilm formation model. J Infect Dis 2014 Aug;210(4):593-6.

53. Patterson JL, Stull-Lane A, Girerd PH, Jefferson KK. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial vaginosis-associated anaerobes. Microbiology 2010 Feb;156(Pt 2):392-9.

54. Machado A, Jefferson KK, Cerca N. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. Int J Mol Sci 2013 Jun;14(6):12004-12.

55. Criswell BS, Ludwig CL, Gardner HL, Dukes CD. Vaginitis by inoculation from culture. Obstet Gynecol 1969 Feb;33(2):195-9.

56. Cassone A. Vulvovaginal *Candida albicans* infections: pathogenesis, immunity and vaccine prospects. BJOG 2015 May;122(6):785-94.

57. Toh ZQ, Kosasih J, Russell FM, Garland SM, Mulholland EK, Licciardi PV. Recombinant human papillomavirus nonavalent vaccine in the prevention of cancers caused by human papillomavirus. Infect Drug Resist 2019 Jul;12:1951-1967.

58. Niu XX, Li T, Zhang X, Wang SX, Liu ZH. *Lactobacillus crispatus* modulates vaginal epithelial cell innate response to *Candida albicans*. Chin Med J (Engl) 2017 Feb;130(3):273-79.

59. Wiggins R, Hicks SJ, Soothill PW, Millar MR, Corfield AP. Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract. Sex Transm Infect 2001 Dec;77:402-8.

60. Spear GT, Kendrick SR, Chen HY, Thomas TT, Bahk M, Balderas R, et al. Multiplex immunoassay of lower genital tract mucosal fluid from women attending an urban STD clinic shows broadly increased IL1ß and lactoferrin. PLoS One 2011 May;6(5):e19560.

61. Ferreira CS, Marconi C, Parada CM, Duarte MT, Gonçalves AP, Rudge MV, et al. Bacterial vaginosis in pregnant adolescents: proinflammatory cytokine and bacterial sialidase profile. Cross- sectional study. Sao Paulo Med J 2015 Out;133(6):465-70.

62. Marconi C, Santos-Greatti MM, Parada CM, Pontes A, Pontes AG, Giraldo PC, et al. Cervicovaginal levels of proinflammatory cytokines are increased during chlamydial infection in bacterial vaginosis but not in lactobacilli-dominated flora. J Low Genit Tract Dis 2014 Jul;18(3):261-5.

63. Cauci S, Guaschino S, De Aloysio D, Driussi S, De Santo D, Penacchioni P, et al.. Interrelationships of interleukin-8 with interleukin-1beta and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women. Mol Hum Reprod 2003 Jan;9(1):53-8.

64. Sturm-Ramirez K, Gaye-Diallo A, Eisen G, Mboup S, Kanki PJ. High levels of tumor necrosis factor- alpha and interleukin-1beta in bacterial vaginosis may increase susceptibility to Human Immunodeficiency Virus. J Infect Dis 2000;182:467-73.

4. Artigo I

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RESEARCH ARTICLE

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Treatment failure of bacterial vaginosis is not associated with higher loads of *Atopobium vaginae* and *Gardnerella vaginalis*

Carolina Sanitá Tafner Ferreira,[1] Gilbert Gerard Donders,[2,3] Cristina Maria Garcia de Lima Parada,[4] Andrea da Rocha Tristão,[5] Thaiz Fernandes,[6] Márcia Guimarães da Silva[1] and Camila Marconi[1,6,*]

Abstract

Purpose. Cervicovaginal *Atopobium vaginae* and *Gardnerella vaginalis* are strongly associated with bacterial vaginosis (BV) and are the main components of vaginal biofilms. The low efficacy of BV treatment with metronidazole may be due to the presence of such biofilms. Thus, the aim of this study was to compare the pretreatment cervicovaginal loads of *A. vaginae* and *G. vaginalis* for women who restored normal flora and those who persisted with BV after a full course of oral metronidazole.

Methodology. In this cross-sectional study, 309 reproductive-aged women were recruited in a primary health care service in Botucatu, Brazil. Cervicovaginal samples were tested for genital tract infections, microscopic classification of local microbiota and molecular quantification of *A. vaginae* and *G. vaginalis*.

Results. All the participants with concurrent cervicovaginal infections (n=64) were excluded. A total of 84 out of 245 (34.3 %) women had BV at enrolment and 43 (51.2 %) of them completed the treatment and returned for follow-up. Evaluation of the vaginal microbiota at follow-up showed that 29 (67.4 %) women restored normal vaginal flora, while 14 (32.6 %) still had BV. The pretreatment loads of *G. vaginalis* were lower in women with treatment failure (P=0.001) compared to those who successfully restored normal flora. The loads of *A. vaginae* did not differ between the groups.

Conclusion. Although *G. vaginalis* produces several virulence factors and its loads correlate positively with those of *A. vaginae*, higher cervicovaginal quantities of these bacteria are not associated with treatment failure of BV after oral metronidazole.

INTRODUCTION

Bacterial vaginosis (BV) is the most frequent type of abnormal vaginal flora in reproductive-aged women [1, 2]. Microbiologically, BV is defined by the shift from a normal lactobacilli-dominated flora to an excessive growth of anaerobic bacteria. In fact, several bacterial species have already been shown to be strongly associated with BV, such as *Atopobium vaginae, Gardnerella vaginalis, Prevotella* spp. and *Mobiluncus* spp., among others [3–5]. Women with BV have increased risk for acquisition for several sexually transmitted infections, such as human immunodeficiency virus (HIV), *Neisseria gonorrhoeae* and *Chlamydia trachomatis* [6–8].

The microbial features of BV are not completed understood, but studies have already shown that bacterial diversity [3–5] and loads [9, 10] are increased in this condition compared to normal vaginal microbiota. When assessed individually, the loads of *A. vaginae* and *G. vaginalis* are also higher in BV [11]. Regarding *A. vaginae,* it is detected in nearly all women with BV and correlates positively with vaginal pH and Nugent scores [9]. In addition, independently of the presence of BV, higher vaginal loads of *A. vaginae* in pregnant women are associated with preterm birth [10]. Similarly, *G. vaginalis* can be detected in up to 100 % of women with BV [12, 13]. It has already been shown that strains of *G. vaginalis* isolated from women with BV are more virulent compared to strains recovered from women with normal microbiota [14]. Several virulence factors are produced by *G. vaginalis*, including hydrolytic enzymes such as sialidases and biofilms [15–18]. Sialidases produced by *G. vaginalis*

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Author affiliations: [1]Department of Pathology, Botucatu Medical School, UNESP – Univ Estadual Paulista, Botucatu, Brazil; [2]Femicare vzw, Clinical Research for Women, Gasthuismolenstraat 31, 3300, Tienen, Belgium; [3]Department of Obstetrics and Gynecology, Antwerp University Hospital, Edegem, Belgium; [4]Department of Nursing, Botucatu Medical School, UNESP – Univ Estadual Paulista, Botucatu, Brazil; [5]Department of Gynecology and Obstetrics, Botucatu Medical School, UNESP – Univ Estadual Paulista, Botucatu, Brazil; [6]Department of Basic Pathology, Setor de Ciências Biológicas, UFPR – Univ Federal do Paraná, Curitiba, Brazil.

***Correspondence:** Camila Marconi, marconi.cml@gmail.com *or* marconi@ufpr.br

Keywords: bacterial vaginosis; *Gardnerella vaginalis*; *Atopobium vaginae*; metronidazole.

Abbreviations: BV, bacterial vaginosis; CDC, Centers for Disease and Control and Prevention; CI, confidence interval.

degrade immunoglobulin A, impairing the local immune response [15]. Sialidases-producing strains of *G. vaginalis* also have increased capability of biofilm production [18, 19]. *A. vaginae* and *G. vaginalis* are the main components of vaginal biofilms [16, 17, 20].

Currently, one of the most common treatments for BV consists of a 1 g oral dose of metronidazole administered daily for 7 days. This regimen is in accordance with the guidelines from the Centers for Disease and Control and Prevention (CDC) [21]. This is the standard treatment for nearly all non-pregnant women attending gynaecology outpatient clinics in the Brazilian public health system. The overall prevalence of BV in this population is 30.0 % [2]. However, the metronidazole regimen has demonstrated low efficacy, with high rates of BV persistence and recurrence after treatment [22, 23]. The question of whether the poor response to metronidazole therapy is due to biofilm formation by *A. vaginae* and *G. vaginalis* and, consequently, their higher vaginal loads, is currently under discussion [24]. Other aspects of BV may also play a role in its treatment failure, such as the high diversity and heterogeneity of vaginal bacterial species [4, 5, 25]. Moreover, antimicrobial resistance genes have already been described in bacterial species associated with BV [26]. Further, other conditions of vaginal flora that are associated with loss of *Lactobacillus*, but are less responsive to metronidazole, such as aerobic vaginitis [27], may have been overlooked in some studies [28–30], leading to a false negative assessment of the treatment efficacy of metronidazole in such cases.

Taken together, these recent findings reinforce the importance of elucidating the microbiological features involved in the failure of BV treatment in order to seek strategies to increase its efficacy. Given that the growth of *A. vaginae* and *G. vaginalis* is expected to be higher in the presence of vaginal biofilms, the aim of this study was to compare their pretreatment cervicovaginal loads in women with BV who had successfully restored normal vaginal microbiota after oral metronidazole with those in women who had persisted with BV despite proper treatment.

METHODS

A total of 309 non-pregnant reproductive-aged women attending a public primary health care service in Botucatu, São Paulo, Brazil, were invited to participate in this prospective study. All of the women enrolled in the study attended outpatient clinics during a 6-month period in 2014 for routine cervical cancer screening (Pap test). Women were not included if they reported urinary loss, menstrual period, antibiotics in the previous 30 days and sexual intercourse in the last 48 h.

