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Lactobacillus paracasei 28.4 reduces *in vitro* hyphae formation of *Candida albicans* and prevents the filamentation in an experimental model of *Caenorhabditis elegans*

Patrícia Pimentel de Barros[a], Liliana Scorzoni[a], Felipe de Camargo Ribeiro[a], Luciana Ruano de Oliveira Fugisaki[a], Beth Burgwyn Fuchs[b], Eleftherios Mylonakis[b], Antonio Olavo Cardoso Jorge[a], Juliana Campos Junqueira[a], Rodnei Dennis Rossoni[a,*]

[a] Department of Biosciences and Oral Diagnosis, Institute of Science and Technology, UNESP – Univ Estadual Paulista, Francisco Jose Longo 777, Sao Dimas, Sao Jose dos Campos, CEP: 12245-000, SP, Brazil

[b] Division of Infectious Diseases, Rhode Island Hospital, Alpert Medical School of Brown University, Providence, RI, 02903, USA

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ABSTRACT

The objective of this study was to evaluate the influence of microbe-microbe interactions to identify a strain of *Lactobacillus* that could reduce the filamentation of *Candida albicans* ATCC 18804 using *in vitro* and *in vivo* models. Thus presenting a probiotic effect against the fungal pathogen. First, we analyzed the ability of 25 clinical isolates of *Lactobacillus* to reduce filamentation in *C. albicans in vitro*. We found that *L. paracasei* isolate 28.4 exhibited the greatest reduction of *C. albicans* hyphae ($p = 0.0109$). This reduction was confirmed by scanning electron microscopy analysis. The influence of *C. albicans* filamentation was found to be contributed through reduced gene expression of filament associated genes (*TEC1* and *UME6*). In an *in vivo* study, prophylactic provisions with *L. paracasei* increased the survival of *Caenorhabditis elegans* worms infected with *C. albicans* ($p = 0.0001$) by 29%. Prolonged survival was accompanied by the prevention of cuticle rupture of 27% of the worms by filamentation of *C. albicans,* a phenotype that is characteristic of *C. albicans* killing of nematodes, compared to the control group. *Lactobacillus paracasei* isolate 28.4 reduced the filamentation of *C. albicans in vitro* by negatively regulating the *TEC1* and *UME6* genes that are essential for the production of hyphae. Prophylactic provision of *Lactobacillus paracasei* 28.4 protected *C. elegans* against candidiasis *in vivo*. *L. paracasei* 28.4 has the potential to be employed as an alternative method to control candidiasis.

1. Introduction

Approximately 1.4 million people have suffered from fungal infections worldwide during the last decade [1]. Some of these mycoses are difficult to diagnose and treat and may cause sequelae [2]. *Candida* species are highlighted because of their high incidence of opportunistic fungal infections, *Candida albicans* being the main causative agent of these infections [3,4]. The polymorphic fungi *C. albicans* belongs to the human microbiome and, in normal conditions, does not affect the host [5,6]. However, under some circumstances, such as incidence of host immunosuppression, these fungi can cause infections ranging from superficial (oral and vaginal candidiasis) to systemic infection [5–7].

Various mechanisms contribute to the virulence of *C. albicans* including adhesion/invasion molecules, secreted enzymes such as hydrolases, biofilm formation, and yeast-to-hyphae transition [6,8,9]. The dimorphism of *C. albicans* is the ability of this fungus to transition from yeast to hyphae morphology, and both forms are important for host infection: the yeast is considered to be important for the dissemination of the infection, while the hyphae have an essential role during tissue invasion [6,10,11]. The morphological transition is influenced by some factors such as pH, temperature (37 °C), nutrients, and serum [12]. In addition, the morphological transition relies on a complex pathway with multiple steps and is synchronized by transcription factors such as the genes *UME6* and *TEC1* that correspond to positive regulators of the process [13–15].

The limited arsenal of antifungal drugs and their toxicity, together with the antifungal resistance mechanisms developed by this microorganism, led to the necessity of developing efficient strategies for

* Corresponding author.
E-mail addresses: barrosdnapp@yahoo.com.br (P.P. de Barros), liliscorzoni@yahoo.com.br (L. Scorzoni), felipe_c_ribeiro@hotmail.com (F.d.C. Ribeiro), luciana.fugisaki@ict.unesp.br (L.R.d.O. Fugisaki), helen_fuchs@brown.edu (B.B. Fuchs), emylonakis@lifespan.org (E. Mylonakis), olavojorge@fosjc.unesp.br (A.O.C. Jorge), juliana@fosjc.unesp.br (J.C. Junqueira), rodnei.rossoni@fosjc.unesp.br (R.D. Rossoni).

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antifungal therapy [16]. One of these strategies is to target the fungal virulence mechanisms using anti-virulence agents [12,17]. The development of anti-virulence agents would bring new and a high number of potential targets, preserve the host normal flora, and potentially decrease the antifungal resistance index [12,17].

