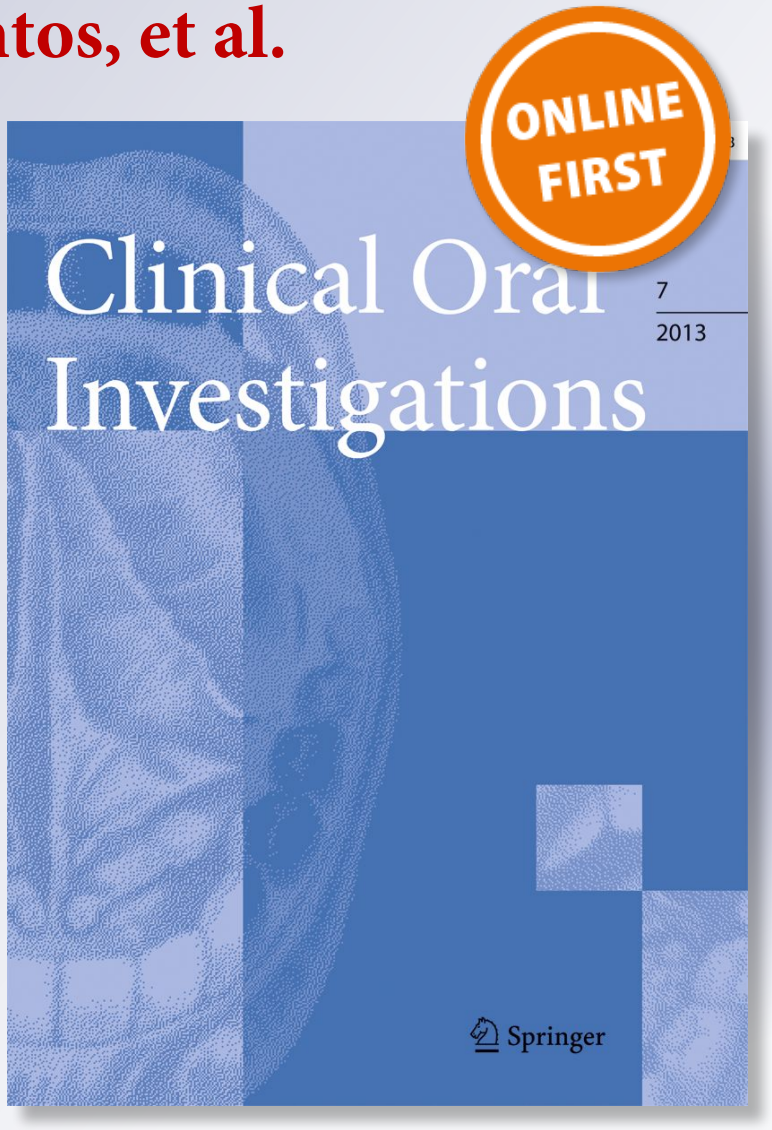
# Lactobacillus rhamnosus *intake can prevent the development of Candidiasis*

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**Clinical Oral Investigations**

ISSN 1432-6981

Clin Oral Invest
DOI 10.1007/s00784-018-2347-8




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https://doi.org/10.1007/s00784-018-2347-8

ORIGINAL ARTICLE

# *Lactobacillus rhamnosus* intake can prevent the development of Candidiasis

Mariella Vieira Pereira Leão[1] · Talita Angelina Aureliano Tavares[2] · Célia Regina Gonçalves e Silva[1] · Silvana Soleo Ferreira dos Santos[1] · Juliana Campos Junqueira[2] · Luciane Dias de Oliveira[2] · Antonio Olavo Cardoso Jorge[2]

Received: 22 February 2017 / Accepted: 17 January 2018
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## Abstract

**Objective** This study aimed to investigate the influence of *Lactobacillus rhamnosus* intake on the development of candidiasis and cytokines release.

**Material and methods** *Candida* suspensions were inoculated into the oral cavity of experimentally immunosuppressed mice for candidiasis induction. The animals were divided into experimental groups: candidiasis with no probiotic intake (F), candidiasis with probiotic intake during *Candida* inoculation (FP), and candidiasis with probiotic intake 14 days before inoculation with *Candida* (FPP); and control groups: (C), (CP), and (CPP) without inducing candidiasis with probiotic intake in the same manner as groups F, FP, and FPP, respectively. After these periods, samples were collected from the oral cavity for yeast counts and, after euthanasia, the tongues of the animals were removed for histological analysis. Sera samples were also collected for analysis of IL-1 beta, TNF-alpha, INF-gamma, IL-12, IL-4, and IL-10.

