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Probiotic lactobacilli inhibit early stages of *Candida albicans* biofilm development by reducing their growth, cell adhesion and filamentation

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Abstract

We evaluated the inhibitory effects of the probiotic *Lactobacillus* species on different phases of *Candida albicans* biofilm development. Quantification of biofilm growth and ultrastructural analyses were performed on *C. albicans* biofilms treated with *Lactobacillus rhamnosus*, *L. casei* and *L. acidophilus* planktonic cell suspensions as well as their supernatants. Planktonic lactobacilli induced a significant reduction ($p<0.05$) in the number of biofilm cells (25.5–61.8%) depending on the probiotic strain, and the biofilm phase. *L. rhamnosus* supernatants had no significant effect on the mature biofilm ($p>0.05$), but significantly reduced the early stages of *Candida* biofilm formation ($p<0.01$). Microscopic analyses revealed that *L. rhamnosus* suspensions reduced *Candida* hyphal differentiation, leading to a predominance of budding growth. All lactobacilli negatively impacted *C. albicans* yeast-to-hyphae differentiation and biofilm formation. The inhibitory effects of the probiotic *Lactobacillus* on *C. albicans* entailed both cell-cell interactions, and secretion of exometabolites that may impact upon pathogenic attributes associated with *C. albicans* colonization on host surfaces, and yeast filamentation. This study clarifies, for the first time, the mechanics of how *Lactobacillus* species may antagonize *C. albicans* host colonization. Our data elucidate the inhibitory mechanisms that define the probiotic candicidal activity of lactobacilli, thus supporting their utility as an adjunctive therapeutic mode against mucosal candidal infections.

Key words

biofilm, *Candida albicans*, candidiasis, *Lactobacillus*, probiotics

Introduction

Candida albicans, an opportunistic pathogen, is a dimorphic fungus that colonizes the oral mucosal surfaces of approximately 30–45% of healthy adults (Samaranayake, 2012). Gastrointestinal and urogenital tracts are also common sites where *Candida* species colonize and cause opportunistic infections (Falagas et al. 2006). Immunosuppressed individuals, transplant recipients, low-birth weight neonates and patients under chemotherapy are more susceptible to invasive diseases caused by *Candida*, most often as bloodstream infections (candidaemia) with a risk to disseminate to different organs, such as liver, spleen, bones and heart (Arendrup 2010).

Candida spp. inhabit humans predominantly in the biofilm phase, which is defined as organized cell communities attached to surfaces and encased in a matrix of extracellular polymeric substances (Samaranayake et al. 2002). The formation of *Candida* biofilm involves the adhesion of planktonic cells (adhesion phase), cell growth and aggregation (initial colonization phase), production of extracellular material, and the eventual development of a mature biofilm matrix (maturation phase) (Bandara et al. 2013). Biofilm formation is an important virulence attribute of *Candida* spp., as the biofilm cells exhibit greater resistance to antifungals and host defenses compared to their planktonic or suspended counterparts (Alcazar-Fuoli and Mellado 2014). This is partially caused by the production of the exopolymeric matrix that restricts penetration of antifungal drugs through the biofilm (Taff et al. 2013).

For some decades, systemic and local antifungal agents such as fluconazole, nystatin, and amphotericin B have been successfully used as therapeutic and prophylactic agents to obviate colonization, as well as invasive fungal infections (Ericson and Benjamin 2014; Pappas 2014). However, their efficacy is compromised due to an alarming increase in the emergence of drug resistant *Candida* strains worldwide (Sanguinetti et al. 2015). Hence, alternative or adjunctive therapies have been explored for candidal infections including the use of natural products such as peptides, oils and phytochemicals (Coleman et al. 2010; Sardi et al. 2013; Sherry et al. 2012). Although promising, the toxicities of these compounds and their bio-tolerance are of concern and they are yet in the experimental stages of development (Nett 2014).

Due to these concerns, the use of probiotic bacteria has been proposed as an alternative prophylactic and therapeutic mode of treatment against human *Candida* infections (Matsubara et al. 2016; Meurman 2005). The use of probiotics to reduce *Candida* infections on mucosal surfaces has been extensively studied in clinical trials lately, not only against urogenital and gastrointestinal infections (Hu et al. 2013; Kovachev and Vatcheva-Dobrevska 2015; Roy et al. 2014), but also against oral infections (Ishikawa et al. 2014; Kraft-Bodi et al. 2015; Li et al. 2014; Matsubara et al. 2012). To date, most of the latter studies have indicated that several probiotics are safe, effective and efficacious as antifungal agents for prophylaxis or indeed, as treatment adjuvants in the management of mucosal candidiasis.

