Research Article

Gardnerella vaginalis and *Neisseria gonorrhoeae* Are Effectively Inhibited by Lactobacilli with Probiotic Properties Isolated from Brazilian Cupuaçu (*Theobroma grandiflorum*) Fruit

Nathan das Neves Selis ⓘ,[1] Hellen Braga Martins de Oliveira ⓘ,[1] Yan Bento dos Anjos ⓘ,[2] Hiago Ferreira Leão ⓘ,[2] Beatriz Almeida Sampaio ⓘ,[2] Thiago Macêdo Lopes Correia ⓘ,[2] Mariane Mares Reis ⓘ,[2] Thamara Louisy Santos Brito ⓘ,[2] Carolline Florentino Almeida ⓘ,[2] Larissa Silva Carvalho Pena ⓘ,[2] Laís Ferraz Brito ⓘ,[2] Roberta Maria Ornelas ⓘ,[1,3] Tizá Teles Santos ⓘ,[1,3] Guilherme Barreto Campos ⓘ,[2] Jorge Timenetsky ⓘ,[4] Mariluze Peixoto Cruz ⓘ,[2] Andréa Miura da Costa ⓘ,[1,3] Regiane Yatsuda ⓘ,[2] Ana Paula Trovatti Uetanabaro ⓘ,[1,3] and Lucas Miranda Marques ⓘ[1,3]

[1]*Programa de Pós-Graduação em Biologia e Biotecnologia de Microrganismos, Pavilhão Max de Menezes, Universidade Estadual de Santa Cruz, Campus Soane Nazaré de Andrade, Salobrinho, Rodovia Jorge Amado, Km 16, CEP 45662-900 Ilhéus, BA, Brazil*
[2]*Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Campus Anísio Teixeira, Rua Hormindo Barros, 58, CEP 45029-094 Vitória da Conquista, BA, Brazil*
[3]*Departamento de Ciências Biológicas, Laboratório de Microbiologia da Agroindústria, Universidade Estadual de Santa Cruz, Campus Soane Nazaré de Andrade, Salobrinho, Rodovia Jorge Amado, Km 16, CEP 45662-900 Ilhéus, BA, Brazil*
[4]*Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenida Professor Lineu Prestes, 2415, CEP 05508-900 São Paulo, SP, Brazil*

Correspondence should be addressed to Lucas Miranda Marques; lmirandamarques@gmail.com

In recent years, certain *Lactobacillus* sp. have emerged in health care as an alternative therapy for various diseases. Based on this, this study is aimed at evaluating in vitro the potential probiotics of five lactobacilli strains isolated from pulp of cupuaçu fruit fermentation against *Gardnerella vaginalis* and *Neisseria gonorrhoeae*. Our lactobacilli strains were classified as safe for use in humans, and they were tolerant to heat and pH. Our strains were biofilm producers, while hydrophobicity and autoaggregation varied from 13% to 86% and 13% to 25%, respectively. The coaggregation of lactobacilli used in this study with *G. vaginalis* and *N. gonorrhoeae* ranged from 15% to 36% and 32% to 52%, respectively. Antimicrobial activity was present in all tested *Lactobacillus* strains against both pathogens, and the growth of pathogens in coculture was reduced by the presence of our lactobacilli. Also, all tested lactobacilli reduced the pH of the culture, even in incubation with pathogens after 24 hours. The cell-free culture supernatants (CFCS) of all five lactobacilli demonstrated activity against the two pathogens with a halo presence and CFCS characterization assay together with gas chromatography revealed that lactic acid was the most abundant organic acid in the samples (50% to 62%). Our results demonstrated that the organic acid production profile is strain-specific. This study revealed that cupuaçu is a promising source of microorganisms with probiotic properties against genital pathogens. We demonstrated by in vitro tests that our *Lactobacillus* strains have probiotic properties. However, the absence of in vivo tests is a limitation of our work due to the need to evaluate the interaction of our lactobacilli with pathogens in the vaginal mucosa. We believe that these findings may be useful in developing a product containing our lactobacilli and their supernatants in order to support with vaginal health.

1. Introduction

The World Health Organization (WHO) describes probiotics as live microorganisms that, when administered in adequate amounts, confer health benefits to the host [1]. Regarding women's health, probiotics have already shown that they can assist in recovering vaginal homeostasis. Generally, results from traditional therapies are optimized when associated with these microorganisms [2]. The benefits attributed to probiotics are related to (a) production of organic acids, (b) modulating the composition of the intravaginal microbiota [3], (c) antioxidant activity and reducing damages caused by oxidation [4], (d) effects on the functionality of the mucosal and systemic immune systems, (e) reducing anti-inflammatory reactions, (f) competition with pathogens [5], and (g) production of hydrogen peroxide and bacteriocins [6].

In the last decade, health professionals have become increasingly interested in new treatment alternatives as a result of consumer demands for better therapies, due to problems resulting from antibiotic resistance and undesirable side effects [7]. Reports from the literature show that lactobacilli isolated from functional foods demonstrate promising probiotic effects beneficial to human health. A number of studies focused on new strains that have probiotic characteristics illustrate that the beneficial properties are strain-specific and can act on different pathogens [8–11]. The administration of probiotics that colonize the vaginal tract can be important in maintaining normal urogenital health and also preventing and treating infections [12], such as bacterial vaginosis (BV) and gonorrhea.

BV is the most commonly reported microbiological syndrome among women of childbearing age, where the genital microbiota changes from the dominant *Lactobacillus* sp. to a polymicrobial anaerobic population [13]. Currently, with uncertain pathogenesis, BV is no longer considered an infection but rather as a dysbiosis, a microbial imbalance in the vaginal microbiota. Many microbial pathogens have been associated with BV; however, *Gardnerella vaginalis* is the bacteria most related to this clinical condition [14]. BV recurrence rates are high in symptomatic women who present vaginal malodor, discharge, itching, and increased vaginal pH [15]. BV is related to obstetric and gynecological complications, including pelvic inflammatory disease, premature labor, and increased incidences of sexually transmitted infections, such as cervicitis by *Neisseria gonorrhoeae*, bacteria that causes gonorrhea [16].

Gonorrhea is a sexually transmitted infection of global proportions associated with pelvic inflammatory disease, infertility, urethritis in men, and mucopurulent cervicitis in women [17]. The gonococci colonize the genital, anal, ocular, and nasopharyngeal mucosa. With a wide variety of niches, *N. gonorrhoeae* expresses a repertoire of factors that allow its replication, survival and modulation, and evasion of the host immune system [18]. In recent years, even with effective antibiotics, gonorrhea remains a serious and common public health problem, and the emergence of resistant strains has hampered and limited treatment options [19]. Thus, considering that researchers around the world have sought alterna-

tive solutions against genital pathogens of medical importance, our study is aimed at evaluating in vitro the potential probiotic effects of five *Lactobacillus* strains isolated from cupuaçu (*Theobroma grandiflorum*) fruit fermentation against *G. vaginalis* and *N. gonorrhoeae*.

2. Material and Methods

2.1. Microorganisms and Growth Conditions. *Lactobacillus* sp. were isolated from originate spontaneous pulp of cupuaçu (*Theobroma grandiflorum*) fruit fermentation from Ilhéus and Itabuna region, Bahia, Brazil, and donated by the Laboratory of Applied Microbiology from the State University of Santa Cruz, Brazil [20]. The five lactobacilli strains used in this study were *Lactobacillus casei* (Lc24), *Lactobacillus fermentum* (Lf38 and Lf47), and *Lactobacillus plantarum* (Lp81 and Lp90). The lactobacilli strains were grown in de Man, Rogosa, and Sharpe (MRS) agar or broth (Acumedia, Lansing, USA) for 18 to 24 hours, at 37°C, under microaerophilic conditions (5% CO_2 atmosphere)—overnight cultures.

Gardnerella vaginalis ATCC 49154 was grown on 5% blood agar (HiMedia Laboratories, Mumbai, India) or Brain and Heart Infusion (BHI) broth (HiMedia) for 18 to 24 hours, at 37°C, under microaerophilic conditions (5% CO_2 atmosphere)—overnight culture.

Neisseria gonorrhoeae (clinical isolate) was grown on chocolate agar (HiMedia) or BHI broth for 18 to 24 hours, at 37°C, under microaerophilic conditions (5% CO_2 atmosphere)—overnight culture.

2.2. Preparation of Cell-Free Culture Supernatant (CFCS). The assay for obtaining the CFCS was adapted from Pessoa et al. [21]. After overnight cultures of *Lactobacillus* strains were centrifuged ($3,000 \times g$, 15 min), the supernatants were discarded; cell pellets (lactobacilli) were washed twice with sterile saline (0.9% NaCl) and resuspended to a final concentration (10^8 CFU.mL^{-1}). After this, suspensions (1.5 mL) of each *Lactobacillus* sp. were inoculated (10%, *v/v*) in sterile MRS broth (15 mL), and after incubation (24 h, 37°C, 5% CO_2 atmosphere), the cultures were centrifuged ($3000 \times g$, 15 min); supernatants were aspirated using sterile syringes and sterilized by filtration (0.22 μm nitrocellulose filter; Merck, Darmstadt, Germany) for obtaining CFCS.

2.3. Technological Evaluation of Lactobacilli

2.3.1. Heat Tolerance Assay. Heat resistance of our lactobacilli strains was evaluated according to Paéz et al. [22] with modifications. Initially, suspensions (10^8 CFU.mL^{-1}) of lactobacilli strains were obtained as previously described. An aliquot (100 μL) was resuspended in volume (500 μL) of 10% skim milk (Nestlé, Araçatuba, Brazil). Then, each cell suspension was incubated in a water bath (60°C, 5 min), followed by cooling in an ice bath. Aliquots (10 μL) of each strain were plated on MRS agar, and after incubation (48 h, 37°C, 5% CO_2 atmosphere), the colonies were counted and enumerated considering CFU.mL^{-1}. As a control, aliquots (10 μL) of the same samples were plated under the same conditions before exposure to heat.

2.3.2. pH Tolerance Assay. The analysis of bacterial growth under varied pH was adapted from Melo et al. [10]. MRS broth solutions of pH 3, pH 4, pH 5, pH 6, pH 7, and pH 8 were prepared by addition of 1 mol.L^{-1} of hydrochloric acid or 1 mol.L^{-1} of sodium hydroxide. The trials were performed in 96-well microplates, where in $180 \mu L$ of MRS broth at each pH was inoculated with $20 \mu L$ of active culture (10^8 CFU.mL^{-1}) or saline as a control. The microplate was incubated overnight, and the optical density (600 nm) was determined every 8 hours using a spectrophotometer (Tp-reader, Thermoplate, USA).