Sociodemographic, behavioural and clinical history data were assessed by applying an individual standardized questionnaire. During the physical examination for Pap test collection, the vaginal pH was measured by allowing 1 min of direct contact between pH strips (4.0–7.0; Merck, Darmstadt, Germany) and the vaginal wall. A potassium hydroxide test was performed by placing a drop of 10 % KOH (v/v) on a swab with vaginal content. Results were then expressed as negative, doubtful and positive (whiff test). Microscopic analyses of vaginal wet-mount smears were performed to detect the presence of *Candida* sp.-like morphotypes, and *Trichomonas vaginalis*. The other vaginal smear was Gram-stained and used for vaginal flora classification according to the Nugent criteria as normal (score 0 to 3), intermediate (score 4 to 6) and BV (score 7 to 10) [31]. Endocervical samples were also taken to test for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using end-point PCR and real-time PCR (RT-PCR), respectively, as described previously [27, 32, 33]. Finally, cervicovaginal samples were obtained by rinsing the vaginal wall using a 3 ml sterile NaCl 9.5 % solution according to a standardized technique [27].

The *A. vaginae* and *G. vaginalis* loads were determined by RT-PCR for cervicovaginal samples from women with BV and intermediate and normal flora that tested negative for other concurrent infections. Total bacterial DNA was extracted from the pellets of cervicovaginal samples using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions for Gram-negative and Gram-positive bacteria using a 100 µl final elution step. The reactions consisted of a 13 ul volume of Maxima SYBR Green/ROX mix (Fermentas, St Leon-Rot, Germany) and the pairs of primers specifically designed to amplify 16S rRNA regions of *A. vaginae* (81 bp) and *G. vaginalis* (332 bp) in separate reactions with 2 ul of DNA template [34, 35]. Amplification cycles were performed in LineGeneK (Bioer, China) equipment in 40 repetition cycles. The bacterial loads were calculated by the interpolation of cycle threshold values for each sample on the standard curve obtained with 10-fold serial dilution of plasmid DNA. The plasmids contained the amplified sequences of *A. vaginae* and *G. vaginalis* 16S rRNA genes that were constructed according to methods described elsewhere [11]. All samples were tested in duplicate and if a difference of more than one cycle threshold was observed between them, reactions were repeated. The mean values of the duplicates were calculated and the bacterial loads were expressed by number of copies ml^{-1} of cervicovaginal sample. Final bacterial loads were obtained by multiplying the mean value by 50 in order to reach the elution volume of 100 µl that corresponded to 1 ml of the sample used for DNA extraction.

Treatment was offered to the 84 women who had BV at the time of enrolment. The treatment consisted of oral metronidazole 1 g administered daily for 7 days, as recommended by the CDC [21]. The 32 women with intermediate flora were only treated when they were symptomatic, and those who had no symptoms were advised to undergo re-evaluation within 30 days (this further visit was not part of this study protocol). Follow-up visits for BV-treated women were scheduled between 30 to 45 days after the end of treatment, depending on their menstrual period. During this follow-up visit, women were asked if they had completed the

treatment correctly, without skipping days. They were also asked if they had abstained from sexual intercourse during this period. Vaginal flora was then reassessed using Nugent's method [31]. Women who had taken an incomplete course of metronidazole or had had intercourse during treatment were not re-evaluated.

Comparison of discrete and continuous socio-demographics and clinical variables between the women with normal vaginal flora and BV was performed, respectively, by the Chi-squared and non-parametric Mann–Whitney tests. Comparison of the *A. vaginae* and *G. vaginalis* loads in patients classified as normal flora, intermediate flora and BV was performed using the non-parametric Kruskal–Wallis followed by Dunn's post-test. The Mann–Whitney non-parametric test was used to compare the bacterial loads of women who restored normal flora after metronidazole and those who persisted with BV. Spearman's correlation coefficient was determined for the the *A. vaginae* and *G. vaginalis* loads of the study groups. All of these analyses were performed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA), with $P<0.05$ considered to be significant.

RESULTS

From the total of 309 women initially recruited, those with vaginal candidosis (n=26, 8.4 %), trichomoniasis (n=1, 0.3 %), *C. trachomatis* (n=22, 7.1 %), *N. gonorrhoeae* (n=2, 0.6 %) or both *C. trachomatis* and *N. gonorrhoeae* infection (n=1, 0.3 %) were excluded from the study (Fig. 1). Additionally, samples from 12 (3.9 %) women were excluded because they were not suitable for analysis. Sociodemographic, behavioural and clinical data for the 245 women enrolled are shown in Table 1, according to the microscopic classification of the vaginal flora. Women

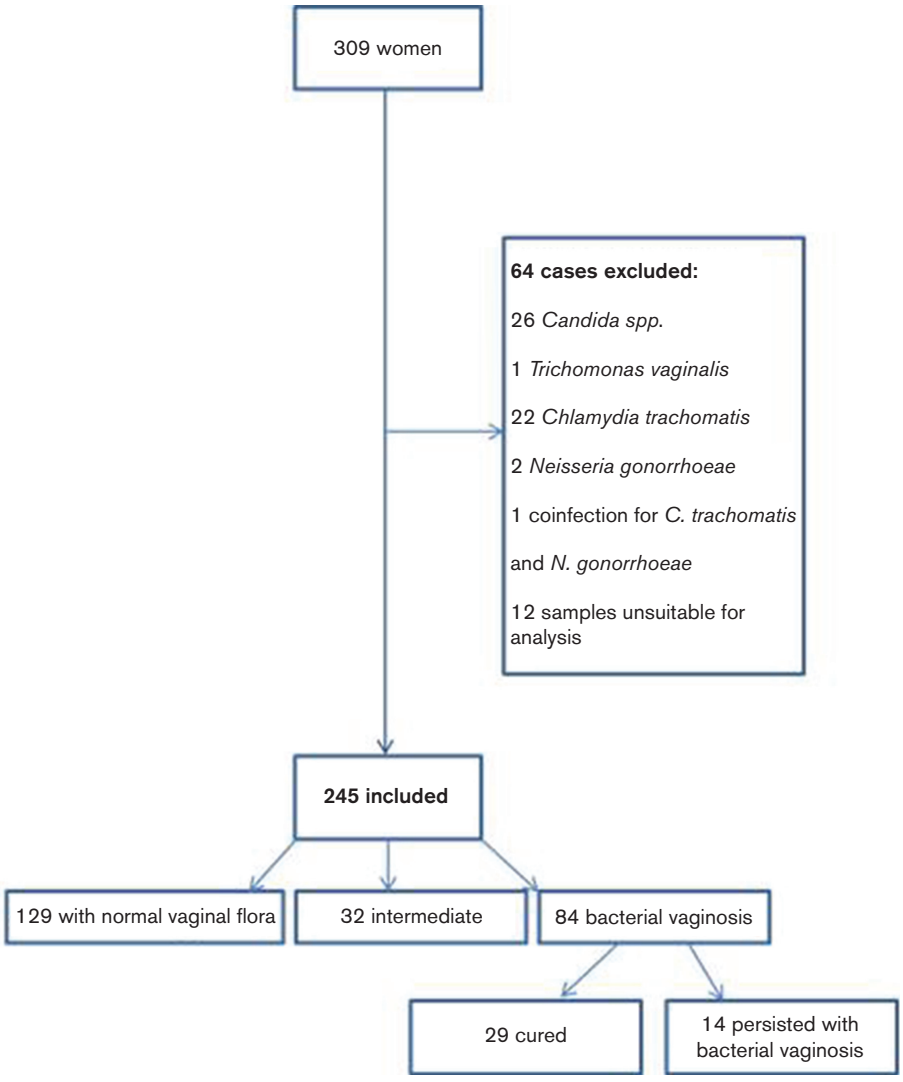

Fig. 1. Flow chart for participants in the study.

Table 1. Demographic, behavioural and clinical characteristics of the 245 women included in the study according to their vaginal flora type at enrolment

Continuous variables are expressed in median values (range) and categorized as total numbers (percentage). BV, bacterial vaginosis; STI, sexually transmitted infection.

Variables	Normal (*n*=129)	Intermediate (*n*=32)	Bacterial vaginosis (*n*=84)	*P* value
Age (years)*				
18–21 (*n*=34)	15 (44.1)	6 (17.6)	13 (38.2)	
22–45 (*n*=193)	106 (54.9)	22 (11.4)	65 (33.7)	
46+ (*n*=18)	8 (44.5)	4 (22.2)	6 (33.3)	0.53
Ethnicity*				
White (*n*=142)	82 (57.7)	19 (13.4)	41 (28.9)	
Non-white (*n*=103)	47 (45.6)	13 (12.6)	43 (41.7)	0.10
Marital status*				
Single (*n*=79)	36 (45.6)	6 (7.6)	37 (46.8)	
Married/living with partner (*n*=166)	93 (56.0)	26 (15.7)	47 (28.3)	0.01‡
Years at school‡	9 (0–16)	5.2 (1–11)	8 (0–15)	0.21
Paid job*				
Yes (*n*=133)	72 (54.1)	15 (11.3)	46 (34.6)	
No (*n*=112)	57 (50.9)	17 (15.2)	38 (33.9)	0.65
Smoking habit*				
Yes (*n*=47)	22 (46.8)	1 (2.1)	24 (51.1)	
No (*n*=198)	107 (54.0)	31 (15.7)	60 (30.3)	0.005§
Body mass index†	26.1 (16.4–49.7)	26.9 (15.6–38.1)	26.7 (16.0–53.0)	0.84
Number of sexual partners (1 year)*				
0 or 1 (*n*=216)	117 (54.2)	29 (13.4)	70 (32.4)	
2 or more (*n*=29)	12 (41.4)	3 (10.3)	14 (48.3)	0.23
Incidences of vaginal intercourse/week†	2 (0–7)	3 (0–7)	2 (0–7)	0.26
Previous BV*				
Yes (*n*=115)	53 (46.1)	17 (14.8)	45 (39.1)	
No (*n*=130)	76 (58.5)	15 (11.5)	39 (33.9)	0.15
Previous STI*				
Yes (*n*=21)	10 (47.6)	4 (19.0)	7 (33.3)	
No (*n*=224)	119 (53.1)	28 (12.5)	77 (34.4)	0.69
Consistent condom use*				
Yes (*n*=44)	18 (40.9)	7 (15.9)	19 (43.2)	
No (*n*=201)	111 (55.2)	25 (12.4)	65 (32.3)	0.23
Hormonal contraceptive (last 4 months)*				
Yes (*n*=170)	58 (58.0)	11 (11.0)	31 (31.0)	
No (*n*=145)	71 (49.0)	21 (14.5)	53 (36.6)	0.37
Vaginal pH‡	4.4 (4.0–5.0)	4.7 (4.0–5.8)	4.7 (4.0–7.0)	<0.001§
Whiff test*				
Positive (*n*=75)	14 (18.7)	8 (10.7)	53 (70.7)	
Doubtful or negative (*n*=170)	115 (67.6)	24 (14.1)	31 (18.2)	<0.001§

*Chi-square test. *P*<0.05 considered as significant.

