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RESEARCH PAPER

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Lactobacillus acidophilus ATCC 4356 inhibits biofilm formation by *C. albicans* and attenuates the experimental candidiasis in *Galleria mellonella*

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Keywords: biofilm, candidiasis, *Candida albicans*, filamentation, probiotic, *Galleria mellonella*, *Lactobacillus acidophilus*

Abbreviations: ATCC, American type culture collection; YNB, Yeast nitrogen base; MRS, Man, Rogosa and Sharpe; PBS, phosphate buffered saline; BHI, Brain heart infusion; CFU, colony-forming unit; SEM, Scanning electron microscopy; PAS, periodic acid-Schiff; HE, hematoxylin-eosin; pH, potential hydrogen ion; NIH, National Institutes of Health.

Probiotic strains of *Lactobacillus* have been studied for their inhibitory effects on *Candida albicans*. However, few studies have investigated the effect of these strains on biofilm formation, filamentation and *C. albicans* infection. The objective of this study was to evaluate the influence of *Lactobacillus acidophilus* ATCC 4356 on *C. albicans* ATCC 18804 using *in vitro* and in vivo models. In vitro analysis evaluated the effects of *L. acidophilus* on the biofilm formation and on the capacity of *C. albicans* filamentation. For *in vivo* study, *Galleria mellonella* was used as an infection model to evaluate the effects of *L. acidophilus* on candidiasis by survival analysis, quantification of *C. albicans* CFU/mL, and histological analysis. The direct effects of *L. acidophilus* cells on *C. albicans*, as well as the indirect effects using only a *Lactobacillus* culture filtrate, were evaluated in both tests. The *in vitro* results showed that both *L. acidophilus* cells and filtrate were able to inhibit *C. albicans* biofilm formation and filamentation. In the *in vivo* study, injection of *L. acidophilus* into *G. mellonella* larvae infected with *C. albicans* increased the survival of these animals. Furthermore, the number of *C. albicans* CFU/mL recovered from the larval hemolymph was lower in the group inoculated with *L. acidophilus* compared to the control group. In conclusion, *L. acidophilus* ATCC 4356 inhibited in vitro biofilm formation by *C. albicans* and protected *G. mellonella* against experimental candidiasis in vivo.

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Introduction

Fungal infections are one of the most common diseases caused by microorganisms, especially in hospitalized and immunocompromised patients. Among species of the genus *Candida*, *C. albicans* is the pathogen most frequently isolated from the human body, including the oral cavity and gastrointestinal and genitourinary tract. This species can cause infections that range from superficial lesions of the mucosa or skin to severe systemic infections.[1,2] *C. albicans* shows a great capacity of biofilm formation on oral structures and its presence in the oral cavity may serve as a reservoir of this fungus for infections in other parts of the body.[3]

Current treatment for candidiasis consists of the administration of topical antifungal agents such as nystatin, amphotericin B and clotrimazole, or systemic antifungal agents such as fluconazole, ketoconazole and itraconazole. However, the use of these drugs can cause side effects and lead to microbial development of resistance.[4,5] The increase in the resistance of microorganisms to conventional antifungal drugs has encouraged studies designed to discover new treatments for infections caused by *Candida* spp.[6,7] The use of probiotics to prevent or treat *Candida* infections may be an interesting strategy. In this respect, certain *Lactobacillus* strains have been shown to exert anti-*Candida* activity by producing antimicrobial molecules, such as organic acids, hydrogen peroxide and bacteriocins.[8]

Probiotics are defined by the World Health Organization as live microorganisms that confer health benefits on the host when administered in adequate amounts. They are included in a variety of products such as foods, dietary supplements and medications. In addition to *Lactobacillus*, other microorganisms have been used as probiotics, including *Bifidobacterium*, *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus*.[9]

The anti-*Candida* effects of *Lactobacillus* have been investigated in several in vitro studies using different strains of *L.*

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plantarum, *L. rhamnosus*, *L. paracasei*, *L. reuteri* and *L. acidophilus*. All of these strains were able to inhibit the growth of *C. albicans*, but the inhibitory effects were dependent on the strain tested, dose administered and duration of treatment.[10-12] Therefore, further studies are needed to elucidate the inhibitory activity of *Lactobacillus* strains against *C. albicans*, particularly in terms of biofilm formation, filamentation capacity and infection potential.

In addition to the antifungal activity of probiotic strains, some *Lactobacillus* strains have been shown to stimulate the host immune response in *C. albicans* infections by interacting with intestinal epithelial cells and cells of the immune system, releasing cytokines involved in the regulation of the immune response.[13] These data suggest that the effect of *Lactobacillus* on *C. albicans* needs to be studied in vivo using host models of experimental infection.

Although mice are the gold standard for the study of experimental candidiasis, economic, logistic and ethical issues limit the use of mammals in these experiments, especially when a large number of strains need to be analyzed.[14] In the last decades, invertebrate models have been used to study the molecular basis of microbial pathogenicity and pathogen-host interactions, which provided considerable insight into different aspects of microbial infection.[15] In this respect, *Galleria mellonella* has been found to be an interesting invertebrate model for the study of experimental candidiasis.[16-23] This larvae has a sufficient size for injection of a standard inoculum of the microorganism and its hemolymph contains various types of hemocytes that play an important role in the defense against pathogens.[16,17] In these models, the experiments are conducted at temperatures ranging from 25 to 37°C, conditions that simulate the natural environment of mammalian hosts.[18] In addition, *C. albicans* produces filaments and can form a biofilm structure inside the *G. mellonella* larvae that are useful tools in evaluating the pathogenicity of *C. albicans* and new antifungal therapies.[24]

So far, no studies have used *G. mellonella* to evaluate the effects of probiotic bacteria on experimental infection, which would permit to standardize an in vivo model for future studies on the antimicrobial and immunomodulatory activity of probiotics in experimental candidiasis.

Therefore, the objective of the present study was to evaluate the in vitro effects of *L. acidophilus* ATCC 4356 on the biofilm formation and filamentation capacity of *C. albicans* ATCC 18804, as well as to determine its activity on experimental candidiasis in the *G. mellonella* model.

Results

Effects of *L. acidophilus* ATCC 4356 on in vitro biofilm formation by *C. albicans*: CFU/mL count

First, we determined the best growth phase of the *L. acidophilus* culture (4, 6, 18 and 24 h) capable of inhibiting *C. albicans* cells in the biofilms formed in vitro. A reduction in the number of *C. albicans* CFU/mL compared to the control group was observed at all time points tested. The highest inhibition (57.52%) was observed after 24 h of culture of *L. acidophilus* (**Fig. 1A**).

