Characteristics of *Lactobacillus* and *Gardnerella vaginalis* from women with or without bacterial vaginosis and their relationships in gnotobiotic mice

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The objectives of the present study were to evaluate *in vitro* the production of antagonistic compounds against *Gardnerella vaginalis* by *Lactobacillus* strains isolated from women with or without bacterial vaginosis (BV), and to select one of the better *Lactobacillus* producers of such a substance to be tested *in vivo* using a gnotobiotic animal model challenged with one of the more sensitive *G. vaginalis* isolates. A total of 24 isolates from women with and without BV were identified as *G. vaginalis*. A higher frequency (P<0.05) of this bacterium was observed in women with BV (56.7%) when compared to healthy women (17.6%). A total of 86 strains of *Lactobacillus* were obtained from healthy women and women with BV. *Lactobacillus* strains were more frequently present (P<0.05) in healthy women (97.5%) than in women with BV (76.7%). *Lactobacillus crispatus* was the predominating strain in both healthy women and women with BV. *Lactobacillus johnsonii*, *Lactobacillus gasseri* and *Lactobacillus vaginalis* were isolated with an intermediate frequency in the two groups. *In vitro* antagonism assays were performed using as indicators 17 reference strains and the *G. vaginalis* strains isolated from women with BV and from healthy women. *Lactobacillus* isolated from healthy women showed the higher antagonistic activity against all the indicator strains when compared with isolates from women with BV. Concerning the indicator strains, *G. vaginalis* found in women with BV was more resistant to the antagonism, particularly when *Lactobacillus* isolates from women with BV were used as producer strains. A high vaginal population level of *G. vaginalis* was obtained by intravaginal inoculation of germ-free mice, and this colonization was accompanied by vaginal histopathological lesions. A tenfold decrease in vaginal population level of *G. vaginalis* and a reduction of histological lesions were observed when the pathogenic challenge was performed in mice previously monoassociated with an *L. johnsonii* strain. Concluding, results of the present study suggest that progression of *G. vaginalis*-associated BV depends in part on a simultaneous presence of *Lactobacillus* populations with a low antagonistic capacity and of a *G. vaginalis* strain with a high resistance to this antagonism. The results could also explain why *G. vaginalis* is frequently found in the vaginal ecosystem of healthy women.

**INTRODUCTION**

Bacterial vaginosis (BV) is a polymicrobial syndrome where indigenous *Lactobacillus* populations, which are usually dominant in the vagina of healthy women (Hillier, 2005), are replaced by a mixture of bacteria that generally includes *Gardnerella vaginalis*, *Prevotella/Bacteroides* species, *Peptostreptococcus* species, *Mycoplasma hominis*, *Ureaplasma urealyticum* and/or *Mobiluncus* species (Atassi et al., 2006). All of these bacteria can be present in low populations in
healthy women, but during BV their concentrations are generally increased 100–1000-fold over normal levels (Eschenbach, 2007).

Factors that lead to the decline of the lactobacilli and the overgrowth of an atypical microbiota, as well as the sequence of bacterial population changes leading to BV, remain unknown. Microbial interactions, including synergism, commensalism and antagonism, can probably modify the environment so that it becomes adverse for some microorganisms by production of inhibitory compounds, but suitable for other organisms by releasing specific growth factors. Pybus & Onderdonk (1999) described in vitro nutritional inter-relationships that can explain commensalism between Prevotella bivia and both G. vaginalis and Peptostreptococcus anaerobius. However, production of antagonistic substances may also be an important factor in the competitive colonization of the vaginal ecosystem.

The lactobacilli metabolize glucose essentially into lactic acid, which contributes to the maintenance of a low vaginal pH (4.0–4.5) and reduces the growth of most pathogenic micro-organisms (Aroucheva et al., 2001). Many isolates of vaginal lactobacilli also produce H₂O₂, a compound having broad antimicrobial activity (Cherpes et al., 2008). Among other antagonistic mechanisms, bacteriocinogenic activity is one of the most studied, and seems to contribute to the intra- and inter-regulation of the human microbiota, influencing microbial invasion and defence (Riley & Wertz, 2002).

