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Immunomodulatory effects and anti-*Candida* activity of lactobacilli in macrophages and in invertebrate model of *Galleria mellonella*

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ABSTRACT

Due to the growing number of multi-resistant *Candida* spp., adjuvant treatments that may help combat these fungal pathogens are relevant and useful. This study evaluated the immunomodulation and anti-*Candida* activity of *Lactobacillus rhamnosus* (LR), *Lactobacillus acidophilus* and *Lactobacillus paracasei* suspensions, either single- or multiple-strain, in mouse macrophages (RAW 264.7) and *Galleria mellonella* (GM). Mouse macrophages were activated by different lactobacilli suspensions and challenged with *C. albicans* (CA). Tumor necrosis factor (TNF)-α, interleukin IL-1β, IL-6 and IL-17 production and cell viability were investigated. LR was the best suspension for stimulating all evaluated cytokines and thus was used in subsequent *in vivo* assays. Two *C. albicans* clinical strains, CA21 and CA60, were then added to the GM assays to further confirm the results. LR suspension was injected into the larvae 24 h before challenging with CA. Survival curve, CFU per larva and hemocytes were counted. In the GM, the LR suspension increased the survival rate and hemocyte counts and decreased the CFU per larva counts for all groups. Lactobacilli suspensions presented strain-dependent immunomodulation; however, single suspensions showed better results. Anti-*Candida* activity was demonstrated by decreased *Candida* counts in the GM with the use of LR.

1. Introduction

Candida yeasts are the primary agents of fungal infection in patients who are immunosuppressed from the use of immunosuppressive drugs, advanced age and systemic diseases [1–3]. Over 90% of all opportunistic mycoses are caused by *Candida* species [4–7]. Since fungi invade tissues, they are subjected to opsonization in which phagocytes are activated by receptors and intracellular signals for phagocytosis [8]. These include the standard receptors for host cell recognition, such as C-type lectin receptors and Toll-like receptors [9,10]. In healthy, immunocompetent individuals, the innate immune system (neutrophils and macrophages) provides protection against fungal species through phagocytosis and cytokines such as interleukin (IL)-1β, IL-6, IL-17 and tumor necrosis factor (TNF)-α, which are important in targeting immune responses to fungi [11,12].

T-lymphocytes (T-cells) are integral to the host's adaptive immune response to *C. albicans* infection and provide direct and indirect means of controlling fungal proliferation. Th17 cells release cytokines, including IL-17 and IL-22, and are critical for immune protection against *C. albicans* in most mucosal sites. In addition, Th17 cells are regarded as the predominant cells for protecting against *C. albicans* in oral and dermal candidiasis [13,14]. Th17 differentiation is influenced by IL-6, which is produced by epithelial cells in response to *C. albicans* infections [15]. Notably, the production of both IL-6 and IL-23 by antigen presenting cells results from recognition of mannan in *C. albicans* [14].

The frequency of invasive fungal infections and resistance to antifungal therapy continue to increase despite the introduction of

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new antifungal agents; therefore, the emergence of resistant fungal strains is a growing concern [16]. Due to the need for alternative anti-fungal methods, probiotics offer a potential resolution. Probiotics are involved in modulating the immune response by increasing nonspecific phagocytic activity through macrophage activation [17,18], altering the release of pro- and anti-inflammatory cytokines [19,20], competing with pathogens for nutrients and receptors, preventing the harmful effects of pathogens and stimulating the release of bacteriocins, hydrogen peroxide and lactase [21,22]. Moreover, probiotic suspensions can be constituted by a single species or by the association of two or more species [19–21], and their beneficial effects are related to the strain and dose [22].

In vitro and *in vivo* studies are necessary to better understand the interaction among hosts, pathogens and probiotics. *In vitro* studies are used to elucidate the role of probiotics [19,23–25], and they represent an important step in this field. For *in vivo* studies, invertebrate models are used [26,27] to investigate fungal pathogenicity and the role of probiotics. An advantage of this model is that it meets the ethical and legal requirements of the 3 Rs (reduction, refinement and replacement) in addition to being an alternative to mammalian use [28,29].

Although there are many probiotic studies in the literature, it is necessary to examine the effects of different probiotic suspensions, isolated or combined, on the immune response to fungal infections. Different strains can induce different responses, and their interactions may influence the immune response and, consequently, the type of immunomodulation provided. This study evaluated the production of TNF-α, IL-1β, IL-6 and IL-17 in macrophages activated by different lactobacilli suspensions and challenged by *C. albicans* to verify the immunomodulatory role of probiotic bacteria on a *G. mellonella* model.

2. Materials and methods

2.1. Macrophage cell culture

RAW 264.7 macrophages were obtained from the cell bank of the Paul Ehrlich Technical Scientific Association (APABCAM, Rio de Janeiro, Rio de Janeiro). These cells were grown in tissue culture flasks with DMEM culture medium (Dulbecco's Modified Eagle - Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (complete medium) and incubated at 37 °C at an atmospheric humidity of 5% CO_2.

2.2. Microorganisms

Initially, *C. albicans* (CA) ATCC 18804 and 3 species of *Lactobacillus*: *L. rhamnosus* (LR) ATCC 7469, *L. acidophilus* (LA) LA 14 and *L. paracasei* (LP) 19.1 were used in this study. All fungal strains and LR were from the Laboratory of Microbiology and Immunology of the Institute of Science and Technology of São José dos Campos/UNESP (São Paulo State University, São José dos Campos, São Paulo, Brazil). LA was isolated from a commercial product (Prolive-Ache®, Guarulhos, São Paulo, Brazil) and LP was isolated from the human oral cavity of an individual without tooth decay, per the approval of the Ethics Committee under protocol 560.479.