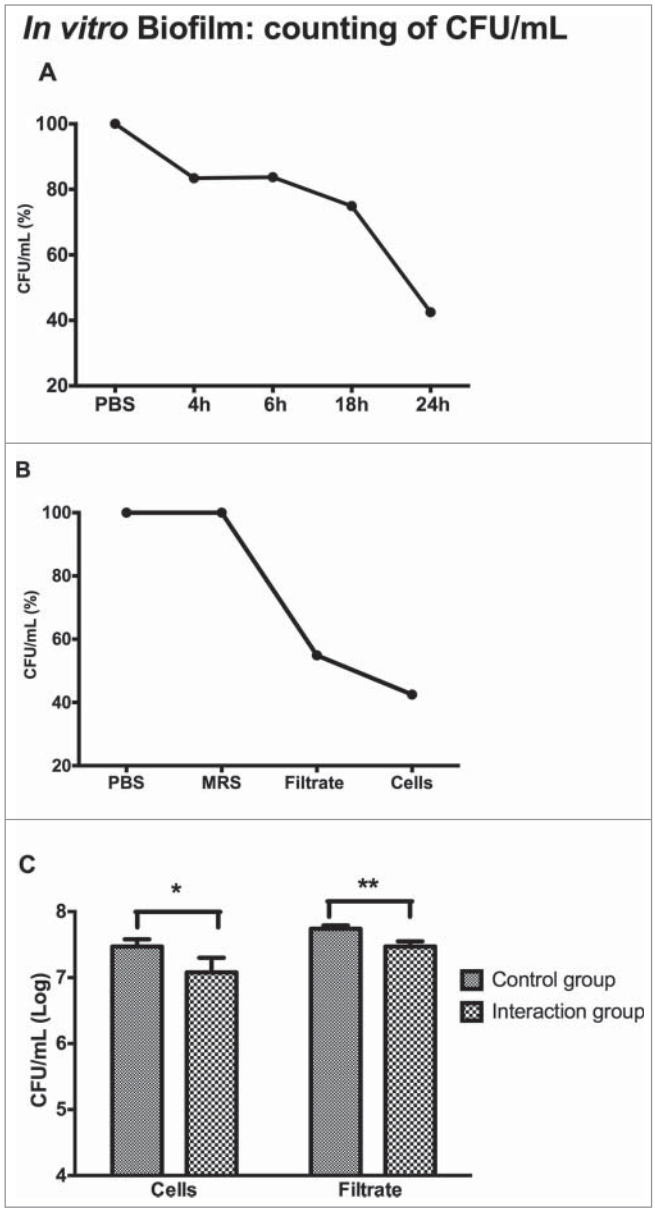

Figure 1. *Candida albicans* biofilm formed in vitro*:* (**A**) Percentage of reduction, expressed as mean values (CFU/mL), in the viability of *C. albicans* in the groups treated with *L. acidophilus* cells obtained from cultures in different phases of growth (4, 6, 18 and 24 h) in relation to the control group (PBS). (**B**) Percentage of reduction, expressed as mean values (CFU/mL), in the viability of *C. albicans* in the groups treated with *L. acidophilus* cells or culture filtrate obtained from 24-h cultures in relation to the control groups (PBS or MRS broth). (**C**) Number of *C. albicans* CFU/mL (log) in biofilms of the control group (PBS or MRS broth) and groups treated with *L. acidophilus* cells or culture filtrate. *Significant difference between the control group (PBS) and *C. albicans* + *L. acidophilus* cell group ($p = 0.0001$). **Significant difference between the control group (MRS broth) and *C. albicans* + *L. acidophilus* culture filtrate group ($p = 0.0001$). Student *t*-test, $P \leq 0.05$.

In addition to the effects of *L. acidophilus* cells on *C. albicans* biofilms, the indirect effects of *L. acidophilus* were also analyzed using only the culture filtrate of *Lactobacillus* obtained after growth for 24 h. In order to determine whether the MRS broth

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of the *L. acidophilus* culture exerts an effect on *C. albicans*, and hence interferes with the culture filtrate experiment, a control group consisting of *C. albicans* and MRS broth was included. The results showed that *L. acidophilus* culture filtrate reduced the growth of *C. albicans* cells by 45.10% (**Fig. 1B**), suggesting that *L. acidophilus* produced substances with anti-*Candida* activity.

Next, the *C. albicans* CFU/mL results obtained for biofilms treated with *L. acidophilus* cells or culture filtrate obtained from 24-h cultures were log transformed and analyzed statistically. A significant reduction in the number of CFU/mL (log) of *C. albicans* was observed for both biofilms treated with *L. acidophilus* cells and biofilms treated only with the culture filtrate (**Fig. 1C**). Therefore, a culture period of *L. acidophilus* of 24 h was adopted for all subsequent tests of this study.

Effects of *L. acidophilus* ATCC 4356 on in vitro *C. albicans* filamentation

The induction of filamentation was tested in vitro to determine whether *L. acidophilus* is able to inhibit hyphal formation by *C. albicans*. The light microscopy images showed a smaller number of hyphae in the interaction groups (*C. albicans* + *L. acidophilus* cells and *C. albicans* + *L. acidophilus* culture filtrate) when compared to the control groups (PBS and MRS broth). The light microscopy images and the results of hyphal quantification are shown in **Figures 2** and **3**, respectively.

Effects of *L. acidophilus* ATCC 4356 on experimental candidiasis: *Galleria mellonella* survival curve

Since there are no studies in the literature about inoculating *L. acidophilus* into *G. mellonella* used as an experimental model, the susceptibility of *G. mellonella* to infection with *L. acidophilus* was evaluated prior to the study of experimental candidiasis in order to determine the sub-lethal concentration for these animals.

Standard suspensions of *L. acidophilus* at 10^4 to 10^8 cells/larva concentrations were inoculated into *G. mellonella* and survival curves were constructed. The results showed that *L. acidophilus* was not pathogenic for *G. mellonella*, since none of the larvae died during the experiment at any of the concentrations tested. On the basis of these results, a concentration of 10^5 cells/larva was adopted for all subsequent assays, since it is the same concentration as that used for infection of *G. mellonella* with *C. albicans*.

Next, *G. mellonella* larvae were used to study the interaction between *C. albicans* and *L. acidophilus*. In some groups, called therapeutic, the standard suspension of *C. albicans* was first injected into *G. mellonella*, followed 1 h later by the inoculation of *L. acidophilus* cells or culture filtrate. In other groups, called prophylactic, the *L. acidophilus* cells or culture filtrate was inoculated 1 h before the inoculation of *C. albicans*.

In the control group with no infected larvae and inoculated with MRS broth was observed 100% of the survival rate indicating that the MRS broth was innocuous to *G. mellonella* larvae (data not shown). In the control groups infected with *C. albicans* and treated with PBS or MRS broth, 100% of the larvae died within 48 h. The interaction of *C. albicans* with *L. acidophilus* cells or culture filtrate significantly increased the survival of the insects, with approximately 20% of the larvae being still alive at the end of the experiment. The increase in the survival of *G. mellonella* larvae infected with *C. albicans* was observed both in the groups treated therapeutically with *L. acidophilus* and in the groups treated prophylactically with *L. acidophilus* (**Fig. 4**).

In the study of the interaction between *C. albicans* and *L. acidophilus*, although no significant difference in the survival curve of *G. mellonella* was observed between the prophylactic and therapeutic groups, the latter presented greater median survival (48 h) when compared to the prophylactic groups (36 h). Therefore, the therapeutic groups were studied in the subsequent assays.

Effects of *L. acidophilus* ATCC 4356 on experimental candidiasis: CFU/mL count of *C. albicans* in the hemolymph of *Galleria mellonella*

For quantification of the presence of *C. albicans* in infected *G. mellonella*, CFU/mL count in the hemolymph was measured at 0, 4, 8, 12, 18 and 24 h after infection. Quantification of *C. albicans* CFU/mL during the first 24 h of infection of *G. mellonella* showed a similar growth of the fungus in all experimental groups. A significant difference between groups was only observed at 24 h of infection, with higher growth of *C. albicans* in the MRS control group compared to the groups treated with *L. acidophilus* (**Fig. 5**).

