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BIOMEDICAL SCIENCES

Antagonistic activity of *Lactiplantibacillus plantarum* 6.2 extracted from cocoa fermentation and its supernatant on *Gardnerella vaginalis*

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Abstract: Search for alternative methods for the treatment of bacterial vaginosis has been growing, and probiotics being among them. The most well-known probiotic microorganisms are lactobacilli, which are naturally present in the vaginal microenvironment. Cocoa fermentation is a source of lactic acid bacteria, with lactobacilli being the most prominent. The aim of this study was to evaluate the antagonistic activity of *Lactiplantibacillus plantarum 6.2* a strain of lactobacilli isolated from cocoa fermentation, and its cell-free supernatant on *Gardnerella vaginalis.* It was shown that *Lpb. plantarum* 6.2 and its supernatant, used at three concentrations, i.e., 40, 20 and 10 mg/mL, have a strong antagonistic activity against *G. vaginalis*, with a probable action of proteinaceous bacteriocins; the activity was lost after heat treatment. The ability to exclude and displace *G. vaginalis* from the adhesion site to vaginal HMVII epithelial cells was also demonstrated by the lactobacilli and the supernatant, with the latter showing a bactericidal effect. Thus, the *Lpb. plantarum* 6.2 strain presents itself as a good probiotic with potential to be used not only as a therapeutic alternative for vaginosis but also as a complement to existing therapies.

Key words: bacterial vaginosis, biotechnological properties, lactobacilli, probiotics.

INTRODUCTION

Vaginosis is a syndrome characterized by populations of pathogenic microorganisms present in the vaginal microenvironment, whose multiplication is exacerbated by changes in the local microbiota. This microbiota is mainly composed of lactobacilli, and its quantity is decreased in vaginosis. With the change in this microenvironment, pathogens can emerge and multiply causing dysbiosis bringing uncomfortable symptoms in the host (Hillier 2005, Eschenbach 2007, Nejad & Shafaie 2008). Lactobacilli present in the vaginal microenvironment are believed to play a protective role. Alteration in the composition of this microbiota are mostly related to sexually transmitted infections, pregnancy, menstrual cycle, childbirth and hormonal changes. Further, these changes may even result in pelvic inflammatory disease and bacterial vaginosis (Amabebe & Anumba 2018, Kroon et al. 2018, Vaneechoutte 2017).

Pathogens responsible for causing vaginosis include, *Candida albicans*, *Prevotella bivia*, *Mycoplasma hominis*, and *Gardnerella vaginalis* (Kroon et al. 2018, Melgaço et al. 2018, Nunn & Forney 2016). *G. vaginalis* is a facultative anaerobic, gram-variable bacterium, with a coccobacillus form. It has the ability to produce a pore-forming toxin called vaginolysin, which affects only human cells. The toxin is able to induce cell death and lyse erythrocytes, and

it is an important virulence factor, playing a prominent role in the pathogenesis of bacterial vaginosis (Jarosik et al. 1998, Gelber et al. 2008, Castro et al. 2018). *G. vaginalis* plays an important role in the pathogenesis of bacterial vaginosis and the development of vaginal biofilm, in which it makes up 90% of the bacteria that form it in the vaginal epithelium (Swidsinski et al. 2005). In the vaginal microenvironment, *G. vaginalis* adheres to the surface of vaginal epithelial cells, leading to the formation of "clue cells" and the development of a thin biofilm that promotes the fixation of other species of pathogens (Kalia et al. 2020).

The search for simpler and more effective treatments for bacterial vaginosis has grown significantly in an attempt to replace or assist traditional antibiotic treatments, which may cause unwanted side effects (Kaur et al. 2013, Vicariotto et al. 2014, Nagaraja 2008). Lactobacilli are a part of the group of lactic acid bacteria (LAB) and are naturally present in the microenvironment of the oral, intestinal, and vaginal mucosa of healthy individuals (Giraffa et al. 2010). For some time, these microorganisms have been used as probiotics in foods and drugs, along with bifidobacteria and some yeast species (Williams 2010, Hill et al. 2014). Probiotics are defined as live microorganisms that can benefit the host, when administered in adequate quantities (FAO/WHO 2001), and this definition is still suitable after 20 years. But Hill et al. (2014) suggested that to be considered as probiotic the microorganism must have an appropriate viable count, suitable evidences for its health benefits and defined contents.

Several studies have demonstrated the effectiveness of microorganisms when used as an alternative for the prevention and treatment of enteric and urogenital infections, as long as they show essentials characteristics to be considered a good probiotic (Giraffa et al. 2010, Reid & Bruce 2001). Various parameters are taken into account for a microorganism to be considered a probiotic and should support they probiotic activity, testing important characteristics including the ability to adhere to epithelial cells and maintain their viability, inhibition of the growth and spread of pathogens, production of substances such as lactic acid and/or bacteriocins that can cause damage to pathogens and modulation of the immune response, that are important for ensure probiotic efficacy (Binda et al. 2020). These interactions are related to a diversity of specific and non-specific factors, including components of the cell and bacterial surface (Carmo et al. 2016, Jørgensen et al. 2017). Probiotics used as part of the treatment for bacterial vaginosis have shown good results and tolerance when used as formulations for topical use as for oral (Han et al. 2014, Rostok et al. 2019)