G. vaginalis is a variable Gram-staining, facultatively anaerobic, non-motile, rod-shaped bacterium (Jarosik et al., 1998), and is commonly found in the vaginal mucosa of asymptomatic women, but shows high concentrations in BV (Ingianni et al., 1997). Recently, our laboratory reported the production of synergistic and/or antagonistic compounds by G. vaginalis (Teixeira et al., 2010). In a second study, we confirmed the importance of high population levels of Lactobacillus (essentially Lactobacillus crispatus) and the quality of these lactobacilli as producers of antagonistic substances for a healthy vaginal environment (Branco et al., 2010). These results showed that the quantity of beneficial bacteria as well as their qualities as producers of antagonistic compounds seem to be pivotal factors in understanding BV and the ecological role of these bacteria in the vaginal environment. However, these results were obtained in in vitro experiments, and it is not certain whether it occurs in vivo. The confirmation of the antagonistic phenomenon in an animal model could lead to the selection and development of probiotic Lactobacillus strains for the prevention and/or treatment of BV (Kaewsrichan et al., 2006). The likely contribution of this mechanism is difficult to determine in the wider presence of a complex vaginal microbial ecosystem. For these reasons, the gnotobiotic mouse provides an in vivo simplified system that allows the observation of ecological interactions in the vaginal tissue among few microbial strains inoculated in this ecosystem.

The objectives of the present study were to evaluate in vitro the production of antagonistic compounds against G. vaginalis by Lactobacillus strains isolated from women with or without BV, and to select one of the best Lactobacillus producers of such a substance to be tested in vivo using a gnotobiotic animal model challenged with one of the most sensitive G. vaginalis isolates.

METHODS

Ethical aspects. This project was approved by the Ethics Committee of the Federal University of Minas Gerais for the experiments with humans (COEP, protocol 062/03) and written informed consent was obtained from all subjects before inclusion in the study. The study was also approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFMG, protocol no. 227/2009).

Patients. The bacterial samples were isolated from healthy women (n=40) and patients with BV (n=30). Patients were screened at the Serviço de Ginecologia, Hospital das Clínicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Bacterial vaginosis was diagnosed when three out of four Amsel criteria were observed (Amsel et al., 1983). Inclusion criteria were: women 18–45 years old, eumenorrheic, with normal blood glucose levels and with or without BV. The use of oral or topical antimicrobials 30 days before the sampling, pregnancy, menstruation, virginity and women in the puerperal phase or under immunosuppressive therapy were considered as exclusion criteria.

Vaginal sampling and sample processing. Vaginal samples were obtained using two 10 µl sterile loops which, after sampling, were introduced under a CO₂ flux in a tube containing 1 ml Ringer-PRAS solution and in a tube containing 1 ml Gardnerella transport medium, respectively. The samples were processed in a time range from 2 to 4 h after collection. The samples were introduced into an anaerobic chamber (Forma Scientific) containing an atmosphere of 85% N₂, 10% H₂ and 5% CO₂ and submitted to serial decimal dilutions until 10⁻⁶. Aliquots of 0.1 ml from dilutions were spread onto Vaginalis agar (using Columbia agar as base; Difco) and de Man, Rogosa and Sharp agar (MRS; Merck). After 48 h to 7 days of incubation at 37 °C, bacterial counts were determined and expressed as log₅ [c.f.u. (ml vaginal fluid)⁻¹]. Colonies with distinct morphology were isolated on the respective medium. The bacterial samples were maintained at −80 °C in medium supplemented with 10% glycerol.

Animals. Germ-free 28-day-old female mice (NIH, Taconic, Germantown, USA) were used in this study. The animals were housed in flexible plastic isolators (Standard Safety Equipment) and handled according to established procedures. Experiments with gnotobiotic mice were carried out in micro-isolators (UNO Roestvaststaal). Water and commercial autoclavable diet (Nuvital) were sterilized by steam and administered ad libitum, and animals were maintained in an animal house with controlled lighting (12 h light, 12 h dark). All experimental procedures were carried out according to the standards set forth in the ‘Colegio Brasileiro de Experimentação Animal’ rules (COBEA, 2006).

G. vaginalis isolates. The isolates from Vaginalis agar were identified as G. vaginalis according to Piot et al. (1982), using the results from Gram stain, catalase and oxidase tests, starch and hippurate hydrolysis, and α- and β-galactosidase activity. Activity of fructose-6-phosphate phosphoketolase was also evaluated (Orban & Patterson, 2000).