In the *C. albicans* and *L. acidophilus* cells group, the number of *Lactobacillus* was also counted and similar results of CFU/mL were observed at all times tested. Statistical analysis revealed no significant difference between the times (**Fig. 6**).

Effects of *L. acidophilus* ATCC 4356 on experimental candidiasis: *C. albicans* filamentation in *Galleria mellonella* tissues

Microscopic analysis was used to evaluate the effects of *L. acidophilus* on *C. albicans* filamentation in *G. mellonella.* Prior to this analysis, the histomorphological structures found in the perivisceral portion of the fat body of uninfected *G. mellonella* larvae were analyzed. The fat body of the larva basically consists of 2 types of cells: trophocytes (or adipocytes) and oenocytes. In the present study, the trophocytes had a globular appearance and nucleus was present in the center of the cells. Oenocytes were found in smaller numbers scattered throughout the fat body and contained a round or slightly elliptical nucleus (**Fig. 7**).

Next, the effects of *L. acidophilus* on experimental candidiasis were studied. In all groups, the hyphae and yeast cells were not uniformly spread throughout the fat body, but rather formed clusters in certain areas of the histological sections. These clusters of hyphae and yeast cells were located close to the organs or scattered throughout the adipose tissue (**Fig. 8**).

For analysis of tissue infection with *C. albicans*, all areas containing hyphae and yeast cells were photographed at 1,000x magnification and the area occupied by these structures (in μm) was measured in each image. The areas of each section were summed and the results were analyzed statistically. The mean size of the areas occupied by yeast cells/hyphae was smaller in the groups treated with *L. acidophilus* (cells or culture filtrate) when compared to the control groups (PBS or MRS broth), but no

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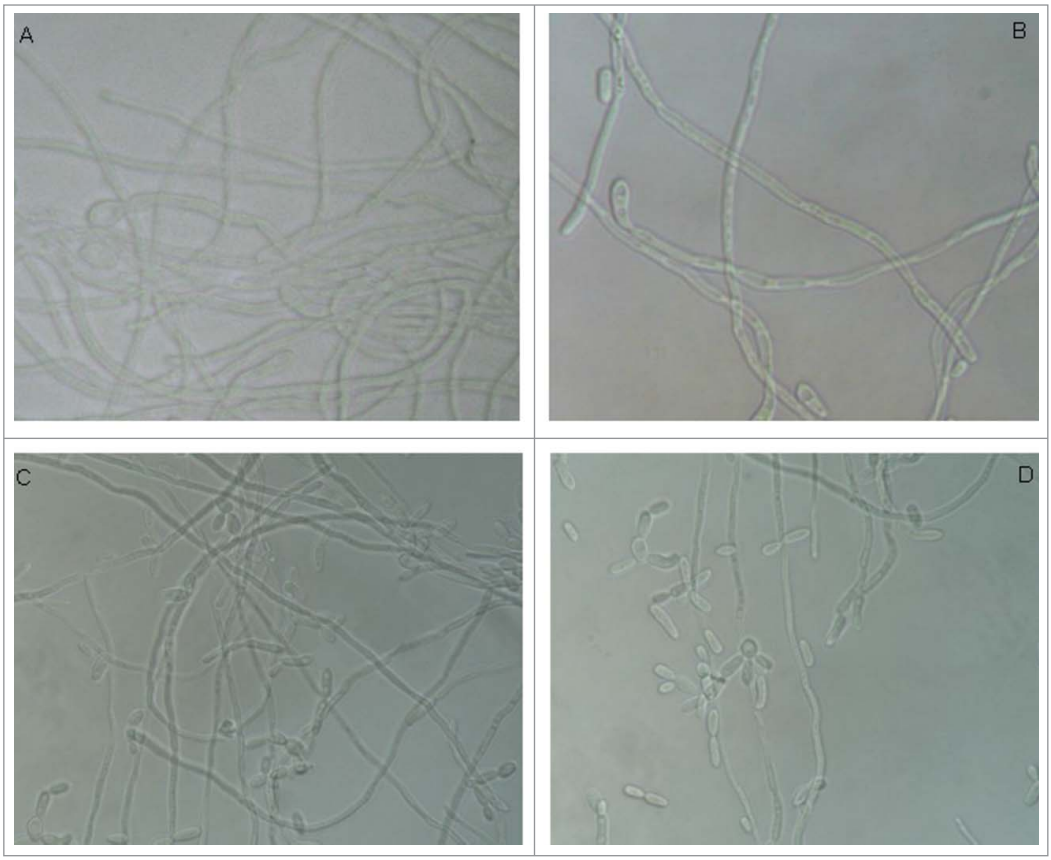

Figure 2. Light microscopy photomicrographs of in vitro *Candida albicans* filamentation. (**A**) Control group (PBS): intense formation of hyphae. (**B**) *C. albicans* + *L. acidophilus* cells group: presence of some hyphae. (**C**) Control group (MRS broth): intense formation of hyphae. (**D**) *C. albicans* + *L. acidophilus* culture filtrate group: note the presence of few hyphae. Original magnification: 400×.

significant differences were observed between the 4 groups studied (**Fig. 9**).

Discussion

Some strains of *Lactobacillus* are cited in the literature as microorganisms that confer probiotic effects when incorporated into fermented food products, such as *L. acidophilus* ATCC 4356, *L. casei* ATCC 393 and *L. paracasei* subsp. paracasei ATCC BAA52.[25-26] The prevention of biofilm formation by such natural lactobacilli-derived agents is one possible therapy currently tested.[27] The present study showed a reduction of *C. albicans* CFU/mL counts in in vitro polymicrobial biofilms with *L. acidophilus* compared to the control group at all times tested; however, this difference was only significant for the 24-h culture of *L. acidophilus* (reduction of 57.5%). Similar results have been reported by Smith et al.[12] who showed a reduction of *C. albicans* cell counts in biofilms during application of a synbiotic containing *L. acidophilus*.

Interaction of the *L. acidophilus* culture filtrate with the biofilm formed by *C. albicans* also led to a significant reduction in the CFU/mL count of this fungus. This finding demonstrates a competitive relationship between the 2 species determined by the presence of substances that render the medium hostile for the development of the fungus. In certain interactions, molecules secreted by bacteria into the external medium can inhibit the growth of a second species as demonstrated in different studies.[27-29] Walencka et al.[27] investigated the effect of a substance obtained from the culture filtrate of *L. acidophilus* on *S. aureus* and *S. epidermidis* biofilms. Confocal microscopy revealed that this substance resulted in the formation of biofilms that covered a smaller surface area, with a reduction in biofilm volume and total thickness. Pascual et al.[30] showed that a bacteriocin present in the culture filtrate of *L. fermentum* L23 was responsible for a broad spectrum of inhibition when inoculated together with Gram-negative and Gram-positive pathogenic bacteria and *Candida* species.