2.4. Safety Evaluation of Lactobacilli

2.4.1. Antibiotic Susceptibility Assay. Susceptibility of *Lactobacillus* strains to antimicrobials was determined by the modified agar diffusion method of Clinical and Laboratory Standards Institute (CLSI). Overnight cultures of *Lactobacillus* strains were adjusted to 0.5 McFarland standards. Then, an aliquot of this suspension was swabbed onto MRS agar plates, followed by the arrangement of antibiotic disks. Plates were incubated overnight, and the diameters of the halos were measured and classified as sensitive (S), moderately sensitive (MS), and resistant (R), according to Charteris et al. [23]. The antimicrobials (Laborclin, Pinhais, Brazil) tested were ampicillin ($10 \mu g$), ceftriaxone ($30 \mu g$), ciprofloxacin ($5 \mu g$), clindamycin ($2 \mu g$), chloramphenicol ($30 \mu g$), erythromycin ($15 \mu g$), nitrofurantoin ($300 \mu g$), penicillin ($10 \mu g$), and vancomycin ($30 \mu g$).

2.4.2. Hemolytic Activity Assay. The hemolytic activity assay was adapted from Abouloifa et al. [9]. Initially, suspensions (10^8 CFU.mL^{-1}) were spot-inoculated ($10 \mu L$) on 5% blood agar. After incubation (48 h, 37°C, 5% CO_2 atmosphere), the hemolytic activity was detected by observing a clear zone of hydrolysis around the colonies (β-hemolysis), partial hydrolysis with green-hued zones around colonies (α-hemolysis), or no zone around colonies (γ-hemolysis). The γ-hemolysis was considered negative hemolysis.

2.5. Evaluation of the Cell Surface Properties of Lactobacilli

2.5.1. Biofilm Formation Assay. Biofilm formation assay was adapted from Ouarabi et al. [24]. Initially, an aliquot ($10 \mu L$) of each *Lactobacillus* (10^8 CFU.mL^{-1}) was inoculated in MRS broth ($200 \mu L$) in a 96-well polystyrene plate and then incubated overnight. After incubation, the plate was washed twice with sterile saline to remove nonadherent cells. The cells were fixed with 96% ethanol ($200 \mu L$) and incubated (15 min, room temperature). After this, the plates were emptied and then filled with violet crystal ($200 \mu L$, 0.1%) and incubated (15 min, room temperature). Then, the plate was washed twice with sterile saline, and the wells were resuspended with 96% ethanol ($200 \mu L$). The absorbance (650 nm) of the samples was immediately measured and taken as an indication of biofilm formation. Sterile medium was included as a negative control to ensure that the influence on biofilm formation was not attributed to a nonspecific binding effect to the violet crystal. Based on the optical densities of the isolates (DO_I) and the negative control (DO_C),

the formation of biofilm by lactobacilli was classified according to their adherence: nonadherent, $DO_I \leq DO_C$; weakly adherent, $DO_C < DO_I \leq (2 \times DO_C)$; moderately adherent, ($2 \times DO_C) < DO_I \leq (4 \times DO_C)$; and strongly adherent, ($4 \times DO_C) < DO_I$.

2.5.2. Hydrophobicity Assay. The hydrophobicity of the *Lactobacillus* strains was verified by testing microbial adherence to hydrocarbons (MATH), using a method adapted from Rodríguez et al. [25], and using xylene as solvent. Initially, suspensions (10^8 CFU.mL^{-1}) of lactobacilli strains were measured (OD 660 nm). The solvent (xylene, 0.4 mL) was then added to each bacterial suspension (1 mL), and the mixtures were vortexed vigorously and incubated (2 h, 37°C). Then, the lower aqueous phase was carefully removed and read in a spectrophotometer. The percentage of hydrophobicity was calculated using Equation (1), where A_0 indicates the absorbance at time 0 hour and A_2 indicates the absorbance after 2 hours. MATH can be classified as low (MATH < 33%), medium (33% < MATH < 66%), or high (MATH > 66%). The hydrophobicity can also be presented as microbial adhesion to solvents (MATS), classifying the bacterial surface as hydrophobic (MATS $\geq$ 55%), amphiphilic (45% $\leq$ MATS $\leq$ 55%), or hydrophilic (MATS $\leq$ 45%).

$$H\% = \frac{A0 - A1}{A0} \times 100. \qquad (1)$$

2.5.3. Autoaggregation Assay. Autoaggregation was adapted from Kos et al. [26]. Initially, suspensions of lactobacilli (10^8 CFU.mL^{-1}) were vortexed (10 s) and incubated (5 h, room temperature). The absorbance (660 nm) was measured at time 0 hour (A_0) and after 5 hours (A_5). The percentage of autoaggregation was calculated using Equation (2).

$$AA\% = \frac{A0 - A5}{A0} \times 100. \qquad (2)$$

2.5.4. Coaggregation Assay. Coaggregation was adapted from Kos et al. [26]. Initially, cell suspensions (10^8 CFU.mL^{-1}) containing mixed suspensions containing equal volumes (1 mL) of each *Lactobacillus* and pathogens were vortexed (10 s) and incubated (4 h, 37°C). The absorbance (660 nm) was measured before and after incubation. The percentage of coaggregation was calculated using the formula below, where A_{LAC} indicates the absorbance of lactobacilli, A_{PAT} indicates the absorbance of pathogen, and A_{MIX} indicates the absorbance of the mixtures.

$$CA\% = \frac{((Alac + Apat)/2) - Amix}{(Alac + Apat)/2}. \qquad (3)$$

2.6. Evaluation of Antimicrobial Activity of Lactobacilli

2.6.1. Deferred Inhibition Assay. The antimicrobial activity evaluated by deferred inhibition assay was tested according to Nardi et al. [27]. Initially, an aliquot ($5 \mu L$) of each lactobacilli strain suspension (10^8 CFU.mL^{-1}) was pipetted in the center of the plate with MRS agar. After incubation (48 h,

$37°C$, 5% CO_2 atmosphere), colony cells were killed by exposure to chloroform (100 mL, 30 min). Residual chloroform was evaporated off, and the Petri dish overlayed with BHI semisolid agar (3.5 mL, 0.75%, w/v) previously inoculated with pathogens (1%, v/v, 10^8 CFU.mL^{-1}). After overnight incubation (18–24 h, $37°C$, 5% CO_2 atmosphere), the presence or absence of inhibition halos was observed, followed by measuring the inhibition halos (millimeters). Sterile MRS broth was considered a negative control.

2.6.2. Microdiffusion Assay. The presence of diffusible inhibitory substances was also evaluated by the microdiffusion assay on semisolid agar adapted from Rodrigues et al. [28]. Initially, suspensions of pathogens (10^8 CFU.mL^{-1}) were added (1%, v/v) on semisolid BHI agar (0.75%, w/v) and plated. After solidification, sterile PVC cylinders (8 mm) were placed centrally on the plates, and inside them, aliquots (100 μL) of CFCS from each *Lactobacillus* were added. After incubation (18–24 hours, $37°C$, 5% CO_2 atmosphere), the presence or absence of inhibition halos was observed, followed by measuring the inhibition halos (millimeters). Sterile MRS broth was considered a negative control.

2.6.3. pH Modulation Assay by Lactobacilli. To evaluate the ability of lactobacilli to modulate the pH of the growth medium by producing organic acids, the methodology of Melgaço et al. [29] was used with modifications. The modulation of the pH of the growth medium by lactobacilli was evaluated under two conditions: isolated growth of *Lactobacillus* or growth of *Lactobacillus* in coincubation with pathogen. Initially, the microorganism suspensions (10^8 CFU.mL^{-1}) were obtained as previously described, and the pH of the MRS or MRS + BHI (v/v) broth was measured and adjusted to 6.5. Then, an aliquot of each *Lactobacillus* was added (10%, v/v) to the MRS or MRS + BHI broth. After that, the same volume of pathogens was added to the MRS + BHI broth. After incubation (24 h, $37°C$, 5% CO_2 atmosphere), the cultures were centrifuged ($3,000 \times g$, 15 min); the supernatant was separated from the bacterial pellet, and the pH was measured (HMMPB-210, Highmed, Tatuapé, Brazil).

2.6.4. Inhibition Assay by Coculture. The antimicrobial activity of lactobacilli against pathogens was tested by coculture assay adapted from Hütt et al. [30]. Initially, activated cultures of pathogens and lactobacilli (10^8 CFU.mL^{-1}) were inoculated together (1%, v/v) in mixed growth medium (0.5 mL BHI broth + 0.5 mL MRS broth) and were incubated overnight. Then, serial dilutions were performed, and aliquots (10 μL) were seeded on blood or chocolate agar, and the plates were incubated again overnight. Cultures performed with the pathogen alone were used as negative controls. The growth of the pathogen with each *Lactobacillus* strain was compared with the growth of the control.

2.7. Evaluation and Characterization of the CFCS

2.7.1. Amplex Red Hydrogen Peroxide Assay. Hydrogen peroxide levels present in CFCS were measured using the Amplex Red Hydrogen Peroxide/Peroxidase kit according to the manufacturer's recommendations (Thermo Fisher Sci-

entific, Waltham, USA). After preparing the kit stock solutions, aliquots (50 μL) of the standard curve samples, controls, and experimental samples were added to individual wells on a microplate. After that, the Amplex® Red reagent/HRP working solution (50 μL) was added to the wells previously plotted. After incubation (30 min, room temperature, protected from light), absorbance was measured in a microplate reader (550 nm) to construct the standard curve and measure the H_2O_2 concentration (μM) of the CFCS.

2.7.2. Detection of Organic Acids, Thermotolerant Antimicrobial Substances, and Bacteriocin in the CFCS. The lactobacilli strains were assayed for production of organic acids, thermotolerant antimicrobial substances, and bacteriocins using the agar well diffusion technique described by Touré et al. [31] with modifications. Initially, *G. vaginalis* suspension (10^8 CFU.mL^{-1}) was swabbed onto 5% blood agar plates, and *N. gonorrhoeae* suspension (10^8 CFU.mL^{-1}) was swabbed onto chocolate agar plates. After that, the plates were incubated (30 min, room temperature). Concomitantly, CFCS aliquots were distributed in fractions for treatment. For the organic acid assay, the CFCS was adjusted to pH 6.5 ± 0.1 using 1 mol.L^{-1} of sodium hydroxide; for the thermotolerant substance assay, the CFCS was incubated at high temperature (5 min, $100°C$), and for bacteriocin assay, the CSCF was treated with trypsin (1%, v/v; Gibco, Mississauga, Canada) or proteinase K (1%, v/v, Invitrogen, Darmstadt, Germany). Then, aliquots (100 μL) of treated CFCS and untreated CFCS were added into the wells (8 mm diameter) previously made on the chocolate and 5% blood agar plates. The plates were incubated overnight, and diameters of inhibition zones (including the 8 mm-well diameter) were measured.