Recent reports demonstrate the importance of the filamentation process of *C. albicans* for the development of anti-virulence agents [12]. The search for C. *albicans* anti-filamentation substances can be found in different sources such as chemical libraries of small molecules [18], natural compounds obtained from plants [19], peptides isolated from bacteria [20], and through microorganism-microorganism interactions [21,22]. We focus on the latter.

Lactobacillus is a Gram-positive bacterium known to interact with other microbes through probiotic effects. The *Lactobacillus* spp.–*C. albicans* interaction is described in different studies [23–25]. The possible mechanisms that *Lactobacillus* spp. could use to prevent *C. albicans* colonization include the secretion of substances that decrease fungal adhesion (biosurfactants), the production of bacteriocin-like compounds to suppress fungal proliferation, host immunomodulation, and the production of hydrogen peroxide and lactic acid that interfere with hyphal formation [22,23,26,27]. Based on this, the use of probiotics would be an interesting strategy to develop anti-filamentation agents.

In vivo studies can be valuable in providing a better understanding of the mechanisms involved during these microorganism-microorganism interactions. *Caenorhabditis elegans* is described as an infection model for *Candida* spp. in the study of new antifungal compounds, anti-virulence agents, and immunomodulatory activity [28–32]. Additionally, *C. elegans* permits the study of *C. albicans* filamentation because hyphae formation can be observed after the ingestion of this yeast; the hyphae grow through the worm cuticle causing *C. elegans* death [33,34].

In this context, the aim of this study was to evaluate the effect of *Lactobacillus* isolates on *C. albicans* in relation to the filamentation process, analyzing *in vitro* and *in vivo* approaches. In addition, the regulation of this mechanism was analyzed, evaluating genes that are important for the C. *albicans* filamentation process.

2. Material and methods

2.1. Strains and growth conditions

In this study, we used the reference strain *C. albicans* ATCC 18804 and 25 clinical strains of *Lactobacillus:* 20 strains of *L. paracasei*, 4 strains of *L. rhamnosus* and 1 strain of *L. fermentum* (Table 1). The *Lactobacillus* strains were isolated from the human oral cavity of an individual without caries, per the approval of the Ethics Committee under protocol 560.479 and identified in a previous study of our group [35]. *C. albicans* was cultured for 18 h at 37 °C in yeast nitrogen base broth (YNB; Difco, Detroit, MI, USA) supplemented with 100 mM of glucose. The *Lactobacillus* strains were cultured in *Lactobacillus* Man-Rogosa-Sharpe broth (MRS broth; Difco, Detroit, USA) for 24 h at 37 °C under microaerophilic conditions. The suspension densities were determined with a spectrophotometer (B582, Micronal, Sao Paulo, Brazil) and were then diluted to concentrations of 10^7 cells/mL. The number of cells in the inoculum was confirmed by counting the CFU/mL following plating on Sabouraud dextrose agar (SDA; Himedia, Mumbai, India) for *C. albicans* and MRS agar (Difco, Detroit, MI, USA) for *Lactobacillus*.

2.2. Selection of the best Lactobacillus strain to reduce filamentation: In vitro study

2.2.1. Induction of filamentation by C. albicans

To select the *Lactobacillus* strain with the prominent anti-*Candida* activity, a filamentation assay was used to compare the effects posed by the 25 isolates from the oral cavity. This assay was performed in 24-well microtiter plates (TPP®, Trasadingen, Switzerland) following a methodology described previously [36,37] with some modifications. The following groups were evaluated: *C. albicans* + Phosphate-buffered saline (PBS/control group) and *C. albicans* + *Lactobacillus* spp. The PBS buffer was diluted to a 1X working concentration that contains 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4. The experiment was performed as three biological replicates independently in triplicate.

In a 24-well culture plate, 1 mL of distilled water was mixed with 10% fetal bovine serum, 50 µL of BHI broth (Difco, Detroit, MI, USA) and 100 µL of standardized *C. albicans* suspension. Fifty microliters of standardized *Lactobacillus* spp. suspension was also added to the experimental group, while 50 µL of PBS was added to the control group. The plates were incubated at 37 °C under a partial pressure of 5% CO_2. After 24 h of incubation, 50 µL of the inoculum was transferred to glass slides with 10 previously demarcated fields on the back of the slide and observed under a light microscope at 40X magnification. The images were analyzed for the *C. albicans* morphology, and 10 microscopic fields per slide were chosen to quantify the hyphae. The following scores were used to delineate the number of hyphae present in each microscopic field: 0 (no hyphae), 1 (1–15 hyphae), 2 (16–30 hyphae) and 3 (more than 30 hyphae).

Table 1
Clinical strains of *Lactobacillus* used in this study.