In the present study, analysis of in vitro filamentation showed that the interaction between *C. albicans* and *L. acidophilus* reduced the number of hyphae when compared to the control group inoculated only with *C. albicans*. The formation of hyphae seems to be a critical step in the development of *C. albicans* biofilms since studies have shown that mutations in the genes related to hyphal formation cause severe defects in biofilm formation in vitro.[21,31,32] Metabolites released by *Lactobacillus* species, such as sodium butyrate, have been shown to inhibit biofilm formation, to potentiate the effect of antifungal agents, and to suppress *C. albicans* filamentation, reducing fungal pathogenicity.[33] Studying the interactions between *C. albicans* and *P. aeruginosa*, Morales et al.[34] demonstrated that phenazines produced by *P. aeruginosa* can modulate the metabolism of *C. albicans*. The presence of low concentrations of these substances permitted the growth of *C. albicans*, but affected biofilm formation and inhibited the transition from the yeast to the hyphal form. Noverr and Huffnagle[10] investigated the effect of live cultures, culture supernatants and dead cultures of probiotic bacteria on the morphogenesis of *C. albicans*. The authors observed that supernatants obtained from 2-h cultures of these bacteria inhibited germ tube formation in *C. albicans* and the addition of 24-h cultures completely inhibited germination, suggesting that the accumulation of a soluble

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compound in the culture supernatant is responsible for this inhibition.

Another factor that plays a role in filamentation is the pH of the medium. In the case of *C. albicans*, pH serves as a strong signal for morphological differentiation. Acid conditions favor the growth of the yeast form, whereas alkaline conditions favor the growth of hyphae.[35] In this respect, the production of lactic acid and other organic acids by *Lactobacillus* can substantially decrease the pH, which plays an important role in fungal growth.[36] Köhler et al.[36] investigated the potential of *L. rhamnosus* and *L. reuteri* to control *C. albicans*. The reduction in cell viability was greater when *C. albicans* was incubated with the *Lactobacillus* culture filtrate in MRS broth at pH 4.5. Furthermore, the incubation of *C. albicans* with MRS broth alone did not reduce cell viability.

Analysis of the virulence of *L. acidophilus* for *G. mellonella* larvae showed that the bacterium did not cause the death of the animals tested, thus demonstrating its lack of pathogenicity in this experimental model. Studies in the literature have only used strains of *Lactococcus lactis*, which is used as a control since it does not cause infection in this experimental model.[37-38] Similarly, *L. acidophilus* is not pathogenic for *Caenorhabditis elegans*, another invertebrate model, with the bacterium being unable to colonize the intestine of this species. Furthermore, inoculation of *L. acidophilus* significantly reduced infection with *Enterococcus faecalis* and prolonged survival of the nematode when exposed to strains of *E. faecalis* and *S. aureus*.[39]

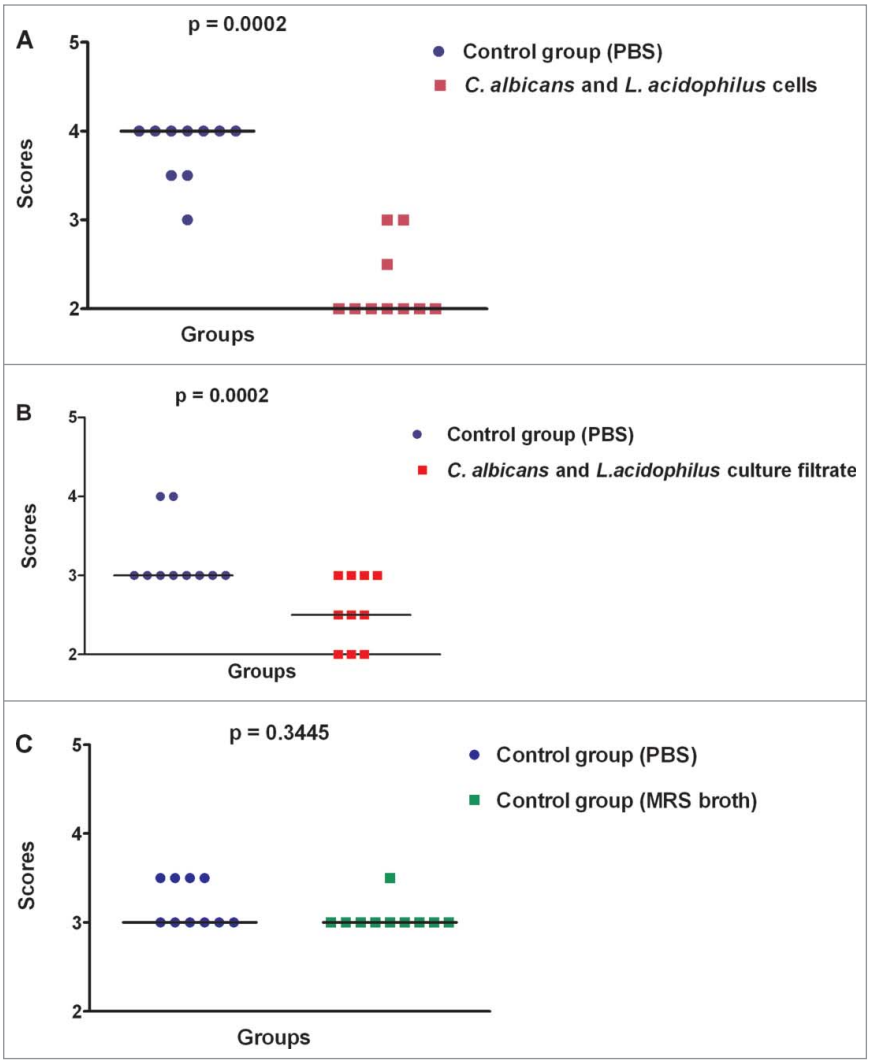

Figure 3. Quantification of hyphae in the in vitro *C. albicans* filamentation assays. Scores were attributed to the number of *C. albicans* hyphae formed in each group. (**A**) Comparison between the control group (PBS) and *C. albicans* + *L. acidophilus* cells group. (**B**) Comparison between the control group (PBS) and *C. albicans* + *L. acidophilus* culture filtrate group. (**C**) Comparison between the PBS and MRS control groups. Mann-Whitney test, $p \leq 0.05$.

In the in vivo study, experiments were conducted to determine whether the presence of *L. acidophilus* injected prophylactically or therapeutically into *G. mellonella* has an effect on systemic infection with *C. albicans*. We showed that both prophylactic and therapeutic inoculation significantly increased the survival of the infected larvae. These results were obtained for interactions with cells and with the culture filtrate of *L. acidophilus*, demonstrating that the metabolites produced by the bacterium also exert an effect on *C. albicans* virulence in vivo.

Similar results have been reported by Matsubara et al.[40] who evaluated oral colonization with *C. albicans* in immunocompromised mice. *Candida* counts were significantly reduced in the groups treated with probiotic bacteria (*L. acidophilus* and *L. rhamnosus*) compared to untreated animals. Furthermore, treatment with the probiotic resulted in greater reduction of *C. albicans* than treatment with nystatin. McCann et al.[41] treated *G. mellonella* larvae infected with *C. albicans* with ketoconazole antifungal modified by silver nanoparticles which were administered prophylactically or therapeutically. Similar to the present study, survival of the larvae increased in the presence of the compound with survival rates of up to 90% after 72 h in prophylactic treatment and of 75% in therapeutic treatment after 1 h of infection with *C. albicans*.