2.7.3. Analysis of the CFCS Metabolome by GC-MS. The composition analysis of CFCS was performed by gas chromatography-mass spectrometry (GC-MS) according to the method described by Rodrigues et al. [28]. First, CFCS were lyophilized for this assay. Lyophilized CFCS were submitted to derivatization reaction by silylation. In this reaction, each sample (3 mg) was diluted in pyridine (60 μL), and 100 μL of *N,O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane was added to this solution (Sigma-Aldrich, Darmstadt, Germany). The solutions were heated ($70°C$, 30 min) in a water bath, and after that, an aliquot (1 μL) of the diluted samples was injected into the QP2010SE-GC2010 Plus (Shimadzu, Kyoto, Japan) chromatograph with Rtx-5MS (30 m, 0.25 mm internal diameter, 0.25 μm film). For chromatographic analysis, helium gas was used as the carrier gas. The temperature employed in the injector, detector, and interface GC-MS system was $290°C$. The analysis of the initial temperature was $80°C$ (5 min), progressively increasing up to $285°C$ in a ratio of $4°C$/minutes. The final temperature remained at $285°C$ (20 min). The detector mass operated ionization electron impact (70 eV), and the scan mass operated 30 to 600 Da. The identification of compounds by GC-MS was performed by comparing the mass spectra of the samples with existing

spectra in the device database (NIST 08, FFNSC1.3 and WILEY8).

2.8. *Statistical Analysis.* The GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, USA) was used for statistical analysis. Quantitative data are presented by means and standard deviations. Normality was tested by D'Agostino and Pearson, Shapiro-Wilk, and KS tests. The statistical differences between the mean values were determined by the t-test, Mann–Whitney test, or Kruskal-Wallis test with Dunn's posttest. Data were considered statistically significant when $* = P < 0.05$, $**P < 0.01$, $*** = P < 0.001$, and $**** = P < 0.0001$. Except for CFCS metabolome, all assays were performed in triplicate.

3. Results and Discussion

3.1. *Technological Characteristics of Lactobacilli.* Figure 1 shows the resistance of five lactobacilli strains to high temperature. According to our data, all lactobacilli tested had a significant reduction in viable cells ($P < 0.05$). Nevertheless, all *Lactobacillus* strains remained viable after the heat shock. Many species of lactobacilli are known to tolerate a rather wide temperature range, with most species capable of growing to at least 45°C [32]. The use of thermotolerant strains is advantageous for the pharmaceutical, beverage, and food industries, as their genetic features are available, adjustable, and better developed to face stressful processes [33–35]. In general, our data showed that five lactobacilli strains were considered thermotolerant, demonstrating their ability to grow at 50°C.

Our strains were also tested for their ability to survive and grow at different pH ranges, as can be seen in Figure 2. With the exception of the Lp90 strain that did not grow at pH 3, all other lactobacilli strains grew at all pHs. The resistance at pH 3 is the standard for acid tolerance of probiotic culture, and the ability of lactobacilli to adapt to the acidic environment can be influenced by growth in MRS broth [36, 37]. Using lactobacilli that grow in a wide pH range (3–8), as occurred with our strains, is of relevant and useful for intravaginal applications in patients with genital infections dominated by anaerobes that raise the pH. The introduction of lactobacilli that survive in this environment may, after adaptation, lower the pH through the production of organic acids and reduce the population of uropathogens [2].

3.2. *Safety Assessment of Lactobacilli for Use in Humans as Probiotics.* Probiotics are safe microorganisms, and nowadays, they are consumed as food, dietary supplements, and medicine all around the world. However, in recent years, many researchers have warned of an increase in antibiotic resistance in lactobacilli strains [38]. Furthermore, the WHO recommended that classifying a microorganism as a probiotic should be based on a series of tests including checking the safety of the strains, such as resistance to antibiotics and hemolytic activity [39]. Thus, we consider it necessary to guarantee the safety of our strains, since there is the possibility of transferring resistance genes from lactobacilli to pathogens or commensal bacteria [40]. In this study, the anti-
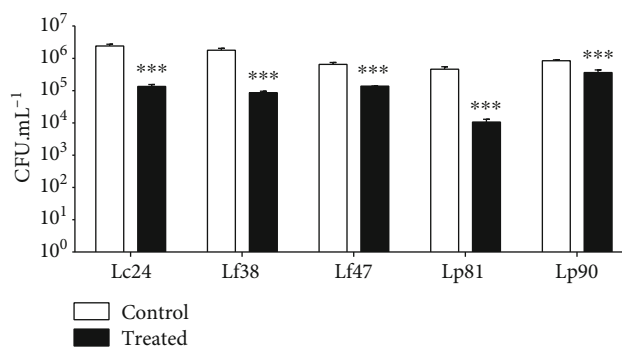

FIGURE 1: Heat resistance standard of lactobacilli isolated from cupuaçu fermentation. Lc24: *L. casei* 24; Lf38: *L. fermentum* 38; Lf47: *L. fermentum*; Lp81: *L. plantarum* 81; Lp90: *L. plantarum* 90. ***Statistically significant differences compared to control ($P < 0.001$). Presented values represent the mean and the standard deviation from triplicate determinations.

microbial susceptibility profile of lactobacilli isolated from pulp of cupuaçu fruit was tested against nine antibiotics belonging to different classes (Table 1). All lactobacilli tested were resistant to vancomycin and sensitive to ampicillin, ceftriaxone, penicillin G, clindamycin, chloramphenicol, erythromycin, and nitrofurantoin.

Regarding ciprofloxacin, Lc24 and Lf47 showed resistance, and Lf38, Lp81, and Lp90 strains were classified as moderately sensitive. Resistance to quinolones (ciprofloxacin) and glycopeptides (vancomycin) exhibited by our lactobacilli strains may be related to the intrinsic resistance mechanism of this genus in relation to these antibiotics [41]. Intrinsic resistance is generally harmless when present in lactobacilli because it is not a transferable characteristic. Some authors have shown that lactobacilli may be resistant to ciprofloxacin due to the modified topoisomerase IV present in lactobacilli and that they are the main target of quinolone [42, 43]. In relation to vancomycin, lactobacilli are naturally resistant due to the presence of peptidoglycan precursors with d-alanyl-d-lactate termination that prevent the binding of the antibiotic to the cell wall [44].

Together with these data, the results in Table 2 show that all *Lactobacillus* strains were considered nonhemolytic. Previously, it was believed that this virulence factor was related only to pathogens and that lactobacilli were unable to cause hemolysis [45, 46]. However, there are reports in the literature that have demonstrated the existence of lactobacilli with hemolytic activity [47]. A study conducted by Kaktcham et al. [48], for example, described lactobacilli isolated from food and dairy products with α-hemolysis. Many authors, however, reported lactobacilli without hemolytic activity [48–51], similar to our data. Regarding hemolytic activity, our *Lactobacillus* strains have proven to be safe due to the absence of this characteristic.

3.3. *Cell Surface Properties of Lactobacilli.* The results of the biofilm formation and hydrophobicity of the *Lactobacillus* strains are reported in Table 2. Producing biofilm is a fundamental microbial survival mode, and the evaluation of biofilm formation and its time-dependent mechanisms are
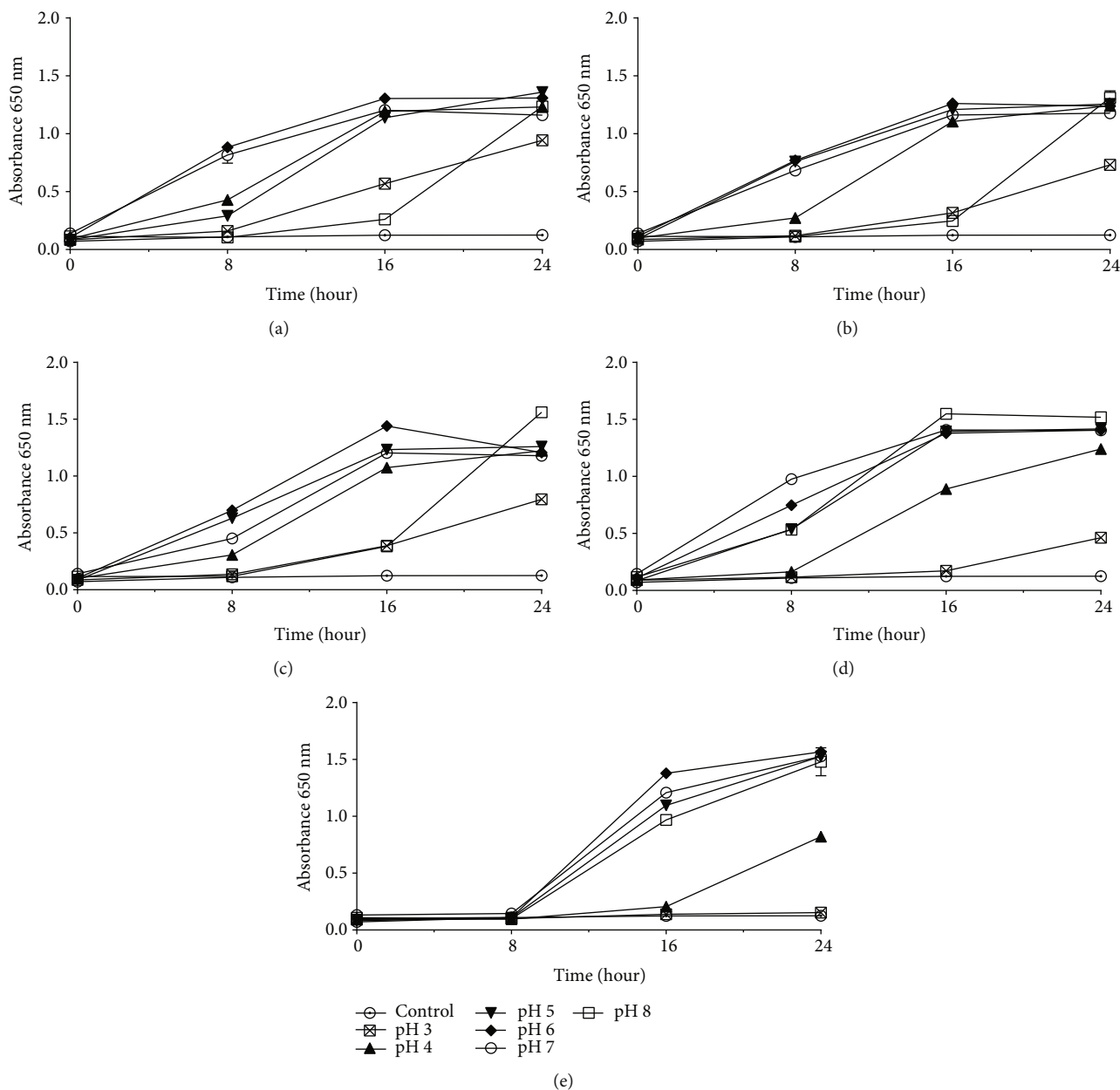
Figure 2: Growth of lactobacilli in different pH ranges: (a) *L. casei* 24; (b) *L. fermentum* 38; (c) *L. fermentum*; (d) *L. plantarum* 81; (e) *L. plantarum* 90. Presented values represent the mean and the standard deviation from triplicate determinations.

