Anti-inflammatory effect of two *Lactobacillus* strains during infection with *Gardnerella vaginalis* and *Candida albicans* in a HeLa cell culture model

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Abstract

Lactobacilli are the dominant bacteria of the vaginal tract of healthy women and they play a major role in the maintenance of mucosal homeostasis, preventing genital infections, such as bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC). It is now known that one mechanism of this protection is the influence that lactobacilli can exert on host immune responses. In this context, we evaluated two *Lactobacillus* strains (*L. plantarum* 59 and *L. fermentum* 137) for their immunomodulatory properties in response to *Gardnerella vaginalis* (BV) or *Candida albicans* (VVC) infections in a HeLa cell infection model. *G. vaginalis* and *C. albicans* triggered the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) and the activation of NF-κB in HeLa cells, in contrast to *L. plantarum* 59 and *L. fermentum* 137. Treatments with the *Lactobacillus* strains or their cell-free supernatants before (pre-treatment) or after (post-treatment) the challenge with the pathogens resulted in decreased secretion of pro-inflammatory cytokines and decreased activation of NF-κB. The treatments with *Lactobacillus* strains not only decreased the secretion of IL-8, but also its expression, as confirmed by gene reporter luciferase assay, suggesting transcription-level control by lactobacilli. In conclusion, *L. plantarum* 59 and *L. fermentum* 137 were confirmed to have an anti-inflammatory effect against *G. vaginalis* and *C. albicans* and they were able to influence signalling in NF-κB pathway, making them interesting candidates as probiotics for the prevention or treatment of BV and VVC.

INTRODUCTION

Lactobacilli are usually the prevalent micro-organisms in the healthy vaginal ecosystem of women of reproductive age. These bacteria have an important role in protecting the mucosa against pathogenic and opportunistic organisms [1]. Vaginal symptoms are seen as one of the most common reasons for women to seek medical care, and the most common gynaecological diagnoses in medical offices are bacterial vaginosis (BV) or vulvovaginal candidiasis (VVC) [2]. Together with urinary tract infections, BV and VVC affect about one billion women worldwide each year [3]. BV is a polymicrobial disorder characterized by the disruption of the vaginal ecosystem, resulting in a reduction of lactobacilli and an overgrowth of obligate or facultative anaerobic bacteria, especially *Gardnerella vaginalis*. BV has been associated with many serious health problems, including many inflammatory reproductive diseases, as well as complications in pregnancy [4]. VVC is defined as the signs and symptoms of inflammation in the vulva and vagina in the presence of *Candida* spp. and in the absence of other infectious aetiology [5].

There is a pressing need for the development of novel and more effective preventive strategies/treatments for BV and VVC due to the problems associated with the use of antimicrobial drugs (ecosystem disturbances, difficulties in adapting to treatment, adverse effects and selection of resistant strains) and the high recurrence rates within the currently recommended therapies [6]. It is also known that the disruption of local microbiological equilibrium constitutes the basis of these infections, which implies that the treatment should be based on the correction of the dysbiosis by...

reposition of the initial resident \textit{Lactobacillus} microbiota [7]. For this reason, there is a growing interest in the selection and use of a probiotic product, which can be considered to be an ecological therapy, since it improves a natural mechanism of vaginal ecosystem protection [6].

The mechanism of action of selected probiotic strains of \textit{Lactobacillus} is multifactorial and the modulation of cellular immune responses has emerged as a new and important indirect mechanism that contributes to human health. The epithelial cells of the human genital tract are the first to come into contact with the local microbiota and besides providing a physical barrier to pathogenic micro-organisms, they also express Toll-like receptors (TLR) that respond to microbial pathogen molecules and initiate the transcription of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6 and IL-8 and tumour necrosis factor alpha (TNF-α), by way of the nuclear factor-kappa B (NF-κB) signal transduction pathway [8]. Certain probiotic lactobacilli have proven to be effective immunomodulators and some in vivo and in vitro studies have demonstrated that they are associated with the modulation of vaginal defence mechanisms. Lactobacilli were able to down-regulate the immune response to inflammatory stimuli in BV and VVC, reducing the levels of pro-inflammatory cytokines induced by pathogens in the genital epithelium [9].

