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Oral treatment with *Saccharomyces cerevisiae* strain UFMG 905 modulates immune responses and interferes with signal pathways involved in the activation of inflammation in a murine model of typhoid fever

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ABSTRACT

Salmonella spp. are Gram-negative, facultative, intracellular pathogens that cause several diarrheal diseases ranging from self-limiting gastroenteritis to typhoid fever. Previous results from our laboratory showed that *Saccharomyces cerevisiae* strain UFMG 905 isolated from 'cachaça' production presented probiotic properties due to its ability to protect against experimental infection with *Salmonella enterica* serovar Typhimurium. In this study, the effects of oral treatment with *S. cerevisiae* 905 were evaluated at the immunological level in a murine model of typhoid fever. Treatment with *S. cerevisiae* 905 inhibited weight loss and increased survival rate after *Salmonella* challenge. Immunological data demonstrated that *S. cerevisiae* 905 decreased levels of proinflammatory cytokines and modulated the activation of mitogen-activated protein kinases (p38 and JNK, but not ERK1/2), NF-κB and AP-1, signaling pathways which are involved in the transcriptional activation of proinflammatory mediators. Experiments in germ-free mice revealed that probiotic effects were due, at least in part, to the binding of *Salmonella* to the yeast. In conclusion, *S. cerevisiae* 905 acts as a potential new biotherapy against *S.* Typhimurium infection due to its ability to bind bacteria and modulate signaling pathways involved in the activation of inflammation in a murine model of typhoid fever.

Introduction

In recent years, worldwide interest in the use of functional foods containing probiotic bacteria for health promotion and disease prevention has increased significantly. According to the currently adopted definition by the World Health Organization, probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO, 2002). Previous results obtained in our laboratory showed that *Saccharomyces cerevisiae* strain UFMG 905, isolated from 'cachaça' (a Brazilian typical beverage) production, was able to colonize and survive in the gastrointestinal tract of germ-free and conventional mice and to protect them against experimental infections with *Salmonella enterica* serovar Typhimurium and *Clostridium difficile* (Martins et al., 2005). Our results also showed that protection was not due to a reduction of the intestinal population of the pathogenic bacteria. Additional results showed that *S. cerevisiae* 905 was able to reduce the translocation of *S.* Typhimurium and to stimulate the immune system in mice (Martins et al., 2007). At the histological level, *S. cerevisiae* 905 conferred protection to intestine and liver tissues, decreased inflammatory foci in liver, and promoted an increase in the number of Kupffer cells after experimental infection with *S.* Typhimurium (Martins et al., 2005). Recent data demonstrated that this yeast protected against bacterial translocation, preserved gut barrier integrity, and stimulated the immune system in a murine model of intestinal obstruction (Generoso et al., 2010).

With an estimated 16–33 million annual cases which result in 500,000–600,000 deaths in endemic areas, the World Health Organization identifies typhoid fever as a serious public health problem

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(Crump et al., 2004). *Salmonella* spp. are Gram-negative, facultative, intracellular pathogens that cause several diarrheal diseases ranging from self-limiting gastroenteritis to typhoid fever. In humans, typhoid fever is caused by *Salmonella enterica* serovar Typhi. An essential feature of the pathogenicity of *Salmonella* is their ability to engage the host cell in a two-way biochemical interaction, or cross-talk, which leads to responses from both the bacteria and the host cell (Galán and Bliska, 1996). The bacterium induces its own uptake through virulent proteins delivered into cytoplasm of infected cells by a specialized mechanism known as type III protein secretion system (TTSS) (Collazo and Galán, 1996; Zaharik et al., 2002) that activates signaling pathways involved in cytoskeleton rearrangements and cellular uptake processes (Galán and Collmer, 1999). This interaction between invading pathogen and host epithelium also leads to activation of a program of epithelial gene expression, such as those with proinflammatory functions (Kagnoff and Eckmann, 1997). Consequently, initial invasion results in the activation of various transcription factors which ultimately result in the production of proinflammatory cytokines such as IL-8, in response to *Salmonella*-mediated activation of mitogen-activated protein kinase (MAPK) cascade and activation of transcription factors such as AP-1 (activator protein 1) and NF-κB (nuclear factor kappa B) (Hobbie et al., 1997). This is an important event in *Salmonella* pathogenesis, since one of the hallmarks of salmonellosis is the stimulation of a profuse inflammatory diarrhea induced by pro- and inflammatory cytokines.