**Results** FP group showed lower *Candida* counts in the oral cavity, and the presence of *Candida* was almost not detected in FPP group. In tissues, the counts of fungi were significantly lower in FPP group, followed by FP. Groups that consumed probiotics also had lower histological and inflammatory infiltrates compared to F. Cytokines analysis demonstrated low concentrations of TNF-α, IL-12, IL-4, and IL-10 in all the groups, and no statistical difference between them. The production of IL-6 could be better detected, and the experimental groups that consumed the probiotic showed significant lower levels of this cytokine.

**Conclusions** The results suggest that *L. rhamnosus* intake, especially preventively, may avoid or decrease the development of candidiasis in immunosuppressed mice.

**Clinical relevance** This work adds scientific evidences that probiotics intake can avoid the development of candidiasis.

**Keywords** Probiotics · *Candida* · *Lactobacillus rhamnosus* · Cytokine

## Introduction

Probiotics are emerging as new strategy to counteract diseases. They are defined as live microorganisms which when used in adequate amounts can confer health benefits to the host [1]. The most common genus with probiotic properties are *Lactobacillus* and *Bifidobacterium*, found on intestinal microbiota in a variable manner [2]. The prophylactic and therapeutical benefits of probiotics have been clearly reported in different conditions as infections, allergy, gastrointestinal, and metabolic disturbances [3, 4]. The mechanisms they use to promote these benefits are, mainly, by improvement of immune response and microbiota balance, avoiding pathogens colonization [5, 6].

Immunity imbalance affects the relation between microbiota and host, favoring the development of opportunistic infections, such as candidiasis, caused by *Candida* genus [7]. Most of the individuals develop immunological defense, which avoid the growth and progression of *Candida*. However, in some situations as antibiotic therapy, use of prosthesis and

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Published online: 25 January 2018

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orthodontic devices and immunosuppression, *Candida* may grow and cause different superficial or systemic diseases, especially in the oral and vaginal mucosae [8–10].

Shalev et al. [11] observed that diary intake of foods enriched with *Lactobacillus acidophilus* increased the colonization of the vagina and recto with these bacteria, reducing *Candida* infections. It has been shown that the consumption of different probiotic microorganisms may reduce the amounts and the prevalence of yeasts in the oral cavity of young and older individuals [12–14].

Villena et al. [15] verified that animals which received probiotics were more resistant against *Candida albicans*, and had their immune response improved, with an effective delivered of pro-inflammatory cytokines and an increase in production of IL-10, avoiding damages caused by inflammatory response.

Oliveira et al. [16] observed that *Lactobacillus rhamnosus* was able to influence the expression of virulence factors by *C. albicans* and could alter its antifungal sensitivity profile.

Since opportunistic diseases are difficult to treat because of the high frequency of resistant microorganisms, probiotic intake may take place as an adjuvant therapy. Taking these points in consideration, this study aimed to investigate the influence of *L. rhamnosus'* intake on candidiasis development and cytokines profile.

## Material and methods

### Microbial strains and suspensions

*C. albicans* clinical strain was isolated from a patient with prosthetic stomatitis and identified in the Laboratory of Microbiology and Immunology from São Paulo State University. It was grown in Sabouraud dextrose Agar (Difco, Detroit, USA), incubated at 37 °C for 24 h. Aliquots of the colony growth were added to sterile saline until the suspension reached $10^9$ or $10^6$ cells/mL, standardized in spectrophotometer at 530 nm. This strain was used based on its greater capacity to induce candidiasis, compared to standard strains.

*L. rhamnosus* ATCC 7469 (Cefar Diagnóstica, São Paulo, Brasil) was grown on Man-Rogosa-Shape Agar (MRS-Oxoid, Basingstroke, Hampshire, England), incubated at 37 °C and 5% $CO_2$ during 36 h. Then, a suspension containing $10^7$ cells/mL was obtained.

### Animals

This study was approved by the local Ethics Committee (n°016/2010-PA/CEP) and the National Research Council's guide, for the care and use of laboratory animals.

