ORIGINAL ARTICLE

Bacteroides fragilis INDUCES CONFORMATIONAL ALTERATIONS IN *Trichomonas vaginalis* ULTRASTRUCTURE IN AN *IN VITRO* INTERACTION

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ABSTRACT

Bacteroides genus are commonly found on mucous membranes, including the female genital tract, acting as agents for several site infections. Anaerobic infections are usually polymicrobial and endogenous. *Trichomonas vaginalis*, the trichomoniasis etiologic agent, is a facultative anaerobic flagellated parasite spread worldwide. The purpose of this study was to explore the association between vaginal bacteria and *T. vaginalis,* as well as to understand factors that may favour the infection of *T. vaginalis*. We have, therefore, used *T. vaginalis* trophozoites and the species *Bacteroides fragilis*, which is considered the most important in its genus, once it is the most commonly isolated bacteria from endogenous infections. The parasite-bacteria interaction was performed in different proportions in periods varying from 1 to 12 hours applying viability tests. The data were analyzed to compare the parasite viability *in vitro* in the presence and absence of *B. fragilis*. The results indicate that in the 1:100 proportion post-interaction analysis, ultrastructural alterations were noticeable after 6 hours. After 8 hours, *T. vaginalis* viability decreased, and after 12 hours of interaction no viable trophozoites were found. These data suggest that the parasite can deal with *B. fragilis* in short interaction periods. However, in longer interaction periods the trophozoites collapse, indicating that *B. fragilis* may produce toxic metabolites against *T. vaginalis* activity.

KEY WORDS: *Bacteroides fragilis; Trichomonas vaginalis*; parasite-bacteria interaction; parasite environment; electron microscopy; gynecologic infections.

INTRODUCTION

The family Bacteroidaceae is composed of anaerobic bacteria that colonize the human gastrointestinal and female genital tracts. It is composed of non-spore-forming Gram-negative bacilli (Cato & Johnson, 1976), including the genus *Bacteroides*. *Bacteroides* species are bile-resistant obligate anaerobes

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(Song et al., 2004). *Bacteroides fragilis* is clinically considered the most important in its genus, since it is the most commonly isolated from endogenous infections, usually associated with infections in the gastrointestinal, respiratory and female genital tracts (Giamarellou, 2000).

The intact mucosa is an important barrier which prevents microorganism proliferation from spreading to other sites. Therefore, anaerobic infections usually occur in the presence of predisposing factors (Brook, 1987). Anaerobic bacteria have become clinically relevant over recent years as a result of advances in the detection and identification techniques of this microbial group, confirming the involvement of these microorganisms in several infections of the female genital tract. In the infectious process, the bacteria act both as a unique pathogen and in association with facultative microorganisms (Ferreira et al., 2003), classifying the anaerobic infections as polymicrobial (Onderdonk et al., 1977). In this context, species of the genus *Bacteroides* have received special attention due to frequent isolation in clinical cases (Koeth et al., 2004).

Interest in bacterial microbiota in the female genital tract has increased due to its relation with gynecological infections, since many of the bacteria found in the vaginal and cervical secretions are anaerobic (Engelkirk et al., 1992). Gorbach et al., (1973), demonstrated the predominance of *Bacteroides* spp. in the cervical microbiota, composed of species that are frequently associated with infections in the female genital tract, showing the relevance of studies involving these microorganisms (Finegold, 1995; Szoke et al., 1996).

An important factor has also emerged: *Bacteroides* spp. can inhibit the phagocytosis of certain polymorphonuclear cells killing co-existing microorganisms (Beena et al., 1997). This ability to resist phagocytosis and kill other microorganisms might be a contributing factor to their prevalence in mixed infections.

Trichomonas vaginalis is the parasite that causes trichomoniasis, a sexually transmitted disease that is common in humans (da Costa et al., 2005). It is also cosmopolitan and the World Health Organization (WHO) estimated 156 million cases in 2016 (Rowley et al., 2019). Street et al., (1984) reported the interaction of *Trichomonas* spp. with other pathogenic microorganisms of the genital tract and observed that *Neisseria gonorrhoeae* and *Mycoplasma hominis* bacterial species are quickly inactivated and phagocyted, suggesting a persistence mechanism in the parasite infection. This discovery highlights the importance of studying *T. vaginalis* phagocytic mechanism with other infectious agents such as *B. fragilis*.