Cocoa is one of the main sources of income for agribusiness in Brazil, with the country being among the leading producers of cocoa and chocolate in the world (Santana et al. 2018). Cocoa fermentation is a source of LAB, which are mainly responsible for this process, and include lactobacilli (Schwan & Wheals 2004, Vuyst & Weckx 2016). Using cocoa fermentation, Passos et al. (1984) were able to isolate strains of *Lactobacillus acidophilus*, *Levilactobacillus brevis*, *Lacticaseibacillus casei*, *Lactobacillus delbrueckii* and *Lactiplantibacillus plantarum*. Our research group was able to identify 68 strains of *Limosilactobacillus fermentum* and 12 of *Lpb. plantarum*, through fine cocoa fermentation, a controlled fermentation process (Santos et al. 2011).

Since the probiotic effects of lactobacilli can be strain, species or genus-specific, studies are required to evaluate newly isolated strains. Based on this assessment, our group observed that some of the species of lactobacilli isolated from

cocoa fermentation showed anti-inflammatory activity in an experimental rat colitis model and in a cell model (Santos et al. 2016a, b); moreover, they had anti-biofilm effects against clinical isolates of staphylococci (Melo et al. 2016). They even presented promising results when used in a vaginal mucosa model as follows: capable of adhering to the epithelium of vaginal cells, self-aggregating and co-aggregating with pathogens and producing antagonistic molecules (Pessoa et al. 2017, Melgaço et al. 2018).

In a pioneering way, our group has investigated the use of lactobacilli extracted from fine cocoa fermentation in models of vaginal infection by *G. vaginalis*, with the first studies showing promising results (Pessoa et al. 2017). However, only a few studies have analyzed the mechanisms involved in the beneficial effect of using lactobacilli, especially their supernatants in dysbiosis. Thus, the aim of the present study was to continue the investigation and expand the understanding by evaluating one strain of these lactobacilli as a probiotic, with a focus on testing the possible antagonistic response to *G. vaginalis*.

MATERIALS AND METHODS

Strains, cell lines and growth conditions

The strain used in this study was previously isolated and characterized by our research group as follows: *Lpb. plantarum* 6.2 (Santos et al. 2016a) (Lp 6.2; GenBank: KU291427.1). The strain was grown in Man, Rogosa and Sharpe (MRS) medium for 24 h at 37°C under microaerophilic conditions.

The *Gardnerella vaginalis* ATCC 49154 was grown on 5% blood agar plates (HiMedia, India) or Brain and Heart Infusion (BHI) broth (HiMedia, India) for 24 h at 37°C in a 5% CO_2 atmosphere.

HMVII vaginal epithelial cell line (BCRJ No. 0316), from the Rio de Janeiro Cell Bank was also used in the study. The cells were grown in RPMI 1640 medium (HyClone, EUA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Australia) and 1% antibiotic (penicillin and streptomycin) (Gibco, EUA) at 37°C in a 5% CO_2 atmosphere untilconfluence.

Obtaining the culture supernatant

Lpb. plantarum 6.2 was inoculated in MRS broth and incubated for 48 h at 37°C. After the incubation period, the culture was centrifuged for 15 min at 8,000 × g to obtain the supernatants; the pellet was discarded. The supernatant was lyophilized (Lyophilizer LS3000, Terroni) for 5 days, and subsequently, its yield was measured by weighing. The lyophilized supernatant was kept refrigerated at -20 °C until use.

Agar overlay assay

To qualitatively assess the antagonistic effect of *Lpb. plantarum* 6.2 an agar overlay assay was performed adapted from Lima et al. (2007) for the intact lactobacilli and Teixeira et al. (2012) for the inactivated lactobacilli. *Lpb. plantarum* 6.2 ($1x10^8$ CFU mL^{-1}) was inoculated in MRS broth and incubated at 37 °C for 24 h. After growth, a 20 µL aliquot of the culture was placed at three points on two plates containing MRS agar (Acumedia, EUA), and incubated at 37 °C for 24 h under anaerobic conditions; one plate was used to evaluate intact lactobacilli and the other for inactivated lactobacilli. To inactivate the lactobacilli, the cells were exposed to chloroform PA for 20 min. After the chloroform residue on the plate had evaporated, it was overlaid with BHI agar (Difco, USA) previously inoculated with *G. vaginalis* ($1x10^8$ CFU mL^{-1}). The same process was performed on a plate containing intact lactobacilli, excluding the chloroform inactivation step. Both plates with the agar overlay were incubated at 37 °C for 24 h. After incubation, the antagonist activity

was determined by the presence of a zone of inhibition around the seeding point.

