UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Ciencias Biológicas y Ambientales

Inhibition of *Candida albicans* by lactobacilli on initial adhesion assays: A case report

.

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Inhibition of *Candida albicans* by lactobacilli on initial adhesion assays: A case report

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RESUMEN

Lactobacillus spp. es el género que domina la microbiota vaginal, encargados de la protección del tracto vaginal inhibiendo la adhesión inicial y colonización de patógenos oportunistas. *Candida albicans* es un microorganismo comensal en condiciones normales, pero puede convertirse en un patógeno oportunista. Su capacidad de formar biofilm le otorga gran resistencia y virulencia. Debido a uso de antimicrobianos sin consulta médica, han provocado un desbalance en la microbiota vaginal, permitiendo que patógenos como *C. albicans* puedan colonizar y causar infecciones. El uso de *Lactobacillus* spp. se presenta como una alternativa de bajo riesgo para combatir la adhesión inicial de patógenos oportunistas. Este estudio se centró en la evaluación de la actividad probiótica de lactobacilos vaginales (*L. gasseri*) y no vaginal (*L. plantarum*) contra *C. albicans* en ensayos de adhesión inicial. El protocolo fue realizado en conjunto con Bryan Guachi. Se evalúo la inhibición de las cepas de *C. albicans* en una superficie de vidrio en distintos escenarios experimentales (ES). *L. gasseri* JCM1131 (27-85%) fue la cepa vaginal que mayor inhibición produjo entre los aislados de *L. gasseri* (IMAUFB014: 28-80%; H59.2: 1-89%), sin significancia estadística. *L. plantarum* produjo mayor inhibición que las cepas de *L. gasseri*, con inhibición superior de C. albicans en ES1 (81%) y ES2 (58%) en comparación con las cepas de *L. gasseri* (ES1: 27-73%, $P < 0,001$; y ES2: 1-49%, $P < 0,001$; ANOVA de dos vías). Los resultados demostraron una variabilidad en la inhibición entre diferentes especies de *Lactobacillus*, así como, entre diferentes cepas de la misma especie. Se encontró la viabilidad en el uso de lactobacilos no vaginales como probióticos para la colonización de la mucosa humana, siendo congruente con estudios anteriores. Es necesario estudios posteriores que en diferentes modelos *in vitro* e *in vivo.*

Palabras clave: *Lactobacillus gasseri*$_1$, *Lactobacillus plantarum*$_2$, *Candida albicans*$_3$, Inhibición$_4$, Adhesión Inicial$_5$, Escenarios experimentales (ES)$_6$.

ABSTRACT

Lactobacillus spp. is the genus that dominates the vaginal microbiota. These microorganisms are responsible for the protection of the vaginal tract by inhibiting the initial adhesion and colonization of opportunistic pathogens. *Candida albicans* is a commensal microorganism under normal conditions, but it can become an opportunistic pathogen. Its ability to form biofilm gives it great resistance and virulence. Due to the use of antimicrobials without medical observation, they have caused an imbalance in the vaginal microbiota, allowing pathogens such as *C. albicans* to colonize and cause infections. The use of *Lactobacillus* spp. is presented as a low-risk alternative to combat the initial adhesion of opportunistic pathogens. This study focused on the evaluation of the probiotic activity of vaginal (*L. gasseri*) and non-vaginal (*L. plantarum*) lactobacilli against *C. albicans* on initial adhesion assays. The methodology was realized with Bryan Guachi. The inhibition of *C. albicans* strains was evaluated on a glass surface in different experimental settings (ES). *L. gasseri* JCM1131 (27-85%) was the vaginal strain that produced the highest inhibition among *L. gasseri* isolates (IMAUFB014: 28-80%; H59.2: 1-89%), without statistical significance. *L. plantarum* produced greater inhibition than *L. gasseri* strains, with a higher inhibition of *C. albicans* in ES1 (81%) and ES2 (58%) compared to *L. gasseri* strains (ES1: 27-73%, $P < 0.001$; and ES2: 1-49%, $P < 0.001$; two-way ANOVA). These results demonstrated variability in the inhibition between different *Lactobacillus* species, as well as, between different strains of the same species. The feasibility of the use of non-vaginal lactobacilli as probiotics for the colonization of the human mucosa was demonstrated, being consistent with previous studies. Further studies are needed than in different *in vitro* and *in vivo* models.

Key words: *Lactobacillus gasseri*$_1$, *Lactobacillus plantarum*$_2$, *Candida albicans*$_3$, Inhibition$_4$, Initial Adhesion$_5$, Experimental Settings (ES)$_6$.

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INTRODUCTION

A normal vaginal microbiota from a healthy woman is a complex ecosystem that is made up of more than 200 species of bacteria, depending on genetic, ethnic, environmental, and behavioral factors (Mendling, 2016). It consists of beneficial microorganisms and commensal microorganisms, which could become opportunistic pathogens in certain conditions (Chee, et al., 2020). It has been found that this microbiota is dominated by the genus *Lactobacillus,* and alterations can lead from an asymptomatic dysbiosis to a serious infection (Van De Wijgert, et al., 2014).

The importance of *Lactobacillus* lies in its protective role in the vaginal tract, being able to achieve concentrations of 10^7-10^9 colony forming units (CFU)/g in vaginal secretions (Mikamo, et al., 2000). These microorganisms produce lactic acid, lowering the pH of the vaginal tract and preventing the growth of pathogens (Dasari, 2018; Matsubara, et al., 2016). Furthermore, they compete for adhesion sites and produce antimicrobial compounds (such as, bacteriocins, immune system stimulation, and hydrogen peroxide), inhibiting pathogens to colonize the vaginal epithelium (Borges, et al., 2014). Its probiotic activity has already been demonstrated against bacterial vaginosis, aerobic vaginitis, candidiasis, and other opportunistic pathogens (Zangl, et al., 2020).

Candida is a genus of commensal microorganisms under normal conditions; however, it can become an opportunistic pathogen due to adhesion to cells, leading to a morphological change from yeast to hyphae, to its growth and to invade tissues (Allonsius, et al., 2019). Also, *Candida* species are able to acquire great resistance and virulence due to its ability to form a biofilm (Matsubara, et al., 2016). Vulvovaginal candidiasis (VVC) is a disease that 75% of women have once in their life, being; *C. albicans* the most predominant species in this infection (De Seta, et al., 2014; Pacha-Herrera, et al., 2020).

There are several reports related to the probiotic activity of *Lactobacillus* against *Candida albicans* (De Seta, et al., 2014). This bacterium inhibits the adhesion and growth of the fungus through natural defense mechanisms. It has already been proven in many studies *in vitro*, *in vivo*, and clinical trials. In addition, an inhibition in *Candida* gene expression related to biofilm formation has been identified (Matsubara, et al., 2016). *Candida* is not the only microorganism to be affected by lactobacilli interaction, as already reported in various studies (Gueimonde, et al., 2006; Millsap, et al., 1994). Nonetheless, it is important to know this interaction in-depth, and understand how to integrate lactobacilli into the human vaginal mucosa for greater protection. The first step is carried out through initial adhesion assays on abiotic surfaces (Machado, et al., 2013; Matsubara, et al., 2016; Matsuda, et al., 2018).