Lactobacillus isolates. Bacteria isolated from the subjects were selected as presumptive Lactobacillus based on the following
characteristics: Gram-positive, microaerophilic and catalase-negative rods isolated on MRS agar (Merck) at 37 °C. The selected strains were identified by amplified rDNA restriction analysis as described by Moreira et al. (2005).

DNA extraction. Chromosomal DNA was isolated from overnight cultures of all isolates in 10 ml MRS broth. After washing the cells with deionized water, the pellet was obtained by centrifugation at 14 000 g for 5 min at 4 °C, suspended in 1 ml M LiCl, and incubated for 1 h with constant shaking. After a second washing with 1 ml deionized water, the pellet was suspended in 1 ml protoplasting buffer (50 mM Tris/HCl, pH 8.0; 10 mM EDTA; 10 mg lysozyme ml⁻¹; 100 µg RNase ml⁻¹). After incubation for 1 h at 37 °C and centrifugation at 14 000 g for 5 min at 4 °C, the pellet was suspended in 500 µl protoplasting buffer without lysozyme, and 100 µl 10 % SDS was added to allow cells to lyse. After lysis, the mixture was extracted once with phenol, phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). After 2-propanol precipitation, the DNA was dissolved in 100 µl TE buffer (10 mM Tris/HC1, 1 mM EDTA, pH 8.0).

PCR amplification of the 16S–23S rRNA gene intergenic spacer. The 16S–23S rRNA gene intergenic spacer region was amplified using the primer 16-1A (GAATTCGATATTAGTC), which anneals to a conserved region of the 16S rRNA gene, and primer 23-1B (GGGTTCCCCATTCGGA), which anneals to a conserved region of the 23S rRNA genes, using a PTC-100 Thermal cycler (MJ Research). The reaction mixture (50 µl) contained 10 µM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate, reaction buffer containing 1 mM EDTA, pH 8.0). & Serviço (2005). Lactobacilli were inoculated onto TMB agar plates and medium, which oxidizes TMB, causing the colonies to turn blue. The reaction occurred with horseradish peroxidase (Sigma) present in the reaction mixture. The 16S–23S rRNA gene sequences were amplified by the method described by Hillier (2003). Lactobacilli were inoculated onto TMB agar plates and incubated in the anaerobic chamber at 37 °C. After 40 h, the plates were exposed to ambient air. If the lactobacilli produced H₂O₂, a reaction occurred with horseradish peroxidase (Sigma) present in the medium, which oxidizes TMB, causing the colonies to turn blue. Lactobacillus acidophilus ATCC 4356 was used as a control.

In vitro antagonism assay. The Lactobacillus johnsonii strain used in this assay was selected based on its high antagonistic activity, and the G. vaginalis strain on its high sensitivity to this antagonism. In a first experiment, two groups of germ-free mice were submitted to the following protocols: group LJGV received by intravaginal inoculation a unique dose of 0.1 ml containing 9.0 log c.f.u. L. johnsonii ml⁻¹, and 10 days later the monoxenic animals were inoculated intravaginally with 0.1 ml of a suspension containing 7.0 log c.f.u. G. vaginalis ml⁻¹. The second group, GV, received only sterile saline by vaginal application 10 days before the pathogenic challenge. In a second experiment, two groups were submitted to the same protocols described above, but both were also injected subcutaneously with two doses of 0.5 ml oestradiol benzoate (0.5 mg in 0.1 ml peanut oil) 72 h before the challenge and 72 h after the challenge, respectively (LJGVH and GVH). One week after the challenge, all the mice were sacrificed by cervical dislocation and vaginal tissues were removed. In a third experiment, two groups were treated only with the lactobacillus, with or without the hormonal treatment, and were sacrificed 17 days after the monoassociation with the lactobacillus (LJ and LJHV). Three germ-free mice were used in each group.

Statistical analysis. Data were analysed using the Minitab Release 14.20 software. Fisher’s exact test and Kruskal–Wallis one-way analysis of variance followed by pair-wise multiple comparisons using the Student–Newman–Keuls method were used. P < 0.05 was considered to be statistically significant.