In a previous study, we demonstrated that eight \textit{Lactobacillus} strains had great probiotic potential and that two specific strains, \textit{L. plantarum} 59 (LP59) and \textit{L. fermentum} 137 (LF137), were more interesting because of their simultaneous antagonistic activity against \textit{G. vaginalis} and \textit{C. albicans} [10]. These strains showed excellent adhesive (biofilm formation, auto-aggregation, adhesion in HeLa cells) and co-aggregation properties, produced hydrogen peroxide and lactic acid, excluded/displaced \textit{G. vaginalis} and \textit{C. albicans} in interference tests within HeLa cells, and prevented apoptosis induced by the pathogens.

The aim of this study was to evaluate the immunomodulatory properties of LP59 and LF137 in a HeLa epithelial cell model infected with \textit{G. vaginalis} or \textit{C. albicans}. We wanted to assess whether, in addition to the beneficial properties that have already been verified, there is a modulation of cytokine production in response to BV or VVC, which would increase its therapeutic potential.

**METHODS**

**Micro-organisms and culture conditions**

\textit{Lactobacillus plantarum} 59 (LP59) and \textit{Lactobacillus fermentum} 137 (LF137) were isolated from the vaginal tract of healthy women from Cuba (Centro Nacional de Sanidad Agropecuaria, San José de las Lajas, Mayabeque, Cuba) and selected as potential probiotics for the prevention/treatment of BV and VVC in a previous study [10]. These bacterial isolates were grown in de Man, Rogosa and Sharpe broth (MRS, Acumedia, Lansing, USA) for 18 h at 37 °C in an anaerobic chamber (Forma Scientific Company, Marietta, USA) containing an atmosphere of 85 % N₂, 10 % H₂ and 5 % CO₂. Cell-free supernatants from LP59 and LF137 MRS cultures were obtained by centrifugation of the cultures at 5500 g for 15 min, followed by filtration through a sterile Millex 0.22 μm filter (Merck Millipore, Darmstadt, Germany). \textit{Gardnerella vaginalis} ATCC 14018 was cultured in Brucella broth (Difco, Sparks, USA) supplemented with 1 % proteose peptone no. 5 (Difco), 0.2 % of 10 % Tween 80, 0.3 % meat extract (Acumedia), 0.5 % hemin, 0.1 % menadione and 3 % horse serum (Sigma-Aldrich, Saint Louis, USA), also under anaerobic conditions. \textit{Candida albicans} ATCC 18804 was grown in Sabouraud dextrose broth (Acumedia) for 18–20 h at 37 °C under aerobic conditions. All micro-organisms were maintained in their respective culture media supplemented with 20 % glycerol and stored at −80 °C.

**HeLa cell culture conditions**

Human cervical HeLa cells (ATCC CCL-2) were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) medium (Sigma-Aldrich) supplemented with 10 % foetal bovine serum (Gibco, Waltham, USA), 1 % 10 000 U ml⁻¹ penicillin, 10 mg ml⁻¹ streptomycin, 25 μg ml⁻¹ amphotericin B solution (Sigma-Aldrich) and 1 % non-essential amino acids (Sigma-Aldrich) at 37 °C in an atmosphere of 5 % CO₂ (Water Jacketed CO₂ Incubator, Thermo Scientific, Waltham, USA). For all assays, 1×10⁵ HeLa cells were seeded to each well of a 24-well tissue culture plate (TPP, Sigma-Aldrich) and incubated for 24 h at 37 °C in an atmosphere of 5 % CO₂ to reach a density of 2×10⁴ cells per well (−90 % confluence). Before each assay, the cells were washed three times with PBS to remove non-adherent cells and culture medium. All HeLa cells used in the experiments were negative for \textit{Mycoplasma} spp.

**HeLa cell viability tests**

A possible cytotoxic effect of LP59 and LF137 cultures was discarded in our previous study by an apoptosis assay [10]. In addition, a Trypan blue exclusion assay was also used to access the cytotoxicity of lactobacilli for HeLa cells [11]. HeLa cells were incubated with LP59 and LF137 cultures (prepared as described below) or their cell-free supernatants for 18 h at 37 °C in an atmosphere of 5 % CO₂. HeLa cells receiving no treatment were used as a control. The enumeration of viable cells was performed under a light microscope (Leica DMIL Inverted Phase Contrast Microscope, Leica Microsystems, Lincolnshire, IL, USA). The results were expressed as the percentage of viable cells in relation to the total number of cells and were the average of three independent experiments performed in triplicate.