important for understanding developing therapeutic interventions and host-microbial interaction [52]. When assessing the ability of strains to form biofilm, all lactobacilli tested were biofilm producers. Our data revealed that the strains Lc24 and Lf38 were classified as moderately adherent, and the strains Lf47, Lp81, and Lp90 were considered strong biofilm producers. The types of biofilms formed by lactobacilli may be strain specific and may be related to the specific bacterial properties of the surface encoded by each genome [53]. The formation of biofilm by lactobacilli is a key factor for maintaining these microorganisms in stable in vivo in vivo ecosystems. Thus, it is clear that the formation of biofilm by beneficial strains is a desirable probiotic property,

since it can promote both the colonization and the long-term presence of lactobacilli in vaginal mucosa [54].

In the present study, our lactobacilli strains showed great variation in relation to hydrophobicity, ranging from 12.56% to 85.9%. Similarly, other authors have observed great variation in the hydrophobicity of their *Lactobacillus* strains [55, 56]. According to the MATH classification, Lc24 and Lf38 showed low hydrophobicity, Lp81 showed moderate hydrophobicity, and Lf47 and Lp90 strains showed high hydrophobicity. Regarding MATS classification, Lf47 and Lp90 were considered hydrophobic, Lc24 and Lf47 were considered hydrophilic, and the Lp81 strain was considered amphiphilic. It is known that hydrophobicity is an important

TABLE 1: Susceptibility profile of five lactobacilli isolated strains from cupuaçu fermentation.

Antimicrobial Type	Name	Disc contents	Susceptibility				
			Lc24	Lf38	Lf47	LP81	Lp90
Inhibitors of cell wall synthesis	Ampicillin	$10 \, \mu g$	S	S	S	S	S
	Ceftriaxone	$30 \, \mu g$	S	S	S	S	S
	Penicillin G	$10 \, \mu g$	S	S	S	S	S
	Vancomycin	$30 \, \mu g$	R	R	R	R	R
Inhibitors of protein synthesis	Clindamycin	$2 \, \mu g$	S	S	S	S	S
	Chloramphenicol	$30 \, \mu g$	S	S	S	S	S
	Erythromycin	$15 \, \mu g$	S	S	S	S	S
Inhibitors of nucleic acid synthesis	Ciprofloxacin	$5 \, \mu g$	R	MS	R	MS	MS
Other urinary tract antiseptics	Nitrofurantoin	$300 \, \mu g$	S	S	S	S	S

Susceptibility expressed as sensitive (S), moderately sensitive (MS), or resistant (R).

TABLE 2: Hemolytic activity and characterization of physiological and antimicrobial properties of five lactobacilli strains isolated from cupuaçu fermentation.

Strain	Hemolytic activity	Biofilm formation	Hydrophobicity (%)	Autoaggregation (%)	Coaggregation (%)	
					G. vaginalis	N. gonorrhoeae
Lc24	γ-Hemolytic	Moderately adherent	12.56 ± 1.11^a	25.18 ± 0.72^a	18.25 ± 0.73^{a1}	36.82 ± 2.40^{a2}
Lf38	γ-Hemolitic	Moderately adherent	28.03 ± 0.76^b	23.33 ± 1.93^a	15.83 ± 1.44^{b1}	32.11 ± 1.53^{b2}
Lf47	γ-Hemolitic	Strongly adherent	85.90 ± 2.91^c	13.13 ± 0.44^b	19.66 ± 0.83^{a1}	39.71 ± 0.00^{a2}
Lp81	γ-Hemolitic	Strongly adherent	40.29 ± 1.25^d	20.30 ± 1.30^a	14.93 ± 2.24^{b1}	36.13 ± 3.53^{a2}
Lp90	γ-Hemolitic	Strongly adherent	81.54 ± 2.78^c	23.57 ± 0.72^a	35.55 ± 2.98^{c1}	51.70 ± 2.26^{c2}

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (±standard deviation) of coaggregation within the same column followed by different superscript letters differ significantly ($P < 0.05$). Mean values (±standard deviation) of coaggregation within the same line followed by different superscript numbers differ significantly ($P < 0.05$).

physicochemical property that interferes with adhesion of bacteria to host cells, which varies between strains [57, 58]. In this way, adherence is an important probiotic criterion for selecting probiotic strains, as it is also involved in the modulation of the host's immune response [59, 60]. Our data for Lf47 and Lp90 strains showed a significant increase in hydrophobicity compared to the Lc24 strain ($P < 0.05$), suggesting that these strains may remain in the vaginal mucosa longer than other lactobacilli tested.

The ability of lactobacilli to aggregate with strains of the same species was performed by the autoaggregation assay. Table 2 shows that the autoaggregation did not vary between *Lactobacillus* species (20.30% to 23.57%), except for Lf47, which presented less aggregation than the others (13%) ($P < 0.05$). In a study done by Chen et al. [61], *L. casei* and *L. plantarum* were evaluated for autoaggregation for 24 hours. Up to the time of 4 hours, all lactobacilli strains showed 7.8% to 12.8% of autoaggregation, lower values than those presented by our strains after 5 hours. However, the authors demonstrated that after 24 hours of incubation, all strains increased the percentage of autoaggregation to values between 32.12% and 47.04%, suggesting that aggregation among lactobacilli tends to increase over time. In fact, the autoaggregation capacity of lactobacilli is time-dependent and strain-specific, highlighting the importance of this mechanism as an association factor with other probiotic character-

istics such as biofilm formation and mucosal adhesion [62–64].

In a similar way to autoaggregation, the direct interaction of lactobacilli strains with genital pathogens was evaluated by the coaggregation assay. The Lp90 strain showed the highest percentages of coaggregation with *G. vaginalis* and *N. gonorrhoeae* with 35.55% and 51.70%, respectively. Our data (Table 2) demonstrate that our lactobacilli were able to aggregate more efficiently with *N. gonorrhoeae* compared to *G. vaginalis* ($P < 0.05$). Coaggregation is a key event for eliminating pathogens. It has been suggested that, through this mechanism, the direct interaction of lactobacilli with pathogenic microorganisms creates an antagonistic microenvironment for pathogens, in which antimicrobial substances are secreted locally, impairing epithelial colonization [65]. Similarly, other authors have also demonstrated the ability of lactobacilli to coaggregate with *N. gonorrhoeae* [66, 67]. Other authors, however, report coaggregation data with *G. vaginalis* with a highly variable percentage, something that may indicate that the coaggregation of lactobacilli with this pathogen must be strain-specific [68, 69].

3.4. Detection of Inhibitory Activity of Lactobacilli. Table 3 shows the identification of antimicrobial compounds against *G. vaginalis* and *N. gonorrhoeae*. Two different assays were used to evaluate inhibiting activity of the lactobacilli strains

TABLE 3: Antimicrobial evaluation of bioactive compounds produced by five lactobacilli strains isolated from cupuaçu fermentation.

Strain/broth	Deferred inhibition (mm)		Microdiffusion (mm)		pH		
	G. vaginalis	N. gonorrhoeae	G. vaginalis	N. gonorrhoeae	Isolated growth	Coincubation (Gv)	Coincubation (Ng)
Lc24	15.00 ± 0.00	26.33 ± 0.58	Contact	Contact	4.38 ± 0.09^{a1}	4.23 ± 0.02^{a1}	4.48 ± 0.01^{a1}
Lf38	15.67 ± 1.53	20.67 ± 1.16	Contact	Contact	4.37 ± 0.13^{a1}	4.20 ± 0.02^{a1}	4.68 ± 0.27^{b1}
Lf47	14.33 ± 1.16	25.00 ± 0.00	Contact	Contact	4.37 ± 0.13^{a1}	4.26 ± 0.01^{a1}	4.37 ± 0.15^{a1}
Lp81	21.00 ± 0.00	23.33 ± 3.51	Contact	Contact	3.93 ± 0.21^{b1}	4.04 ± 0.04^{b1}	4.13 ± 0.03^{c1}
Lp90	21.00 ± 0.00	31.67 ± 2.89	17.33 ± 0.58	13.67 ± 0.58	3.78 ± 0.05^{b1}	3.85 ± 0.02^{c1}	3.75 ± 0.02^{d1}
MRS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.50 ± 0.00^{c1}	6.50 ± 0.00^{d1}	6.50 ± 0.00^{e1}

