Selection of *Lactobacillus* strains as potential probiotics for vaginitis treatment

Carolina M. A. Santos, Maria C. V. Pires, Thiago L. Leão, Zulema P. Hernández, Marisleydys L. Rodríguez, Ariane K. S. Martins, Lilian S. Miranda, Flaviano S. Martins and Jacques R. Nicoli

1Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
2Centro Nacional de Sanidad Agropecuaria, Dirección de Producciones Biofarmacéuticas, San José de las Lajas Mayabeque, Cuba

Lactobacilli are the dominant bacteria of the vaginal tract of healthy women, and imbalance of the local microbiota can predispose women to acquire infections, such as bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC). Although antimicrobial therapy is generally effective, there is still a high incidence of recurrence and increase of microbial resistance due to the repetitive use of antimicrobials. Thus, it has been suggested that administration of probiotics incorporating selected *Lactobacillus* strains may be an effective strategy for preventing vaginal infections. Accordingly, the *in vitro* probiotic potential of 23 lactobacilli isolated from the vaginal ecosystem of healthy women from Cuba was evaluated for use in BV and VVC treatments. Eight strains were selected based on their antagonist potential against *Gardnerella vaginalis*, *Candida albicans* both. *In vitro* assays revealed that all these strains reduced the pathogen counts in co-incubation, showed excellent adhesive properties (biofilm formation and auto-aggregation), were able to co-aggregate with *G. vaginalis* and *C. albicans*, yielded high amounts of hydrogen peroxide and lactic acid and demonstrated high adhesion rates to epithelial HeLa cells. Interference tests within HeLa cells showed that all strains were able to reduce the adherence of pathogens by exclusion or displacement. Lactobacilli were able to inhibit HeLa cell apoptosis caused by pathogens when the cells were incubated with the probiotics prior to challenge. These results suggest that these strains have a promising probiotic potential and can be used for prevention or treatment of BV and VVC.

INTRODUCTION

More than 250 species of bacteria have already been detected by genomic sequencing in a healthy vaginal environment and lactobacilli are usually the prevalent microorganisms in women in reproductive age (Martin, 2012). The species most frequently found are *Lactobacillus crispatus, L. gasseri, L. jensenii, L. johnsoni* and *L. iners*, which protect the mucosa against the establishment of pathogenic microorganisms, such as human immunodeficiency virus (HIV) and those associated with bacterial vaginosis (BV), vulvovaginal candidiasis (VVC) and gonorrhea (Danielsson et al., 2011). This protection is mainly due to the ability of lactobacilli to adhere to vaginal epithelial cells and inhibit the growth of pathogens. For this purpose, different mechanisms are employed, including immunomodulation, production of antimicrobial substances, such as organic acids, hydrogen peroxide and bacteriocins, competition for nutrients and inhibition of pathogens adhesion to epithelial receptors by steric exclusion/displacement, biofilm production and/or co-aggregation (Petrova et al., 2015).
Vaginal symptoms are seen as one of the most common reasons for women seeking medical care and vaginitis is the most common gynecologic diagnosis in the primary health care setting. Among the urogenital infections, BV, VVC and urinary tract infections, together, affect about one billion women worldwide each year. It is estimated that about 75% of women will have at least one episode of lower genital tract infection in their life and that about half of them will present new occurrences (ACOG, 2006; Mashburn, 2006).

Some of the strategies used to manage vaginal tract infections are frequently ineffective in solving the problem. These methods involve the continued use of antibiotics, which can lead to ecosystem disturbances, difficulties in adapting to treatment, adverse effects and selection of resistant strains. Additionally, the high recurrence rates within the currently recommended therapies encouraged the search for new and more effective treatments for BV and VVC. For this reason, there is a growing interest in the study of the vaginal ecosystem. The loss of these bacteria may predispose women to urogenital infections. For this reason, the use of probiotics is recommended in maintaining their respective culture medium supplemented with 20% glycerol and stored at 80°C.

Identification of Lactobacillus spp. by 16S rDNA sequencing. DNA extraction of Lactobacillus strains was carried out according to Teixeira et al. (2012). The 16S rDNA was amplified using the oligonucleotides SAdir (5’AGAGTTTGATCCTGGCTCAG3’) and S1rev (5’GTTACCTTGTAGACTTCT3’), which correspond to the positions 8–28 and 1493–1508, respectively, of the 16S rDNA gene of E. coli (Wery et al., 2001). PCR reactions were performed in a thermocycler PTC-100 Thermal Cycler (MJ Research Inc, Saint Bruno, CAN) using the Taq DNA polymerase enzyme (Phoneutria Biotechnology and Services Ltd, Belo Horizonte, BRA) following the manufacturer’s recommendations. PCR fragments were sequenced using the BigDye® Terminator Cycle Sequencing Kit v.3.1 (Life Technologies, Carlsbad, USA) in an ABI 3130 sequencer (Life Technologies-Animal Genetics Laboratory, Department of Animal Science, Veterinary School, UFMG). The sequences were assembled and edited using the CodonCode Aligner v.6.0.2 software (CodonCode Corporation, Massachusetts, USA). The alignment was manually checked and identification of bacterial species was made with the support of the GenBank database of the National Center for Biotechnology Information (NCBI, 2015) based on the BLAST tools (Basic Local Alignment Search Tool – Altschul et al., 1990). The criteria adopted for species identification were those described by Stackebrandt & Ebers (2006).