The main goal of the present study was to evaluate the probiotic activity of lactobacilli (vaginal and non-vaginal) against *Candida albicans* on initial adhesion assays. The initial adhesion was evaluated on an inert surface (glass surface), where lactobacilli were placed and then evaluated their initial probiotic activity against with *Candida albicans.*

METHODS

Strains and culture conditions

There were used 3 strains of *Lactobacillus gasseri* (H59.2, IMAUFB014, and JCM1131) and 3 strains of *Candida albicans*, 2 strains isolated and sequenced from patients (one with candidiasis, and another with healthy microbiota) and *C. albicans* ATCC® 10231 ™, obtained from a previous study on the vaginal microbiota of the Microbiology Institute at USFQ (Pacha-Herrera, et al., 2020; Salinas, et al., 2020). A non-vaginal strain of lactobacilli, *L. plantarum* ATCC® 14917 ™, was used in this experiment. Each microorganism was respectively labeled: *L. plantarum* ATCC® 14917 ™ (LB14917P), *L. gasseri H59.2* (V130 B), *L. gasseri* JCM1131 (V140 B), *L. gasseri* IMAUFB014 (V254 A), *C. albicans* ATCC® 10231 ™ (ATCC10231), *C. albicans* from candidiasis (V535 A) and *C. albicans* from healthy vaginal microbiota (V251 A) (see Table #1). A strain collection was established, 4 cryovials were made for each microorganism. They were cultured in Brain Heart Infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA) with 15% glycerol at -80 °C.

Lactobacilli were cultured in Man, Rogosa and Sharpe agar (MRS, Becton, Dickinson and Company, Sparks, MD, USA) under microaerophilic conditions (5-10% CO_2) at 37 °C for 48 h (Matsubara, et al., 2016; Ribeiro, et al., 2017). *C. albicans* strains were grown in BBL Sabouraud Dextrose Agar (SD, Becton, Dickinson and Company, USA) for 18 h at 37 °C (Matsubara, et al., 2016; Ribeiro, et al., 2017; Vilela, et al., 2015). In mixed cultures of *Lactobacillus* spp. and *C. albicans* strains BHI broth was used in the initial adhesion assays (Matsubara, et al., 2016).

Initial adhesion assays

The inhibition of *C. albicans* strains caused by lactobacilli was verified by initial adhesion assays. After the growth of each microorganism, they were concentrated in 5 ml of sterile

phosphate-buffered saline (PBS) solution. The solutions were centrifuged (400 *x* g for 12 min, at room temperature), and washed twice with PBS. The pellet was resuspended in PBS, obtaining a stock solution for each microorganism. Their concentration was adjusted to 1.0E+03 colony forming units (CFU)/ ml and 1.00E+09 CFU/ml according to the calibration curves (see Appendix A and B) by the spectrophotometer GENESYS ™ 20 (Thermo Scientific, New York, USA) at an optical density (OD) of 600 nm. The combinations of the obtained solutions were carried out, resulting in 4 experimental settings (ES) (see Table #2). These scenarios were aimed to simulate high and low levels of lactobacilli and *C. albicans* in the colonization of the human mucosal epithelium (Castro, et al., 2013; Fidel, et al., 2004; Machado, et al., 2013; Seneviratne, et al., 2016). They were centrifuged (400 *x* g for 12 min, at room temperature), PBS was discarded, and the pellet was resuspended in 13 ml of BHI broth (Matsubara, et al., 2016).

Two 6-well plates were used for the mixed culture. Controls and samples were performed in triplicate (see Figure #1). Sterile glass coverslips were placed in each well, 2 ml of *Lactobacillus* solution was placed for each sample and adhesion control of *Lactobacillus*; and 2 ml of BHI broth was applied to the negative controls (culture medium without microorganisms) and adhesion controls of *Candida albicans*. The 6-well plates were incubated at 37 °C for 4 h, with 120 revolutions *per* minute (rpm) under anaerobic conditions (Machado, et al., 2013; Nishiyama, et al., 2014). The plates were washed with 2 ml of PBS to remove non-adherent lactobacilli. Subsequently, 2 ml of *Candida albicans* solution was added for each sample and adhesion control of *Candida albicans*; and 2 ml of BHI broth was placed in negative controls and adhesion controls of *Lactobacillus*. The plates were cultivated under anaerobic conditions at 37 °C for 30 min with 120 rpm. A wash with 2 ml of PBS was carried out to eliminate non-adherent microorganisms (Machado, et al., 2013; Nishiyama, et al., 2014).

Microscopy analysis and cell quantification

Absolute ethanol (96%, v/v) was used to fix the microorganisms over the coverslips, and they were stained with 1 ml of crystal violet at 3% for 1 minute (Weerasekera, et al., 2016). In every experimental assay, 15-20 random fields of each coverslip were observed in the OLYMPUS BX50 microscope under 1000x, as reported in previous studies (Chauviere, et al., 1992; Machado, et al., 2013). A picture of each random field observed was taken using the AmScope Digital Camera MU633-FL camera and the AmScope program, version 4.8.15934 (https://www.amscope.com/software-download#toup1). The number of lactobacilli and *C. albicans* for each image was counted (see Appendix C), obtaining the number of cells over the total area of the abiotic surface (glass surface). This data was obtained from the division of the area of the coverslip (4.84E + 08 μm^2) over the image area (12880 μm^2), which was multiplied by the average number of cells (bacteria or yeast) of the 15-20 random fields. As shown in Figures #2 and #3, the results were expressed as the number of cells per glass surface ± standard deviation (No. of cells *per* glass surface ± SD). All experimental assays were carried out in triplicate and on different days.

Statistical analysis

The experimental assays of the present study were analyzed by statistical analysis. Statistically significant differences were evaluated through a two-tailed ANOVA (ANalysis Of VAriance) analysis with post-hoc Tukey HSD (Honestly Significant Difference) test and Student *t*-test. In summary, the evaluation of the differences in and between experimental settings (ES) were performed by ANOVA analysis. The post-hoc Tukey HSD test allowed to evaluate the differences between *Lactobacillus* species and *C. albicans* strains in the same ES. Student *t*-test allowed to see the statistical significance between each species analyzed with their respective control. Statistical analysis was completed using the computer software JASP

version 0.13 (http://www.jasp-stats.org, JASP, Amsterdam, The Netherlands), considering all *P* values equal or less than 0.050 ($P \leq 0.050$) as statistically significant.

RESULTS

The main objective of the present work was to evaluate the inhibition of *C. albicans* strains produced by the probiotic activity of *Lactobacillus* species (*L. gasseri* and *L. plantarum*) during initial adhesion assays. The inhibition of the adhesion of *C. albicans* on the abiotic surface was evaluated in different experimental settings. The inhibition produced by vaginal lactobacilli (*L. gasseri*) was compared to non-vaginal lactobacilli (*L. plantarum*).