Here, we evaluated the effects of oral treatment with *S. cerevisiae* 905 on *Salmonella*-induced infection in mice. We show that probiotic treatment reduced weight loss and mortality and modulated signaling pathways involved in the activation of inflammatory responses induced by *Salmonella*.

Materials and methods

Microorganisms and growth conditions

The bacterial strain *Salmonella enterica* serovar Typhimurium (ATCC 14028) was kindly provided by Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, RJ, Brazil. The bacterium was stored at −80 °C in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD, USA) with 15% glycerol and grown in BHI broth at 37 °C during 18 h under aerobic conditions without shaking for reactivation.

S. cerevisiae strain UFMG 905 belongs to the Yeasts Bank of Dr. Carlos A. Rosa (Laboratory of Ecology and Biotechnology of Yeasts, Dept. of Microbiology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil). Identity of the strain was determined as *S. cerevisiae* using the computer program YEASTCOMPARE (Ciriello and Lachance, 2001). The yeast was grown overnight at 37 °C, with shaking, in YPD (yeast extract 1%, peptone 1%, and dextrose 2%) broth. The culture was then concentrated to obtain 9.0 log of colony-forming units (CFU) ml^{-1}.

Mice, treatment, and infection procedures

Germ-free 21–23-day-old NIH mice (Taconic, Germantown, NY, USA) were used in this work. The animals were housed in flexible plastic isolators (Standard Safety Equipment Company, McHenry, IL, USA) and handled according to established procedures (Pleasants, 1974). Experiments with gnotobiotic mice were carried out in microisolators (UNO Roestvaststaal B.V., Zevenaar, The Netherlands). Conventional NIH mice were derived from the germ-free colony and only used after at least 2 generations following the conventionalization. Water and commercial autoclavable diet (Nuvital, Curitiba, PR, Brazil) were sterilized by steam and administered ad libitum, and animals were maintained in an open animal house with controlled lighting (12 h light, 12 h dark). All experimental procedures were carried out according to the standards set forth in the 'Guide for the Care and Use of Laboratory Animals' (National Research Council, 1996). The study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFMG, protocol no. 197/2007).

For probiotic treatment, conventional experimental mice received by oral gavage a daily dose of 0.1 ml containing 9.0 log CFU ml^{-1} 10 days before infection, and treatment was continued during all the experimental infection. Germ-free mice received by oral gavage a unique dose of 0.1 ml containing 9.0 log CFU ml^{-1} 10 days before infection. Control mice received only sterile water by oral gavage following the same procedure that their experimental counterparts. For *S.* Typhimurium experimental infection, conventional mice were inoculated intragastrically with 0.1 ml of a bacterial suspension containing 5.0 log CFU ml^{-1}.

Experimental design

To evaluate the effects of the treatment with the yeast on the morbidity and mortality during an experimental bacterial challenge, 30 conventional animals were divided into 3 groups ($n = 10$ in each group): (C) control 1 group (mice receiving only sterile water by oral gavage), (ST) mice receiving sterile water by oral gavage and challenged with *S.* Typhimurium, and (905 + ST) mice treated by oral gavage with *S. cerevisiae* 905 and challenged with *S.* Typhimurium. During 38 days (10 days of yeast pretreatment before challenge and 28 days post-challenge) mice were analyzed for clinical signs, weight, and mortality induced by *S.* Typhimurium infection. Clinical signs were evaluated by diarrhea (consistency and presence of feces on cages wall), morbidity, and fecal blood (Hemaccult cards, INLAB-Diagnostica, São Paulo, SP, Brazil).

