Institut Pasteur
Microbes and Infection 15 (2013) 270–279
www.elsevier.com/locate/micinf

Original article

# Inhibition of tissue inflammation and bacterial translocation as one of the protective mechanisms of *Saccharomyces boulardii* against *Salmonella* infection in mice

Flaviano S. Martins [a,b,*], Angélica T. Vieira [a], Samir D.A. Elian [b], Rosa M.E. Arantes [c], Fabiana C.P. Tiago [b], Lirlândia P. Sousa [d], Helena R.C. Araújo [e], Paulo F. Pimenta [e], Cláudio A. Bonjardim [b], Jacques R. Nicoli [b], Mauro M. Teixeira [a]

[a] *Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[b] *Department of Microbiology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[c] *Department of Pathology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[d] *Department of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[e] *Laboratory of Medical Entomology, Institute René Rachou, Fiocruz, Belo Horizonte, MG, Brazil*

Received 29 June 2012; accepted 3 December 2012
Available online 30 January 2013

**Abstract**

Growing evidences suggest that *Saccharomyces boulardii* (SB) is efficacious against bacterial infections and inflammatory bowel diseases. This study investigated the effects of treatment with SB provided in a murine model of typhoid fever. Mice were divided into two groups: (1) control animals challenged with *Salmonella* Typhimurium (ST), and (2) animals receiving SB, and then challenged with ST. At days 0, 1, 5, 10 and 15 post-challenge, animals were euthanized and tissues collected to analyze bacterial translocation, cytokines, signaling pathways and histological analysis. Survival rate and animal weight were also evaluated. Treatment with SB increased survival rate and inhibited translocation of bacteria after ST challenge. Histological data showed that SB also protected mice against liver damage induced by ST. SB decreased levels of inflammatory cytokines and activation of mitogen-activated protein kinases (p38, JNK and ERK1/2), phospho-IκB, p65-*RelA*, phospho-jun and c-fos in the colon, signal pathways involved in the activation of inflammation induced by ST. Further experiments revealed that probiotic effects were due, at least in part, to the binding of ST to the yeast. Such binding diminishes ST translocation, resulting in decreased activation of signaling pathways which lead to intestinal inflammation in a murine model of typhoid fever.


*Keywords:* *Saccharomyces boulardii*; *Salmonella enterica* serovar Typhimurium; Bacterial translocation; Cytokines; Mitogen-activated protein kinases

## 1. Introduction

*Salmonella enterica* is a Gram-negative facultative intracellular anaerobe of worldwide importance causing as many as 1.3 billion cases of disease annually. Over 2500 serovars of *S. enterica* have been identified belonging to six subspecies [1]. In humans, ingestion of various *Salmonella* serovars gives rise to infection of the small intestine and to gastroenteritis. A small number of *Salmonella* serovars can lead to systemic infection and enteric fever. Typhoid fever in humans, caused by *S. enterica* serovar Typhi, is the prototype of such disease. Without treatment, it poses a threat to human health, and in developing countries typhoid fever is still an important cause of morbidity and mortality, especially for children [2]. In 2000, typhoid fever caused an estimated 21.7 million illnesses and 217,000 deaths [3].

* Corresponding author. Laboratório de Agentes Bioterapêuticos - Bloco J4, sala 255, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 Pampulha, 31270-901 Belo Horizonte, MG, Brazil. Tel.: +55 31 3409 2738; fax: +55 31 3409 2730.
*E-mail addresses:* flaviano@icb.ufmg.br, flavianosmartins@yahoo.fr (F.S. Martins).

1286-4579/$ - see front matter © 2013 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.micinf.2012.12.007

All *Salmonella* serotypes share the ability to invade the host by inducing their own uptake into intestinal epithelial cells. This uptake is induced by virulence proteins delivered into cytoplasm of infected cells by a specialized mechanism known as type III protein secretion system (TTSS) [4]. These proteins activate signaling pathways involved in cytoskeleton rearrangements and cellular uptake processes [5]. Besides facilitating the invasion process, the interaction between invading pathogen and host epithelium also leads to activation of a program of epithelial gene expression, including of genes with pro-inflammatory functions [6–9]. Consequently, initial invasion results in inflammation and production of pro-inflammatory 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) [10].

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 FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" [11]. Among the best studied and characterized probiotics are specific strains of the species *Saccharomyces boulardii*. *S. boulardii* is a tropical strain of yeast first isolated from lychee fruit in 1923 by French scientist Henri Boulard [12]. Although its taxonomy is a matter of debate, it is related to, but distinct from, *Saccharomyces cerevisiae* in several taxonomic, metabolic, and genetic properties [13,14].

*S. boulardii* is used worldwide for the prevention and treatment of a variety of diarrheal diseases and inflammatory bowel diseases. In the case of infectious diarrhea, administration of *S. boulardii* to animals provides protection against intestinal lesions caused by several diarrheal pathogens [15]. The mechanisms by which *S. boulardii* exerts its protective effects include antitoxin effects [16–19], trophic effects on enterocytes [20–22], anti-inflammatory effects [23,24], increased immune response [25–27], binding and elimination of bacterial toxins [28], binding to and elimination of pathogenic bacteria [29,30], interference on bacterial-induced signaling pathways [30–32], actions on bacterial virulence factors [33], and interference on bacterial motility [34].

We have previously shown that the preventive treatment with *S. boulardii* diminishes mortality of mice challenged with *S.* Typhimurium [30,35]. Using an in vitro model we have investigated some mechanisms involved in this protection due to the interaction of *S. boulardii* with *S.* Typhimurium [30]. In the present study, we have followed-up such mechanisms using an in vivo model. We examined the effect(s) of *S. boulardii* on infection by *S.* Typhimurium utilizing a murine model of infection simulating human typhoid fever.

## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

The bacterial strain *S. enterica* serovar Typhimurium (ATCC 14028) was kindly provided by Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, 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. Cultures of *S. boulardii* were obtained by inoculating a commercial lyophilized preparation of the yeast (Floratil®, MERCK, Rio de Janeiro, Brazil) and growing overnight at 37 °C, with shaking, in YPD (yeast extract 1%, peptone 1%, and dextrose 2%) broth. The culture was then concentrated in the same medium to obtain 9.0 log of colony-forming units (CFU) $ml^{-1}$. For animal oral administration the yeast was centrifuged and re-suspended in phosphate buffered saline (PBS) at the time of inoculation [35].

### 2.2. Mice and infection procedure

Germ free 21-23 day-old NIH Swiss outbred 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 [36]. Experiments with gnotobiotic mice were carried out in micro-isolators (UNO Roestvaststaal B.V., Zevenaar, The Netherlands). Conventional NIH Swiss outbred mice were derived from the germ free colony and only used after at least two generations following the conventionalization [37]. Water and commercial autoclavable diet (Nuvital, PR, Brazil) were sterilized by steam and administered *ad libitum*, and animals were maintained in a ventilated animal caging system (Alesco Ltda., Campinas, SP, Brazil) with controlled lighting (12 h light, 12 h dark). All experimental procedures were carried out according to the standards set forth by the Brazilian College for Animal Experimentation [38]. The study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFMG, protocol n° 197/2007).

For probiotic treatment, conventional 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 [30,35]. For *S.* Typhimurium experimental infection (bacterial challenge), mice were inoculated intragastrically with 0.1 ml of a bacterial suspension containing 5.0 log CFU $ml^{-1}$ (based on a LD50 previous experiment).