Lactobacilli strains were grown on Man-Rogosa-Shape agar (Himedia, Mumbai, India) for 4 days at 37 °C under microaerophilic conditions (5% CO_2). Isolated colonies were seeded in MRS broth. To prepare the suspensions, the species cultures were centrifuged for 5 min at 8300×*g* separately, and the supernatant was discarded. The sediments were resuspended in sterile and pyrogen-free 0.9% NaCl and centrifuged twice. After centrifugation, the pellet was resuspended to a ratio of 25:1 (lactobacilli cells:macrophages) [30] and standardized in a spectrophotometer with a wavelength of 540 nm and optical density of 0.4. Three monospecies (LR, LA and LP) and four multispecies (LR + LA, LR + LP, LA + LP and LR + LA + LP) suspensions were obtained. The multispecies suspensions were obtained by combining the monospecies suspensions in equal parts.

The *C. albicans* suspension was prepared from overnight cultures in 5 mL of yeast-nitrogen base (YNB) broth (Difco Laboratories Inc, Detroit, MI, USA) at 37 °C for 24 h. The cells were then centrifuged at 2.000×*g* for 10 min and the supernatant was discarded. The sediment was resuspended in sterile and pyrogen-free 0.9% NaCl and mixed in shaker tubes for 30 s. Cell washing was repeated twice. Fungal cells were adjusted to a ratio of 2:1 (fungal cells:macrophages) using a hemocytometer.

2.3. Selection of the best lactobacillus strain to stimulate cytokines: In vitro study

2.3.1. Macrophage activation

Macrophages were distributed into 24-well polystyrene microplates (5×10^5 viable cells per well) with DMEM supplemented with 10% fetal bovine serum to a final volume of 1 mL. After 24 h of incubation (37 °C and 5% CO_2) for cell adhesion, the wells were washed three times with pyrogen-free, sterile saline to remove nonadherent cells. Aliquots of 500 μL of DMEM and 500 μL of pyrogen-free 0.9% NaCl with either the different lactobacilli suspensions or pyrogen-free 0.9% NaCl (control group) were added to each well, resulting in a ratio of 25:1 (125×10^5 lactobacilli: 5×10^5 macrophages). The plates were incubated at 37 °C in 5% CO_2 for 2.5 h. After incubation, the wells were washed three times with pyrogen-free, sterile 0.9% NaCl to remove lactobacilli. Next, 500 μL of DMEM with streptomycin (2% $v.v^{-1}$) (Gibco®, São Paulo, Brazil) and 500 μL of pyrogen-free 0.9% NaCl with either *C. albicans* or pyrogen-free 0.9% NaCl (control group) were added to each well, resulting in a ratio of 2:1 (fungal cells:macrophages). The plates were again incubated at 37 °C in 5% CO_2 for 2.5 h.

After incubation, the plates were washed three times with pyrogen-free, sterile saline to remove microbial cells, and 1 mL of DMEM with streptomycin and amphotericin B (1% $v.v^{-1}$) (Gibco®, São Paulo, Brazil) was added. The plates were incubated 37 °C in 5% CO_2 for 16 h to release the cytokines, and the supernatants were collected and maintained at −80 °C to quantify the cytokines.

2.3.2. Cytokine quantification

A commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to determine the concentration of TNF-α, IL-1β, IL-6 and IL-17 per the manufacturer's instructions. The optical density for the cytokine quantification tests was measured using a microplate reader (EL808 - BioTek Instruments Inc., Winooski, Vermont, USA).

2.3.3. Cell viability assay

This experiment verified whether macrophage viability would be affected by probiotics or *C. albicans*. Macrophages were distributed into 96-well polystyrene microplates at a ratio of 25:1 (1×10^6 lactobacilli: 4×10^4 macrophages) with DMEM to a final volume of 100 μL. The rest of the procedure was performed as previously described in section 2.3.1, maintaining the same ratios and incubation periods.

After the 16 h incubation period, the wells were washed and the test was performed per the manufacturer's instructions (Biotool, Houston, Texas, USA). The microplates were read in a spectrophotometer (Cambrex Elx808cse – Lonza, Basel, Switzerland) at a wavelength of 570 nm, and the optical densities were converted into a viability percentage.

It is important to note that at the end of the 16 h incubation

period, all microbial species were dead due to the streptomycin and amphotericin B in the medium, removing any possibility of a false-positive result in the macrophage viability. In addition, three washes were performed between each step of the protocol to remove any microbial cells left in the wells.

2.3.4. Selection of the lactobacilli suspension

The best lactobacilli suspension, either in combination or as a single species, for *C. albicans* was chosen based on the results of the cytokine and viability assays. The probiotic suspension used to conduct the *in vivo* tests was chosen from the suspensions containing only one species. This was because the concentrations were the same in all suspensions for both the combined species and the single species (125×10^5 viable cells per well in the macrophage activation assay and 1×10^6 viable cells per well in the cell viability assay).

2.4. Lactobacillus rhamnosus interference in different strains of C. albicans: in vivo study

2.4.1. Susceptibility of Galleria mellonella to lactobacilli suspensions

We determined the sub-lethal inoculum concentration of *Lactobacillus* by injecting *G. mellonella* larvae with serial dilutions of the probiotic bacteria. Different concentrations of each *Lactobacillus* strain (10^4 to 10^7 cells/larvae) were inoculated into the larvae through the last left proleg. The larvae were kept on petri dishes at 37 °C, and they were monitored daily for survival.