Antagonism assay

For quantitative evaluation of the antagonist activity of the *Lpb. plantarum* culture supernatant, an antagonism assay was performed following Vicarioto et al. (2014). *G. vaginalis* was inoculated in BHI broth (HiMedia, India) and incubated at 37 °C for 24 h. The culture was then centrifuged for 15 min at 8,000 × g; the cell pellets were resuspended, washed twice with 0.9% saline, and resuspended to 1×10^8 CFU mL^{-1}. The lyophilized supernatant was weighed, diluted in BHI broth (at concentrations of 40, 20, and 10 mg/mL), and filtered through 0.22 µm membranes. In a well plate, *G. vaginalis* was inoculated in BHI broth along with the supernatants. For control wells, only BHI broth + *G. vaginalis* and BHI broth + *G. vaginalis* + MRS were added. The plate was incubated at 37 °C, and after 24 and 48 h, growth was quantified using a spectrophotometer (EZ Read 400, Biochrom) at a wavelength of 600 nm. The final values were calculated using the following formula: bacterial viability (%) = $[(OD_{neg.\ cont.} - OD_{blank}) / (OD_{posit.\ cont.} - OD_{blank})] \times 100$, where the OD negative control was made with BHI + *G. vaginalis* broth and OD blank with only BHI broth.

Evaluation of thermolabile substances

To assess the heat sensitivity of the substances present in the *Lpb. plantarum* 6.2 supernatant, the supernatant was denatured by autoclaving for 15 min at 121 °C and the quantitative antagonism test was performed, as described above.

FTIR-ATR analysis of functional groups

To analyze the structural chemical composition of the intact and denatured *Lpb. plantarum* 6.2 culture supernatant, infrared spectroscopy was performed using a PerkinElmer Spectrum 100 FTIR spectrometer, equipped with an ATR accessory containing a zinc selenide (ZnSe) prism, according to the protocol described by Ammann & Brandl (2011), with a range between 400 - 4000 cm^{-1} for detecting the functional groups present in both samples. Fifty scans, at a resolution of 2 cm^{-1}, were performed and used for chemometric analyses.

Exclusion and displacement assay

To evaluate whether the lactobacilli and/or their supernatant were able to promote the exclusion and displacement of *G. vaginalis* from the adhesion site in HMVII cells, exclusion and displacement tests were performed. Both assays were adapted from Santos et al. (2016c). The exclusion assay was performed on a 24-well cell culture plate where lactobacilli (1×10^8 CFU mL^{-1}) and the supernatant, at concentrations of 40, 20, and 10 mg/mL were added along with *G. vaginalis* (MacFarland scale - 1×10^8 CFU mL^{-1}) in RPMI 1640 medium (HyClone, EUA) were added to wells containing HMVII cells previously adhered for 24 h (1×10^6 cells mL^{-1}); the plate was incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. Subsequently, all the medium was removed from the wells, and the cell monolayer was washed with PBS twice to remove non-adherent bacteria. The medium containing *G. vaginalis* was added to the wells where only lactobacilli were previously present, and the plate was incubated for another 2 h, under the same conditions as before. After incubation, all the medium was removed and the wells were washed twice with PBS and treated with 0.25% trypsin-EDTA for 15 min. Subsequently, saline was added to the same amount of medium used previously, and a serial dilution was made, followed by plating on blood agar. The plates were incubated for 48 h at 37 °C, for determination o microbial count. The displacement assay was performed

in a manner similar to the exclusion assay but with *G. vaginalis* incubated with HMVII cells first. The control consisted of HMVII cells incubated with *G. vaginalis*. The percentage of *G. vaginalis* adherence, after treatment with lactobacilli and the supernatant, was obtained using the formula: adhesion (%) = (CFU_{end} / $CFU_{initial}$) × 100. It was compared to the control (considered as 100% adhesion).

Statistical analysis

All experiments were performed in triplicates. Quantitative data are presented as mean and standard deviation, calculated using GraphPad Prism 7.04. Statistical differences between mean values were determined using One-way ANOVA test followed by Dunnet post-test, with $p < 0.05$.

RESULTS

Agar overlay assay

Both intact and inactivated lactobacilli were able to inhibit the growth of *G. vaginalis*, as shown in Figure 1, where the presence of zones of inhibition around the previously formed colonies was verified. Despite being a qualitative assay, it was possible to verify a reduction in the area of halos of inactivated lactobacilli (Figure 1b) when compared to intact lactobacilli (Figure 1a). This reduction in the inhibitory capacity of the inactivated *Lpb. plantarum* 6.2 suggests that the viability of the bacteria increases its antagonistic capacity against *G. vaginalis*. Further, the metabolic products of these lactobacilli seem to be sufficient to act antagonistically on the pathogenic bacteria. Thus, these data strongly suggest that the antagonistic action of *Lpb. plantarum* 6.2 is exerted by both, the bacteria and its metabolism products.

Antagonism assay

Supernatants of lactobacilli and other microorganism cultures contain the products of bacterial metabolism in a concentrated manner. It was observed in the previous experiment that the *Lpb. plantarum* 6.2 strain, even when

