Appl Microbiol Biotechnol (2015) 99:7953–7961
DOI 10.1007/s00253-015-6696-9

Pichia pastoris X-33 has probiotic properties with remarkable antibacterial activity against *Salmonella* Typhimurium

Rodrigo Correa França[1,2] · Fabricio Rochedo Conceição[1] · Marcelo Mendonça[1] · Louise Haubert[1,2] · Gabriela Sabadin[1] · Patrícia Diaz de Oliveira[1] · Marta G. Amaral[1] · Wladimir Padilha da Silva[2] · Ângela Nunes Moreira[1,3]

Received: 4 March 2015 / Revised: 13 May 2015 / Accepted: 15 May 2015 / Published online: 19 June 2015

Abstract Probiotics are live microorganisms which are beneficial for the host when ingested at high enough concentrations. The methylotrophic yeast *Pichia pastoris* is widely used as heterologous protein production platform. However, its use as probiotic is poorly studied. The objective of this study was to evaluate some probiotic properties of the *P. pastoris* strain X-33 wild type. The resistance to in vitro and in vivo gastrointestinal conditions, stability in feed, safety, and antibacterial activity against *Salmonella* Typhimurium were evaluated. The yeast remained viable and persisted at appropriate concentration in the diet for at least 2 months, survived the stresses of the gastrointestinal tract in vitro and in vivo, caused no behavioral changes or lesions when administered to mice, inhibited the growth of *S.* Typhimurium in culture media, and reduced adhesion of the bacteria to the intestinal cells HCT-116. In the challenge experiment with a LD_{50} of virulent *S.* Typhimurium strain, mice supplemented with the yeast had a higher survival rate (50 % when administered by gavage and 80 % via the diet, compared with 20 and 50 %, respectively, in the control group). In addition, the *S.* Typhimurium concentration in the intestine of the surviving mice was lower; the score of intestinal lesions, lower; and the pathogen, not detected in the liver, spleen, and feces when compared to the control group ($p < 0.05$). It was concluded that the yeast *Pichia pastoris* X-33 has probiotic properties with remarkable antibacterial activity against *S.* Typhimurium.

Keywords Probiotics · Yeast · Translocation · Intestinal lesions · Adhesion inhibition · Salmonellosis

Introduction

Probiotics are defined by the World Health Organization (WHO) as living microorganisms that ingested in adequate concentrations, are beneficial for the host (FAO/WHO 2002), e.g., by the regulation of intestinal microbiota, improved feed conversion, protection from pathogenic microorganisms (Brandao et al. 1998; Czerucka et al. 1994; Czerucka et al. 2000; Martins et al. 2009), and modulation of immune response (Bron et al. 2012; Kaila et al. 1992). To have an effect on the host, probiotics must survive the adversity of the gastrointestinal (GI) tract, be safe for human and animal use, resist the industrial processing, and remain viable for long periods of storage and transportation (Saad 2006).

Probiotics have attracted attention in the prevention and treatment of food-borne diseases and as an alternative to the antibiotics used as growth promoters in animal production (Bron et al. 2012; Fang et al. 2012; FAO/WHO 2002; Le Blanc et al. 2010; Martins et al. 2009). Among the commonly used probiotics in humans and animals, some species of lactic acid bacteria of the genus *Lactobacillus* and *Bifidobacterium* and the yeasts *Saccharomyces boulardii* and *Saccharomyces cerevisiae* are particularly promising, in view of the advantages over bacteria, for not being inhibited by the antibacteria.

✉ Fabricio Rochedo Conceição
fabricio.rochedo@ufpel.edu.br

[1] Nucleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário, Capão do Leão, Caixa Postal 354, Pelotas, RS 96010-900, Brazil

[2] Departamento de Ciência e Tecnologia de Agroindustrial, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, RS, Brazil

[3] Departamento de Nutrição, Faculdade de Nutrição, Universidade Federal de Pelotas, Pelotas, RS, Brazil

This property is interesting because some therapies associate the administration of probiotics with antibacterials against gastric infections, e.g., those caused by *Helicobacter pylori* (Martins et al. 2005). Moreover, no transfer of genetic material was detected between yeast and commensal bacteria (Czerucka et al. 2007). Other advantages are the high multiplication from low-cost substrates and the fact that they can be used as protein source in animal feeds (Blehaut et al. 1989; Boddy et al. 1991).

