UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

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Lactobacilli displacement by *Candida albicans* on initial adhesion assays: A case report

.

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RESUMEN

Ciertas especies de *Lactobacillus* con carácter probiótico son los que al agregarse al epitelio vaginal constituyen una barrera protectiva en contra de microorganismos patógenos. *Candida albicans* es una levadura que en bajas concentraciones (1.00E+03 CFU/ml) forma parte de la microbiota vaginal, pero al desencadenarse un desequilibrio ecológico puede generar candidiasis vulvo vaginal (CVV), la cual se reconoce que al menos el 75% de las mujeres lo ha experimentado en su edad fértil. El presente estudio evalúa el nivel de desplazamiento de tres cepas de *L. gasseri* (origen vaginal) y una de *L. plantarum* (no vaginal) ya adheridos a una superficie abiótica (vidrio) por parte de tres cepas de *Candida albicans*. Junto con Robert Rodríguez en el instituto de Microbiología USFQ se desarrollaron y estandarizaron ensayos de adhesión inicial, bajo cuatro condiciones experimentales específicas (ES1-ES4) que variaron la concentración de los microorganismos, siendo un inóculo alto (1.00E+09 CFU/ml) o bajo (1.00E+03 CFU/ml). Cada experimento enfrentó una de las cuatro cepas de *Lactobacillus* sp. frente a una de las tres cepas de *C. albicans*. De acuerdo con los seteos experimentales *L. plantarum* fue desplazado por *C. albicans* en un 23%, 31% y 54% para los niveles ES1, ES2 y ES3, respectivamente. Por su parte *L. gasseri* sufrió un desplazamiento mayor siendo: 61-84%, 82-96% y 83-95% para los niveles ES1, ES2 y ES3, respectivamente. Mostrando diferencias estadísticas (ES1: $P = 0.002$, ES2: $P = 0.007$ y ES3: $P = 0.031$; ANOVA de dos factores). Los datos respaldan investigaciones previas en los que se ha empleado *Lactobacillus* no humanos como potenciales agentes probióticos para colonizar el epitelio de la mucosa e inhibir la colonización inicial de patógenos. Se deben realizar más estudios *in vitro* e *in vivo* para caracterizar la colonización longitudinal de lactobacilos no vaginales.

Palabras clave: Probióticos, *Candida albicans*, *Lactobacillus gasseri*, *Lactobacillus plantarum*, Candidiasis, Desplazamiento, Adhesión inicial.

ABSTRACT

Certain species of Lactobacilli with a probiotic character are those that, when added to the vaginal epithelium, constitute a protective barrier against pathogenic microorganisms. *Candida albicans* is a yeast that in low concentrations (1.00E + 03 CFU / ml) is part of the vaginal microbiota, when an ecological imbalance is triggered, it can generate vulvo vaginal candidiasis (VVC), it is recognized that at least 75% of women they have experienced it in their childbearing years. The present study seeks to evaluate the level of displacement (by three strains of *C. albicans*) of three strains of *L. gasseri* (vaginal origin) and one of *L. plantarum* (non-vaginal) already adhered to an abiotic surface (glass). Together with Robert Rodríguez at the Institute of Microbiology (USFQ), initial adhesion tests were developed and standardized under four specific experimental settings (ES1-ES4) that varied the concentration of the microorganisms, being an inoculum high (1.00E + 09 CFU/ml) or low (1.00E + 03 CFU/ml). Each experiment faced one of the four strains of *Lactobacillus* sp. against one of the three strains of *C. albicans*. According to the experimental settings *L. plantarum* was displaced by *C. albicans* 23%, 31% and 54% for the ES1, ES2 and ES3 levels, respectively. On the other hand, *L. gasseri* suffered a greater displacement being: 61-84%, 82-96% and 83-95% for levels ES1, ES2 and ES3, respectively. Showing statistical differences (ES1: $P = 0.002$, ES2: $P = 0.007$ and ES3: $P = 0.031$; two-factor ANOVA). The data support previous research in which non-human Lactobacilli have been used as potential probiotic agents to colonize the mucosal epithelium and inhibit initial colonization of pathogens. Further *in vitro* and *in vivo* studies should be performed to characterize the longitudinal colonization of non-vaginal lactobacilli.

Keywords: Probiotics, *Candida albicans*, *Lactobacillus gasseri*, *Lactobacillus plantarum*, Candidiasis, Displacement, Initial adhesion.

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INTRODUCTION

There are more than 1,000 bacterial species that live commensally in human microbiota, such as oral cavity, respiratory tract, gastrointestinal tract, vagina, skin, and other tissues. These microorganisms are acquired shortly after birth and remain relatively stable until the death of the host. For the human body, commensal microorganisms represent multiple benefits, since they play an important role in relation to their physiology, participating in mechanisms, such as: digestion and assimilation of nutrients; protection against the colonization of pathogens; modulation of the immune response; the regulation of fat storage; and intestinal angiogenesis (Lebeer et al., 2008).

The vaginal microbiota is typically made up of a polymicrobial diversity formed by anaerobic and aerobic microorganisms (Borges et al., 2014). Certain species of lactobacilli are the most predominant in the vaginal tract (Hickey et al., 2012). It is commonly characterized a healthy vaginal microbiota through a dominance of certain *Lactobacillus* species, more exactly, *Lactobacillus crispatus, L. inners, L. jensenii* and *L. gasseri* (Lebeer et al., 2008; Martín et al., 2008).

Lactobacilli are crucial in several fields due to their metabolic activity, allowing the generation of fermented products for centuries. In the last decades, their reputation has increased as they started to be consider ¨health promoters¨, being used as probiotics in food for animals and humans (Charteris et al., 1998; Coeuret et al., 2004; Lebeer et al., 2008). However, the question is still remain about the etiology of a probiotic. In the USA, Food and Agriculture Organization (FAO) defines probiotic as "*a living microorganism that, when administered in adequate amounts, confers a benefit for the health of the host*" (2001).