For molecular and immunological analysis, 60 conventional animals were divided into 2 groups ($n = 30$ in each group): (ST) control mice receiving sterile water and then challenged with *S.* Typhimurium, and (905 + ST) experimental mice receiving *S. cerevisiae* 905 and then challenged with *S.* Typhimurium. By days 0, 1, 5, 10, and 15 post-challenge, 5 animals of each group were sacrificed by cervical dislocation. Colons were collected for ELISA and Western blot.

For experiments of probiotic–pathogen binding, germ-free mice were used. Mice were pretreated with *S. cerevisiae* 905 during 10 days and then inoculated intragastrically with 0.1 ml of a bacterial suspension containing 7.0 log CFU ml^{-1}. After 2 h, mice were sacrificed by cervical dislocation, and the small intestine tissues were fixed and processed for scanning electron microscopy.

Cytokines and chemokine determinations

The concentration of CXCL-1/KC, IL-6, IL-10, TGF-β, TNF-α, and IFN-γ were measured by ELISA in colons of animals using commercially available antibodies according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN, USA). Aliquots of the colon (100 mg) were homogenized in 1 ml PBS (0.4 M NaCl and 10 mM $NaPO_4$) containing antiproteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 10,000 rpm, and the supernatant was collected, diluted at 1:3 in PBS, and used immediately for assays, as previously described (Souza et al., 2004).

Cytosolic and nuclear extracts and Western blotting analysis

Nuclear extracts were obtained from powdered colon and prepared as described by Dignam et al. (1983) with minor modifica-

tions (Souza et al., 2009). Briefly, 30 mg of tissue were homogenized in ice-cold hypotonic lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.002% NaN_3, 1 mM PMSF, 0.1 mM EGTA, 10 μM aprotinin, 20 μM leupeptina, 0.5 mM DTT, 25 mM NaF) chilled on ice for 15 min and then 5% NP-40 added for further 5 min. The supernatant containing the cytosolic fraction was removed and stored at −80 °C. The nuclear pellet was ressuspended in 200 μl of high salt extraction buffer (20 mM HEPES pH 7.4, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.01% NaN_3, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM PMSF, 10 μM aprotinin, 20 μM leupeptin, 0.5 mM DTT) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 rpm, and supernatant was aliquoted and stored at −80 °C. Whole-cell extracts were prepared as previously described (Sousa et al., 2005). Protein was quantified using the Bradford assay reagent from Bio-Rad (Hercules, CA, USA).

Nuclear (30 μg) or whole- (60 μg) cell extracts were separated by electrophoresis on a denaturing 10% polyacrylamide-SDS gel and transferred to nitrocellulose membranes, as previously described (Sousa et al., 2005). Membranes were blocked overnight at 4 °C with PBS containing 5% (w/v) non-fat dry milk and 0.1% Tween 20, washed 3 times with PBS containing 0.1% Tween 20, and then incubated at a dilution of 1:1000 with specific primary antibodies: anti-p65/*RelA* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-p38, anti-phospho-ERK1/2, anti-phospho-JNK, anti-phospho-IκB-α, anti-phospho-jun, anti-fos (Cell Signaling Technology, Beverly, MA, USA), or β-actin (Sigma-Chemicals, St. Louis, MO, USA) in phosphate-buffered saline containing 5% (w/v) BSA and 0.1% Tween 20. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:3000, Cell Signaling Technology). Immunoreactive bands were visualized by using an enhanced chemiluminescence detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ, USA).

Scanning electron microscopy (SEM)

Tissues (cecum) were fixed overnight at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. Then, they were treated with 1% osmium tetroxide solution plus 0.2% potassium ferrocyanide in the same buffer for 1 h. After dehydration with increasing acetone concentrations (30–100%), tissues were dried by the critical point device with CO_2, as previously described (Pimenta and De Souza, 1985). The next steps were mounting in SEM stubs, coating with gold particles in a sputtering, and analyzing in a JEOL 5600 SEM.