Studies have demonstrated the involvement of probiotic bacteria in the regulation of the host immune system.[39] Immune system of insects basically comprises 2 types of immune responses, a humoral and a cellular response. The immune responses are triggered through the recognition of microorganisms by specific host cell receptors. These immune reactions have been shown to be similar to those that occur in vertebrates, and insect models are therefore useful for the study of fungal and bacterial virulence.[42]

The results of the in vivo study of *C. albicans* cell counts (CFU/mL) in the hemolymph of *G. mellonella* showed the occurrence of an immune response of the larva after infection with the

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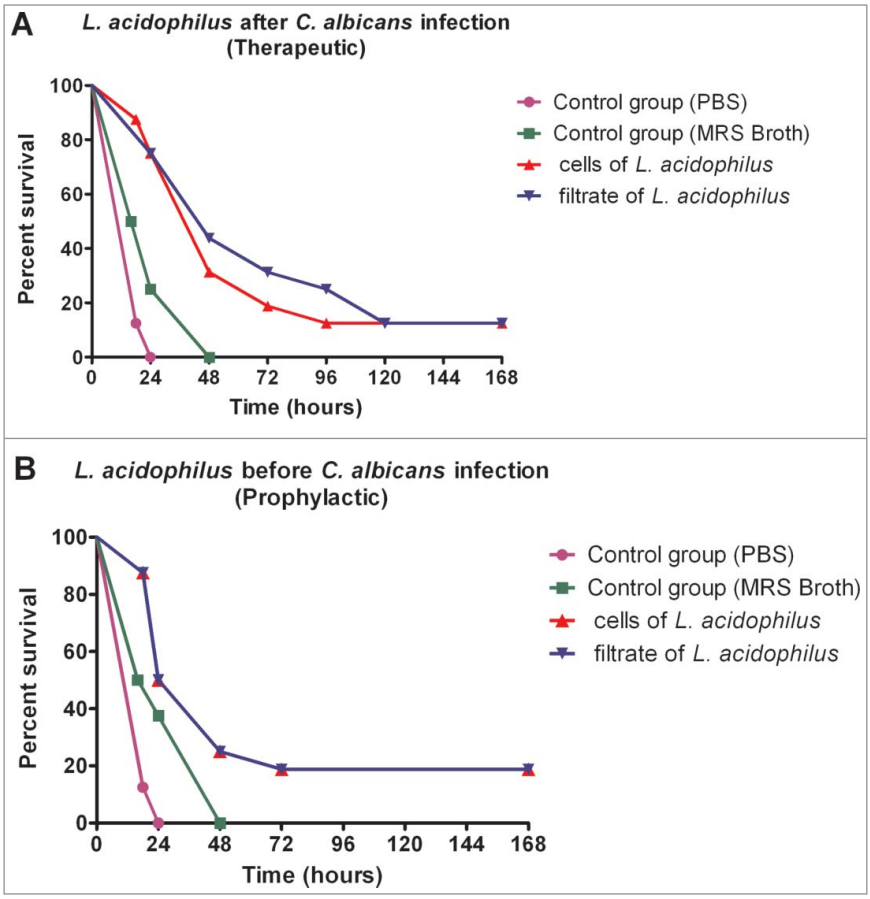

Figure 4. Effects of *L. acidophilus* on experimental candidiasis based on the analysis of survival curves of *G. mellonella* larvae (**A**) Therapeutic groups: significant differences were observed between the *C. albicans* + *L. acidophilus* cells group and PBS control group (p=0.0001) and between the *C. albicans* + *L. acidophilus* culture filtrate group and MRS control group (p=0.0002) (**B**) Prophylactic groups: significant differences were observed between the *C. albicans* + *L. acidophilus* cells group and PBS control group (p=0.0001) and between the *C. albicans* + *L. acidophilus* culture filtrate group and MRS control group (p=0.0490) Log-rank test, $p \leq 0.05$.

fungus. The number of microorganisms recovered from the larval hemolymph immediately after inoculation (time 0) did not differ from the number used at the beginning of infection. However, a reduction in the number of recovered cells was noted after 8 h. After this period, the larval immune system was probably no longer able to combat the infection and the number of *C. albicans* cells gradually increased, with the highest count being observed after 24 h. The same trend of microbial growth was seen in the interaction and control groups. However, growth of *C. albicans* was significantly lower in the interaction groups after 24 h when compared to control. These findings suggest that *L. acidophilus* renders the larva more resistant to infection when compared to the control group. Another interesting observation was the constant presence of bacteria throughout the period analyzed, demonstrating again that *L. acidophilus* is not pathogenic in this experimental model and that the bacterium was interacting with *C. albicans* in vivo.

Evans and Rozen[43] evaluated the growth of *Streptococcus pneumoniae* strains that differed in virulence in the *G. mellonella* model after 0, 0.5, 1, 4 and 24 h of infection. As observed in the present experiment, the number of bacteria recovered from the larval hemolymph during the first hours did not differ from the number used at the beginning of infection. However, the mean number of cells of the most virulent strain increased after 4 h, whereas the bacterial count of the least virulent strain decreased significantly within the first 30 min of infection. A small number of bacteria were observed in some larvae after 4 h. These results suggest that high mortality may be associated with the proliferation of bacteria inside the host as a result of a deficient immune response.

In relation to the study of *C. albicans* filamentation in *G. mellonella* tissues, the results from microscopic analysis showed that the areas occupied by hyphae were smaller in the groups treated with *L. acidophilus* (cells or culture filtrate) when compared to the control groups (PBS or MRS broth), however, no significant differences were observed between the groups. Probably, the lack of a significant reduction in the *C. albicans* filamentation can be attributed to the selected time for observation (18 h after infection) and not 24 h as in the previous experiments. The time of observation for microscopic analysis was limited to 18 h because we wanted to remove the body fat with the larvae still alive and the results from survival curve assays showed death of larvae 24 h post infection in the control group (PBS).

The interactions between bacteria and fungi are highly complex and a series of factors need to be analyzed in conjunction, such as the virulence of microorganisms associated with environmental factors and the interactions between different species present in a given niche. The development of appropriate in vitro and in vivo models is necessary to characterize these interactions. In this study, we verified that *G. mellonella* is an adequate model for the study of the interaction between *C. albicans* and *L. acidophilus*. Here, we conducted a large number of in vitro and in vivo experiments in order to determine the conditions of *Lactobacillus* inoculation (growth phase, sub-lethal concentration, cells and culture filtrate, and prophylactic or therapeutic) required to achieve a significant reduction of *C. albicans* and experimental candidiasis. Since this model is established, future studies can be conducted for evaluating other strains and dosages of *Lactobacillus*, as well as the molecular changes and dynamics of the immune response in this interaction to better understand human disease and to develop new therapies for candidiasis.

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In summary, in the present study both cells and culture supernatant of *L. acidophilus* ATCC 4356 were able to inhibit biofilm formation and filamentation by *C. albicans* in vitro, with 24-h cultures of *L. acidophilus* showing the greatest inhibitory effect on *C. albicans* biofilm formation. The prophylactic or therapeutic inoculation of *L. acidophilus* into *G. mellonella* infected with *C. albicans* reduced the number of yeast cells in the larval hemolymph and increased the survival of these animals.