The protective properties that make certain lactobacilli as potential probiotics are the following reasons: the ability to adhere to the cells of the vaginal epithelium and minimize adherence by

pathogens; maintain and multiply, modulate the immune response; produce antimicrobial compounds (bacteriocins, hydrogen peroxide, lactic acid); resist vaginal microbicides and spermicides; being safe (non-invasive, non-carcinogenic) for human and animals; and lastly, constitute part of a normal and healthy microbiota (Borges et al., 2014; Lebeer et al., 2008; Martín et al., 2008; Reid, 1999). Thus, certain *Lactobacillus* species are essential to achieve vaginal homeostasis and to prevent opportunistic infections in the human host, such as bacterial vaginosis, yeast vaginitis urinary tract infections and sexually transmitted diseases (Borges et al., 2014; Burton et al., 2003; Martín et al., 2008).

More thoroughly, the lactobacilli adherence to the vaginal epithelium is driven by the recognition of receptors in the epithelium (such as fibronectin) by their adhesins. The union between the lactobacilli and the vaginal epithelium triggers the scaffolding of a biological surfactant or even biofilm that serves as a protective barrier for certain microorganisms, such as *E. coli, G. vaginalis, C. albicans* (Martín et al., 2008).

Some pathogens are capable of overcoming this lactobacilli protection and as a result of a significant displacement or decrease in levels of *Lactobacillus* sp., leading to imbalance microbiota and to a pathological state (Parolin et al. 2015). At diagnostic level, the observation of this imbalance vaginal microbiota on the slides (from a vaginal swab) is commonly associated with vaginitis or dysbiosis, such as bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), trichomoniasis and lower urinary tract infections (Martín et al., 2008). It is noteworthy that 75% of all women experience a CVV episode in their childbearing years, being *Candida albicans* the main etiological agent. *Candida albicans* is a dimorphic fungus (from Phylum Ascomycota) with the ability to establish pseudohyphae and hyphae. The immune system is responsible for controlling the concentration of the fungus. However, its augmentation in the vaginal microbiota is associated to multiple reasons, such as sporadic or immunosuppressive symptoms, deterioration of the vaginal microbiota, intake of antibiotics,

hormonal changes, pregnancy, supply of antifungals (e.g., clotrimazole and fluconazole) (Achkar & Fries, 2010; J. Sobel, 2013; J. D. Sobel, 2007).

In order to better understand the different probiotic properties and their intrinsic and potential effects on the vaginal microbiota, the present study seeks to evaluate the level of displacement of three strains of *Lactobacillus gasseri* (vaginal lactobacilli) and one *Lactobacillus plantarum* (non-vaginal lactobacilli) by three strains of *Candida albicans* (from different origins). Initial adhesion evaluation were realized in the present study, using several experimental settings (high and low inocula of both microorganisms) on an abiotic surface (glass). Thus, in lactobacilli attempt to block the adherence of the *C. albicans*, the present work evaluated the displacement suffered by these lactobacilli against the opportunistic pathogen, as previously reported in the literature (Zarate & Nader-Macias, 2006).

METHODS

Strains and culture conditions

From previous vaginal microbiota studies conducted by the Microbiology Institute at USFQ (Pacha-Herrera et al., 2020; Salinas et al., 2020; Montalvo, 2018), the present work selected the following microorganisms: three strains of *Lactobacillus gasseri* (H59.2, IMAUFB014 and JCM1131), one *L. plantarum* ATCC® 14917 ™ and three isolates of *Candida albicans* (*C. albicans* ATCC® 10231 ™; one isolate of *C. albicans* from a patient with a healthy microbiota, and another *C. albicans* isolate from a patient with candidiasis). These microorganisms were preserved in Brain Heart Infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA) with 15% glycerol in an ultra-freezer at -80°C with their respective label: *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).

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

Initial adhesion assays

Lactobacillus spp. was tested against *C. albicans* isolates on initial adhesion assays. Each microorganism was concentrated in 5 ml of sterile phosphate-buffered saline (PBS) solution. Both suspensions were collected by centrifugation (4000 *g*, 12 min, at room temperature),

washed twice with PBS. The pellet was resuspended in PBS and its concentration was adjusted according to the standard growth curves to 1.0E+03 colony-forming unit (CFU)/ml (low inocula) and 1.00E+09 CFU/ml (high inocula; see Appendix A and B) in both microorganisms, by the optical density at 600 nm (OD600) using the visible spectrophotometer GENESYS ™ 20 (Thermo Scientific, New York, USA). Four experimental settings (see Table 1) were obtained from the combination of the concentrations (Castro et al., 2013; Fidel et al., 2004; António Machado, Jefferson, et al., 2013; Seneviratne et al., 2016). Each solution was then centrifuged (400 *g* for 12 min), PBS was discarded, and the pellet was resuspended in 13 ml of BHI broth (Matsubara et al., 2016).

In each experimental assay, the controls and the samples were elaborated with triplicate. Sterile glass coverslips were placed in two 6-well plates, 2 ml of the *Lactobacillus* solution was placed for each adhesion control of *Lactobacillus* and sample; 2 ml of BHI broth was added to the wells designated for the adhesion control of *C. albicans* and negative control (culture media without bacteria or yeast). The plates were incubated for 4h at 37°C, in anaerobic conditions, and 120 revolutions per minute (rpm) (António Machado, Jefferson, et al., 2013; Nishiyama et al., 2014). Non-adherent lactobacilli were removed by washing with 2 ml of PBS, and subsequently a second adhesion step was performed, 2 ml of the *C. albicans* solution was added for each adhesion control of *Candida* and sample; 2 ml of BHI broth was added to the designated wells for *Lactobacillus* control and negative control. The plates were then cultured for 30 min at 37°C, in anaerobic conditions, and 120 rpm (António Machado, Jefferson, et al., 2013; Nishiyama et al., 2014) (see Figure #1).

Microscopy analysis and cell quantification

Prior to the recovery of the coverslips, a careful washing with 2 ml of PBS was carried out. They were fixed with absolute ethanol (96%; v/v) and were stained with 1 ml of crystal violet

at 3% for 1 minute (Weerasekera et al., 2016). Finally, from each coverslip, 15 random fields were observed in the OLYMPUS BX50 microscope under 1000x, as previously realized in other studies (CHAUVIERE et al., 1992; António Machado, Jefferson, et al., 2013). One picture was taken for each field, 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 cells from *Lactobacillus* spp. and *C. albicans* was counted in each field (see Appendix C) to obtain the number of cells over the total area of the abiotic surface. Briefly, the coverslip area (4.84E + 08μm^2) was divided by the area of the picture (12,880 μm^2) and the average of cells (bacteria and or yeast) of the 15 fields was multiplied by the previous relationship, obtaining the estimated number of cells over the total area of the abiotic glass surface (approximately 4.84 cm^2 = 4.84E + 08μm^2). As shown in Tables 2 and 3, the results were expressed as number of cells per glass surface ± standard deviation (N. of cells per glass surface ± SD). All experimental assays carried out with triplicate samples and each assay was repeated three times on different days.