Wistar male mice, 11–12 weeks old, were separated into six groups:

1. F: with experimental candidiasis (fungal infection) ($n = 15$);
2. FPP: with probiotic intake for 2 weeks before experimental candidiasis (fungal infection + probiotic before + probiotic after) ($n = 15$);
3. FP: with probiotics intake since experimental candidiasis (fungal infection + probiotic after) ($n = 15$);
4. CPP: with only probiotic intake, such as FPP group (control + probiotic before + probiotic after) ($n = 15$);
5. CP: with only probiotic intake, such as FP group (control + probiotic after) ($n = 15$);
6. C: with no probiotic intake or candidiasis (control) ($n = 15$).

Sample size was calculated using the formula: $n = 1 + [2C*(s/d)^2]$, considering $C = (z\alpha + z\beta)^2$ ($z = 1.96$, significance level of 0.05 ($\alpha$) and power of test of 90% ($\beta$)), maximum deviation of 20% ($s$), and expected difference of 25% between the groups ($d$) [17], using as parameter *Candida* counts, observed by Mendonça et al., [14].

All the animals were verified about absence of previous colonization with *Candida*.

### Experimental candidiasis

Candidiasis was induced according to Dongari-Bagtzoglou et al. [18]. One day before the infection, mice were immunosuppressed by intraperitoneal injection with dexamethasone (65 mg/kg) (Dexium, Chemitec, São Paulo, Brasil). For *C. albicans* challenge, mice were anesthetized with an intramuscular injection of ketamine: xylazine (90–100 mg/kg and 10 mg/kg of body weight, respectively) and a swab, soaked with 100 μL of *C. albicans* suspension ($10^9$ cells/mL), was introduced in the oral cavity. The swab was left for 80 min under the tongue and removed before the animals awoke. This procedure was repeated during 2 days and mice were sacrificed after 4 days of total exposure to *C. albicans*. During this period, animals were also given drinking water containing fresh suspension of *C. albicans* ($10^6$/mL).

The control groups (C, CP, and CPP) received the same procedures but the swab introduced in the oral cavity was soaked with PBS, and they did not receive *C. albicans* in drinking water.

### Probiotic intake

The animals were given drinking water containing fresh suspension of *L. rhamnosus* at diary consumption mean of $10^6$ cells/mice. Fifteen animals consumed lactobacilli during 14 days before candidiasis induction (FPP group), 15 since

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the first day of candidiasis induction (FP group), 15 during 21 days (CPP group), and 15 during 7 days (CP group).

### Samples collections

The last day of experiment, after anesthesia, samples of the oral cavity were collected using a swab wet in sterile PBS. After 1 min of rotatory movements, the swabs were transferred to 0.9 mL of sterile PBS and homogenized during 60 s. It was considered a 10-fold dilution. After that, sequential dilutions were performed and from each dilution, 0.1 mL was seeded in Sabouraud dextrose agar with chloramphenicol. After incubation at 37 °C for 48 h, *Candida* colonies were counted and CFU/mL values calculated.

Also after anesthesia, blood samples were collected by retro-orbital bleeding and cardiac puncture. After coagulation and centrifugation, sera were obtained for cytokine analysis.

Euthanasia was performed using excessive dose of anesthesia. After that, the tongues were dissected and analyzed macro- and microscopically.

### Microscopic analysis

The tongues were formalin-fixed and embedded in paraffin. Slices of 5 μm thick were stained with hematoxylin and eosin (HE) and periodic acid–Schiff (PAS). The presence of candidiasis was investigated and the presence of yeasts and hyphae was quantified by observation of 20 fields of each histological section.

The lesions and inflammatory infiltrates were also investigated by observation of 20 fields of each histological section. The alterations investigated were epithelial hyperplasia, basement membrane changes, exocytosis, spongiosis, filiform papillae loss, hyperkeratosis, and presence of microabscess in epithelium. For the inflammatory infiltrate, the following scores were attributed: 0 (absence), 1 (mild), 2 (moderate), and 3 (pronounced).

### Cytokines quantification

The cytokines levels (TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-12, and INF-γ) were quantified using enzyme-linked immunosorbent assay (ELISA). The DuoSet ELISA detection kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions. In all cases, detection antibody binding was visualized using the streptavidin-horseradish peroxidase conjugate and TMB (trimethyl benzidine) substrate system at a wavelenght of 450 nm. After determining optical densities, the cytokine concentrations (pg/mL) in sera were calculated, based on a standard curve, using GraphPad Prism 5.0 software.