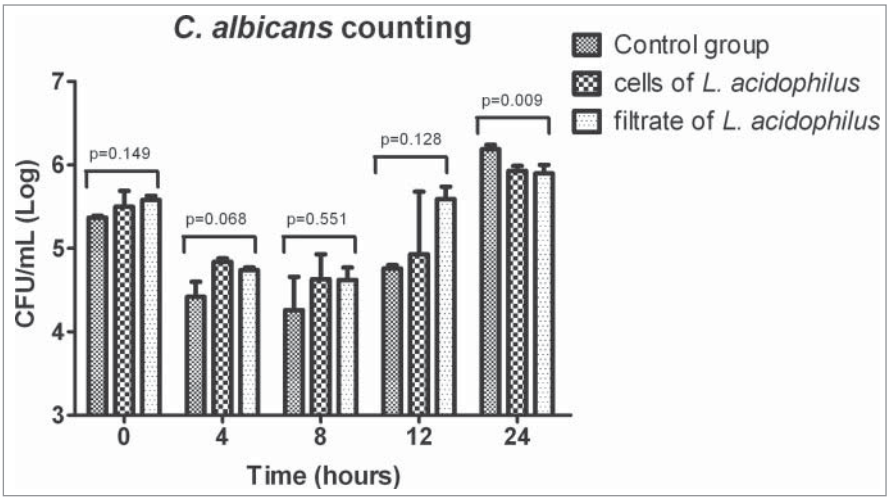

Figure 5. Mean and standard deviation of *C. albicans* counts (CFU/mL) in the hemolymph of *Galleria mellonella* immediately after inoculation and after 4, 8, 12 and 24 h of experimental infection. The following groups were compared at each time of infection: control group (MRS broth), *C. albicans* + *L. acidophilus* cells group, and *C. albicans* + *L. acidophilus* culture filtrate group. A significant difference between groups was only observed after 24 h of infection (ANOVA, $P \leq 0.05$), with a larger number of CFU/mL in the control group compared to the *C. albicans* + *L. acidophilus* cells ($p = 0.0205$) and *C. albicans* + *L. acidophilus* culture filtrate groups ($p = 0.0135$). No significant difference was observed between the last 2 groups ($p = 0.9251$). Tukey test, $P \leq 0.05$.

Material and Methods

Interaction between *L. acidophilus* ATCC 4356 and *C. albicans* ATCC 18804 - in vitro assays

In the in vitro study, the direct and indirect effects of *Lactobacillus* on biofilm formation by *C. albicans* and its filamentation capacity were evaluated.

First, the direct effect was evaluated during different phases of growth of the bacterial culture (4, 6, 18 and 24 h). Ten assays were performed per experimental group and repeated twice on different occasions.

Reference strains of *Candida albicans* ATCC 18804 and *Lactobacillus acidophilus* ATCC 4356 were used in the experiments. *C. albicans* was cultured for 18 h at 37°C in yeast nitrogen base broth (YNB; Difco, Detroit, USA) supplemented with 100 mM glucose. *L. acidophilus* was grown in Lactobacillus MRS Broth (Himedia, Mumbai, India) for 4, 6, 18 and 24 h at 37°C in a bacteriological incubator under microaerophilic conditions. The number of cells in suspension was determined with a spectrophotometer (B582, Micronal, São Paulo, Brazil) at a concentration of 10^7 cells/mL. The optical density and wavelength used were 0.381 and 530 nm for *C. albicans*, respectively, and 0.296 and 600 nm for *L. acidophilus*. Cells densities of the inoculum were confirmed by CFU/mL counting after plating in Sabouraud dextrose agar for *C. albicans* and Rogosa agar for *L. acidophilus*.

For preparation of the *L. acidophilus* culture filtrate, 1 mL of the standard suspension was transferred to a Falcon tube containing 6 mL MRS broth and incubated for 24 h at 37°C in a bacteriological incubator under microaerophilic conditions. After this period, the broth was centrifuged (2000 x *g* for 10 min) and filtered through a membrane with a 0.22-μm pore size (MFS, Dublin, USA).

Biofilm Formation

The method described by Thein et al.,[44] with some modifications, was used for the study of biofilm formation. Briefly, 100 μL aliquot of the standard *C. albicans* suspension was inoculated into each well of a 96-well flat-bottom microtiter plate (Costar Corning, New York, USA) and the plate was incubated for 90 min at 37°C under shaking at 75 rpm. Each well was then washed twice with PBS, 100 μL fetal bovine serum was added, and the plate was again incubated for 2 h. The plates

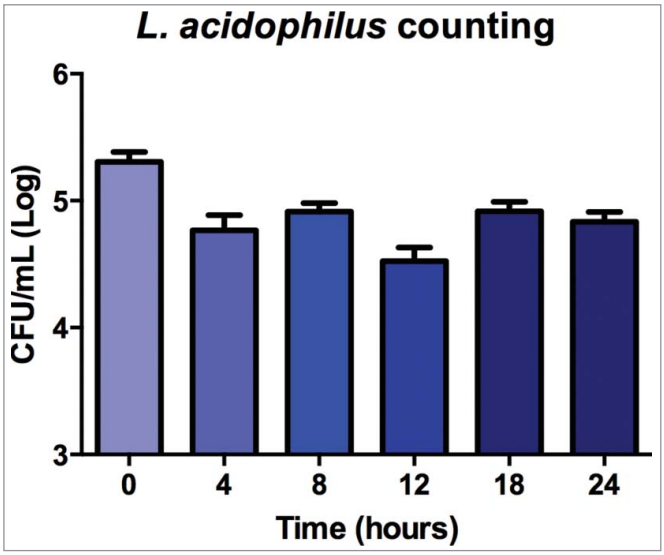

Figure 6. Mean and standard deviation of *L. acidophilus* counts (CFU/mL) in the hemolymph of *G. mellonella* at times 0, 4, 8, 12, 18 and 24 h for the group formed by interaction of *C. albicans* + *L. acidophilus* cells. No significant difference between the times was observed ($p = 0.840$). ANOVA, $P \leq 0.05$).

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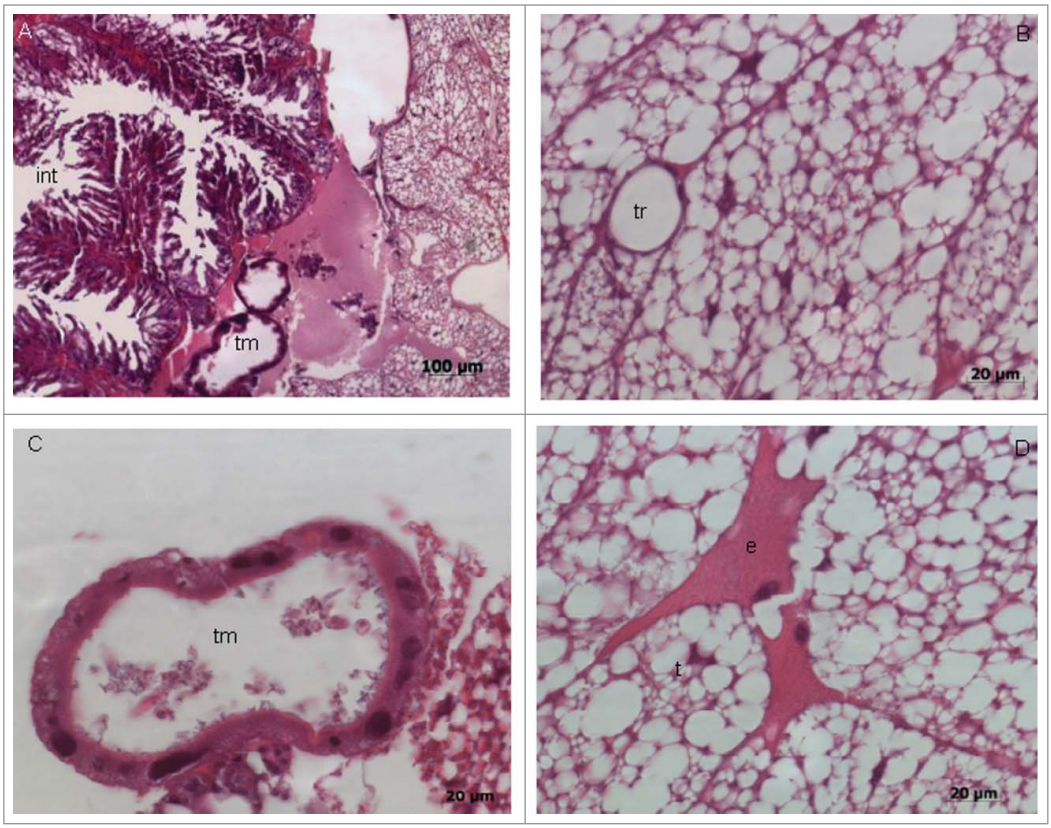