**Experimental designs**

Two experimental designs were established to collect HeLa cell supernatants for cytokine determination. For both, LP59 and LF137 cultures were centrifuged at 5500 g for 15 min, washed twice with PBS and resuspended in fresh MRS medium. The same procedure was performed for \textit{G. vaginalis} and \textit{C. albicans} cultures, which were resuspended in DMEM.
Lactobacillus spp., G. vaginalis and C. albicans were used at m.o.i.s of 5000:1, 500:1 and 50:1, respectively, as already established in our first study [10].

In the first design (pre-treatment model), HeLa cells were incubated with LP59, LF137 or their cell-free supernatants for 2 h at 37 °C in an atmosphere of 5 % CO2 and then challenged with G. vaginalis or C. albicans for 6 h under the same incubation conditions. In the second design (post-treatment model), HeLa cells were challenged for 2 h with the pathogens and then treated with the lactobacilli for 6 h under the same incubation conditions described above. In the first model (pre-treatment) we attempted to mimic prior treatment with lactobacilli before the occurrence of the disease (preventive manner), while in the second one (post-treatment) we simulated the onset of the disease first and then the treatment with lactobacilli (curative manner). The choice of the incubation periods was based on our knowledge that 2 h was a suitable time for the total adhesion and internalization of all micro-organisms to HeLa cells [10] and that a total time of 8 h was suitable for the measurement of all micro-organisms to HeLa cells [10] and that a total time of 8 h was suitable for the measurement of the analysed cytokines [12]. In both experimental designs, HeLa cells were also challenged with G. vaginalis or C. albicans alone. HeLa cells in DMEM were used as controls.

Cytokine determination in supernatants of HeLa cell cultures

The supernatants collected from HeLa cell cultures were used to determine the levels of TNF-α, IL-1β, IL-6, IL-8 and IL-10 cytokines, measured by enzyme-linked immunosorbent assay (ELISA) using the commercial Duo Set ELISA kit (R&D Systems, Minneapolis, USA), according to the manufacturer’s instructions. When the cell-free supernatants of LP59 and LF137 cultures were tested on HeLa cell cultures, only IL-8 levels were determined. The results were expressed in pg ml−1 and were the average of three independent experiments performed in triplicate.

IL-8 and NF-κB luciferase assay

HeLa cells were transfected with 200 ng of pIL8-Luc (a generous gift from Dr Charalabos Pothoulakis, Department of Medicine, University of California, Los Angeles, USA) [13] or pNFκB-luc (a generous gift from Dr Bryan R.G. Williams, The Cleveland Clinic Foundation, Cleveland, USA) [14] and 50 ng of pRL-TK (Promega, WI, USA), using Lipofectamine 2000 (Invitrogen, Waltham, USA) according to manufacturer’s protocol. The transfected cells were incubated with the lactobacilli and pathogens as in the two experimental designs described above. The supernatants were removed at the end of the treatment period, the cells were rinsed with PBS and lysed by shaking on ice for 15 min with 100 µl of passive lysis reagent (Promega). Twenty microlitres of each cell lysate was assayed for firefly and renilla luciferase activities using a LumiCount Microplate Reader Luminometer (Packard Bio-Sciences, CT, USA) and Dual-Luciferase Reporter Assay System (Promega), according to the protocol provided by the manufacturer. HeLa cells in DMEM and those induced with 1 µl of recombinant TNF-α (20 ng ml−1, Sigma-Aldrich) were used as negative and positive controls, respectively. The results were obtained as the ratio of firefly luciferase/renilla luciferase activities (relative luciferase activity, RLA) and expressed as fold change in relation to the negative control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.03 (GraphPad Software, La Jolla, USA) and the statistical test used was the one-way ANOVA followed by Tukey’s post-test. Results were considered to be statistically different for at least P<0.05.

RESULTS

The LP59 and LF137 cultures and cell-free supernatants showed no evidence of cytotoxicity to HeLa cells after an 18 h incubation (Table 1). In our previous study [10] we also demonstrated that LP59 and LF137 cultures adhered to HeLa cell monolayers with no signs of apoptosis.