Presented values of pH assay are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (± standard deviation) within the same column followed by different superscript letters differ significantly ($P < 0.05$). Mean values (±standard deviation) within the same line followed by different superscript numbers differ significantly ($P < 0.05$). Gv: G. vaginalis; Ng: N. gonorrhoeae. The measurement of the inhibition halos of the microdiffusion assay includes the diameter of the PVC cylinder (8 mm). Contact means that there was no growth of the pathogen just inside the PVC cylinder.
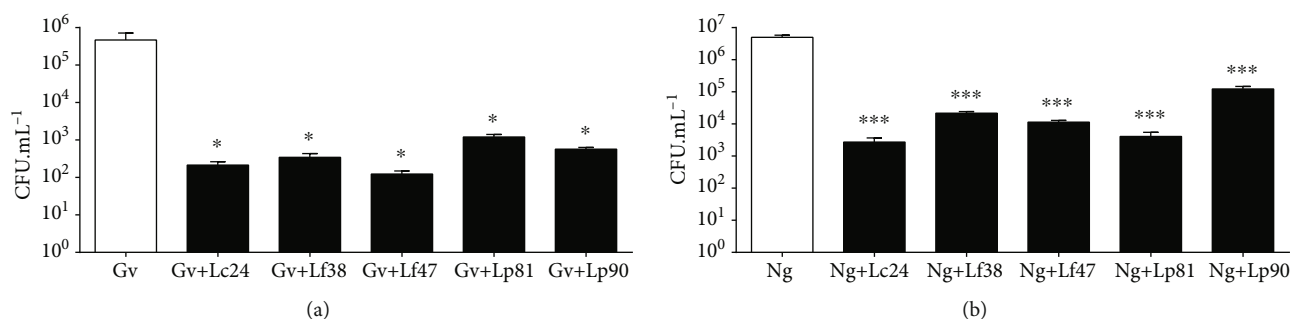

(a)

(b)

FIGURE 3: Growth inhibition of pathogens by lactobacilli after 24 h of culture. The growth of pathogenic microorganism is expressed as $\log_{10}$ CFU.mL^{-1}. Each value corresponds to the mean ± standard deviation of triplicate determinations. Control: (Gv) G. vaginalis or (Ng) N. gonorrhoeae. The different lactobacilli isolates are represented by their respective numbers: (a) represents coculture of G. vaginalis with lactobacilli; (b) represents coculture of N. gonorrhoeae with lactobacilli. *Statistically significant differences compared to control ($P < 0.05$). ***Statistically significant differences compared to control ($P < 0.001$). Presented values represent the mean and the standard deviation from triplicate determinations.
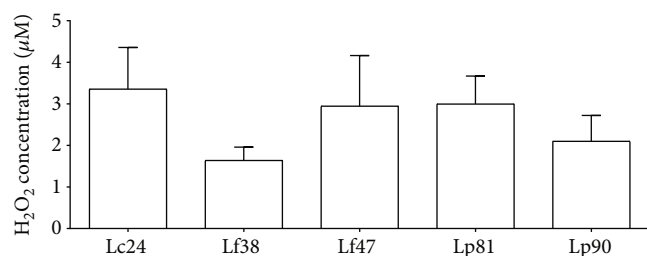

FIGURE 4: Hydrogen peroxide levels in CFCS of lactobacilli isolated from cupuaçu fermentation. The standard curve ($R^2 = 0.9927$) was performed together with the experimental samples in a controlled environment protected from light. Presented values represent the mean and the standard deviation from triplicate determinations.

against these genital pathogens. In the deferred inhibition assay, our results demonstrated that all lactobacilli strains were bioactive compound producers. Regarding G. vaginalis, the substances produced by lactobacilli formed halos, varying from 14.33 to 21.00 mm. With respect to N. gonorrhoeae, the compounds secreted by lactobacilli formed larger inhibition halos in relation to the first pathogen, ranging from 20.67 to 31.67 mm in diameter. In another similar study, Pessoa et al. [21] evaluated three strains of lactobacilli isolated from

cocoa fermentation against G. vaginalis ATCC 49154 (same strain of this study) and reported halos with diameters ranging from 11 to 12 mm using the agar diffusion assay.

In the second assay, microdiffusion showed that the bioactive compounds inhibited the growth of pathogens by contact or by the presence of halos. The strains Lc24, Lf38, Lf47, and Lp81 inhibited the growth of genital pathogens by contact. Only the Lp90 strain exhibited inhibition halo by this methodology. The halo of inhibition against G. vaginalis was larger (17.33 mm) than the halo presented against N. gonorrhoeae (13.67 mm). Coincidentally, Qian et al. [70] also applied two methodologies similar to the tests carried out in this study to identify the presence of antimicrobial compounds. According to the authors, the five tested Lactobacillus strains displayed the ability to inhibit G. vaginalis, and the halo formation was higher in the deferred assay (10.00–13.67 mm) compared to microdiffusion (1.00–3.02 mm). In another study, Carmo et al. [66] evaluated seven reference strains of Lactobacillus against three genital pathogens, including N. gonorrhoeae. Six of these lactobacilli showed antimicrobial activity with zones of inhibition ranging from 9.0 to 19.3 mm in diameter. Our results agree with these findings, because all supernatants presented antipathogenic

TABLE 4: Inhibitory activity of treated and untreated CFCS of *Lactobacillus* strains against *G. vaginalis* and *N. gonorrhoeae*.

Strain	MRS	Untreated CFCS	Neutralized CFCS	Boiled CFCS	CFCS + trypsin	CFCS + proteinase K
			Growth of *G. vaginalis*			
Lc24	+	+	+	+	+	+
Lf38	+	+	+	+	+	+
Lf47	+	+	+	+	+	+
Lp81	+	Inhibited	+	Inhibited	Inhibited	Inhibited
Lp90	+	Inhibited	+	Inhibited	Inhibited	Inhibited
			Growth of *N. gonorrhoeae*			
Lc24	+	+	+	+	+	+
Lf38	+	Inhibited	+	Inhibited	Inhibited	Inhibited
Lf47	+	+	+	+	+	+
Lp81	+	+	+	+	+	+
Lp90	+	Inhibited	+	Inhibited	Inhibited	Inhibited

(+) means growth of pathogen.

activity; however, larger inhibition diameters were found in the present study.

3.5. Antimicrobial Properties of Lactobacilli in Coculture with G. vaginalis and N. gonorrhoeae. The pH of isolated culture of lactobacilli and coculture of *Lactobacillus* strains with pathogen was measured, and the data are shown in Table 3. There was no significant difference ($P > 0.05$) between the pH of *Lactobacillus* isolated culture and cocultures. This demonstrates that lactobacilli are able to reduce the pH of the culture (initial pH of 6.5) and keep it low, even with the presence of genital pathogens. In this study, the highest pH was from the coculture of Lf38 with diplococcus (4.68), and the lowest pH recorded was from Lf90 with diplococcus (3.75). In our study, we also evaluated the inhibitory effect of *Lactobacillus* on the growth of pathogens in coculture (Figure 3). Noting the inhibition caused by a direct interaction between our lactobacilli strains and pathogens, we observed that the growth of *G. vaginalis* and *N. gonorrhoeae* decreased compared to the control. In relation to *G. vaginalis*, all lactobacilli tested were able to decrease its growth ($P < 0.05$). In the coculture with *N. gonorrhoeae*, all lactobacilli tested also decreased the growth of diplococcus after 24 hours ($P < 0.001$).

The acidification potential of lactobacilli under anaerobic conditions is an important characteristic conferring protection against pathogens, such as *N. gonorrhoeae* [71]. Considering our data, we also believe that the reduction in pH promoted by the secretion of organic acids by our lactobacilli strains constituted a fundamental mechanism for inhibiting the growth of *N. gonorrhoeae* and *G. vaginalis*. According to our study, Bertuccini et al. [72] demonstrated that *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were able to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Atopobium vaginae*, and *G. vaginalis* at different incubation times. We demonstrated that our *Lactobacillus* strains are potential probiotic candidates because they were able to maintain a low pH and fight the growth of the pathogens tested, corroborating the literature that points to the genus

Lactobacillus as a potential source of bacteria with safe probiotic characteristics [73].

3.6. Production of H$_2$O$_2$ by Lactobacilli. Figure 4 demonstrates the production of hydrogen peroxide by lactobacilli isolated from cupuaçu pulp. In this study, all five lactobacilli tested were able to produce hydrogen peroxide, and the H$_2$O$_2$ concentration varied between 1 and 5 μM. There was no significant difference ($P > 0.05$) between strains or species studied. Classically, lactobacilli are considered biomarkers of women's health, as they protect vaginal mucosa against genital infections due to the presence of lactic acid and H$_2$O$_2$ [74–76]. It is known that hydrogen peroxide-producing lactobacilli are commonly isolated from the healthy vagina of women of childbearing age, which may make this type of *Lactobacillus* an important element in maintaining genital homeostasis [77], since the presence of these microorganisms reduces the risk of BV and infections associated with BV [78]. The literature suggests that the main advantages associated with H$_2$O$_2$ activity are its ability to cross the cell membrane of the pathogen, to damage the membrane, proteins, DNA, and enzymes by the formation of free radicals and the anti-inflammatory stimulus of the vaginal mucosa [79–81]. Furthermore, the joint action of metabolites, such as H$_2$O$_2$, organic acids, bacteriocins, and biosurfactants, has already been attributed as a potent antibiotic-assisting tool to contain genital infections and minimize resistance to antibiotics [82].

3.7. Characterization of Antimicrobial Substances in CFCS. After verifying that our five lactobacilli strains showed antimicrobial activity against pathogens, we identified these substances. Treatments were applied with the CFCS responsible for inhibiting pathogens in the previous tests. The treatments of CFCS included neutralization of possible organic acids, boiling, and inactivation of possible bacteriocins through the enzymatic action of trypsin and proteinase K. Our results are shown in Table 4. Our data showed that only CFCS from *L. plantarum* (Lp81 and Lp90) inhibited the growth of *G. vaginalis* when untreated or treated by boiling or enzymatic action. The neutralized CFCS allowed the growth of the

TABLE 5: Metabolomic analysis of CFCS.