The antifungal effect of probiotic bacteria, especially *Lactobacillus* spp, has been evaluated in a number of *in vitro* studies using conventional agar diffusion assays on solid media, biofilm assays and microscopy (Chew et al. 2015b; Verdenelli et al. 2014; Vilela et al. 2015). The underlying fungicidal or fungistatic effects of probiotics may involve the production of secondary metabolites with antimicrobial activity (Ceresa et al. 2015; Vilela et al. 2015; Zakaria Gomaa 2013), the competition for nutrients and adhesion sites (Servin and Coconnier 2003) and the stimulation of the immune system (Fidan et al. 2009). Others have also shown that probiotic bacteria have the ability to suppress biofilm formation by *C. albicans* on various surfaces,

such as silicone and related biomaterials (Ceresa et al. 2015; Murzyn et al. 2010; Orsi et al. 2014; Rodrigues et al. 2006b). Such effects of probiotic bacteria on *Candida* biofilm development may also entail genetic interference. A number of studies have demonstrated that various bacteria may induce down-regulation of genes associated with *Candida* biofilm formation, such as PHR1 and ALS12 in *C. albicans* (Kohler et al. 2012), and EPA6 and YAK1 in *C. glabrata* (Chew et al. 2015b). *C. albicans* hypha-specific genes (ECE1, HWP1 and HYR1) were also down-regulated by *E. coli* biofilm supernatant (Bandara et al. 2013).

Despite such burgeoning information on the effect of probiotics on *C. albicans* biofilms, it is still unclear whether the interference with the fungal biofilm development is dependent on a direct interaction between fungal cells and probiotic bacterial cells and/or the secretion of exometabolites by the probiotics.

The aim of this study, therefore, was to evaluate the effect of *Lactobacillus* planktonic cell suspensions and cell-free bacterial supernatants on the *C. albicans* biofilm development during the adhesion, initial colonization and maturation phases of the yeast using quantitative analyses and ultrastructural visualization. We demonstrate here, for the first time, that *Lactobacillus* species antagonize *C. albicans* biofilm formation, particularly in the critical, early colonization phase of the yeast, through cell-cell interactions and likely secretion of exometabolites that inhibit their filamentation.

Materials and methods

Experimental design

This study comprised two stages (Figure 1). First, we evaluated the direct effect of probiotic bacterial cells on the *C. albicans* biofilm development. Probiotic cells suspensions of *Lactobacillus* were added to *C. albicans* at two different moments of biofilm formation: the initial colonization phase and the secondary maturation phase. The cell viability was determined by viable colony forming unit (CFU) quantification at each phase (Figure 1a).

The second stage was performed to evaluate the effects of probiotic bacterial products (exometabolites) in *Lactobacillus* cell supernatants on *C. albicans* biofilm formation. After three different time intervals of incubation with bacteria cells supernatants (1.5 h, 25.5 h and 49.5 h), *C. albicans* cell viability was determined by a tetrazolium salt (XTT) reduction assay (Figure 1b).

Finally, the morphology *C. albicans* biofilms exposed to the probiotics was visualised by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) and compared with control *C. albicans* biofilms with no probiotic treatment.

Microorganisms

Candida albicans ATCC SC5314, isolated from a human clinical infection (Jones et al. 2004) and a clinical isolate of *C. albicans*, denominated *C. albicans* 75, were used in this study. The probiotic bacteria *Lactobacillus rhamnosus* LR32 (Danisco, Madison, WI, USA), *Lactobacillus acidophilus* NCFM (Danisco, Madison, WI, USA) and *Lactobacillus casei* L324m (a clinical isolate, Institute of Biomedical Sciences, University of São Paulo, Brazil) were selected as the probiotics to be tested against *Candida* spp. All strains were stored in 20% glycerol at -80°C prior to the experiments.

Growth media and culture conditions

Sabouraud dextrose agar (SDA; BD Biosciences, San Jose, CA, USA) and Sabouraud dextrose broth (SDB; BD Biosciences, San Jose, CA, USA) were used for culturing *C. albicans*. Brain heart infusion agar and

broth (BHI, BD Biosciences, San Jose, CA, USA) were used for culturing probiotic strains, and the latter was also used for culturing *Candida* and *Lactobacillus* mixed biofilm.