Figs 1 and 2 show the influence of treatments with LP59 and LP137 on the secretion of TNF-α, IL-1β, IL-6 and IL-8 by HeLa cells challenged or not with the pathogens, in pre- or post-treatment models. HeLa cells did not secrete detectable amounts of IL-10 in any model used (data not shown). As expected, the infections with G. vaginalis and C. albicans induced an increase in the production of all these pro-inflammatory cytokines. The treatment with both lactobacilli before or after the challenge with G. vaginalis reduced the secretion of TNF-α, IL-1β and IL-8 to basal levels (P<0.05), except for the last cytokine, in which the post-treatment with LF137 resulted in a lower level of reduction, but one that was also significant. IL-6 secretion was also significantly reduced during the pathogenic challenge in both models (P<0.05). Further, incubation with LP59 and LF137 alone did not alter TNF-α, IL-1β and IL-8 levels, but resulted in increased secretion of IL-6, which was at lower levels when compared to the induction by pathogens. In both models, the treatments with LP59 or LF137 were also able to reduce the production of IL-1β, IL-6 and IL-8.

Table 1. Viability of HeLa cells incubated with lactobacilli or their cell-free supernatants for 18 h

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Viable cells after incubation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.11±3.37</td>
</tr>
<tr>
<td>MRS</td>
<td>90.33±3.20</td>
</tr>
<tr>
<td>L. plantarum 59</td>
<td>89.00±2.74</td>
</tr>
<tr>
<td>L. fermentum 137</td>
<td>89.11±2.47</td>
</tr>
<tr>
<td>L. plantarum 59s</td>
<td>88.67±2.18</td>
</tr>
<tr>
<td>L. fermentum 137s</td>
<td>88.56±3.40</td>
</tr>
</tbody>
</table>

HeLa cells incubated for 18 h in DMEM (control), MRS medium (MRS), or with lactobacilli or their cell-free supernatants (S). The values are the percentage of viable cells in relation to the total number of cells and correspond to the mean±standard deviation of the experiments (P<0.05).
(P<0.05) in cells challenged with C. albicans, whereas for TNF-α this reduction led to basal levels.

The influence of cell-free supernatants from each Lactobacillus culture on IL-8 secretion by HeLa cells challenged with G. vaginalis or C. albicans is shown in Fig. 3. As observed in the previous results, there was only an increase in IL-8 concentration in the groups challenged with the pathogens. This secretion was reduced by the treatment with the supernatants from LP59 and LF137 in both models utilized (P<0.05). Further, there was a decrease or no IL-8 transcription when HeLa cells challenged with the pathogens were treated in the same models with each Lactobacillus or their supernatants, as shown in Fig. 4a. Reductions in IL-8 transcription ranging from 3- to 552-fold, as well as inhibition (LF137 supernatant), were observed for the treatments in the G. vaginalis challenge. For C. albicans, these reductions varied from 4- to 29-fold.

It was verified that NF-κB activation was induced by the incubation of HeLa cells with G. vaginalis or C. albicans alone and that pre- or post-treatment of these cells with
LP59, LF137 or its supernatants were able to reduce this activation (Fig. 4b). Further, neither lactobacilli nor its supernatants induced NF-\(\kappa\)B activation. There were 29- to 42-fold and 23- to 72-fold reductions of NK-\(\kappa\)B activity in cells treated and challenged with \textit{G. vaginalis} or \textit{C. albicans}, respectively.

**DISCUSSION**

The pathogenesis of BV is still poorly understood, given its complex polymicrobial aetiology, but \textit{G. vaginalis} is still the bacterium that is found most frequently in women with this disease. Despite the fact that BV is not characterized by acute inflammation, as shown by the absence or scarcity of inflammatory cells in vaginal discharge, a pro-inflammatory immune response is clearly associated with this diagnosis [15]. The majority of cross-sectional and longitudinal studies reported an increase of pro-inflammatory cytokines in the cervicovaginal secretions of women with BV, especially IL-1\(\beta\), IL-6 and TNF-\(\alpha\) [9, 16]. TNF-\(\alpha\) is known to play a key role in increasing the release of pro-inflammatory cytokines and is thought to be a pivotal cytokine. Together with IL-1\(\beta\), it is particularly effective in initiating a cascade of