†Kruskal–Wallis, followed by Dunn's post-test.

‡BV versus normal flora, BV versus intermediate flora.

§Differs among the three groups.

with normal flora (*n*=129, 52.6 %), intermediate flora (*n*=84, 34.3 %) and BV (*n*=84, 34.3 %) did not differ for most of the variables evaluated, except with regard to the smoking habit, which was reported more frequently by women with BV. In addition, the subset of women who were married or lived with a partner at enrolment were more likely to have normal flora. A positive whiff test and higher vaginal pH were more frequent in the BV group, followed by the intermediate and normal flora groups.

Cervicovaginal samples obtained at baseline had significantly higher loads of both *A. vaginae* and *G. vaginalis* for women with BV compared to those for women with intermediate and normal vaginal flora, as shown in Fig. 2. As shown in Table 2, there was a strong correlation between loads of *A. vaginae* and *G. vaginalis* in women with BV [Spearman r=0.69; 95 % confidence interval (CI)=0.55–0.79, P<0.01;], while in women with intermediate flora this correlation was weak and non-significant (Spearman r=0.25; 95 % CI=0.11–0.56; P=0.16), and in women with normal vaginal flora it was weak but significant (Spearman r=0.23; 95 % CI=0.06–0.40; P=0.01).

Of the 84 women who had BV at enrolment, 46 (54.8 %) finished the treatment and returned for a follow-up visit. At follow-up, two participants reported that they had forgotten to take the medication at some point, and one had had sexual intercourse during treatment, and these subjects were excluded from the analysis. The remaining 43 women who had completed the treatment properly were then divided in two groups: those who had shifted from BV to normal vaginal flora (n=29, 67.4 %) and those who had persisted with BV (n=14, 32.6 %). In the latter group, 13 women had Nugent scores higher than or equal to 7, and 1 had an intermediate vaginal flora (score=5). The participant with intermediate vaginal flora was included in the group of women who persisted with BV because she had a vaginal pH equal to 5.0 and continued to have abnormal vaginal discharge complaints. As shown in Fig. 3, the baseline cervicovaginal loads of *A. vaginae* did not differ between the group of women who restored normal flora after treatment and those who persisted with BV. However, the cervicovaginal *G. vaginalis* loads at baseline were significantly lower in women who persisted with BV after treatment. A strong correlation between *A. vaginae* and *G. vaginalis* cervicovaginal loads was observed in women with restored normal flora after metronidazole treatment (Spearman r=0.68; 95 % CI=0.42–0.84; P<0.01) and a slightly weaker correlation in women who persisted with BV (Spearman r=0.58; 95 % CI=0.05–0.85; P<0.01) (Table 3).

Table 2. Number of positive samples, [*n* (%)], Spearman correlation coefficient (*r*) and confidence interval (CI) [*r* (CI range)] for *A. vaginae* and *G. vaginalis* presented at enrolment by the 245 women included in the study

	Vaginal flora		
	Normal (*n*=129)	**Intermediate (*n*=32)**	**Bacterial vaginosis (*n*=84)**
A. vaginae positivity (%)	30 (23.3)	13 (40.6)	74 (88.1)
G. vaginalis positivity (%)	4 (31.0)	8 (25.0)	66 (78.6)
Spearman correlation for *A. vaginae*/*G. vaginalis*	0.23 (0.06–0.40)*	0.25 (0.11–0.56)	0.69 (0.55–0.79)*

*P<0.01.

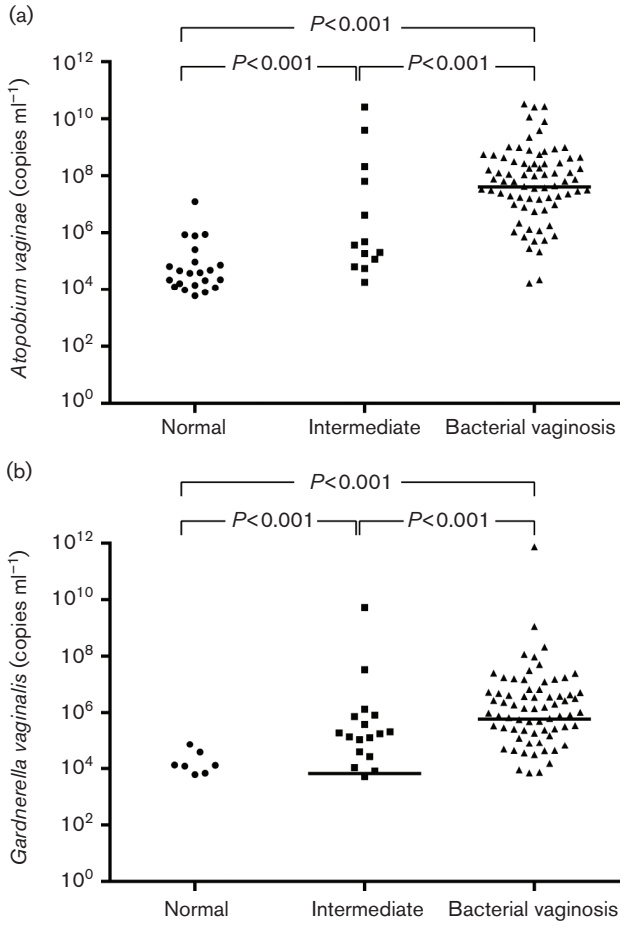

Fig. 2. Baseline cervicovaginal loads of *A. vaginae* and *G. vaginalis* in 129 women who presented normal vaginal flora, 32 with intermediate vaginal flora and 84 with bacterial vaginosis at enrolment. Horizontal bars represent the median values of copies ml^{-1} of cervicovaginal fluid. Comparison of bacterial load among the groups was performed using the Kruskal–Wallis test followed by Dunn's post-test

DISCUSSION

To the best of our knowledge, this is the first report to show that pretreatment cervicovaginal *G. vaginalis* loads are significantly lower in women who persist with BV after a proper regimen of oral metronidazole compared to those with restored normal flora after treatment. This finding is particularly relevant, since it may contribute to a better understanding of the role of the microbiological composition associated with BV persistence after metronidazole therapy. This regimen for BV treatment, with exclusive use of oral metronidazole, is still widely used in many developing countries, such as Brazil, in which BV prevalence is quite high [2].

When comparing the absolute loads of *A. vaginae* and *G. vaginalis* in different patterns of vaginal flora, the highest loads for both bacteria were observed in BV, followed by intermediate flora and normal vaginal flora. These results

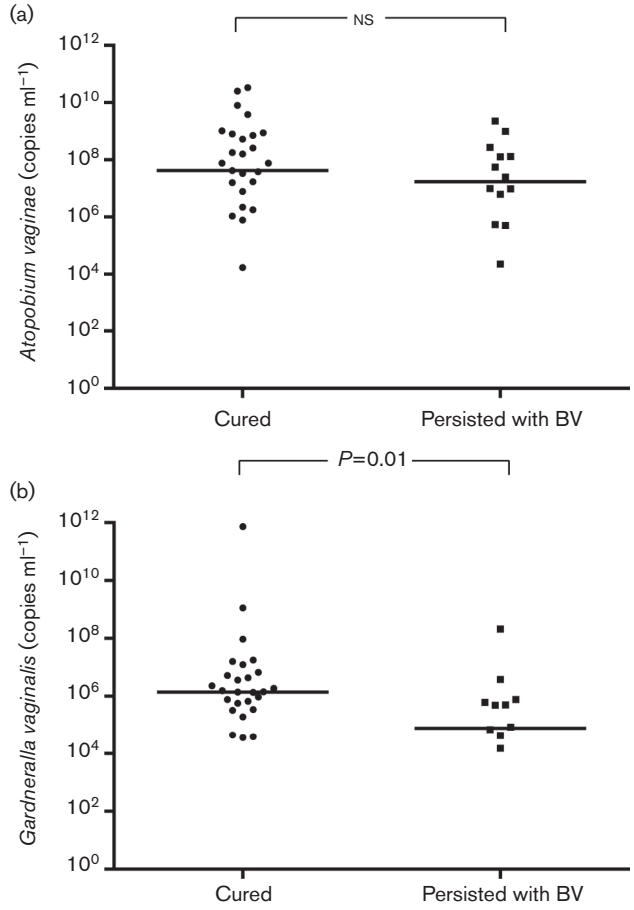

Fig. 3. Pretreatment cervicovaginal *A. vaginae* and *G. vaginalis* loads for 29 women with BV who restored *Lactobacillus*-dominant flora after metronidazole treatment and 14 BV-positive women who who persisted with BV after treatment. Horizontal bars represent the median values of copies ml^{-1} of cervicovaginal fluid. Comparison of bacterial load between the groups was performed using Mann–Whitney test.

were expected and they are in agreement with the findings of previous studies [36, 37]. Although *A. vaginae* and *G. vaginalis* are well recognized as organisms associated with BV, they are also detected in the cervicovaginal samples of women with normal vaginal flora [38, 39], and this was also found in the current study. Thus, as has already been pointed out by other authors, the question of whether these species present more virulent genotypes when they are found in BV and, as a result, are more capable of disrupting vaginal flora, is a matter for discussion [14, 19, 40].