### 2.3. Experimental design

Sixty conventional animals were divided into two groups ($n = 30$ in each group): (1) control animals receiving sterile water and then challenged with *S.* Typhimurium, and (2) experimental animals receiving *S. boulardii* (treated group) and then challenged with *S.* Typhimurium. At the days 0, 1, 5, 10 and 15 post-challenge, five animals of each group were killed by cervical displacement. The liver was aseptically collected for bacterial translocation. Colons were collected for ELISA (distal colon) and Western blot (median colon). Colon (proximal part), ileum and liver were used for histological analysis.

For experiments of probiotic-pathogen binding, germ free mice were used, as previously described [39]. Mice received a single dose of *S. boulardii* 10 days before challenge, and then inoculated intragastrically with 0.1 ml of a bacterial suspension containing 5.0 log CFU $ml^{-1}$. After 1, 3 and 6 h mice were euthanized and the intestinal epithelium was fixed and processed for scanning electron microscopy.

### 2.4. Determination of S. boulardii protective effect

To analyze the protective effect of *S. boulardii* another group of conventional animals were divided into four groups ($n = 10$ in each group): (1) control 1 group (mice receiving only sterile PBS by oral gavage), (2) control 2 group (mice receiving *S. boulardii* daily by oral gavage), (3) mice challenged with *S.* Typhimurium, and (4) mice pre-treated (during 10 days) with *S. boulardii* and challenged with *S.* Typhimurium. During 38 days (10 days before challenge and 28 days post-challenge) mice were analyzed for clinical signs, weight and mortality induced by *S.* Typhimurium infection.

To verify the therapeutic effect of *S. boulardii* another group of conventional animals were divided into eight groups ($n = 10$ in each group): (1) mice challenged with *S.* Typhimurium, (2) mice pre-treated with *S. boulardii* and then infected with *S.* Typhimurium, (3) mice infected with *S.* Typhimurium and treated with *S. boulardii* at the same day; mice infected with *S.* Typhimurium and treated with *S. boulardii* after 2 (group 4), 4 (group 5), 6 (group 6), 8 (group 7), and 10 (group 8) days of infection.

### 2.5. Measurement of bacterial translocation

After sacrifice, liver was aseptically collected, weighed, and homogenized in sterile PBS (1:10, w/v). Serial decimal dilutions were prepared and 100 μl aliquots were plated onto MacConkey agar (Difco). Colonies were counted after incubation at 37 °C for 24 h.

### 2.6. Cytokines and chemokine determinations

Distal colon (100 mg) was homogenized in 1 ml PBS (0.4 M NaCl and 10 mM $NaPO_4$) containing anti-proteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 Kallikrein Inhibitor Units of aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 10,000 rpm, and the supernatant was used immediately for assays, as previously described [39]. The concentration of CXCL-1/KC, IL-6, TNF-α, IFN-γ IL-10 and TGF-β, were measured by ELISA using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN, USA).

### 2.7. Cytosolic and nuclear extracts and Western blot analysis

Nuclear cell extracts were obtained from homogenized intestine (median colon), as previously described [40]. Briefly, 30 mg of colon were homogenized in ice-cold hypotonic lyses 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 addiction of 5% NP-40 for further 5 min. The supernatant containing the cytosolic fraction was removed and stored at −80 °C. The nuclear pellet was resuspended 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 [39]. Protein was quantified using the Bradford assay reagent from Bio-Rad (Hercules, CA, USA). Nuclear (30 μg) or cytosolic (60 μg) extracts were separated by electrophoresis on a denaturing 10% SDS-PAGE and transferred onto nitrocellulose membranes, as previously described [39]. Membranes were blocked overnight at 4 °C, and then incubated with anti-p65/*RelA* antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with specifics antibodies: anti-phospho-p38, anti-phospho-ERK1/2, anti-phospho-JNK, anti-phospho-IκB-α, anti-phospho-jun, anti-fos, and β-actin (Cell Signaling Technology, Beverly, MA, USA) according to the procedures supplied by the manufacturer. 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).

Levels of protein were quantified by densitometry using the LabImage software (LabImage, Leipzig, Germany). Levels of cytosolic proteins were normalized to the levels of β-actin in the same sample and results expressed as Protein/β-actin ratio in arbitrary units.

### 2.8. Histological analysis

Tissue samples from small and large intestine and liver were fixed in buffered 4% formaldehyde and processed for paraffin embedding. The histopathological sections (3–5 μm) were stained with hematoxylin-eosin (H&E). The slides were coded and examined by a single pathologist, who was unaware of the experimental conditions of each group. The representative histopathological aspects of intestines and liver were documented using a digital camera and the software Image-Pro (Media Cybernetics, Bethesda, MD, USA).

For morphometric examination of the liver, the images were obtained by a JVC TK-1270/RGB microcamera and the KS 300 Software built into a Kontron Elektronick/Carl Zeiss image analyser (Oberkohen, Germany). The inflammatory foci were considered as a damage index for hepatic tissue. Inflammatory focus is defined as an accumulation of inflammatory cells in number higher than 10 cells, accompanied by necrotic alterations of the associated parenchyma. The unit of focus area measured in all animals is the sum of ten camps

(10×). The number of granulocytes was counted per 100 inflammatory cells at histological sections [41].

### 2.9. Scanning electron microscopy (SEM)

Tissues 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 (20–100%), tissues were dried by the critical point device with $CO_2$, as previously described [42]. The next steps were mounting in SEM stubs, coating with gold particles in a sputtering and analyzing in a JEOL 5600 SEM.

### 2.10. Statistical analysis

The results were expressed as the average of at least two independent experiments. Results are shown as the mean ± SEM. Differences were evaluated by using the Student's *t*-test, Analysis of Variance (ANOVA) or Survival Log Rank test. The test performed for each experiment is indicated in the figures and the results with $P < 0.05$ were considered significant. Statistical analysis were performed using the programs Sigma Stat (Jandel Scientific Software, version 1.0, San Rafael, CA, USA) for the survival analysis and GraphPad Prism v. 5.00 for Windows (GraphPad Software, San Diego, CA, USA) for the other tests.

## 3. Results

### 3.1. Prophylactic administration of S. boulardii inhibited weight loss and increased survival of mice after S. Typhimurium challenge

In order to confirm our previous data [30,35], we have evaluated weight loss and survival after *S.* Typhimurium challenge. As seen in Fig. 1A, *S.* Typhimurium challenge caused significant weight loss when compared to control animals, especially between 4 and 21 days post infection (d.p.i.). When *S. boulardii* was present, it prevented weight loss and body weight stabilized at the same level of naive mice, although no statistical differences were observed. Cumulative survival was also evaluated during this period. As seen in Fig. 1B, treatment of *S.* Typhimurium-infected mice with *S. boulardii* enhanced survival (70%) in relation to mice not given the probiotic (40%). Yeast administration alone did not interfere on mice body weight and mortality (data not shown).