### Statistical analyze

*Candida* counts and ELISA results were statistically analyzed by ANOVA and Tukey's test, since the data showed a normal distribution and tissue alterations by Kruskal-Wallis and Dunn's test. The level of significance considered was 0.05 for all the tests.

## Results

### *Candida* counts

It was observed that probiotics intake for 7 days (FP) did not influence significantly CFU/mL counts ($p = 0.87$), when compared with the group with candidiasis with no probiotic intake (F). In the group with probiotic intake during 21 days (FPP), *Candida* was not detected in 11 of the 15 studied animals, showing a significant elimination of these fungi in their oral cavity ($p < 0.01$). The same results were observed when the yeasts and hyphae were analyzed in the tissues. The mean of scores attributed to amounts of *Candida* was significant lower in group FPP, and reduced in group FP, when compared with group F (Fig. 1).

### Tissue alterations

All types of alterations were verified on tissue of mice's tongues, stained with HE. The groups that consumed probiotics (FP and FPP) showed lower alterations when compared with group F, especially FPP, which reduction was significant ($p < 0.01$). Small alterations could be verified in some of the animals of groups C, CP, and CPP, but the numbers were significant lower when compared with the other groups ($p < 0.01$). Some of these alterations are demonstrated in Fig. 2, and the differences of the amounts in each group are presented in Fig. 3.

Regarding the presence of inflammatory cells, it was observed inflammatory infiltrate ranging from moderate to pronounced in mice of groups F and FP, from moderate to mild in mice of FPP group, and when present, mild inflammatory infiltrate in mice with no candidiasis induction (group C, CP, and CPP).

### Cytokine analysis

Probiotic intake induced different effects on cytokines release in animals of control groups (with no *Candida* inoculation) and experimental groups (with *Candida* inoculation). Generally, all the groups presented low concentrations of TNF-α (≤ 12.6 pg/mL), IL-12 (≤ 25.1 pg/mL), IL-4 (≤ 2.9 pg/mL), IL-10 (≤ 22.8 pg/mL), IL-1β (≤ 17.2 pg/mL), and INF-ɣ (≤ 4.2 pg/mL) (Fig. 4).

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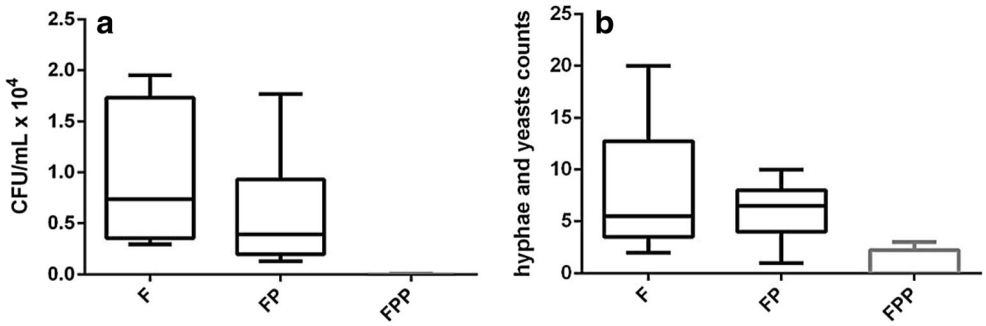



**Fig. 1** **a** Average of CFU/mL counts of *Candida* isolated from the oral cavity of mice with experimental candidiasis without consuming probiotic (F), with candidiasis and consumption of probiotic during *Candida* inoculation, for 7 days (FP), and with *Candida* inoculation and consumption of probiotic from 14 days before first inoculation, totaling 21 days of consumption (FPP). **b** Total yeasts and hyphae counted in 20 fields of histological sections, stained with PAS, of animals' tongues of groups F, FP, and FPP

In the analysis of TNF-α, IL-12, IL-4, and IL-10, no significant differences among studied groups were verified.

In the production of IL-1β, probiotic intake showed dose-dependent stimulatory effect in control animals and in experimental animals, but with no statistical difference within each group. Statistical difference was only observed in the concentrations of group C (with no probiotic or *Candida*) compared with FPP group (with *Candida* and probiotic for 21 days) ($p < 0.01$).