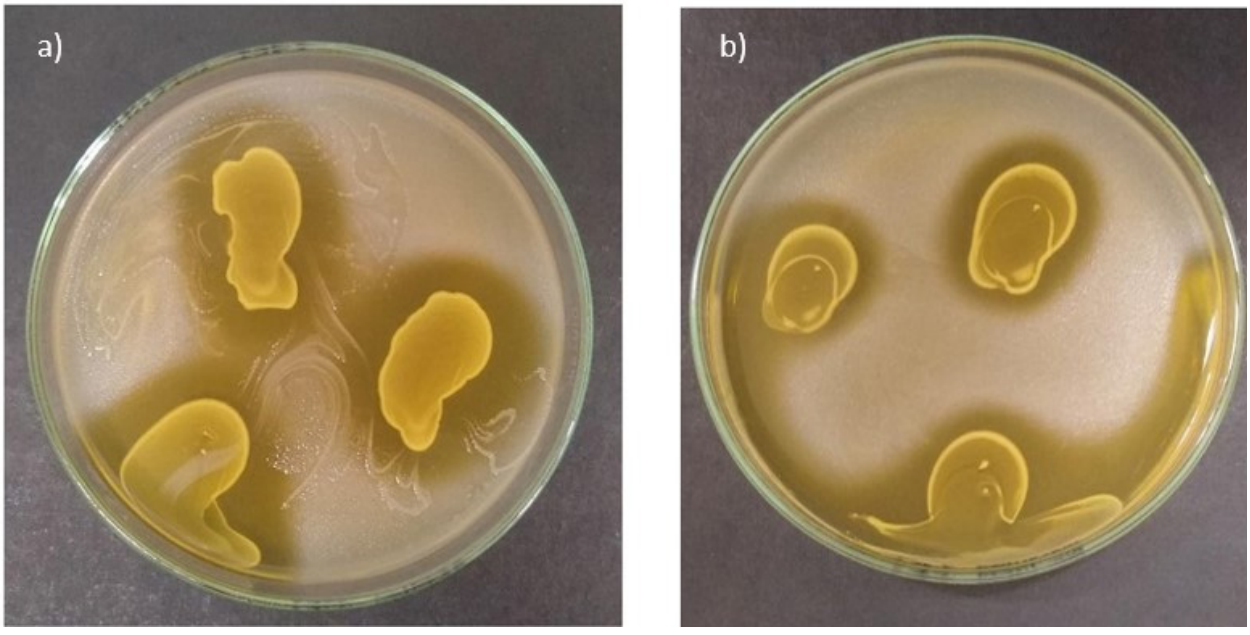

Figure 1. Antimicrobial activity of intact (a) and chloroform-inactivated (b) *Lactiplantibacillus plantarum* 6.2 against *Gardnerella vaginalis* demonstrated by the formation of a zone of inhibition at the inoculation points by the agar overlay antagonism evaluation method.

inactivated, maintained an antagonistic action, which came from the products of its metabolism. Thus, a quantitative assay was also carried out to assess the antagonistic action of three different concentrations of *Lpb. plantarum* 6.2 supernatants on *G. vaginalis* at 24 and 48 h. Analysis of the percentage of viable bacteria after 24 h of incubation with the supernatant (Figure 2a), indicated an inhibitory effect at all the three concentrations, showing a dose-dependent effect, in which the highest concentration, i.e., 40 mg/mL, resulted 90.61% inhibition, followed by 20 mg/mL, with 54.28% inhibition and 10 mg/mL, with 37.79%. Analysis of bacterial viability after 48 h of incubation (Figure 2b) showed a smaller but significant inhibition, with 76.75% inhibition at 40 mg/mL, followed by 37% at 20 mg/mL, and 34,97% at 10 mg/mL. Thus, it is possible to infer that the best inhibition values were obtained in the first 24 h of incubation, where the supernatant prevented or attenuated the exponential growth phase or even the stationary phase, as a slightly higher viability of bacteria was observed after 48 h.

Evaluation of thermolabile substances

To determine whether the antagonistic effect presented by the culture supernatant could be due to the presence of some proteins, the supernatant was subjected to denaturation to assess the influence of thermolabile substances on the inhibition of *G. vaginalis* growth. Upon analysis of the percentage of viable *G. vaginalis* after 24 h of incubation (Figure 3a) with the denatured supernatant, it was possible to observe that at all three concentrations tested, there was no reduction in bacterial growth. And with the analysis after 48 h of incubation (Figure 3b), i.e., there was no inhibition of bacterial growth but an increase of it. Thus, it was observed that there was a total loss of inhibitory activity of the supernatant after it had been subjected to a high-temperature treatment.

Analysis of functional groups by FTIR-ATR

The presence of a peak between 3500 - 3200 cm^{-1} in the spectrum indicates the presence of OH groups, with NH groups that, may be primary or secondary amines, which is related to the presence of proteins groups (Morais et al. 2017, Coates 2006). In our result, stretching of the spectrum at 3326.6 cm^{-1} was observed for the intact supernatant and 3362.8 cm^{-1} for the denatured supernatant (Figure 4), indicating the presence of possible protein groups. However, there was a difference of more than 22% in the