The methylotrophic yeast *Pichia pastoris* is widely used as a production platform for heterologous proteins (Cregg et al. 1985; Cregg et al. 2000; Love et al. 2012; Torres and Moraes 2000), also distinguished by being able to grow from low-cost substrates, including industrial wastes and effluents (Çelik et al. 2008; Santos et al. 2012), a characteristic that caused some groups to test it as probiotic in animal production. However, this application has been little exploited, addressed by only one study on broilers (Gil de los Santos et al. 2012). In this study, chickens fed with a diet supplemented with *P. pastoris* gained more weight. However, other important properties of a probiotic, such as harmlessness and ability to survive the mammalian GI tract conditions, stability in the feed, and antimicrobial action against enteropathogens, have not yet been evaluated. The objective of this study was to evaluate some probiotic properties of *P. pastoris*, strain X-33, emphasizing the antibacterial action against *S.* Typhimurium.

Methodology

Microorganisms and culture conditions

The microorganisms *Salmonella enterica* serovar Typhimurium strain 29630 was obtained from ATCC, *S. boulardii* was isolated from a commercial lyophilized product (Floratil®, Merck, Brazil), and *P. pastoris* strain X-33 (wild type) was purchased from Invitrogen (CA, USA). *Salmonella* Typhimurium ATCC 29630 was grown in Luria-Bertaine broth (LB) for 16 to 18 h at 37 °C, under shaking at 200 rpm in an orbital shaker. The bacteria were quantified in a Petroff-Hausser chamber by plating on agar Xylose lysine deoxycholate (XLD) (37 °C for 18 h) to determine colony-forming units per milliliter ($CFU.mL^{-1}$). The yeasts *P. pastoris* X-33 and *S. boulardii* Cenbiot were cultured in yeast peptone dextrose (YPD) broth for 16 to 18 h at 28 °C, under shaking at 200 rpm in an orbital shaker. Five hundred milliliters of inoculum were added to 7 L of YPD broth and incubated in a bioreactor (Bioflo 110, New Brunswick) at 28 °C, 500 rpm, 1 vvm air for 24 h. Antifoam 204 (Sigma) was used during fermentation. After fermentation, the cells were centrifuged at 5000×*g* for 15 min, washed in 0.9 % saline solution at 4 °C, concentrated 10 times, and quantified. Viable counts were determined by plating decimal yeast dilutions on YPD agar after incubation at 28 °C for 48 h.

Feed preparation and stability

Approximately 7 log $CFU.g^{-1}$ of *P. pastoris* X-33 or *S. boulardii* were added to the rodent-specific, antibiotic-free, pre-ground diet. These formulations were pelleted and maintained in an oven with forced air circulation for 18 h at 40 °C. The feeds were stored at 4 °C until use and every 7 days, the yeast was quantified ($CFU.g^{-1}$) to assess viability and stability.

Animals

Male BALB/c mice, 6 to 8 weeks old, weighing between 15 and 20 g, were placed in micro-isolators with five animals each and maintained on a ventilated rack at an average temperature of 22 °C and light cycle of 12 h. Rodent-specific, antibiotic-free feed was provided, and water ad libitum.

Animal welfare

Animal welfare and experimental procedures were carried out according to the ethical regulations of the Brazilian College of Animal Experimentation (COBEA), and the project was approved by the Committee for Ethics in Animal Experimentation (EAEC) of the UFPel.

Resistance of *P. pastoris* X-33 to simulated gastrointestinal conditions (in vitro)

The resistance of the yeast *P. pastoris* X-33 to simulated gastrointestinal conditions was assessed using the methodology described by Duc et al. (2004), with modifications. Approximately 8 log $CFU.mL^{-1}$ yeast were suspended in 10 mL of a solution that simulates gastric fluid (acidified YPD broth at pH 2 with 0.1 N HCl and supplemented with 1 $mg.mL^{-1}$ pepsin). The tubes, in triplicate, were incubated at 37 °C under constant shaking to simulate peristalsis. Tubes containing YPD broth adjusted to pH 7.2 were included as controls. After 1 h of incubation, the suspensions were centrifuged at 7000×*g* for 10 min and 10 mL of the solution which simulates the intestinal fluid (YPD broth supplemented with 1 $mg.mL^{-1}$ pancreatin, 0.2 % bile salts, and pH adjusted 8 with 0.1 N NaOH) were added to the cells collected by centrifugation. After incubation for 5 h, aliquots of 0.1 ml were collected, decimal diluted in 0.9 % saline (pH 7.2), and viable yeast cells counted as described above. The survival rate was calculated as the percentage of the log CFU number grown on the plates after exposure to simulated gastrointestinal conditions in relation to the initial yeast concentration. The experiment was performed in triplicate.