In this regard, trichomoniasis has been associated with the heightened risk of multiple adverse outcomes in women and a deeper understanding is essential of the factors associated with *T. vaginalis* acquisition. Some authors have demonstrated that the vaginal microbiota and some specific vaginal bacteria are associated with an increased risk of *T. vaginalis* infection (Brotman et al., 2010; Martin et al., 2013; Balkus et al., 2014). It is unknown whether

other vaginal bacterial species or overall microbiota diversity contribute to the increased risk of *T. vaginalis* infection. To explore the association between vaginal bacterial infection and female risk of *T. vaginalis* acquisition, we have investigated the association of *T. vaginalis* with *B. fragilis* in an *in vitro* system.

METHODS

Cultivation of Bacteroides fragilis

B. fragilis (ATCC25285) specimens were reactivated in Brain Heart Infusion (BHI) - Pre-Reduced Anaerobically Sterilized (PRAS) broth from BHI Agar stock, and incubated for 24 hours at 37ºC. Viability and purity criteria were used after seeding in blood agar plates supplemented with vitamin K and hemin. After the incubation period in an anaerobic media, the colonies were re-cultivated in BHI-PRAS broth.

Cultivation of Trichomonas vaginalis

Trophozoites of *T. vaginalis* were cultivated in 8mL glass tubes filled with 7 mL of Trypticase-Yeast Extract-Maltose (TYM) media, as described by Diamond (1957) with 10% fetal bovine serum (FBS). The cultures were kept at 37ºC for 24 hours and the media was changed after this period, matching the logarithmic growth phase.

Parasite-bacteria interaction

T. vaginalis was counted in a Neubauer chamber and 10^5 cells were placed in microtubes. The number of *B. fragilis* was estimated using the McFarland scale. Interactions occurred in TYM medium supplemented with FBS and incubated at 37ºC. The protozoa-bacteria interaction was performed in 1:10, 1:50 and 1:100 proportions, in periods varying from 1 to 12 hours.

Scanning Electron Microscopy

After different periods of interaction, the samples were fixed in a solution containing glutaraldehyde 2.5% and 4% formaldehyde in sodium cacodylate 0.1 M with pH 7.4, dehydrated in acetone solutions (from 30% to 100%), dried using the CO_2 critical point and mounted on stubs. The samples were covered with gold (20-30 nm), for observation by Scanning Electron Microscope Jeol JSM 6490LV.

Transmission Electron Microscopy

The sample suspension was fixed with 2.5% glutaldehyde and 4%, recently prepared formaldehyde in sodium cacodilate buffer (0.1M, pH 7.2) for 2 h. Cells were washed with sodium cacodilate buffer and post-fixed with 1% osmium tetroxide, 1.6% potassium ferrocyanide and 5 mM calcium chloride in sodium cacodilate buffer for 1 h. Cells were dehydrated with acetone serial concentrations of 30%, 40%, 50%, 70% and 100%. Inclusion was performed with epoxy resin. Ultrafine sections were contrasted with uranyl acetate and lead citrate and observed in a Transmission Electron Microscope FEI SPIRIT 120 Kvolts.

Trichomonas vaginalis *viability analisys*

After different interaction periods, the samples were colored with trypan blue 0.4% for the cell viability assay and the living cells were counted with a Neubauer chamber.

Bacteroides fragilis *viability analisys*

Aliquots of medium containing bacteria after interaction were cultivated on blood agar plates supplemented with vitamin K and hemin and incubated in an anaerobic environment at 37°C. *B. fragilis* was considered viable if growth occurred after 24 hours.

Statistical analysis

The results were expressed as means and standard deviation. Variance between three independent experiments was analyzed using the One Way ANOVA test, followed by the Tukey post-test in the Graph-Pad Prism 5.0 statistical program.