Inhibition of *Candida albicans* on initial adhesion by *Lactobacillus gasseri*

This study evaluated the probiotic activity of *L. gasseri* to inhibit the initial adhesion of *C. albicans* isolates (see Figure #2). In low levels of both microorganisms (ES1, similar to a dysbiosis condition), *L. gasseri* JCM1131 and *L. gasseri* IMAUFB014 showed statistically significant differences among *C. albicans* isolates (*L. gasseri* JCM1131 $P = 0.006$, and *L. gasseri* IMAUFB014 $P = 0.002$; two-way ANOVA). *L. gasseri* JCM1131 evidenced the lowest inhibition rate against *C. albicans* ATCC10231 (27%), illustrating statistically significant values when compared against *C. albicans* isolated from candidiasis (60%; $P = 0.016$, using Tukey's post hoc) and *C. albicans* isolated from healthy vaginal microbiota (67%; $P = 0.006$, using Tukey's post hoc). While *L. gasseri* IMAUFB014 showed the lowest inhibition rate against *C. albicans* isolated from healthy vaginal microbiota (28%), demonstrating statistically significant values when compared against *C. albicans* ATCC10231 (56%; $P = 0.025$, using Tukey's post hoc) and *C. albicans* isolated from candidiasis (76%; $P = 0.002$, using Tukey's post hoc). No statistically inhibition differences were detected among *C. albicans* isolates by *L. gasseri* H59.2 in ES1.

However, against high levels of *C. albicans* (in ES2, similar to a candidiasis infection), *L. gasseri* H59.2 was the only *L. gasseri* strain to show statistically inhibition values among *C. albicans* isolates ($P = 0.008$; two-way ANOVA), revealing no inhibition ability against *C.*

albicans ATCC10231 (1%; *P* = 0.010, using Tukey's post hoc) and *C. albicans* isolated from candidiasis (6%; *P* = 0.019, using Tukey's post hoc) when compared to *C. albicans* isolated from healthy vaginal microbiota (43%).

In high levels of lactobacilli against low inocula of *C. albicans* (ES3, similar to healthy vaginal microbiota), only *L. gasseri* H59.2 demonstrated a statistically significant difference in its inhibition ability (*P* = 0.030; two-way ANOVA) against *C. albicans* ATCC10231 (61%) and *C. albicans* isolated from candidiasis (89%; *P* = 0.034, using Tukey's post hoc). However, none of *C. albicans* isolates evidenced any statistical differences in their inhibition rates among *L. gasseri* strains.

In high levels of lactobacilli and *C. albicans* (ES4, similar to a dysbiosis condition), all *C. albicans* isolates showed statistically significant inhibition rates from *L. gasseri* strains (*C. albicans* ATCC10231: *P* = 0.010; *C. albicans* isolated from candidiasis: *P* = 0.011; *C. albicans* isolated from healthy microbiota: *P* = 0.025, using two-way ANOVA analysis). *L. gasseri* H59.2 poorly inhibited *C. albicans* ATCC10231 (28%; *P* = 0.007, using Tukey's post hoc) and *C. albicans* isolated from candidiasis (37%; *P* = 0.025, using Tukey's post hoc) when compared to *C. albicans* isolated from healthy microbiota (68%). On the other hand, *L. gasseri* IMAUFB014 showed the highest inhibition rate against *C. albicans* isolated from healthy microbiota (80%), being statistically different to *C. albicans* ATCC10231 (47%; *P* = 0.016, using Tukey's post hoc). However, this statistical difference was not found in the inhibition of *C. albicans* isolated from candidiasis by *L. gasseri* IMAUFB014 (59%; *P* = 0.096, using Tukey's post hoc). Only *L. gasseri* JCM1131 maintained a similar inhibition rate among all *C. albicans* isolates (70-72%) and no statistically significant differences were found on ES4.

As expected, high levels of lactobacilli showed a superior inhibition rate (between 60 and 89%) of all *C. albicans* isolates when compared to their low levels (between 1 and 77%). When comparing low (ES1) and high (ES3) levels of lactobacilli against low inocula of *Candida*

albicans, significant statistical differences were found in the inhibition rates of *L. gasseri* IMAUFB014 against *C. albicans* isolated from healthy vaginal microbiota ($P = 0.003$; two-way ANOVA), and *L. gasseri* JCM1131 against *C. albicans* ATCC10231 ($P = 0.011$; two-way ANOVA) and *C. albicans* isolated from candidiasis ($P = 0.019$; two-way ANOVA). No significant statistical differences were found in inhibition rates of any isolates of *C. albicans* induced by *L. gasseri* H59.2 between ES1 and ES3 assays (see Figure #2).

On high inocula of *C. albicans*, discrepancies were detected between low (ES2) and high (ES4) levels of lactobacilli. In ES2 and ES4, *L. gasseri* H59.2 possessed the lowest inhibition rates (ES2: 1–43%; ES4: 28–68%) in all *C. albicans* isolates, which its maximum inhibition rates were obtained against *C. albicans* isolated from healthy vaginal microbiota. In fact, *L. gasseri* H59.2 demonstrated statistically significant differences in all *C. albicans* isolates between ES2 and ES4 (more exactly, two-way ANOVA analysis: *C. albicans* ATCC10231 $P = 0.044$; *C. albicans* isolated from candidiasis $P = 0.029$; *C. albicans* isolated from healthy vaginal microbiota $P = 0.033$). *L. gasseri* JCM1131 also evidenced significant statistically differences in its inhibition ability between ES2 and ES4 against *C. albicans* ATCC10231 ($P = 0.037$; two-way ANOVA) and *C. albicans* isolated from candidiasis ($P = 0.009$ with ANOVA analysis). At last, *L. gasseri* IMAUFB014 only revealed this significant statistically difference against *C. albicans* isolated from healthy vaginal microbiota ($P = 0.018$; two-way ANOVA). In ES2 and ES4, both *L. gasseri* IMAUFB014 and *L. gasseri* H59.2 showed their maximum inhibition rates against *C. albicans* isolated from healthy vaginal microbiota, more exactly, 50–80% and 43–68%, respectively. While *L. gasseri* JCM1131 constantly maintained its inhibition ability against all *C. albicans* isolates (ES2: 32–43%; ES4: 70–72%).

Finally, most *C. albicans* isolates on high and low inocula showed a statistical reduction in their initial adhesion by lactobacilli when compared to their control ($P < 0.05$ with Student t-test; see Figure #2). Overall results evidenced several statistically significant differences in

lactobacilli ability to inhibit *C. albicans* colonization between their low and high levels. Although low levels of *Candida albicans* did not show statistical differences in their inhibition against low and high levels of lactobacilli (ES1 and ES3), high levels of *Candida albicans* showed statistical differences in their inhibition between low and high levels of lactobacilli (ES2 and ES4; $P = 0.030$, using Tukey's post hoc).

Inhibition of *Candida albicans* on initial adhesion by *Lactobacillus plantarum*

The probiotic activity of *L. plantarum* ATCC 14917 against *C. albicans* ATCC 10231 was evaluated and compared with the experimental data obtained from Montalvo (2018) on the vaginal strains of *L. gasseri* and then statistically analyzed in the present work. The same analyzes and experimental settings (ES) were performed. However, there was a contamination problem in the initial adhesion assays of ES4, and so it was not possible to present these results on time in the present study. The remaining experimental settings allowed to obtain the inhibition values of *C. albicans* ATCC 10231 by *L. plantarum* ATCC 14917.