Methods

Microorganisms and culture conditions. Twenty-three Lactobacillus spp. strains isolated from the vaginal tract of healthy women from Cuba (Centro Nacional de Sanidad Agropecuaria, San José de las Lajas, Mayabeque, Cuba) were used in the present study (Sanchez et al., 2011). 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.

The following indicator strains were used for the antagonist assays: Gardnerella vaginalis ATCC 14018, Mobilincus mulieris ATCC 35239 and Prevotella intermedia ATCC 29561 were cultured in Brucella broth (Difco, Sparks, USA) supplemented with 1% protease peptone No. 5 (Difco), 0.2% Tween 80 10%, 0.3% meat extract (Acumedia), 0.5% hemin, 0.1% menadione and 3% horse serum (Sigma-Aldrich, Saint Louis, USA). Bacteroides fragilis ATCC 25285 and B. vulgatus ATCC 8482 were grown in Brain Heart Infusion broth (BHI, Acumedia) supplemented with 0.5% hemin and 0.1% menadione. All these bacteria were incubated for 18–24h at 37°C in the anaerobic chamber. Other indicator strains were Streptococcus agalactiae ATCC 1381, Enterococcus faecalis ATCC 19433, Escherichia coli ATCC 25922, Proteus mirabilis ATCC 25933 and Staphylococcus aureus ATCC 29213, which were grown in BHI broth for 18–20h at 37°C under aerobic conditions. The yeasts Candida albicans ATCC 18804, C. parapsilosis ATCC 20019, C. krusei ATCC 20298, C. tropicalis ATCC 15126 and C. glabrata ATCC 750 were grown in Sabouraud dextrose broth (Acumedia) for 18–20h at 37°C also under aerobic conditions. All microorganisms were maintained in their respective culture medium supplemented with 20% glycerol and stored at 80°C.

Determination of antagonist activity in vitro: double layer agar diffusion assay and co-incubation. The antagonist activity of the 23 Lactobacillus isolates was evaluated by the double layer agar diffusion assay as described by Teixeira et al. (2012). Aliquots of 5 µl from Lactobacllus cultures containing 105 colony forming units (CFU) ml−1 were spotted on plates containing MRS agar. After incubation for 24h in the anaerobic chamber, lactobacilli were killed by exposure to chloroform steam for 30 min, and the residual chloroform was allowed to evaporate for an equal period of time. The presence of antagonistic substances was revealed by overlaying the plate with 3.5 ml of soft agar medium (0.75%) – according to the requirements of each microorganism – supplemented with 10 µl or 100 µl of cultures of indicator bacteria.
(10^9 CFU ml^{-1}) or yeasts (10^7 CFU ml^{-1}), respectively. The plates were incubated for 24 h at 37 °C, under anaerobic or aerobic conditions according to the incubation conditions of each microorganism.

A co-incubation assay using cultures or supernatants of selected Lactobacillus strains was performed as described by Hütt et al. (2006) with some modifications. Lactobacillus spp., G. vaginalis and C. albicans cultures were washed in phosphate buffered saline (PBS) and resuspended in their respective fresh broths to obtain 10^6 CFU ml^{-1} or 10^8 CFU ml^{-1} for Lactobacillus, 10^6 CFU ml^{-1} for G. vaginalis and 10^6 CFU ml^{-1} for C. albicans. From these suspensions, a 5% inoculum (v/v) for each microorganism was prepared in fresh culture media. The supernatants obtained from the initial Lactobacillus cultures were stored and subsequently passed through a sterile Millex 0.22 µm filter (Merck Millipore, Darmstadt, Germany). Aliquots of 2 ml of each Lactobacillus culture or culture supernatant were co-incubated with an equal volume containing the same concentration of G. vaginalis or C. albicans for 18 h at 37 °C under anaerobic or aerobic conditions, respectively. After this period, the number of CFU ml^{-1} of G. vaginalis, C. albicans and lactobacilli was determined by serial dilution and plating onto Vaginalis agar (Columbia agar Base, Acumedia, supplemented with 10 g l^{-1} cycloheximide (Sigma-Aldrich) or MRS agar supplemented with 100 mg l^{-1} clindamycin (Sigma-Aldrich) or MRS agar supplemented with 100 mg l^{-1} clindamycin (Sigma-Aldrich), respectively. The controls of the experiments were aliquots of G. vaginalis or C. albicans incubated with equal aliquots of fresh MRS medium. The results were expressed as log_{10} CFU ml^{-1}.

**Hydrogen peroxide production.** The qualitative test of hydrogen peroxide production by Lactobacillus isolates was performed as described by Rabe & Hillier (2003). Lactobacillus cultures (10^6 CFU ml^{-1}) were seeded on tetramethylbenzidine (TMB)-plus agar plates using a Steers replicator. After incubation at 37 °C for 24 h in anaerobiosis, the plates were removed from the chamber and exposed to air for about 30 min. Hydrogen peroxide-producing lactobacilli were characterized as blue colonies.

The production of hydrogen peroxide was quantified by oxidation-reduction volumetric analysis. Preparation of samples and calculations were performed according to Edema & Sanni (2008). L. acidophilus ATCC 4356 was used as positive control in both assays.

**Acidifying capacity: hydrogenic potential and production of organic acids.** Medium acidification by lactobacilli was determined by measuring the hydrogenic potential (pH) of the supernatants obtained from the centrifugation of cultures (10^6 CFU ml^{-1}) for 5500 g for 20 min at 4 °C. L. acidophilus ATCC 4356 was used as positive control.

The amount of organic acids produced by lactobacilli was determined by acid–base titration, following Edema & Sanni (2008). L. acidophilus ATCC 4356 was used as positive control and results were expressed as g l^{-1}.