2.4.2. Survival of G. mellonella after inoculation with L. rhamnosus suspension and being challenged by different C. albicans strains

To determine the survival curve of *G. mellonella*, *L. rhamnosus* cells were injected into the last right proleg of the larvae. Twenty-four hours after inoculation with *L. rhamnosus* cells, *C. albicans* cells were injected into the last left proleg of the larvae.

Two other *C. albicans* clinical strains were added to the GM assays to further confirm the results: *C. albicans* 21 (CA21) and *C. albicans* 60 (CA60). Both strains were from the Laboratory of Microbiology of the Institute of Science and Technology of São José dos Campos/UNESP (São Paulo State University) and were isolated from oropharyngeal candidiasis lesions of HIV-positive patients from Emílio Ribas Infectology Institute (Instituto de Infectologia Emílio Ribas, São Paulo, Brazil), with the approval of the Ethics Committee (Protocol 051/2009-PH/CEP). The samples were cultured in chromogenic HiCrome *Candida* medium (Himedia, Mumbai, India), identified by biochemical methods (API20C System- BioMérieux, Paris, France) and confirmed via molecular methods (PCR Multiplex) according Junqueira et al. [31].

C. albicans inocula were prepared by growing 50 mL YPD cultures overnight at 30 °C. Cells were pelleted at 1.300×*g* for 10 min followed by three washes in PBS. Cell densities were determined by hemocytometer count. Sixteen randomly chosen *G. mellonella* larvae (330 ± 25 mg) were infected with 10 μL of a suspension containing *Candida* in 10^6 CFU per larva in the last right proleg with a Hamilton syringe. In brief, *G. mellonella* larvae in the final instar larval stage were stored in the dark and used within 7 days from the date of shipment. Before injection, the area was cleaned using an alcohol swab, and after injection, larvae were incubated in petri dishes (37 °C). The number of dead *G. mellonella* was scored every 24 h for 7 days. Larvae were considered dead when they did not respond to touch.

2.4.3. CFU per larva counts in the G. mellonella hemolymph

To quantify the presence of *C. albicans* in the infected *G. mellonella*, experiments were conducted on the euthanized larvae at 12 and 24 h post-infection. The experimental groups were divided by the interaction of *L. rhamnosus* with each *C. albicans* strain, as well as the control group with each *C. albicans* strain. The experiment was performed in triplicate.

Each larva was sectioned with a scalpel in the cephalo-caudal direction and compressed to remove the hemolymph, which was placed in a microtube. Serial dilutions (10^{-1} and 10^{-2}) were made from the hemolymph and seeded in petri dishes containing Sabouraud dextrose agar with chloramphenicol, a culture medium selective for *C. albicans*. The plates were incubated at 37 °C for 48 h, and the colonies were counted to calculate the CFU/mL^{-1}.

2.4.4. Hemocyte density

Larvae were infected by injecting *C. albicans* at the last left proleg 24 h after the *L. rhamnosus* was injected at the last right proleg. Hemocytes were collected from the hemocoel at 24 h post-

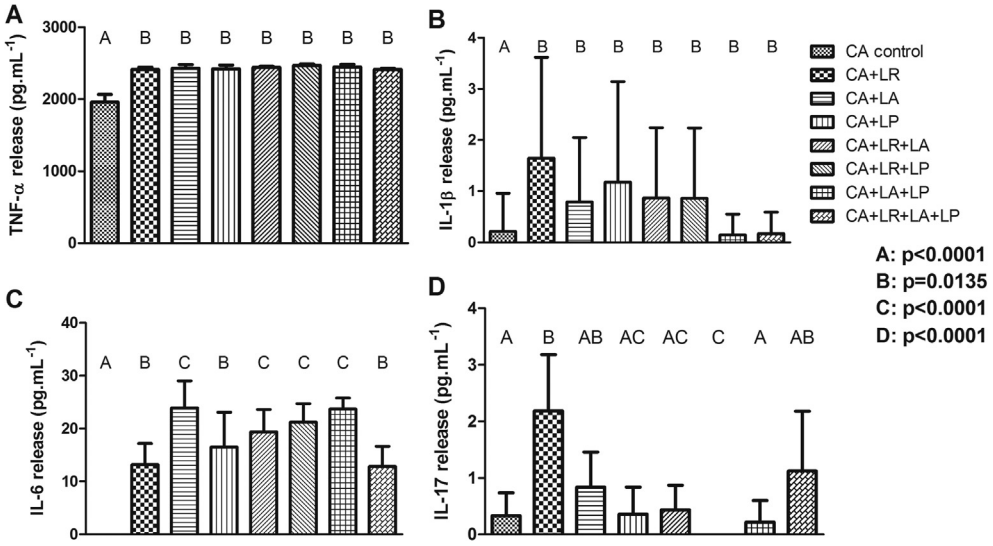

Fig. 1. Production of proinflammatory cytokines TNF-α, IL-1β, IL-6 and IL-17 by RAW 264.7, in the presence or absence of *L. rhamnosus*. Mean values and standard deviation of TNF-α, IL-1β, IL-6 and IL-17 (pg/mL) production by RAW 264.7 after contact with *L. rhamnosus* for 2.5 h. For the *C. albicans* control group, the cells were in sterile saline for 2.5 h. Statistically significant differences among experimental groups can be observed with different superscript letters (A, B and C in the graph). The *p* value in the figure corresponds to the comparison of all groups. ANOVA and Tukey Test, $p = 0.05$.