### 3.2. Administration of S. boulardii inhibits bacterial translocation and protects mice against intestinal and liver damage after S. Typhimurium challenge

Histopathological sections of intestine and liver were analyzed at 0, 1, 5, 10 and 15 d.p.i. Significant pathological alterations, especially in the liver, were clearly observed at 10 d.p.i. At 10 d.p.i., *Salmonella*-infected mice showed

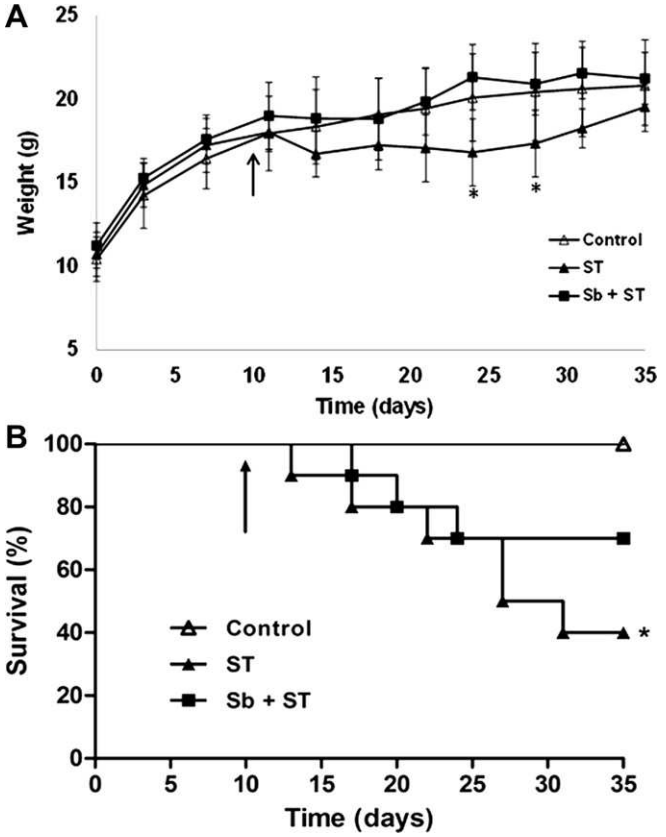


Fig. 1. *Saccharomyces boulardii* prevents mice weight loss (A) and increases survival of mice (B) after *S.* Typhimurium challenge. Weight loss (A) and survival (B) were evaluated at control (non-treated and non-challenged) mice (6 ), *S.* Typhimurium-challenged mice (: ) and mice treated with *S. boulardii* and then challenged (- ). Statistical test performed: A) two-way ANOVA followed by Tukey's test; B) Survival Log Rank test. $N = 10$ in each group. $^*P < 0.05$ in relation to control. ST (*S.* Typhimurium), Sb (*S. boulardii*). Arrow indicates the day of challenge.

discrete architectural changes of the colon when compared to the probiotic group (data not shown), confirming our previous data [30,35]. In the liver (Fig. 2), infection was associated with large areas of intense hepatocyte degeneration and necrosis. There were mono- and polymorphonuclear cells (arrows Fig. 2) which were present in foci or "palisade", delimiting extensive necrotic areas (thicker arrow Fig. 2D). In inflammatory foci, there were at least 100 inflammatory cells per foci (Fig. 2G) and they were also spread over the intralobular parenchyma.

Overall, liver histopathology of the probiotic-treated group (Fig. 2E and F) was very similar to the control group (Fig. 2A and B). There were very rare inflammatory foci (2–3 foci per liver) (data not shown), with fewer granulocytes per 100 inflammatory cells (Fig. 2G). Differently from untreated animals, leukocytes were all restricted to inflammatory foci in probiotic-treated animals. Necrotic areas were absent and there were few cells with degenerative changes (arrow Fig. 2F).

Bacterial translocation could be observed from 5 d.p.i. and persisted until day 15 in the liver (Fig. 2H). Treatment with probiotic prevented bacterial translocation and this was

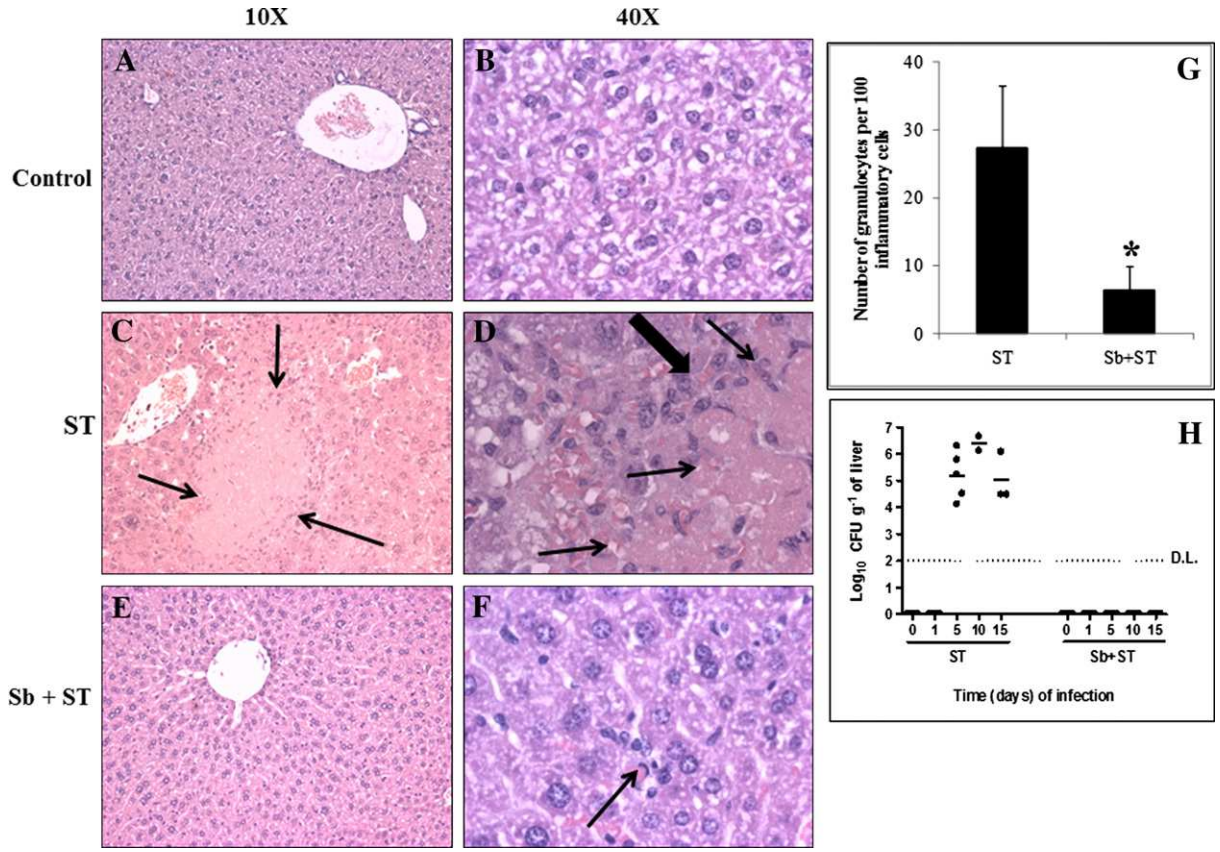


Fig. 2. *Saccharomyces boulardii* inhibits *S.* Typhimurium translocation to liver in mice. Representative aspects of liver in control (A, B), ST (C, D) and Sb + ST (E, F) groups, (G) show the number of granulocytes counted per 100 inflammatory cells at histological sections, and (H) shows the number of bacteria, in CFU, per gram of liver tissue. Arrows indicate mono- and polymorphonuclear cells which were present in foci or "palisade", delimiting extensive necrotic areas (thicker arrow in D) and degenerative changes (in F). ST (*S.* Typhimurium), Sb (*S. boulardii*), DL (detection limit). In (H) each dot represents the result of one single animal. Broken line represents the detection limit. H & E staining. Statistical test performed: G) Student's t-test; H) two-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ in relation to control.

associated with overall amelioration of liver damage in these animals. It is clear, therefore, that *S. boulardii* has a protective effect in this murine model of typhoid fever, preventing *Salmonella* translocation.