Species	Strain designation
L. paracasei	1.1
L. paracasei	3.1
L. paracasei	4.2
L. paracasei	6.2
L. paracasei	7.5
L. paracasei	8.4
L. paracasei	11.6
L. rhamnosus	13.1
L. paracasei	15.8
L. paracasei	16.4
L. paracasei	17.1
L. rhamnosus	19.3
L. rhamnosus	19.9
L. paracasei	21.4
L. paracasei	23.4
L. paracasei	24.1
L. paracasei	25.4
L. paracasei	26.1
L. paracasei	27.1
L. paracasei	28.4
L. paracasei	30.1
L. fermentum	31.4
L. rhamnosus	36.4
L. paracasei	37.1
L. paracasei	39.2

2.2.2. Measurement of pH values

The pH of the media during the filamentation process were tested under the same conditions as the filamentation assay described above. After 24 h of incubation in the 24-well culture plates, the supernatant from each well was collected, and the pH value was measured using a pH meter (Mettler, Toledo, Ohio, USA). Four wells were measured per group, and the experiment was done at three different times.

2.2.3. Analysis of filamentation assay by scanning electron microscopy (SEM)

The L. *paracasei* 28.4 strain demonstrated the greatest ability to reduce hyphae production, and all the experiments of this study were conducted with this strain. Acrylic resin discs measuring 8 mm in diameter were placed on a 24-well plate for filamentation assay as previously described. After 24 h, the specimens were fixed in 1 mL of 2.5% glutaraldehyde for 1 h. The specimens were then dehydrated in an increasing ethanol series (10, 25, 50, 75 and 90%) for 20 min each, followed by immersion in 100% alcohol for 1 h. The plates were kept in an

oven at 37 °C for 24 h to permit complete drying of the specimens. After drying, the specimens were transferred to aluminum stubs and sputtered with gold for 160 s at 40 mA (Denton Vacuum Desk II). The specimens were examined and photographed using a JEOL JSM5600 scanning electron microscope at the Institute of Science and Technology, UNESP (Univ Estadual Paulista). These experiments were performed at two different times with three assays per group.

2.2.4. Quantitative real-time polymerase chain reaction (qPCR)

The qReal-time PCR assay was performed in the same conditions described in the *in vitro* filamentation assay. Total RNA was isolated with the TRIzol reagent (Ambion, Inc., Carlsbad, CA, USA). The extracted total RNA (700 ng) was treated with DNase I (Turbo DNase Treatment and Removal Reagents - Ambion Inc., Carlsbad, CA, USA) and transcribed into complementary DNA (cDNA) using SuperScript® IV First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen™, Carlsbad, CA, USA), according to the manufacturer's instructions.

Transcribed cDNAs were amplified for the relative quantification of *TEC1* and *UME6* gene expression levels in relation to the concentration of the reference gene (*ACT1*). In our study, three reference genes, *ACT1, PMA1* and *RPP2B*, were tested in all the experimental groups. The results were analyzed at http://www.leonxie.com/referencegene.phpe, and the reference gene chosen was *ACT1*. All the primers used in the present study were described previously [38,39].

Quantitative real-time PCR was conducted using a Platinum® SYBR® Green qPCR SuperMix-UDG Kit (Applied Biosystems, Framingham, MA, USA) in the StepOnePlus™ apparatus (Applied Biosystems, Framingham, MA, USA). The $2^{-}\Delta\Delta^{CT}$ method was used to analyze the relative changes in gene expression from the quantitative RT-qPCR experiment [40].

2.3. Lactobacillus paracasei 28.4 interferes in filamentation of C. albicans: in vivo study

2.3.1. C. elegans strain

C. elegans glp-4, sek-1 strain was used in this study with the methodologies described by Oh et al. [41], Kim et al. [42] and Peleg et al. [43]. Mixed infections with *Lactobacillus* and *C. albicans* were induced in *C. elegans* in all experiments (survival and filamentation assays). As control groups, a monotypic infection was induced in *C. elegans* by inoculation only of *C. albicans*, *Lactobacillus*, or *E. coli* OP50.

2.3.2. Preparation of conditioning plates with probiotic bacteria

To prepare probiotic-conditioning plates, *L. paracasei* 28.4 was grown in Man-Rogosa-Sharpe (MRS) medium (Difco, Detroit, MI, USA) at 37 °C for 24 h. Following five washes with M9 medium, 500 µL of *L. paracasei* 28.4 suspension (1.0×10^9 CFU/mL) was spread on a Nematode Growth Medium (NGM) plate and dried for 3 h at room temperature. Conditioning plates were stored at 4 °C and used within 1 week. *E. coli* strain OP50 was used as the control, and the same protocols were followed. The M9 medium consists of 3 g KH_2PO_4, 6 g Na_2HPO_4, 0.5 g NaCl, 1 g NH_4Cl and 0.25 g $MgSO_4{\cdot}7H_2O$ in 1000 mL of water. The NGM plate consists of 2.5 g peptone, 3 g NaCl, 20 g agar, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KPO_4, 5 µg/mL cholesterol in 1000 mL of water.

2.3.3. Effects of Lactobacillus on experimental candidiasis in C. elegans: survival assay

First, *C. elegans* nematodes were cultured on NGM plates with a 12 h culture of *E. coli* OP50 (10^8 CFU/mL) for 48 h at 25 °C. After this time, the nematodes were washed twice with M9 buffer using centrifugation.