Table 3. Number of positive samples, [*n* (%)], Spearman correlation coefficient (*r*) and confidence interval (CI), [*r* (CI range)] for *A. vaginae* and *G. vaginalis* presented pretreatment by the 45 women with BV who completed the metronidazole course and returned for follow-up.

	Treatment outcome	
	Normal flora (*n*=29)	**Persisting bacterial vaginosis (*n*=14)**
A. vaginae positivity (%)	24 (85.7)	13 (92.9)
G. vaginalis positivity (%)	23 (82.1)	10 (71.4)
Correlation of *A. vaginae*/*G. vaginalis* loads	0.68 (0.42–0.84)*	0.58 (0.05–0.85)*

*$P<0.01$.

The current data showing the positive correlation found between *A. vaginae* and *G. vaginalis* in BV and normal vaginal flora are supported by previous findings on the co-detection of these two species in different types of flora [41]. However, in women with intermediate flora only a weak and non-significant correlation was found. This observation offers more evidence of the fact that the bacterial composition of intermediate flora is not classifiable as intermediate between normal and BV flora. In fact, the literature has consistently shown that the classification of intermediate flora is very heterogeneous, since other abnormal conditions of vaginal flora, such as aerobic vaginitis, may readily be classified as 'intermediate' when the Nugent criteria are used [13, 42]. As aerobic vaginitis scoring was not performed for these samples, its influence in intermediate and BV flora cannot be completely ruled out.

Even though *G. vaginalis* is one of the most frequently found species in BV [12, 40, 43], the current data show that women who have achieved a successful cure after treatment with metronidazole had significantly higher baseline loads of this organism compared to women who failed to restore lactobacilli-dominated flora (treatment failure). Thus, it is not possible to assign the treatment failure to an increased load of *G. vaginalis*. In fact, women with high loads of *G vaginalis* respond better to metronidazole treatment. This finding highlights the importance of assessing the pathogenic diversity among the different strains of *G. vaginalis* for further determination of whether it plays a role in the treatment outcome.

On the other hand, the bacterial diversity of vaginal flora may also be a factor that influences BV treatment. What has been learnt so far is that the correlation between *G. vaginalis* and *A. vaginae* is weaker in the group of women who persisted with BV after treatment. Thus, it can be hypothesized that other micro-organisms may be interfering with this synergism and establishing a bacterial community that is less susceptible to metronidazole, leading to treatment failure. In a previous study performed by Bradshaw *et al.* [12], the co-detection of *G. vaginalis* and *A. vaginae* was associated with recurrent BV. However, despite showing quantitative data for *A. vaginae* and *G. vaginalis*, the former study did not provide the baseline pretreatment loads for these bacteria. The fact that the lower sensitivity of *A. vaginae* to metronidazole favours a cure in women with higher *G. vaginalis* loads could also be discussed, as a higher proportion of *A. vaginae* compared to *G. vaginalis* may be triggering treatment failure in such cases [44].

The development of more refined molecular methods for determining vaginal flora allowed the recognition of new bacterial species associated with BV, such as BV-associated bacteria (BVAB)-1, BVAB-2 and BVAB-3, which belong to the *Clostridiales* order. It was recently suggested that these micro-organisms can produce endospores that could lead to fast recolonization after the antibiotics regimen ends [45]. In addition, these newly recognized species are associated with *Mobiluncus sp.* suggestive morphotypes in BV. *Mobiluncus* sp. strains that have already showed significant resistance rates to metronidazole *in vitro* [46, 47].

A previous study by this group that used flow cytometry showed that the total bacterial count in BV does not determine the outcome to metronidazole treatment [48]. Thus, given that the bacterial count is not associated with BV treatment failure, while the *G. vaginalis* loads decrease in such cases, the poor treatment outcome may be associated with the replacement of *G. vaginalis* by other bacteria in the niche it had occupied in the BV bacterial community. Further, as the higher loads of *G. vaginalis* and *A. vaginae* were shown to be useless in predicting treatment failure for BV, future studies are essential to determine the microbiological aspects involved in this troublesome issue. Then, more efficient alternatives may be developed to protect these women from the serious consequences of persisting with abnormal vaginal flora for long periods of time.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was reviewed and approved by the Ethics Committee Board of Botucatu Medical School, São Paulo State University (no. 478.483), and all participants provided a signed consent form.

References

1. Spiegel CA. Bacterial vaginosis. *Clin Microbiol Rev* 1991;4:485–502.
2. Marconi C, Duarte MT, Silva DC, Silva MG. Prevalence of and risk factors for bacterial vaginosis among women of reproductive age attending cervical screening in Southeastern Brazil. *Int J Gynaecol Obstet* 2015;131:137–141.
3. Sobel JD. Bacterial vaginosis. *Annu Rev Med* 2000;51:349–356.
4. Pereira L, Culhane J, Mccollum K, Agnew K, Nyirjesy P. Variation in microbiologic profiles among pregnant women with bacterial vaginosis. *Am J Obstet Gynecol* 2005;193:746–751.
5. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK *et al.* Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA* 2011;108:4680–4687.
6. Sewankambo N, Gray RH, Wawer MJ, Paxton L, Mcnaim D *et al.* HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet* 1997;350:546–550.
7. Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL. Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin Infect Dis* 2003;36:663–668.
8. Myer L, Denny L, Telerant R, Souza M, Wright TC *et al.* Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. *J Infect Dis* 2005;192:1372–1380.
9. Marconi C, Cruciani F, Vitali B, Donders GG. Correlation of *Atopobium vaginae* amount with bacterial vaginosis markers. *J Low Genit Tract Dis* 2012;16:127–132.
10. Bretelle F, Rozenberg P, Pascal A, Favre R, Bohec C *et al.* High *Atopobium vaginae* and *Gardnerella vaginalis* vaginal loads are associated with preterm birth. *Clin Infect Dis* 2015;60:860–867.
11. Marconi C, Donders GG, Parada CM, Giraldo PC, Da Silva MG. *Do Atopobium vaginae*, *Megasphaera* sp. and *Leptotrichia* sp. change the local innate immune response and sialidase activity in bacterial vaginosis? *Sex Transm Infect* 2013;89:167–173.
12. Bradshaw CS, Tabrizi SN, Fairley CK, Morton AN, Rudland E *et al.* The association of *Atopobium vaginae* and *Gardnerella vaginalis* with bacterial vaginosis and recurrence after oral metronidazole therapy. *J Infect Dis* 2006;194:828–836.
13. Srinivasan S, Liu C, Mitchell CM, Fiedler TL, Thomas KK *et al.* Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS One* 2010;5:e10197.
14. Schwebke JR, Muzny CA, Josey WE. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *J Infect Dis* 2014;210:338–343.
15. Cauci S, Driussi S, Monte R, Lanzafame P, Pitzus E *et al.* Immunoglobulin a response against *Gardnerella vaginalis* hemolysin and sialidase activity in bacterial vaginosis. *Am J Obstet Gynecol* 1998;178:511–515.
16. Swidsinski A, Mendling W, Loening-Baucke V, Ladhoff A, Swidsinski S *et al.* Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 2005;106:1013–1023.
17. Swidsinski A, Mendling W, Loening-Baucke V, Swidsinski S, Dörffel Y *et al.* An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* 2008;198:97.e1–97.e6.
18. Patterson JL, Stull-Lane A, Girerd PH, Jefferson KK. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 2010;156:392–399.
19. Santiago GL, Deschaght P, El Aila N, Kiama TN, Verstraelen H *et al.* *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. *Am J Obstet Gynecol* 2011;204:450.e1–450.e7.
20. Hardy L, Jespers V, Dahchour N, Mwambarangwe L, Musengamana V *et al.* Unravelling the bacterial vaginosis-associated biofilm: a multiplex *Gardnerella vaginalis* and *Atopobium vaginae* fluorescence *in Situ* hybridization assay using peptide nucleic acid probes. *PLoS One* 2015;10:e0136658.
21. Workowski KA, Bolan GA. Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recomm Rep* 2015;64:1–137.
22. Larsson PG, Forsum U. Bacterial vaginosis-a disturbed bacterial Flora and treatment enigma. *APMIS* 2005;113:305–316.
23. Bradshaw CS, Morton AN, Hocking J, Garland SM, Morris MB *et al.* High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis* 2006;193:1478–1486.
24. Watnick P, Kolter R. Biofilm, city of microbes. *J Bacteriol* 2000;182:2675–2679.
25. Ravel J, Brotman RM, Gajer P, Ma B, Nandy M *et al.* Daily temporal dynamics of vaginal Microbiota before, during and after episodes of bacterial vaginosis. *Microbiome* 2013;1:29.
26. Bostwick DG, Woody J, Hunt C, Budd W. Antimicrobial resistance genes and modeling of treatment failure in bacterial vaginosis: clinical study of 289 symptomatic women. *J Med Microbiol* 2016;65:377–386.
27. Donders GG, Vereecken A, Bosmans E, Dekeersmaecker A, Salembier G *et al.* Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. *BJOG* 2002;109:34–43.