The probiotic activity of *L. plantarum* ATCC14917 to inhibit the initial adhesion of *C. albicans* ATCC10231 was also evaluated in ES1, ES2, and ES3 (see Figure #3). As expected, *L. plantarum* ATCC14917 was able to induce a superior inhibition of *C. albicans* ATCC10231 in ES1 (81%) and ES2 (58%) when compared to *L. gasseri* strains (ES1:27-73%; $P < 0.001$, using two-way ANOVA; ES2:1-49%; $P < 0.001$, using two-way ANOVA). In ES1, the inhibition induced by *L. plantarum* ATCC14917 on *C. albicans* ATCC10231 was statistically significantly superior against *L. gasseri* IMAUFB014 (Tukey's post hoc, $P = 0.050$) and *L. gasseri* JCM1131 (Tukey's post hoc, $P < 0.001$). No statistically significant differences were found in the inhibition values between *L. plantarum* ATCC14917 and *L. gasseri* H59.2 (Tukey's post hoc, $P = 0.735$). In ES2, *L. plantarum* ATCC14917 has only evidenced statistically differences against *L. gasseri* H59.2 (Tukey's post hoc, $P < 0.001$) on the inhibition

of *C. albicans* ATCC10231, although lower values of inhibition were achieved by *L. gasseri* IMAUFB014 (49%; Tukey's post hoc, $P = 0.937$) and *L. gasseri* JCM1131 (43%; Tukey's post hoc, $P = 0.598$).

Finally, in ES3, *L. plantarum* ATCC14917 showed the lowest inhibition rate on the initial adhesion of *C. albicans* ATCC10231 (56%), but no statistically significant differences were found against *L. gasseri* strains (ES3:60-67%; $P = 0.764$, using two-way ANOVA). When comparing low (ES1) and high (ES3) levels of lactobacilli against low inocula of *Candida albicans*, no statistically significant differences were detected in the inhibition rates of *L. plantarum* ATCC14917 against *C. albicans* ATCC10231 ($P = 0.072$; two-way ANOVA).

In all experimental settings, *L. plantarum* ATCC14917 showed a statistical reduction in their colonization by *C. albicans* ATCC10231 when compared to their control ($P < 0.05$ with Student *t*-test; see Figure #3). *L. plantarum* ATCC14917 was able to induce a superior inhibition of *C. albicans* ATCC10231 in ES1 and ES2.

DISCUSSION

The vaginal microbiota is also constituted by fungi, hence the difficulty to eradicate *Candida albicans* from the human epithelium. Many treatments have been recommended against *Candida* species (Santos, et al., 2018); however, they can lead to increase the antimicrobial resistance of the pathogen or even side effects in the host (Graf, et al., 2019). Intravaginal antifungals can cause irritation, burning, and pain in the vaginal area. Oral antifungals are metabolized in the liver, being risky for people who suffer from diseases related to this organ, and they can interact with other medications, causing side effects (Jeavons, 2003). These treatments do not eradicate *Candida* spp. from the rectal area, which could lead to reinfection. On the other hand, some conditions increase the possibility of colonization of this fungus, such as a state of immunosuppression, use of antibiotics, contraceptives, diabetes mellitus, clothing (causing irritation or wet environment), and sexual activity. The application of lactobacilli has been demonstrated as an alternative with a low probability of generating resistance and producing side effects, as well as a more accessible and attractive natural methodology (Jeavons, 2003). Several researchers have studied the application of lactobacilli as a treatment against *Candida* spp. infections finding promising results (Chew, et al., 2015; De Seta, et al., 2014; Macklaim, et al., 2015).

This study evaluated the intrinsic probiotic activity of lactobacilli against strains of *C. albicans* in initial adhesion assays on an abiotic surface (coverslip). The inhibition produced in *C. albicans* due to lactobacilli was evaluated through different experimental settings. These scenarios were intended to aim different conditions of the human vaginal mucosa, more exactly: ES1 and ES4 simulated vaginal dysbiosis, where the human microbiota is altered; ES2 mimicked the concentration of these microorganisms in candidiasis; and ES3 exemplified a healthy microbiota, being *Lactobacillus* spp. in high concentrations and *Candida albicans* in low concentrations (Pacha-Herrera, et al., 2020; Salinas, et al., 2020). Few studies focus on the

inhibition of pathogen initial adhesion (Alves, et al., 2014; Machado, et al., 2013; Machado, et al., 2013; Matsuda, et al., 2018). This research study reported on the initial inhibition of *C. albicans*, having a great impact through its evaluation in the early stages of colonization on a glass surface (abiotic surface).

Some studies previously characterized the inhibition of the initial adhesion of certain pathogens, such as *Gardnerella vaginalis*, *Prevotella bivia*, *Mobiluncus mulieris* (Machado, et al., 2013), *Listeria monocytogenes* (Lezzoum-Atek, et al., 2019), *Streptococcus mutans*, and *C. albicans* (Hasslöf, et al., 2010; Matsuda, et al., 2018). He et al. (2020) evaluated the probiotic activities of certain *Lactobacillus* species (two *L. gasseri* and three *L. crispatus* strains) on the inhibition of the initial adhesion of several pathogens, including *C. albicans in vitro* assays, using vaginal epithelial cell lines (VK2/E6E7 and primary VECs). On the initial adhesion assays using high levels of lactobacilli (10^8 CFU/ml), *L. gasseri* 1 # strain showed the strongest initial adhesion inhibition against *C. albicans*, 56%. *L. crispatus* 3 # were able to inhibit the initial adhesion of *S. aureus* with greater efficiency, while *L. crispatus* 4 # evidenced a larger inhibition proficiency against multiple opportunistic pathogens (*Gardnerella, Mobiluncus, E.* coli and *E. faecalis*). However, only *L. gasseri* demonstrated a more probiotic activity against *C. albicans*, when compared to *L. crispatus* strains. Indeed, *L. crispatus* 4 # only inhibited around 39% of the initial adhesion of *C. albicans*. Our results are in agreement with He et al. (2020), reporting differences in probiotic activity among *Lactobacillus* species and strains against *C. albicans* isolates.