In control animals, the consumption of lactobacilli reduced the production of INF-ɣ, and in groups with candidiasis induction, the consumption for 7 days increased the production of INF-ɣ while the consumption for 21 days significantly reduced its synthesis. There was statistical difference only between C and FPP ($p < 0.01$) and CP and FPP ($p = 0.049$) groups.

The cytokine IL-6 was the one with the highest serum concentrations and, therefore, was better detected (Fig. 5). Among the experimental groups (with candidiasis induction), those that consumed probiotics had lower amounts of this cytokine and, the difference between F and FPP groups was statistically significant ($p = 0.03$). Regarding the control groups (with no candidiasis induction), consumption of probiotics induced different effects. The intake for 7 days induced decrease in production of IL-6, while drinking for 21 days induced a significant increase in the this cytokine release (CP and CPP groups, $p = 0.01$).

## Discussion

The maintenance and restoration of the microbiota balance are the best alternatives to avoid the development of candidiasis and other opportunistic diseases. Several studies have been demonstrating that the consumption of probiotics could help in recuperation of this balance and in preventing *Candida* prevalence and growth [12–14].

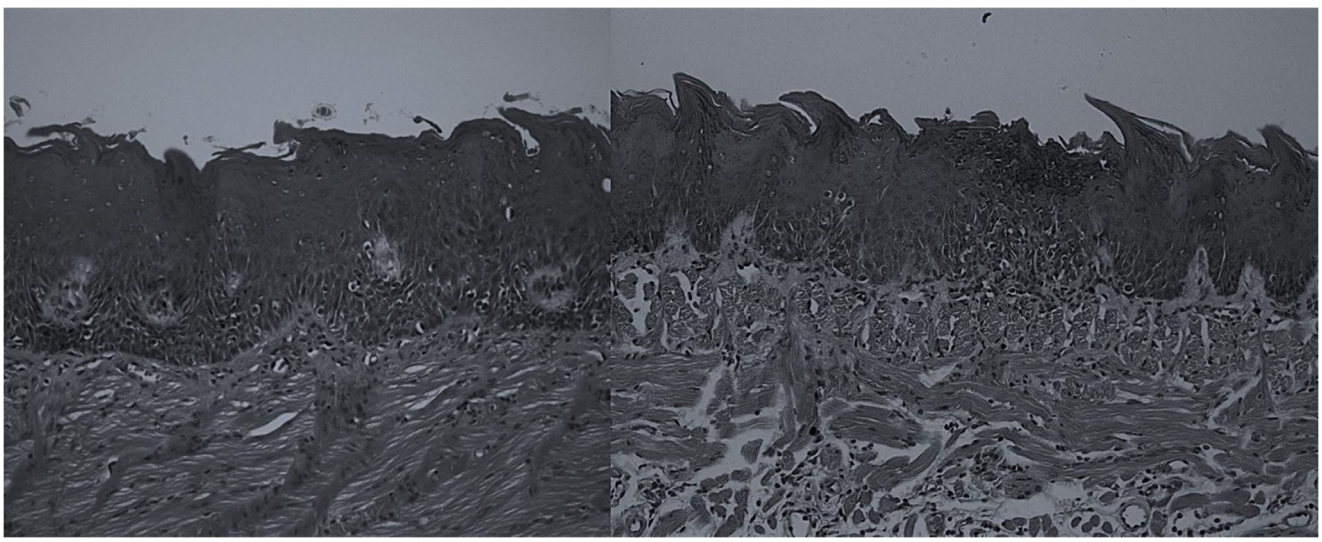


**Fig. 2** Sagittal section of animal's tongue of groups F and FP, showing different alterations as epithelial hyperplasia, basement membrane changes, exocytosis, spongiosis, filiform papillae loss, and intraepithelial microabscesses. Pronounced inflammatory infiltration is observed in the lamina propria. Histological sections stained with HE; ×200