Statistical analysis

All statistical analysis was based on the experimental assays realized by our research group. The evaluation of statistically significant differences was realized through a two-tailed ANOVA (ANalysis Of VAriance) analysis with post-hoc Tukey HSD (Honestly Significant Difference) test and Student *t* test. More exactly, ANOVA analysis was applied to evaluate differences in and between experimental settings (ES), post-hoc Tukey HSD test was performed to evaluate differences between different species of lactobacilli and isolates of *Candida albicans* on the same ES, and finally Student *t* test evaluated differences between each analyzed specie and their respective control. Statistical analysis was performed using the

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

RESULTS

The main objective of the present study was to evaluate the probiotic activity, in relation to the displacement induced by *Candida albicans*, of various strains of *Lactobacillus gasseri* (vaginal lactobacilli) and one reference strain of *L. plantarum* (non-vaginal lactobacilli) through initial adhesion assays. On initial adhesion assays, displacement of the adhered lactobacilli was evaluated on abiotic surface (glass surface) through different experimental settings, mimicking different vaginal microbiota conditions. These different experimental settings (ES) were realized with low and high levels of lactobacilli and *C. albicans* on the initial colonization of a surface.

Displacement of *Lactobacillus gasseri* by *Candida albicans*

The evaluation of the lactobacilli displacement was firstly evaluated on low levels (ES1 and ES2) against low and high concentrations of *C. albicans* and then on high levels (ES3 and ES4), as shown on Table 2. On low levels of lactobacilli, the range of displacement was between 15 and 99%, evidencing the greatest displacement percentages against *C. albicans* at high levels (range of 72-99%). Moreover, at low levels of both microorganisms (ES1, similar to a dysbiosis condition), *C. albicans* ATCC10231 induced bigger displacement of *L. gasseri* IMAUFB014 (84%) and H59.2 (83%) but showing a reduced displacement of *L. gasseri* JCM1131 (61%). In fact, the displacement of *L. gasseri* IMAUFB014 ($P = 0.010$; two-way ANOVA) and H59.2 ($P < 0.001$; two-way ANOVA) proved to be statistically significant among the different *C. albicans* isolates, being both lactobacilli vulnerable against *C. albicans* ATCC10231 (Tukey's post hoc, $P < 0.05$; when comparing their displacement against the remaining *Candida* isolates). Likewise, all *C. albicans* isolates showed to be statistically different in their displacement ability among the evaluated *L. gasseri* strains (*C. albicans* ATCC10231: $P = 0.051$; *C. albicans* isolated from candidiasis: $P = 0.001$; *C. albicans* isolated

from healthy microbiota: $P = 0.002$, using two-way ANOVA analysis). However, when low levels of lactobacilli were exposed to high levels of *Candida albicans* (ES2, similar to a candidiasis infection), no statistically significant differences were found in the displacement ability of any *C. albicans* isolates among *L. gasseri* strains. In ES2, only *C. albicans* isolated from candidiasis was able to reach the highest displacement values (more than 90%) in all strains of *L. gasseri*. It is important also to mention that the displacement of *L. gasseri* IMAUFB014 ($P = 0.049$; two-way ANOVA) proved again to be statistically significant among the different *C. albicans* isolates. However, no statistically significant differences in displacement of *L. gasseri* IMAUFB014 were found through Tukey's post hoc analysis.

On high levels of lactobacilli (ES3 and ES4), the range of displacement was between 15 and 98%, and certain differences were found in lactobacilli displacement between low and high levels of *C. albicans*. At low levels of *C. albicans* (ES3, similar to healthy vaginal microbiota), *C. albicans* ATCC10231 demonstrated again the highest displacement values in all *L. gasseri* strains and without statistical significance among them. While *C. albicans* isolated from candidiasis showed statistically differences in lactobacilli displacement, evidencing a greater ability to displace *L. gasseri* H59.2 (90%; $P < 0.001$ using two-way ANOVA). In addition, *L. gasseri* H59.2 was the most susceptible to be displaced by all isolates of *C. albicans* at both levels (ES3 and ES4) and without statistical significance among them. However, the displacement of *L. gasseri* JCM1131 showed to be statistically different among the evaluated *C. albicans* isolates in low ($P < 0.001$; two-way ANOVA in ES3) and high levels ($P = 0.039$, using two-way ANOVA in ES4). In ES3, *L. gasseri* JCM1131 showed only 15% of displacement by *C. albicans* isolated from candidiasis, being statistically different when compared to *C. albicans* ATCC10231 (83%; $P = 0.001$, using Tukey's post hoc) and *C. albicans* isolated from healthy vaginal microbiota (84%; $P < 0.001$, using Tukey's post hoc). When high levels of lactobacilli and *Candida albicans* were simultaneously evaluated in

adhesion assays (ES4, similar to a dysbiosis condition), *L. gasseri* JCM1131 showed 65% of displacement by *C. albicans* isolated from candidiasis, but it only evidenced statistically significant different against *C. albicans* isolated from healthy vaginal microbiota (93%; P = 0.045, using Tukey's post hoc). In ES4, the remaining lactobacilli did not evidence statistically significant differences among *Candida* isolates through two-way ANOVA nor Tukey's post hoc analyses. Likewise, in ES4, most *C. albicans* isolates did not show statistically differences in their ability to displace *L. gasseri* strains, excepting for *C. albicans* isolated from candidiasis (P = 0.030; two-way ANOVA). More exactly, *C. albicans* isolated from candidiasis showed a lower ability to displace *L. gasseri* JCM1131 when compared to *L. gasseri* H59.2 (P = 0.037; Tukey's post hoc). It is important to mention that only *C. albicans* isolated from healthy vaginal microbiota induced the highest average of displacement values (more than 90%) among all strains of *L. gasseri* in ES4. Also, *L. gasseri* H59.2 was the only lactobacilli to simultaneously suffer maximum displacement values against all *C. albicans* in ES4, more exactly between 93 and 97%.