### 3.3. *Administration of* S. boulardii *decreases inflammatory cytokines in mice after* S. *Typhimurium challenge*

The interaction between *S.* Typhimurium and host epithelium leads to activation of a program of epithelial gene expression, such as those with pro- and inflammatory functions, as chemokines and cytokines with inflammatory properties. We next examined the ability of *S. boulardii* to reduce or prevent key inflammatory responses associated to *S.* Typhimurium infection in the colon. In Fig. 3, data obtained at day 0 for ST and Sb + ST groups were used as basal control values for each cytokines (not-treated and not-infected mice). As shown in Fig. 3A higher levels of KC (the mouse ortholog of GRO-α) were found at 10th d.p.i. When *S. boulardii* was present, KC levels were similar to uninfected mice. Similar results were observed for IL-6 (Fig. 3B), and TNF-α (Fig. 3C), i.e., infection resulted in enhanced cytokine production which was decreased in the presence of probiotic. No difference was observed in the levels of IFN-γ at the 10th d.p.i. (Fig. 3D). In contrast, evaluation of the levels of cytokines with major anti-inflammatory activity (TGF-β and IL-10, Fig. 3E and F, respectively), showed that presence of *S. boulardii* did not decrease cytokine levels and actually had an increase in the early stage of the infection reinforcing the anti-inflammatory effects of *S. boulardii* treatment.

By day 15 p.i. we observed that all inflammatory cytokines returned to its baseline level in mice challenged with *Salmonella* (Fig. 3A–D). This could be explained by the fact that at 15 d.p.i., all the mice that got sick have already died and the remaining animals that survived the infection probably came back to a normal immune status (the critical time of infection is between days 10–15). In the case of the mice treated with the yeast, this was not observed for some of the cytokines. By day 15 p.i., some inflammatory cytokines are higher, but less than in *Salmonella* group at day 10 (Fig. 3A–D), and such results suggest that the yeast treatment not only retarded some symptoms of the disease, but also induced a less severe disease, diminishing mortality (Fig. 1B) and morbidity (Fig. 1A).

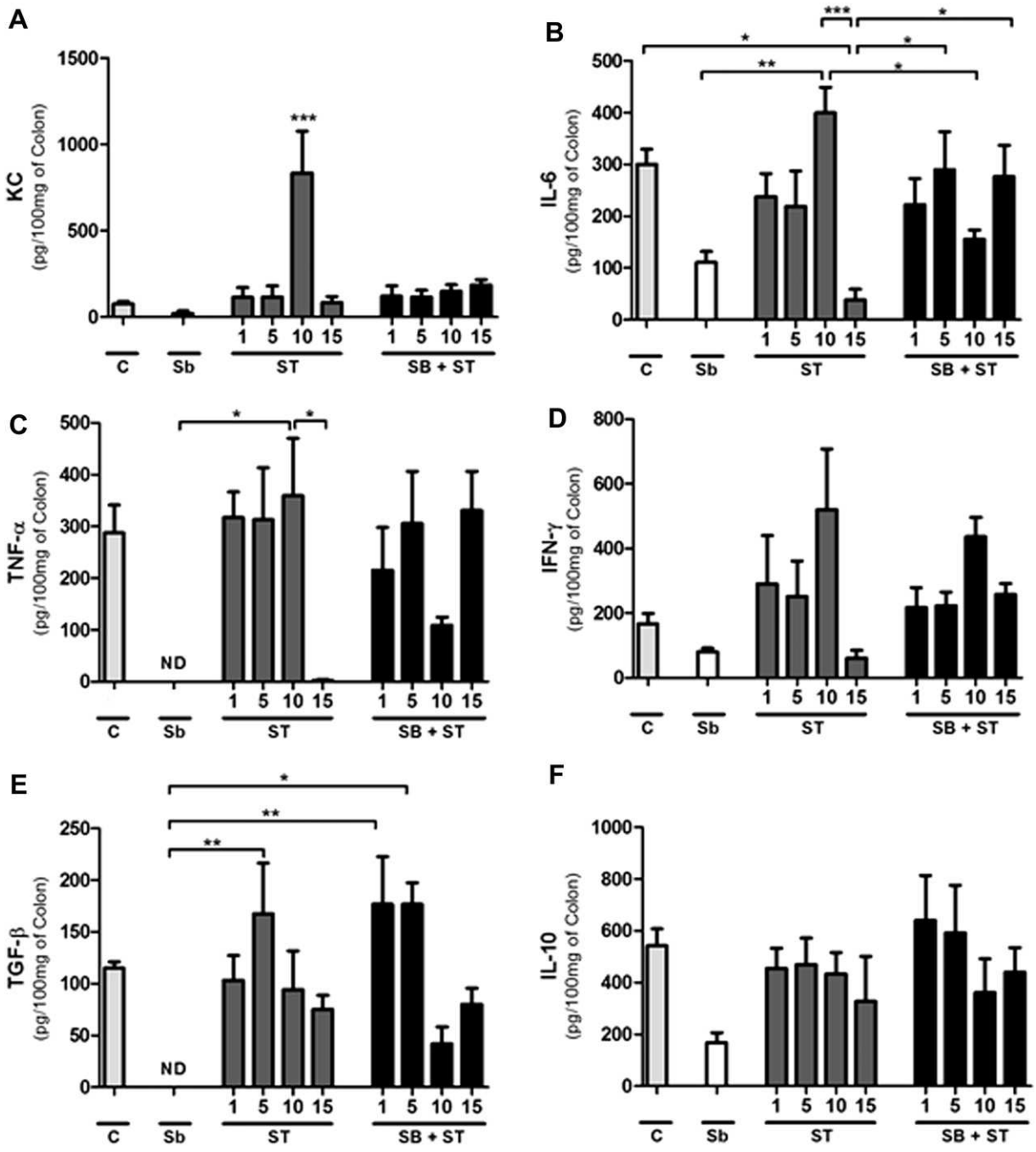


Fig. 3. Effects of *S. boulardii* on KC (A), IL-6 (B), TNF-α (C), IFN-γ (D), TGF-β (E) and IL-10 (F) levels after *S.* Typhimurium infection in colon tissue. Notice that *S. boulardii* lowered the activation of (pro) inflammatory cytokines (A–D) when present at *Salmonella* infection. Cytokines contents were estimated by ELISA. Data obtained at day 0 for ST and Sb + ST groups were used as basal control values for each cytokines (not-treated and not-infected mice). The statistical test performed was one-way ANOVA followed by Student-Newman–Keuls post-hoc analysis. $N = 5$ in each point. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. KC (A) was statistically significant between ST day 10 and all the other groups. ST (*S.* Typhimurium), Sb (*S. boulardii*).