Statistical analysis

The results were expressed as the average of at least 2 independent experiments. The data were statistically analyzed using the exact Fisher or Student's *t*-test at a probability level of 0.05. Statistical analyses were performed using the program Sigma Stat (Jandel Scientific Software, version 1.0, San Rafael, CA, USA).

Results

S. cerevisiae 905 inhibited weight loss and increased survival of mice after S. Typhimurium infection

Mice only infected with *S.* Typhimurium (ST) stopped gaining body weight just after infection and failed to recover until 20 days when compared to the uninfected (C) group (Fig. 1A). In addition, *Salmonella* infection caused the death of 60% of mice by day 28 of the experiment (Fig. 1B). Mice with an administration of *S. cerevisiae* 905 (905) 10 days before *S.* Typhimurium infection maintained a similar body weight gain after the pathogenic challenge when compared to the uninfected group (Fig. 1A) and induced a lower mortality rate (20%) than in ST group ($P < 0.05$). Diarrhea and fecal blood were mainly observed in animals in the ST group (data not shown).

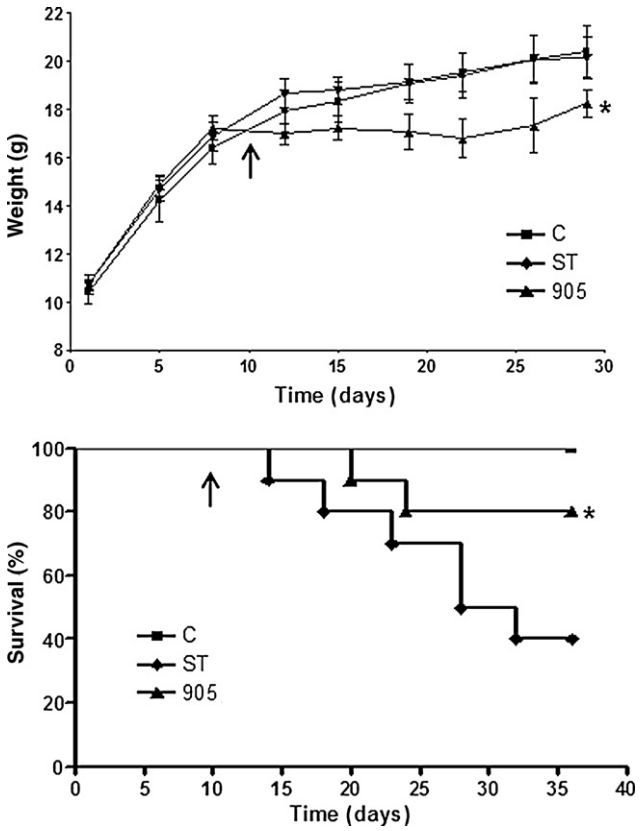

Fig. 1. Effect of treatment with *S. cerevisiae* 905 on weight gain (A) and survival (B) of mice non-treated and non-challenged (Control), mice receiving orally sterile water and challenged with *S.* Typhimurium (ST), or mice pretreated orally with *S. cerevisiae* 905 and challenged with *S.* Typhimurium (905). $n = 10$ in each group. $^*P < 0.05$ in relation to control group. Arrows indicates the day of *Salmonella* infection.

S. cerevisiae 905 diminished inflammatory cytokines induced in mice challenged with S. Typhimurium

The interaction between *S.* Typhimurium and host epithelium leads to activation of a program of epithelial gene expression, such as those with proinflammatory functions, including chemokines and cytokines with inflammatory properties. To assess the effects of *S. cerevisiae* 905 on cytokine production in the colon of mice challenged with *S.* Typhimurium, animals were divided into 2 groups, as described above, and the chemokine CXCL-1/KC (the mouse ortholog of GRO-α) and cytokines IL-6, TNF-α, IFN-γ, IL-10, and TGF-β were measured at 0, 1, 5, 10, and 15 days post-infection. As shown in Fig. 2, *S. cerevisiae* 905 diminished basal levels of all cytokines measured at the day of the infection with *Salmonella* (i.e., 10 days after the beginning of treatment with *S. cerevisiae* 905). Basal levels of TNF-α (Fig. 2C) and TGF-β (Fig. 2E) were undetectable in treated animals. *S.* Typhimurium infection induced an increase in several cytokines, particularly for higher levels of KC, IL-6, TNF-α, and INF-γ in ST mice by day 10 after infection (Fig. 2). In animals which were given *S. cerevisiae* 905 prior to infection, levels of KC were similar to those found in uninfected mice during all the experimental period (Fig. 2A), and a statistically significant decrease was observed for levels of IL-6 (Fig. 2B) and TNF-α (Fig. 2C) by day 10 post-challenge with *Salmonella*.