Almost all lactobacilli on high and low levels showed a statistically reduction on their colonization by *C. albicans* when compared their control (P <0.05 with Student *t* test; see Table 2). Overall results showed several statistical differences in the displacement of *L. gasseri* strains between *C. albicans* isolates at low levels (ES1).

Preliminary analysis of the probiotic activity of *Lactobacillus plantarum*

A preliminary analysis of the potential probiotic activity of *Lactobacillus plantarum* ATCC14917 was also realized against *C. albicans* ATCC10231 to compare with previous experimental data obtained from Montalvo (2018) on *L. gasseri* strains and then statistically analyzed in the present work. Although all experimental settings (ES) were also realized in initial adhesion assays between these two species, contamination issues occurred in ES4

adhesion assays, and these results were not achieved in the present study (data not shown). However, the remaining experimental settings were successively performed, evidencing significant displacement values of *L. plantarum* ATCC14917 (see Table 3).

On low levels of *L. plantarum*, the displacement values were 23% and 54% against low (ES1) and high (ES2) levels of *C. albicans*, respectively. The displacement values of *L. plantarum* by *C. albicans* ATCC10231 were considerably inferior to all *L. gasseri* strains at the same experimental settings (ES1: 61-84% and ES2: 82-96%). In fact, *L. plantarum* ATCC14917 showed statistically differences in relation to the displacement of *L. gasseri* strains (ES1: $P = 0.002$ and ES2: $P = 0.007$; two-way ANOVA), more exactly, *L. gasseri* IMAUFB014 (Tukey's post hoc, ES1: $P = 0.003$ and ES2: $P = 0.006$), *L. gasseri* JCM1131 (Tukey's post hoc, ES1: $P = 0.039$ and ES2: $P = 0.056$), and *L. gasseri* H59.2 (Tukey's post hoc, ES1: $P = 0.003$ and ES2: $P = 0.030$).

On high levels of lactobacilli against low levels of *C. albicans* (ES3), *L. plantarum* was only displaced by 31% evidencing a better resistance against *C. albicans* ATCC10231, when compared with *L. gasseri* strains (ES3: 83-95%). Likewise, *L. plantarum* ATCC14917 showed statistical differences with the displacement of *L. gasseri* strains in ES3 ($P = 0.031$; two-way ANOVA), more exactly, *L. gasseri* IMAUFB014 (Tukey's post hoc, $P = 0.038$), and *L. gasseri* H59.2 (Tukey's post hoc, $P = 0.054$). However, no statistically significant differences were found in the displacement values between *L. plantarum* ATCC14917 and *L. gasseri* JCM1131 (Tukey's post hoc, $P = 0.090$) in ES3.

DISCUSSION

Certain strains of *Lactobacillus gasseri* (vaginal lactobacilli) and one reference strain of *L. plantarum* (non-vaginal lactobacilli) were confronted with *Candida albicans* in initial adhesion assays. The main objective of the study was to evaluate the displacement of lactobacilli already attached to an abiotic surface using different experimental settings. The different experimental settings simulated high and low concentration levels of lactobacilli and *C. albicans* in the initial colonization of a surface, values that could be found in different well-being conditions in the vaginal epithelium, more exactly, healthy microbiota, dysbiosis and candidiasis (Pacha-Herrera et al., 2020; Salinas et al., 2020). One of the pathogenic characteristics of *C. albicans* is its ability to initially adhere to host cells and its subsequent morphological transition from yeast to hyphae, allowing the fungus to invade host cells and move across epithelial barriers (Graf et al., 2019). This study focused exclusively on the intrinsic capacity of resistance to displacement of lactobacilli against *C. albicans* on an abiotic surface, specifically glass surfaces were used for the initial adhesion assays. Previous studies exclusively analyzed the activity of biofilms and biosurfactants of certain *Lactobacillus* species against opportunistic pathogens (De Gregorio et al., 2020; Itapary Dos Santos et al., 2019; Jalilsood et al., 2015; Martinez et al., 2020). However, there are few studies that evaluated the susceptibility of lactobacilli to be displace by the initial adhesion of pathogens (P. Alves et al., 2014; António Machado, Almeida, et al., 2013; António Machado, Jefferson, et al., 2013; Matsuda et al., 2018). The present work reported the displacement of lactobacilli by various strains of *Candida albicans* in the first step of a surface colonization.

The interaction of established biofilms of lactobacilli against pathogens has also been evaluated in several studies (Jalilsood et al., 2015; Martinez et al., 2020; Matsubara et al., 2016). These probiotic approach is usually used for the treatment of established infections but, it is possible

that these biofilms would not be persistent or assimilate in the vaginal microbiota (Di Cerbo et al., 2016). The vaginal environment can be colonized by newer and more probiotic lactobacilli (Zangl et al., 2020). Upon assimilation into the vaginal microbiota, certain lactobacilli could be able to form biofilms and to produce supernatants, such as *L. plantarum* (R. Alves et al., 2020; Zangl et al., 2020). Thus, the initial adhesion is a crucial step for further colonization of the vaginal epithelium and deserves to be fully understood, evaluating different *Lactobacillus* species as a probiotic and its resistance to displacement against the initial adhesion of opportunistic pathogens (such as *C. albicans*). Moreover, He et al. (2020) also evaluated the initial adherence of five *Lactobacillus* strains (two *L. gasseri* and three *L. crispatus*) on two epithelial cell lines, more exactly, immortalized vaginal cells (VK2/E6E7) and primary valvular endothelial cells (VECs). Initial adhesion assays with high levels of lactobacilli (10^8 CFU/ml) were realized on primary VECs and VK2/E6E7 cell lines. Although both *L. gasseri* and *L. crispatus* evidenced high values of initial adhesion, *L. crispatus* strains showed stronger adhesion index in both VECs and VK2/E6E7 cells. However, lactobacilli displacement values were not quantified on cell lines or against pathogens during *in vivo* and *in vitro* assays. It is also important to compare the intrinsic variability of lactobacilli to avoid its displacement by several strains of a certain pathogen. Despite several initial adhesion assays may be done (such as competition, displacement, and exclusion assays) (De Gregorio et al., 2020; Graf et al., 2019; Gueimonde et al., 2006), this study evaluated the ability of three *L. gasseri* strains and one *L. plantarum* strain to protect an abiotic surface in the initial adhesion step, assessing the lactobacilli displacement and the inhibition of three *C. albicans* strains. Another study realized by Parolin et al. (2015), several vaginal lactobacilli (*L. crispatus* B1-BC8, *L. gasseri* BC9-BC14 and *L. vaginalis* BC15-BC17) were isolated from 15 healthy premenopausal women and further tested against *Candida* species on HeLa cells. At high levels of lactobacilli (5×10^7 CFU/ml), the highest adhesion values on HeLa cells were observed among *L. crispatus* BC1,

