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The effect of probiotic *Lactobacillus rhamnosus* GG on *Candida albicans* and *Candida tropicalis* biofilm formation: A preliminary study

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Abstract

Lactobacillus rhamnosus is Gram-positive and lactic acid-producing bacterium. Meanwhile, *Candida albicans* and *Candida tropicalis* are opportunistic fungi that cause oral candidiasis. This study aimed to determine the effect of *L. rhamnosus* GG (LGG) on the biofilm formation of *C. tropicalis* and *C. albicans* with the hypothesis that LGG inhibits the biofilm of the yeasts. C. albicans ATCC MYA-4901 and C. tropicalis ATCC 13803 were standardised to 1x106 cells to form a mono-species biofilm. LGG was standardised to $1x10^7$ cells, equivalent to absorbance 0.5 at OD620nm. The microorganisms were cultivated in nutrient broth in a 96-well plate and incubated at 37°C for 24 h and 48 h. Co-culture biofilm was developed by combining *Candida* spp. with LGG in the same well at a similar concentration as the mono-culture. Crystal violet assay was conducted to assess the biofilm biomass with absorbance measured at OD_{620nm} wavelength. After 24 hours, polymicrobial biofilms of *C. albicans* with LGG decreased by 37.1 ± 9.2%. At 48 hours, it further decreased to 44.7 ± 5.9%. For *C. tropicalis*, co-culture biofilms with LGG decreased by 16.3 ± 5.9% and 35.7 ± 7.6% after 24 h and 48 h incubation, respectively. LGG significantly reduced *C. albicans* biofilm compared to *C. tropicalis* ($P<0.05$). In conclusion, LGG has antibiofilm activity against *C. albicans* and *C. tropicalis.* However, further study is needed to conclude the effect against other species strains.

Keywords: *biofilm, Candida albicans, Candida tropicalis, Lactobacillus rhamnosus GG, probiotic*

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Introduction

Candida species are common human opportunistic pathogens and capable of colonising the oral cavity, despite being the normal oral microbiome. The fungi commonly infect immunocompromised persons, causing severe mucosal and systemic infections such as oral candidiasis (Hernday *et al*., 2010; Geng *et al.*, 2018). Candidiasis is the most common disease associated with *Candida* spp. infection. The disease results from the overgrowth of fungi and is frequently observed in patients with HIV and those under steroid (Martins *et al.*, 2014; Erdogan *et al.*, 2015).

Although *C. albicans* is the most common aetiology for oral candidiasis, other *Candida* spp. such as *Candida tropicalis*, contribute to the infection (Hu *et al*., 2019). In oral infection, *C. tropicalis* is commonly found in a mixed culture with *C. albicans, C. glabrata* and *C. parapsilosis* (Miranda-Cadena *et al.*, 2018). *C. tropicalis* has been identified as the leading cause of candidemia in Algeria, followed by *C. albicans* (Megri *et al.*, 2020). Furthermore, the formation of true hyphae is also observed in *C. tropicalis,* increasing the virulence of the species (Zuza-Alves *et al.*, 2017).

Biofilm formation is a virulent factor of *Candida* spp. It begins with the adhesion of a single yeast cell to the substrate base, forming a groundwork of the basal yeast layer. The initiation step begins as proliferation and filamentation of the yeast cells occur, as they spread across the surface and form elongated projections, arising to filamentous hyphal form. Then, the accumulation of the extracellular polysaccharide matrix follows, indicating the maturation of the biofilm. Finally, non-adherent yeast cells are dispersed to colonise other mucosal surfaces. It is clinically significant, broadening the infection by forming new biofilm or disseminating it into the host tissue (Andes *et al.*, 2004; Uppuluri *et al.*, 2010; Tournu *et al*., 2012; Tsui *et al.*, 2016).

Probiotics can benefit human health through three mechanisms of action which are 1) inhibition or exclusion of pathogen through direct inhibitory/bactericidal/fungicidal action or by influencing the commensal microbiome; 2) improvement of the epithelial barrier through modulation of signalling pathways; and 3) modulation of host's immune response by exercising strain-specific local and systemic immune response (Mack *et al.*, 2003; Lebeer *et al.,* 2008; Corr *et al.*, 2009; Segers *et al.*, 2014). Furthermore, the biofilm formed by probiotics can also act as a protective barrier against oral pathogens from colonising the cavity (Alok *et al.*, 2017).