![Fig. 2. Effect of \textit{L. plantarum} 59 and \textit{L. fermentum} 137 on the secretion of IL-6 (a) and IL-8 (b) in HeLa cells infected with \textit{G. vaginalis} or \textit{C. albicans} in pre-treatment (grey bars) or post-treatment (black bars) models. The values are expressed in pg ml\(^{-1}\) and correspond to the mean±standard deviation of the experiments. HeLa cells in DMEM (M); with sterile MRS medium (MRS); inoculated with \textit{L. plantarum} 59 or \textit{L. fermentum} 137 (LP59 or LF137); challenged with \textit{G. vaginalis} (GV) or \textit{C. albicans} (CA); pre-treated with LP59 or LF137 and challenged with \textit{G. vaginalis} (LP59+GV or LF137+GV) or \textit{C. albicans} (LP59+CA or LF137+CA); challenged with \textit{G. vaginalis} or \textit{C. albicans} and post-treated with LP59 or LF137 (GV+LP59 or GV+LF137; CA+LP59 or CA+LF137). Different letters indicate significant differences (\(P<0.05\)).](image-url)
inflammatory mediators by inducing the expression of pro-inflammatory genes and may act in synergy in this process [17]. IL-6 is a multifunctional cytokine and a potent inducer of the acute-phase protein response that regulates inflammation [18]. The increase in cervicovaginal IL-1β level is associated with the production of the hydrolytic enzymes sialidase and prolidase by BV-associated bacteria. These enzymes may be able to degrade some mucosal protective factors, such as cAMPs [19]. The IL-8 cytokine, a powerful chemoattractant and activator of neutrophils, is still a subject for discussion. Several studies have reported no increase in vaginal IL-8 concentrations or vaginal neutrophils in woman with BV, in spite of an increase in IL-1β [20]. However, in vitro studies with epithelial cell monolayers showed that G. vaginalis elicits a pro-inflammatory response by inducing the production of IL-1β, IL-6, TNF-α and also IL-8 [21, 22], as did our results. It appears that, in vivo, one or more BV-associated bacteria can short-circuit the immune response by inhibiting IL-8 production or stability, or by secreting some factors that can promote IL-8 degradation. This only occurs in vivo and results from the interaction of all BV-associated bacteria with the vaginal tract during the disease [15].

Some studies have identified the importance of innate immunity in regulating VVC symptomatology. To explain the vulvovaginal symptoms, a common theory suggests that the normal regulated mucosal pro-inflammatory cytokine response, which is dependent on intracellular inflammasome regulation, is enhanced, resulting in an exaggerated inflammatory reaction (hyper-reactive). A family of calcium-binding proteins, called S100A8 and S100A9 alarmins, has been implicated in the innate vaginal epithelial cell response to C. albicans [23]. The mucosal damages are associated with an aggressive neutrophil migration into the vagina and a subsequent acute host inflammatory response. This neutrophil response is initiated by the interaction of C. albicans with vaginal epithelial cells, which have no apparent ability to clear the pathogen [24]. Epithelial cell triggers are ultimately considered to be dependent on a threshold level of C. albicans, such that under sensitive conditions, C. albicans will stimulate the epithelial cells to produce alarmins and pro-inflammatory cytokines that ultimately lead to neutrophil migration and the inflammatory symptomatic condition [23]. In the present study, we demonstrated that C. albicans induced the secretion of IL-1β, IL-6, IL-8 and TNF-α in HeLa cells, similarly to what was observed in other in vitro studies with epithelial VK2 and HeLa cell cultures [25, 26].