28. **Donders GG.** The prevalence of bacterial vaginosis and aerobic vaginitis in young finish women. *APMIS* 2011;119:224–225.
29. **Rezeberga D, Lazdane G, Kroica J, Sokolova L, Donders GG.** Placental histological inflammation and reproductive tract infections in a low risk pregnant population in Latvia. *Acta Obstet Gynecol Scand* 2008;87:360–365.
30. **Vieira-Baptista P, Lima-Silva J, Pinto C, Saldanha C, Beires J *et al.*** Bacterial vaginosis, aerobic vaginitis, vaginal inflammation and major Pap smear abnormalities. *Eur J Clin Microbiol Infect Dis* 2016;35:657–664.
31. **Nugent RP, Krohn MA, Hillier SL.** Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* 1991;29:297–301.
32. **Marconi C, Donders GG, Martin LF, Ramos BR, Duarte MT *et al.*** Chlamydial infection in a high risk population: association with vaginal flora patterns. *Arch Gynecol Obstet* 2012;285:1013–1018.
33. **Ho BS, Feng WG, Wong BK, Egglestone SI.** Polymerase chain reaction for the detection of *Neisseria gonorrhoeae* in clinical samples. *J Clin Pathol* 1992;45:439–442.
34. **Dezzutti CS, Hendrix CW, Marrazzo JM, Pan Z, Wang L *et al.*** Performance of swabs, lavage, and diluents to quantify biomarkers of female genital tract soluble mucosal mediators. *PLoS One* 2011;6:e23136.
35. **Spear GT, Kendrick SR, Chen HY, Thomas TT, Bahk M *et al.*** Multiplex immunoassay of lower genital tract mucosal fluid from women attending an urban STD clinic shows broadly increased IL1ß and lactoferrin. *PLoS One* 2011;6:e19560.
36. **Menard JP, Fenollar F, Henry M, Bretelle F, Raoult D.** Molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae* loads to predict bacterial vaginosis. *Clin Infect Dis* 2008;47:33–43.
37. **Kusters JG, Reuland EA, Bouter S, Koenig P, Dorigo-Zetsma JW.** A multiplex real-time PCR assay for routine diagnosis of bacterial vaginosis. *Eur J Clin Microbiol Infect Dis* 2015;34:1779–1785.
38. **Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM.** Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol* 2007;45:3270–3276.
39. **Mendes-Soares H, Krishnan V, Settles ML, Ravel J, Brown CJ *et al.*** Fine-scale analysis of 16S rRNA sequences reveals a high level of taxonomic diversity among vaginal *Atopobium* spp. *Pathog Dis* 2015;73:ftv020.
40. **Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler TL *et al.*** Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of Microbiota to clinical criteria. *PLoS One* 2012;7:e37818.
41. **de Backer E, Verhelst R, Verstraelen H, Alqumber MA, Burton JP *et al.*** Quantitative determination by real-time PCR of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae* indicates an inverse relationship between *L. gasseri* and *L. iners*. *BMC Microbiol* 2007;7:115.
42. **Donders GG.** Definition and classification of abnormal vaginal flora. *Best Pract Res Clin Obstet Gynaecol* 2007;21:355–373.
43. **Catlin BW.** *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clin Microbiol Rev* 1992;5:213–237.
44. **Donders GG, Zodzika J, Rezeberga D.** Treatment of bacterial vaginosis: what we have and what we miss. *Expert Opin Pharmacother* 2014;15:645–657.
45. **Marrazzo JM, Thomas KK, Fiedler TL, Ringwood K, Fredricks DN.** Relationship of specific vaginal bacteria and bacterial vaginosis treatment failure in women who have sex with women. *Ann Intern Med* 2008;149:20–28.
46. **Skarin A, Holst E, Mårdh PA.** Antimicrobial susceptibility of comma-shaped bacteria isolated from the vagina. *Scand J Infect Dis Suppl* 1983;40:81–84.
47. **Spiegel CA, Eschenbach DA, Amsel R, Holmes KK.** Curved anaerobic bacteria in bacterial (nonspecific) vaginosis and their response to antimicrobial therapy. *J Infect Dis* 1983;148:817–822.
48. **Luchiari HR, Ferreira CS, Golim MA, Silva MG, Marconi C.** Cervicovaginal bacterial count and failure of metronidazole therapy for bacterial vaginosis. *Int J Gynaecol Obstet* 2016;132:297–301.

5. Artigo II

Shifts due to bacterial sialidase in microbioma components*

Carolina Sanitá Tafner Ferreira,[a] Jacques Ravel,[b] Márcia Guimarães da Silva,[a] Camila Marconi[a,c]

[a]UNESP – Sao Paulo State University, Botucatu Medical School, Department of Pathology, Botucatu, Sao Paulo, Brazil

[b]University of Maryland School of Medicine, Institute of Genomic Science, Baltimore, MD, USA

[c]UFPR – Federal University of Parana, Sector of Biological Sciences, Department of Basic Pathology, Curitiba, Paraná, Brazil

* Corresponding author: Camila Marconi

Department of Basic Pathology, Setor de Ciências Biológicas, UFPR – Univ Federal do Paraná, Curitiba, Paraná, Brazil. Tel.: +55 41 3361 1691; fax: +55 41 3266 2042. Zipcode: 81531-980

E-mail address: marconi.cml@gmail.com

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ABSTRACT

Introduction: Bacterial sialidases are detected in the cervicovaginal samples in more than 50% women with abnormal vaginal microbiota (AVM) and degrade IgA. *Gardnerella vaginalis* is strongly associated with AVM and some strains are capable of produce sialidases. However, it is still unknown if other vaginal bacterial species contribute for sialidase production. Given the impact of sialidase at local immunity, it may have an impact in microbial composition, which was not addressed so far. Objective: To test if sialidases can be detected in cervicovaginal fluid in the absence of *G. vaginalis* sialidase-encoding gene and also to compare the AVM-microbiome components according to the status of cervicovaginal sialidases. Methods: We cross-sectionally enrolled 114 women seeking routine Pap-test. Vaginal microbiota was assessed by microscopy for AVM detection (Nugent scores 4 to 10) and by V3-V4 16S rRNA sequencing. Detection of *G. vaginalis* sialidase-encoding gene was performed by real time-PCR, while sialidase measurements in cervicovaginal fluid were performed using fluorometric assay. Results: We detected sialidases in the cervicovaginal fluid of 28 out the 40 women with AVM, of which 4 (14.3%) were negative for the *G. vaginalis* sialidase-enconding gene. Microbiome analysis showed that among the 30 most prevalent bacterial taxa in AVM, 17 were significantly enriched in sialidase-positive samples ($P<0.05$), while only one taxon was enriched in sialidase negative-group. Conclusion: *G. vaginalis* is likely to be the main but not the exclusive source of cervicovaginal sialidases. Bacterial sialidases is associated with changes in components of vaginal microbiome, although a causality link could not be addressed.

Keywords: Vaginal microbiome; Bacterial vaginosis; *Gardnerella vaginalis*; Sialidase.

1. Introduction

Over decades, bacterial vaginosis (BV) has been considered the most common type of abnormal vaginal microbiota (AVM), affecting about 30% of women in reproductive age [1, 2]. It is a condition that has a high recurrence rates even after appropriate treatment [3] and is characterized by the replacement of normal *Lactobacillus*-dominated microbiota by an overgrowth of *Gardnerella vaginalis, Atopobium vaginae, Prevotella bivia, Bacteroides* spp., among other species [1, 2]. The presence of virulence factors produced by abnormal vaginal microbiota (AVM)-associated bacteria as the sialidases are associated to increased risk to acquisition of sexually transmitted infections (STI), including the human immunodeficiency virus (HIV), *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and recently were associated with the persistence of cervical infection by human papillomavirus (HPV) [4, 5].

Sialidases can impair the host immunity local by degrade sialic acid of immunoglobulin A, mucins, membrane receptors, peripheral blood mononuclear cells and of inflammatory cytokines, which constitute the first line of defense against invasion of pathogenic microorganisms in the cervicovaginal mucosa [6, 7]. Some bacteria isolated from AVM have already been demonstrated capacity of producing and releasing sialidases in culture media [8]. The *G. vaginalis* strains with sialidase-encoding gene have been associated with biofilm formation [9]. Vaginal biofilm hinders antibiotic action and is associated with BV recurrence [10]. Sialidases are enzymes that facilitate the attachment of BV-associated bacteria to epithelial cells by hydrolyzing the sialic acid on the terminal glycans of cellular membranes in mucous layer [11].

Sialidases production by *G. vaginalis* in vaginal environment is well documented, while its production by other BV-associated bacteria as *Prevotella bivia* and *Bacteroides* spp. was only demonstrated *in vitro* [8, 9, 11]. Since *G. vaginalis* is strongly associated with BV and some strains present the sialidase-encoding gene, other possible sources of sialidase have been overlooked.

Considering the impact of cervicovaginal sialidase to the local immunity and the lack of

information regarding its source, the aims of this study were to verify if bacterial sialidases can be detected in cervicovaginal fluid in the absence of *G. vaginalis* sialidase-encoding gene and if this enzyme may lead to changes in vaginal microbiome of women with AVM.

2. Materials and methods

Study design and population

From March to September of 2014, a total of 141 non-pregnant reproductive aged women attending a primary healthcare clinic in Botucatu, São Paulo, Brazil, for routine Pap-testing was cross-sectionally enrolled. This study was reviewed and approved by the Ethics Committee of UNESP - São Paulo State University, Botucatu Medical School (Approval number 3.095.119). All women that agreed to participate of this study signed a consent term. Those women that reported urinary loss, being at menstrual period, use of antibiotics in the previous 30 days and sexual intercourse in the last 48 hours were not included. An individual structured questionnaire was applied by face-to-face interview to assess sociodemographic, behavioral and clinical characteristics of the population.