**Auto-aggregation and co-aggregation assays.** The auto-aggregation and co-aggregation assays were performed following Kos et al. (2003). The cultures were centrifuged at 5500 g for 15 min, washed twice and resuspended in PBS to a final concentration of 10^6 CFU ml^{-1} or 10^8 CFU ml^{-1} (lactobacilli), 10^6 CFU ml^{-1} (G. vaginalis) or 10^6 CFU ml^{-1} (C. albicans). Cell suspensions containing each microorganism alone (4 ml) (auto-aggregation) or mixed suspensions containing equal volumes (2 ml) of each Lactobacillus and the pathogens in the same concentration (co-aggregation) were vortexed for 10 s and incubated for 5 h at room temperature. The absorbance at 600 nm (A_{600}) was measured at time 0 (A_0) and after 5 h (A_5). Multiskan Spectrum, Thermo Scientific, Waltham, USA. The percentage of auto-aggregation was calculated by the equation: 1− (A_5 / A_0) × 100 and co-aggregation was calculated as follows:

\[
\text{Co-aggregation} (%) = \left( \frac{[Ax + Ay] - [A(x + y)]}{[Ax + Ay]/2} \right) \times 100
\]

Where x and y represent each of the strains in the control tubes (each microorganism alone), and x + y the mixture.

A visual co-aggregation assay was also performed as described below. An aliquot of 500 µl of each Lactobacillus isolate was mixed with aliquots of 500 µl containing the same concentration of G. vaginalis or C. albicans. After brief vortexing, the samples were left on a rotary shaker (50 rpm) at room temperature for 2 h. Then, a drop of this suspension was submitted to microscopy examination after Gram-staining for observation of aggregates defined as visible clumps of bacteria and classified according to the presence of large and dense visible clumps of microorganisms (+ +), small and sparsely distributed clumps (+) or no visible clumps or bound microorganisms (−) (Younes et al., 2012).

**Biofilm formation assay.** The biofilm development by lactobacilli was evaluated as described by Ibarreche et al. (2014). Lactobacillus cultures (10^6 CFU ml^{-1}) were used as inoculum (5% v/v) for 200 µl of fresh MRS medium and then added to 96-well polystyrene microplates. These plates were left under anaerobiosis at 37 °C for 72 h. To quantify biofilm formation, the wells were washed three times with PBS and the remaining attached bacteria in each well were stained for 30 min with 200 µl of 0.1% crystal violet (w/v) in an isopropanol–methanol–PBS solution (1:1:18 v/v/v). The excess of dye was rinsed twice with sterile distilled water and the plates were allowed to dry at room temperature for about 2–3 h. Then, the dye that had adhered to the cells was extracted with 200 µl of 30% glacial acetic acid. The optical density (OD) of 135 µl of each well was measured at 570 nm using a microplate reader (Multiskan Spectrum). Sterile MRS medium was included as a negative control. As a selection criterion for biofilm formation, a cut-off OD (OD...) for the test was defined as three standard deviations above the mean OD of the negative control. The strains were considered as: non-biofilm producers (OD<OD_1); weak biofilm producers (OD_1<OD<OD_2<OD_3); moderate biofilm producers (2×OD<OD_4<5×OD_3); strong biofilm producers (4×OD<OD_5<8×OD_3) and very strong biofilm producers (8×OD_5<OD) (Dubravski et al., 2016, with minor modifications).

**Adhesion and invasion assays in HeLa cells.** Human cervical HeLa cells (ATCC CCL-2) were routinely cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, Waltham, USA), 1% of 10000 U ml^{-1} penicillin, 10 mg ml^{-1} streptomycin and 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% CO_2 (Water Jacketed CO_2 Incubator, Thermo Scientific). For all assays, 1×10^5 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% CO_2 to reach a density of 2×10^5 cells per well (~90% confluence). Before each assay, the cells were washed three times with PBS to remove non-adherent cells and culture medium.

For the adhesion assay, Lactobacillus cultures were centrifuged at 5500 g for 15 min, washed twice with PBS and resuspended in RPMI 1640 medium (Gibco). An aliquot of 500 µl of each suspension (MOH of 5000: 1) was mixed with 500 µl of RPMI 1640 medium, added to wells containing HeLa cells and incubated at 37 °C for 2 h in an atmosphere of 5% CO_2 (Atassi et al., 2006). After incubation, the monolayers were washed three times with PBS to remove non-adherent bacteria, 500 µl of 0.25% trypsin-EDTA solution (Sigma-Aldrich) were added to each well, the cells were homogenized and appropriate dilutions were plated on MRS agar to determine the bacterial counts. The results were expressed as log_{10} CFU of cell-adhered lactobacilli per well.

The same procedure described in the earlier paragraph was followed for the internalization assay. Lactobacillus spp., G. vaginalis and C. albicans were incubated with HeLa cells (1×10^5) in a 24-well tissue culture plate (TPP). After 2 h of incubation at 37 °C, the monolayers were washed three times with PBS and fixed with 100% methanol. The cells were stained with DAPI (4',6-diamidino-2-phenylindole) and imaged under a confocal microscope. The percentage of internalized cells was calculated by the equation: 1− (N_2 / N_0) × 100 and co-aggregation was calculated as follows:

\[
\text{Co-aggregation} (%) = \left( \frac{[Ax + Ay] - [A(x + y)]}{[Ax + Ay]/2} \right) \times 100
\]
Interference assays: blockage by exclusion or displacement of G. vaginalis and C. albicans. Both assays were performed as described by Castro et al. (2013). In the blockage by exclusion tests, aliquots of 250 µl (MOI of 5000:1) containing: (1) Lactobacillus cultures in MRS (bacteria plus culture supernatant); (2) Lactobacillus cells resuspended in fresh MRS (only bacteria); or (3) supernatant of Lactobacillus cultures in MRS obtained by filtration through a sterile Millex 0.22-µm filter, were mixed with 500 µl of RPMI 1640 medium and added to each well of tissue culture plates containing HeLa cells. The plates were incubated at 37 °C for 2 h in an atmosphere of 5% CO2. Subsequently, 250 µl of G. vaginalis (MOI of 500:1) or C. albicans (MOI of 50:1) cultures washed and resuspended in RPMI 1640 medium were added to the plates, which were incubated again for 2 h in the same conditions as described earlier. After incubation, cell monolayers were washed three times with PBS, 250 µl of 0.25% trypsin-EDTA solution were added to each well, the cells were homogenized and appropriate dilutions were plated on Vaginalis agar, SDA supplemented with 100 µg/ml amphotericin B for G. vaginalis and 0.025 µg/ml amphotericin B for C. albicans was added. The plates were incubated for 2 h at 37 °C in an atmosphere of 5% CO2 (Bouchard et al., 2013, with modifications). Then, cells were washed three times with PBS, lysed with 500 µl of sterile distilled water and appropriate dilutions were plated on microbial specific-medium. Results were expressed as log10 CFU of cell-internalized microorganisms per well.

Cytotoxic effects and apoptosis assay. To assess the integrity of HeLa cell monolayers when exposed to lactobacilli or pathogens, 500 µl of suspensions containing each Lactobacillus isolate (MOI of 5000:1) in fresh MRS, G. vaginalis (MOI of 500:1) or C. albicans (MOI of 50:1) in RPMI 1640 medium were mixed with 500 µl of RPMI 1640 and added to 24-well tissue culture plates containing HeLa cells. The plates were incubated for 18 h at 37 °C in an atmosphere of 5% CO2. HeLa cells receiving no treatment were used as control. After incubation, the plates were washed three times with PBS, fixed with 10% formalin for 30 min, washed with sterile distilled water, stained with a 1% crystal violet in 7% ethanol solution for 1 h and washed with sterile distilled water to remove the excess of dye. The cytotoxic effects of lactobacilli, G. vaginalis and C. albicans were evaluated by detection of cellular apoptosis when HeLa cells were exposed to the microorganisms alone or in combination as described by Vlachos et al. (2007). Aliquots of 250 µl containing Lactobacillus cells in fresh MRS (MOI of 5000:1) were mixed with 500 µl of RPMI 1640 medium and added to 24-well tissue culture plates containing HeLa cells. After incubation for 2 h at 37 °C in an atmosphere of 5% CO2, 250 µl of G. vaginalis (MOI of 500:1) or C. albicans (MOI of 50:1) suspensions in RPMI 1640 were added to the plates, which were incubated for 16 h under similar conditions. Lactobacillus, G. vaginalis and C. albicans incubated alone were also analysed. HeLa cells receiving no treatment were used as control. After incubation, plates were washed three times with PBS and the DNA was stained with 0.1 µg/ml Hoechst 33342 (Life Technologies) in 0.5% Tween 20 solution for 15 min. The number of apoptotic cells was determined by counting cells with condensed or fragmented nuclei in 10 different fields with at least 50 cells each, and expressed as a percentage of apoptotic cells in relation to the total number of cells. In both experiments the plates were examined and photographed using a fluorescence microscope (Evos FL Imaging system, Life Technologies).

Statistical analysis. For all assays, the results were the average of three independent experiments performed in duplicate or triplicate, except for the biofilm formation assay, which was evaluated in six independent experiments with eight replicates each. Statistical analysis was done using the GraphPad Prism 6.00 (GraphPad software, La Jolla, USA) and the statistical tests used for each assay are indicated on their respective results. Results were considered statistically different for at least p<0.05.

RESULTS AND DISCUSSION

Identification and in vitro antagonism against vaginal pathogens of Lactobacillus spp. strains

Table 1 shows the results of partial 16S rDNA sequencing of the 23 Lactobacillus isolates. All isolates could be classified to the species level as follows: nine L. plantarum, eight L. fermentum, four L. gasseri and two L. reuteri. These results may seem unexpected since among lactobacilli species normally found in the dominant healthy vaginal ecosystem, L. iners, L. crispatus, L. gasseri, L. johnsonii and L. jensenii are the most frequent. However, L. acidophilus, L. fermentum, L. plantarum, L. brevis, L. casei, L. vaginalis, L. delbrueckii, L. salivarius, L. reuteri and L. rhamnosus have also been isolated from healthy women (Cribby et al., 2008). Individual differences in the Lactobacillus spp. composition of the vaginal tract of women from different locations, races and ethnicities have already been noted in multiple studies (reviewed by Borges et al., 2014). This could explain our findings, recalling that there are virtually no previous reports of isolation and molecular identification of vaginal lactobacilli in Cuba. Additionally, most of the well conducted clinical trials used for treatment of BV, as an example, have been performed with strains of species such as L. rhamnosus, L. reuteri, L. brevis, L. salivarius, L. plantarum or L. acidophilus (Mastromarino et al., 2013).