Liquid killing assay and the different times of infection were performed using published methods with some modifications [43]. A synchronous population of worms is necessary in order to eliminate variation in results due to age differences. Thereby, the worms were synchronized in young adult stage. Young adult worms were placed on conditioning plates with *L. paracasei* 28.4 at 25 °C for 2 h. After this period, the nematodes were washed with M9 buffer and placed in brain heart infusion (BHI - Difco, Detroit, MI, USA) plates containing *C. albicans* for 2 h. Infected worms were then washed twice with M9 medium and transferred into wells of a 6-well microtiter dish (50 worms per well). Each well contained 2 mL of liquid assay medium (20% BHI and 80% M9). For the control groups (*E. coli* + *E. coli*, *E. coli* + *C. albicans* and *L. paracasei* + *E. coli*), the worms were infected with two subsequent infections of 2 h each as in the *L. paracasei* + *C. albicans* group described above. The plates were sealed with a breathe easy membrane (Sigma Aldrich, St. Louis, MO) and incubated at 25 °C for 10 days. Nematodes were observed daily under a stereomicroscope and were considered dead when they did not move after being touched with a platinum loop. This assay was performed as three independent experiments with n = 6 wells per group.

2.3.4. Study of C. albicans filamentation in C. elegans treated with Lactobacillus

The filamentation assay was performed according Peleg et al. [43]. The *C. elegans* nematodes were provided with *L. paracasei* 28.4 as a food source for 4 h, and then *C. albicans* was used to infect the worms. For this, the nematodes were fed *C. albicans* that were seeded on the surface of BHI agar (Difco, Detroit, MI, USA) and incubated at 25 °C for 4 h. The nematodes were then washed with M9 buffer and transferred by pipette into each well of a 6-well culture of cells containing 2 mL of liquid medium (80% M9 and 20% BHI) plates. The plates were incubated at 25 °C and analyzed after 60 h to identify nematodes with *C. albicans* filamentation. This analysis was conducted using a stereomicroscope (Nikon SMZ645, Minato, Tokyo, Japan) to observe a rupture in the cuticle of the nematode by the filamentation of *C. albicans*. The differences in filamentation were compared between groups of nematodes infected with *L. paracasei* 28.4 and *C. albicans* and infected only with *C. albicans*. This assay was performed in triplicate.

2.4. Statistical analysis

A statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The scores obtained through *in vitro* filamentation analysis were compared using the Mann-Whitney test. The percent survival of *C. elegans* was plotted, and statistical analysis was performed using the Kaplan-Meier test. Student's *t*-test was used to compare the filamentation assay with *C. elegans*. A P value ≤ 0.05 was considered significant.

3. Results

First, we carried out an *in vitro* assay as a screening with 25 clinical isolates of *Lactobacillus* spp. to select the strain with the greatest capacity to interfere with the formation of *C. albicans* hyphae. Among the 25 strains analyzed, only 8 could significantly reduce the formation of *C. albicans* hyphae, as shown in Fig. 1.

We observed many *C. albicans* hyphae in the control group with PBS. However, we verified that hyphal formation was significantly reduced when *C. albicans* was incubated with *Lactobacillus* strains 13.1 ($p = 0.0209$), 19.3 ($p = 0.0420$), 26.1 ($p = 0.0420$), 25.4 ($p = 0.0290$), 30.1 ($p = 0.0209$), 27.1 ($p = 0.0290$), 19.9 ($p = 0.0290$) and 28.4 ($p = 0.0109$) compared to the control group. Because L. *paracasei* strain 28.4 resulted in lower *C. albicans* filamentation scores compared to the other strains, it was chosen for subsequent assays.

To invalidate the hypothesis that the *C. albicans* hyphae were inhibited by the reduction in the pH of the medium due to the action of *Lactobacillus*, which are lactic acid bacteria, we compared the pH of the control group and the group associated with strain 28.4. Although the pH value decreased in the interaction group with *L. paracasei* 28.4, it was not significant ($p = 0.0643$), suggesting that there are metabolites secreted by the probiotic strain capable of interfering with the