Retention time (min)	Substance	MRS area (%)	CFCS area (%) Lc24	CFCS area (%) Lf38	CFCS area (%) Lf47	CFCS area (%) Lp81	CFCS area (%) Lp90
4.164	Carbodiimide	—	1.48	0.74	0.89	1.51	1.37
4.700	N.N-Dimethylglycine	0.04	—	0.03	—	0.08	—
7.295	Lactic acid	0.85	61.77	49.18	56.43	57.64	61.05
7.524	Acetic acid	—	—	0.05	0.07	—	0.04
7.531	2-Hydroxyetanoic acid	—	—	—	—	0.04	—
7.768	Valine	0.05	—	—	—	—	—
8.405	Alanine	1.14	2.15	4.09	3.40	2.24	2.00
9.020	Glycine	0.33	0.81	1.11	0.82	0.99	0.77
9.873	β-Lactate	—	—	0.08	0.10	0.06	0.07
10.168	Leucine	0.17	—	—	—	—	—
10.445	3-Hydroxybutyric acid	—	—	—	—	0.04	—
10.638	α-Hydroxyvaleric acid	—	0.17	—	—	0.10	0.16
12.465	Valine	1.02	1.71	2.85	2.95	2.12	2.06
13.203	4-Methyl-2-hydroxypentanoic acid	—	0.35	0.87	0.71	0.29	0.58
13.395	3-Methyl-2-hydroxypentanoic acid	—	—	—	—	0.07	0.10
14.510	Leucine	2.48	2.55	3.45	4.49	3.50	3.43
14.733	Glycerol	—	0.71	—	1.34	—	—
14.769	Phosphoric acid	7.29	7.56	11.13	9.73	7.85	7.86
15.236	Isoleucine	1.18	1.89	2.65	2.74	2.17	2.15
15.308	γ-Amino butyric acid	—	—	0.10	—	0.14	—
15.839	Butanoic acid	0.20	0.28	0.97	1.00	0.46	0.50
16.220	2-Methyl-2.3-dihydroxypropanoic acid	0.10	0.13	0.27	0.36	0.15	0.16
16.705	Pyrimidine	—	—	0.13	—	—	—
17.644	Serine	0.75	0.85	2.23	1.19	0.61	0.47
18.148	Butanoic acid	0.02	0.12	0.09	0.11	0.06	0.07
18.272	3-Methyl-1.4-dihydroxypiperazine-2.5-dione	—	0.79	0.08	—	0.07	—
18.496	Butyric acid	—	—	2.07	—	—	—
18.531	Threonine	0.62	—	—	1.71	1.38	1.24
18.555	Lactic acid dimer	—	0.52	—	—	0.25	0.27
19.332	2.4-Dihydroxybutanoic acid	—	—	0.12	0.15	0.07	0.07
19.494	Aspartic acid	—	1.04	—	0.64	—	0.64
21.091	Trisiloxane	—	0.11	0.15	0.15	0.11	0.13
21.769	Malic acid	—	0.12	—	—	0.10	0.24
21.865	2-Pyrrolidone-5-carboxylic acid	—	0.34	—	0.58	—	0.81
22.262	Glutamic acid	—	—	—	0.15	—	—
22.458	Methionine	0.22	—	0.27	—	0.31	—
22.588	Proline	1.40	1.17	3.55	0.84	2.20	1.52
22.708	Aspartic acid	0.56	—	0.63	—	0.90	0.20
23.099	Phenylalanine	—	0.92	—	1.29	—	0.59
24.376	Benzenepropanoic acid	—	0.09	0.57	0.45	0.28	0.50
25.371	Ornithine	—	—	—	—	0.21	—
25.583	Glutamine	1.76	—	3.81	—	3.06	1.40
26.434	Tartaric acid	0.34	0.40	0.67	0.47	0.49	0.54
26.995	Hydroxy 4-oxo-2.4-di(hydroxylamine)butanoate	—	—	—	—	0.16	—

TABLE 5: Continued.

Retention time (min)	Substance	MRS area (%)	CFCS area (%) Lc24	CFCS area (%) Lf38	CFCS area (%) Lf47	CFCS area (%) Lp81	CFCS area (%) Lp90
27.048	Asparagine	0.04	—	0.36	—	—	—
27.895	Lysine	—	—	1.22	—	1.11	—
28.638	Arabinitol	—	—	0.10	—	—	—
28.805	Ribitol	—	—	—	—	0.11	0.12
29.803	2.3-Dihydroxypropylphosphoric acid	0.13	0.11	0.30	0.26	0.26	0.25
29.920	D-Ribo-Hexonic acid	—	—	—	—	0.09	0.11
30.946	2-Keto-D-gluconic acid	0.7	—	—	—	0.06	—
31.133	1,2,3-Propanetricarboxylic acid	—	6.15	0.11	—	—	—
31.259	Citric acid	7.49	—	—	—	6.66	5.91
32.345	Pentanedioic acid	—	0.34	0.75	0.16	0.29	—
32.451	Benzenepropanoic acid	0.10	—	—	—	—	—
32.879	4-Hydroxyphenyllactic acid		—	—	0.07	0.10	0.16
33.891	Tyrosine	0.13	—	—	—	—	0.40
33.955	Glucitol	0.04	0.16	3.65	2.76	—	
35.660	Inositol	31.07	—	0.07	—	—	0.07
39.845	Tryptophan	0.06	—	0.26	—	0.16	—
—	Sugars	38.12	3.38	4.07	2.96	0.83	1.54
—	Unidentified compounds	1.60	1.81	1.20	3.83	0.62	0.46
—	Identified compounds (except sugars)	60.28	94.81	94.73	93.21	98.55	98.00
—	Total	100.00	100.00	100.00	100.00	100.00	100.00

pathogen. Regarding *N. gonorrhoeae*, only the CFCS of the Lf38 and Lp90 strains were able to inhibit the bacterial growth of the diplococcus. The inhibition and growth profile of these bacteria were similar to that of the first. CFCS treated with trypsin or proteinase K did not affect the growth of both bacteria, suggesting that the compound for the antibacterial activity is not related to the class of bacteriocins. We inferred from our data that the bioactive compounds present in the CFCS responsible for the antimicrobial action probably are the organic acids. This is explained by the fact that bacterial growth was only possible when the CFCS was neutralized. Also, we realized that organic acids were thermostable compounds, since there was no growth of pathogens after boiling.

The analysis of the CFCS metabolome of our lactobacilli strains demonstrated a wide variety of organic compounds. Among the main substances, there were organic acids, alcohols, sugars, and other biological compounds (Table 5). In this study, however, we evaluated only the profile of organic acids produced by five lactobacilli strains, and the CFCS characterization revealed that lactic acid was the most prevalent organic acid in the sample, ranging from 49.18% to 61.77% of the analyte. The secretion of organic acids was quite diverse, even within the same species. In this study, we conclude that the types of acids produced are strain-specific and are not species-specific. Other organic acids were also produced by the strains, including acetic acid, butyric acid, 4-methyl-2-hydroxypentanoic acid, 3-methyl-2-hydroxypentanoic acid, malic acid, 2-pyrrolidone-5-carboxylic acid, benzenepropanoic acid, and pentanedioic acid. We also believe that strain-specific production of organic acids is responsible for the different patterns of inhibition presented against *G. vaginalis* and *N. gonorrhoeae*.

Lactic acid plays a complex and critical role in maintaining of homeostasis in the healthy vaginal environment, because it is believed that an important function of lactic acid is inhibiting the growth of genital pathogens, such as *G. vaginalis* [83]. Many studies have reported that lactic acid produced by lactobacilli favors the promotion of vaginal health and has been shown to inhibit the growth of pathogenic organisms in vitro [84, 85] The literature indicates that women with a predominance of lactobacilli in the vaginal microbiota are less susceptible to infection by *N. gonorrhoeae* [86]. Besides that, many studies have shown that a complex vaginal microenvironment with lactic acid and hydrogen peroxide inhibits genital pathogens, such as *N. gonorrhoeae* [67, 71, 87]. Finally, we understand that the growth of genital pathogens depends on the pH, and the production of lactic acid by our lactobacilli strains must be sufficient to reduce the pH and thus inhibit the growth of anaerobes [88].

4. Conclusion

The current understanding of the complex interaction between lactobacilli, genital pathogens, and vaginal mucosa of the human host remains incomplete. Our strains proved to be promising probiotic candidates due to the different characteristics analyzed. All five *Lactobacillus* strains isolated from Brazilian cupuaçu were considered safe for use in humans according to trials recommended by the WHO. In general, our strains were resistant to temperature and pH

stresses, desirable requirements for strains targeted in the industry. Besides this, the five lactobacilli strains were biofilm producers and inhibited the growth of two pathogens in different methodologies. In addition, our strains produce H_2O_2 and are able to reduce the pH of the medium through the production of organic acids, especially lactic acid, an important mechanism to combat genital pathogens. Collectively, our data demonstrated that our lactobacilli strains are promising probiotic candidates. We understand that tests on living models will be necessary to confirm our findings and, possibly, to develop mixed or separate products of lactobacilli and supernatants in order to contribute to vaginal homeostasis. In the future, we believe that our strains can be applied as probiotics usable in clinical conditions.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

References

[1] D. Gawkowski and M. L. Chikindas, "Non-dairy probiotic beverages: the next step into human health," *Beneficial Microbes*, vol. 4, no. 2, pp. 127–142, 2013.

[2] J. M. Kim and Y. J. Park, "Probiotics in the prevention and treatment of postmenopausal vaginal infections: review article," *Journal of Menopausal Medicine*, vol. 23, no. 3, pp. 139–145, 2017.

[3] M. Falagas, G. I. Betsi, and S. Athanasiou, "Probiotics for the treatment of women with bacterial vaginosis," *Clinical Microbiology and Infection*, vol. 13, no. 7, pp. 657–664, 2007.

[4] Y. Wang, Y. Wu, Y. Wang et al., "Antioxidant properties of probiotic bacteria," *Nutrients*, vol. 9, no. 5, p. 521, 2017.

[5] C. Maldonado Galdeano, S. I. Cazorla, J. M. Lemme Dumit, E. Vélez, and G. Perdigón, "Beneficial effects of probiotic consumption on the immune system," *Annals of Nutrition & Metabolism*, vol. 74, no. 2, pp. 115–124, 2019.

[6] J. P. Lepargneur, "Lactobacillus crispatus as biomarker of the healthy vaginal tract," *Annales de Biologie Clinique*, vol. 74, no. 4, pp. 421–427, 2016.

[7] G. Reid, "The development of probiotics for women's health," *Canadian Journal of Microbiology*, vol. 63, no. 4, pp. 269–277, 2017.

[8] S. D. Todorov, M. B. Wachsman, H. Knoetze, M. Meincken, and L. M. T. Dicks, "An antibacterial and antiviral peptide produced by Enterococcus mundtii ST4V isolated from soya beans," *International Journal of Antimicrobial Agents*, vol. 25, no. 6, pp. 508–513, 2005.

[9] H. Abouloifa, Y. Rokni, R. Bellaouchi et al., "Characterization of probiotic properties of antifungal Lactobacillus strains isolated from traditional fermenting green olives," *Probiotics and Antimicrobial Proteins*, vol. 12, no. 2, pp. 683–696, 2020.

[10] T. A. Melo, T. F. dos Santos, L. R. Pereira, H. M. Passos, R. P. Rezende, and C. C. Romano, "Functional profile evaluation of Lactobacillus fermentum TCUESC01: a new potential probiotic strain isolated during cocoa fermentation," *BioMed Research International*, vol. 2017, Article ID 5165916, 7 pages, 2017.