RESULTS

A total of 24 isolates from women with (17 isolates) and without (7 isolates) BV were identified as G. vaginalis. Table 1 shows a higher frequency (P < 0.05) of this bacterium in women with BV (56.7 %) when compared to healthy women.
(17.6 %). However, when present, the vaginal population levels of *G. vaginalis* were similar in both groups, and reached about 8.0 log c.f.u. (ml vaginal fluid)$^{-1}$. A total of 86 strains of *Lactobacillus* were obtained from healthy women (58 isolates) and women with BV (28 isolates). *Lactobacillus* strains were more frequently present ($P, 0.05$) in healthy women (97.5 %) than in women with BV (76.7 %), but as for *G. vaginalis*, vaginal population levels were similar in both groups and reached about 6.8 log c.f.u. (ml vaginal fluid)$^{-1}$.

*L. crispatus* was the predominating strain in both healthy women (22 isolates) and women with BV (7 isolates). *Lactobacillus jensenii, L. johnsonii, Lactobacillus gasseri* and *Lactobacillus vaginalis* were isolated with an intermediate frequency in the two groups.

A total of 2549 assays of antagonism were performed in the present study, using as indicators the reference strains and the *G. vaginalis* isolates from women with BV and from healthy women. The *Lactobacillus* isolates from healthy women showed a higher antagonistic activity against the indicator strains when compared with isolates from women with BV (Table 2). Additionally, all the 24 *G. vaginalis* isolates used as indicator were sensitive to at least one of the *Lactobacillus* isolates from healthy women and tested in the antagonistic assay (data not shown). However, 64.7 % of the *G. vaginalis* isolated from patients with BV and 42.8 % of those from healthy women were resistant to all the 17 *Lactobacillus* isolates from women with BV (data not shown). Concerning the indicator strains, *G. vaginalis* found in women with BV was more resistant to the antagonism, particularly when *Lactobacillus* species from healthy women were tested. The higher antagonistic activity frequency (of about 70 %) was observed when *L. johnsonii* isolates were assayed against *G. vaginalis*, both from healthy women.

Table 3 shows that 57.4 % of the *Lactobacillus* isolates produced H$_2$O$_2$, with a slightly higher frequency (45.5 %) for strains isolated from healthy women than from women with BV (35.3 %). However, this difference was not statistically

<table>
<thead>
<tr>
<th>Bacterium (total number of isolates)</th>
<th>Women with BV (30)</th>
<th>Healthy women (40)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gardnerella vaginalis</em> (24)</td>
<td>56.7</td>
<td>17.6</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>7.9 ± 1.4</td>
<td>8.0 ± 1.2</td>
<td>0.834†</td>
</tr>
<tr>
<td>Total <em>Lactobacillus</em> spp. (86)</td>
<td>76.7</td>
<td>97.5</td>
<td>0.009*</td>
</tr>
<tr>
<td></td>
<td>6.8 ± 1.2</td>
<td>6.9 ± 1.4</td>
<td>0.644†</td>
</tr>
<tr>
<td><em>L. crispatus</em> (29)</td>
<td>26.7</td>
<td>35.0</td>
<td>0.316*</td>
</tr>
<tr>
<td></td>
<td>7.4 ± 0.4</td>
<td>6.2 ± 1.5</td>
<td>0.099†</td>
</tr>
<tr>
<td><em>L. jensenii</em> (11)</td>
<td>10.0</td>
<td>17.5</td>
<td>0.298*</td>
</tr>
<tr>
<td></td>
<td>7.7 ± 2.0</td>
<td>8.0 ± 1.4</td>
<td>0.798†</td>
</tr>
<tr>
<td><em>L. gasseri</em> (10)</td>
<td>16.6</td>
<td>12.5</td>
<td>0.437*</td>
</tr>
<tr>
<td></td>
<td>6.1 ± 0.8</td>
<td>7.1 ± 1.3</td>
<td>0.198†</td>
</tr>
<tr>
<td><em>L. johnsonii</em> (12)</td>
<td>3.4</td>
<td>17.5</td>
<td>0.067*</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>7.1 ± 1.3</td>
<td></td>
</tr>
<tr>
<td><em>L. vaginalis</em> (6)</td>
<td>6.8</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td><em>L. fermentum</em> (3)</td>
<td>6.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>L. salivarius</em> (4)</td>
<td>3.4</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td><em>L. reuteri</em> (2)</td>
<td>0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td><em>L. acidophilus</em> (3)</td>
<td>0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td><em>L. delbrueckii</em> (1)</td>
<td>3.4</td>
<td>0</td>
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<tr>
<td></td>
<td>5.0</td>
<td>0</td>
<td></td>
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<tr>
<td><em>L. colehominis</em> (1)</td>
<td>0</td>
<td>2.5</td>
<td></td>
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<tr>
<td></td>
<td>0</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td><em>L. hilgardii</em> (1)</td>
<td>0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus</em> spp. (3)</td>
<td>3.4</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s exact test.
†Student t-test.
significant \( P > 0.05 \), but this can be due to the too low number of strains tested. \textit{L. johnsonii} from healthy women was the highest producer of \( \text{H}_2\text{O}_2 \) in terms of intensity of production.