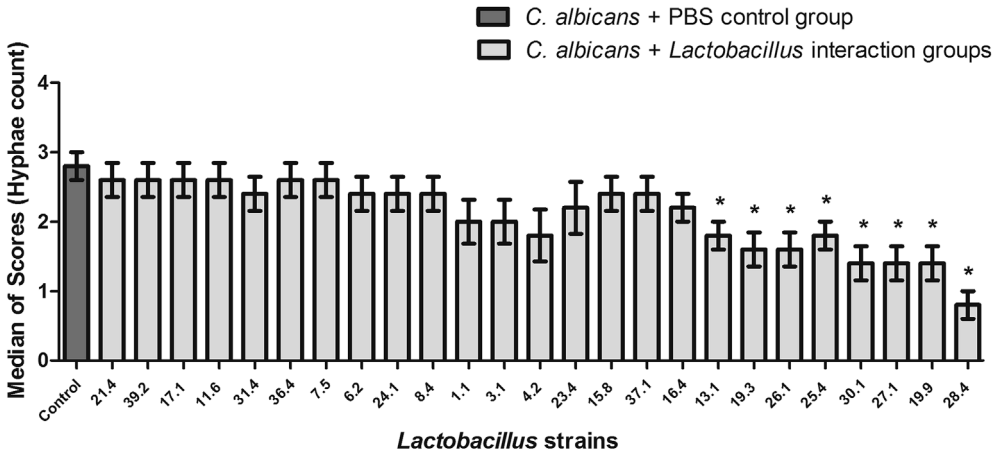

Fig. 1. Quantification of hyphae in the *in vitro C. albicans* filamentation assays. Scores were attributed to the number of *C. albicans* hyphae formed in association with clinical strains of *Lactobacillus* or with PBS (control group). Mann-Whitney test, $p \leq 0.05$.

Fig. 2. The reduction of hyphae was not caused by pH change in the medium. Mean and standard deviations for the pH value per well in the filamentation assay. Student's *t*-test, $p \leq 0.05$.

formation of hyphae (Fig. 2). In addition, the SEM analysis revealed a large number of hyphae in the control group of *C. albicans*, as opposed to large numbers of yeast in the group associated with *L. paracasei* 28.4, confirming the results obtained in the previous assay (Fig. 3).

To elucidate the mechanisms involved in the action of *L. paracasei* 28.4 on the filamentation of *C. albicans*, we did quantification of transcriptional genes such as *TEC1* and *UME6* by qPCR. These genes were downregulated with 2.5- and 3.0-fold decreases, respectively, when compared to the control (Fig. 4). Both *TEC1* and *UME6* are required for the morphogenesis of *C. albicans* and play key roles in this important virulence factor.

In the *in vivo* assays, we first examined whether *L. paracasei* strain 28.4 could influence the life span of *C. elegans*. As a control, we employed the Gram-negative bacterium *E. coli* strain OP50 that is non-pathogenic to *C. elegans*. We found no significant difference in the viability of *C. elegans* exposed to *E. coli* OP50 or 28.4 strain in the liquid assays (Fig. 5A) ($p = 0.1270$ for *L. paracasei* + *E. coli* group compared with worms fed only OP50). Therefore, we concluded that 28.4 strain does not significantly reduce the lifespan of *C. elegans*.

We hypothesized that despite the ability of *C. albicans* to kill *C. elegans* individually, a combined infection with *L. paracasei* might lead to attenuated killing compared with *C. albicans* alone due to the fact that hyphae formation by *C. albicans* inhibition of *C. elegans* contributes to the death of the worms when filaments puncture the cuticle. We observed that when *C. elegans* was sequentially exposed for 2 h with *L. paracasei*, followed by *C. albicans* on separate agar plates, and then transferred into standard liquid medium, the death of the nematodes was significantly attenuated (29% increase in survival) compared with that observed with *C. albicans* infection alone ($p = 0.0001$) (Fig. 5A).

Given the importance of filamentation in the pathogenesis of *C. albicans* infection in mammals and *C. elegans*, we sought to assess the consequences of prior exposure to *L. paracasei* on the filamentation of *C. albicans* within the worms. When *C. elegans* was infected with *C. albicans* and exposed to a liquid environment, the majority of the nematodes died due to the penetration of *C. albicans* filaments through the nematode cuticles (Fig. 5B). *C. albicans* filamentation within *C. elegans* begins within 24 h of liquid-medium exposure and peaks by 60 h. Remarkably, when nematodes were exposed to *L. paracasei* prior to C. albicans infection, there was a reduction in the number of worms exhibiting filaments (27%), but it was not statistically significant ($p = 0.1012$) (Fig. 5B; Fig. 6).

4. Discussion

A variety of commercially available probiotic preparations are

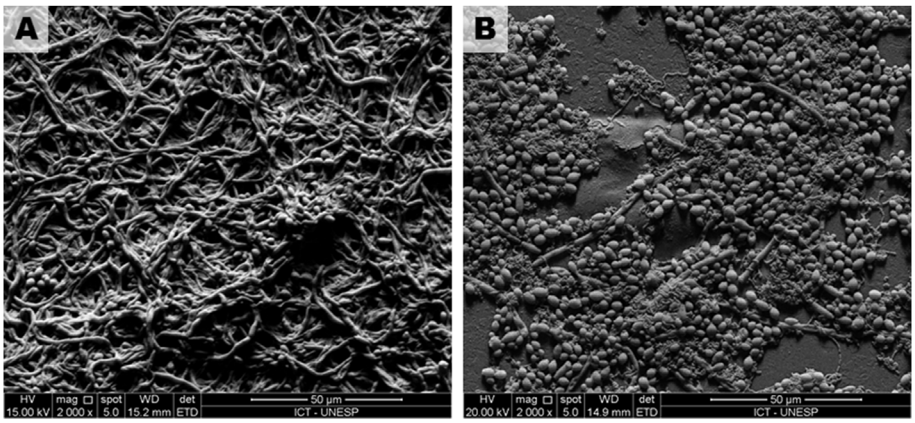

Fig. 3. SEM of filaments formed *in vitro*. A. Control group of *C. albicans* + PBS: the presence of numerous hyphae is verified, B. Group interaction of *C. albicans* + *L. paracasei* 28.4 cells. In the interaction group, it is possible to observe a reduction in the number of hyphae of *C. albicans* and an intimate interaction between *C. albicans* and *Lactobacillus* cells. Magnification: 2000X.