Candida and *Lactobacillus* cells were inoculated into SDB and BHI broth, respectively, and incubated at 37°C, for 18 h in an orbital shaker, under ambient conditions for *Candida*, whereas the probiotic strains were grown under strict anaerobic conditions in an anaerobic chamber (85% N_2, 10% CO_2, 5% H_2). Cells were harvested by centrifugation (2,000 Xg for 5 min), washed twice in phosphate buffered saline (PBS, pH 7.2) and resuspended in BHI. *Candida* and bacterial cells suspensions were adjusted to 1 x 10^7 cells/mL by spectrophotometry. The number of *C. albicans* cells was confirmed by hemocytometric counting. *Lactobacillus* suspensions were serially diluted in 1:2, ranging from 1 x 10^7 to 6.25 x 10^5 viable cells/ml.

Candida albicans biofilm formation

Presterilized, polystyrene, flat bottom 96-well microtiter plates (Corning Incorporated, New York, NY, USA) were used to develop *C. albicans* biofilms. At first, 100 µl of a standard cell suspension of *Candida* spp. (1 x 10^7 cells/mL) were transferred into each well and incubated for 1.5 h at 37 °C under agitation at 80 rpm. After incubation, the cell suspensions were removed and each well was washed twice with PBS to remove loosely adherent cells. A total of 200 µl of BHI was transferred to each well and the plates were further incubated for different periods according to the study group (Bandara et al. 2013).

Fungi-Probiotic cells interaction assay

Each of the three probiotics strains was tested on the biofilms formed by *C. albicans* ATCC SC5314 and *C. albicans* 75. *C. albicans* biofilms were developed for 7.5 h and 25.5 h, in different plates (Figure 1a). Afterwards, biofilm supernatants were aspirated, a total of 100 µl of fresh BHI and 100 µl of *Lactobacillus* cells suspensions at different cell densities (1 x 10^7, 5 x 10^6, 2.5 x 10^6, 1.25 x 10^6, and 6.25 x 10^5 cells/ml) were added individually to the wells (Bandara et al. 2013). The plates with the 7.5 h old and 25.5 h old *C. albicans* biofilms were incubated for further 18 h and 24 h, respectively, both at 37 °C, 75 – 80 rpm. Fresh BHI were used as control in place of the probiotic suspension. After the final incubation, the wells were washed twice with PBS and *C. albicans* cells viability was determined by CFU quantification on SDA plates. The pH of the coculture supernatants was monitored over the initial colonization phase (9-18 h of coculture) and maturation phase (8-24 h of coculture).

Probiotic supernatant assay

L. rhamnosus biofilms were obtained by inoculating wells of 6-well microtiter plates (Corning Incorporated, New York, NY, USA) with 3 ml of cell suspension at 1 x 10^7 CFU/ml, followed by anaerobic incubation at 37 °C /80 rpm for 1.5 h. After this incubation period, the supernatant Phase-1 was collected. Alternatively, supernatants Phase-2 and Phase-3 were collected from biofilms cultivated with *L. rhamnosus* suspensions with an initial cell density at 1 x 10^6 CFU/ml, after incubation for 24 h and 48 h, respectively. Cell-free supernatants were obtained by centrifugation followed by filtration through a 0.2 µm membrane filter (Life Sciences, Ann Arbor, MI, USA). All supernatants were freshly prepared and their cell-free nature validated by viable counts prior to each experiment.

The following assay was performed to assess the effect of *L. rhamnosus* supernatants on the adhesion (T^1), initial colonization (T^2) and maturation (T^3) phases of *Candida* biofilm formation. *L. rhamnosus* supernatants obtained at Phase-1, Phase-2 and Phase-3 were added to *C. albicans* ATCC SC5314 biofilms in 96-well plates. In T^1, *C. albicans* cells were resuspended in probiotic supernatants at 1 x 10^7 cells/ml, and aliquots of 100 µl of these suspensions were added to wells and incubated for 1.5 h (37 °C, 80 rpm). In T^2, *L. rhamnosus* supernatants (200 µl/well) were added to 1.5 h old *Candida* biofilms and incubated for 24 h (37°C, 80 rpm). In T^3, *L. rhamnosus* supernatants (200 µl/well) were added to 25.5 h old *C. albicans* biofilms and incubated for further 24 h (37°C, 80 rpm). Fresh BHI was used in place of *L. rhamnosus* supernatants as control.