Figure 7. Photomicrographs of a histologically normal fat body of *Galleria mellonella* larvae not infected with the microorganisms. (**A**) Note the presence of Malpighi tubules (tm), part of the intestine (int), and trophocytes (t). HE; original magnification: 100x. (**B**) Presence of the trachea (TR) surrounded by trophocytes of the fat body. HE; original magnification: 630×. (**C**) Malpighi tubule (tm) responsible for the removal of excreta from the hemolymph. HE; original magnification: 630×. (**D**) Presence of trophocytes (t) with irregular nuclei and oenocytes (e). HE; original magnification: 630×.

were then washed twice and 50 µL of the standard suspension or the *L. acidophilus* culture filtrate was added. In the control group, 50 µL PBS was added. For promotion of biofilm growth, 70 µL YNB supplemented with 100 mM glucose and 30 µL BHI broth were added to each well and the plate was incubated for 48 h at 37°C under shaking at 75 rpm. The broth was partially changed at intervals of 24 h. After this period, the wells were washed and the biofilm adhered to the bottom of the plate was scraped off with a sterile wooden toothpick. One hundred µL of this inoculum was transferred to a Falcon tube containing 6 mL PBS and homogenized for 30 s. Next, decimal dilutions were prepared and seeded onto Petri dishes containing selective culture media. The plates were incubated for 48 h at 37°C. After this period, the number of colonies was counted for the calculation of colony-forming units per mL (CFU/mL). The study was supported by 2 experiments at different times with 10 biofilms per group.

Study of *L. acidophilus* effects of on *C. albicans* filamentation

The following groups were formed for the study of in vitro filamentation: PBS control group, MRS control group, *C. albicans* + *L. acidophilus* cell group, and *C. albicans* + *L. acidophilus* culture filtrate group. One mL distilled water supplemented with 10% fetal bovine serum and 100 µL of the standard *C. albicans* suspension were added to the wells of a 24-well cell culture plate (Costar Corning, New York, USA). Next, 50 µL of the standard suspension or the *L. acidophilus* culture filtrate was inoculated into each well. In the control groups, 50 µL PBS or MRS broth was used. The plates were incubated for 24 h at 37°C under microaerophilic conditions.

After this period, 50 µL of the inoculum was spread on glass slides and observed under a light microscope at 400x magnification. The images were analyzed regarding morphological features and quantification of the number of hyphae. For the latter, 10 microscopic fields were analyzed per slide and a score (0 to 4) was attributed to each field according to the number of hyphae present: score 0: no hyphae; score 1: 1 to 3 hyphae; score 2: 4 to 10 hyphae; score 3: 11 to 20 hyphae; and score 4: more than 20 hyphae.

Interaction between *L. acidophilus* ATCC 4356 and *C. albicans* ATCC 18804 - in vivo model

For this study, the methodology described by Cowen et al.[45] was used with some modifications.

G. mellonella (Embrapa Gado de Leite, Juiz de Fora, MG 36038-330, Brazil) in the final larval stage were stored in the dark and used within 7 days from shipment. Sixteen randomly chosen *G. mellonella* larvae with similar weight and size (250-350mg) were used in each group in all assays. Two control groups with no infected larvae were included for all assays: one group was inoculated with PBS to observe physical trauma, and the other received no injection as a control for general viability.

Before the study of the interaction between *C. albicans* and *L. acidophilus*, the susceptibility of *G. mellonella* to infection with *L. acidophilus* was tested to determine the sub-lethal concentration to be administered to these animals. For this purpose, 5 µL of different concentrations (10^4 to 10^8 cells/mL) of the standard *L. acidophilus* suspension was inoculated into *G. mellonella*. A group of 16 larvae was used per concentration.

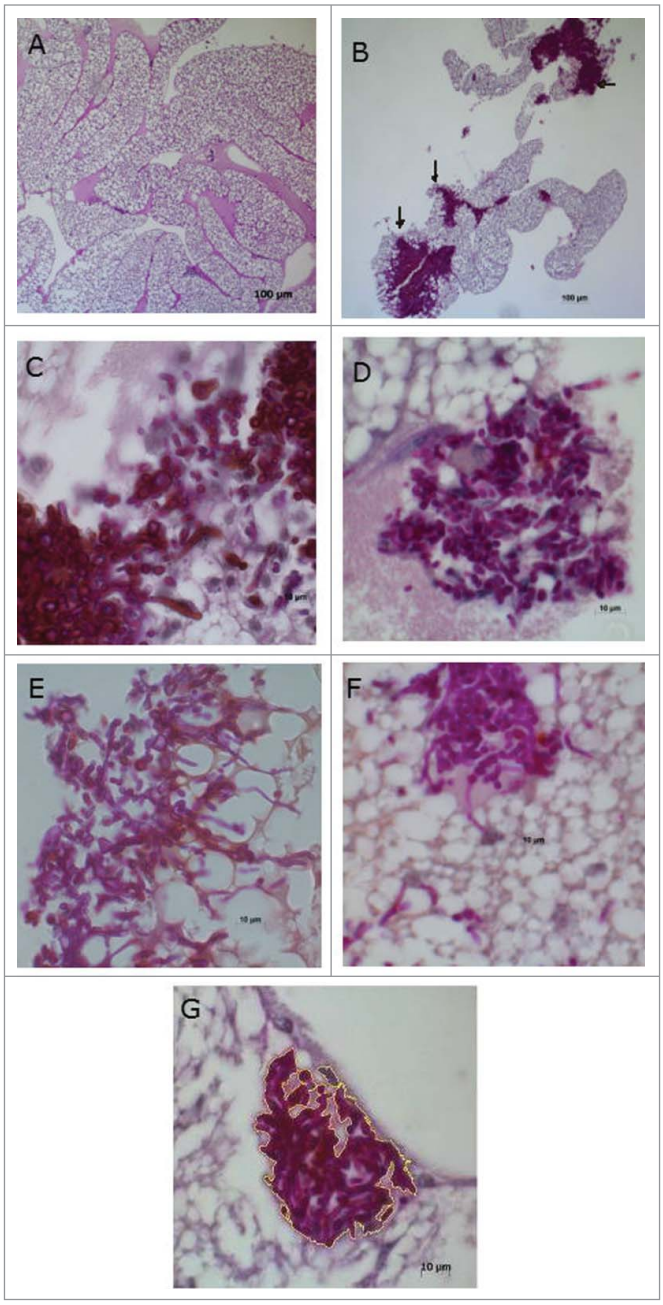

Figure 8. Histological sections of the fat body of *Galleria mellonella*. (**A**) Normal appearance of the fat body of *G. mellonella* not infected with *Candida albicans*. (**B**) *C. albicans* and PBS control group: observe the presence of clusters of hyphae and yeast cells (arrow). PAS; original magnification: 100×. (**C**) *C. albicans* and MRS control group. (**D**) *C. albicans* + *L. acidophilus* culture filtrate group. (**E**) *C. albicans* and PBS control group. (**F**) *C. albicans* + *L. acidophilus* cells group. (**G**) *C. albicans* + *L. acidophilus* cells group: with demarcation taken by Image J program for obtaining occupied by hyphae and yeasts area. PAS; original magnification: 1000×.