L. crispatus BC3, and *L. gasseri* BC8 strains. This study is in agreement with our results, demonstrating the variability in the probiotic activity and initial adhesion ability among different *Lactobacillus* species or even between strains of the same species against the same opportunistic pathogen. Our results evidenced statistically differences between the displacement values of these *L. gasseri* strains by the same *C. albicans* isolate (see Table 2).Therefore, it is plausible to assume that the remaining *Lactobacillus* species of the normal vaginal microbiota could also evidence discrepancies in their displacement resistance against different isolates of *C. albicans*, as proposed by Zangl et al. (2020), being consequently vulnerable to certain strains of opportunistic pathogens. On the other hand, the application of different lactobacilli species from other biological sources or ecosystems in the colonization of human mucosa could increment the probiotic activity of the remaining commensal microbiota, as suggested in other studies (Hasslöf et al., 2010; Wang et al., 2018; Wasfi et al., 2018). Wang et al. (2018) previously demonstrated a greater adhesion of *L. plantarum in vitro* assays, proposing this species as an ideal probiotic *Lactobacillus* sp. for a mucosa with acidic pH (such as vaginal epithelium). In addition, Kang et al. (2018) also reported the effective adhesion ability of *L. plantarum* when evaluating the hydrophobicity and the probiotic properties of *Lactobacillus fermentum* MG901 and *L. plantarum* MG989 on initial adhesion assays. In this study, both lactobacilli evidenced an excellent hydrophobicity index against organic solvents (e.g., chloroform and ethyl acetate), indicating a good potential to adhere on epithelial cells. Also, *Lactobacillus fermentum* MG901 and *L. plantarum* MG989 revealed highest rates of adhesion on HT-29 cells (Kang et al., 2018), more exactly, 97 and 99%, respectively. In all these studies, *L. plantarum* showed a great displacement resistance against *C. albicans*. In 2018, Garcia-Gonzalez and colleagues evaluated the impact on cell viability and the adhesion ability of 22 *L. plantarum* strains (mainly isolated from fermented foods) on a mucosa cell line (Garcia-Gonzalez et al., 2018). Indeed, all *L. plantarum* strains were able to adhere to the cell

line with an adhesion percentage ranging from 77 to 98%. Also, certain *L. plantarum* strains led to a reduction of interleukin-8 (IL-8; a chemoattractant cytokine and inflammation stimulator) levels from mucosal cells, showing reduction percentages between 56.18 and 75.56%. These authors demonstrated the strong abilities of *L. plantarum* strains to adhere to host cells and suggesting a potential cross-talk with the host immune system based on the IL-8 release of mucosal cells. So, these previous studies together with our results of low displacement values (23-54%) in *L. plantarum* by *C. albicans* suggested the application of non-human lactobacilli strains in the colonization of human's mucosal epithelia. In 2018, Abdou and colleagues isolated naturally occurring probiotic *Lactobacillus* species in numerous animals with a different environmental background (food, plants, and animals) and studied interspecies differences in probiotics on the species level. Their results indicated that the diversity of probiotic strains isolated from different animal species implies different types of benefits to the host (Abdou et al., 2018).

Furthermore, Jalilsood et al. (2015) evaluated the ability of *L. plantarum* ATCC 14917 and PA21 isolate to form a strong biofilm, showing a strong resistance effect against several spoilage and pathogenic bacteria, such as *Salmonella enterica*, *Bacillus cereus*, *Pseudomonas fluorescens*, and *Aeromonas hydrophila*. In fact, a biofilm is a fundamental microbial survival mode that naturally proceeds an initial adhesion, colonization, and maturation of continuous growth on a surface or an epithelium (António Machado & Cerca, 2015). Although the dynamic of biofilm growth of *L. plantarum* was already showed by Martinez et al. (2020), there is still scarce information about the initial adhesion and colonization of lactobacilli on biotic and abiotic surfaces. The present study showed the initial adhesion phase of vaginal and non-vaginal lactobacilli on a glass surface (abiotic surface). This preliminary phase is vital for the further colonization of the abiotic or biotic surface (such as vaginal epithelium), avoiding its displacement by opportunistic pathogens, as *C. albicans*. As shown in our results, *L. plantarum*

ATCC 14917 demonstrated low displacement values against the initial adhesion of *C. albicans* ATCC 10231, being more resilient and statistically different in its probiotic activity when compared to the others *L. gasseri* strains against the same *C. albicans* isolate. Only 23 and 54% of *L. plantarum* ATCC 14917 were displaced by *C. albicans* at low and high levels, differing from the displacement values obtained by vaginal *L. gasseri* strains evaluated in the present study (61–97% on ES1 and ES2 against *C. albicans* ATCC 10231; see Table 2). It is important to mention that few studies showed the displacement values of the evaluated lactobacilli against opportunistic pathogens, reporting only displacement values of the pathogens by lactobacilli or their biosurfactants (Allonsius et al., 2017; De Gregorio et al., 2020; Zarate & Nader-Macias, 2006). However, our previous studies already evaluated displacement values of *L. crispatus* and *L. iners* against several opportunistic BV-associated anaerobes (*G. vaginalis* 101, *A. vaginae* FA, *M. mulieris* ATCC 26-9, *P. bivia* ATCC 29303, and *F. nucleatum* 718BVC) in biotic and abiotic surfaces (A Machado et al., 2013; António Machado, Jefferson, et al., 2013). When evaluating both lactobacilli species pre-adhered to ME-180 epithelial cells (biotic surface), *L. cripatus* showed a greater displacement values (4–25%) by BV-associated anaerobes when compared to *L. iners* (4–13%) at high levels in initial adhesion assays (10^9 CFU/mL) (A Machado et al., 2013). Further evaluation on a glass surface, *L. crispatus* demonstrated variability of displacement values against BV-associated anaerobes at low and high levels in initial adhesion assays (10^3 and 10^9 CFU/mL), more exactly, 1–32% and 1–23% (António Machado, Jefferson, et al., 2013), respectively. These previous studies agree with the variability of displacement values found in the present study among *L. gasseri* strains and *L. plantarum*, suggesting *C. albicans* as a more aggressive opportunistic pathogen to displace lactobacilli species when compared to BV-associated anaerobes. Further studies should characterize long-term colonization between vaginal and non-vaginal lactobacilli and evaluate their probiotic activities.