The quantitative analysis of the biofilm was performed using standard XTT reduction assay to measure the metabolic activity of biofilms (Bandara et al. 2013). Cells supernatants were removed, and the wells were washed twice with PBS to remove loosely adherent cells. 79 µL of PBS, 20 µL of XTT solution (1 mg/mL) and 1 µL of fresh prepared menadione solution (0.4 mM) were then added to each well and incubated in dark for 3 h at 37°C. The resultant solution in each well was transferred to a clean well prior to measuring the color change of the solution using a microtiter plate reader (Spectra Max 340 tunable microplate reader; Molecular Devices Ltd, Sunnyvale, CA, USA) at 492 nm. All assays were carried out in triplicate.

Microscopic analyses

Confocal laser scanning microscopy (CLSM)

C. albicans ATCC SC5314 biofilms were prepared on sterilized plastic cover slips (1cm; Thermanox plastic cover slips; Nulge Nunc International, Rochester, NY, USA). *L. rhamnosus* supernatants (Phase-2 and Phase-3) were added to *C. albicans* biofilms during the adhesion (T^1) and initial colonization phases (T^2). Furthermore, *L. rhamnosus* cell suspension (1 x 10^7 cell/ml) was added to 7.5 h old *Candida* biofilm, as described above. Fresh BHI was used instead of *L. rhamnosus* supernatants or bacteria suspensions, as controls. Biofilms were stained with SYTO® 9 dye and Propidium iodide (Live/Dead BacLight Bacterial Viability kit; Invitrogen, Eugene, OR, USA) (Jin et al. 2005). The stained biofilms were visualized under a Nikon C2 confocal laser scanning microscope (Nikon Corp., Tokyo, Japan).

Scanning electron microscopy (SEM)

C. albicans biofilms (ATCC SC5314) in the initial colonization phase (7.5 h old), with or without treatment with *L. rhamnosus* cells suspensions, were grown on pre-sterilized plastic cover slips (1 cm), fixed with 4% glutaraldehyde for 30 min and freeze dried. The specimens were sputtered with gold prior to being visualized under a Sigma VP Field Emission Scanning Electron Microscope (Carl Zeiss Inc., Oberkocken, Germany) in high-vacuum mode at 10 kV.

Statistical analysis

All assays were carried out in triplicate on three different occasions with independently grown cultures unless otherwise stated. All results obtained were expressed as mean ± standard deviation. A one-way ANOVA, Tukey Honestly Significant Difference (HSD) test, or a student's *t*-test was performed on all data sets to compare the data of the treated groups and the control groups. All analysis was conducted using GraphPad Software (GraphPad Prism® Version 6.0c, La Jolla, CA, USA) at a 95% confidence level.

Results

Effect of probiotic suspension on C. albicans biofilm formation

L. rhamnosus, *L. casei* and *L. acidophillus* suspensions were found to significantly reduce ($p<0.05$) CFU levels of *C. albicans* SC5314 biofilms at both 24 h and 48 h time intervals (Figure 2A, 2B, 2C) suggesting that the presence of *Lactobacillus* cells could not only inhibit the initial colonization of *C. albicans*, but also suppress the development of a mature biofilm. However, no correlation ($p>0.05$) was found between the density of *Lactobacillus* cells and the inhibitory effects, suggesting that the maximum inhibitory activity was achieved even with the lowest concentration of probiotic bacteria.

On the other hand, the two lower densities of all probiotics suspensions tested (1.25×10^6 and 6.25×10^5 cells/ml) had no significant effect ($p>0.05$) on the number of biofilm cells of the clinical isolate *C. albicans* 75, at both 24 h and 48 h (Figure 2D, 2E, 2F), indicating that this strain is less susceptible to the inhibitory activity of lactobacilli than *C. albicans* SC5314. Furthermore, a significant inhibitory effect ($p<0.05$) on the maturation phase of *C. albicans* 75 biofilms was only observed at the highest density of *L. acidophilus* (1×10^7 cells/ml; Figure 2F), whereas the inhibitory effect promoted by *L. rhamnosus* and *L. casei* was also observed with lower concentrations, 5×10^6 cells/ml, in both studied phases (Figure 2D, 2E).