Table 1 also shows that the in vitro antagonistic activity was quite variable between the Lactobacillus isolates, as well as the sensitivity of bacteria and yeasts. The mean diameter of the inhibitory zone was of 14.07 mm for C. albicans and 19.29 mm for G. vaginalis. For the next steps, eight isolates were selected based on their antagonistic activity against G. vaginalis (LF195 and LP198), C. albicans (LP138, LP174, LF197 and LG202) or against both (LP59 and LP137). The selection of these eight Lactobacillus isolates was based on the fact that G. vaginalis is the most frequently isolated bacterium in cases of BV (Borges et al., 2014) and C. albicans is responsible for the majority (80–90%) of VVC and RVVC (recurrent vulvovaginal candidiasis) cases (Sobel, 2007). Unfortunately, no Lactobacillus isolate showed antagonist activity against C. glabrata, a yeast also responsible for several cases of RVVC (Sobel, 2007). The antagonism of lactobacilli against other bacteria is very frequent and well described in scientific literature and relies on the
production of organic acids, hydrogen peroxide and bacteriocins. However, inhibition of yeast growth by lactobacilli is rarely found and the mechanisms of action generally unknown. The results obtained here can therefore be considered very interesting (26% of the unknown. The results obtained here can therefore be confirmed by the double layer agar diffusion method. This method is widely used in microbiology to detect antagonistic substances or by direct interaction between lactobacilli and pathogen cells.

The results obtained by the double layer agar diffusion method are only qualitative, since the halo diameter is not directly related to the amount of inhibitory substances produced; it also depends on other parameters such as size and spatial configuration of the compound. Therefore, co- incubation experiments were performed to quantify growth inhibition of \textit{C. albicans} and \textit{G. vaginalis} by the selected \textit{Lactobacillus} isolates, and also to evaluate a possible inhibition caused by a direct interaction between lactobacilli and pathogen cells (co-culture). Fig. 1 shows that all \textit{Lactobacillus} cultures or their cell-free supernatants had inhibitory effects on both \textit{C. albicans} and \textit{G. vaginalis} when co- incubated. For \textit{C. albicans}, the best results were obtained when the yeast was incubated with the supernatants from \textit{Lactobacillus} cultures, especially for LF197 strain, which completely inhibited the growth of \textit{C. albicans}. In co-culture, the reduction of \textit{C. albicans} counts was quite similar for all \textit{Lactobacillus} strains, with slightly better results for LP59, LF137 and LP138. For \textit{G. vaginalis}, excellent results were obtained with the culture supernatants from LP59, LF195 and LP198 and in co-culture with LP59 and LF137; the latter being able to inhibit totally the growth of the pathogen. The \textit{Lactobacillus} counts for all strains reached $10^8$ CFU ml$^{-1}$ or $10^6$ CFU ml$^{-1}$ when they were co- incubated with \textit{G. vaginalis} or \textit{C. albicans}, respectively. Shortly, the results showed that the lactobacilli were able to reduce the viability of \textit{C. albicans} and \textit{G. vaginalis} with different levels of effectiveness, either by the production of antagonistic substances or by direct interaction between lactobacilli and pathogen cells.

### Hydrogen peroxide and lactic acid production

The qualitative analysis demonstrated that all the eight selected \textit{Lactobacillus} isolates produced H$_2$O$_2$, and quantitiative analysis by titration (Table 2) showed that the best
Auto-aggregation, co-aggregation and biofilm formation

Table 3 shows that all microorganisms presented some level of auto-aggregation, ranging from medium to high values (43.06–100%). *C. albicans* showed the highest auto-aggregation ability, reaching surprisingly a percentage of 100%. *G. vaginalis* showed a moderate level of auto-aggregation (44.0%) and amongst the strains of lactobacilli, LP174 (96.58%), LG202 (94.83%), LF137 (93.28%) and LF195 (85.21%) were emphasized for their high levels of auto-aggregation. The auto-aggregation ability of vaginal lactobacilli is an intrinsic characteristic that can substantially increase the colonization of the vaginal tract (Owen & Clenney, 2004). This ability is higher in acid environments and represents the first step during the formation of biofilms by lactobacilli (Juarez-Thomas et al., 2005). As in other studies (Gil et al., 2010; Kassaa et al., 2014), the present results revealed different levels of auto-aggregation of the lactobacilli, suggesting that these levels depend on the strain and not on the species.

The ability of *Lactobacillus* strains to co-aggregate with *C. albicans* or *G. vaginalis* was evaluated by visual and quantitative methods. Table 3 shows a correspondence between the visual index and the percentage of co-aggregation obtained. All lactobacilli tested showed co-aggregation with *C. albicans* and/or *G. vaginalis*. LP138 exhibited the best result (59.04%) for *C. albicans* and the other isolates showed levels of co-aggregation ranging from 20.98 to 43.0%. Most strains showed high co-aggregation rates with *G. vaginalis*: LF137 (97.5%), LF195 (83.05%) and LP198 (70.46%) with only LP59 showing a medium level (45.1%). The co-aggregation ability is an important property of lactobacilli, because it can create a microenvironment around the pathogen with a higher concentration of inhibitory substances and also be a trapping mechanism that prevents pathogen adhesion to the vaginal epithelium (Mastromarino et al., 2002).
Table 2. Production of lactic acid and H₂O₂ by the Lactobacillus isolates