P. pastoris X-33 resistance of the gastrointestinal tract of mice and persistence

An oral dose containing approximately 7 log CFU of *P. pastoris* X-33 was administered to mice fed an antibiotic-free diet by gavage. Feces were collected 24, 48, and 72 h after administration of the yeast and decimal diluted in 0.9 % saline. To evaluate the resistance of the yeast *P. pastoris* X-33 to the GI mouse tract and its persistence, counts were determined by plating decimal dilutions of feces on YPD agar. Mice not inoculated with yeast served as controls.

Growth inhibition capacity of *P. pastoris* X-33 on *S.* Typhimurium in culture media

The inhibitory effect of *P. pastoris* X-33 on growth of *S.* Typhimurium was assessed by a methodology described by Drago et al. (1997) with modifications. About 6 log CFU of *S.* Typhimurium and yeast were incubated in one tube containing 10 mL of YPD broth and 10 mL of LB broth in another. Pure cultures of each microorganism were used as controls. The tubes were incubated at 37 °C under shaking at 200 rpm, in an orbital shaker. After 0, 4, 8, and 24 h, aliquots were taken, decimal diluted, and plated in duplicate to determine the number of viable cells of each microorganism. For the yeast count, potato dextrose agar (PDA) pH 5.2 was used and XLD agar to count *S.* Typhimurium. The growth time of *S.* Typhimurium was also determined in the presence and absence of *P. pastoris* X-33.

Inhibition capacity of *P. pastoris* X-33 of adhesion of *S.* Typhimurium to intestinal cells HCT-116

Human colorectal HCT-116 cells were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % fetal bovine serum (FBS) in 12-well plates at a concentration of 6 log cells/well, and incubated at 37 °C with 5 % CO_2 until confluence reached 90 %. *S.* Typhimurium and the yeasts *P. pastoris* X-33 and *S. boulardii* were washed twice (8000×g for 15 min) in phosphate-buffered saline (PBS) and suspended in RPMI medium with 10 % FBS. About 6 log CFU/well of *S.* Typhimurium were added to three wells, pure or in combination with about 5 log CFU/well of *P. pastoris* X-33 or *S. boulardii* (positive control). After 1 h incubation with the cells, the culture medium was removed, the wells were washed three times with PBS containing 137 mMNaCl, 5.4 mMKCl, 3.5 mM Na_2HPO_4, 4.4 mM NaH_2PO_4, and 11 mM glucose (cell-PBS, pH 7.2), and the plates incubated with 100 μL of cell-PBS containing 0.1 % Triton X-100 for 5 min at 37 °C. Finally, 100 μL were removed, decimal diluted and plated on XLD agar. The results were expressed as percentage of adhesion to intestinal cells HCT-116.

Antibacterial action of *P. pastoris* X-33 in vivo and harmlessness

The lethal dose of *S.* Typhimurium for 50 % of the mice population (LD50) was determined by the method described by Reed and Muench (1938). Briefly, BALB/c mice were divided into groups of five animals and treated by gavage with 300 μL of each of the decimal dilutions, containing from 1 to 6 log CFU of *S.* Typhimurium. The animals were monitored daily and deaths recorded to determine the LD50.

The antibacterial activity of *P. pastoris* X-33 on *S.* Typhimurium was evaluated in two ways. A first group consisting of 15 BALB/c mice received daily oral doses of 1 mL of a 0.9 % saline suspension containing 7 log CFU *P. pastoris* X-33 per animal via gavage for 20 days and the control group received the same volume of 0.9 % saline solution. In the other experiment, a group of 10 BALB/c mice was treated with antibiotic-free diet ad libitum containing 7 log $CFU.g^{-1}$ of *P. pastoris* X-33, for the same period. The negative control group received only the diet, and the positive control group was treated with a diet supplemented with 7 log $CFU.g^{-1}$of *S. boulardii*.