### *3.4. Administration of* S. boulardii *inhibits inflammatory signal transduction pathways in mice after* S. *Typhimurium challenge*

We investigated the effect of the yeast on different signaling pathways involved in pro- and inflammatory cytokines production. For this purpose, mice were infected with *S.* Typhimurium in the presence, or not, of *S. boulardii*, and MAPKs (p38, JNK and ERK1/2) (Fig. 4A), p65-*RelA* and phospho-IκB (Fig. 4B), and phospho-jun and c-fos (Fig. 4C) were analyzed at 0, 1, 5, 10 and 15 d.p.i. in the colon. As it can be observed in Fig. 4, infection with *S.* Typhimurium was associated with activation of all MAPKs (Fig. 4A), translocation of p65 to the nucleus and phosphorylation of IκB (Fig. 4B), and the two main subunits of AP-1 transcription factor (phospho-jun and c-fos) (Fig. 4C). In the presence of *S.*

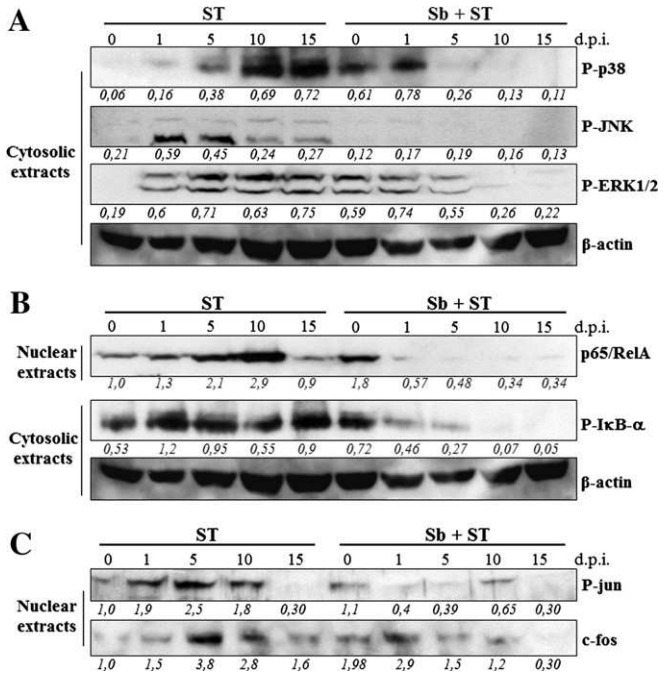


Fig. 4. Effects of *S. boulardii* on *S.* Typhimurium-induced p38, JNK and ERK1/2 MAPKs activation (A), NF-κB (p65/RelA and phospho-IκB) activation (B), and AP-1 (phospho-jun and c-fos) activation (C) in colon tissue. Samples were resolved by SDS-PAGE and analyzed by immunoblotting with specific antibodies in total or nuclear extracts. Western blottings shown represent 1 of 5 independent animals. Control (not-treated and not-infected mice) is represented by ST day 0, and Sb-treated group (not challenged mice) is represented by Sb + ST day 0. Densitometric analysis was performed by using LabImage software. ST (*S.* Typhimurium), Sb (*S. boulardii*).

*boulardii*, activation of JNK was abolished, and p38 and ERK1/2 were decreased (Fig. 4A). The yeast also decreased phosphorylation of IκB and completely inhibited the translocation of p65 to the nucleus (Fig. 4B), and also decreased activation of AP-1 (through phospho-jun and c-fos analysis) (Fig. 4C).

In Fig. 4A and B *S. boulardii* seems to increase phospho-p38 and phospho-ERK1/2 and p65-*RelA* and phospho-IκB signaling pathways at the beginning of the experiments. This effect could be due to the yeast itself since glucan components (Zymozan) present in the yeast wall may have a pro-inflammatory effect [43].

### 3.5. *Binding of* Salmonella *to S. boulardii cells in vivo*

To investigate in vivo if the binding of *S.* Typhimurium to *S. boulardii* cells instead of to intestinal epithelial cells could be one of the mechanisms explaining how the yeast inhibited *Salmonella* translocation and the associated inflammatory response, we used a gnotobiotic model (germ free mice only associated with the two microorganisms of interest). This simplified animal model allows the visualization of a possible bacterium–yeast interaction without the interference of the very complex indigenous microbiota. Fig. 5 depicts the interactions between yeast, bacteria and tissues. In monoxenic mice (*Salmonella* associated animals), bacteria are randomly distributed in close contact with the cecal epithelium (Fig. 5A and B). In dixenic mice (*Saccharomyces/Salmonella* associated animals), bacterial cells were found adhered more frequently to the yeast than to the epithelium cells (Fig. 5C–F), suggesting that this phenomenon may lead to a decreasing in bacteria translocation and inflammation.

### 3.6. *Therapeutic administration of* S. boulardii *reduced mortality of mice challenged with* S. *Typhimurium*

Because in the experiments describe so far, *S. boulardii* was given in a preventive manner, we investigated whether therapeutic administration of the yeast would also benefit *Salmonella*-infected mice. To this end, the yeast was given 10 days before, when *Salmonella* was inoculated (day 0) or treatment was started at days 2, 4, 6, 8, and 10 after inoculation. As seen in Fig. 6, *Salmonella* infection induced a lethality rate of 60% in untreated mice and this was greatly decreased by preventive treatment with the probiotic (approximately 30% lethality rate). Similar protection was also observed when the yeast was given to animals from day 0 (25% lethality) or from days 2–6 (35% lethality). Thereafter, protection was not marked (day 8, 45% and day 10, 55% lethality) (Fig. 6).

## 4. Discussion

In humans, *Salmonella* is believed to cause over one billion infections annually, with consequences ranging from self-limiting gastroenteritis to typhoid fever. In contrast to the severe outcome of disease in humans, *S.* Typhi is avirulent in most animals, including mice. However, the disease associated with *S.* Typhimurium infection of mice closely resembles that of *S.* Typhi in humans [44]. *S.* Typhimurium infection in mice is therefore widely accepted as an experimental model for typhoid fever in humans. A wide range of antibiotics are used to treat human salmonellosis [45]. However, genetic mutations and selective pressure have pushed *Salmonella* spp., as well as other bacteria, to become resistant or multi-resistant to antibiotics [3,46,47]. Development of alternative processes for the treatment and prevention of gastrointestinal disorders, such as probiotics, has become an attractive option.