RESULTS

The interaction of the protozoan *T. vaginalis* with *B. fragilis* in the proportion of 1:100 was analyzed after 2, 6 and 12 hours, through scanning electron microscopy. In the images of the 2 h period (Figure 1 B), no modifications in the ultrastructure of the parasite are noticeable in comparison to the control with no bacteria (Figure 1 A), suggesting that the parasite presents no apparent disturbance. It is also possible to observe bacterial adherence to the surface of the parasite (Figure 1 B). After the 6 h incubation (Figure 1 C), the same morphological profile was noted with no sign of disturbance, but the bacteria can be seen strongly adhered to the surface of the parasite (Figure 1C).

After the 12 h interaction period (Figure 1 D), few modifications can be seen in the ultrastructure of the parasite, especially the loss of the ellipsoidal shape of the protozoan. Modifications in the disposition of the bacteria can also be seen, suggesting the formation of biofilms (Figure 1 D), due to the seemingly high level of organization of *B. fragilis*.
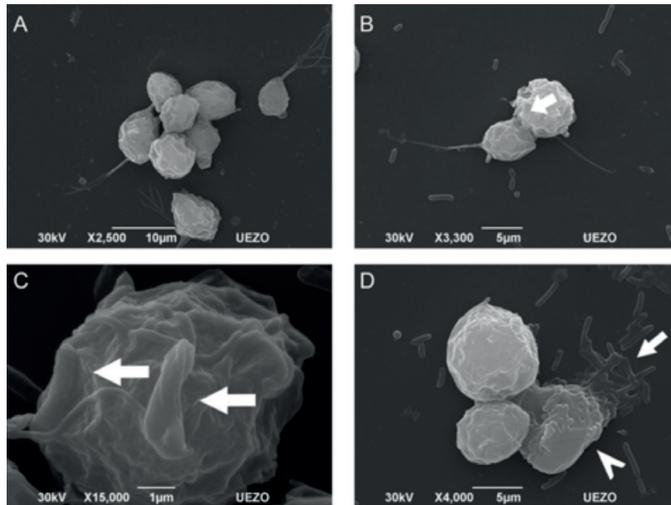

Figure 1. Scanning Electron Microscopy showing assays of interactions of *T. vaginalis* with *B. fragilis* in the 1:100 proportion, after 2 (B), 6 (C) and 12 (D) hours of interaction. Note *B. fragilis* strongly attached to *T. vaginalis* cell body (B and C: arrow) and the alteration in the parasite's ultrastructure, which has lost its rounded shape (D: arrowhead). It was possible to notice a biofilm formation by *B. fragilis* (D: arrow). A: Control; B: 2 h; C: 6 h; D: 12 h.

To better understand the relationship between *B. fragilis* and *T. vaginalis*, an ultrastructural analysis was performed of several sections of the incubation at the 6 h moment when the analysis through transmission electron microscopy showed *B. fragilis* internalized by *T. vaginalis* (Figure 2).