G. mellonella survival assay

The cell densities of *C. albicans* were adjusted to 10^5 cells/mL with a hemocytometer. The standard *L. acidophilus* suspension (10^5 cells/mL) was obtained with a spectrophotometer as described above.

An inoculum of 5 µL of the standard *C. albicans* suspension was injected into the hemolymph of each larva through the last left proleg and 5 µL of the standard cell suspension or the *L. acidophilus* culture filtrate was inoculated into the last right proleg. For the groups infected with only one microorganism, 5 µL of the microbial suspension was inoculated into the last left proleg and the same volume of PBS or MRS broth into the last right proleg. In order to verify the toxicity of MRS broth for *G. mellonella* larvae, a control group with no infected larvae and inoculated with MRS broth was included in this study.

Sixteen larvae were used per experimental group. The interaction groups were subdivided into prophylactic and therapeutic. In the prophylactic group, the *L. acidophilus* cells or supernatant were injected 1 h before the inoculation of *C. albicans* cells, whereas in the therapeutic group *C. albicans* cells were inoculated 1 h before the inoculation of *L. acidophilus* cells or supernatant. The larvae were kept on Petri dishes and incubated at 37°C in a bacteriological oven. The number of dead larvae was recorded daily for 7 days.

Colony-Forming Unit Count

For quantification of the presence of *C. albicans* in infected *G. mellonella*, the larvae were euthanized 0, 4, 8, 12, 18 and 24 h after infection in the following groups: MRS control group, *C. albicans* + *L. acidophilus* cell group, and *C. albicans* + *L. acidophilus* culture filtrate group. A pool of 6 larvae was used per group and time. The experiment was carried out in triplicate using 18 larvae per group, for a total of 324 infected larvae. A control group was included for each time point, which was injected with 5 µL PBS into the last right and left proleg.

At each time point, the larvae were cut in the cephalocaudal direction with a scalpel blade and squeezed to remove the hemolymph, which was transferred to an Eppendorf tube. Serial dilutions were prepared from the hemolymph pool, seeded onto Petri dishes containing Sabouraud dextrose agar (Difco, Detroit, USA) supplemented with chloramphenicol (100 µg/mL), and incubated for 48 h at 37°C. In the *C. albicans* + *L. acidophilus* cell group, dilutions were also seeded on Rogosa agar (Himedia, Mumbai, India) and incubated under microaerophilic conditions (48 h at 37°C) to count the number of *Lactobacillus* in the hemolymph of larvae. After this period, the colonies were counted for the calculation of CFU/mL.

G. mellonella histological analysis

Histology was used to analyze histological structures present in the fat body of the larva and to evaluate the effects of *L. acidophilus* on *C. albicans* filamentation in *G. mellonella*. The following groups were formed: PBS control group, MRS control group, *C. albicans* + *L. acidophilus* cell group, *C. albicans* + *L. acidophilus* culture filtrate group, and control group not inoculated with any of the microorganisms. Five larvae were used per group.

Eighteen hours after infection, an incision was made in the midline of the ventral part of the animal, the hemolymph was

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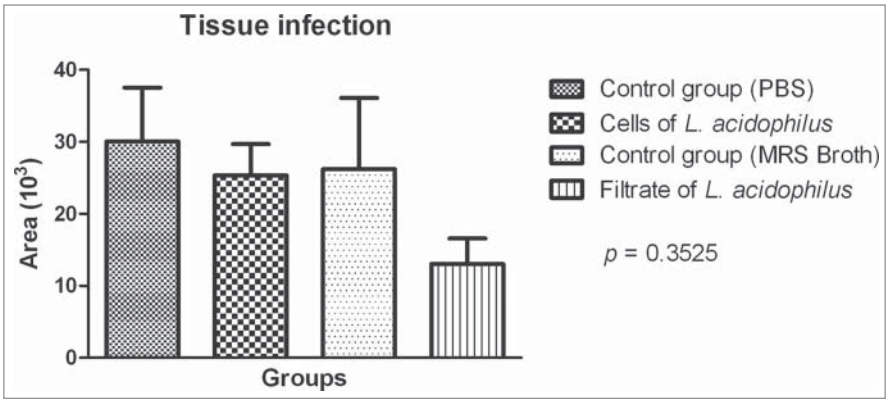

Figure 9. Mean and standard deviation of the area occupied by *Candida albicans* yeast cells and hyphae in histological sections of *Galleria mellonella* with experimental candidiasis. No significant difference was observed between groups: control group (PBS), *C. albicans* + *L. acidophilus* cells group, control group (MRS broth), and *C. albicans* + *L. acidophilus* culture filtrate group. ANOVA, $P \leq 0.05$.

discarded, and the fat body was removed. The fat body was placed in 10% formalin and stored for 24 h at 4°C. The tissue was then immersed in different alcohol concentrations (50, 70 and 90% for 1 h and 100% for 3 h), incubated in xylene for 3 h, and mounted in paraffin blocks.

The blocks were cut and the sections were stained with hematoxylin-eosin (HE) and periodic acid Schiff (PAS). Hyphae and yeast cells were visualized under a light microscope at 100, 630 and 1,000x magnification. For analysis of filamentation, all areas of the histological section that contained hyphae and yeast cells were photographed at an original magnification of 1,000× with a Cyber Shot DSC-585 digital camera (Sony Corporation) coupled to a Zeiss Axiophot 2 light microscope (Carl Zeiss, Oberkochen, Germany). The area occupied by hyphae and yeast cells (in μm) was determined for each image using the ImageJ program (version 1.32 for Windows), a public domain image processing program developed at the National Institutes of Health (NIH), Bethesda, USA. All areas of hyphae and yeast cells in each section were summed and the result was $\log_{10}$ transformed.

Statistical analysis

The CFU/mL results of in vitro biofilm formation were analyzed by the Student *t*-test. Analysis of variance and the Tukey test were used for the analysis of CFU/mL recovered from *G. mellonella* and microscopic analysis of the presence of hyphae. The Mann-Whitney test was applied to compare the scores obtained in the analysis of in vitro filamentation. For survival analysis of *G. mellonella*, survival curves were constructed and differences were estimated by the log-rank method (Mantel-Cox test) using the GraphPad Prism program. A level of significance of 5% was adopted for all tests.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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