For the \textit{in vivo} study of the inter-relationships between \textit{Lactobacillus} and \textit{G. vaginalis}, a highly antagonistic and \( \text{H}_2\text{O}_2 \) producer strain of \textit{L. johnsonii} from a healthy woman was tested against a \textit{G. vaginalis} isolate selected for its high sensitivity to this antagonism. Fig. 1(a) shows that treatment with oestradiol benzoate was necessary for the colonization of germ-free mice by \textit{L. johnsonii}, but not for \textit{G. vaginalis} (Fig. 1b). When the colonization was obtained, both bacteria reached vaginal population levels ranging from 4.0 to 5.0 log c.f.u. (g tissue\(^{-1}\)). Curiously, a vaginal colonization by \textit{L. johnsonii} [between 2.0 and 3.0 log c.f.u. (g vaginal tissue\(^{-1}\))] was observed when the monoassociated mice were posteriorly challenged with \textit{G. vaginalis} (Fig. 1c). A tenfold reduction in vaginal population levels of \textit{G. vaginalis} was observed when the pathogenic challenge was performed in mice previously monoassociated with \textit{L. johnsonii}, and this reduction was statistically significant in the mice not treated with hormone (from 5.01 to 3.62 log c.f.u. g\(^{-1}\); \( P < 0.009 \)). A tendency for a similar effect was also noted in animals treated with the hormone (from 4.40 to 3.34 log c.f.u. g\(^{-1}\); \( P = 0.078 \)).

\begin{table}
\centering
\caption{Frequency (%) of antagonism of \textit{Lactobacillus crispatus}, \textit{Lactobacillus jensenii}, \textit{Lactobacillus gasseri} and \textit{Lactobacillus johnsonii} isolated from healthy women and women with BV against the reference strains and the strains of \textit{Gardnerella vaginalis} isolated in the present study from healthy women and women with BV}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textit{Lactobacillus} & \textit{Reference strains} (17) & \textit{Isolated G. vaginalis} (24) & & \\
\hline
& & Women with BV (17) & Healthy women (7) & \\
\hline
\textit{Healthy women} & & & & \\
\textit{L. crispatus} (22) & 24.6 & 32.6 & 57.8 & \( 10^{-8} \) \textit{P*} \\
\textit{L. jensenii} (9) & 17.0 & 30.7 & 44.4 & 0.039 \\
\textit{L. gasseri} (5) & 21.2 & 22.3 & 54.3 & 0.001 \\
\textit{L. johnsonii} (9) & 30.1 & 31.4 & 69.8 & \( 10^{-7} \) \\
\hline
\textit{Women with BV} & & & & \\
\textit{L. crispatus} (7) & 12.6 & 7.6 & 16.3 & 0.079 \\
\textit{L. jensenii} (2) & 8.8 & 5.9 & 21.4 & 0.140 \\
\textit{L. gasseri} (5) & 12.9 & 4.7 & 8.6 & 0.332 \\
\textit{L. johnsonii} (3) & 17.6 & 3.9 & 14.3 & 0.113 \\
\hline
\end{tabular}
\footnotesize{\textit{P*} Values of \textit{P} from Fisher’s exact test for comparisons of antagonism frequencies between the same isolates of one \textit{Lactobacillus} species against \textit{G. vaginalis} isolated from healthy women or from women with BV.}
\end{table}