Most probiotic studies evaluated the ability of lactobacilli supernatants to downregulate the levels of gene expression in biofilm formation and even in cells' adhesion (Dos Santos, et al., 2019; Matsubara, et al., 2016; Matsuda, et al., 2018; Wasfi, et al., 2018). For instance, Jang et al. (2019) evaluated the inhibition of *Candida albicans* initial growth with several lactobacilli supernatants. After the initial evaluation of 51 *Lactobacillus* strains, selected three species to

evaluate the probiotic activity of their supernatants on an abiotic surface (polystyrene) for growth inhibition (*L. gasseri* SNUV281, *L. crispatus* SNUV220, and *L. fermentum* SNUV175). The maximum inhibition growth of *C. albicans* was induced by *L. gasseri* strain SNUV281 (75%), being only surpassed by the combination of both supernatants from *L. fermentum* SNUV175 and *L. crispatus* SNUV220 (77%). In another study, vaginal lactobacilli (*L. crispatus* B1-BC8, *L. gasseri* BC9-BC14, and *L. vaginalis* BC15-BC17) were isolated from 15 healthy premenopausal women. The probiotic activity of these lactobacilli (5×10^7 CFU/ml) against different *Candida* species (5×10^7 yeast/ml) was analyzed in an *in vitro* model by the inhibition in HeLa cells. The most probiotic *Lactobacillus* spp. were *L. crispatus* BC2, *L. gasseri* BC10, and *L. gasseri* BC11 against *C. albicans* (Parolin, et al., 2015).

The present study also compared the probiotic activity of vaginal lactobacilli strains (*L. gasseri*) and a non-vaginal specie (*L. plantarum*) against different strains of *C. albicans*. According to our data, a difference in relation to the inhibition can be seen for each *Lactobacillus* species. Likewise, among the *L. gasseri* strains, there is a difference on the probiotic activity against each of the *C. albicans* strains. It was possible to verify a variability in the probiotic activity of lactobacilli, not only between different species, but also against different strains of the same *Lactobacillus* species when they are analyzed against the same pathogen. This variability agrees with a recent study realized with *Lactobacillus crispatus* BC1 and a vaginal *L. crispatus* strain on the adhesion of *Candida* species among HeLa cell line. Although this study evaluated the activity of biosurfactants on yeast adhesion, it evidenced that the probiotic activity of *L. crispatus* was *Candida* strain-specific due to different results on inhibition among strains of the same species (*C. albicans*), and also between different *Candida* species (such as, *C. tropicalis*, *C. krusei,* and *C. glabrata*) (De Gregorio, et al., 2020).

However, these studies did not consider the colonization of these probiotics in the vaginal epithelium in the long term (Di Cerbo, et al., 2016; Jalilsood, et al., 2015; Martinez, et al.,

2020; Matsubara, et al., 2016). A healthy vaginal environment is usually associated to *L. gasseri*, *L. crispatus*, and *L. jensenii*. Therefore, the present study evaluated *Lactobacillus plantarum* probiotic activity in the initial adhesion of an abiotic surface, aiming for a future and a different approach. An alternative approach could be realized in the vaginal colonization of a non-vaginal lactobacilli with greater probiotic activity, which could be assimilated and persisted in the vaginal microbiota over time and being capable to produce biosurfactants or to form biofilms with a protective role. This approach could increase the probiotic activity of the remaining commensal microbiota, as previously postulated in several studies (Alves, et al., 2020; Burton, et al., 2003; Petrova, et al., 2017; Zangl, et al., 2020). It is also important to mention that several studies reported the use of different lactobacilli and their multiple benefits for the same host (Hasslöf, et al., 2010; Wang, et al., 2018; Wasfi, et al., 2018).

In 2010, Hasslöf and colleagues evaluated the probiotic activity of several lactobacilli at concentrations ranging from 10^9 to 10^5 CFU/ml against *Streptococcus mutans* and *C. albicans*, showing the strongest inhibition on *C. albicans* by *L. plantarum* and *L. reuteri* (Hasslöf, et al., 2010). No significant differences in the inhibition scores of *C. albicans* were detected by Hasslöf et al. (2010) between the reference strains and the lactobacilli isolates, differing from our results. However, *L. plantarum* showed a great inhibition ability against *C. albicans*. In 2018, Wasfi and colleagues demonstrated a serious reduction in the initial adhesion and biofilm growth of *Streptococcus mutans* ATCC 25175 by untreated supernatants of *L. plantarum* ATCC 14917 and *L. reuteri* ATCC 23272, reporting an adherence inhibition of 80.5–81.7% and even a biofilm inhibition of 24.7–26.5% (Wasfi, et al., 2018).

The activity of *L. plantarum* ATCC 14917 to form biofilm has been evaluated with an impressive results, showing a great capacity to inhibit opportunistic bacteria (Jalilsood, et al., 2015; Martinez, et al., 2020). The biofilm is a post-colonization step after a successful initial adhesion, allowing a continuous colonization on the surface or epithelium. It consists of an

initial adhesion on the cells of the epithelium of the human mucosa, colonization, and maturation, where extracellular polysaccharides are going to be produced. In the final stage, microorganisms are dispersed as planktonic cells in search of other zones to repeat this process (Machado & Cerca, 2015; Salas-Jara, et al., 2016). Not all lactobacilli possess the ability to form a biofilm (Salas-Jara, et al., 2016). However, there is still limited information on lactobacilli biofilms on different abiotic and biotic surfaces. The present study confirmed the probiotic activity of lactobacilli against *C. albicans* on a glass surface, demonstrating higher percentages of *C. albicans* inhibition (56-81%) by *L. plantarum* ATCC 14917 and showing statistically significant differences when compared to *L. gasseri* strains (1-73%).

The results of *L. plantarum* surpassed the rates of inhibition adhesion on *C. albicans* reported by Dos Santos et al. (2019) with biosurfactants produced by vaginal lactobacilli, such as *L. gasseri* strains (22–67%), *L. fermentum* ATCC 23271 (33%), *L. paracasei* (44%), *L. debrueckii* ATCC 9645 (44%), *L. acidophilus* ATCC 4356 (78%), and *L. rhamnosus* ATCC 9595 (78%) on antagonism assays for 4h. In the present study, low levels of *L. plantarum* ATCC 14917 (10^3 CFU/mL) efficiently inhibited the initial adhesion of low and high levels of *C. albicans* (81 and 58% on ES1 and ES2, respectively), disagreeing with previous studies that stipulated a higher dose of *L. plantarum* (> 10^8 CFU) to treat candidiasis and bacterial vaginosis (De Seta, et al., 2014; Di Cerbo, et al., 2016; Vicariotto, et al., 2014).

In summary, the existing variability on the inhibition was demonstrated in this study. In addition, current work demonstrated the potential probiotic benefits of *L. plantarum* as a non-vaginal lactobacilli, which could be used against other opportunistic microorganisms associated in different vaginal infections, such as bacterial vaginosis (BV) and as aerobic vaginitis (AV). However, further studies are necessary to fully evaluate the colonization over time on abiotic and biotic surfaces by vaginal and non-vaginal lactobacilli.

CONCLUSIONS

The present work was able to find variability in the probiotic activity of different species of *Lactobacillus*, as well as between different strains of *L. gasseri* isolated from vaginal microbiota against the same pathogen (different isolates of *C. albicans*) on initial adhesion assays. *L. plantarum* had the highest inhibition capacity when compared to *L. gasseri* strains. More exactly, a greater inhibition was found in ES1 (81%) and ES2 (58%) by *L. plantarum* while *L. gasseri* strains evidenced lower levels of inhibition against *C. albicans* ATCC 10231 (ES1: 27-73%; ES2: 1-49%). These findings agree with previous studies. Also, *L. plantarum* showed better probiotic activity on initial adhesion, when compared to other lactobacilli analyzed in previous studies. These results support the possibility to use non-vaginal lactobacilli as possible candidates for human colonization and greater inhibition of opportunistic pathogen colonization, as already postulated by other authors.