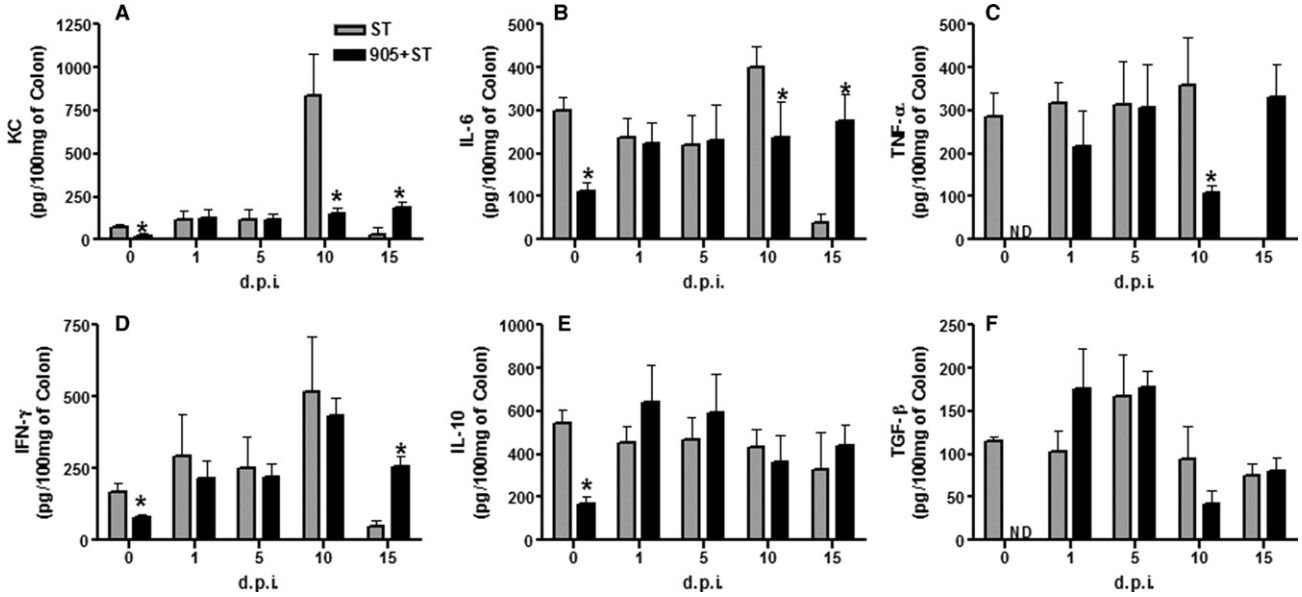

Fig. 2. Effects of *S. cerevisiae* 905 on KC (A), IL-6 (B), TNF-α (C), IFN-γ (D), IL-10 (E), and TGF-β (F) levels in mice receiving orally sterile water and challenged with *S.* Typhimurium (ST) and mice pretreated orally with *S. cerevisiae* 905 and challenged with *S.* Typhimurium (905 + ST). Cytokines contents were estimated by ELISA in different days after infection. $n = 5$ for each point. $^*P < 0.05$ in relation to *Salmonella*-infected group for the same day.