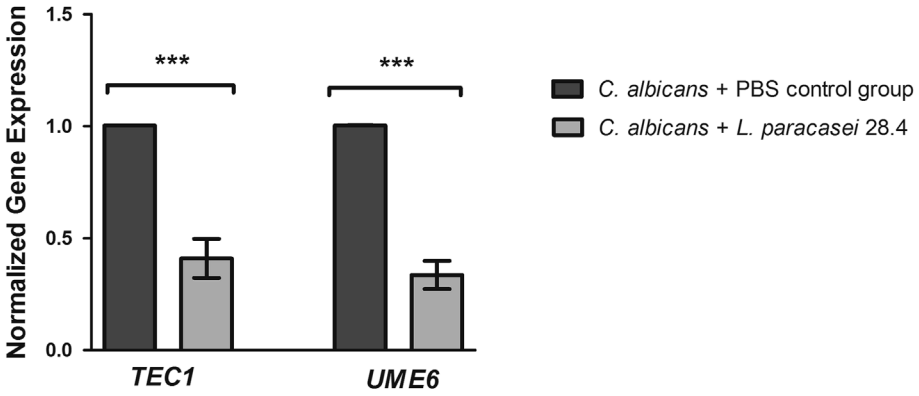

Fig. 4. Relative quantification of *TEC1* and *UME6* in monotypic and mixed groups of *C. albicans* associated with *L. paracasei 28.4*. Each gene, *UME6* or *TEC1*, was normalized and compared with *C. albicans* + PBS (control). Values were expressed as the mean and standard deviation. Student's *t*-test was used to compare gene expression among the groups studied ($p \leq 0.05$).

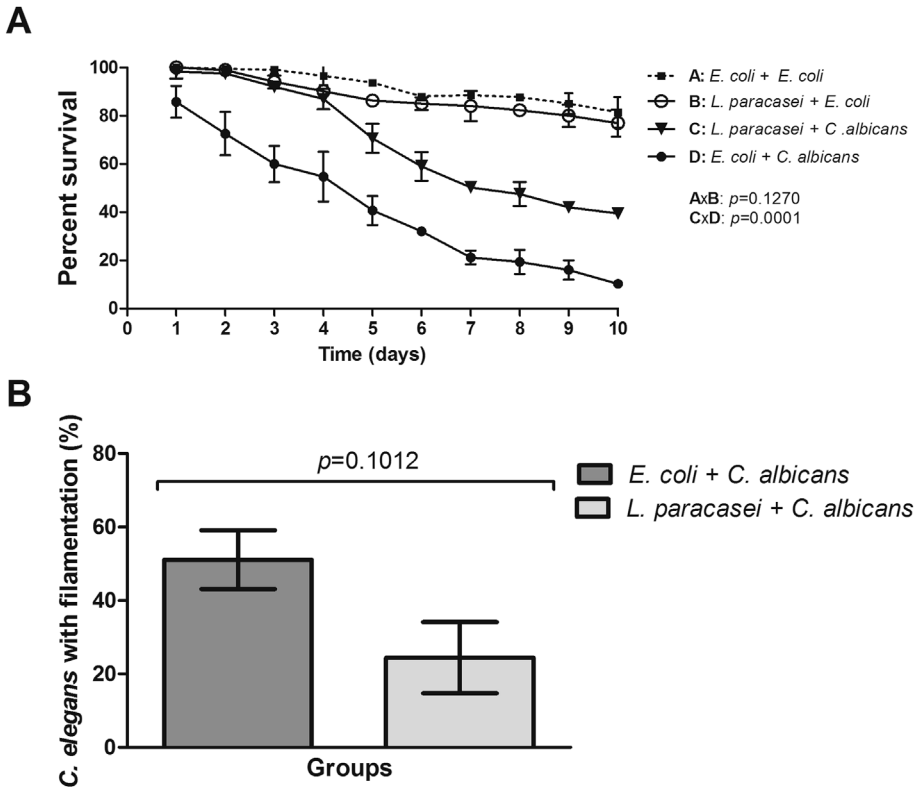

Fig. 5. The probiotic bacterium *L. paracasei* 28.4 prolonged the survival of *C. elegans* nematodes infected with *C. elegans* and decreased the frequency of nematodes observed with filaments protruding through the cuticle. A. Liquid killing assays of *C. elegans* (n = 50 per well) *glp-4* worms infected with *C. albicans* ATCC 18804 after conditioning with *L. paracasei* 28.4. Survival statistic: $p = 0.0001$ for *L. paracasei* + *C. albicans* group compared to worms that were only fed *C. albicans* (*E. coli* + *C. albicans* group). *P* values were obtained from the repetition of the most representative experiment. Kaplan-Meier test, $p \leq 0.05$. B. Mean and standard deviation of worms with *C. albicans* filaments in the liquid medium assay. Student's *t*-test was used to compare the worms with *C. albicans* filaments between the groups studied ($p \leq 0.05$).