[11] T. Teles Santos, R. M. Santos Ornellas, L. Borges Arcucio et al., "Characterization of lactobacilli strains derived from cocoa fermentation in the south of Bahia for the development of probiotic cultures," *LWT- Food Science and Technology*, vol. 73, pp. 259–266, 2016.

[12] S. Borges, J. Silva, and P. Teixeira, "The role of lactobacilli and probiotics in maintaining vaginal health," *Archives of Gynecology and Obstetrics*, vol. 289, no. 3, pp. 479–489, 2014.

[13] A. B. Onderdonk, M. L. Delaney, and R. N. Fichorova, "The human microbiome during bacterial vaginosis," *Clinical Microbiology Reviews*, vol. 29, no. 2, pp. 223–238, 2016.

[14] J. R. Schwebke, C. A. Muzny, and W. E. Josey, "Role of Gardnerella vaginalis in the pathogenesis of bacterial vaginosis: a conceptual model," *The Journal of Infectious Diseases*, vol. 210, no. 3, pp. 338–343, 2014.

[15] M. S. Coudray and P. Madhivanan, "Bacterial vaginosis–A brief synopsis of the literature," *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 245, pp. 143–148, 2020.

[16] M. A. O. Ignacio, J. Andrade, A. P. F. Freitas, G. V. . S. Pinto, M. G. Silva, and M. T. C. Duarte, "Prevalence of bacterial vaginosis and factors associated among women who have sex with women," *Revista Latino-Americana de Enfermagem*, vol. 26, article 3077, 2018.

[17] A. P. R. Costa-Lourenço, K. T. Barros dos Santos, B. M. Moreira, S. E. L. Fracalanzza, and R. R. Bonelli, "Antimicrobial resistance in _Neisseria gonorrhoeae_ : history, molecular mechanisms and epidemiological aspects of an emerging global threat," *Brazilian Journal of Microbiology*, vol. 48, no. 4, pp. 617–628, 2017.

[18] S. J. Quillin and H. S. Seifert, "Neisseria gonorrhoeae host adaptation and pathogenesis," *Nature Reviews Microbiology*, vol. 16, no. 4, pp. 226–240, 2018.

[19] A. Lovett and J. A. Duncan, "Human immune responses and the natural history of Neisseria gonorrhoeae infection," *Frontiers in Immunology*, vol. 9, article 3187, 2019.

[20] R. M. S. Ornellas, T. T. Santos, L. B. Arcucio et al., "Selection of lactic acid bacteria with probiotic potential isolated from the fermentation process of "cupuaçu" (Theobroma grandiflorum)," *Advances in Experimental Medicine and Biology*, vol. 973, pp. 1–16, 2017.

[21] W. F. Pessoa, A. C. Melgaço, M. E. de Almeida, L. P. Ramos, R. P. Rezende, and C. C. Romano, "In vitro activity of lactobacilli with probiotic potential isolated from cocoa fermentation against Gardnerella vaginalis," *BioMed Research International*, vol. 2017, Article ID 3264194, 10 pages, 2017.

[22] W. P. Charteris, P. M. Kelly, L. Morelli, and J. K. Collins, "Antibiotic susceptibility of potentially probiotic Lactobacillus species," *Journal of Food Protection*, vol. 61, no. 12, pp. 1636–1643, 1998.

[23] R. Paéz, L. Lavari, G. Vinderola et al., "Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion," *Food Research International*, vol. 48, no. 2, pp. 748–754, 2012.

[24] L. Ouarabi, Y. A. Chait, H. A. Seddik, D. Drider, and F. Bendali, "Newly isolated lactobacilli strains from algerian human vaginal microbiota: Lactobacillus fermentum strains relevant probiotic's candidates," *Probiotics and Antimicrobial Proteins*, vol. 11, no. 1, pp. 43–54, 2019.

[25] C. Rodríguez, J. V. Cofré, M. Sánchez, P. Fernández, G. Boggiano, and E. Castro, "Lactobacilli isolated from vaginal vault of dairy and meat cows during progesteronic stage of estrous cycle," *Anaerobe*, vol. 17, no. 1, pp. 15–18, 2011.

[26] B. Kos, J. Šušković, S. Vuković, M. Šimpraga, J. Frece, and S. Matošić, "Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92," *Journal of Applied Microbiology*, vol. 94, no. 6, pp. 981–987, 2003.

[27] P. Hutt, J. Shchepetova, K. Loivukene, T. Kullisaar, and M. Mikelsaar, "Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero and uropathogens," *Journal of Applied Microbiology*, vol. 100, no. 6, pp. 1324–1332, 2006.

[28] A. C. C. Melgaço, W. F. Blohem Pessoa, H. P. Freire et al., "Potential of maintaining a healthy vaginal environment by two Lactobacillus strains isolated from cocoa fermentation," *BioMed Research International*, vol. 2018, Article ID 7571954, 14 pages, 2018.

[29] R. D. Nardi, A. R. M. Santos, M. A. R. Carvalho, L. M. Farias, L. C. Benchetrit, and J. R. Nicoli, "Antagonism against anaerobic and facultative bacteria through a diffusible inhibitory compound produced by a Lactobacillus sp. isolated from the rat fecal microbiota," *Anaerobe*, vol. 5, no. 3-4, pp. 409–411, 1999.

[30] J. Z. S. Rodrigues, M. R. Passos, N. Silva de Macêdo Neres et al., "Antimicrobial activity of _Lactobacillus fermentum_ TcUESC01 against _Streptococcus mutans_ UA159," *Microbial Pathogenesis*, vol. 142, article 104063, 2020.

[31] R. Toure, E. Kheadr, C. Lacroix, O. Moroni, and I. Fliss, "Production of antibacterial substances by bifidobacterial isolates from infant stool active against listeria monocytogenes," *Journal of Applied Microbiology*, vol. 95, no. 5, pp. 1058–1069, 2003.

[32] E. F. Bosma, J. Forster, and A. T. Nielsen, "Lactobacilli and pediococci as versatile cell factories - evaluation of strain properties and genetic tools," *Biotechnology Advances*, vol. 35, no. 4, pp. 419–442, 2017.

[33] E. F. Bosma, J. van der Oost, W. M. de Vos, and R. van Kranenburg, "Sustainable production of bio-based chemicals by extremophiles," *Current Biotechnology*, vol. 2, no. 4, pp. 360–379, 2013.

[34] M. Taylor, L. van Zyl, I. Tuffin, D. J. Leak, and D. A. Cowan, "Genetic tool development underpins recent advances in thermophilic whole-cell biocatalysts," *Microbial Biotechnology*, vol. 4, no. 4, pp. 438–448, 2011.

[35] M. A. Correa Deza, M. Grillo-Puertas, S. Salva, V. A. Rapisarda, C. L. Gerez, and G. Font de Valdez, "Inorganic salts and intracellular polyphosphate inclusions play a role in the thermotolerance of the immunobiotic Lactobacillus rhamnosus CRL 1505," *PLoS One*, vol. 12, no. 6, article 179242, 2017.

[36] M. T. Liong and N. P. Shah, "Acid and bile tolerance and cholesterol removal ability of lactobacilli strains," *Journal of Dairy Science*, vol. 88, no. 1, pp. 55–66, 2005.

[37] H. Hassanzadazar, A. Ehsani, K. Mardani, and J. Hesari, "Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese," *Veterinary Research Forum*, vol. 3, no. 3, pp. 181–185, 2012.

[38] L. Sukmarini, A. Z. Mustopa, M. Normawati, and I. Muzdalifah, "Identification of antibiotic-resistance genes from lactic acid bacteria in Indonesian fermented foods," *HAYATI Journal of Biosciences*, vol. 21, no. 3, pp. 144–150, 2014.

[39] G. Huys, N. Botteldoorn, F. Delvigne et al., "Microbial characterization of probiotics-advisory report of the Working Group "8651 Probiotics" of the Belgian Superior Health Council (SHC)," *Molecular Nutrition & Food Research*, vol. 57, no. 8, pp. 1479–1504, 2013.

[40] G. Dixit, D. Samarth, V. Tale, and R. Bhadekar, "Comparative studies on potential probiotic characteristics of Lactobacillus acidophilus strains," *EurAsian Journal of Bioscience*, vol. 7, no. 1, pp. 1–9, 2013.

[41] M. Nawaz, J. Wang, A. Zhou et al., "Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products," *Current Microbiology*, vol. 62, no. 3, pp. 1081–1089, 2011.

[42] C. Sharma, S. Gulati, N. Thakur et al., "Antibiotic sensitivity pattern of indigenous lactobacilli isolated from curd and human milk samples," *3 Biotech*, vol. 7, no. 1, p. 53, 2017.

[43] A. S. Hummel, C. Hertel, W. H. Holzapfel, and C. M. A. P. Franz, "Antibiotic resistances of starter and probiotic strains of lactic acid bacteria," *Applied and Environmental Microbiology*, vol. 73, no. 3, pp. 730–739, 2007.

[44] M. Gueimonde, B. Sánchez, C. G. de los Reyes-Gavilán, and A. Margolles, "Antibiotic resistance in probiotic bacteria," *Frontiers in Microbiology*, vol. 4, article 202, 2013.

[45] N. M. Jose, C. R. Bunt, and M. A. Hussain, "Comparison of microbiological and probiotic characteristics of lactobacilli isolates from dairy food products and animal rumen contents," *Microorganisms*, vol. 3, no. 2, pp. 198–212, 2015.

[46] S. A. Palaniyandi, K. Damodharan, J. W. Suh, and S. H. Yang, "In vitro characterization of Lactobacillus plantarum strains with inhibitory activity on enteropathogens for use as potential animal probiotics," *Indian Journal of Microbiology*, vol. 57, no. 2, pp. 201–210, 2017.

[47] A. M. O. Leite, M. A. L. Miguel, R. S. Peixoto et al., "Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains," *Journal of Dairy Science*, vol. 98, no. 6, pp. 3622–3632, 2015.

[48] N. F. Kaktcham, F. M. Zambou, F. M. Tchouanguep, M. el-Soda, and M. I. Choudhary, "Antimicrobial and safety properties of lactobacilli isolated from two Cameroonian traditional fermented foods," *Scientia Pharmaceutica*, vol. 80, no. 1, pp. 189–203, 2012.