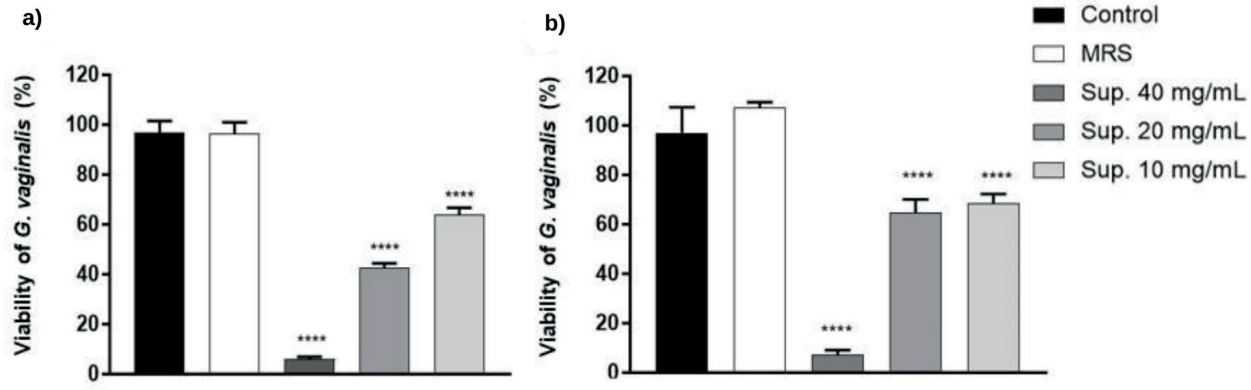

Figure 2. Antagonistic effect of the culture supernatant of *Lactiplantibacillus plantarum* 6.2 in different concentrations against *Gardnerella vaginalis* after 24 h (a) and 48 h (b) of incubation. Each value corresponds to the mean ± standard deviation of triplicates. Significant differences from the control are indicated by asterisks (** p <0.0001).**

transmittance of this group when comparing the percentage of transmittance between intact and denatured sample, indicating a possible reduction in the amount of protein due to heat denaturation. Bands observed between approximately 1220 - 900 cm^{-1} indicate the presence of carbohydrates (C – O) in both samples, but being more in the intact supernatant. Futhermore, CH_2 and CH_3 groups were also present in both samples.

The process of heat denaturation translates into an increase in molecular movements, which affect hydrogen bonds and other covalent bonds, causing the protein to lose its tertiary structure, but maintain the primary structure, which is rich in peptide bonds. The presence of a band observed near 1650 cm^{-1} in the denatured supernatant corresponds to the C=O stretching of peptide bonds, which did not change during the denaturation process. In the intact supernatant, bands indicating nitro groups (NO_2) at 1576.0 cm^{-1}, alkanes, at 2970.9 cm^{-1}, alkyl halides at ~1400 cm^{-1}, and aromatic hydrocarbon (C_6H_6), at 855.3 cm^{-1}, were observed.

Exclusion and displacement assay

Analysis of the percentage of bacteria adhering to the HMVII cells in the exclusion assay (Figure 5a) indicated that lactobacilli were able to exclude *G. vaginalis*, with 75.95% inhibition of adhesion. The culture supernatant, at all the three concentrations used, were also significantly effective in excluding *G. vaginalis* from the cell adhesion site, presenting a 66.67% inhibition for the concentration of 40 mg/mL, 68.07% for 20 mg/mL and showing the best exclusion result at a concentration of 10 mg/mL which inhibited the adhesion of 90.12% of the bacteria. In the displacement analysis (Figure 5b), the culture supernatant was significantly more effective than the lactobacilli. After contact with the *G. vaginalis* already adhered to HMVII cells, lactobacilli decreased adherence by up to 67.61%, with only 32.9% of bacteria adhering till to the end. However, all three concentrations of supernatant reduced the number of bacteria adhering to the cells drastically, showing more than 90% inhibition.

DISCUSSION

One of the essential conditions for a microorganism to be considered as a possible probiotic is that it can inhibit the growth of the target pathogen, demonstrating an antagonistic action by inhibiting it when both are in the same

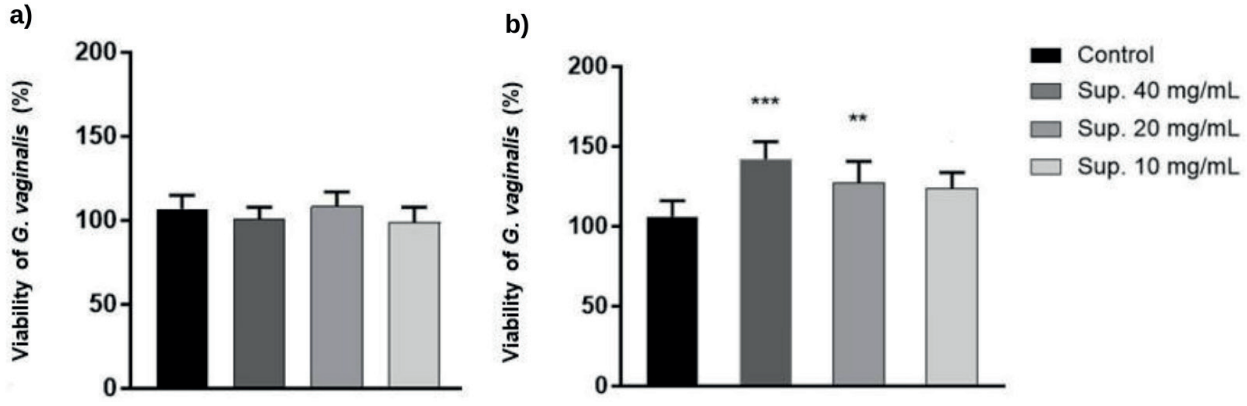

Figure 3. Effect of thermolabile substances on the antagonist action of the culture supernatant of *Lactiplantibacillus plantarum* 6.2 used at different concentrations against *Gardnerella vaginalis* after 24 h (a) and 48 h (b) of incubation. Each value corresponds to the mean ± standard deviation of triplicates. Significant differences from the control are indicated by asterisks (*p < 0.0005, **p < 0.0057).**