A reduction in the levels of viable *Candida* cells was observed after the biofilms were exposed to growth with all tested probiotics at the highest cell density (1×10^7 cells/ml) (Table 1). The effect of *L. rhamnosus* against *C. albicans* ATCC SC5314 was significantly higher ($p<0.05$) on the maturation phase of *Candida* biofilm (29.8% higher) as compared to the initial colonization phase. On the other hand, *L. acidophillus* showed a significantly weaker effect ($p<0.05$) at 48 h (22.1% lower) in comparison with the 24 h biofilms. No difference ($p>0.05$) was observed for *L. casei* between the two incubation times. For *C. albicans* 75, the percentages of *Candida* biofilm reduction were not significantly different ($p>0.05$) between the initial colonization and the maturation phases for all three probiotic strains. In general, all three probiotic strains showed a better anti-biofilm effect against *C. albicans* SC5314 as compared to the clinical isolate. For instance, *L. rhamnosus* promoted a reduction of 61.8% in *C. albicans* SC5314 viable cells during the maturation phase whereas this reduction was of 39.8% when *C. albicans* 75 was exposed to the identical conditions ($p<0.05$). These results indicate that the probiotic effect of lactobacilli was both bacterial strain-specific, and fungal strain-specific.

Coculture of *C. albicans* and all three *Lactobacillus* strains led to a reduction in the pH of the coculture supernatants, when compared to the control culture inoculated only with *C. albicans*, although this difference in pH reduction was not significant ($p>0.05$) at all time points of analysis (Figure 3). The pH values of the supernatants increased significantly ($p<0.05$) overtime in all studied groups, during both the initial colonization and the maturation phases of *C. albicans* biofilms.

Effect of probiotic supernatant on C. albicans biofilm development

The supernatant collected from 1.5 h old (Phase-1) *L. rhamnosus* biofilm was found to have no effect on all three phases of *C. albicans* biofilm development ($p>0.05$) (Figures 4A, 4B and 4C). On the other hand, the supernatants collected from 24 h (Phase-2) and 48 h (Phase-3) old *L. rhamnosus* biofilm were found to significantly inhibit ($p<0.01$) the growth of *C. albicans* biofilms from 0 min to 1.5 h (adhesion phase) (Figure

4A) and from 1.5 h to 24 h (initial colonization phase) (Figure 4B). This suggests that the metabolites produced by *L. rhamnosus* cells could inhibit the formation and development of *C. albicans* biofilms.

All three *L. rhamnosus* supernatants did not inhibit the growth of *Candida* biofilm ($p>0.05$) at the maturation phase (from 24 h to 48 h) (Figure 4C), suggesting that no soluble bacterial exometabolites impacted on the mature biofilm growth of *C. albicans*.

Confocal laser scanning microscopy

Visualisation with confocal laser scanning microscopy (with the live and dead stains), at the end of *C. albicans* adhesion period (1.5 h), showed a reduction in *C. albicans* ATCC SC5314 blastospores after treatment with *L. rhamnosus* biofilm supernatant, which is in accordance with the data obtained from the XTT reduction assay (Figure 5b and 5c). In general, the biofilms presented with a poorly developed architecture and a reduction in hyphal elements (Figure 5c), as compared with the control group (Figure 5a).

At the end of the initial colonization phase (24 h) of the *C. albicans* biofilm, a significant reduction of yeast-to-hyphae transition was observed when the biofilm was treated with *L. rhamnosus* supernatant. The blastospore form (yeast phase) of *C. albicans* predominated in the biofilm e exposed to *L. rhamnosus* supernatant (Figure 5 e and 5f), whereas the control *C. albicans* biofilm comprised mainly filamentous structures and a scanty growth of budding yeasts (Figure 5d).

In both phases of *C. albicans* biofilm development, the inhibition of candidal adhesion and hyphae formation was greater with the addition of the Phase-3 *L. rhamnosus* supernatant (Figure 5c and 5f) in comparison with that of the Phase-2 supernatant (Figure 5b and 5d). This suggests that supernatants collected from older probiotic cells presented the greatest concentration of the inhibitory product that modified the biofilm architecture of *C. albicans*.

Furthermore, the addition of the cell suspensions of *L. rhamnosus* to the *Candida* biofilm during its initial colonization phase inhibited *C. albicans* filamentation. For instance, the biofilm had a predominance of yeast instead of hyphae, while the control biofilm presented a denser distribution of filamentous cells (Figures 6e and 6f). Similar effects were observed with the probiotic supernatant (Figure 5).