Table 1
Cell viability, expressed in percentage, according to the lactobacilli suspension and fungal strain.

Cell Viability (%)	CA	Sterile saline
LR	102.94	109.42
LA	95.64	95.55
LP	96.84	109.09
LR + LA	99.83	98.25
LR + LP	100.95	92.32
LA + LP	93.60	89.78
LR + LA + LP	91.98	103.03
Negative control	97.65	100

CA = *C. albicans*; LR = *L. rhamnosus*; LA = *L. acidophilus*; LP = *L. paracasei*.

injection with *C. albicans*. Four larvae were bled into four tubes per group, containing cold sterile insect physiologic saline (IPS) (150 mM sodium chloride; 5 mM potassium chloride; 100 mM Tris–hydrochloride, pH 6.9 with 10 mM EDTA and 30 mM sodium citrate). The hemocytes were counted using a hemocytometer. We did not differentiate between the six types of hemocytes, and the results were averaged from three replicates.

2.5. Statistical analysis

Cytokine counts were analyzed by a one-way ANOVA and Tukey's test when the results passed a normality test. When they failed to pass normality, Kruskal-Wallis and Dunn's tests were used. Killing curves were plotted, and statistical analysis was performed by the log-rank (MantelCox) test. Student's t-test was used to hemocyte densities and CFU per larva assays. Statistical analysis was performed using the software GraphPad Prism 5 (GraphPad Software, Inc., California, CA, USA). A P value < 0.05 was considered significant.

3. Results

All lactobacillus suspensions stimulated macrophages to produce more TNF-α than the *C. albicans* control group (Fig. 1A). There was no difference between the different suspensions of probiotics tested ($p = 0.0460$), but all suspensions stimulated more TNF-α production than the control group ($p < 0.0001$). The IL-1β production results were strain-dependent, although *L. rhamnosus* was able to stimulate more cytokine production (1.64 ± 1.97 pg mL^{-1}) than the other probiotic groups as well as the *Candida* control group ($p = 0.0283$) (Fig. 1B). The macrophages challenged by *C. albicans* produced significantly more IL-6 in all lactobacilli suspensions, both mono and multispecies ($p < 0.0001$) (Fig. 1C). The best lactobacilli suspension for stimulating IL-17 production was *L. rhamnosus* (2.18 ± 0.99 pg/mL^1) when compared with the other probiotic groups ($p < 0.0001$) as well as the *Candida* control group ($p < 0.0001$) (Fig. 1D). This cytokine plays a key role in protecting mammals from fungal infections.

Macrophage cell viability is expressed as percentages in Table 1. The negative control group, which did not receive any microorganisms, was considered 100% viable. Cell viability was not affected by the treatments, since no group showed cell viability lower than 70%.

Based on the *in vitro* results, the best lactobacilli suspension against *C. albicans* was *L. rhamnosus*; therefore, this suspension was used in the subsequent *in vivo* assays. First, we determined the sublethal concentration for *G. mellonella* by inoculating it with L. *rhamnosus* ATCC 7469, since no previous studies have been reported on this strain in a *G. mellonella* model. Standard suspensions of *L. rhamnosus* at a 10^4 to 10^7 cells/larva concentration were inoculated into *G. mellonella*, and survival curves were constructed (Fig. 2). The results showed that only concentrations greater than 10^6 cells/larva were pathogenic to *G. mellonella*, since none of the larvae died at this concentration. Therefore, the concentration of 10^6 cells/larva was adopted for all subsequent assays, since it is the same concentration as that used for *C. albicans* infections.

We then extended our *in vivo* assays to laboratory reference strain, *C. albicans* ATCC 18804, and two clinical isolates, CA21 and CA60, as previous studies demonstrated that these isolates exhibited intra-species variability in relation to pathogenicity in animal models [32,33]. In addition, we could exclude the hypothesis that the probiotic would be strain specific.

After the prophylactic treatment with *L. rhamnosus* and infection with the *C. albicans* strains, the survival curve of the larvae was evaluated. There was a statistically significant difference for all *Candida* strains tested between the following: *L. rhamnosus* + *C. albicans* ATCC 18804 and PBS + *C. albicans* ATCC 18804 control group ($p = 0.0001$); *L. rhamnosus* + *C. albicans* 21 and PBS + *C. albicans* 21 control group ($p = 0.0008$) and *L. rhamnosus* + *C. albicans* 60 and PBS + *C. albicans* 60 control group ($p = 0.0008$) (Fig. 3). Per these results, the survival rates increased by 68, 81 and 75% for the groups infected with *C. albicans* ATCC 18804, *C. albicans* 21 and *C. albicans* 60, respectively.

Additionally, we investigated the ability of *L. rhamnosus* to reduce the fungal load in the hemolymph of *G. mellonella* by counting the *C. albicans* CFU per larva. In the groups infected with *C. albicans* ATCC 18804, there was a statistically significant difference between the group treated with *L. rhamnosus* and the untreated group at both 12 h ($p = 0.0099$) and 24 h ($p < 0.0001$), with reductions of 0.93 log and 1.41 log in the CFU per larva