environment. In previous studies, lactobacilli extracted from cocoa fermentation have shown a good ability to inhibit clinically important pathogens tested via the minimum inhibitory concentration (MIC) method, demonstrated by Melo et al. (2016), a strain of *Li. fermentum* was able to inhibit the growth of *Staphylococcus aureus*. Further, Pessoa et al. (2017) demonstrated that the minimal inhibitory effect exerted by two strains of *Lpb. plantarum* on *G. vaginalis* was bactericidal. Some studies, analyzing the antagonistic activity of lactobacilli species, specifically through the formation of zones of inhibition by the agar overlay methodology, have shown that *Lpb. plantarum* strains stand out with better inhibition results, when compared to other LAB used. They showed extensive zones of inhibition against pathogens in both the digestive tract and the urogenital tract. This effect can be attributed to the presence of antagonistic factors, such as bacteriocins or similar substances which can vary in production, as well as to action between different strains and species (Xu et al. 2008, Dubourg et al. 2015).

It is important to obtain reproducibility of an effect when more than one technique is applied to assess and define the in vitro antimicrobial effect of lactobacilli species when selecting it as a possible probiotic candidate, as different factors, such as the state of the culture medium, can influence the outcome (De Gregorio et al. 2019). Studies have shown that a better bactericidal activity of some *Lactobacillus* strains and their supernatant against urogenital pathogens, including *G. vaginalis*, usually appears within the first eight hours of interaction (Coudeyras et al. 2008, Atassi et al. 2006). From the evaluation of the growth curve of the pathogen of interest in our study, it was observed that *G. vaginalis* presents an exponential growth phase in broth, which begins only after 5 to 8 h of incubation, reaching its maximum between 15 and 24 h, and subsequently entering the stationary phase up to 48 h for further decline (Pleckaityte et al. 2012,

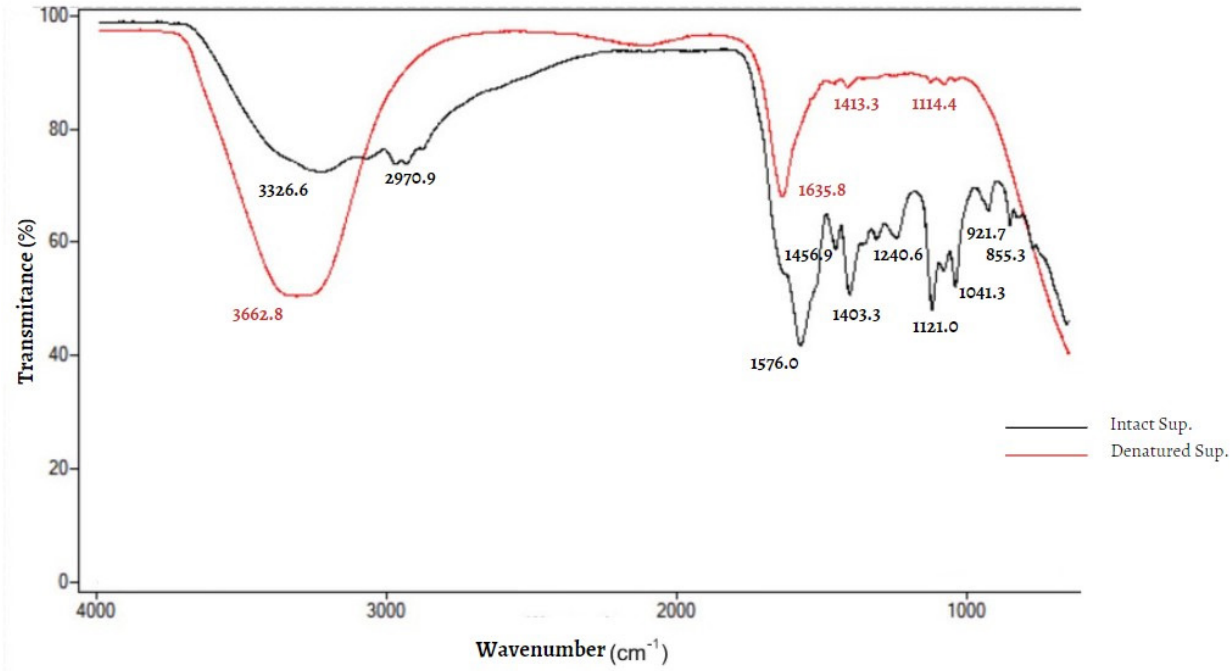

Figure 4. Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) of the intact and denatured supernatant of *Lactiplantibacillus plantarum* 6.2.

Anukam & Reid 2008). In our study, after 48 h of incubation, a smaller reduction in bacterial viability was noted with the use of 40 mg/mL *Lpb. plantarum* supernatant, while at the lowest concentrations, *G. vaginalis* was able to proliferate. Catlin (1992) inferred that *G. vaginalis* may have longer latency periods depending on the environment in which it is found, such as a more acidic one, extending the time necessary for colony formation, showing growth even after 24 h. This behavior reinforces the need for future studies to investigate the reproducibility of the antagonistic effect in higher concentration of supernatant with shorter interaction times.