<table>
<thead>
<tr>
<th>Lactobacillus strain</th>
<th>pH</th>
<th>Lactic acid (g L⁻¹)</th>
<th>H₂O₂ (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. plantarum 59</td>
<td>4.10 ± 0.18</td>
<td>1.47 ± 0.04abc</td>
<td>0.23 ± 0.01b</td>
</tr>
<tr>
<td>L. fermentum 137</td>
<td>4.12 ± 0.17</td>
<td>1.62 ± 0.07abc</td>
<td>0.07 ± 0.00c</td>
</tr>
<tr>
<td>L. plantarum 138</td>
<td>4.11 ± 0.11</td>
<td>1.38 ± 0.04c</td>
<td>0.18 ± 0.02bc</td>
</tr>
<tr>
<td>L. plantarum 174</td>
<td>4.13 ± 0.10</td>
<td>1.68 ± 0.04b</td>
<td>0.15 ± 0.02bc</td>
</tr>
<tr>
<td>L. fermentum 195</td>
<td>4.11 ± 0.18</td>
<td>1.50 ± 0.04bc</td>
<td>0.15 ± 0.02bc</td>
</tr>
<tr>
<td>L. fermentum 197</td>
<td>4.14 ± 0.17</td>
<td>1.41 ± 0.04c</td>
<td>0.08 ± 0.01c</td>
</tr>
<tr>
<td>L. plantarum 198</td>
<td>4.10 ± 0.17</td>
<td>1.44 ± 0.00c</td>
<td>0.24 ± 0.03b</td>
</tr>
<tr>
<td>L. gasseri 202</td>
<td>4.10 ± 0.19</td>
<td>1.35 ± 0.13c</td>
<td>0.26 ± 0.03b</td>
</tr>
<tr>
<td>L. acidophiles ATCC 4356</td>
<td>3.77 ± 0.01</td>
<td>2.51 ± 0.04a</td>
<td>0.67 ± 0.11a</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate significant differences by One-way ANOVA test, followed by Tukey post-test (p<0.05). Positive control: L. acidophiles ATCC 4356. The results were obtained from three independent experiments performed in triplicate.

Table 3 also shows that all lactobacilli were able to form biofilms and two of them stand out for being strong (LF137) and very strong (LG202) biofilm producers. As already seen in the previous tests, these strains have also a high rate of auto-aggregation (93.28% and 94.83%, respectively), a property that is important and correlated with the initial development of biofilms. Biofilm formation is important to promote colonization and permanence of pathogens (Lepargneur & Rousseau, 2002). Different approaches of in vitro formation of biofilms by vaginal lactobacilli conclude that virtually all strains tested are able to form biofilms on polystyrene surfaces at different levels (Leccese Terraf et al., 2014).

Adhesion to and internalization in HeLa cells

The adhesion of microorganisms to epithelial cells is an essential step in the colonization and persistence in a specific site; thus, we investigated the ability of lactobacilli to bind to HeLa cells, a human cell line originated from a cervix carcinoma. The results showed that all strains had an ability to adhere to monolayers of HeLa cells (Fig. 2a), and among them LF197 strain showed the highest capacity (7.76 ± 0.15 log₁₀ CFU per well). The other strains showed adhesion values which ranged from 6.17 ± 0.30 to 7.12 ± 0.22 log₁₀ CFU per well. The results of the adhesion of Lactobacillus strains to HeLa cells reinforce that their adhesive properties (specific or not) are rather strain- than species-specific. The excellent adhesion values found for all

Table 3. Auto-aggregation, biofilm formation and co-aggregation of Lactobacillus isolates, C. albicans and G. vaginalis

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Auto-aggregation (%)</th>
<th>Biofilm formation</th>
<th>Co-aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. albicans</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>(%)</td>
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<td></td>
<td></td>
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<td>Visual index</td>
</tr>
<tr>
<td>L. plantarum 59</td>
<td>43.06 ± 3.34 c</td>
<td>Moderate</td>
<td>27.46 ± 4.88 cd</td>
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<tr>
<td>L. fermentum 137</td>
<td>93.28 ± 0.64 ab</td>
<td>Strong</td>
<td>43.00 ± 2.40 bc</td>
</tr>
<tr>
<td>L. plantarum 138</td>
<td>64.03 ± 1.54 d</td>
<td>Moderate</td>
<td>59.04 ± 0.41 a</td>
</tr>
<tr>
<td>L. plantarum 174</td>
<td>96.58 ± 3.42 ab</td>
<td>Moderate</td>
<td>20.98 ± 2.68 e</td>
</tr>
<tr>
<td>L. fermentum 195</td>
<td>85.21 ± 4.35 ab</td>
<td>Moderate</td>
<td>NP</td>
</tr>
<tr>
<td>L. fermentum 197</td>
<td>80.78 ± 4.36 bc</td>
<td>Moderate</td>
<td>NP</td>
</tr>
<tr>
<td>L. plantarum 198</td>
<td>66.58 ± 3.38 cde</td>
<td>Moderate</td>
<td>NP</td>
</tr>
<tr>
<td>L. gasseri 202</td>
<td>94.83 ± 3.68 ab</td>
<td>Very strong</td>
<td>42.90 ± 1.04 b</td>
</tr>
<tr>
<td>C. albicans</td>
<td>100.00 ± 0.00 a</td>
<td>NP</td>
<td>-</td>
</tr>
<tr>
<td>G. vaginalis</td>
<td>44.00 ± 0.60 d</td>
<td>NP</td>
<td>-</td>
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</table>

Each value of auto-aggregation and co-aggregation assays corresponds to the mean ± standard deviation of three independent experiments performed in duplicate. For biofilm formation, six independent experiments were performed with eight replicates each and the classification was performed following Dubravka et al. (2010). Different letters in the same column indicate significant differences by One-way ANOVA test, followed by Tukey post-test (p<0.05). NP, not performed. Only strains that showed antagonism against G. vaginalis were evaluated for the ability to co-aggregate with G. vaginalis. The same criterion was adopted for C. albicans.
lactobacilli, along with the data from other adhesive properties are strong indications that these strains may be able to successfully colonize the human vaginal epithelium.