Scanning electron microscopy

After 18 h of incubation with *L. rhamnosus* cells, the *C. albicans* biofilm (Figure 6b and 6d) presented a lower degree of hyphal development than did the control group (Figure 6a and 6c). A stratified architecture, with relatively large water channels (Figure 6b), and budding cells were noted throughout the treated biofilm (Figure 6d) after 18 h of coculture. The control group exhibited a high density of hyphae with relatively smaller water channels (Figure 6a and 6b). *Lactobacillus* cells were found in close contact with *Candida* cells surrounding the hyphae and the yeasts (Figure 6c and 6d).

Discussion

The mechanisms by which probiotic bacteria such as lactobacilli exert their antifungal effect on different phases of candidal biofilm development are still unclear. Hence, we performed quantitative and qualitative analyses using lactobacilli, the most widely used probiotic and *C. albicans*, an opportunistic human fungal pathogen, to shed light on the underlying mechanisms involved in their interactions.

The probiotics *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Lactobacillus acidophillus*, with proven candicidal effect in humans (Hatakka et al. 2007; Ishikawa et al. 2014; Mendonça et al. 2012) were assessed in our study. Both planktonic cell suspensions as well as supernatants of *Lactobacillus* biofilms were incubated with *C. albicans* biofilms under differing circumstances to verify the direct and indirect effect of probiotics on the fungal biofilms.

Each of the three *Lactobacillus* planktonic cell suspensions exerted an anti-biofilm effect on initial colonization and maturation stages of *C. albicans* biofilm development. However, the effects were found to be probiotic species-specific, as the lactobacilli-mediated inhibition of *C. albicans* varied among the three *Lactobacillus* species tested. Organic acid production by the probiotic bacterial metabolism and consequent reduction in the final pH of the growth medium have been suggested as reasons for such growth inhibition of *Candida* (Simark-Mattsson et al. 2009). In a previous similar study Hasslof et al. (2010) noted that the lactobacilli that induced the lowest pH after incubation were the most effective in inhibiting candidal growth. However, our results revealed that none of the tested lactobacilli induced an acidic growth milieu ($pH < 6.0$) in coculture, suggesting that the species-specific candicidal effect of the tested lactobacilli is unrelated to a pH change in the culture medium, and is likely be due to exometabolites of the probiotic.

We also noted that the degree of growth inhibition exerted by the probiotics against *C. albicans* 75 correlated well with the planktonic cell density. Thus, only a relatively high cell density of lactobacilli significantly reduced ($p<0.05$) the biofilm (CFU) of *C. albicans* 75, implying that the total quantum of exometabolites produced by the probiotic may be critical for the antimicrobial effect observed. Additionally, quorum-sensing molecules, such as autoinducers, are known to regulate the production of antimicrobial peptides in broth cultures of lactobacilli (Rizzello et al. 2014). It is known that lactobacilli, as other Gram-positive bacteria, use cell-cell communication circuits to regulate the production and release of autoinducers, chemical signal molecules that increase in concentration as a function of cell density (Miller and Bassler 2001). Another reason for the suppression of *C. albicans* biofilms by planktonic lactobacilli could be the production of bacteriocins by lactobacilli in such cocultures (Maldonado-Barragan et al. 2013).

The cell-free supernatants of *Lactobacillus* biofilm demonstrated a limited capacity for suppressing the development of *C. albicans* biofilms as they were effective only during the early biofilm developmental phase, and were unable to suppress/eliminate the mature biofilms in a manner akin to the planktonic *Lactobacillus* suspensions. This indicates that the effects of the *Lactobacillus* supernatants on the biofilms might be physicochemical or, more specifically, interfacial in nature. It is tempting to speculate, therefore, that some components of the supernatants, presumably the exometabolites of lactobacilli, may have modified the surface energies of the *Candida* blastospores and prevented them from clumping and forming an organized network (Ceresa et al. 2015). Such exometabolites of lactobacilli may include hydrogen peroxide (H_2O_2) (Strus et al. 2005), proteinaceous elements (Atanassova et al. 2003), low molecular compounds such as reuterin, carboxylic

acids, fatty acids, cyclic dipeptides and nucleosides (Li et al. 2012; Ryu et al. 2014; Wang et al. 2012), and biosurfactants with antimicrobial activity (Kheradmand et al. 2014; Zakaria Gomaa 2013).