The acute inflammatory response that ensues *Salmonella* invasion and production of inflammatory molecules causes diarrhea and may lead to ulceration and destruction of the mucosa [48]. The bacteria can then disseminate from the intestine to cause liver injury and systemic disease [49]. Here, we showed that *Salmonella* infection activates relevant intracellular signaling pathways associated with inflammatory cytokines production. Activation of these pathways is associated with local production of pro-inflammatory chemokines and cytokines, including CXCL1/KC and TNF-α [1,50]. There is consequent inflammation, ulceration and translocation of the bacteria, with consequent systemic inflammation and injury to various organs, including the liver. Preventive treatment with *S. boulardii* greatly decreased activation of inflammation-associated signaling pathways and the inflammatory response and injury in the gut. This was accompanied by

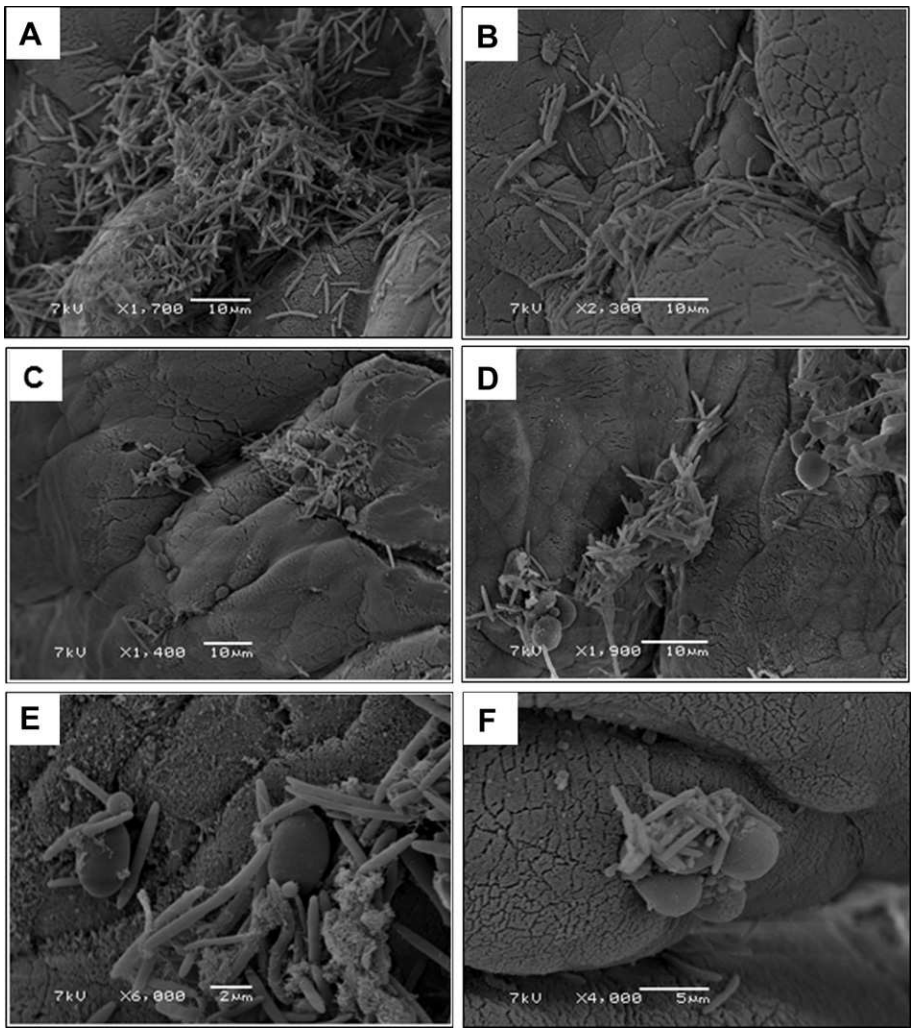


Fig. 5. Scanning electron microscopy showing *S.* Typhimurium adhesion on *S. boulardii* cells in tissue (cecum) of mice. (A–B) *S.* Typhimurium infected-mice, (C–F) mice previously treated with *S. boulardii* and then infected with *S.* Typhimurium. Magnification is shown in each figure.

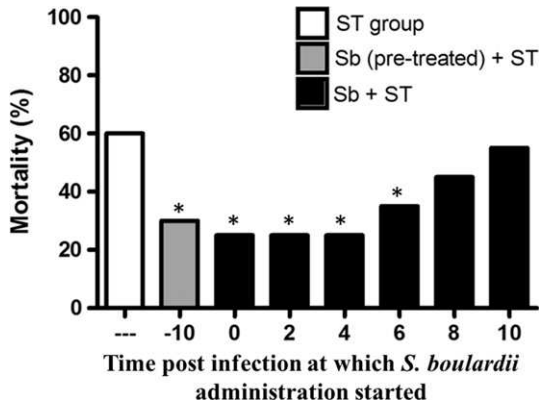


Fig. 6. Efficacy of *S. boulardii* on *S.* Typhimurium-induced mortality in mice treated after pathogen challenge. White row shows *S.* Typhimurium-infected mice; gray row represents mice pre-treated (during 10 days) with *S. boulardii* and then infected with *S.* Typhimurium; and black rows shows mice infected with *S.* Typhimurium and treated with *S. boulardii* after 0, 2, 4, 6, 8 and 10 days of infection. Each row indicates an average of 2 independent experiments ($n = 10$ in each group) after 28 days of infection. Statistical test performed: one-way ANOVA followed by Tukey's test. $^*P < 0.05$ in relation to ST group. ST (*S.* Typhimurium), Sb (*S. boulardii*).

decreased bacterial translocation, decreased systemic inflammation, decreased liver injury and lethality. Importantly, protective effects of *S. boulardii* were also observed when the yeast was given 6 days after infection with *Salmonella*. Altogether, these results confirm the protective effects of *S. boulardii* in the context of *Salmonella* infection.

The effects observed were not associated with bacteria antagonism, since the number of *Salmonella* in the feces remained the same in the presence – or not – of the yeast (data not shown). In an attempt to define how *S. boulardii* could protect mice from *Salmonella* infection, we evaluated whether the yeast could interact with bacteria and, hence, decrease the number of bacteria free to invade and activate epithelial cells. Experiments were conducted in germ free mice to facilitate the study of the direct interaction between the yeast and *Salmonella*. It was clear from these experiments that bacteria associated more frequently with *S. boulardii* than with epithelial cells when the yeast was present. This is in agreement with our previous in vitro studies, where it was demonstrated that *S. boulardii* inhibited *Salmonella* invasion

in T84 cells via binding of bacteria to yeast cells and decreasing of Rac1 pathway [30]. Some authors have already demonstrated in vitro 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 [29,30,51,52]. To our knowledge, this is the first study to show that *Salmonella* bind to *S. boulardii* in an in vivo model, and the results suggest that such binding decreases the number of bacteria associated to the epithelium and, consequently, translocation and decrease of inflammation and signaling pathways involved in inflammatory response. Despite the binding of *Salmonella* to *S. boulardii* in conventional mice was not demonstrated, we can speculate that the capacity of *Salmonella* to bind to *S. boulardii* and prevent activation of epithelial cells may explain how this yeast prevents disease and death in this murine model of typhoid fever.

The major findings of this study can be summarized as follows: first, infection of mice with *Salmonella* induced significant clinical manifestations, tissue damage, and lethality. These manifestations are well known and described during experimental infection of murine models with *S.* Typhimurium and enteroinvasive *E. coli*, and are the results of inflammation in the gut and liver of animals induced by the pathogenic bacteria through inflammation-associated signaling pathways. Second, preventive treatment with *S. boulardii* greatly decreased activation of signaling pathways with consequent reduction of inflammation, clinical manifestations, tissue damage and death. Third, bacteria-yeast binding could be responsible for a diminished number of free bacteria able to bind to epithelial cells what could be, in turn, decrease activation of pro-inflammatory signaling pathways in vivo in the gut. This alternative adhesion of pathogenic bacteria onto the surface of probiotics instead onto their intestinal receptors could explain part of the probiotic effect. Importantly, *S. boulardii* was effective to diminish death even when given 6 days after infection with *Salmonella*.