CONCLUSIONS

In summary, the present study showed that different strains of *L. gasseri* isolated from human vaginal microbiota displayed a variability of probiotic activity among several *C. albicans* isolates, through their displacement resistance abilities against opportunistic pathogens on initial adhesion assays. Also, *L. plantarum* ATCC 14917 demonstrated higher probiotic ability against *C. albicans* ATCC 10231, evidencing statistically significant differences when compared to *L. gasseri* strains. Our results supported previous studies, indicating non-human lactobacilli as possible probiotic candidates to colonize human mucosal epithelia and to inhibit the initial colonization of human opportunistic pathogens. However, there are some major limitations of the present study: (1) it is a preliminary study realized on an abiotic surface and therefore unable to establish an efficient report on human epithelial colonization, (2) the study did not evaluate the continuous colonization and interaction between lactobacilli and *C. albicans* isolates, (3) this study only evaluated the probiotic activity of *L. gasseri* strains and a reference strain of *L. plantarum* against a single *Candida* species, and (4) the probiotic activity was only evaluated through displacement resistance of the initial adhesion. Further studies should be conducted to establish a longitudinal relationship in colonization between non-vaginal lactobacilli and *Candida* species through *in vitro* and *in vivo* models.

TABLES

Table # 1

Experimental settings (ES) realized in the initial adhesion assays.

C. albicans / ***Lactobacillus* sp.**	1.00E+03 CFU/ml	1.00E+09 CFU/ml
1.00E+03 CFU/ml	**1**	**2**
	Dysbiosis	**Candidiasis**
1.00E+09 CFU/ml	**3**	**4**
	Healthy microbiota	**Dysbiosis**

Table # 2

Displacement of *Lactobacillus gasseri* by *Candida albicans* obtained through initial adhesion assays, adapted to this work from Montalvo (2018).

Microorganisms		Experimental setting (ES) 1		2		3		4	
		SAMPLE (N. of cells per glass surface)	DISPL. (%)	SAMPLE (N. of cells per glass surface)	DISPL. (%)	SAMPLE (N. of cells per glass surface)	DISPL. (%)	SAMPLE (N. of cells per glass surface)	DISPL. (%)
	C. albicans ATCC® 10231™	2.25E+04 (±2.66E+00) b	84 (±0.08)	5.01E+03 (±8.85E-11) b	96 (±0.08)	7.27E+04 (±4.34E+03) a	95 (±0.09)	3.58E+05 (±3.19E+04) a	73 (±0.09)
L. gasseri IMAUFB014	*C. albicans* from candidiasis	8.35E+04 (±8.05E+03) a,b,c	44 (±0.10)	5.01E+03 (±7.23E-11) b	97 (±0.08)	3.43E+05 (±5.01E+03) a,c	75 (±0.08)	1.39E+05 (±1.77E+03) a,c	90 (±0.07)
	C. albicans from healthy vaginal microbiota	6.60E+00 (±1.45E+03) a,b,c	55 (±0.08)	4.09E+04 (±1.45E+03) a,b	72 (±0.08)	9.10E+04 (±7.65E+03) a	93 (±0.09)	2.13E+04 (±1.77E+03) a	98 (±0.09)
	C. albicans ATCC® 10231™	1.27E+05 (±1.13E+04) a	61 (±0.07)	6.01E+04 (±3.54E+03) a	82 (±0.06)	9.33E+05 (±8.67E+04) a,b	83 (±0.08)	5.90E+05 (±5.49E+04) a,b	89 (±0.08)
L. gasseri JCM1131	*C. albicans* from candidiasis	7.27E+00 (±3.54E+03) a,c	78 (±0.06)	2.38E+04 (±1.77E+03) a	93 (±0.06)	4.69E+06 (±4.32E+05) b,c	15 (±0.11)	1.94E+06 (±1.57E+05) a,b,c	65 (±0.09)
	C. albicans from healthy vaginal microbiota	6.93E+04 (±5.21E+03) a,c	79 (±0.06)	6.14E+04 (±1.77E+03) a	81 (±0.06)	8.96E+05 (±7.27E+04) a,b	84 (±0.08)	3.82E+05 (±3.98E+04) a,b	93 (±0.08)
	C. albicans ATCC® 10231™	3.01E+04 (±5.11E-12) a,b	83 (±0.09)	2.63E+04 (±1.77E+03) a	86 (±0.09)	3.73E+05 (±3.90E+04) a	90 (±0.08)	1.00E+05 (±4.34E+03) a	97 (±0.08)
L. gasseri H59.2	*C. albicans* from candidiasis	1.54E+05 (±1.77E+03) a,b,c	15 (±0.09)	2.51E+03 (±0.00E+00)	99 (±0.09)	3.73E+05 (±2.71E+04) a,c	90 (±0.08)	2.58E+05 (±2.13E+04) a,c	93 (±0.08)
	C. albicans from healthy vaginal microbiota	1.44E+05 (±1.16E+04) a,b,c	21 (±0.10)	1.84E+04 (±1.45E+03)	90 (±0.09)	6.91E+05 (±3.65E+04) a	81 (±0.08)	1.44E+05 (±1.77E+03) a	96 (±0.08)

Sample: the amount of *L. gasseri* adhered to the abiotic glass surface after initial adhesion assays of *L. gasseri* vs *C. albicans*.
DISPL %: percentage of *L. gasseri* displaced at the end of the initial adhesion assays.
ES 1: *L. gasseri* (1.00E+03 CFU/ml) & *C. albicans* (1.00E+03 CFU /ml).
ES 2: *L. gasseri* (1.00E+03 CFU /ml) & *C. albicans* (1.00E+09 CFU /ml).
ES 3: *L. gasseri*. (1.00E+09 CFU /ml) & *C. albicans* (1.00E+03 CFU /ml).
ES 4: *L. gasseri*. (1.00E+09 CFU /ml) & *C. albicans* (1.00E+09 CFU /ml).