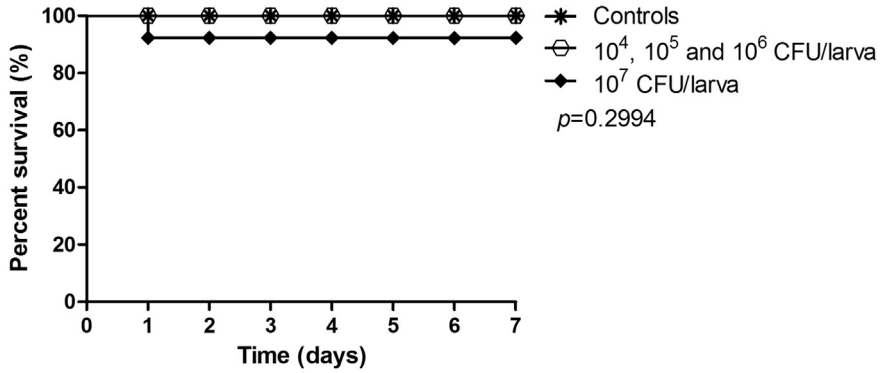

Fig. 2. Susceptibility of *G. mellonella* to infection with *L. rhamnosus* using larvae not infected by *C. albicans*. *G. mellonella* larvae were infected with serial concentrations of *L. rhamnosus* (CFU/larva). Controls were untreated *G. mellonella* larvae that received only a PBS injection or the No injection group. There was no difference between the groups ($p = 0.2994$). Log-rank test, $p \leq 0.05$.

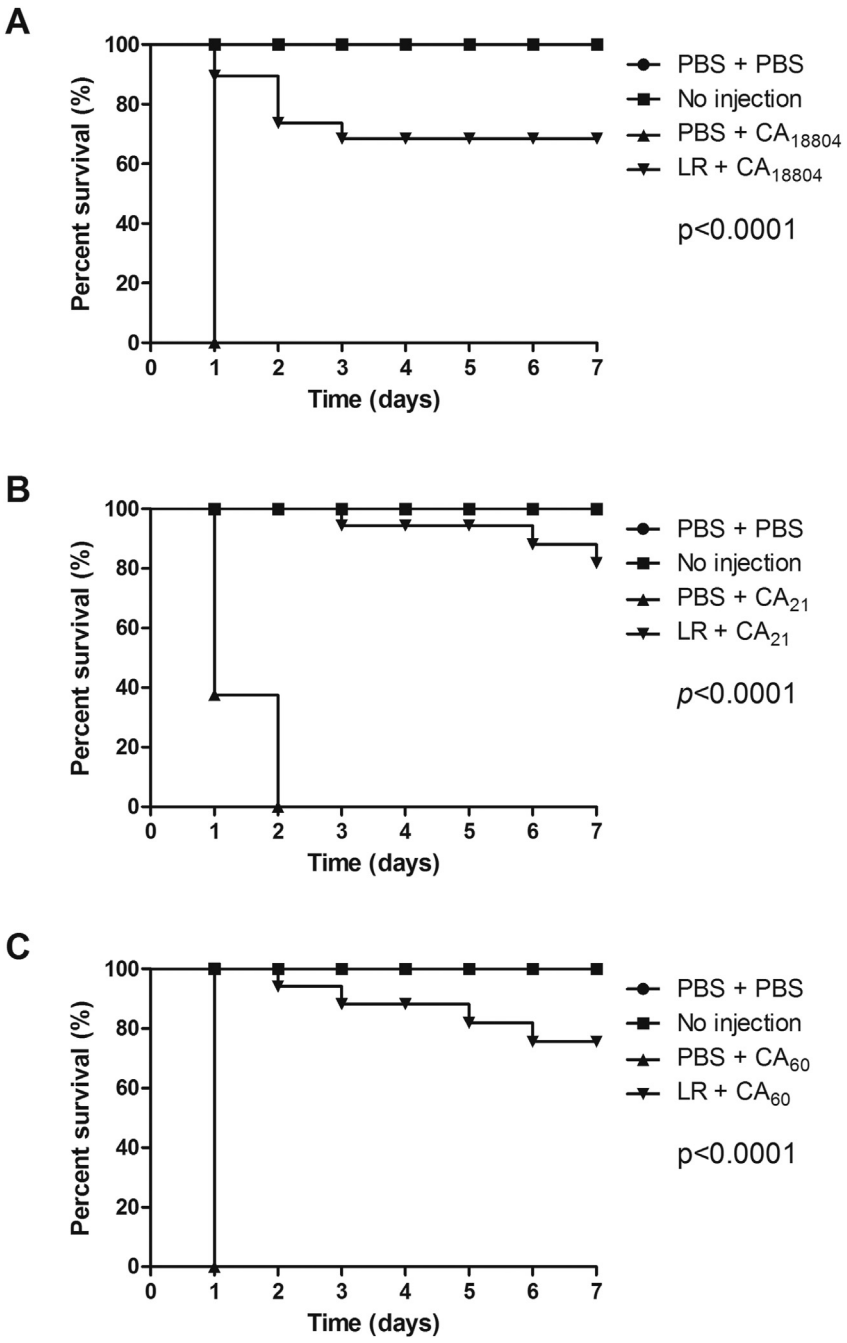

Fig. 3. *L. rhamnosus* prolongs the survival of *G. mellonella* larvae infected with *C. albicans*. **A.** *C. albicans* ATCC 18804: There was a significant difference between *L. rhamnosus* + *C. albicans* and PBS + *C. albicans* control group ($p = 0.0001$). **B.** *C. albicans* 21: There was a significant difference between *L. rhamnosus* + *C. albicans* and PBS + *C. albicans* control group ($p = 0.0008$). **C.** *C. albicans* 60: There was a significant difference between *L. rhamnosus* + *C. albicans* and PBS + *C. albicans* control group ($p = 0.0008$). Log-rank test, $p = 0.05$.

counts, respectively (Fig. 4A). For the groups infected with the clinical strains, there was a statistically significant difference only at 24 h for *C. albicans* 21 ($p = 0.0023$) and *C. albicans* 60 ($p = 0.0001$) and reductions of 1.49 log and 1.85 log, respectively (Fig. 4B and C).