The CLSM images showed that *L. rhamnosus* supernatant reduced the adhesion of *C. albicans* cells to the plastic surface. Biosurfactants, important exometabolite of lactobacilli, could account for the latter observation as they reduce the hydrophobicity of the surface substratum, and interfere with processes related to microbial adhesion and desorption (Rodrigues et al. 2006a). Previous studies have demonstrated that biosurfactants produced by lactic acid bacteria were able to reduce the initial adhesion of yeasts to silicone elastomers (Ceresa et al. 2015) and polystyrene (Rodrigues et al. 2006b). Biosurfactants are also known to thwart the adhesion of bacteria to hard surfaces and to each other, and also to induce detachment of already adhered organisms (Zakaria Gomaa 2013). This mechanism, however, is unlikely to be operative on a mature biofilm matrix as shown in the present study.

The current studies further demonstrate that the supernatants from the early adhesion phase of *Lactobacillus* cells (1.5 h) were unable to inhibit the *C. albicans* adhesion as well as their biofilm development, and the inhibitory effect was observed only with the 24 h and 48 h supernatants of lactobacilli. This tends to suggest that the exometabolites by *L. rhamnosus* that interfered with the *Candida* biofilm development requires a mature probiotic growth to induce this change in the yeast. Our CLSM images also corroborate the above findings, suggesting that the late stage exometabolites of *L. rhamnosus*, possibly regulated by environmental stimuli such as cell density (Chew et al. 2015a), may be more potent in suppressing the yeast biofilm growth. Finally, the decreased number of viable *C. albicans* after exposure to *Lactobacillus,* either through direct cell-cell contact or via the supernatant, could be lactobacilli altering the architecture of *C. albicans* biofilms by down-regulating the genes involved in biofilm development as well as those associated with DNA replication, translation, glycolysis and gluconeogenesis (Chew et al. 2015a; Kohler et al. 2012).

The hyphae formation or filamentation is an essential step in the pathogenesis of candidiasis (Polke et al. 2015). Our ultrastructural analyses with SEM imaging demonstrated that both the supernatant as well as the planktonic forms of *L. rhamnosus* significantly reduced the fungal filamentation and affected their biofilm architecture (Figure 6). It is known that neutral pH favors yeast-to-hyphae transition, which in turn contributes to *C. albicans* cell adhesion (Kucharikova et al. 2011). The stationary phase cells of *Candida* form ellipsoidal buds at low pH and elongated hyphae at high pH (Anderson and Soll 1986). As all the tested *Lactobacillus* strains did not significantly reduce the pH in the cocultures, the environmental acidification by *Lactobacillus* strains is unlikely to be involved in the yeast-to-hyphae inhibition. We surmise therefore that the modulation of hypha-specific genes expression of *Candida albicans* induced by lactobacilli as one likely reason for the suppression of *Candida* filamentation (Bandara et al. 2013).

The presence of buds throughout the probiotic-treated *Candida* biofilm was an indication that the yeasts were proliferating rather than forming hyphae, as the budding cells are a sign of mature blastospore growth and would not be present in large numbers during *Candida* filamentation (Anderson and Soll 1986). On the other hand, a recent *in vitro* study testing the effects of biosurfactants produced by *L. brevis* on *C. albicans* biofilms showed no phenotypic differences in blastoconidia (blastospores), hyphal morphology and budding locations between the control and the test groups (Ceresa et al. 2015). The different probiotic bacteria used in the latter study may explain the divergent result.

When comparing the two strains of yeast we evaluated, *C. albicans* 75 was less susceptible to the inhibition eventuated by lactobacilli than *C. albicans* ATCC SC5314 ($p<0.05$). This implies that the anti-biofilm potential of the tested probiotics was also pathogen-specific. In addition to this intra-species variability, a previous study has also noted interspecies variability for the fungicidal and fungistatic activities of *Lactobacillus* against different *Candida* species (Parolin et al. 2015). The latter workers noted that, the *lactobacilli* they tested were more effective in suppressing *C. albicans* than the other human pathogenic *Candida* species, including *Candida krusei* and *Candida parapsilosis*.