The experimental controls (N. of cells per glass surface) for the high and low inoculums of ***L. gasseri*** are as follows: IMAUFB014 1.34 E + 06 (± 1.45 E + 05) & 1.48 E + 05 (± 1.48 E + 04); JCM1131 5.54 E + 06 (± 5.62 E + 05) & 3.27 E + 05 (± 2.53 E + 04); H59.2 3.70 E + 06 (± 3.51 E + 05) & 1.82 E + 05 (± 1.87 E + 04).
The experimental controls (N. of cells per glass surface) for the high and low inoculums of ***C. albicans*** are as follows: ATCC® 10231 ™ 1.95 E + 06 (± 1.86 E + 05) & 1.60 E + 05 (± 1.49 E + 04); from candidiasis 2.16 E + 06 (± 1.84 E + 05) & 2.30 E + 05 (± 1.70 E + 04); from healthy vaginal microbiota 3.41 E + 06 (± 2.99 E + 05) & 8.52 E + 05 (± 7.60 E + 03).

Statistical analysis:
[a] $P < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of lactobacilli control and sample tested in the adhesion assay;
[b] $P < 0.05$ analyzed using two-tailed ANOVA statistical test (95% confidence interval) for comparison of displacement values from a certain strain of lactobacilli among all *C. albicans* isolates tested in the adhesion assay;
[c] $P < 0.05$ analyzed using a two-tailed ANOVA statistical test (95% confidence interval) for comparison of displacement values from all lactobacilli strains induced by a certain *C. albicans* isolate tested in the adhesion assay.

Table # 3

Displacement of *Lactobacillus plantarum* by *Candida albicans* obtained through initial adhesion assays.

Microorganisms		Experimental setting (ES)					
		1		2		3	
		SAMPLE (N. of cells per glass surface)	DISPL. (%)	SAMPLE (N. of cells per glass surface)	DISPL. (%)	SAMPLE (N. of cells per glass surface)	DISPL. (%)
L. plantarum ATCC 14917	*C. albicans* ATCC® 10231™	1.20E+06 (6.22E+04) [a,b]	23 (±0.17)	7.85E+05 (9.63E+04) [a,b]	54 (±0.10)	7.05E+06 (1.09E+06) [a,b]	31 (±0.34)

Sample: amount of *L. plantarum* adhered to the abiotic glass surface after initial adhesion assays of *L. plantarum* vs *C. albicans*.

DISPL %: percentage of *L. plantarum* displaced at the end of the initial adhesion assays.
ES 1: *L. plantarum* (1.00E+03 CFU/ml) & *C. albicans* (1.00E+03 CFU /ml).
ES 2: *L. plantarum* (1.00E+03 CFU /ml) & *C. albicans* (1.00E+09 CFU /ml).
ES 3: *L. plantarum*. (1.00E+09 CFU /ml) & *C. albicans* (1.00E+03 CFU /ml).

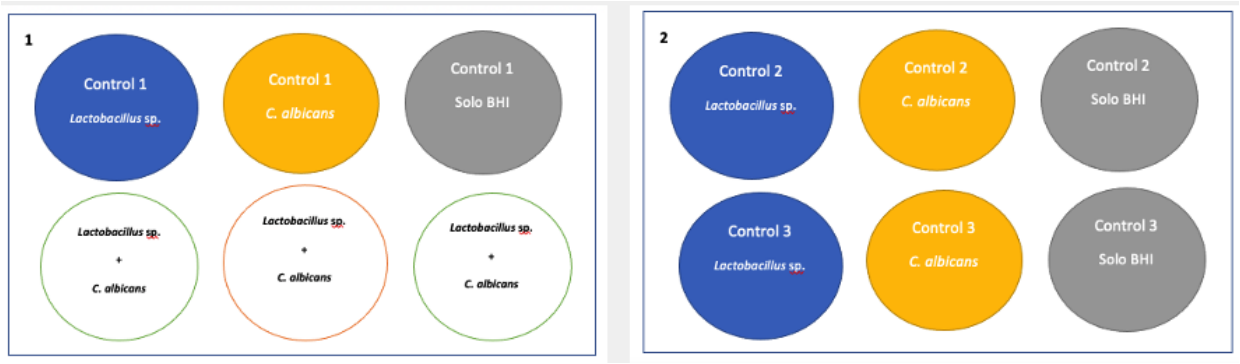
The experimental controls (N. of cells per glass surface) for the high and low inoculums of ***L. plantarum*** are as follows: ATCC 14917 1.03 E + 07 (± 1.01 E + 06) & 1.56 E + 06 (± 1.06 E + 05).
The experimental controls (N. of cells per glass surface) for the high and low inoculums of ***C. albicans*** are as follows: ATCC® 10231 ™ 4.16 E + 07 (± 6.90 E + 06) & 1.24 E + 06 (± 4.62 E + 04).

Statistical analysis:
[a]$p < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of lactobacilli control and sample tested in the adhesion assay;
[b]$p < 0.05$ analyzed using two-tailed ANOVA statistical test (95% confidence interval) for comparison of displacement values between *L. plantarum* and *L. gasseri* strains in the adhesion assay at same experimental setting.

FIGURES

Figure # 1

Six-well plate distribution of the microorganism cultures and their positive and negative controls.



