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Note

Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells

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ABSTRACT

Bacterial vaginosis is the leading vaginal disorder but the transition from health to this dysbiotic condition remains poorly characterized. Our goal was to quantify the ability of BV-associated anaerobes to adhere to epithelial cells in the presence of lactobacilli. *Gardnerella vaginalis* outcompeted *Lactobacillus crispatus* and *Lactobacillus iners* actually enhanced its adherence.

Adhesion to host cells is a necessary early step in the establishment of infection [1]. Bacterial vaginosis (BV) is the most common vaginal disorder in women of reproductive age [2,3], but its etiology is still unclear [4–6]. BV is characterized by a decrease in beneficial vaginal bacteria, specifically *Lactobacillus* spp., and by an increase in the number of anaerobic bacteria, including *Gardnerella vaginalis*, *Mobiluncus mulieris*, *Atopobium vaginae*, *Prevotella bivia* and *Fusobacteria nucleatum* and others [7–9]. In 2005, Swidsinski et al. conducted a study in which vaginal epithelial biopsies from healthy subjects and those with BV were analysed, and found that a multispecies biofilm, predominated by *G. vaginalis* and *A. vaginae* adhered to the surface of the epithelium in BV [9]. They hypothesized that *G. vaginalis* is the initial colonizing species and that its adherence is required before other BV-associated anaerobes are able to colonize the vaginal epithelium. *G. vaginalis* can display resistance to the antimicrobial products produced by *Lactobacillus* spp. including hydrogen peroxide and lactic acid [10,11]. Therefore, it has been proposed that *G. vaginalis* might compete with *Lactobacillus* spp. and enable other anaerobes to incorporate and grow within the biofilm [12]. However, convincing evidence that *G. vaginalis* is an initial colonizer requires further study. Evidence indicates that certain *Lactobacillus* species are capable of blocking adhesion of pathogenic bacteria to the vaginal epithelium, and these have been studied for their potential use as probiotics [10,13–15]. The goal of this study was to characterize and quantify the initial adhesion of several of the most common BV-associated anaerobes to ME-180 cervical epithelial cells in the presence of vaginal lactobacilli. We analysed the ability of these anaerobes to compete for adherence to epithelial monolayers when added simultaneously with lactobacilli and when added after the lactobacilli have adhered.

We first studied the competition between several BV anaerobes and *Lactobacillus crispatus*, a species tends to promote vaginal health and prevents the growth of other species, to determine the effects on initial adhesion in the ME-180 cell line (see some image examples in Fig. A1 at the appendices). The *L. crispatus* strain used was EX533959VC06, a vaginal isolate from a healthy woman (BEI repository). The BV-associates anaerobes tested were *A. vaginae* FA, *M. mulieris* ATCC 26-9, *P. bivia* ATCC 29303, *F. nucleatum* 718BVC, and *G. vaginalis* 101 [16]. *L. crispatus* was grown in Man, Rogosa and Sharpe broth (MRS; Sigma) and the others were grown in Brain Heart Infusion (Oxoid) supplemented as previously described [16]. All strains were incubated at 37 °C under anaerobic conditions for 24–48 h prior to adhesion assays. Strains were washed and resuspended in sterile PBS at concentration adjusted to 2×10^3 CFU/ml (for competition assays) and 1×10^9 CFU/ml (for displacement/

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blockage assays). ME-180 cervical epithelial cells (ATCC) were cultured in McCoy's 5A medium, as described before [16]. Before adding bacteria, the monolayers were washed twice with 300 μl of PBS to remove non-adherent cells and culture media. To assess the competition for adhesion between *L. crispatus* and the anaerobes, 200 μl *L. crispatus* and 200 μl of one anaerobe were added to the monolayers, and the chambers were incubated for 30 min at 37 °C in anaerobic conditions and 120 rpm. Finally, each chamber was carefully washed twice with 300 μl of sterile PBS to remove non-adherent bacteria and was allowed to air-dry before FISH hybridization procedure, as previously described [17]. In each assay, adhesion controls were performed simultaneously in each 8 chamber slide with a monolayer of ME-180 epithelial cells by adding each bacterium individually and maintaining the same experimental conditions. Microscopic visualization was performed using an EVOS*fl* fluorescence microscope (AMG, USA) equipped with a CCD camera (Sony ICX285AQ color) and filters capable of detecting the two PNA probes and DAPI staining. All images were acquired by AMG EVOS*fl* intrinsic software using a total magnification of ×1000. The lactobacilli and anaerobes adhered cells quantification was done with the National Institutes of Health image analysis software ImageJ (version 1.451). All assays were repeated three times, on independent days. The data was analysed using a two-tailed ANOVA or Student's *t*-test with SPSS statistical software (version 17.0) and expressed as mean ± standard deviation (SD). $p < 0.05$ was considered significant.