[49] D. Halder, M. Mandal, S. S. Chatterjee, N. Pal, and S. Mandal, "Indigenous probiotic lactobacillus isolates presenting antibiotic like activity against human pathogenic bacteria," *Biomedicine*, vol. 5, no. 4, p. 31, 2017.

[50] N. Talib, N. E. Mohamad, S. K. Yeap et al., "Isolation and characterization of Lactobacillus spp. from kefir samples in Malaysia," *Molecules*, vol. 24, no. 14, article 2606, 2019.

[51] T. Padmavathi, R. Bhargavi, P. R. Priyanka, N. R. Niranjan, and P. V. Pavitra, "Screening of potential probiotic lactic acid

bacteria and production of amylase and its partial purification," *Journal, Genetic Engineering & Biotechnology*, vol. 16, no. 2, pp. 357–362, 2018.

[52] S. Martinez, J. G. Garcia, R. Williams et al., "Lactobacilli spp.: real-time evaluation of biofilm growth," *BMC Microbiology*, vol. 20, no. 1, article 32209050, p. 64, 2020.

[53] M. C. Leccese Terraf, L. M. Mendoza, M. S. Juárez Tomás, C. Silva, and M. E. F. Nader-Macías, "Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli," *Journal of Applied Microbiology*, vol. 117, no. 6, pp. 1761–1772, 2014.

[54] M. C. L. Terraf, M. S. Juárez Tomás, M. E. Nader-Macías, and C. Silva, "Screening of biofilm formation by beneficial vaginal lactobacilli and influence of culture media components," *Journal of Applied Microbiology*, vol. 113, no. 6, pp. 1517–1529, 2012.

[55] A. Pino, E. Bartolo, C. Caggia, A. Cianci, and C. L. Randazzo, "Detection of vaginal lactobacilli as probiotic candidates," *Scientific Reports*, vol. 9, no. 1, p. 3355, 2019.

[56] D. Y. Ren, C. Li, Y. Q. Qin et al., "Lactobacilli reduce chemokine IL-8 production in response to TNF-α and Salmonella challenge of Caco-2 cells," *BioMed Research International*, vol. 2013, Article ID 925219, 9 pages, 2013.

[57] B. Sánchez, P. Bressollier, and M. C. Urdaci, "Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host," *FEMS Immunology and Medical Microbiology*, vol. 54, no. 1, pp. 1–17, 2008.

[58] C. J. P. Boonaert and P. G. Rouxhet, "Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties," *Applied and Environmental Microbiology*, vol. 66, no. 6, pp. 2548–2554, 2000.

[59] F. Rozgonyi, K. R. Szitha, and A. Ljungh, "Improvement of the salt aggregation test to study bacterial cell-surface hydrophobicity," *FEMS Microbiology Letters*, vol. 30, no. 1-2, pp. 131–138, 1985.

[60] W.-H. Pan, P.-L. Li, and Z. Liu, "The correlation between surface hydrophobicity and adherence of Bifidobacterium strains from centenarians' faeces," *Anaerobe*, vol. 12, no. 3, pp. 148–152, 2006.

[61] X. Chen, F. Tian, X. Liu et al., "In vitro screening of lactobacilli with antagonistic activity against Helicobacter pylori from traditionally fermented foods," *Journal of Dairy Science*, vol. 93, no. 12, pp. 5627–5634, 2010.

[62] S. Y. Chew, Y. K. Cheah, H. F. Seow, D. Sandai, and L. T. L. Than, "Probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 exhibit strong antifungal effects against vulvovaginal candidiasis-causing Candida glabrata isolates," *Journal of Applied Microbiology*, vol. 118, no. 5, pp. 1180–1190, 2015.

[63] A. H. Rickard, A. J. McBain, A. T. Stead, and P. Gilbert, "Shear rate moderates community diversity in freshwater biofilms," *Applied and Environmental Microbiology*, vol. 70, no. 12, pp. 7426–7435, 2004.

[64] E. Zakaria Gomaa, "Antimicrobial and anti-adhesive properties of biosurfactant produced by lactobacilli isolates, biofilm formation and aggregation ability," *The Journal of General and Applied Microbiology*, vol. 59, no. 6, pp. 425–436, 2013.

[65] J. A. Younes, H. C. van der Mei, E. van den Heuvel, H. J. Busscher, and G. Reid, "Adhesion forces and coaggregation between vaginal staphylococci and lactobacilli," *PLoS One*, vol. 7, no. 5, article e36917, 2012.

[66] M. S. do Carmo, F. M. Noronha, M. O. Arruda et al., "Lactobacillus fermentum ATCC 23271 displays in vitro inhibitory activities against Candida spp.," *Frontiers in Microbiology*, vol. 7, article 1722, 2016.

[67] C. Foschi, M. Salvo, R. Cevenini, C. Parolin, B. Vitali, and A. Marangoni, "Vaginal lactobacilli reduce Neisseria gonorrhoeae viability through multiple strategies: an in vitro study," *Frontiers in Cellular and Infection Microbiology*, vol. 7, no. 502, article 29270390, 2017.

[68] P. Mastromarino, P. Brigidi, S. Macchia et al., "Characterization and selection of vaginal Lactobacillus strains for the preparation of vaginal tablets," *Journal of Applied Microbiology*, vol. 93, no. 5, pp. 884–893, 2002.

[69] P. Hütt, E. Lapp, J. Štšepetova et al., "Characterisation of probiotic properties in human vaginal lactobacilli strains," *Microbial Ecology in Health and Disease*, vol. 27, pp. 1–9, 2016.

[70] Z. Qian, D. Zhao, Y. Yin, H. Zhu, and D. Chen, "Antibacterial activity of Lactobacillus strains isolated from Mongolian yogurt against Gardnerella vaginalis," *BioMed Research International*, vol. 2020, Article ID 3548618, 9 pages, 2020.

[71] M. A. Graver and J. J. Wade, "The role of acidification in the inhibition of Neisseria gonorrhoeae by vaginal lactobacilli during anaerobic growth," *Annals of Clinical Microbiology and Antimicrobials*, vol. 10, no. 1, p. 8, 2011.

[72] L. Bertuccini, R. Russo, F. Iosi, and F. Superti, "Effects of Lactobacillus rhamnosus and Lactobacillus acidophilus on bacterial vaginal pathogens," *International Journal of Immunopathology and Pharmacology*, vol. 30, no. 2, pp. 163–167, 2017.

[73] J. van de Wijgert and M. C. Verwijs, "Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs," *BJOG : An International Journal of Obstetrics and Gynaecology*, vol. 127, no. 2, pp. 287–299, 2020.

[74] A. C. Vallor, M. A. Antonio, S. E. Hawes, and S. L. Hillier, "Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production," *The Journal of Infectious Diseases*, vol. 184, no. 11, pp. 1431–1436, 2001.

[75] R. R. Spurbeck and C. G. Arvidson, "Lactobacilli at the front line of defense against vaginally acquired infections," *Future Microbiology*, vol. 5, no. 5, pp. 567–582, 2011.

[76] M. N. Matu, G. O. Orinda, E. N. Njagi, C. R. Cohen, and E. A. Bukusi, "_In vitro_ inhibitory activity of human vaginal lactobacilli against pathogenic bacteria associated with bacterial vaginosis in Kenyan women," *Anaerobe*, vol. 16, no. 3, pp. 210–215, 2010.

[77] S. J. Klebanoff, S. L. Hillier, D. A. Eschenbach, and A. M. Waltersdorph, "Control of the microbial flora of the vagina by H202-generating lactobacilli," *The Journal of Infectious Diseases*, vol. 164, no. 1, pp. 94–100, 1991.

[78] R. H. Beigi, H. C. Wiesenfeld, S. L. Hillier, T. Straw, and M. A. Krohn, "Factors associated with absence of H_2O_2-producing Lactobacillus among women with bacterial vaginosis," *The Journal of Infectious Diseases*, vol. 191, no. 6, pp. 924–929, 2005.

[79] S. J. Quillin, A. J. Hockenberry, M. C. Jewett, and H. S. Seifert, "Neisseria gonorrhoeae exposed to sublethal levels of hydrogen peroxide mounts a complex transcriptional response," *mSystems*, vol. 3, no. 5, p. 156, 2018.

[80] L. C. Seaver and J. A. Imlay, "Are respiratory enzymes the primary sources of intracellular hydrogen peroxide?," *Journal of Biological Chemistry*, vol. 279, no. 47, pp. 48742–48750, 2004.

[81] W. A. Rose II, C. L. McGowin, R. A. Spagnuolo, T. D. Eaves-Pyles, V. L. Popov, and R. B. Pyles, "Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures," *PLoS One*, vol. 7, no. 3, article 32728, p. e32728, 2012.

[82] A. Sgibnev and E. Kremleva, "Influence of hydrogen peroxide, lactic acid, and surfactants from vaginal lactobacilli on the antibiotic sensitivity of opportunistic bacteria," *Probiotics and Antimicrobial Proteins*, vol. 9, no. 2, pp. 131–141, 2017.

[83] S. E. Dover, A. A. Aroutcheva, S. Faro, and M. L. Chikindas, "Natural antimicrobials and their role in vaginal health: a short review," *International Journal of Probiotics and Prebiotics*, vol. 3, no. 4, pp. 219–230, 2008.

[84] A. Aroutcheva, D. Gariti, M. Simon et al., "Defense factors of vaginal lactobacilli," *American Journal of Obstetrics and Gynecology*, vol. 185, no. 2, pp. 375–379, 2001.

[85] S. S. Witkin, H. Mendes-Soares, I. M. Linhares, A. Jayaram, W. J. Ledger, and L. J. Forney, "Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections," *MBio*, vol. 4, p. 460, 2013.

[86] D. C. S. Amant, I. E. Valentin-Bon, and A. E. Jerse, "Inhibition of Neisseria gonorrhoeae by Lactobacillus species that are commonly isolated from the female genital tract," *Infection and Immunity*, vol. 70, no. 12, pp. 7169–7171, 2002.

[87] D. E. O'Hanlon, B. R. Lanier, T. R. Moench, and R. A. Cone, "Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli," *BMC Infectious Diseases*, vol. 10, no. 1, article 20482854, 2010.

[88] L. M. Breshears, V. L. Edwards, J. Ravel, and M. L. Peterson, "Lactobacillus crispatus inhibits growth of Gardnerella vaginalis and Neisseria gonorrhoeae on a porcine vaginal mucosa model," *BMC Microbiology*, vol. 15, no. 1, article 26652855, 2015.