There is a proportional relationship between the amount of the products secreted by lactobacilli and their inhibitory activity, with a possible dose-dependent effect demonstrated in the present study. This relationship was also observed by Vicariotto et al. (2014), in which the inhibitory capacity of different concentrations of neutralized culture supernatant of some strains of lactobacilli against *G. vaginalis* was evaluated. It was observed that using higher concentrations of a *Li. fermentum* strain showed strong inhibitory activity, both at 24 and 48 h. When using another strain of *Li. fermentum*, at lower concentrations, they observed a decrease in the percentage of inhibition between 24 and 48 h, yielding a reduction in the antagonistic effect by more than 10%.

The understanding of the inhibitory mechanisms of probiotic strains on the vaginal microenvironment in dysbiosis has numerous variables and methodological limitations. Some of these factors are as follows: the probiotic strain used, as certain mechanisms can be strain-specific; the state of the culture medium used in the *in vitro* study, as the liquid medium promotes a faster diffusion of the supernatant components; the growth environment, as the growth may be better in a microaerophilic or anaerobic environment depending on the strain; the components produced by lactobacilli (such as hydrogen peroxide, lactic acid); and bacteriocins and similar substances (Coudeyras et al. 2008, Hutt et al. 2006). Conversely, our study is very promising, as both the strain and the culture supernatant of lactobacilli obtained from cocoa fermentation, showed antagonistic properties against *G. vaginalis*. This effect has been only demonstrated by several authors when using probiotic species of lactobacilli isolated from the vaginal microenvironment itself. (Daniele et al. 2014, Breshears et al. 2015, Adreeva et al. 2016).

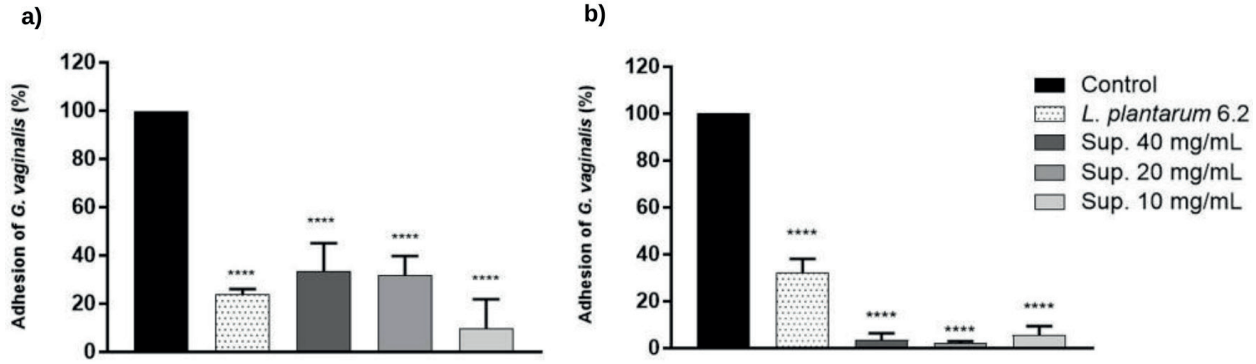

Figure 5. Exclusion (a) and displacement (b) of *Gardnerella vaginalis* adhering to HMVII cells promoted by treatment with *Lactiplantibacillus plantarum* 6.2 and its culture supernatant at different concentrations. Each value corresponds to the mean ± standard deviation of triplicates. Significant differences from the control are indicated by asterisks (**p < 0.0001).**

It is known that thermolabile substances can influence the antimicrobial action of lactobacilli, as demonstrated by some authors. Kang et al. (2017) observed that strains of *Ligilactobacillus salivarius* and *Li. fermentum* had a bactericidal effect on *S. aureus*, an effect that was significantly reduced after the culture supernatant was subjected to heat treatment; no inhibitory activity was observed after 24 h, suggesting that the secretion of proteins with specific antimicrobial properties would be one of the main mechanisms involved in the action of the supernatant. In a study conducted by Matu et al. (2010) for studying the effects of some *Lactobacillus* species (collected from the vaginal mucosa) against pathogens that cause vaginosis, when the culture supernatants were subjected to heat denaturation, none of the tested lactobacilli inhibited the growth of *P. bivia* or *Mobiluncus*; some did not inhibit *G. vaginalis*, with bacteriocins, and acid and hydrogen peroxide production had been suggested as a fundamental part of the antagonistic action of lactobacilli in the vaginal environment.

The functional structure of a bacteriocin is supported by the concept that protein molecules that undergo conformational changes can have their functions modified or neutralized, thereby losing their effect on bacteria (Klaenhammer 1993). Karaoğlu et al. (2003) evaluated the characteristics and antimicrobial properties of bacteriocins produced by lactobacilli of vaginal origin. They observed that six species of lactobacilli showed bacteriocins with inhibitory activity against *G. vaginalis* and *P. aeruginosa*, as well as that two bacteriocins of *L. gasseri* lost their inhibitory activity when subjected to high temperatures. Sabia et al. (2014) observed that an *Li. fermentum* strain produces a protein substance similar to a bacteriocin, which showed strong antagonistic activity against the growth of two other important pathogens in the vaginal tract, namely *C. albicans* and *Streptococcus agalactiae*, and the antimicrobial activity of bacteriocin was totally lost after exposure to a temperature of 121°C, suggesting that this substance is sensitive to heat and that the inhibitory activity is directly related to its presence.