\begin{table}
\centering
\caption{Frequency (%) and intensity of \( \text{H}_2\text{O}_2 \) production by \textit{Lactobacillus crispatus}, \textit{Lactobacillus jensenii}, \textit{Lactobacillus gasseri} and \textit{Lactobacillus johnsonii} isolated from healthy women and women with BV}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textit{Lactobacillus} & Presence and intensity of \( \text{H}_2\text{O}_2 \) production & Frequency of positive strain & & \\
\hline
& \textit{–} & + & ++ & +++ & ++++ & & \\
\textit{Healthy women} & & & & & & & \\
\textit{L. crispatus} (22) & 9 & 1 & 9 & 3 & 59.1 & \\
\textit{L. jensenii} (8) & 4 & 1 & 1 & 2 & 50.0 & \\
\textit{L. gasseri} (5) & 3 & 1 & 1 & & 40.0 & \\
\textit{L. johnsonii} (9) & 4 & 1 & 2 & 2 & 55.5 & \\
\textit{Women with BV} & & & & & & \\
\textit{L. crispatus} (7) & 1 & 3 & 2 & 1 & 85.7 & \\
\textit{L. jensenii} (2) & & & 1 & 1 & 100.0 & \\
\textit{L. gasseri} (5) & 3 & 1 & 1 & & 40.0 & \\
\textit{L. johnsonii} (3) & 2 & & & & 33.3 & \\
\hline
Total frequency of positive and negative strains & 42.6 & 57.4 & & & & \\
\hline
\end{tabular}
\footnotesize{Production of \( \text{H}_2\text{O}_2 \): \textit{–}, absence; +, low; ++, intermediate; ++++, high; ++++, intense.}
\end{table}
Histological examination of the vaginal mucosa from germ-free mice showed no inflammation or hyperaemia of the epithelium. In mice only monoassociated with *L. johnsonii*, a discrete inflammatory infiltrate was observed, as well as a direct transition from columnar to mature squamous epithelium. In mice challenged with *G. vaginalis*, histopathological examination showed inflammatory alterations of the ectocervix and lamina propria epithelium. In mice pre-treated with *L. johnsonii* before the challenge with *G. vaginalis*, the inflammatory infiltrate and oedema were less pronounced. In these mice, no differences were observed in relation to the germ-free animal for the endocervical epithelium aspects. Table 4 presents the results obtained from the histopathological examination and shows a clear

Figure 1. Individual vaginal population levels [log c.f.u. (g vaginal tissue)$^{-1}$] of *Lactobacillus johnsonii* (a, c) and *Gardnerella vaginalis* (b, d) in mice only monoassociated (a, b) or diassociated (c, d), with (■) or without (□) hormone treatment. n=3.

Table 4. Histopathological score for vaginal mucosa of mice only challenged with *Gardnerella vaginalis* (treated, GVH, or not, GV, with hormone), only associated during 17 days with *Lactobacillus johnsonii* (treated, LJH, or not, LJ, with hormone) or mice associated during 10 days with *Lactobacillus johnsonii* and then challenged with *Gardnerella vaginalis* (treated, LJGVH, or not, LJGV, with hormone)

<table>
<thead>
<tr>
<th>Histopathological aspect of vaginal mucosa</th>
<th>Score in each experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GVH</td>
</tr>
<tr>
<td>Reactive area</td>
<td>+++</td>
</tr>
<tr>
<td>Inflammatory alterations</td>
<td>+</td>
</tr>
<tr>
<td>Eosinophil presence</td>
<td>++</td>
</tr>
<tr>
<td>Interstitial oedema</td>
<td>+</td>
</tr>
</tbody>
</table>

Score: -, absence; +, low; ++, intermediate; ++++, high; +++++, intense.
protective effect against the *G. vaginalis* infection when animals were pre-treated with *L. johnsonii*.

**DISCUSSION**

The presence of a high population of *Lactobacillus* species is well known as an important factor for a healthy vaginal ecosystem (Hillier, 2005; Falagas et al., 2007). However, beyond their high local levels, these lactobacilli must also be highly antagonistic against potential pathogenic microorganisms to offer protection. There is little information about the importance of the sensitivity of the pathogenic target to this antagonism. Additionally, the antagonistic property is generally demonstrated by *in vitro* assay, and it is not certain whether this ability can be extrapolated to *in vivo* conditions.