Lactobacillus rhamnosus GG (LGG) is a gram-positive, non-spore-forming and lactic acid-producing bacterium classified as a beneficial probiotic to the gut (Doron *et al.*, 2005). For example, the pre-treatment of LGG to septic mice decreased inflammatory cytokines, reversed colonic proliferation, and recuperated gut microbiota diversity (Chen *et al.,* 2019).

Although probiotic LGG is commonly associated with gastrointestinal health, the effect on the colonisation of *Candida* spp. in the oral cavity remains unclear. Thus, this study aimed to determine the effect of LGG on the biofilm formation of *C. albicans* and *C. tropicalis* with the hypothesis that LGG inhibits the biofilm of the yeasts.

Materials and Methods

Growth of microorganisms

C. albicans ATCC MYA-4901, *C. tropicalis* ATCC 13803 and *Lactobacillus* GG (LGG) were used in the study. The inocula were standardised using a spectrophotometer (UviLine 9400, Secomam, Ales) in nutrient broth to give a final cell density of 10^6 cells/ml for *C. albicans* and *C. tropicalis*, while 10^7 cells/ml for LGG, which equivalent to an absorbance of 0.5 at 620 nm wavelength (OD_{620nm}).

Static biofilm formation

Biofilm formation in a static environment was conducted according to the protocol outlined by Alnuaimi *et al.* (2013). To develop mono-species biofilm, 60 µl of *C. albicans*, *C. tropicalis* or LGG standard suspension was pipetted into different wells of a 96-well plate. Following that, 120 µl of nutrient broth was added to the wells. To develop a dual-culture biofilm, 60 µl of *C. albicans* or *C. tropicalis* and 60 µl of LGG were pipetted into the same well. Then, 60 µl of nutrient broth was added to give a final volume of 180 µl. The plates were incubated for 24 and 48 hours at 37 ℃.

Crystal violet assay

Crystal violet (CV) assay was conducted according to the protocol outlined by Badri *et al.* (2022). Briefly, non-adherent cells were washed with sterile phosphate buffer saline (PBS) (Amresco, Ohio). Fixation of biofilm was done by pipetting 200 µl of ethanol into each well, followed by incubation at room temperature for 15 minutes. The supernatant was removed, and the 96-well plate was air-dried for 45 minutes. Next, 200 µl of CV solution was added to the wells and was incubated for another 20 minutes at room temperature. The plate was washed with distilled water twice, and 200 µl of 33% acetic acid was pipetted into each well, followed by five minutes of incubation. Finally, 100 µl of the solution from each well was transferred into a new sterile 96-well plate, and the absorbance was measured at 620 nm using a microplate reader (Infinite 200 PRO, Tecan, Switzerland).

The inhibitory effect of LGG on *Candida* biofilm was assessed by calculating the percentage of biofilm reduction at 24 and 48 hours. The calculation was performed according to Subramenium *et al.* (2018) using the formula below:

Reduction (%) = [(OD_{620nm}X – OD_{620nm}Y) / OD_{620nm}X] x 100%

Where X is the expected biofilm, and Y is the obtained biofilm. The expected value is the sum of the mono-culture biofilm biomass of *Candida* spp. and LGG, while the obtained value is the observed biofilm biomass of *Candida* spp. when co-cultured with LGG.

Statistical analysis

All experiments were conducted in three biological and three technical replicates (N=9). Statistical analysis was performed using SPSS software version 27.0. An independent t-test was used to compare biofilm biomass between the mono- and co-culture biofilm and between incubation times. Data with a value of $P<0.05$ were considered statistically significant.

Result and Discussion

C. tropicalis had more biofilm biomass than *C. albicans* after 24 h and 48 h incubations in mono-culture and co-culture. However, no significant differences were observed ($P>0.05$; Table 1). In addition, mono-culture *C. albicans* and *C. tropicalis* had significantly decreased biofilm biomass at 48 h incubation compared to 24 h ($P<0.05$). Meanwhile, only *C. albicans* exhibited significantly higher biofilm biomass at 24 h incubation than at 48 h in dual-culture biofilm ($P<0.05$).

The biofilm of both *C. albicans* and *C. tropicalis* was observed to develop as early as 24 h. This is attributed to the secretion of adhesin by both *C. albicans* and *C. tropicalis* that aid in the initial adherence to the host during the infection period (Yang, 2003; Martin et al., 2020).