Besides adhesion assay, internalization of $G$. vaginalis, $C$. albicans and Lactobacillus was also evaluated in HeLa cells (Fig. 2b). All lactobacilli and $G$. vaginalis were internalized at different levels in HeLa cells, while unexpectedly $C$. albicans was not. Among the lactobacilli, internalization varied widely from one isolate to another, ranging from $1.93 \pm 0.01 \log_{10}$ CFU per well (LF197) to $5.14 \pm 0.32 \log_{10}$ CFU per well (LP138), whereas $G$. vaginalis showed the highest internalization value ($5.21 \pm 0.66 \log_{10}$ CFU per well). When the number of bacteria internalized in relation to the number of bacteria initially inoculated is calculated as a percentage, $G$. vaginalis had an internalization of $0.45 \%$, whereas for lactobacilli this percentage was of $0.0004 \%$ (LP59), $0.0014 \%$ (LF137), $0.0166 \%$ (LP138), $0.0010 \%$ (LP174) $0.0007 \%$ (LF195), $0.00001 \%$ (LF197), $0.0001 \%$ (LP198) and $0.0005 \%$ (LG202). In other words, the internalization of $G$. vaginalis in HeLa cells was approximately $27$ to $80,000$ times higher than the internalization of lactobacilli, confirming $G$. vaginalis higher invasiveness. Similar results were found in a study of Marrs et al. (2012) that examined the internalization of $G$. vaginalis, L. crispatus and L. acidophilus in VK2 cells (vaginal epithelium immortalized cells). In fact, there are few data in the literature on Lactobacillus internalization, since this is generally associated with virulence factors of pathogenic microorganisms. However, it would be logical to think that there is a need of lactobacilli to enter in the epithelial cells in order to develop their immunomodulatory effects, with the condition that such internalization is not excessive. Curiously, $C$. albicans was not able to be internalized in HeLa cells under our methodological conditions. It is well known that $C$. albicans invades host epithelial cells by two distinct mechanisms and that its hyphae form has a crucial role in both of them. The factors involved in the hyphae growth are very complex and involve changes in environmental conditions and signal transduction cascades regulated by quorum sensing (Yang et al., 2014). In the present study, it is possible that $C$. albicans remained in its unicellular form under the methodological conditions used, a fact which could explain the lack of internalization.

**Interference with the adhesion of pathogens to HeLa cells**

It can be assumed that vaginal lactobacilli of healthy women prevent the colonization of pathogenic microorganisms by occupying or blocking (by steric hindrance) potential binding sites in the mucosa. However, in cases of dysbiosis, where there is a reduction in the population of lactobacilli, it is expected that exogenous probiotics would be able to compete for the same receptors and/or promote displacement of pathogens already adhered. Previous in vitro studies have already demonstrated the ability of some vaginal Lactobacillus strains to interfere with the adhesion of $G$. vaginalis and $C$. albicans to epithelial cells, by exclusion (preventive) and displacement (curative) mechanisms (Mastromarino...
et al., 2002; Castro et al., 2013; Parolin et al., 2015). These interference abilities were tested for Lactobacillus cultures, bacterial cells resuspended in fresh MRS medium and culture cell-free supernatants. When preventive effect was evaluated, Lactobacillus cultures were able to drastically reduce the adhesion of both pathogens, in values ranging from 87.1% to 91.4% for G. vaginalis and 94.6% to 98.3% for C. albicans, depending on the Lactobacillus strain. G. vaginalis and C. albicans counts were reduced, respectively, from 65.8% to 91.7% and from 92.8% to 98.2% when cells resuspended in fresh MRS medium were tested. MRS supernatants of most isolates were also able to reduce G. vaginalis adhesion from 42.3% to 76.0% and from 37.2% to 75.0% for C. albicans (Fig. 3a, b).

Fig. 3. Adhesion exclusion (a and b) or displacement (c and d) of pathogens adhered to HeLa cells by Lactobacillus isolates. Results are expressed as percentages of C. albicans (a and c) or G. vaginalis (b and d) adhered to the wells in relation to the adhesion of pathogens alone, set as 100% (black bars, control). MRS, MRS medium non-inoculated with lactobacilli (white bars). Each value corresponds to the mean ± standard deviation of three independent experiments performed in triplicate. Significant differences from the control are indicated by asterisks: ****p<0.0001; ***0.0001≤p<0.001; **0.001≤p<0.01; *0.01≤p<0.05 (One-way ANOVA test followed by Dunnet post-test). Only strains that showed antagonism against G. vaginalis were evaluated for the ability to inhibit the adhesion of G. vaginalis. The same criterion was adopted for C. albicans.
When curative effect was tested, all conditions were able to displace or probably kill (if the supernatant was used) *C. albicans* already adhering to HeLa cells (Fig. 3c, d). *Lactobacillus* cultures, cells resuspended in fresh MRS and MRS supernatants were responsible for reductions of *C. albicans* counts ranging from 75 % to 95.7 %, 79.8 % to 93.3 % and from 88.2 % to 97.6 %, respectively. With regard to *C. albicans*, reductions ranged from 41.8 % to 69.9 % when *Lactobacillus* cultures were used, from 36.2 % to 56.0 % for cells resuspended in fresh MRS, and from 43.0 % to 53.7 % for MRS supernatants. For both assays no inhibition of adhesion was observed in the presence of sterile MRS medium.