Since our ultrastructural analysis showed the loss of the ellipsoidal shape of the protozoan, we performed an *in vitro* proliferation assay to determine whether or not, cells are viable after long term exposure to *B. fragilis*. In this assay the number of parasites was analyzed by counting with a Neubauer chamber, using trypan blue to analyze the viability of the protozoan (Figure 3). After each count, the samples were cultivated in blood agar plates supplemented with vitamin K and hemin and incubated in an anaerobic environment for 24h at 37ºC to ensure the viability of *B. fragilis* after 12 hours of the experiment (data not shown).
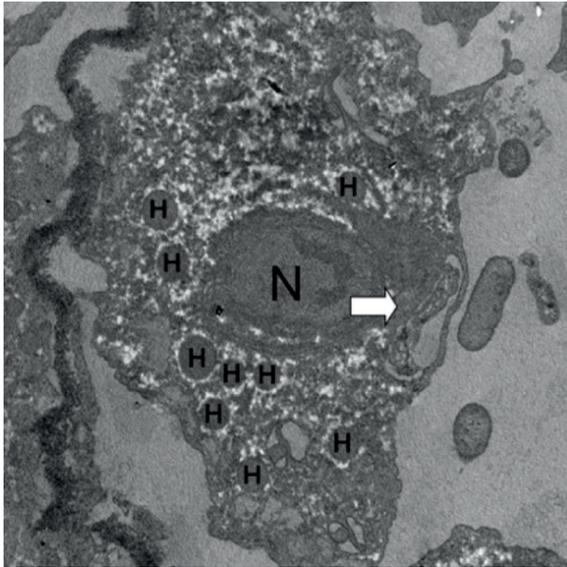
Figure 2. Transmission electron of *T. vaginalis* with *B. fragilis* in the 1:100 proportion, after 6 hours of interaction. Note the apparently internalized and degraded bacteria (arrow). N: Nucleus; H: Hydrogenosome.
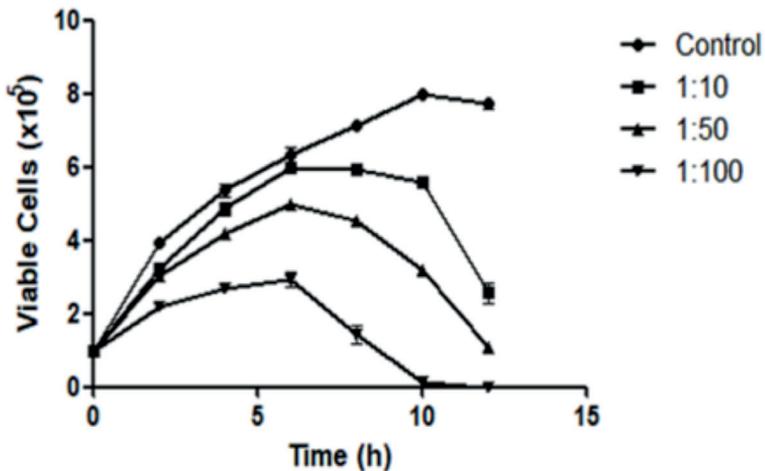

Figure 3. T. vaginalis cell viability in periods from 0 to 12 hours in interaction with *B. fragilis* in the 1:10, 1:50 and 1:100 proportions. Note that the viability of the parasite is severely affected according to interaction time and *B. fragilis* concentration ($p < 0.01$).

The proliferation assay showed that in the 1:10 parasite-bacteria proportion, after 8 hours, the viability of *T. vaginalis* is affected in comparison to the control (Figure 3), corroborating the data obtained by the ultrastructure analysis which shows the loss of the ellipsoidal shape of the parasite after increasing incubation time. In interaction assays in the 1:50 proportion, a more significant drop in the number of viable parasites is observed in comparison to the assay performed in the 1:10 proportion. The interaction in the 1:100 proportion shows a more expressive decrease in the number of viable parasites after 6 hours, in comparison to assays with interaction in smaller proportions. After 10 hours of interaction, viable cells can no longer be observed (Figure 3). In this regard, the results showed that the number of viable *T. vaginalis* cells is directly related to the proportion of *B. fragilis*, reinforcing the results obtained through ultrastructural analysis.

DISCUSSION

Genitourinary infections are split into two main categories: primary infections, due to sexually transmissible pathogenic microorganisms, like parasites (*T. vaginalis*), bacteria (*Treponema pallidum, N. gonorrhoeae, Chlamydia trachomatis, Haemophilus ducreyi*) or viruses (*Herpes simplex*), and infections due to members of the microbiota, such as *B. fragilis* and members of the Enterobacteriaceae family (Ronald & Alfa, 1996). Therefore, studies on microorganisms that reside in the female genital tract have increased due to the relation between microbiota and gynecologic infections.

Bacteria act both as unique pathogens and in association with facultative microorganisms in infections, and many of these found in the vaginal and cervical secretions are anaerobic. These factors highlight the need for studies on anaerobic microorganisms with other existing microorganisms, facultative or not, in the female genital tract (Gorbach et al., 1973; Engelkirk et al., 1992; Ferreira et al., 2003).