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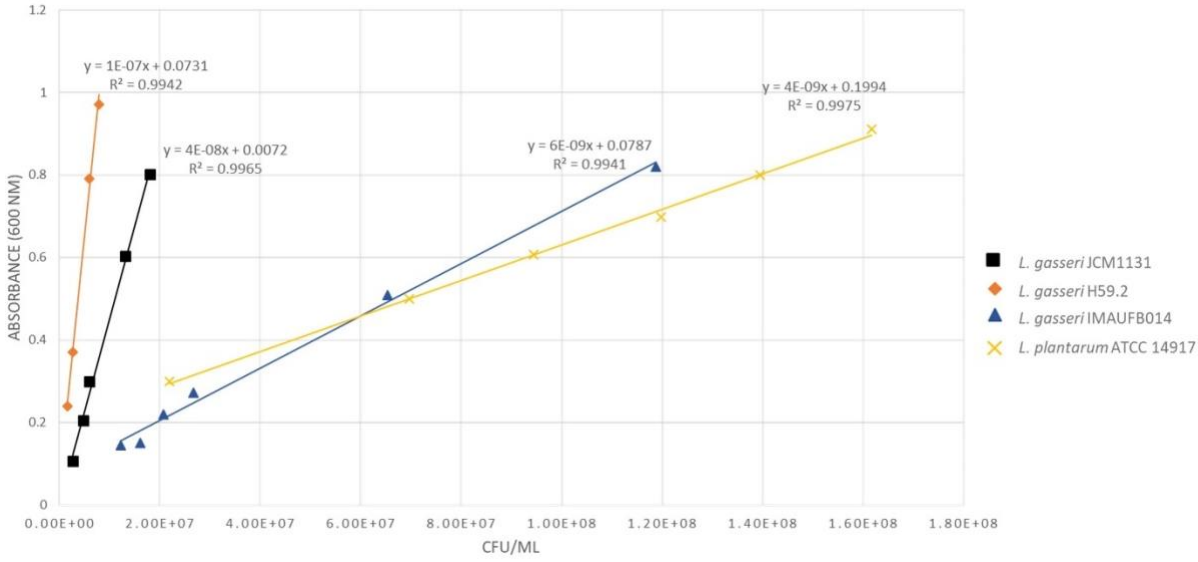
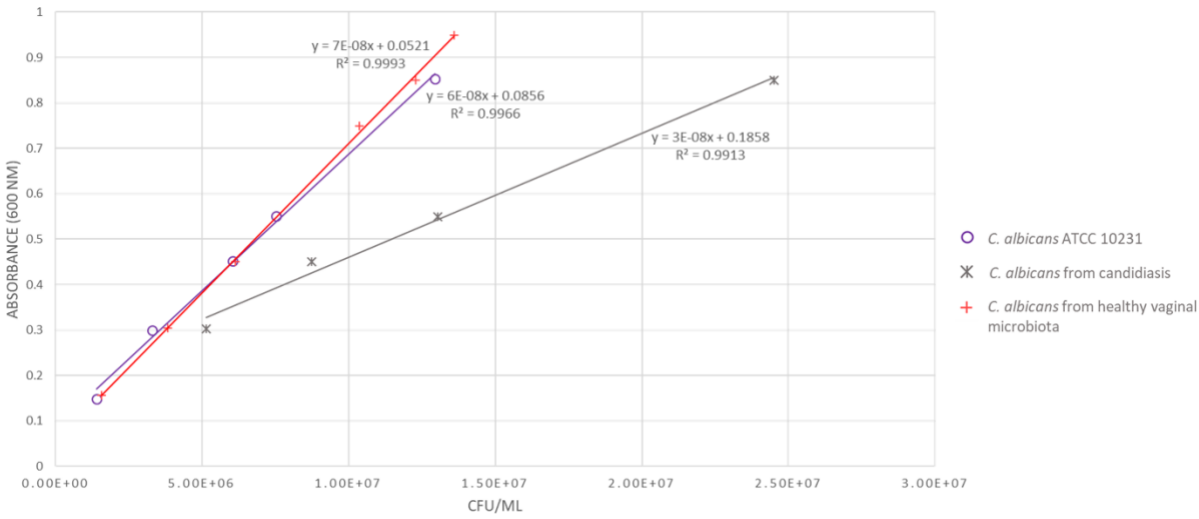
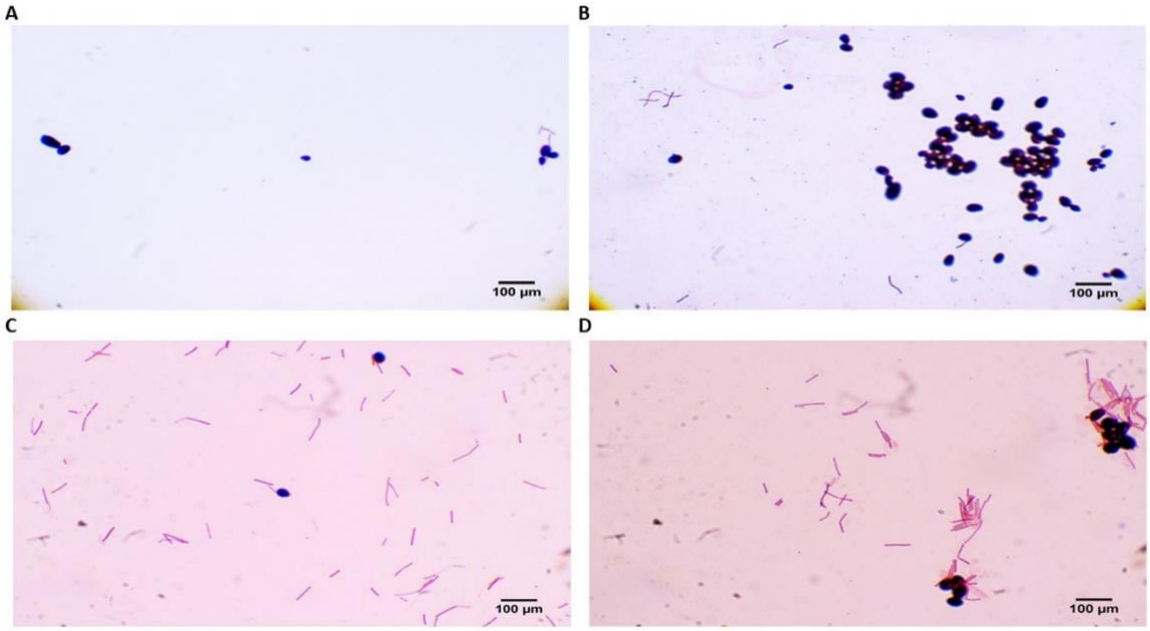
ANEXO A: COMPARISON OF GROWTH CALIBRATION CURVES BETWEEN *LACTOBACILLUS GASSERI* STRAINS AND *LACTOBACILLUS PLANTARUM* ATCC 14917.



ANEXO B: COMPARISON OF GROWTH CALIBRATION CURVES BETWEEN *CANDIDA ALBICANS* ISOLATES.



ANEXO C: COMPARISON OF SAMPLE FOR *L. GASSERI* IMAUFB014 AGAINST *C. ALBICANS* ATCC 10231 OBSERVED IN THE OLYMPUS BX50 MICROSCOPE FOR EACH EXPERIMENTAL SETTING (ES)



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