S. cerevisiae 905 controls inflammation through inhibition of signal pathways involved in inflammatory response

Once *S. cerevisiae* 905 decrease the levels of inflammatory cytokines induced by *Salmonella* infection in mice (Fig. 2), a possible modulation by the yeast of signaling transduction pathways that govern the induction of such cytokines during the course of *Salmonella* infection in mice was investigated. Thus, MAPKs (p38, JNK, and ERK1/2) (Fig. 3A), NF-κB (p65-*RelA* and phospho-IκB-α) (Fig. 3B), and AP-1 (phospho-jun and c-fos) (Fig. 3C) were analyzed at 0, 1, 5, 10, and 15 days post-infection. As it can be observed, *Salmonella* activated p38 and JNK MAPKs (Fig. 3A), as soon as promoted p65/*RelA* nuclear translocation and phosphorylation of its inhibitory protein (IκB-α) (Fig. 3B), and activated AP-1 (indirectly analyzed by phospho-jun and c-fos activation) (Fig. 3C). In the presence of the yeast, activation of JNK was completely abolished, p38

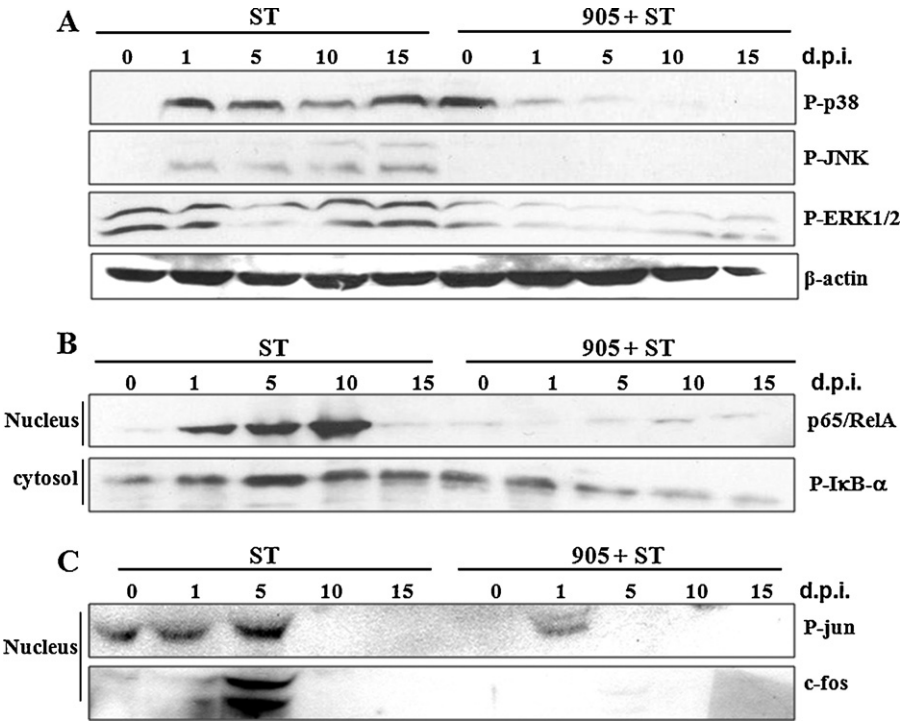

Fig. 3. Effects of *S. cerevisiae* 905 on *S.* Typhimurium-induced p38, JNK, and ERK1/2 MAPKs activation (A), NF-κB (p65/RelA and P-IκB) activation (B), and AP-1 (phospho-jun and c-fos) activation (C) in the colon of mice receiving orally sterile water and challenged with *S.* Typhimurium (ST) or mice pretreated orally with *S. cerevisiae* 905 and challenged with *S.* Typhimurium (905 + ST). Samples were fractioned in SDS-PAGE and analyzed by immunoblotting with specific antibodies in total or nuclear extracts. The Western blots showed are one representative of 3 similarly, but independently performed.