In the *G. mellonella* hemocyte counts, a statistical difference was obtained for all groups ($p < 0.05$). Prophylactic treatment with *L. rhamnosus* increased the number of hemocytes in all groups infected with the fungal strains as well as in the negative control group (*L. rhamnosus* + PBS), indicating that this *Lactobacillus* strain recruited hemocytes into the hemolymph. For the groups treated with *L. rhamnosus* and infected with *C. albicans* ATCC 18804, there was a 2.15-fold increase in the number of hemocytes. The same was observed for the groups infected with *C. albicans* 21 (4.26-fold increase) and *C. albicans* 60 (2.89-fold increase) and for the control group inoculated with PBS instead of the fungal strains (1.5-fold-increase). The means and the *p* value for the comparison between groups are shown in Fig. 5. These results agreed with the survival assay and the CFU count assay, indicating that prior exposure to *L. rhamnosus* activates the *G. mellonella* immune system, which may allow the larvae to combat a lethal infection by *C. albicans*.

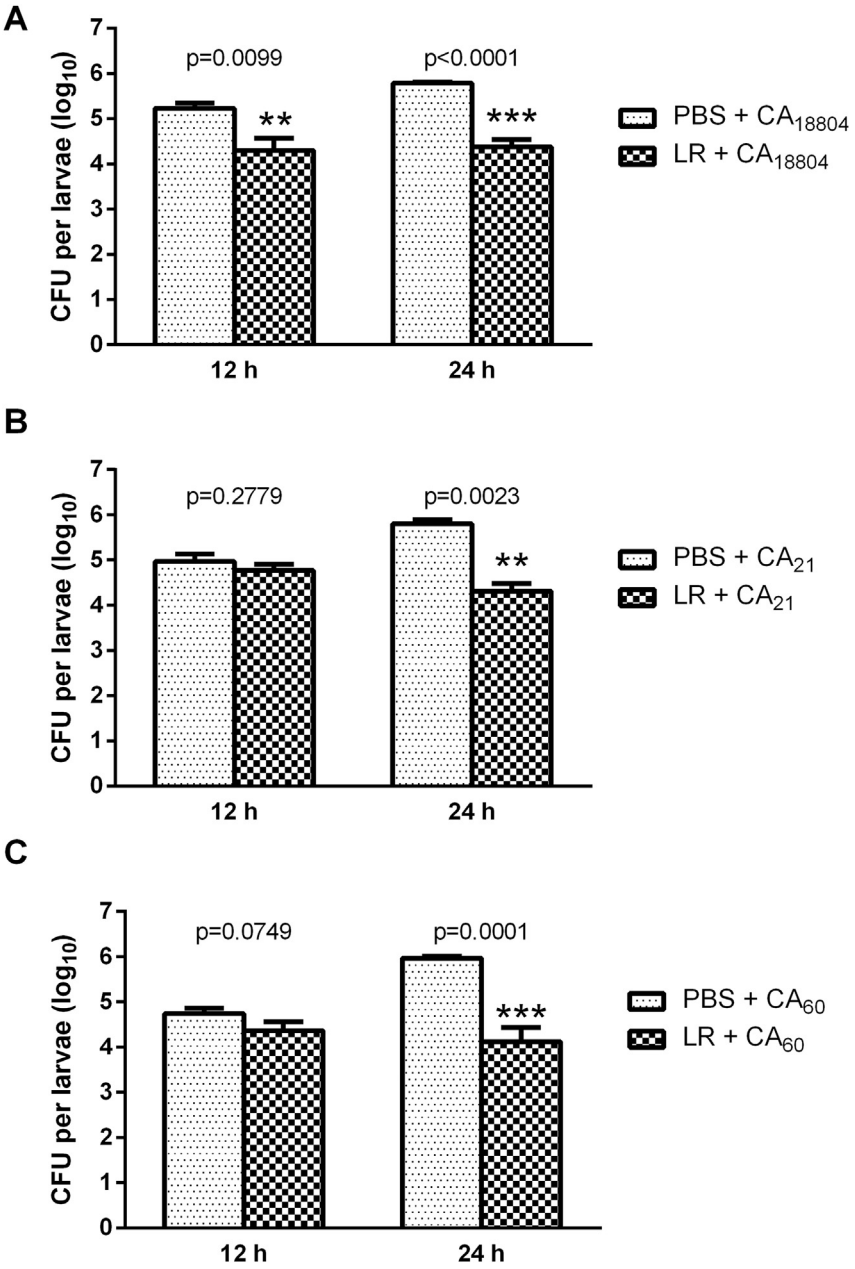

Fig. 4. *L. rhamnosus* decreased the number of *C. albicans* cells in *G. mellonella* hemolymph. Mean and standard deviation (log10) of the CFU per larva count of the different experimental groups at times of 12 and 24 h. Groups infected by **A.** *C. albicans* ATCC 18804; **B.** *C. albicans* 21; **C.** *C. albicans* 60, with and without prophylactic treatment with *L. rhamnosus*. PBS = phosphate buffered saline; CA18804 = *C. albicans* ATCC 18804; CA21 = *C. albicans* 21; CA60 = *C. albicans* 60; LR = *L. rhamnosus*. *P* value refers only to the groups infected with *Candida*. Student's *t*-test, $p \leq 0.05$.