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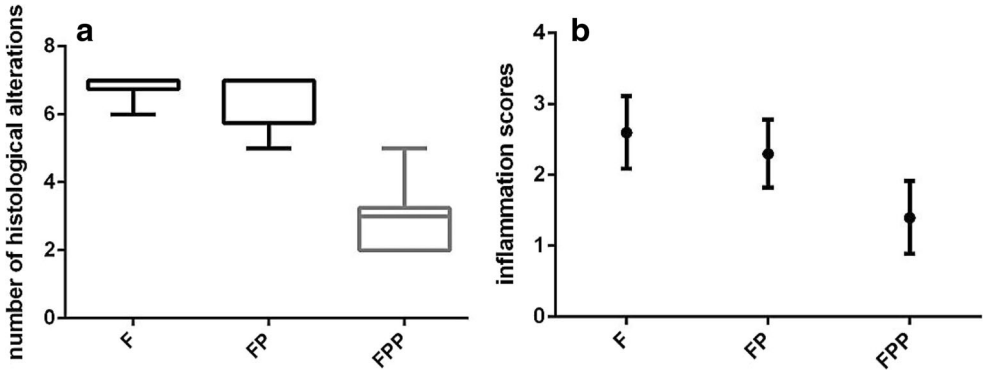



**Fig. 3** **a** Amounts of epithelial alterations in tongues of mice with experimental candidiasis without consuming probiotic (F), with candidiasis and consumption of probiotic during *Candida* inoculation, for 7 days (FP), and with *Candida* inoculation and consumption of probiotic from 14 days before first inoculation, totaling 21 days of consumption (FPP); **b** mean of scores attributed to inflammatory infiltrate: 0 (absence), 1 (mild), 2 (moderate), and 3 (pronounced) observed in animals' tongues of groups F, FP, and FPP, observed in histological sections stained with HE; ×200

In the present study, after immunosuppression and induction of candidiasis and subsequent recovery of yeasts from the oral cavity of the animals, it was noted that the amounts of *Candida* were significantly lower in mice that consumed *L. rhamnosus* suspensions during 21 days. In animals supplemented with probiotic suspension from the first day of candidiasis induction, the average of *Candida* counts was not statically different form the control group, suggesting that the