comprised of strains of *Lactobacillus* or *Bifidobacterium* isolated from human feces to facilitate compatibility with the human intestinal microbiota [44]. Among the bacteria that show promise to be used as probiotics in the oral cavity, *Lactobacillus* spp. has been the subject of much research because it is a microorganism belonging to the normal oral microbiota able to colonize and modulate this microbiota, thus inhibiting the growth of several pathogens such as *Candida* yeasts [27,45]. In this study, we screened the capacity of 25 clinical isolates of

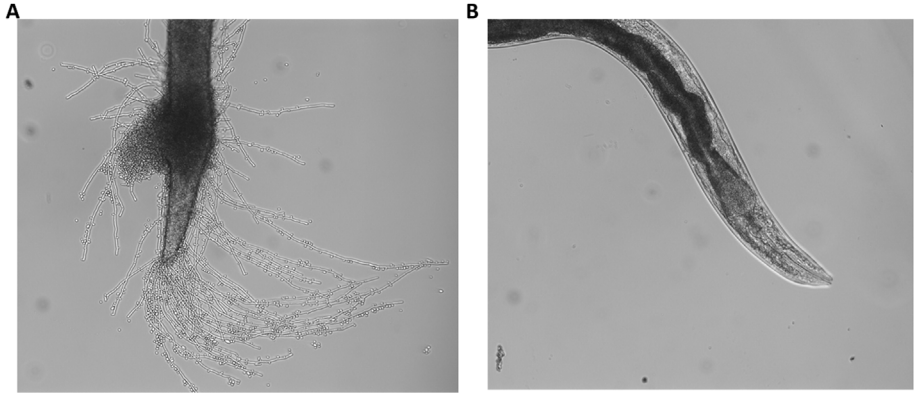

Fig. 6. Prophylactic provisions of *L. paracasei* reduce the filamentation of *C. albicans* in a model of co-infection with *C. elegans*. A. Control group of *C. albicans*: it is possible to observe the formation of hyphae rupturing the cuticle of the nematodes (60 h after infection). B. *L. paracasei* + *C. albicans* group: formation of few or no hyphae of *C. albicans* can be observed after 60 h of infection. Magnification: 100X.

Lactobacillus to reduce filamentation in *C. albicans in vitro* for use as a potential probiotic in the oral cavity.

By analyzing clinical *Lactobacillus* isolates from the oral cavity of healthy patients, we found that some strains provide better reduction of *C. albicans* filamentation than others. We found that eight *Lactobacillus* isolates could reduce *C. albicans* hyphae formation, and *L. paracasei* 28.4 showed the greatest amount of reduction. This fact could be due to the *Lactobacillus* production of antimicrobial molecules such as bacteriocins, since the reduction of the pH was not significant [46,47].

The *in vitro* reduction of hyphae formation by strain *L. paracasei* 28.4 that we observed suggests that this strain could be a highly promising potential probiotic that could be used to control *C. albicans* infections, since the hyphae of *C. albicans* are responsible for invasion into host tissues. Mailänder-Sánchez et al. (2017) performed a study using a multilayer of oral epithelial cells (reconstructed human oral epithelium, RHOE) and provided the first clear evidence for a protective effect of *Lactobacillus rhamnosus* GG (LGG) against infection with *C. albicans*. Pre-incubation of RHOEs with LGG protected the tissue from *C. albicans*-induced cell damage, characterized by a strong reduction in the levels of epithelial lactate dehydrogenase (LDH) released into the RHOE supernatant, which indicates lysis of the epithelial cells. Through histological analysis, the authors found a strong reduction in the signs of damage in the cell layer and hyphae growth into the epithelial cells, while in the absence of LGG, *C. albicans* invaded the whole epithelium and caused vacuoles as signs of damage.

Recent studies have also shown that *Lactobacillus* spp. can reduce the number of hyphae by *C. albicans* formed *in vitro* [36,37]. Lactobacilli can create an adverse microenvironment for pathogens by delivering localized antimicrobial compounds, thus impairing epithelial colonization and subsequent tissue invasion [48–50]. Metabolites released by *Lactobacillus* species such as sodium butyrate have been shown to suppress *C. albicans* filamentation, reducing fungal pathogenicity [51]. Noverr and Huffnagle [52] observed that supernatants obtained from 2 h probiotic bacteria inhibited germ tube formation in *C. albicans,* and complete inhibition of germination was obtained in 24 h supernatants. Other antimicrobial metabolites described in the literature may reduce the survival of the pathogen as a result of their synergistic actions, such as biosurfactants, organic acids, and H_2O_2 [53], bacteriocins [54] and peptide fermenticin HV6b [55].