In the present study, the interaction of *T. vaginalis* and *B. fragilis* was analyzed, to explore the effects of a polymicrobial vaginal bacterial infection. The results showed bacteria adhered to the parasite and seemingly being internalized. This *T. vaginalis* mechanism was demonstrated by Benchimol & de Souza (1995), who performed the interaction of *T. vaginalis* with *Escherichia coli* species and obtained similar results. In this study, *E. coli* was rapidly phagocyted and inactivated by *T. vaginalis*. Moreover, our analysis shows a relationship between phagocytic activity and length of incubation time. Analyses performed by transmission electron microscopy showed that after the 6 h interaction period, unusual stretched structures are found in the interior of the parasite, similar to *B. fragilis*, indicating the internalization of the bacteria by the parasite. Although not completely elucidated, one can also deduce that such phagocytosis of the parasite may be an attempt to balance the media ecologically, as a persistence mechanism of the infection by the parasite, confirming results obtained by Street et al. (1984).

Several studies have evaluated the endocytic ability of *T. vaginalis*. Pereira-Neves & Benchimol (2007) demonstrated that *T. vaginalis* can perform two different types of phagocytosis. The classical phagocytosis, with the emission of pseudopods and phagocytosis by "sinking", where there is no emission of pseudopods. In this study the occurrence of classical phagocytosis was not observed. This probably occurred due to the strain of *T. vaginalis* utilized having been kept in culture since the 1980s. There are several studies that suggest that "wild" strains of *T. vaginalis* perform the classical phagocytosis more often than the "domesticated" strains (Pereira-Neves & Benchimol, 2007).

Subsequently, parasite viability in interaction with *B. fragilis* was analyzed. The assays were performed using different parasite-bacteria proportions verifying that from 2 to 8 hours of interaction, the number of *T. vaginalis* decreased in comparison to the control. Therefore, these data suggest that the parasite phagocytes the bacteria in an attempt to continue the infection, but this mechanism is not sufficient, and *B. fragilis* manages to work around the attack from the parasite. These results differ from those obtained in experiments that performed interactions with other bacterial species, such as the work of Street et al. 1984, seeing that in such experiments the parasite managed to prolong the infection, in contrast to what occurred in the *B. fragilis* interaction.

Studies have shown that *B. fragilis* can generate and release desoxyribonucleases, proteases and lipases in the extracellular medium (Rudek & Haque, 1976). The generation of these metabolites is a possible explanation to the death of *T. vaginalis* when in interaction with *B. fragilis* for long periods. This hypothesis is strongly justified by the reduction in number of viable parasites matching the increase in parasite-bacteria proportion. Another possible explanation for the death of the protozoan is the fact that the interaction occurs in a closed system and this model may cause the competition for nutrients between both microorganisms.

In conclusion, *Trichomonas vaginalis* was able to phagocyte *B. fragilis* in every experiment, in the 1:10, 1:50 and 1:100 proportions. During the interaction assays, the ultrastructure of *T. vaginalis* proved to be drastically modified after a period of 12 hours, but alterations can be noted after 6 hours probably due to metabolites produced by *B. fragilis*. *T. vaginalis* cell viability drops according to incubation time and the proportion of *B. fragilis,* but its viability is maintained even after 12 hours of interaction (data not shown). Evidences that *B. fragilis* creates a biofilm after long periods of incubation were also found.

Future studies will be necessary to verify other important aspects of the phagocytosis of the parasite, such as the probable role of receptors and other mechanisms that are responsible for the adherence of *B. fragilis* to *T. vaginalis* cellular body.

ACKNOWLEDGMENTS

REFERENCES

1. Balkus JE, Richardson BA, Rabe LK, Taha TE, Mgodi N, Kasaro MP, Ramjee G, Hoffman IF, Abdool Karim SS. Bacterial vaginosis and the risk of *Trichomonas vaginalis* acquisition among HIV-1-negative women. *Sex Transm Dis 41*: 123-128, 2014.

2. Beena VK, Nagaraja K, Shivananda PG. Inhibition of Phagocytosis of *E. coli* by *B. fragilis* Group - An In vitro Study. *Indian J Pathol Microbiol 40*: 373-376, 1997.

3. Benchimol M, de Souza W. Carbohydrate involvement in the association of a prokaryotic cell with *Trichomonas vaginalis* and *Trichomonas foetus*. *Parasitol Res 81:* 459-464, 1995.

4. Brook I. Pathogenicity and therapy of anaerobic bacteria in upper respiratory tract infections. *Pediatr Infect Dis J 6*: 131-136, 1987.