In conclusion, our study clearly shows that preventive or therapeutic treatment with *S. boulardii* decreases disease and death caused by challenge with *S.* Typhimurium. Our study suggests that *S. boulardii* may be useful as adjuvant in patients with typhoid fever even when started after the onset of disease, a possibility that should be tested in well controlled clinical trials in humans.

## Acknowledgments

The authors are grateful to Clélia N. Silva and Mirna D'auriol for valuable technical help, and to Antônio M. Vaz for the animal care. This work was funded by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Ministério da Ciência e Tecnologia, Brazil; and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), Brazil. FSM was the recipient of a postdoctoral fellowship from FAPEMIG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## References

[1] B. Coburn, G.A. Grassl, B.B. Finlay, *Salmonella*, the host and disease: a brief review, Immunol. Cell. Biol. 85 (2007) 112–118.
[2] T. Pang, M.M. Levine, B. Ivanoff, J. Wain, B.B. Finlay, Typhoid fever – important issues still remain, Trends Microbiol. 6 (1998) 131–133.
[3] J.A. Crump, E.D. Mintz, Global trends in typhoid and paratyphoid fever, Clin. Infect. Dis. 50 (2010) 241–246.
[4] J.E. Galán, H. Wolf-Watz, Protein delivery into eukaryotic cells by type III secretion machines, Nature 444 (2006) 567–573.
[5] J.E. Galán, Common themes in the design and function of bacterial effectors, Cell Host Microbe 5 (2009) 571–579.
[6] L. Eckmann, J.R. Smith, M.P. Housley, M.B. Dwinell, M.F. Kagnoff, Analysis by high density cDNA arrays of altered gene expression in human intestinal epithelial cells in response to infection with the invasive enteric bacteria *Salmonella*, J. Biol. Chem. 275 (2000) 14084–14094.
[7] D.G. Guiney, The role of host cell death in *Salmonella* infections, Curr. Top. Microbiol. Immunol. 289 (2005) 131–150.
[8] V.M. Bruno, S. Hannemann, M. Lara-Tejero, R.A. Flavell, S.H. Kleinstein, J.E. Galán, *Salmonella* Typhimurium type III secretion effectors stimulate innate immune responses in cultured epithelial cells, PLoS Pathog. 5 (2009) e1000538.
[9] F.C. Huang, Upregulation of *Salmonella*-induced IL-6 production in Caco-2 cells by PJ-34, PARP-1 inhibitor: involvement of PI3K, p38 MAPK, ERK, JNK, and NF-kappaB, Mediators Inflamm. 2009 (2009) 103890.
[10] S. Hobbie, L.M. Chen, R.J. Davis, J.E. Galán, Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells, J. Immunol. 159 (1997) 5550–5559.
[11] FAO/WHO Working Group, Guidelines for the Evaluation of Probiotics in Food (2002). London, Ontario, Canada.
[12] L.V. McFarland, Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients, World J. Gastroenterol. 16 (2010) 2202–2222.
[13] J.L. Fietto, R.S. Araújo, F.N. Valadão, L.G. Fietto, R.L. Brandão, M.J. Neves, F.C. Gomes, J.R. Nicoli, I.M. Castro, Molecular and physiological comparisons between *Saccharomyces cerevisiae* and *Saccharomyces boulardii*, Can. J. Microbiol. 50 (2004) 615–621.
[14] J.Y. Malgoire, S. Bertout, F. Renaud, J.M. Bastide, M. Mallié, Typing of *Saccharomyces cerevisiae* clinical strains by using microsatellite sequence polymorphism, J. Clin. Microbiol. 43 (2005) 1133–1137.
[15] C. Pothoulakis, Review article: anti-inflammatory mechanisms of action of *Saccharomyces boulardii*, Aliment. Pharmacol. Ther. 30 (2009) 826–833.
[16] C. Pothoulakis, C.P. Kelly, M.A. Joshi, N. Gao, C.J. O'Keane, I. Castagliuolo, J.T. Lamont, *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin A binding and enterotoxicity in rat ileum, Gastroenterology 104 (1993) 1108–1115.
[17] I. Castagliuolo, J.T. LaMont, S.T. Nikulasson, C. Pothoulakis, *Saccharomyces boulardii* protease inhibits *Clostridium difficile* toxin A effects in the rat ileum, Infect. Immun. 64 (1996) 5225–5232.
[18] I. Castagliuolo, M.F. Riegler, L. Valenick, J.T. LaMont, C. Pothoulakis, *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins A and B in human colonic mucosa, Infect. Immun. 67 (1999) 302–307.
[19] A. Qamar, S. Aboudola, M. Warny, P. Michetti, C. Pothoulakis, J.T. LaMont, C.P. Kelly, *Saccharomyces boulardii* stimulates intestinal immunoglobulin A immune response to *Clostridium difficile* toxin A in mice, Infect. Immun. 69 (2001) 2762–2765.
[20] J.P. Buts, N. De Keyser, L. De Raedemaeker, *Saccharomyces boulardii* enhances rat intestinal enzyme expression by endoluminal release of polyamines, Pediatr. Res. 36 (1994) 522–527.
[21] J.P. Buts, N. De Keyser, Effects of *Saccharomyces boulardii* on intestinal mucosa, Dig. Dis. Sci. 51 (2006) 1485–1492.
[22] J.P. Buts, N. De Keyser, Transduction pathways regulating the trophic effects of *Saccharomyces boulardii* in rat intestinal mucosa, Scand. J. Gastroenterol. 45 (2010) 175–185.

[23] G. Dalmasso, F. Cottrez, V. Imbert, P. Lagadec, J.F. Peyron, P. Rampal, D. Czerucka, H. Groux, A. Foussat, V. Brun, *Saccharomyces boulardii* inhibits inflammatory bowel disease by trapping T cells in mesenteric lymph nodes, Gastroenterology 131 (2006) 1812–1825.

[24] S. Thomas, I. Przesdzing, D. Metzke, J. Schmitz, A. Radbruch, D.C. Baumgart, *Saccharomyces boulardii* inhibits lipopolysaccharide-induced activation of human dendritic cells and T cell proliferation, Clin. Exp. Immunol. 156 (2009) 78–87.

[25] J.P. Buts, P. Bernasconi, J.P. Vaerman, C. Dive, Stimulation of secretory IgA and secretory component of immunoglobulins in small intestine of rats treated with *Saccharomyces boulardii*, Dig. Dis. Sci. 35 (1990) 251–256.

[26] A.C. Rodrigues, D.C. Cara, S.H. Fretez, F.Q. Cunha, E.C. Vieira, J.R. Nicoli, L.Q. Vieira, *Saccharomyces boulardii* stimulates sIgA production and the phagocytic system of gnotobiotic mice, J. Appl. Microbiol. 89 (2000) 404–414.

[27] F.S. Martins, A.A. Silva, A.T. Vieira, F.H. Barbosa, R.M. Arantes, M.M. Teixeira, J.R. Nicoli, Comparative study of *Bifidobacterium animalis*, *Escherichia coli*, *Lactobacillus casei* and *Saccharomyces boulardii* probiotic properties, Arch. Microbiol. 191 (2009) 623–630.

[28] R.L. Brandão, I.M. Castro, E.A. Bambirra, S.C. Amaral, L.G. Fietto, M.J. Tropia, M.J. Neves, R.G. Dos Santos, N.C. Gomes, J.R. Nicoli, Intracellular signal triggered by cholera toxin in *Saccharomyces boulardii* and *Saccharomyces cerevisiae*, Appl. Environ. Microbiol. 64 (1998) 564–568.