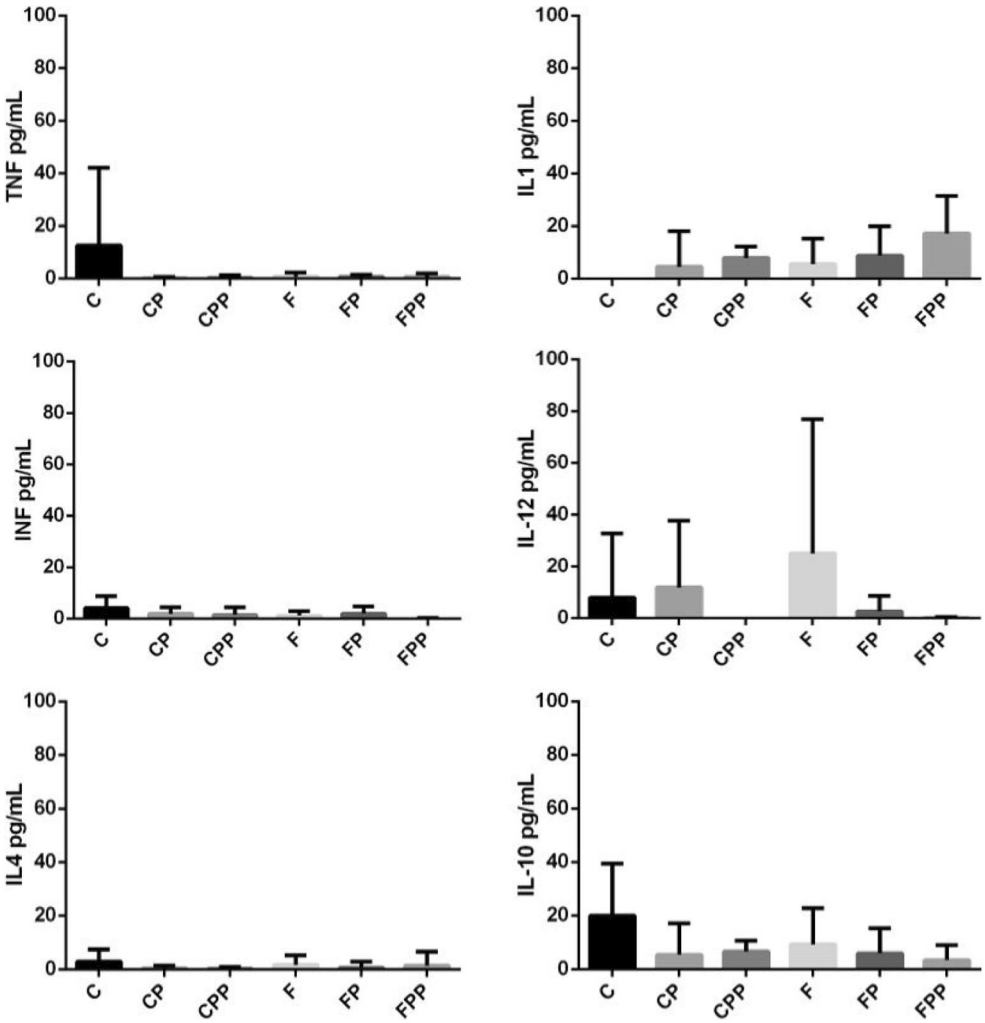



**Fig. 4** Mean of serum concentrations (in pg/mL) of TNF-α, IL-12, IL-4, IL-10, IL-1b, and INF-ɣ, of mice with experimental candidiasis without consuming probiotic (F), with candidiasis and consumption of probiotic during *Candida* inoculation, for 7 days (FP), with *Candida* inoculation and consumption of probiotic from 14 days before first inoculation, totaling 21 days of consumption (FPP), without induction of candidiasis or consumption of probiotics (C), without induction of candidiasis with consumption of probiotic for 7 days (CP), and without induction of candidiasis with consumption of probiotic for 21 days (CPP)

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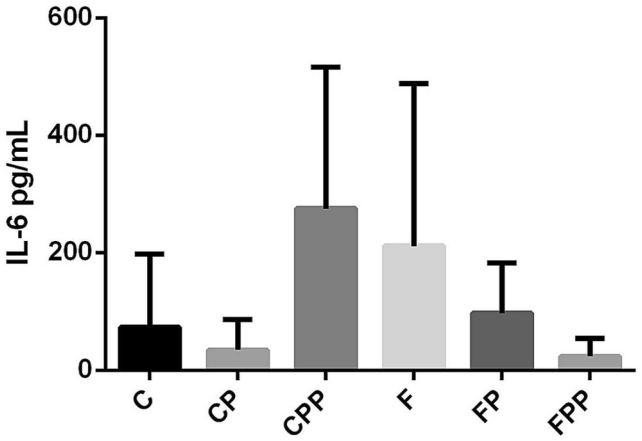



**Fig. 5** Mean of serum concentrations (in pg/mL) of IL-6 of mice with experimental candidiasis without consuming probiotic (F), with candidiasis with consumption of probiotic during *Candida* inoculation, for 7 days (FP), with *Candida* inoculation and consumption of probiotic from 14 days before first inoculation, totaling 21 days of consumption (FPP), without induction of candidiasis or consumption of probiotics (C), without induction of candidiasis with consumption of probiotic for 7 days (CP), and without induction of candidiasis with consumption of probiotic for 21 days (CPP)

presence of *L. rhamnosus*, prior to inoculation with *Candida*, prevented the colonization and, consequently, growth, effectively preventing the development of candidiasis.

Rossoni et al., 2017 [19] also observed the preventive role of probiotic strains of *Lactobacillus* spp. on *Candida* infections in a *Galeria mellonella* invertebrate host model. The authors found that lactobacilli improved the survival of *G. mellonella* infected with a lethal inoculum of *C. albicans*. Their results demonstrated that the immune response of *G. mellonella* could be stimulated with a prophylactic provision of probiotic bacteria, making them more resistant to virulent pathogens. The effects were associated with recruitment of hemocytes into the hemolymph and by stimulating antimicrobial peptide response.

In the present study, the consumption of probiotics may also have stimulated local and systemic immune system, improving the elimination of *Candida* and reducing candidiasis development. However, the observation of probiotics' immunostimulatory or immunomodulatory effects in the mice of this study was difficult, since low concentrations were observed in the majority of the investigated cytokines, with no significant differences among most of them.

Among the pro-inflammatory cytokines, significant changes were only observed when IL-1β and IL-6 were investigated. Consumption of probiotics for 21 days induced higher production of IL-1β, suggesting an immunostimulatory role for *L. rhamnosus*. Wagner and Johnson [20] also found that probiotic lactobacilli were able to induce the expression of IL-1α and IL-1β by NF-kB alternative intracellular signaling pathways, as MAPK/AP-1. In the present study, as significant differences were found just between the group with no *Candida* or *L. rhmanosus* and the group with *Candida* and probiotics for a longer period, probably the double microbial and longer stimulus, contributed for increasing production of this cytokine. This cytokine, in some way, may have exerted beneficial effect on the elimination of *Candida* or aided in preventing its colonization.