5. Brotman RM, Klebanoff MA, Nansel TR, Yu KF, Andrews WW, Zhang J, Schwebke JR. Bacterial vaginosis assessed by Gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. *J Infect Dis 202:* 1907-1915, 2010.

6. Cato EP, Johnson JL. Reinstatement of Species Rank for *Bacteroides fragilis, B. ovatus, B. distasonis, B. thetaiotaomicron,* and *B. vulgatus*: Designation of Neotype Strains for *Bacteroides fragilis* (Veillon and Zuber) Castellani and Chalmers and *Bacteroides thetaiotaomicron* (Distaso) Castellani and Chalmers. *Int J Syst Evol Microbiol 26*: 230-237, 1976.

7. da Costa RF, de Souza W, Benchimol M, Alderete JF, Morgado-Diaz JA. *Trichomonas vaginalis* perturbs the junctional complex in epithelial cells. *Cell Res 15*: 704-716, 2005.

8. Diamond LS. The Establishment of Various Trichomonads of Animals and Man in Axenic Cultures. *J Parasitol 43*: 488-490, 1957.

9. Engelkirk PG, Duben-Engelkirk J, Dowell VR. Principles and Practice of Clinical Bacteriology. 1st edition. Star Pub Co: Belmont, 1992.

10. Ferreira MCS, Domingues RMCP, Uzeda M. *Manual de Bacteriologia de Anaeróbios*. UFRJ: Rio de Janeiro, 2003.

11. Finegold SM. Anaerobic infections in humans: an overview. *Anaerobe 1*: 3-9, 1995.

12. Giamarellou H. Anaerobic infection therapy. *Int J Antimicrob Agents 16*: 341-346, 2000.

13. Gorbach SL, Menda KB, Thadepalli H, Keith L. Anaerobic microflora of the cervix in healthy women. *Am J Obstet Gynecol 117:* 1053-1055, 1973.

14. Koeth LM, Good CE, Appelbaum PC, Goldstein EJ, Rodloff AC, Claros M, Dubreuil LJ. Surveillance of susceptibility patterns in 1297 European and US anaerobic and capnophilic isolates to co-amoxiclav and five other antimicrobial agents. *J Antimicrob Chemother 53:* 1039-1044, 2004.

15. Martin DH, Zozaya M, Lillis RA, Myers L, Nsuami MJ, Ferris MJ. Unique vaginal microbiota that includes an unknown Mycoplasma-like organism is associated with *Trichomonas vaginalis* infection. *J Infect Dis 207*: 1922-1931, 2013.

16. Onderdonk AB, Kasper DL, Cisneros RL, Bartlett JG. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. *J Infect Dis 136*: 82-89, 1977.

17. Pereira-Neves A, Benchimol M. Phagocytosis by *Trichomonas vaginalis:* new insights. *Biol Cell 99:* 87-101, 2007.

18. Ronald AR, Alfa MJ. Microbiology of the Genitourinary System. In: Baron S, ed. Medical Microbiology. 4th ed. University of Texas Medical Branch: Galveston, 1996. Chapter 97.

19. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, Chico RM, Smolak A, Newman L, Gottlieb S, Thwin SS, Broutet N, Taylor MM. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. *Bull World Health Organ 97*: 548-562, 2019.

20. Rudek W, Haque RU. Extracellular enzymes of the genus Bacteroides. *J Clin Microbiol 4:* 458-460, 1976.

21. Song YL, Liu CX, McTeague M, Finegold SM. "*Bacteroides nordii*" sp nov and "*Bacteroides salyersae*" sp nov isolated from clinical specimens of human intestinal origin. *J Clin Microbiol 42:* 5565-5570, 2004.

22. Street DA, Wells C, Taylor-Robinson D, Ackers JP. Interaction between *Trichomonas vaginalis* and other pathogenic micro-organisms of the human genital tract. *Br J Vener Dis 60*: 31-38, 1984.

23. Szoke I, Pascu C, Nagy E, Ljung A, Wadstrom T. Binding of extracellular matrix proteins to the surface of anaerobic bacteria. *J Med Microbiol 45*: 338-343, 1996.