Most of the adverse health outcomes associated with BV and VVC are related to an acute or chronic inflammatory condition that can be a direct consequence of the disease, as in VVC, or an indirect consequence, as with the associated complications in BV [9]. Therefore, understanding whether a Lactobacillus species can ameliorate these inflammations is of crucial importance for their selection as a potential probiotic candidate. It is known that lactobacilli can promote vaginal health by immunomodulatory mechanisms in the female genital tract, but these mechanisms are not completely understood [27]. We observed that G. vaginalis and C. albicans infection induced high levels of IL-1β, IL-6, IL-8 and TNF-α associated with a potent NF-κB activation. Pre- or post-treatment with LP59 and LF137 reduced the

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**Fig. 3.** Influence of L. plantarum 59 and L. fermentum 137 cell-free supernatants on the secretion of IL-8 in HeLa cells infected with G. vaginalis or C. albicans in pre-treatment (grey bars) or post-treatment (black bars) models. The values are expressed in pg ml⁻¹ and correspond to the mean±standard deviation of the experiments. HeLa cells in DMEM (M); with sterile MRS medium (MRS); inoculated with L. plantarum 59 or L. fermentum 137 supernatants (LP59s or LF137s); challenged with G. vaginalis (GV) or C. albicans (CA); pre-treated with LP59 or LF137 supernatants and challenged with G. vaginalis (LP59s+GV or LF137s+GV) or C. albicans (LP59s+CA or LF137s+CA); challenged with G. vaginalis/C. albicans and post-treated with LP59 or LF137 supernatants (GV+LP59 s or GV+LF137s; CA+LP59 s or CA+LF137 s). Different letters indicate significant differences by *(P<0.05).*
secretion of these pro-inflammatory cytokines induced by the challenge with the pathogens, and also reduced NF-κB activation. NF-κB is a major transcription factor that plays a key role in inflammatory diseases, up-regulating many important genes involved in inflammation, such as those of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and...
TNF-\(\alpha\) evaluated in this study [28]. A down-regulation of IL-8 expression when the cells were treated under the same conditions was also verified, which revealed transcription-level control of this cytokine expression in the presence of both lactobacilli and their supernatants. In relation to IL-10, Wagner and Johnson [25] also verified that VK2 epithelial cells (E6/E7) did not secrete detectable amounts of this cytokine, and these authors hypothesized that detection was not possible because IL-10 is not secreted by epithelial cells to the same degree that it is by other cell types, such as lymphocytes.

Recently, some studies have pointed out a specific association between vaginal lactobacilli and lower levels of pro-inflammatory cytokines [22, 29–31]. Other authors also verified that these anti-inflammatory effects were in the presence of either an up- or a down-regulation of NF-\(\kappa\)B activity. It has previously been reported that a Lactobacillus reuteri strain inhibited the translocation of NF-\(\kappa\)B to the nuclei of HeLa cells and prevented the degradation of IkB inhibitory protein [32]. Lactobacillus rhamnosus GR-1 and L. reuteri RC-14 suppressed C. albicans-induced NF-\(\kappa\)B IkB\(\alpha\), TLR2, TLR-6, IL-8 and TNF-\(\alpha\) in VK2/E667 cells, suggesting an inhibition of NF-\(\kappa\)B signalling [25]. In contrast, some vaginal Lactobacillus species were able to cause NF-\(\kappa\)B activity and yet maintain low levels of anti-inflammatory cytokines [12, 33]. The net effect of NF-\(\kappa\)B activation depends on the cell and tissue properties, the interaction of intra- and extracellular factors, and the nature of the activating signal [34]. Thus, each species and strain of lactobacilli is unique and has very specific molecular interactions that can activate different signals in cells, but a final anti-inflammatory effect is an important characteristic that is expected from a candidate for use as a vaginal probiotic.

Three mechanisms, which probably act simultaneously, can be suggested to explain these immunomodulatory effects. Firstly, the pathogenic micro-organisms could be killed by inhibitory compounds produced by LP59 and LF137, and secondly, adhesion to the vaginal epithelium could be prevented by co-aggregation with the lactobacilli or interference mechanisms, such as blockage by exclusion or displacement. In a previous study [10], all these mechanisms were shown to be present in LP59 and LF137. Therefore, it can be suggested that the secretion of pro-inflammatory cytokines triggered by G. vaginalis and C. albicans adhesion was reduced because the lactobacilli totally or partially inhibited the contact of the pathogens with the epithelial cells, preventing the induction of the intracellular signalling that ultimately would lead to inflammation. Recently, it was demonstrated that a L. rhamnosus GR-1 lectin (a family of surface proteins that bind carbohydrates with high specificity and no catalytic activity) not only mediated adhesion to vaginal and ectocervical epithelial cells, but also displayed some immunomodulation ability through a modest up-regulation of some anti-inflammatory cytokines, in addition to inhibiting the adhesion and biofilm formation of uropathogenic Escherichia coli [35]. Similarly, the purified mannose-specific lectin domain from vaginal Lactobacillus plantarum CMPG5300 was also able to bind efficiently to HIV-1 glycoprotein gp120 and C. albicans, and showed inhibitory activity against the biofilm formation of uropathogenic E. coli, Staphylococcus aureus and Salmonella enterica serovar Typhimurium [36].