We explored the specific effect caused by *L. paracasei* 28.4 by examining the expression of genes that are important for the *C. albicans* filamentation process, *TEC1* and *UME6*. During the pathogenicity process of *C. albicans*, several transcriptional regulators are known to play critical roles. Importantly, two of these regulators, *TEC1* and *UME6,* are themselves controlled at the transcriptional level by filament-inducing conditions [56,57]. *UME6*, a zinc finger DNA-binding protein, lies at the center of hyphal regulation in *C. albicans* as a necessary and sufficient factor for hyphae growth [14,58,59] and as a key transcriptional target for the various signal transduction pathways that mediate hyphal induction stimuli [60]. *TEC1* is involved in the regulation of hyphae-specific genes, and it is required for wild-type biofilm formation [57,61]. In addition, *C. albicans TEC1* acts downstream of *EFG1,* a protein that plays a critical role in hyphae morphogenesis [10]. Our results demonstrated that *L. paracasei* 28.4 associated with *C. albicans* significantly downregulate these two genes.

Among the virulence factors of *C. albicans*, filamentation represents an attractive target, already validated at the genetic level, for the development of anti-virulence approaches against candidiasis. The ability of *C. albicans* to respond to a multitude of signals allows its adaptation to the environmental conditions presented by the host. Some signals can stimulate hyphae formation including neutral pH, 5% CO_2, body temperature (37 °C), serum, nutrient availability and embedded and microaerophilic conditions [6,45]. The filamentation process in *C. albicans* is tightly regulated, while simultaneously being highly dynamic, occurring under a wide range of environmental conditions [62,63]. Due to its activity against *C. albicans* filaments, *L. paracasei* strain 28.4 represents a new potential probiotic strain that can be used to control *C. albicans* infections.

The introduction of *C. albicans* in *C. elegans* causes lethal infection with intense proliferation of yeast in the intestinal tract and the subsequent formation of hyphae breaching the cuticle of the nematode [31]. Pukkila-Worley et al. [33] and Tan et al. [64] also demonstrated that the formation of hyphae by *C. albicans* is an important virulence factor to model infection in *C. elegans,* and some *C. albicans* mutant strains incapable of forming hyphae were less virulent to these nematodes. In this study, we also observed a significant inhibition of *C. albicans* filamentation by the influence of *L. paracasei*, agreeing with our *in vitro* results on the specific effect that isolate 28.4 has on filament formation.

The inhibition of various microbial pathogens by *Lactobacillus* spp. was demonstrated in *in vivo* studies using a *C. elegans* model [65–68]. Kim et al. [42] showed that using the probiotic *Lactobacillus acidophilus* NCFM to condition *C. elegans* prolongs the survival of nematodes exposed to resistant *Enterococcus faecium*. Recently, Li et al. [66] explored the role of *Lactobacillus plantarum* in modulating host responses to vancomycin-resistant *Enterococcus faecium* (VRE) using *C. elegans*. The authors found that *L. plantarum* also significantly protected the worms against VRE infection by increasing the transcription of host defense genes, such as cpr-1 and clec-60, reported to have protective roles against infections. Our previous work was consistent with these results and showed that the probiotic strain used in this study (*L. paracasei* 28.4) had immunomodulatory action in the *Galleria mellonella* model and protected the insects against *C. albicans* infection by up-regulating the genes encoding antifungal peptides, such as galiomicin and gallerymicin [27].

More specifically the mechanism of interaction between *L. paracasei* 28.4 and *C. albicans* in *C. elegans* model may be explained by two hypotheses. First, it was verified that the probiotic strain (*L. paracasei* 28.4) used in this study has direct action on *C. albicans* and its mechanisms of pathogenicity. Rossoni et al. [35] observed that *L. paracasei* 28.4 reduced the biofilm, hyphae formation and downregulated genes from different strains of *C. albicans*. Second, it is suggested that this strain has an indirect action by stimulating *C. elegans* defense genes such as FIP (fungus-induced protein), NLP (neuropeptide-like protein), CNC (caenacin) and GRSP (glycine-rich secreted protein) in *C. albicans* infections. According Pujol et al. [69] all these genes could be responsible to encode antimicrobial peptides (AMPs) involved in the innate response against fungi.

The different mechanisms used by probiotics to combat pathogens are still unclear. In addition, the differences in metabolites between *Lactobacillus* species merit further research. Future studies to identify the exact compounds produced by *L. paracasei* 28.4 and their mechanisms underlying the inhibition of *C. albicans* hyphae need to be conducted in more detail.

5. Conclusion

In summary, the clinical strains of *Lactobacillus* isolated from the oral cavity show varied activity against *C. albicans* filamentation. *L. paracasei* 28.4 reduced the filamentation of *C. albicans in vitro* by negatively regulating the *TEC1* and *UME6* genes, which are essential for the production of hyphae. *L. paracasei* 28.4 protected *C. elegans* against experimental candidiasis *in vivo*. *L. paracasei* 28.4 demonstrated the best probiotic profile compared with the other lactobacilli species tested, suggesting that it may be useful as an adjunctive therapeutic mode against oral *Candida* infections.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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