As shown in Fig. 1, *G. vaginalis* 101 exhibited the greatest capacity for adherence to ME-180 cells, confirming our previous observations [12]. Interestingly, the *G. vaginalis* strain also maintained its ability to adhere in the presence of *L. crispatus* better than the other species, and there was only a 10% reduction in adherence with respect to the control. This was statically different from the others BV anaerobes (ANOVA Tukey statistical test values, $p < 0.05$). In the competition assays against *L. crispatus*, *G. vaginalis* adhered approximately 4-fold better than *A. vaginae* or *M. mulieris* and approximately 2-fold better than *P. bivia* (see Fig. 1). Adherence of *L. crispatus* was not statistically significantly inhibited by any of the BV anaerobes tested.

We then simulated the introduction of BV-associated bacteria into a healthy vagina colonized by lactobacilli. To determine the displacement and blockage ability of the tested bacteria, aliquots of 400 μl of either *L. crispatus* or *Lactobacillus iners* were added to the epithelial monolayers in each well of the 8 chamber slides. Then, the chamber slides were incubated for 4 h at 37 °C in anaerobic conditions and 120 rpm. Subsequently a second adhesion step was performed, using one BV-associated anaerobe, for 30 min under the same conditions as before. As can be seen in Table 1, *L. crispatus* inhibited adherence of *G. vaginalis* 101 by approximately 43%. Addition of *G. vaginalis* appeared to cause a slight displacement of adherent *L. crispatus*, but this did not reach statistical significance. *L. crispatus* also reduced adherence of *A. vaginae* and *M. mulieris* by approximately 50%. *P. bivia* and *F. nucleatum* appeared to be less susceptible to inhibition by *L. crispatus*. Interestingly, *L. iners*, which has been shown in previous studies to be less protective against BV relative to other vaginal lactobacilli [7], had a similar inhibitory effect on adherence by all of the BV-associated species except *G. vaginalis* (see Table 2). Adherence of *G. vaginalis* actually increased somewhat in the presence of *L. iners*, although this increase did not reach statistical significance. None of the anaerobes displaced *L. iners*, as shown in Table 2.

While it was already known that vaginal lactobacilli could inhibit the growth of BV anaerobes, largely through the production of lactic acid and hydrogen peroxide [11], the effect of lactobacilli on initial adherence of BV-associated anaerobes, which could be mediated through steric hindrance, competition for receptors, or the secretion of soluble factors, has not been reported, as far as we know. Previously, using a semi-quantitative approach, we determined that *G. vaginalis* had a greater capacity for adhesion to ME-180 cells relative to other known BV-associated bacteria [12]. Here, we confirmed this finding using a quantitative assay to