However, the present research only evaluated the inhibition of one opportunistic pathogen in initial adhesion assays on an abiotic surface. Therefore, some limitations could be found in this study, such as the non-evaluation of long-term colonization, nor the colonization and interaction of lactobacilli and *C. albicans* strains in the human epithelium. A limited number of lactobacilli was used in the experimental assays, more exactly, a single non-vaginal lactobacilli strain and merely three strains of the same vaginal lactobacilli. Further studies should evaluate other species of *Lactobacillus* and strains, as well as *Candida* species. It is necessary to characterize probiotic activities beyond inhibition, as competition and exclusion abilities; as well as to carry out studies *in vitro* and *in vivo* models that evaluate a longitudinal relationship in the colonization of non-vaginal lactobacilli and *C. albicans.*

TABLES

Table # 1

Microorganisms used in this study

Microorganism	**Strains**	**Labeled as:**
Lactobacillus gasseri	JCM1131	V140 B
	IMAUFB014	V254 A
	H59.2	V130 B
L. plantarum	ATCC 14917	LB14917P
Candida albicans	ATCC10231	ATCC10231
	Isolated from candidiasis	V535 A
	Isolated from healthy microbiota	V251 A

Table # 2

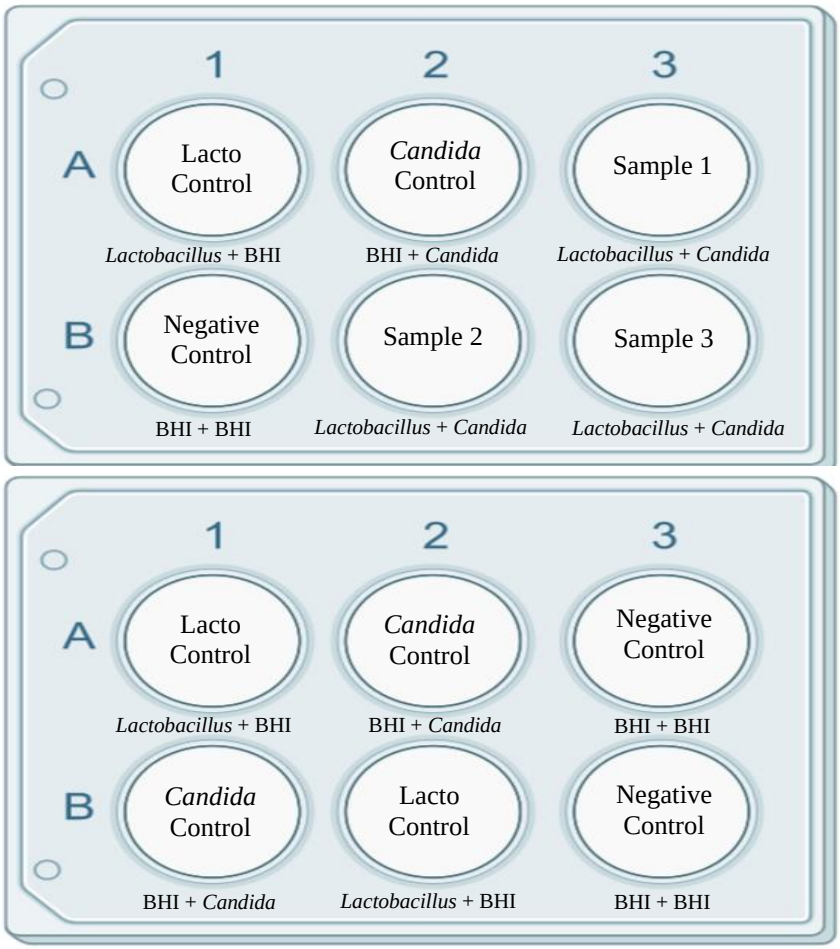
Combinations of Experimental Settings (ES)

Lactobacillus / ***Candida***	**1.0E+03 UFC/ml**	**1.0E+09 UFC/ml**
1.0E+03 UFC/ml	1	3
1.0E+09 UFC/ml	2	4

FIGURES

Figure # 1

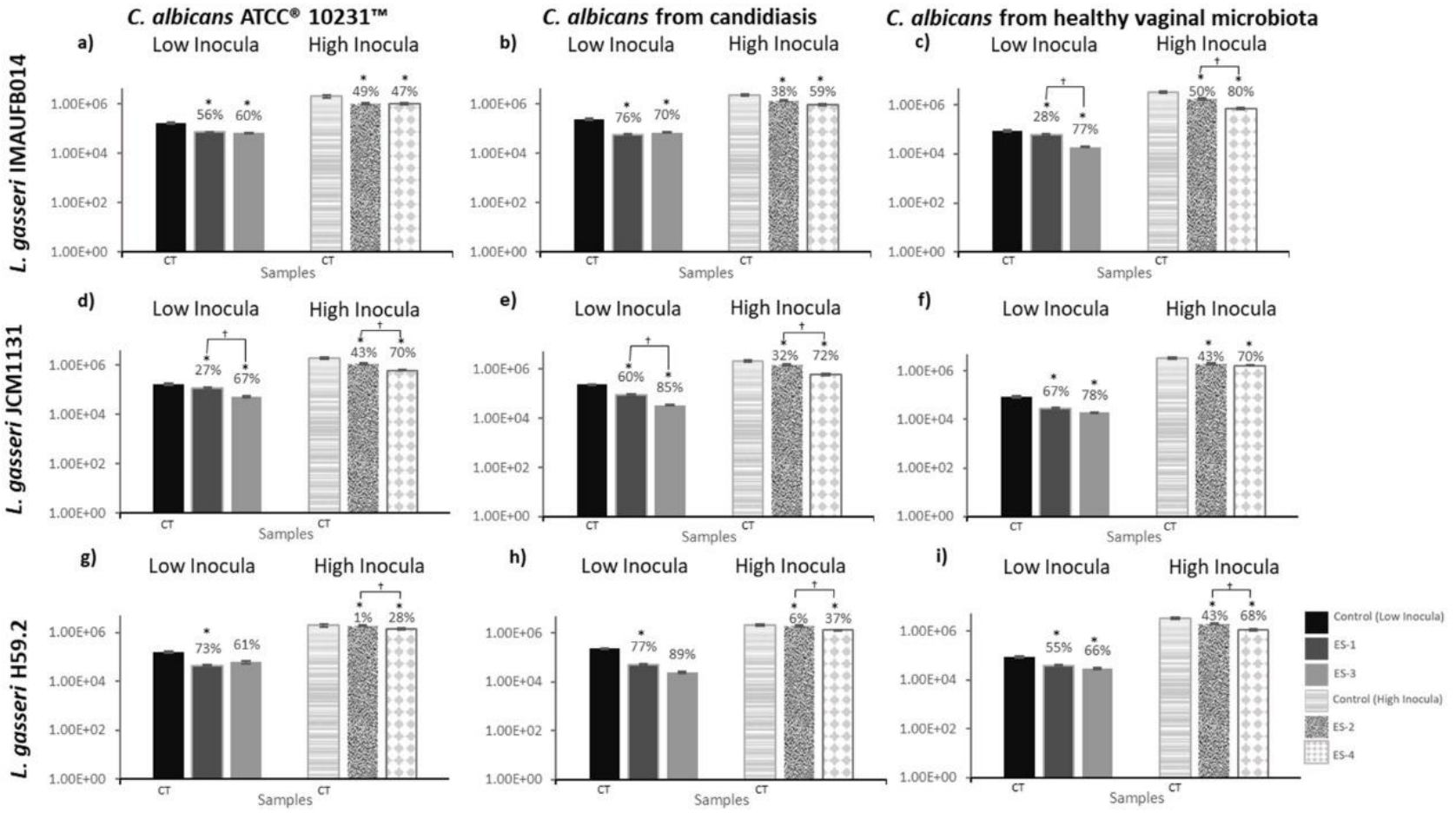
6-well plate distribution on initial adhesion assays