Sampling procedures

During physical exam for Pap-testing, additional procedures were carried out to complete the study protocol. Vaginal pH was measured by allowing one minute of direct contact of the commercial pH strips (range 4.0–7.0, Merck, Darmstadt, Germany) with the vaginal wall. Whiff test was performed adding a potassium hydroxide drop (10% KOH v/v) on a swab with vaginal content and the results were expressed as negative, doubtful and positive. Mid-third of vaginal wall was swabbed and stored at Amies liquid medium (Copan, Brescia, Italy) at -80^{o} C for microbiome analysis. Additional vaginal swab was smeared onto two glass slides for microscopic evaluation. Wet-mount smears were used detect presence of *Candida* sp.-like morphotypes and *Trichomonas vaginalis*.

Gram-stained smears were used for classification of the vaginal microbiota using Nugent scoring system, which is based on the semi-quantification of different bacterial morphotypes, with results expressed as normal (scores 0-3), intermediate (scores 4-6) and BV (scores 7-10) [12]. Participants with scores between 4 and 10 were classified as AVM. Endocervical samples were also taken for detection of *G. vaginalis* sialidase gene and assessments of *C. trachomatis* and *N. gonorrhoeae* status by PCR, according to methods previously described [13-15]. Finally, 3 mL of cervicovaginal fluid samples were obtained using sterile NaCl 9.5% [w/v] solution as standardized previously [16] and were used for measurement of sialidase levels.

Detection of G. vaginalis sialidase gene and measurement of sialidases cervicovaginal levels

Presence of *G. vaginalis* sialidase A gene was assessed by real-time polymerase chain reaction (PCR) on cervicovaginal DNA. Reactions were performed in 13 uL total reaction volume containing DNA template, Maxima SYBR Green/ROX mix (Fermentas, St. Leon-Rot, Germany), the pair of primers for amplification of *G. vaginalis* sialidase gene A (GVSI forward: 5´– GACGACGGCGAATGGCACGA – 3´ and GVSI reverse 5´– AGTCGCACTCCGCGCAAGTC – 3´) [9]. It was included positive samples, in which gene sequence was confirmed by sequencing in 3500xL ABI Genetic Analyzer platform (Applyed Biosystems, Foster, CA). Reactions were incubated at 95ºC for 10 min and 40 cycles of 15 seconds at 95ºC and 30 seconds at 60ºC resulting in melting curve analysis. All they were performed in LineGeneK (Bioer, China) equipment. Measurement of cervicovaginal sialidase levels were determined by the conversion of the fluorogenic substrate 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN; Sigma-Aldrich, St. Louis, MO), according to methods previously described [17]. The detection limit of the assay at this study was 0.1 ng/mL.

Microbiome assessment

Frozen vaginal samples inoculated in transport medium were thawed on ice, shaken vigorously and the swabs were then discarded. The fluid was subjected to DNA extraction using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), according to manufacturer's protocol. Analysis of vaginal microbiome was performed in the Institute for Genomic Sciences of University of Maryland (Baltimore, MD) according to Fadrosh et al. [18]. Briefly, all samples were submitted to quantification of DNA using Picogreen (Invitrogen, Carlsbad, CA) and diluted in order to reach concentration of 5 ng/uL using Qiagility (Qiagen, Germantown, MD). Posteriorly, it was performed an end-point PCR for amplification of a sequence of 532 bp of V3-V4 hypervariable region of 16S rRNA gene using a combination of 48 different primers attached to barcodes for samples identification. Amplicons were sequenced using the 400 PE MiSeq (Illumina Inc., San Diego, Califórnia) equipment. Reads were de-multiplexed and quality trimmed in QIIME (version 1.8.0)[Q], refer to Fadrosh et al.[20] for further details. 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)[UQ] using greengenes database of 16S rRNA sequences (Aug, 2013 vers.) as a reference.

Data analyses

Statistical analysis for comparison of socio-demographics and clinical variables between the groups were performed, by Chi-squared and by the non-parametric Mann-Whitney test. Chi-squared test was also performed to test association between presence of *G. vaginalis* sialidase A gene and sialidases detection. Comparison of sialidases concentration between *G. vaginalis* sialidase A gene-positive and negative samples was performed by the non-parametric Mann-Whitney test, while the association between BV and presence of *G. vaginalis* sialidase A gene was tested by Chi-squared. All analyses were performed in GraphPad Prism 6.0 software (GraphPad, San Diego, CA) with $P<0.05$ considered as significant. Linear discriminant analysis effect size (LDAES) was performed to compare the relative abundance of the top-30 more prevalent microbial taxa between women with

normal and those with AVM. Subsequent LDAES analyses were performed only in AVM women (1) according to the presence of *G. vaginalis* sialidase A gene and (2) according to the presence of detectable levels of cervicovaginal sialidases. Tools used for LDAES analyses are available at http://huttenhower.sph.harvard.edu/galaxy.

3. Results

Of the 141 women initially enrolled, 27 (19.1%) were further excluded of the study by presenting *Candida* sp. (n=17; 12%), *T. vaginalis (*n=2; 1.4%) and *C. trachomatis* (n=8; 5.7%). Microscopic classification of the vaginal microbiota of the 114 remaining samples showed 64 (56.1%) women with normal microbiota, 10 (8.8%) with intermediate and 40 (35.1%) with BV. Therefore, 50 (43.9%) women were classified as AVM.

Sociodemographic, behavioral and clinical history data are displayed in **Table 1**. Bacterial vaginosis was associated with smoking habit and to women with two sex partners or more in the year prior to enrollment. Bacterial vaginosis was also associated to an increased vaginal pH (P<0.05). Intermediate microbiota was marginally associated with BV history in the previous year (P=0.04). No other association was observed for the remaining variables.

As shown in **Table 2**, 24 (33.3%) of the samples with positive results for *G. vaginalis* sialidase A gene also presented sialidases at detectable levels (P<0.01). Other 48 samples were positive for sialidase gene but with enzyme levels below the detection limit of the assay. On the other hand, sialidases were detected in four samples that tested negative for *G. vaginalis* sialidase-encoding gene. When comparing the results of the presence of sialidase gene according to the microscopic classification of vaginal microbiota (**Table 2**), *G. vaginalis* gene was more prevalent in participants with BV (P<0.01). All samples with measurable cervicovaginal sialidases levels were from women with AVM, including intermediate (n=2, 20%) and BV (n=26, 60%).

Of the total of 114 samples included in the study, were successfully sequenced 100 samples for microbiome analysis, resulting in a total of 1.393.522 reads from 108 bacterial taxa identified.

Relative abundance of the most prevalent taxa (top 30) of vaginal microbiome was compared between normal microbiota and AVM. As observed in **Fig. 1A**, *Lactobacillus* spp. *(L. crispatus, L. iners, L. helveticus, L. jensenii, L. vaginalis*) were more abundant in normal group, while 18 other species were enriched in AVM ($P<0.05$). As shown in **Figs. 1B e 1C**, when evaluating top 30 taxa of AVM participants according to the presence of *G. vaginalis* sialidase-encoding gene, five were significantly enriched in gene-positive group (*Atopobium vaginae, Leptotrichia amnionii, Parvimonas micra, Gemella, Prevotella genogroup 2*) and only one in gene-negative group (*Lactobacillus helveticus*). Those five bacterial taxa significantly enriched in *G. vaginalis* sialidase gene-positive group were also significantly more abundant in group with sialidases detection (**Fig. 1C**) in addition to other 12 bacterial taxa (*G. vaginalis*, *Anaerococcus, Dialister, BVAB, Megasphaera, Eggerthella* and others). Only *Pediococcus acidilacti* was significantly more abundant in sialidase-negative group.

4. Discussion

With relation to sociodemographic, behavioral and clinical history data of this population, other studies also could demonstrate that BV is more frequent in smokers and in those with more sex partners. The higher vaginal pH is characteristic of BV [19-21] and the fact of that BV history in the previous year to be significantly most frequent in women with intermediate microbiota compared to normal is comprehensive since it is common women with intermediate microbiota changing to BV and vice versa, what may be associated to BV recurrence [1].

The main findings of this study were of the changes of vaginal microbiome in the presence of *G. vaginalis* sialidase-enconding gene and in detectable levels of sialidases. Another interesting finding observed in this study was that although *G. vaginalis* seems have a most significant portion in sialidases production in cervicovaginal environment, other BV-associated bacteria can also contribute for the production of this enzyme in this milieu, despite of these species were not the specific target of the present study. So far, the production of

sialidases by microorganisms other than *G. vaginalis* had only been demonstrated *in vitro* [8].

The sialidases activity in BV is well described and it was also shown some activity in intermediate microbiota [7, 11, 17, 22]. Indeed, in our study only women with AVM presented concentration levels for the enzyme. Although we did detect sialidase-gene in normal vaginal microbiota in several samples, there was not enzyme production in detectable levels. It may be due to some hypotheses: in these cases sialidase production may be downregulated due to gene silencing or even it may be undergoing by post translational modification. Also, it should be considered that in view of the much smaller amount of *G. vaginalis* found in normal microbiota, sialidases may are being produced at undetectable levels. Even it should be considered that certain substances produced by *Lactobacillus* spp. in normal microbiota ma degrade bacterial sialidases for obtaining growth advantages. It is well known that *Lactobacillus* spp. produce several antimicrobial components such as bacteriocins, hydrogen peroxidase and, especially, acid lactic which maintains a low vaginal pH [23]. It was already demonstrated that in pH 4.5 the toxins activity as vaginolysin produced by *G. vaginalis* is reduced, thus pH may have same effect on sialidase [24].