[29] B.R. Gedek, Adherence of *Escherichia coli* serogroup O157 and the *Salmonella typhimurium* mutant DT 104 to the surface of *Saccharomyces boulardii*, Mycoses 42 (1999) 261–264.

[30] F.S. Martins, G. Dalmasso, R.M. Arantes, A. Doye, E. Lemichez, P. Lagadec, V. Imbert, J.F. Peyron, P. Rampal, J.R. Nicoli, D. Czerucka, Interaction of *Saccharomyces boulardii* with *Salmonella enterica* serovar Typhimurium protects mice and modifies T84 cell response to the infection, PLos One 5 (2010) e8925.

[31] X. Chen, E.G. Kokkotou, N. Mustafa, K.R. Bhaskar, S. Sougioultzis, M. O'Brien, C. Pothoulakis, C.P. Kelly, *Saccharomyces boulardii* inhibits ERK1/2 mitogen-activated protein kinase activation both in vitro and in vivo and protects against *Clostridium difficile* toxin A-induced enteritis, J. Biol. Chem. 281 (2006) 24449–24459.

[32] K.L. Mumy, X. Chen, C.P. Kelly, B.A. McCormick, *Saccharomyces boulardii* interferes with *Shigella* pathogenesis by postinvasion signaling events, Am. J. Physiol. Gastrointest. Liver Physiol. 294 (2008) G599–G609.

[33] X. Wu, B.A. Vallance, L. Boyer, K.S. Bergstrom, J. Walker, K. Madsen, J.R. O'Kusky, A.M. Buchan, K. Jacobson, *Saccharomyces boulardii* ameliorates *Citrobacter rodentium*-induced colitis through actions on bacterial virulence factors, Am. J. Physiol. Gastrointest. Liver Physiol. 294 (2008) G295–G306.

[34] R. Pontier-Bres, F. Prodon, P. Munro, P. Rampal, E. Lemichez, J.F. Peyron, D. Czerucka, Modification of *Salmonella* Typhimurium motility by the probiotic yeast strain *Saccharomyces boulardii*, PLoS One 7 (2012) e33796.

[35] F.S. Martins, L.C. Veloso, R.M. Arantes, J.R. Nicoli, Effects of yeast probiotic formulation on viability, revival and protection against infection with *Salmonella enterica* ssp. *enterica* serovar Typhimurium in mice, Lett. Appl. Microbiol. 49 (2009) 738–744.

[36] J.R. Pleasants, Gnotobiotics, in: E.C. Melby Jr., N.H. Altmann (Eds.), Handbook of Laboratory Animal Science, CRC Press, Cleveland, 1974, pp. 119–174.

[37] F.A. Amaral, D. Sachs, V.V. Costa, C.T. Fagundes, D. Cisalpino, T.M. Cunha, S.H. Ferreira, F.Q. Cunha, T.A. Silva, J.R. Nicoli, L.Q. Vieira, D.G. Souza, M.M. Teixeira, Commensal microbiota is fundamental for the development of inflammatory pain, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 2193–2197.

[38] COBEA. Colégio Brasileiro de Experimentação Animal, Legislação e Ética (2006). Available at: http://www.cobea.org.br/.

[39] F.S. Martins, S.D. Elian, A.T. Vieira, F.C. Tiago, A.K. Martins, F.C. Silva, E.L. Souza, L.P. Sousa, H.R. Araújo, P.F. Pimenta, C.A. Bonjardim, R.M. Arantes, M.M. Teixeira, J.R. Nicoli, 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, Int. J. Med. Microbiol. 301 (2011) 359–364.

[40] D.G. Souza, F.A. Amaral, C.T. Fagundes, F.M. Coelho, R.M. Arantes, L.P. Sousa, M.M. Matzuk, C. Garlanda, A. Mantovani, A.A. Dias, M.M. Teixeira, The long pentraxin PTX3 is crucial for tissue inflammation after intestinal ischemia and reperfusion in mice, Am. J. Pathol. 174 (2009) 1309–1318.

[41] F.S. Martins, R.M. Nardi, R.M. Arantes, C.A. Rosa, M.J. Neves, J.R. Nicoli, Screening of yeasts as probiotic based on capacities to colonize the gastrointestinal tract and to protect against enteropathogen challenge in mice, J. Gen. Appl. Microbiol. (2005) 83–92.

[42] P.F. Pimenta, W. De Souza, Fine structure and cytochemistry of the endoplasmic reticulum and its association with the plasma membrane of *Leishmania mexicana amazonensis*, J. Submicrosc. Cytol. 17 (1985) 413–419.

[43] A.T. Vieira, K.D. Silveira, M.C. Arruda, C.T. Fagundes, J.L. Gonçalves, T.A. Silva, M.J. Neves, M.A. Menezes, J.R. Nicoli, M.M. Teixeira, F.S. Martins, Treatment with Selemax®, a selenium-enriched yeast, ameliorates experimental arthritis in rats and mice, Br. J. Nutr. 108 (2012) 1829–1838.

[44] R.L. Santos, S. Zhang, R.M. Tsolis, R.A. Kingsley, L.G. Adams, A.J. Bäumler, Animal models of *Salmonella* infections: enteritis versus typhoid fever, Microbe. Infect. 3 (2001) 1335–1344.

[45] T. Butler, Treatment of typhoid fever in the 21st century: promises and shortcomings, Clin. Microbiol. Infect. 17 (2011) 959–963.

[46] J.M. Whichard, K. Gay, J.E. Stevenson, K.J. Joyce, K.L. Cooper, M. Omondi, F. Medalla, G.A. Jacoby, T.J. Barrett, Human *Salmonella* and concurrent decreased susceptibility to quinolones and extended-spectrum cephalosporins, Emerg. Infect. Dis. 13 (2007) 1681–1688.

[47] K.L. Hopkins, M. Day, E.J. Threlfall, Plasmid mediated quinolone resistance in *Salmonella enterica*, Emerg. Infect. Dis. 14 (2008) 340–342.

[48] R.A. Giannella (Ed.), Salmonella, fourth ed.,Baron's Medical Microbiology The University of Texas Medical Branch at Galveston, 1996.

[49] D.B. Huang, H.L. DuPont, Problem pathogens: extra-intestinal complications of *Salmonella enterica* serotype Typhi infection, Lancet Infect. Dis. 5 (2005) 341–348.

[50] L. Eckmann, M.F. Kagnoff, Cytokines in host defense against *Salmonella*, Microbe. Infect. 3 (2001) 1191–1200.

[51] T.K. Kornonen, H. Leffler, C. Svanborg Eden, Binding specificity of piliated strains of *Escherichia coli* and *Salmonella typhimurium* to epithelial cells, *Saccharomyces cerevisiae* cells, and erythrocytes, Infect. Immun. 32 (1981) 796–804.

[52] L.S. Pérez-Sotelo, M. Talavera-Rojas, H.G. Monroy-Salazar, S. Lagunas-Bernabé, J.A. Cuarón-Ibargüengoytia, R.M. Jimenez, J.C. Vázquez-Chagoyán, In vitro evaluation of the binding capacity of *Saccharomyces cerevisiae* Sc47 to adhere to the wall of *Salmonella* spp, Rev. Latinoam. Microbiol. 47 (2005) 70–75.