The present study confirms that *Lactobacillus* species are more frequently found in the vaginal ecosystem of healthy subjects than in patients with BV (Mikamo et al., 2000; Eschenbach, 2007; Klomp et al., 2008). Moreover, as described in other studies (Hellberg et al., 2001; Branco et al., 2010; Srinivasan et al., 2010), *G. vaginalis* frequency was higher in patients with BV when compared to healthy women. However, when present in patients with BV, *Lactobacillus* levels were similar to those found in healthy women, suggesting that a beneficial effect of these bacteria depends not only on their presence in high quantities but also on their quality in term of protective ability. These results were different from those in some studies which observed higher levels of *Lactobacillus* in healthy women (Mikamo et al., 2000; Eschenbach, 2007; Klomp et al., 2008). Similarly, *G. vaginalis* population levels when present in healthy women were as high as in patients with BV, suggesting on the other hand that pathological symptoms of infection depend also on the pathogenic capacity of the bacteria and not only on their quantities.

Many studies have suggested that the presence of H$_2$O$_2$-producing vaginal lactobacilli may protect against BV, although some studies do not support this hypothesis (Falagas et al., 2007). In the present study, *L. johnsonii* from healthy women showed better antagonistic properties and H$_2$O$_2$ production when compared to the other *Lactobacillus* species. In fact, the production of lactic acid by lactobacilli, which is mainly responsible for the low vaginal pH, could contribute more than the production of H$_2$O$_2$ to the inhibition of the *G. vaginalis* growth (McLean & McGroarty, 1996). However, in the present study, experiments determining the most acidic culture supernatant after growth of the various *Lactobacillus* isolates have not been performed to correlate this information with their antagonistic abilities. The most interesting information described here is related to the need for a high *Lactobacillus* inhibitory capacity combined with a high *G. vaginalis* susceptibility to direct the vaginal ecosystem to a healthy status and inversely for the BV status.

No reports are available in the literature using animal models to confirm *in vivo* the antagonistic properties demonstrated *in vitro* for vaginal *Lactobacillus* against pathogens. For such finality, it is necessary to obtain animals which can be colonized by human lactobacilli and infected by human *G. vaginalis*. The progression of colonization/infection of body epithelial surfaces by a micro-organism depends mainly on its resistance to the local conditions (such as the mucosal barrier integrity, the indigenous microbiota balance and the immunological status of the host), its ability to use the available nutrients and its capacity to adhere to the mucosa. Few studies describe experimental vaginal inoculation of micro-organisms in animal models, and none of them used germ-free animals for such finality (Johnson et al., 1984; Fidel et al., 2000; de Ruiz et al., 2001; Hamad et al., 2004). In these animal models, the authors recommended an illness administration to induce a pre-oestral stage, which ensures a successful colonization/infection. In healthy human vaginas, it is well known that the presence and number of lactobacilli are influenced by oestrogen production (Keane et al., 1997). Oestrogen converts columnar epithelium into a thick layer of squamous stratified epithelium and increases the glycogen content and other substrates for bacterial growth. As expected from this information, vaginal colonization of germ-free mice by the selected *L. johnsonii* strain was observed in the present study only in animals treated with the hormone. However, this treatment was not necessary for the *G. vaginalis* colonization. Interestingly, vaginal colonization by *L. johnsonii* was obtained without hormonal treatment when the mice were also challenged with *G. vaginalis*. However, the *L. johnsonii* population levels were higher in this dissociated model when the mice were treated with oestradiol when compared to the non-treated group. We have no strong explanation for this phenomenon, but the presence of *G. vaginalis* could provide a synergistic effect for the growth of *L. johnsonii* similar to that described for *Peptostreptococcus anaerobius* in a recent study in our laboratory (Teixeira et al., 2010). In any case, the previous monoassociation with *L. johnsonii* protected the mice against the vaginal challenge with *G. vaginalis* as demonstrated by both lower population levels of the pathogen and the reduced lesions observed from the histopathological examination. However, it was not possible to determine in this animal model whether the protection was due to the production of antagonistic compounds observed in the *in vitro* assays or to other mechanisms such as co-aggregation or competition for shared adhesion receptors or nutrients between the lactobacillus and the pathogen.

Concluding, results of the present study suggest that the success of BV due to *G. vaginalis* depends, at least in part, on a simultaneous presence of *Lactobacillus* populations with low antagonistic capacity and of a *G. vaginalis* strain with a high resistance to this antagonism.

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