The present results show that all eight isolates in culture or their fractions (cells and cell-free supernatants) were able to interfere with the adhesion of pathogens in different degrees, by either exclusion or displacement mechanisms.

**Cytotoxic effects and apoptosis assay**

Microscopy observations of HeLa cell monolayers incubated only with each microorganism individually revealed that cell monolayers incubated with the *Lactobacillus* strains remained intact and showed no visible changes when compared to the non-inoculated control (Fig. S1a, available in the online Supplementary Material). However, *V. parvula* caused an intense desquamation of the HeLa cell monolayers, with little remaining cellular debris. Another feature was noted for *C. albicans*, a large presence of cellular debris wrapped by the fungus either in its yeast, pseudohyphae or hyphae form (data not shown). When HeLa cells were pretreated with lactobacilli before being challenged with the pathogens, cell monolayers remained intact, showing no staining changes when compared to HeLa cells either non-inoculated or incubated only with the *Lactobacillus* strains (Fig. S1b).

In a last experiment, by observing the results in Table 4 and in Fig. 4, it can be noted that none of the *Lactobacillus* strains induced apoptosis in HeLa cells (pretreatment only), showing a percentage of apoptotic cells (from 12.24 ± 4.07 % to 19.41 ± 0.24 %) similar to the non-inoculated control (13.32 ± 3.67 %). In contrast, both *G. vaginalis* and *C. albicans* induced total apoptosis in HeLa cells (100 %). These results confirm the analysis performed previously, where the integrity of HeLa cell monolayers was observed. When HeLa cells were pretreated with lactobacilli before being challenged by the pathogens, the percentage of apoptotic cells dropped sharply to levels only slightly higher than the control ones (from 16.37 ± 6.34 % to 29.80 ± 5.78 %).

**CONCLUSION**

Together, all these results reveal the probiotic potential of eight selected *Lactobacillus* strains, and further studies involving immunomodulation capacity and in vivo tests with animals are necessary for a thorough understanding of the mechanisms by which they exert their beneficial role. Although the *Lactobacillus* isolates LP59 and LF137 seem to be the most interesting potential probiotics because of their simultaneous antagonist activity against both pathogens, the other isolates must be maintained in future studies, considering a possible strategy of administration using a combination of lactobacilli with complementary antagonism.

**Table 4. Percentage of apoptotic nuclei of HeLa Cells treated with lactobacilli and challenged with the pathogens**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Apoptotic nuclei (%)</th>
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<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
</tr>
<tr>
<td></td>
<td>G. vaginalis</td>
</tr>
<tr>
<td>Control</td>
<td>13.32 ± 3.67</td>
</tr>
<tr>
<td>MRS</td>
<td>13.78 ± 4.57</td>
</tr>
<tr>
<td><em>L. plantarum</em> 59</td>
<td>13.98 ± 5.20</td>
</tr>
<tr>
<td><em>L. fermentum</em> 137</td>
<td>19.41 ± 0.24</td>
</tr>
<tr>
<td><em>L. plantarum</em> 138</td>
<td>14.98 ± 4.02</td>
</tr>
<tr>
<td><em>L. plantarum</em> 174</td>
<td>15.25 ± 5.22</td>
</tr>
<tr>
<td><em>L. fermentum</em> 195</td>
<td>12.24 ± 4.07</td>
</tr>
<tr>
<td><em>L. fermentum</em> 197</td>
<td>15.11 ± 3.12</td>
</tr>
<tr>
<td><em>L. plantarum</em> 198</td>
<td>12.97 ± 3.98</td>
</tr>
<tr>
<td><em>L. gasseri</em> 202</td>
<td>14.11 ± 4.21</td>
</tr>
</tbody>
</table>

HeLa cells were pre-incubated with lactobacilli for 2h and then challenged with *G. vaginalis* or *C. albicans* for further 16h. Control, HeLa cells non-inoculated with lactobacilli or only challenged with *G. vaginalis* or *C. albicans*; MRS, HeLa cells treated with non-inoculated MRS; NP, not performed. Pretreatment: incubation with each *Lactobacillus* alone. Asterisks indicate values that are significantly different from the pretreatment control; ****p<0.0001; **0.001≤p<0.01; *0.01≤p<0.05 (One-way ANOVA followed by Dunnet post-test). Only strains that showed antagonism against *G. vaginalis* were evaluated for the ability to prevent HeLa cells apoptosis when challenged with *G. vaginalis*. The same criterion was adopted for *C. albicans.*
Fig. 4. Prevention of HeLa cells apoptosis by LP59. HeLa cell monolayers were stained with Hoechst 33342 and observed in fluorescence microscopy. Results were similar for all the Lactobacillus isolates used for the pretreatment and the microphotographs obtained with LP59 were shown as the representative ones (scale bar, 400 µm). (a) Non-inoculated HeLa cells; (b) HeLa cells inoculated with LP59; (c) HeLa cells pretreated with LP59 and challenged with G. vaginalis; (d) HeLa cells pretreated with LP59 and challenged with C. albicans. Note that these monolayers remain intact and have few apoptotic cells. (e) and (f) HeLa cells challenged with G. vaginalis showing the exfoliation and loss of integrity of the cell monolayers, which remained with few apoptotic cells; (g) HeLa cells challenged with C. albicans showing the destruction of the monolayer cells and the presence of yeast among apoptotic cells. (h) Higher increase of G (scale bar, 100 µm) to better show apoptotic cells and cellular debris surrounded by pseudo-hyphae and hyphae of C. albicans.
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