Controls and samples were carried out by triplicate. The figure shows the solution used for each control and the samples.

Figure # 2

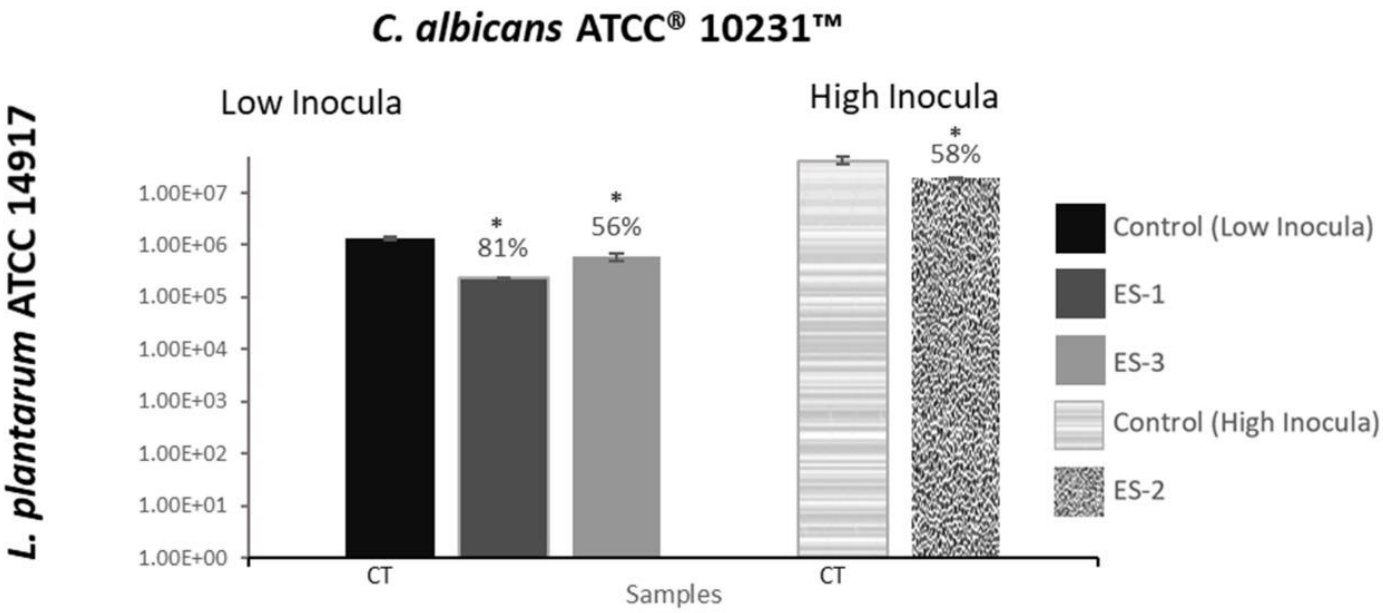
Probiotic activity of Lactobacillus gasseri against Candida albicans evaluated in initial adhesion assays, Adapted from (Montalvo, 2018)



Inhibition of *Candida albicans* by *Lactobacillus gasseri* after initial adhesion treatments with the experimental setting of high and low inoculum. The percentage of adhesion of *Candida albicans* is the result of the variation in the adhesion of *Lactobacillus gasseri* and *C. albicans* strains to coverslip in comparison to controls (CT, 100 % of adhesion) when incubated alone at the same conditions in the glass surface. **Statistical analysis:** $^{*}P < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of candida control and sample tested in the adhesion assay; $^{\dagger}P < 0.05$ analyzed using two-tailed ANOVA statistical test (95% confidence interval) for comparison of inhibition values between experimental setting (ES) for each evaluated *Candida albicans* isolated in the adhesion assays

Figure # 3

Probiotic activity of Lactobacillus plantarum against Candida albicans evaluated in initial adhesion assays



Inhibition of *Candida albicans* by *Lactobacillus gasseri* after initial adhesion treatments with the experimental setting of high and low inoculum in the glass surface. **Statistical analysis:** * $P < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of candida control and sample tested in the adhesion assay; † $P < 0.05$ analyzed using two-tailed ANOVA statistical test (95% confidence interval) for comparison of inhibition values between experimental setting (ES) for each evaluated *Candida albicans* ATCC® 10231 ™ isolated in the adhesion assay. No statistically significant values were found.