Some studies have shown that some species of LAB, such as those belonging to lactobacilli and lactococci, can synthesize low molecular weight antibacterial substances, as well as high molecular weight substances, such as bacteriocins or similar (Klaenhammer 1988). Research on possible bacteriocins present in LAB has been described in the literature for some time; Talarico & Dobrogosz (1989) identified an antimicrobial substance isolated from *Li. reuteri*, a bacteriocin called reuterine, whose molecular structure was confirmed using FTIR.

Research on other isolated bacteriocins and their analysis using FTIR corroborates our hypothesis that the antimicrobial substance present in the *Lpb. plantarum* 6.2 supernatant is a bacteriocin or a bacteriocin-like substance (Fahim et al. 2017, Feliatra et al. 2018). In the last decade, the most studied antimicrobial agents were bacteriocins, mainly those produced by LAB (Song et al. 2014, Adebayo et al. 2014, Borrero et al. 2017). Further, it is already established in the literature that bacteriocins are proteins produced as secondary metabolites, and the greater their antagonistic effect, the better the microorganism's ability to produce them (Nofiani et al. 2009, Feliatra et al. 2018). In this study, the culture supernatant of *Lpb. plantarum* 6.2 demonstrated strong inhibitory capacity in two antagonism assays, with a possible activity of bacteriocins not yet identified, and the activity that was lost when it was submitted to denaturation. This was corroborated by FTIR assay that there was a reduction in the

transmittance of proteinaceous substances, the main group in the structure of a bacteriocin.

It was found that in cases of dysbiosis in the vaginal microenvironment, lactobacilli compete with pathogenic bacteria for nutrients and for the same binding sites in the epithelial cells of the vaginal mucosa, which may promote exclusion of the binding site or a displacement of the adhering pathogen (Santos et al. 2016c). Several studies have shown the ability of lactobacilli and their compounds to prevent the adhesion of pathogenic microorganisms to epithelial cells (Zárate & Nader-Macias 2006, Parolin et al. 2015). This adhesion is the first step towards colonization and biofilm formation, playing a fundamental role in the pathogenesis of bacterial vaginosis, and a greater adhesion capacity presented by *Lactobacillus* strains, in comparison to a pathogen, is among the most important properties for a strain to be considered an effective probiotic. Moreover, there is the possibility that it may induce the production of adhesins (Melgaço et al. 2018). Castro et al. (2013) reported that a strain of *L. crispatus* drastically reduced the adhesion of two strains of *G. vaginalis* on HeLa cells and suggested that lactobacilli may inhibit this adhesion through steric mechanisms or by masking or occupying receptors.

The level of competition between pathogens and lactobacilli can vary depending on the strains used, it can be determined by the affinity of adhesins on the bacterial surface for specific receptors in cells, which both are competing to adhering. Or because of their relative location, in case of a steric impediment, where lactobacilli "fit" better in cell receptors than pathogenic bacteria (Lee et al. 2003). The ability to inhibit the binding of a pathogen by displacement highlights that lactobacilli may have more affinity for specific receptors than other microorganisms (Coman et al. 2015).

Using the supernatant instead of the microorganism itself can show an advantage in having different biologically active substances, with possible synergistic effects, for future use in a single product to treat or prevent a certain pathology (Hartmann et al. 2011). The *Lpb. plantarum* 6.2 strain and its supernatant showed a significant effective action for preventive use by excluding *G. vaginalis* from the vaginal epithelial cell adhesion site and for curative use, by promoting displacement and even death (when the supernatant was used) of the *G. vaginalis* adhered to vaginal epithelial cells. Such action may occur due to the antagonistic properties of the *Lpb. plantarum* 6.2 strain and its culture supernatant as shown in the previous tests, which demonstrated a strong anti-*Gardnerella* antimicrobial effect present in both bacterial cells and the supernatant, with a possible mode of action through bacteriocins.

Using a microorganism or a product of its metabolism to inhibit the development of another microorganism is that this is a healthier alternative measure, as species of lactobacilli are naturally a part of the vaginal microbiota, without causing any apparent damage. However, more studies are needed because this study used lactobacilli extracted from fine cocoa fermentation and the *in vivo* behavior of this bacteria may be different from the *in vitro* behavior. Further, the possibility of a bacteriocin being one of its antimicrobial components present in the metabolites must also be investigated.

CONCLUSIONS

The *Lpb. plantarum* 6.2 strain extracted from fine cocoa fermentation, as well as its culture supernatant, showed a strong antagonistic effect on *G. vaginalis*. With the demonstration of such an effect, it is expected that the *Lpb.*

plantarum 6.2 strain can be considered a probiotic agent for use as a possible alternative (i.e., as a replacement of or complementation to currents treatments) to treat and prevent bacterial vaginosis caused by *G. vaginalis*.

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LR, CR and WP conceived and designed the experiments. LR, HF, MA, WP e CR generated and analyzed the data. RZ contributed reagents and equipment. LR, CR e WP drafted the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.