Thirdly, an immunomodulatory role has been attributed directly to the substances secreted by lactobacilli, such as lactic acid [37], suggesting its role in maintaining vaginal immune homeostasis by modulating cytokine secretion. Mares et al. [38] have previously demonstrated that THP-1 human monocytes cells treated with cervicovaginal lavages (CVLs) from women with BV induced higher levels of NF-\(\kappa\)B activation, TNF-\(\alpha\) and IL-8 secretion than THP-1 cells treated with CVLs from women with healthy microbiota. This study suggested that the different composition of healthy CVLs contributed to the maintenance of a low-inflammatory state in the vaginal tract. In fact, it is known that there are differences in the metabolite concentrations between a healthy vaginal environment dominated by lactobacilli and rich in lactic acid and a BV one that is characterized by decreased lactic acid and increased mixed short-chain fatty acids and amine production [7]. Similarly, Watanabe et al. [39] demonstrated that l-lactic acid produced by L. casei strain Shiroro prevented NF-\(\kappa\)B activation, I-\(\kappa\)B-\(\alpha\) phosphorylation and TNF-\(\alpha\) expression in LPS-stimulated THP-1 cells. In addition, it has been reported that treatment of cervicovaginal cells with lactic acid decreases the production of pro-inflammatory cytokines and chemokines induced by the TLR1/2 agonist Pam3CSK4, which mimics PAMPs from bacterial pathogens associated with BV and viruses such as HIV. The anti-inflammatory effect of lactic acid was distinct from low pH alone, since acidifying the media to the same pH with HCl did not cause the same effects [40, 41]. These studies suggest a role for lactobacilli in maintaining vaginal immune homeostasis by modulating NF-\(\kappa\)B activity and cytokine responses through lactic acid [37]. In addition to lactic acid, other compounds produced by the lactobacilli could also interfere with the pro-inflammatory cellular signalling pathways stimulated by the adhesion of the pathogens. In a study by Chon et al. [42], an active protein fraction of 8.7 kDa of L. plantarum 10hk2 was able to induce IL-10 production, suppress the induction of NF-\(\kappa\)B and inhibit the phosphorylation of I-\(\kappa\)B and p38 MAPK in a RAW 264.7 murine macrophage cell line that was LPS-stimulated. In the present study, a similar anti-inflammatory effect of the cell-free supernatants from the two Lactobacillus cultures was obtained through the reduction of IL-8 secretion and expression, as well as the down-regulation of NF-\(\kappa\)B activity. The choice to only analyse this cytokine in the treatments with cell-free supernatants was based on the fact that the secretion of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 usually culminates in the secretion of IL-8 in response to microbial infection [43]. Then, the evaluation of IL-8 secretion could reflect the secretion of all the analysed cytokines. Further studies are needed to discover
which compound (compounds) is (are) directly involved in this modulation.

In conclusion, *L. plantarum* 59 and *L. fermentum* 137 were confirmed to be potential candidates for use as probiotics for the treatment and prevention of vaginal infections such as BV and VVC. These data reinforce the hypothesis of the simultaneous action of several mechanisms acting at different levels in the course of a pathogenic aggression to explain the protective effect of probiotic micro-organisms. In addition to the mechanisms involved in these beneficial effects that were demonstrated in our previous study, including the production of inhibitory compounds, adhesive properties, the inhibition of pathogen adhesion and cell apoptosis prevention, we verified that LP59 and LF137 have an anti-inflammatory action that is mediated by the reduction of NF-κB activity and pro-inflammatory cytokine secretion. These results suggest that a decreased immune response is maintained in the vaginal and ectocervix epithelium by lactobacillus colonization, which may be to minimize excessive prejudicial inflammation that can be triggered by transient natural cycle changes or diseases that affect vaginal microbiota.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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