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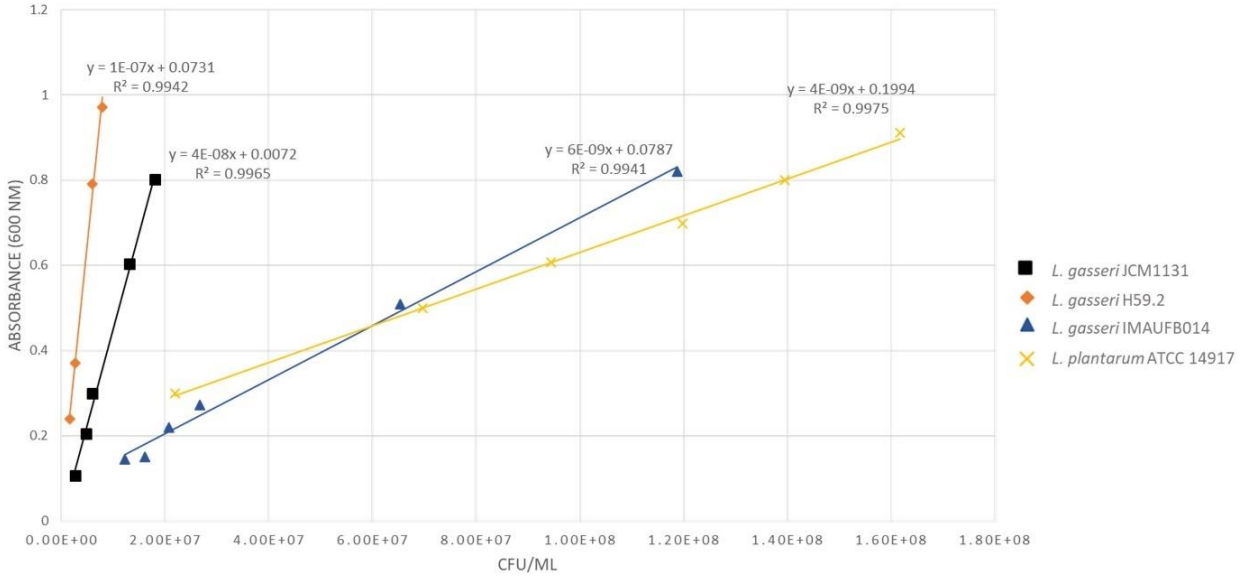
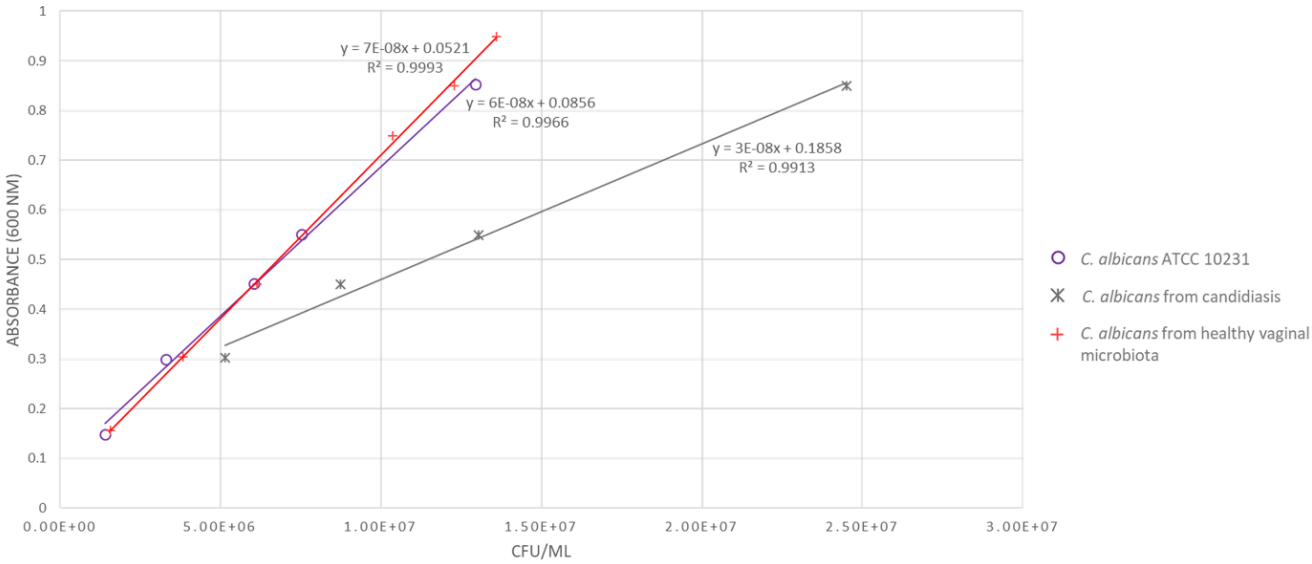
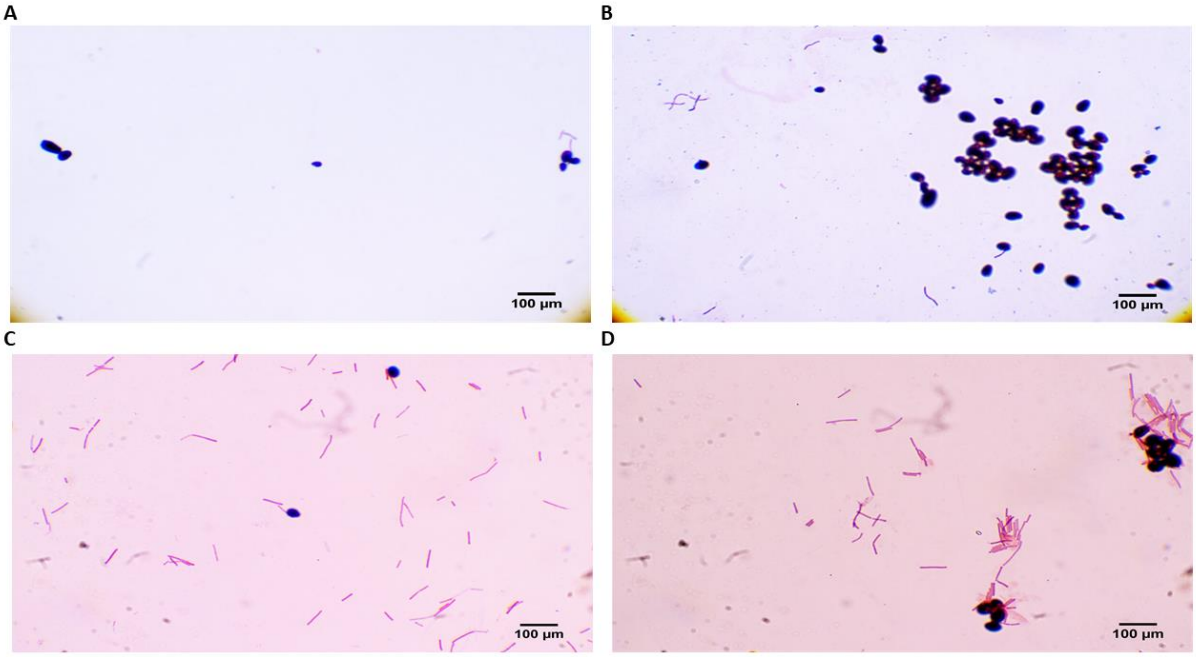
APPENDIX A: GROWTH CALIBRATION CURVES OF *LACTOBACILLUS* SPP. (*L. GASSERI* AND *L. PLANTARUM*) AT OD 600 NM.



APPENDIX B: GROWTH CALIBRATION CURVES OF *CANDIDA ALBICANS* STRAINS AT OD 600 NM.



APPENDIX C: EXPERIMENTAL SETTINGS (ES) OF *L. GASSERI* IMAUFB014 AGAINST *C. ALBICANS* ATCC 10231 IN INITIAL ADHESION ASSAYS.



Description: A Photography of ES1 at 1000x *L. gasseri* IMAUFB014 (1.00E+03 CFU/ml) against *C. albicans* ATCC® 10231 ™ (1.00E+03 CFU/ml). **B** Photography of ES2 at 1000x *L. gasseri* IMAUFB014 (1.00E+03 CFU/ml) against *C. albicans* ATCC® 10231 ™ (1.00E+09 CFU/ml). **C** Photography of ES3 at 1000x *L. gasseri* IMAUFB014 (1.00E+09 CFU/ml) against *C. albicans* ATCC® 10231 ™ (1.00E+03 CFU/ml). **D** Photography of ES4 at 1000x *L. gasseri* IMAUFB014 (1.00E+09 CFU/ml) against *C. albicans* ATCC® 10231 ™ (1.00E+09 CFU/ml).