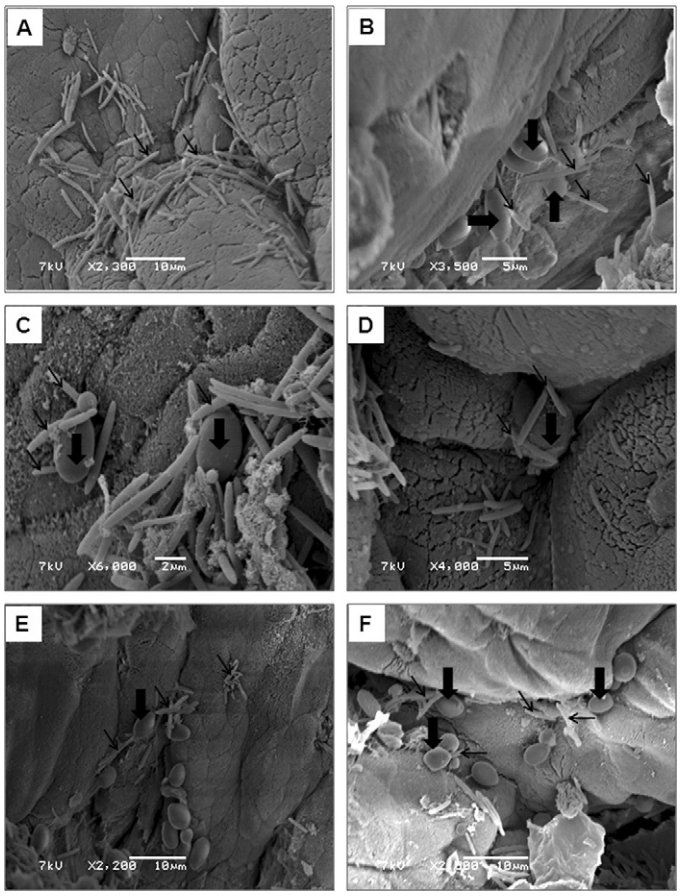

Fig. 4. Scanning electron microscopy showing *S.* Typhimurium adhesion on *S. cerevisiae* 905 cells in cecum tissue. (A) *S.* Typhimurium-infected mice, (B–F) mice previously treated with *S. cerevisiae* 905 and then infected with *S.* Typhimurium. Small arrows indicate *S.* Typhimurium, thick arrows indicate yeast cells. Magnification is shown in each figure.

was diminished, and ERK1/2 was not affected (Fig. 3A). The yeast completely inhibited the translocation of NF-κB p65 to the nucleus and also diminished the phosphorylation of IκB-α, a hallmark of IκB degradation (Fig. 3B), and significantly diminished activation of AP-1 (through phospho-jun and c-fos analysis) as well (Fig. 3C).

S. cerevisiae 905 affects S. Typhimurium translocation via bacteria–yeast binding

Since we have observed that the yeast was able to retard *Salmonella* translocation in a germ-free mouse model and prevented translocation in conventional mice (Martins et al., 2007) and that this effect was not due to lowering in bacterial colonization (Martins et al., 2005), we have hypothesized that the yeast may be protecting mice and preventing translocation via binding to bacteria, as we have previously observed in a cell culture model for *Saccharomyces boulardii* (Martins et al., 2010). To test our hypothesis, an in vivo model using germ-free mice was used to visualize the interaction between the yeast and the pathogenic bacterium without the interference of the indigenous microbiota. As it can be seen in Fig. 4, in *Salmonella* mono-infected mice, bacteria covered the epithelium in a homogeneously distributed way (Fig. 4A), but when mice were mono-associated with the yeast prior to adding bacteria, binding between yeast and bacteria was clearly observed (Fig. 4B–F). Additionally, in the presence of *Saccharomyces*, the bacteria seemed to be attracted to the yeast surface and were not homogeneously distributed.

Discussion

We have previously demonstrated that *S. cerevisiae* 905 had a potential for probiotic use because of its ability to survive in the mammal gastrointestinal tract and to protect mice against *S.* Typhimurium and *C. difficile* infections in mice (Martins et al., 2005) as well as to inhibit bacterial translocation and to modulate both local and systemic immunity (Martins et al., 2007; Generoso et al., 2010). The present study confirms this potential and presents some of the mechanisms which can explain the protective effect of the yeast in a murine model experimentally infected with *S.* Typhimurium. Infection of mice with *Salmonella* induced significant clinical manifestations, tissue damage, and lethality, which were associated with an activation of inflammation-signaling pathways. Previous treatment with *S. cerevisiae* 905 prevented this activation of signaling pathways with consequent reduction of inflammation, clinical manifestations, tissue damage, and death. Mechanistically, this preventing effect could be due, at least in part, to a preferential binding of the *Salmonella* to the yeast than to gut epithelial cells.