In conclusion, the findings of the present study validate the hypothesis that the therapeutic and prophylactic effects of *Lactobacillus* against candidiasis could be attributed to both the interference of interfacial interactions (cell-to-cell and cell-to-surface) (Chew et al. 2015a) and the production of exometabolites that destabilize the biofilm organization and architecture. Our study also reveals that *Lactobacillus* species inhibit the development of *C. albicans* biofilm by suppressing the initial colonization, and hyphae formation, possibly due to the exometabolites produced by lactobacilli. The direct contact of probiotic cells with *C. albicans* biofilms was essential for the anti-biofilm effect at the maturation stage. However, the molecular mechanisms underlying the action of probiotics against *C. albicans* are still unclear and need to be further investigated. These studies may include the isolation and identification of the effective components in the exometabolites produced by *Lactobacillus* using liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. In addition, the isolated exometabolite fractions need to be evaluated for their impact upon *Candida* biofilm development and gene expression responsible for filamentation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends:

Fig. 1 Flowchart of the probiotic cell suspension assays (a) and the probiotic supernatant assays (b)

Fig. 2 Effects of probiotic cell suspensions on 24 h and 48 h old biofilms of *C. albicans* ATCC SC5314 (A,B,C) and *C. albicans* 75 (D,E,F). Data are presented as mean ± SD (n=3). * ($p<0.05$), ** ($p<0.005$), ***($p<0.001$)

Fig. 3 Variation of pH in the *C. albicans* SC5314 biofilm supernatants after incubation with *Lactobacillus* cell suspension (1 x 10^7 cells/ml). *L. rhamnosus* LR32, *L. casei* L324m and *L. acidophulus* NCFM were tested and the pH quantifications performed every 9 and 8 hours, during the initial colonization and the maturation phases of *C. albicans* biofilm formation, respectively. Data are presented as mean ± SD. * ($p<0.05$)

Fig. 4 The effects of *L. rhamnosus* LR32 supernatants on *C albicans* ATCC SC5314 biofilms determined by XTT reduction assays. (A) Effects of Phase-1 (90 min), Phase-2 (24 h) and Phase-3 (48 h) supernatants on *C. albicans* biofilm T^1 (0 – 90 min). (B) Effects of Phase-1, Phase-2 and Phase-3 supernatants on *C. albicans* biofilm T^2 (90 min – 24 h). (C) Effects of Phase-1, Phase-2 and Phase-3 supernatants on *C. albicans* biofilm T^3 (24 h – 48 h). Data are presented as mean ± SD (n=3). * ($p < 0.01$)

Fig. 5 Confocal laser scanning microscopy (CLSM) images. Live cells were stained in green and dead cells in red. (a – c) 90 min old *C. albicans* ATCC SC5314 biofilms (40x): (a) control group; (b) treated with *L. rhamnosus* supernatant Phase-2 (24 h); (c) treated with *L. rhamnosus* supernatant Phase-3 (48 h). Note the weak biofilm architecture of tests group, and the inhibition of *C. albicans* cells adhesion and hyphae formation by *Lactobacillus* supernatant in comparison to its control. The more concentrated the supernatant, the more evident was the inhibition. (d – f) 24h old *C. albicans* ATCC SC5314 biofilms (40x): (d) control group; (e) treated with *L. rhamnosus* supernatant Phase-2 (48 h); (f) treated with *L. rhamnosus* supernatant Phase-3 (48 h). Note the strong inhibition of yeast-to-hyphae transition by *Lactobacillus* supernatant, specially the 48 h supernatant, in comparison to the control

Fig. 6 Scanning electron microscopy (SEM) images of *C. albicans* ATCC SC5314 biofilms in the initial colonization phase (a – d). (a and c) Control group without probiotic cells treatment. (b and d) Experimental group treated with *L. rhamnosus* suspension and cocultured for 18 h. A dense development of hyphae can be visualized in the control group (c), with relatively small water channels dispersed in the biofilm architecture (a). In the experimental group, large channels and more yeast are distributed in the *C. albicans* biofilm (b). *L. rhamnosus* cells (black arrow) are dispersed throughout the biofilm, and many buds are present on the surface of yeast cells (white arrow) (d). Confocal laser scanning microscopy (CLSM) images of *C. albicans* ATCC SC5314 biofilms at the initial colonization phase (e–f). (e) Control group without probiotic cells treatment. (f) Treated with *L. rhamnosus* suspension. Note less hyphae formation after the coculture with probiotic