Furthermore, a previous study reported that the biofilm formation by *C. albicans* and *C. tropicalis* was at the intermediate phase during the first 24 h (Cavalheiro *et al.*, 2018). At this phase, the extracellular polymeric substances (EPS) increased, and the bilayer formation of biofilm, commonly composed of yeast, germ tubes, and/without young hyphae, was formed. This phase lasts for 12 to 30 h. The maturation phase proceeds with a dense network of hyphae and yeast,

forming a thicker EPS, which will take approximately 38 to 72 h (Cavalheiro *et al.*, 2018). It is suggested that the decreased biofilm biomass at 48 h in this study was due to the competition for nutrients and microhabitats by microorganisms in the dense biofilm consortium at the maturation phase.

C. albicans biofilm biomass was reduced by 37.1 ± 9.2% at 24 h when co-cultured with LGG (Figure 1). The biofilm decreased by 44.7 ± 5.9% after 48 h. As for *C. tropicalis*, the biofilm biomass of the yeast was reduced by 16.3 ± 5.9% when co-cultured with LGG after 24 h. The biofilm was further decreased by 35.7 ± 7.6% after 48 h incubation with LGG. These results are suggested due to the antagonistic interaction of LGG with *C. albicans* and *C. tropicalis* that compete for the binding site on the oral surface to initiate colonisation (Jiang *et al*., 2016; *Jørgensen et al.*, 2017; Meurman *et al.*, 2018).

Furthermore, metabolites such as bacteriocin-like peptides that LGG produces have also been reported to contribute to the biofilm reduction (Dimitrijević *et al.,* 2009; Zhang *et al.,* 2018). In addition, these secondary metabolites also act antagonistically against biofilm formation by interfering with the adhesion of *Candida* spp. to the surfaces and by reducing the sturdiness of the formed biofilm (Barzegari *et al.,* 2020).

Table 1. Biofilm biomass produced by *C. albicans*, *C. tropicalis* and LGG in mono-culture and dual-culture biofilms.

Inocula	Incubation period	
	24 h	48 h
C. albicans	0.157 ± 0.046*	0.067 ± 0.006
C. tropicalis	0.217 ± 0.082*	0.109 ± 0.042
LGG	0.077 ± 0.006	0.073 ± 0.009
C. albicans + LGG	0.147 ± 0.033*	0.077 ± 0.006
C. tropicalis + LGG	0.215 ± 0.087	0.120 ± 0.043

The data were CV assay and expressed as mean value ± standard deviation from nine replicates, N = 9. *Significant differences were observed between 24 h and 48 h. Data were considered statistically significant when $P<0.05$.

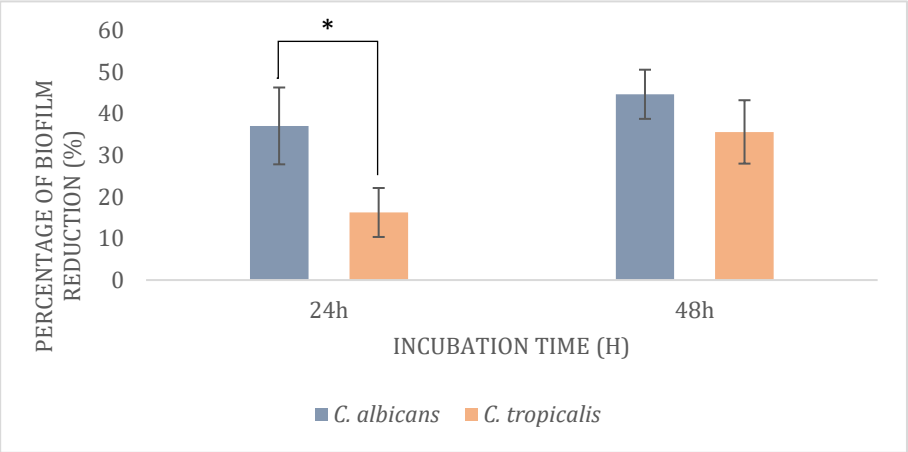

Figure 1. Percentage of biofilm reduction in nutrient broth (NB) at the 24 hours and 48 h incubation, N = 9. *Significant difference was observed between *C. albicans* and *C. tropicalis* (P<0.05).

Conclusion

This preliminary *in vitro* study demonstrated that LGG inhibits the biofilm formation of *C. albicans* and *C. tropicalis* at 24 h and 48 h incubation. However, more *Candida* strains are suggested to elucidate the mechanism of inhibition by LGG to the oral pathogen.

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