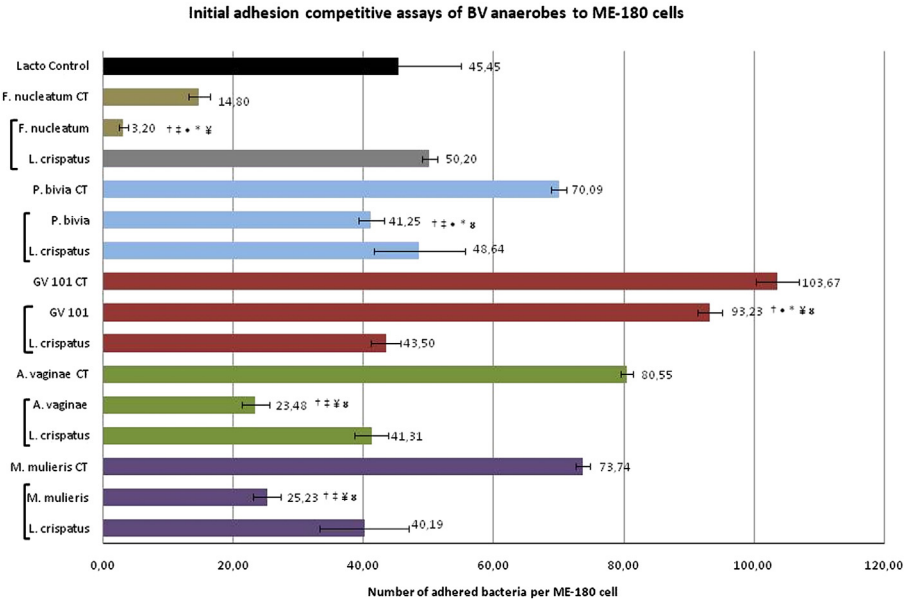

Fig. 1. Initial adhesion competitive assays realized by equal mixture between *L. crispatus* and a BV anaerobe at low level each one (10^3 CFU/ml) to ME-180 cells during 30 min at 100 rpm and anaerobic conditions. † $p < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the adhesion assay. ‡ $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* 101 tested in the adhesion assay. • $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay. * $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay. ¥ $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *P. bivia* strain tested in the adhesion assay. □ $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *F. nucleatum* strain tested in the adhesion assay.

Table 1
Blockage of adherence of BV-associated anaerobes to ME-180 epithelial cells by adherent *L. crispatus* and its displacement by BV-associated anaerobes. The number of each BV-associated anaerobe, when incubated at high level (1E9 CFU/ml), that adhered per ME-180 cell (±standard deviation) is shown on the left and the percentage of bacteria that adhered when the ME-180 monolayer was pre-coated with *L. crispatus* (1E9 CFU/ml) relative to the control (±standard deviation) is shown on the middle. Following the addition of a BV-associated anaerobe, the number of remaining *L. crispatus* was counted and compared to the *L. crispatus* control (62.91 per ME-180 cell ± 1.96) and the percent (±standard deviation) of *L. crispatus* that remained adherent after addition of each BV anaerobe (1E9 CFU/ml) is shown on the right.

	Number of BV anaerobe per ME-180 cell	Percent adherent to *L. crispatus*-coated ME-180 monolayer	Percentage of *L. crispatus* remaining after addition of BV anaerobe
G. vaginalis 101	232.11 (±6.39)	57.15%[a,c,d,e,f] (±2.31)	76.37%[c,d] (±4.93)
A. vaginae FA	16.74 (±1.09)	51.42%[a,b,f] (±7.28)	95.53%[b,e,f] (±4.09)
M. mulieris ATCC 26-9	16.61 (±1.60)	52.85%[a,b,f] (±0.46)	95.62%[b,e,f] (±4.57)
P. bivia ATCC 29303	23.17 (±3.00)	70.11%[b] (±6.17)	75.41%[b,c,d] (±12.70)
F. nucleatum 718BVC	25.79 (±1.16)	74.34%[a,b,c,d] (±8.50)	82.94%[b,c,d] (±12.71)

[a] $p < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the adhesion assay.
[b] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* 101 tested in the adhesion assay.
[c] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay.
[d] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay.
[e] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *P. bivia* strain tested in the adhesion assay.
[f] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *F. nucleatum* strain tested in the adhesion assay.

determine adherence of *G. vaginalis*, *A. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum* and we determined the effects of *L. crispatus*, which has been shown to be a highly protective vaginal lactobacilli [18], and *L. iners*, which has been associated with risk for BV, on initial adherence of these anaerobes to epithelial cells [4,7,18].