On the other hand, the presence of certain BV-associated bacteria seems to contribute to the expression of sialidase gene. One of the most abundant bacteria, *A. vaginae*, is positively correlated with *G. vaginalis* [25] and together they are the main components of the vaginal biofilm in BV [26]. Besides *A. vaginae* is capable of increasing the expression of membrane-associated mucins that are one of the targets of sialidases [27]. So, it may be hypothesized that presence of *A. vaginae* species may induce to the expression of sialidase by *G. vaginalis* due to increase the bioavailability of mucins for its metabolism.

As expected, from comparison of the relative abundance of bacterial taxa according to the microscopic status of vaginal microbiota, only *Lactobacillus* spp. were enriched in normal microbiota (Nugent score 0 to 3). On the other hand, several bacterial taxa bacteria were significantly more abundant in AVM and, all of them had been previously associated with BV [2, 28,

29]. The results from comparison between samples positive and negative for *G. vaginalis* sialidase-encoding gene showed that only *L. helveticus* seem to thrive significantly in the absence of sialidase-positive gene *G. vaginalis* strains, while *A. vaginae, L. aminionii, P. micra, Prevotella* genogroup 2 and *Gemella* sp. are significantly more abundant in the presence of these strains. It is known that the action of sialidases on the sialoglycans of cervicovaginal mucus may provide other substrates that can be cleaved by other glycosidases produced by vaginal bacteria [30]. Also, it must be considered that IgA and interferon-gamma degradation by sialidases hampers local immunity and certainly contribute for BV-associated bacteria overgrowth [7]. In fact, *G. vaginalis* has a positive effect on the growth of other BV-associated bacteria as *Prevotella bivia* and *Fusobacterium nucleatum*, while *G. vaginalis* itself growth is three times greater when a second anaerobe (in special *P. bivia* and *Mobiluncus mulieres*) is included 24 hours after *in vitro* biofilm formation [31]. Therefore, the BV-associated bacteria seem to interact positively among them which may lead to expression of metabolites in order to increase the diversity of vaginal microbiota. Sialidases may be a factor underlying of this microbial interaction, as it may be an essential path to obtain free sialic acid, a carbohydrate source for bacterial growth [32].

This study focused in the presence of sialidases, but other virulence factors are produced by BV-associated species and modulate local microbiota, being necessary further investigation. Even so, this study brings more evidences of the key role of *G. vaginalis* for BV-pathogenesis. However, as other bacteria can also produce sialidases in the cervicovaginal environment, it is important to know better which bacteria additionally to *G. vaginalis* may be in fact acting as pathogenic sialidases sources to adopt strategies to prevent its release in the vaginal environment. Finally, it is fundamental the execution of longitudinal studies in order to establish the impact of bacterial sialidases on the temporal fluctuation of vaginal microbiome.

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Conflicts of interest

The authors have no conflicts of interest to mention.

6. References

1. Marconi C, Duarte MT, Silva DC, Silva MG. Prevalence of and risk factors for bacterial vaginosis among women of reproductive age attending cervical screening in southeastern Brazil. Int J Gynaecol Obstet 2015;131:137-41.

2. Lambert JA, John S, Sobel JD, Akins RA. Longitudinal analysis of vaginal microbiome dynamics in women with recurrent bacterial vaginosis: recognition of the conversion process. PLoS One 2013;8(12):e82599.

3. Bradshaw CS, Morton AN, Hocking J, Garland SM, Morris MB, Moss LM, et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. J Infect Dis 2006;193:1478–86.

4. Ketterer MR, Rice PA, Gulati S, Kiel S, Byerly L, Fortenberry JD, et al. Desialylation of Neisseria gonorrhoeae Lipooligosaccharide by Cervicovaginal Microbiome Sialidases: The Potential for Enhancing Infectivity in Men. JID 2016:214.

5. Di Paola M, Sani C, Clemente AM, Iossa A, Perissi E, Castronovo G, et al. Characterization of cervico-vaginal microbiota in women developing persistent high-risk Human Papillomavirus infection. Sci Rep 2017;7(1):10200.

6. Stamatos NM, Gomatos PJ, Cox J, Fowler A, Dow N, Wohlhieter JA, et al. Desialylation of peripheral blood mononuclear cells promotes growth of HIV-1.Virology 1997;228(2):123-31.7. Cauci S, Culhane JF. High sialidase levels increase preterm birth risk among women who are bacterial vaginosis-positive in early gestation. Am J Obstet Gynecol 2011;204:142.e1-9.

8. Briselden AM, Moncla BJ, Stevens CE, Hillier SL. Sialidases (neuraminidases) in bacterial vaginosis and bacterial vaginosis-associated microbiota. J Clin Microbiol 1992;30:663-6.

9. Santiago GL, Deschaght P, El Aila N, Kiama TN, Verstraelen H, Jefferson KK, et al. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase.

American journal of obstetrics and gynecology 2011;204(450):e451–457.

10. Mendling W. Vaginal microbiota. Adv Exp Med Biol 2016;902:83–93.

11. Patterson JL, Stull-Lane A, Girerd PH, Jefferson KK. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. Microbiology 2010;156(2):392–9.

12. Nugent RP, Krohn MA, Hillier SL. Reability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. J Clin Microbiol 1991;29:297-301.

13. Marconi C, Donders GG, Martin LF, Ramos BR, Duarte MT, Parada CM, et al. Chlamydial infection in a high risk population: association with vaginal microbiota patterns. Arch Gynecol Obstet 2012;285:1013-18.

14. Ho BSW, Feng WG, Wong BKC, Egglestone SI. Polymerase chain reaction for the detection of *Neisseria gonorrhoeae* in clinical samples. J Clin Pathol 1992;45:439-42.

15. Marconi C, Donders GG, Bellen G, Brown DR, Parada CM, Silva MG. Sialidase activity in aerobic vaginitis is equal to levels during bacterial vaginosis. Eur J Obstet Gynecol Reprod Biol 2013;167:205-9.

16. Donders GG, Vereecken A, Bosmans E, Dekeersmaecker A, Salembier G, Spitz B. Definition of a type of abnormal vaginal microbiota that is distinct from bacterial vaginosis: aerobic vaginitis. BJOG 2002;109(1):34-43.

17. Marconi C, Donders GG, Parada CM, Giraldo PC, da Silva MG. Do *Atopobium vaginae, Megasphaera* sp. *and Leptotrichia* sp. change the local innate immune response and sialidase activity in bacterial vaginosis? Sex Transm Infect 2013;89(2):167-73.

18. Fadrosh DW, Ma B, Gajer P, Ott S, Sengamalay N, Brotman RM, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq Platform. Microbiome 2014;24:2-6.

19. Holzman C, Leventhal JM, Qiu H, Jones NM, Wang J, BV Study Group. Factors linked to bacterial vaginosis in nonpregnant women. American Journal of Public Health 2001;91:1664–70.

20. Ness RB, Hillier S, Richter HE, Soper DE, Stamm C, Bass DC, et al. Can known risk factors explain racial differences in the occurrence of bacterial vaginosis? Journal of the National Medical Association 2003;95:201–12.

21. Koumans EH, Sternberg M, Bruce C, McQuillan G, Kendrick J, Sutton M, et al. The prevalence of bacterial vaginosis in the United States, 2001–2004; associations with symptoms, sexual behaviors, and reproductive health. Sexually Transmitted Diseases 2007;34:864–9.

22. Santos-Greatti MM, da Silva MG, Ferreira CS, Marconi C. Cervicovaginal cytokines, sialidase activity and bacterial load in reproductive-aged women with intermediate vaginal microbiota. J Reprod Immunol 2016;118:36–41.

23. Dover SE, Aroutcheva AA, Faro S, Chikindas ML. Natural antimicrobials and their role in vaginal health: a short review. Int J Probiotics Prebiotics 2008;3:219–30.

24. Rampersaud R, Planet PJ, Randis TM, Kulkarni R, Aguilar JL, Lehrer RI, et al. Inerolysin, a cholesterol-dependent cytolysin produced by *Lactobacillus iners*. J Bacteriol 2011;193(5):1034–41.

25. Ferreira CST, Donders GG, Parada CMGL, Tristão ADR, Fernandes T, da Silva MG, et al. Treatment failure of bacterial vaginosis is not associated with higher loads of *Atopobium vaginae* and *Gardnerella vaginalis*. J Med Microbiol. 2017;66(8):1217-24.

26. Hardy L, Jespers V, Dahchour N, Mwambarangwe L, Musengamana V, Vaneechoutte M, et al. Unravelling the bacterial vaginosis-associated biofilm: A multiplex *Gardnerella vaginalis* and *Atopobium vaginae* fluorescence in situ hybridization assay using peptide nucleic acid probes.

27. Doerflinger SY, Throop AL, Herbst-Kralovetz MM. Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner. J Infect Dis 2014;209(12):1989-99.

28. Nunn KL, Forney LJ. Unraveling the dynamics of the human vaginal microbiome. Yale J Biol Med 2016;89(3):331-7.

29. Shipitsyna E, Roos A, Datcu R, Hallén A, Fredlund H, Jensen JS, et al. Composition of the vaginal microbiota in women of reproductive age – sensitive and specific molecular diagnosis of bacterial vaginosis is possible? PLoS One 2013;8(4): e60670.

30. Lewis WG, Robinson LS, Perry J, Bick JL, Peipert JF, Allsworth JE, et al. Hydrolysis of secreted sialoglycoprotein immunoglobulin A (IgA) in ex vivo and biochemical models of bacterial vaginosis. J Biol Chem 2012;287:2079–89.

31. Machado A, Jefferson KK, Cerca N. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. Int J Mol Sci 2013;14:12004-12.