4. Discussion

Alternative treatments against resistant pathogens are gaining increasing attention in the scientific field. Among the most studied treatments, is the use of probiotics [27,34]. Probiotics are commensal microorganisms that benefit their host by providing anti-infective [35] and immunomodulatory properties [36,37].

In this study, the tests performed using mouse macrophages (RAW 264.7) directly reflect the central role of the innate immune system's defense mechanisms, including the inflammatory response [38] that produces chemical mediators such as cytokines, which are essential for host defense [39]. After activation and infection of the macrophages, their viability was verified to validate the other tests, as a high rate of cell death would make the results suspect. According to Li et al. [40], 70% is the standard rate for determining whether the treatment is cytotoxic or not to the macrophages. Considering this, the treatments did not interfere with the macrophage viability.

In this study, there were statistically significant differences in cytokine production, and these differences varied by the species of lactobacilli in the suspension. Some authors have previously described lactobacilli modulation as being controversial, since it may increase, decrease or cause no change in cytokine levels [41,42].

Romani [12] found that virulent strains of *Cryptococcus neoformans* can inhibit TNF-α production and induce IL-10 production, thus inhibiting macrophage activation. Although different fungal species were used in this study, we noted this low cytokine

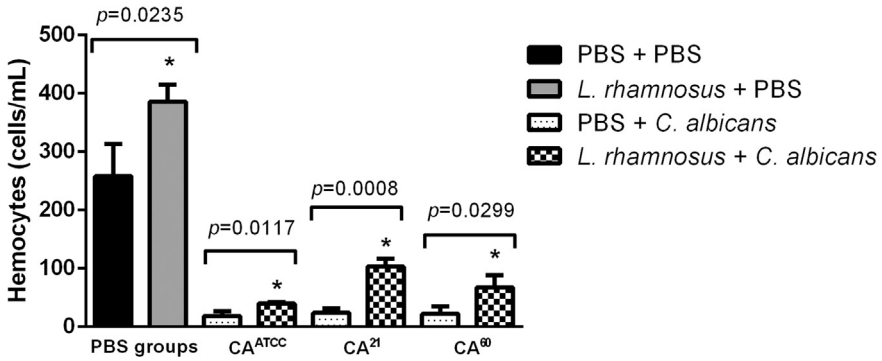

Fig. 5. *G. mellonella* hemocyte density increased with the injection of *L. rhamnosus*. Mean and standard deviation of hemocyte counts in the hemolymph of *G. mellonella*. Student's *t*-test was used to compare hemocyte densities between the experimental groups. A p value ≤ 0.05 was considered significant.

production when macrophages were only challenged with *Candida*. However, in the groups previously treated with lactobacilli suspensions, there was an increase in TNF-α production, indicating the immunomodulatory effect of the suspensions toward this cytokine.

Vonk et al. [43] and Hise et al. [44] showed that IL-1β-deficient rats had a reduced survival and an increased fungal load when compared with healthy rats, showing the importance of this cytokine in host defense against fungal infections. In our results, the groups infected only with *Candida* presented low IL-1β production. When the macrophages were previously treated with lactobacilli suspensions, these averages either increased or remained similar in most cases, based on the fungal strain and lactobacilli suspension used. Therefore, the stimulation of IL-1β production provided by lactobacilli suspensions may play an essential role against fungal infection, since this cytokine is crucial for host defense in response to infection and injury [45].

Interleukin-6 levels in the *Candida*-infected cells were modulated when treated with lactobacilli, with a significant increase in all groups. IL-6 has a multifunctional role [46], mediating the direction of the inflammatory response, including the mechanisms of transition from innate to acquired immune response [47,48]. Genetic deletion of the of IL-6 production mechanisms in mice was examined in 1994 [49], and the deletion resulted in an impaired response to infections, defects in the production of T-cell regulated antibodies and an absence of the acute phase response after infection or trauma. This suggests that IL-6 production is important for the control of fungal infections, as well as serving as a signal to coordinate macrophages. In our study, the results showed that all lactobacillus suspensions are capable of increasing IL-6 production, an important aspect, since the group infected only by *Candida* showed very low production of this cytokine in response to the *Candida*.

We also analyzed the levels of IL-17 produced by macrophages. The results showed that in all experimental groups, only the group treated prophylactically with *L. rhamnosus* showed a statistically significant increase in IL-17 production. These results are promising for treatment against *Candida* infections, since *C. albicans* is the most pathogenic of the genus, and IL-17 plays an important role in innate and adaptive responses against infections at the mucosal and cutaneous interfaces. This was exemplified by the occurrence of chronic mucocutaneous candidiasis, in patients with genetic defects that impaired IL-17-related immune responses [50]. In addition, in patients treated with IL-17 antagonists, a higher incidence rate of *Candida* infections is expected [51]. These recent studies show the importance of finding a new alternative to improve the host defenses without inducing the emergence of resistant strains.

A cellular immune response against fungi is essential, and macrophages are the most important cells in the control of fungal growth [52]. When macrophages, challenged only by *Candida*, were evaluated in this study, the concentration of cytokines was lower than those in the groups previously treated with probiotics, indicating a low inflammatory mediator release profile. This suggests the pathogen's ability to escape recognition by the immune system and not activate host defense mechanisms.

Another important aspect is the composition of the microbial wall of lactobacilli. Lipoteichoic acid (LTA) and peptidoglycan are important components of the Gram-positive bacterial cell wall, capable of stimulating an immune response. However, the ability to influence responses is strain-dependent, since LTA and peptidoglycan may undergo species-dependent modifications [53–55]. Modifications in these structures may reflect different patterns of cytokine modulation.