Regarding IL-6, among the groups with candidiasis induction, those with intake of probiotics for 21 days showed lower concentrations of cytokines, suggesting an immunomodulatory action of *L. rhamnosus*. IL-6 is among the most potent mediators of the acute response and participates in a cascade of reactions that starts with tissue damage and aims, ultimately, the restoration of tissue. Thus, modulation in the production of IL-6 could prevent the development of more exacerbated tissue injury. In fact, these animals had lower tissue alterations and inflammatory scores. Likewise, Habil et al. [21] reported that different strains of probiotic bacteria, including *L. rhamnosus*, were able to suppress the production of IL-6 in LPS-stimulated macrophages, also indicating an immunomodulatory role. These authors concluded that probiotic strains might perform activation, suppression, and/or modulation of immune functions, i.e., different effects, in different situations. In fact, in the control groups, probiotic intake induced different effects in IL-6 production. In these groups, probiotics seemed to favor the synthesis of this pro-inflammatory cytokine. High levels of IL-6 could provide a condition of resistance against *Candida*, favoring elimination and the restoration of homeostasis.

As IL-6, IFN-ɣ production was significantly reduced in animals with induced candidiasis and probiotic intake during 21 days. IFN-ɣ is required for efficient activation of phagocytes and contributes to a protective humoral and Th1 response, important mechanisms in the acquired defense against *Candida* [22]. Zhang et al. [23] reported that animals previously immunized against *Candida* had higher levels of Th1 cytokines and were more resistant to development of candidiasis or, when the disease was present, it was softer and more quickly solved. In this study, it was expected that the production of IFN-ɣ would be higher in the animals challenged with pathogens with better clinical condition. Probably the fact that animals of FPP group did not develop candidiasis had dismissed the necessity of an acquired immune response and the production of this cytokine.

It is important to consider that all animals were treated with dexamethasone, which is a potent inhibitor of cytokines production, by inhibiting the regulatory gene NF-kB [24]. Due to their anti-inflammatory, pro-apoptotic, and antiemetic properties, dexamethasone have been widely used in the treatment of inflammatory disorders and cancer. However, there is also some evidence to indicate that Dex could have a stimulatory effect on immune cells, including macrophages, T cells, and NK cells [25]. Therefore, the analysis of the different serum cytokine levels could have been severely influenced by the

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dexamethasone medication, limiting the conclusion regarding the real influence of probiotic consumption on cytokine production.

The protocol with dexamethasone was chosen since it was the best tested one to induce candidiasis. Mice models require the use of immunosuppressive agents or other predisposing factors to establish persistent infection since these animals are not naturally colonized by *C. albicans* [26]. The protocols that use antibiotics are also effective; however, they could interfere with the viability of the probiotic suspension and further impair the interpretation of the results. Thus, although the use of dexamethasone has contributed to the low and unexpected levels of cytokines observed, similar effects of probiotic consumption would be expected in patients requiring long-term use of immunosuppressors. Even so, other studies avoiding immunosuppression in the induction of candidiasis or with others sample sizes (since *Candida* counts were used as a parameter in the sample calculation) could clarify the real impact of the probiotic lactobacilli on cytokine serum levels.

*C. albicans* is an important opportunistic pathogen in the oral cavity. Besides candidiasis, most verified in immunosuppressed patients, it is considered the main etiological agent of denture stomatitis [8] and has being associated with caries development and/or progression [27]. The relationship of *Candida* and host is complex, involving other microorganisms and components of innate and adaptive immune response. Although Th1/Th17 response seems to be protective against candidiasis, Th1/Th2/Th17 balance may be important for better antifungal protection and reduction of the damages induced by cellular immunity [28, 29]. Thus, the consumption of probiotics could contribute to achieve this balance, even in immunosuppressed conditions, by stimulating the synthesis of certain cytokines and suppressing others.

The results obtained in this study suggest that consumption of *L. rhamnosus*, especially in a preventive manner, may reduce the development of candidiasis in immunosuppressed host. This work adds scientific evidences about the probiotic benefits.

**Funding** The work was supported by Fundação de Amparo à Pesquisa de São Paulo (FAPESP).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Informed consent** For this type of study, formal consent was not required.

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