32. Sudhakara P, Sellamuthu I, Aruni AW. Bacterial sialoglycosidases in virulence and pathogenesis. Pathogens 2019;8(1):39.

Q. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. NIH Public Access 2011;**7**:335–6.

UQ. Edgar RC, Haas BJ, Clemente JC, Quince C & Knight R. UCHIME improves sensitivity and speed of chimera detection. 2011; 27:2194–2200.

Table 1. Demographic, behavioural and clinical characteristics of the 114 participants of the study according to the microscopic classification of vaginal microbiota.

Variables	Normal (n=64)	Intermediate (n=10)	Bacterial vaginosis (n=40)	P-value[a]
Age, years; median (range)	35 (18-51)	31 (18-43)	35 (18-47)	0.65
Age, years				
<35	31 (48.4)	6 (60.0)	20 (50.0)	
≥35	33 (51.6)	4 (40.0)	20 (50.0)	0.79
Ethnicity				
White	41 (64.1)	4 (40.0)	17 (42.5)	
Non-White	23 (35.9)	6 (60.0)	23 (57.5)	0.06
Marital status				
Single	18 (28.1)	2 (20.0)	17(42.5)	
Married/living together with partner	46 (71.9)	8 (80.0)	23(57.5)	0.21
Years at school	9 (0-16)	8 (2-11)	8 (1-15)	0.23
Remunerated activity (n/total)				
Yes	40 (62.5)	3 (30.0)	24 (60.0)	
No	24 (37.5)	7 (70.0)	16 (40.0)	0.15
Smoking				
Yes	9 (14.1)	1 (10,0)	14 (35.0)	
No	55 (85.9)	9 (90.0)	26 (65.0)	0.03[d]
Number of sex partner[b]				
0 or 1	62 (96.9)	9 (90.0)	31 (77.5)	
2 or more	2 (3.1)	1 (10.0)	9 (22.5)	<0.01[d]
Number of vaginal intercourse/week	2 (0-7)	3 (1-7)	3 (0-7)	0.70

Previous Bacterial vaginosis				
Yes	21 (32.8)	7 (70.0)	20 (50.0)	
No	43 (67.2)	3 (30.0)	20 (50.0)	0.04[e]
Previous STI				
Yes	7 (10.9)	2 (20.0)	3 (7.5)	
No	57 (89.1)	8 (80.0)	37 (92.5)	0.51
Consistent condom use				
Yes	22 (34.4)	2 (20.0)	10 (25.0)	
No	42 (65.6)	8 (80.0)	30 (75.0)	0.46
Hormonal contraceptive[c]				
Yes	32 (50.0)	5 (50.0)	13 (32.5)	
No	32 (50.0)	5 (50.0)	27 (67.5)	0.20
Vaginal pH	4.7 (4.0-5.0)	4.7 (4.4-5.5)	4.7 (4.4-5.3)	<0.01[d]
<4.5	24 (37.5)	4 (20.0)	2 (5.0)	
≥4.5	40 (62.5)	8 (80.0)	38 (95.0)	<0.01[f]
Whiff test				
Positive	17 (26.6)	2 (20.0)	7 (17.5)	
Doubtful or negative	47 (73.4)	8 (80.0)	33 (82.5)	0.21

STI: Sexually transmitted infection.

[a]Categorical variables were made by Chi-squared or Fisher exact test when applicable and continuous by Kruskall-Wallis test, considering P<0.05 as significant.

[b]One year prior to enrollement.

[c] last 4 months.

[d] Normal group differed from BV.

[e] Intermediate microbiota differed from normal and BV.

[f] Normal and intermediate microbiota differed from BV.

Table 2. Number of cervicovaginal samples (%) with *G. vaginalis* sialidase A gene-positive and sialidases-positivity in the assay.

	G. vaginalis sialidase A gene			P-value[a]
Detectable sialidases	**Yes**	**No**	**Overall**	
	(n=72)	**(n=42)**	**(n=114)**	
Negative	48 (66.7)	38 (90.5)	86 (82.7)	<0.01
Positive	24 (33.3)	4 (9.5)	28 (26.9)	
Microscopic classification				
Normal	34 (53.1)	30 (46.9)	64 (100.0)	
Intermediate	5 (50.0)	5 (50.0)	10 (100.0)	<0.01[c]
Bacterial vaginosis	33 (82.5)	7 (17.5)	40 (100.0)	

[a] *P*<0.05 considered significant.

[b] Mean of sialidase values

[c] Normal and intermediate microbiota differed from BV by Chi-squared test.

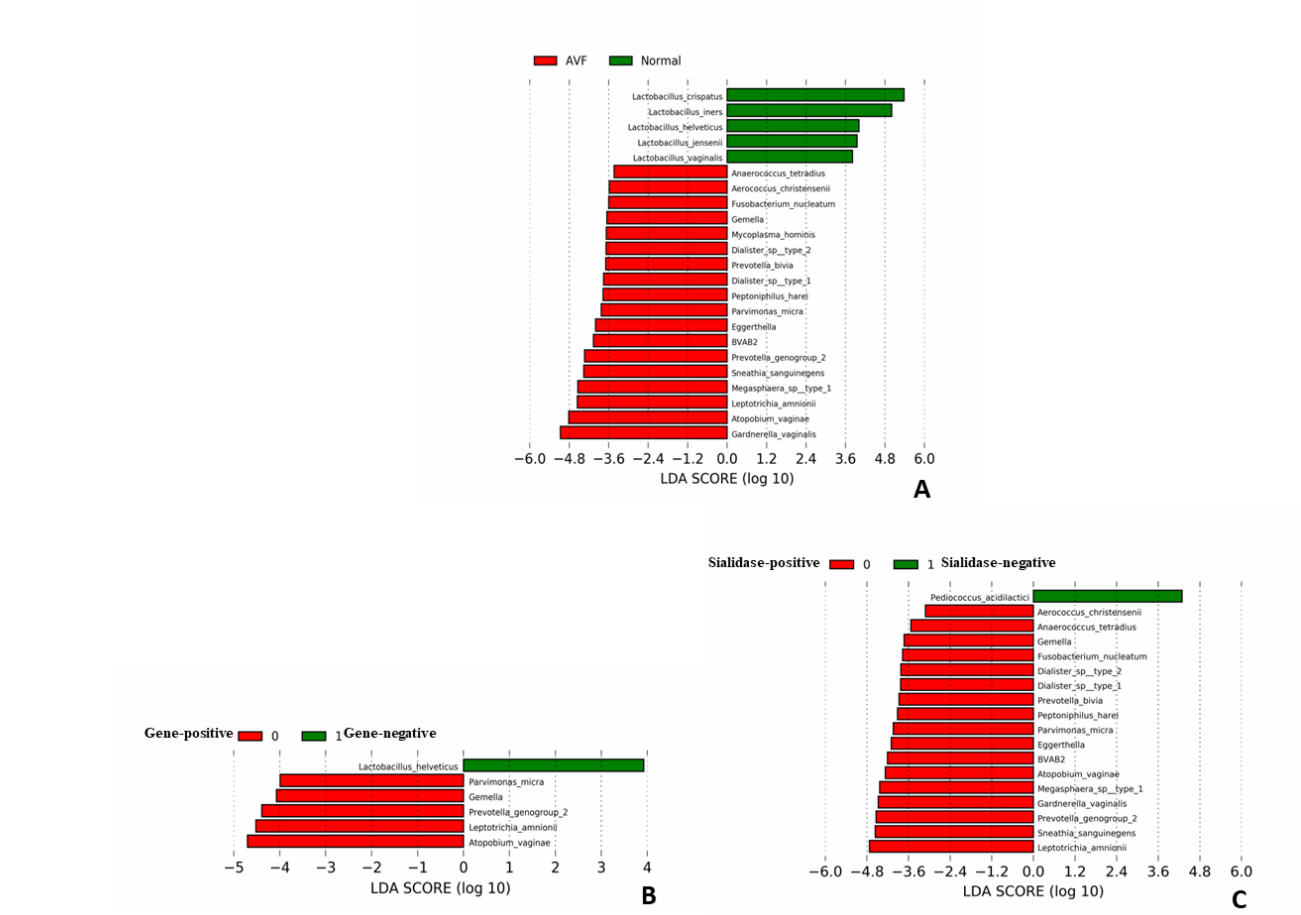

Figure 1. A: Relative abundance of top 30 most frequent taxa according to the pattern of vagina microbiota [normal (n=53) versus abnormal vaginal microbiota - AVM (n=47)]; **B:** Relative abundance of top 30 most frequent taxa according to the detection of *G. vaginalis* sialidase-encoding gene in the cervicovaginal fluid of women with AVM [0=gene-positive (n=36) versus 1=gene-negative (n=11)]; **C:** Relative abundance of top 30 most frequent taxa according to the presence of detectable levels of sialidases in the cervicovaginal fluid of women with AVM [0=sialidase-positive (n=26) versus 1=sialidase-negative (n=21)].

6. Conclusão

A falha no tratamento da vaginose bacteriana (VB) não pode ser atribuída a maiores quantidades pré-tratamento das bactérias mais comumente associadas à VB: *Atopobium vaginae* e *Gardnerella vaginalis*. Portanto, a falha no tratamento deve estar envolvida com outros fatores, como a totalidade do *core* microbiológico da VB, bem como sua diversidade. Ainda, devem ser considerados os aspectos individuais do hospedeiro, bem como a presença de fatores de virulência nas diferentes linhagens de espécies bacterianas que colonizam microbiota vaginal, em especial as sialidases. O papel das sialidases deve ser mais profundamente investigado, uma vez que esse trabalho demonstrou que sua presença no ambiente cérvico-vaginal determina mudanças profundas de composição e diversidade bacteriana entre as mulheres com alteração de microbiota vaginal.