The best lactobacilli suspension with immunomodulatory effects against *C. albicans* was *L. rhamnosus* (single species), and the study of this suspension was extended to *in vivo* assays using a *G. mellonella* model. In this study, the single *Lactobacillus* strain suspension produced better results than the multi-strain suspension. Agreeing with our results, MacPherson et al. [56] evaluated the immune modulation of TLR3-mediated inflammation in intestinal epithelial cells with single and multi-strain probiotic combinations, and they did not find statistically significant differences between the groups for either TNF-α or IL-8. In addition, IL-1β and IL-6 revealed no detectable protein modulation for all probiotic combinations, suggesting that the success of the probiotic combination varies with the strain studied.

Insects, such as *G. mellonella* larvae, possess only an innate immune system, which comprises humoral and cellular branches. Cellular response involves hemocytes, whose main function is phagocytosis of foreign bodies or sequestration of these bodies in the insect body in structures called nodules. As part of this humoral response, insects synthesize antimicrobial peptides, small molecules with bactericidal and fungicidal activity, by destabilizing the microbial membranes [57,58].

However, new studies have been changing this paradigm by suggesting that invertebrate immune responses are more complex and specific than previously thought. Primary exposure of invertebrates to pathogens increases their resistance to subsequent pathogenic challenges [59]. This mechanism has been reported in other invertebrate models [60,61]. Our results corroborate those of the previous studies, although the microorganism injected first cannot be considered pathogenic, since it did not affect the survival rate of the larvae in any of the tested concentrations. Moreover, previous studies have shown that the results derived from *G. mellonella* assays were positively correlated with results from studies with *Caenorhabditis elegans* and mice, demonstrating the

potential that this invertebrate model has as an alternative to *in vivo* studies of probiotics strains [62–64].

In all survival assays, our study found statistically significant differences between the groups treated prophylactically with *L. rhamnosus* and those not treated. The lactobacilli suspension significantly increased the survival rate of the larvae infected with *C. albicans* ($p < 0.05$) in all cases, suggesting that *L. rhamnosus* ATCC 7469 is a modulator in the immune response. Our study evaluated the survival of *G. mellonella* larvae similarly to Vilela et al. [26], Grounta et al. [65] and Ribeiro et al. [27], who prophylactically treated the larvae with *Lactobacillus* prior to the injection of fungal or bacterial pathogens. In all studies, they observed an increase in the larval survival rate that varied by strain and type of infection.

CFU counts for the *G. mellonella* hemolymph showed a decrease in the fungal load for all the strains evaluated when the larvae received prophylactic treatment with *L. rhamnosus*, in all time periods. However, we obtained statistically significant reductions in all groups, ranging from 96.2 to 98.6%, only after 24 h. Our results are similar to those of other authors. Ribeiro et al. [27] found a reduction of 52.2% in the CFU count in the *C. albicans* ATCC 18804 biofilm with the addition of *L. rhamnosus* ATCC 9595 cells. Vilela et al. [26] verified a reduction in the *G. mellonella* hemolymph fungal load in the groups treated with *L. acidophilus* ATCC 4356 cells only at 24 h in accordance with our results. The result for the 12-h period in the group infected by *C. albicans* ATCC 18804 differs from those found by Vilela et al. [26]. In our study, there was a significant reduction in the fungal load of *C. albicans* ATCC 18804 in the 12-h time period. It is possible that this difference is due to the use of different strains of lactobacilli. As previously stated, modulation of the immune response is strain-dependent, i.e., it depends on both the pathogen and lactobacilli strains used.

Recently, members of our research group developed a study to evaluate the immunomodulatory action of the strain *L. paracasei* 28.4 in experimental candidiasis in *G. mellonella*. Rossoni et al. [66] showed that prior exposure to *L. paracasei* 28.4 activates the *G. mellonella* immune system, which may allow the larvae to combat a lethal infection by *C. albicans*. This effect was mediated by an increase of circulating hemocytes and the production of elevated levels of antimicrobial peptides that consequently reduced *Candida* cells in *G. mellonella* hemolymph. These data demonstrate the need to invest in the strains used in this study as potential probiotics.

To understand the survival curve and CFU counts, a hemocyte-counting assay was performed with the infected larvae, which plays a key role in the defense against fungal pathogens. Hemocyte density and survival of *Galleria* are indicators of pathogenicity [67,68]. Non-pathogenic fungi result in high hemocyte densities, while pathogenic fungi reduce hemocyte levels [68]. Our results demonstrate the modulatory power of the immune response of the *L. rhamnosus* suspension used. In all prophylactically treated groups, there was a significant increase in hemocyte density.

Density and survival of *Galleria* were related because they indicate fungal pathogenicity, which was verified by our results. For example, the groups treated with *L. rhamnosus* and infected with *C. albicans* ATCC 18804, had the lowest increase in survival, at approximately 37.5%, and the lowest increase in hemocyte density (2.15-fold). It was the opposite for the clinical strains. The increase in survival may be related to the increased hemocyte density, confirming our results.

The results of this study corroborate other *G. mellonella* studies in the literature while simultaneously indicating the need for further studies using different assays (phagocytosis, production of phenoloxidase and antimicrobial peptides) to elucidate how the immunomodulation of probiotics acts on the host and eliminates pathogens.

5. Conclusion

Based on these results, Lactobacilli suspensions showed strain-dependent immune modulation, and single-strain suspensions showed better results than multiple-strain suspensions. *L. rhamnosus* ATCC 7469 showed anti-*Candida* activity, demonstrated by the decreased *C. albicans* infection in *G. mellonella*.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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