As further evidence of its role in BV, *G. vaginalis* exhibited the greatest capacity for adherence to ME-180s, and while adherence was inhibited somewhat by *L. crispatus*, it actually increased slightly in the presence of *L. iners*. Effects of *L. crispatus* on initial adherence to epithelial cells could be related to steric hindrance, receptor blockage, or the secretion of soluble factors. Confirming our first experiments, *G. vaginalis* was better able (relative to the other BV-associated species) to adhere to ME-180 cells when *L. crispatus* was allowed to attach to the cells first. In addition, *P. bivia* and *F. nucleatum* were proportionally less affected by *L. crispatus* early colonization (Table 1). Interestingly, adherence of *L. iners* to the ME-180 cells did not prevent secondary colonization by *G. vaginalis* (Table 2), but it prevented adherence of the other anaerobes as effectively as *L. crispatus*. Evidence suggests that *L. iners* is not very protective against BV, but the reason for this lack of apparent protection role is not clear [18,19]. Our results show that *L. iners* did not have an antagonistic effect on *G. vaginalis*, which may partially explain its failure to prevent BV. Our data also suggest that *L. iners* was not displaced by *G. vaginalis* suggesting that the two species may be tolerant of one another. These results support the idea that *G. vaginalis* is an early colonizer in BV that may contribute to the colonization of the vagina by other BV-associated species. However, this is a simplified model system and lacks many of the bacteria-specific and host-specific factors that would be present in the vagina.

We also found that *F. nucleatum* adhered poorly in the competitive initial adhesion assays. However, it was able to adhere more efficiently when it was added after the lactobacilli had adhered to the ME-180 cells. This result is in agreement with a study elaborated by Foster and Konlenbrander [20], demonstrating that *F. nucleatum* is a weak initial adherent bacteria but it is capable of co-aggregating with other pre-adhered bacteria. Our study is the first to quantify initial adhesion per epithelial cell and demonstrated clearly the greater capacity of *G. vaginalis* for initial adhesion even in presence of high levels of *L. crispatus* and *L. iners*. Also, it appears that the species of vaginal lactobacilli plays an important role not only in preventing the growth of BV-associated anaerobes but also in impairing the adherence of certain species to vaginal epithelial cells as well.

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Table 2
Blockage of adherence of BV-associated anaerobes to ME-180 epithelial cells by adherent *L. iners* and its displacement by BV-associated anaerobes. The number of each BV-associated anaerobe, when incubated at high level (1E9 CFU/ml), that adhered per ME-180 cell (±standard deviation) is shown on the left and the percentage of bacteria that adhered when the ME-180 monolayer was pre-coated with *L. iners* (1E9 CFU/ml) relative to the control (±standard deviation) is shown on the middle. Following the addition of a BV-associated anaerobe, the number of remaining *L. iners* was counted and compared to the *L. iners* control (126.92 per ME-180 cell ± 2.25) and the percent (±standard deviation) of *L. iners* that remained adherent after addition of each BV anaerobe (1E9 CFU/ml) is shown on the right.

	Number of BV anaerobe per ME-180 cell	Percentage of BV adherent to *L. iners*-coated ME-180 monolayer	Percentage of *L. iners* remaining after addition of BV anaerobe
G. vaginalis 101	411.91 (±52.90)	116.90%[c,d,e,f] (±18.34)	96.29%[c,d] (±4.90)
A. vaginae FA	43.82 (±3.18)	48.62%[a,b] (±3.86)	87.23%[b,e,f] (±2.72)
M. mulieris ATCC 26-9	47.54 (±3.62)	79.13%[b] (±8.97)	94.61%[b,e,f] (±5.83)
P. bivia ATCC 29303	145.34 (±8.38)	79.99%[b] (±1.24)	93.26%[b,c,d] (±2.38)
F. nucleatum 718BVC	206.32 (±3.44)	48.42%[a] (±0.15)	94.60%[b,c,d] (±0.43)

[a] $p < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the adhesion assay.
[b] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* 101 tested in the adhesion assay.
[c] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay.
[d] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay.
[e] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *P. bivia* strain tested in the adhesion assay.
[f] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *F. nucleatum* strain tested in the adhesion assay.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2013.07.007.

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