In the majority of cases, infectious diarrhea is treated through rehydration or an eventual use of antibiotics. However, the World Health Organization has recommended the search for alternative treatments for infection, and probiotics have been proposed for this purpose (Vieira et al., 2008). Although no proof of efficacy of such treatment against salmonellosis has been demonstrated in humans, results obtained in murine models have indicated that some probiotic microorganisms may be efficient against *Salmonella* infection (Jain et al., 2008, 2009; Truusalu et al., 2008; Martins et al., 2009).

In mice, infection with *S.* Typhimurium gives rise to enteric fever, with symptoms similar to those observed in humans after infection with *S.* Typhi (Eisenstein, 1999; Santos et al., 2001), such as intense inflammation characterized by the release of proinflammatory cytokines (IL-1 and TNF-α) and chemokines (KC). Other proinflammatory cytokines involved in host defense against *S.* Typhimurium infection include IFN-γ, IL-12, and IL-18. In the anti-inflammatory group of cytokines, IL-4, IL-10, and TGF-β have been shown to down-regulate inflammatory responses (reviewed by Eckmann and Kagnoff, 2001; Coburn et al., 2007). The release of inflammatory cytokines is under the control of many signal transduction pathways, including NF-κB and AP-1 transcription factors, and MAPK pathway (Hobbie et al., 1997; Hoffmann et al., 2002). In the present study, the inhibitory effect of the yeast on the inflammatory response induced by *Salmonella* infection was demonstrated, and this seems to be a major mechanism by which the yeast prevented inflammation and disease after *Salmonella* infection. To explain why the treatment with the yeast, before *Salmonella* challenge, diminished all the cytokines evaluated, it could be speculated that the yeast could: (i) down-regulate the baseline inflammation and/or the complex balance between Th1 and Th2 responses, as already observed by Jawhara and Poulain (2007), (ii) modulate the population levels of some members of the intestinal microbiota, which in turn could, in part, down-regulate the immune system, as reviewed by Wohlgemuth et al. (2010) and Reiff and Kelly (2010), and (iii) up-regulate anti-inflammatory cytokines at the beginning of infection.

In an attempt to define the mechanisms by which *S. cerevisiae* 905 prevented the activation of inflammation-signaling pathways normally induced by the *Salmonella* infection, a possible binding between the yeast and the bacteria was evaluated. Experiments were conducted in germ-free mice to facilitate the visualization of the interaction between the yeast and bacteria. The electronic microscopy showed that bacterial cells bound preferentially to *S. cerevisiae* 905 than to intestinal epithelial cells when the yeast was present. Some authors have already demonstrated that bacteria expressing type 1 fimbria, such as *Salmonella* and *Escherichia coli*, are able to bind to *S. boulardii* and some strains of *S. cerevisiae*

through mannose residues (Kornonen et al., 1981; Gedek, 1999; Perez-Sotelo et al., 2005; Martins et al., 2010). It is very reasonable to hypothesize that the binding of *S.* Typhimurium to *S. cerevisiae* 905 surface instead of to mice epithelium surface could be responsible for the diminution of activation of MAPKs, NF-κB, AP-1, and consequently of inflammatory cytokine production.

In conclusion, *S. cerevisiae* 905 acts as a potential new biotherapy against *S.* Typhimurium infection in part due to its interference on signal pathways involved in the activation of inflammation in a typhoid fever murine model. Electronic microscopy data suggest that preferential binding of the bacteria to the yeast prevents activation of proinflammatory signal transduction pathways in epithelial cells with consequent diminishing of inflammation.

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