On the 20th day after initiating gavage administration of the yeast, five mice per group were euthanized to assess its harmlessness. Samples of different tissues and organs were collected and subjected to histopathological analysis. The animals were observed daily to assess the behavior. The remaining 10 mice of both experiments were challenged by gavage with 1 LD50 (5 log CFU/mouse) of *S.* Typhimurium and 10 days later, survival rates were calculated. On the 13th day after challenge, fresh feces of the surviving animals were collected, the animals euthanized, and intestines, livers, and spleens collected aseptically. The protection against intestinal colonization of *S.* Typhimurium was measured by viable cell counts in the feces and small intestine. To evaluate the translocation, two thirds of the liver and spleen were ground, suspended in 1 ml of 0.9 % sterile saline, decimal diluted in 0.9 % saline, and plated on XLD agar. The results were expressed in log CFU per organ and in percentage of animals per group with pathogen symptoms in the organs. Samples of spleen, liver, and intestine were fixed in 10 % formalin and paraffin-embedded. Fragments of 5–6 μm were stained with hematoxylin-eosin. The samples were prepared at the Department of Histology of the UFPel and examined by pathologists for the presence of lesions. Leukocyte infiltration in the mucosa and submucosa lumen and the presence of isolated follicles were evaluated by histological analyses. The presence or absence of morphological changes in the villi was assessed in the absence of the coating epithelium and with a reduced

number of villi. In the mucosa and submucosa, the infiltrated leukocytes were classified as mild (+), moderate 2 (+), and severe 3 (+) lesions.

Statistical analysis

A survival test was applied to find significant differences ($p < 0.05$) between the survival rates in the challenge experiment with *S.* Typhimurium. Analysis of variance (ANOVA) and Tukey's test were used to determine significance levels ($p < 0.05$) between treatment means. All statistical analyses were performed using software Statistic 9.

Results

Stability of *P. pastoris* X-33 in the feed

The concentration of viable *P. pastoris* X-33 cells remained stable in the feed for at least 2 months (7 log CFU.g^{-1}). It was not possible to assess its stability for longer since the feed was antibiotic-free and contaminated by molds shortly after the study period.

Resistance of *P. pastoris* X-33 to gastrointestinal conditions in vitro and in vivo

P. pastoris X-33 survived the simulated GI tract conditions at a sufficiently large number to have beneficial effects in situ because their survival rate to consecutive exposure to gastric and intestinal conditions was 76.8 % (results not shown).

P. pastoris X-33 TGI also survived the simulated mouse GI tract conditions and after 24 h of administration, about 6.84 log CFU.g^{-1} was found in fresh feces. Then, 48 h after treatment application, 2.87 log CFU.g^{-1} was obtained, and no growth was observed after 72 h (Fig. 1).

Safety of *P. pastoris* X-33

No behavioral changes were observed in the mice supplemented with *P. pastoris* X-33. In the histopathological analysis, no injury was observed. No changes were observed either in intestinal microvilli and lamina propria.

Growth inhibition capacity of *P. pastoris* X-33 on *S.* Typhimurium in culture media

P. pastoris X-33 inhibited *S.* Typhimurium growth in both culture media after 24 h of incubation. The *S.* Typhimurium population was reduced by approximately 43 % (from 2.1×10^8 to 1.2×0^8 CFU.mL^{-1}) in LB broth and 86 % (from

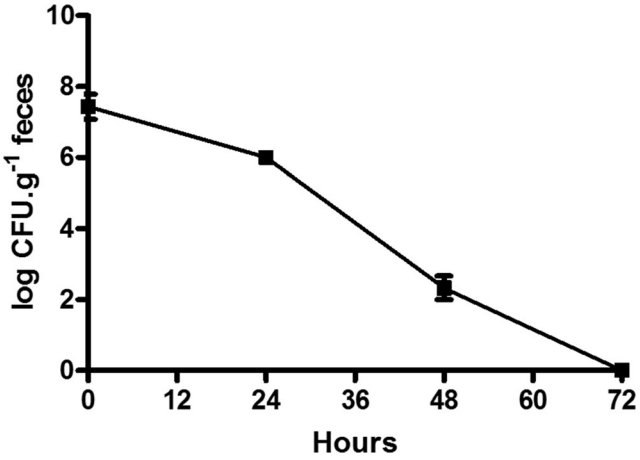

Fig. 1 Count of viable *P. pastoris* X-33 cells (in log CFU.g^{-1} of feces) after passing through the mouse GI tract. The counts were performed 24, 48, and 72 h after administration of an oral dose containing approximately 7 log CFU of yeast

1.0×10^9 to 1.4×10^8 CFU.mL^{-1}) in YPD broth when the bacteria were co-cultivated with yeast, compared to the growth of the control group (bacteria only). The time of generation of bacteria increased also when grown in the presence of *P. pastoris* X-33. *S.* Typhimurium grew fast when cultured alone (24 min) in both broths, but in the presence of *P. pastoris* X-33, this period was extended to 36 min (results not shown).

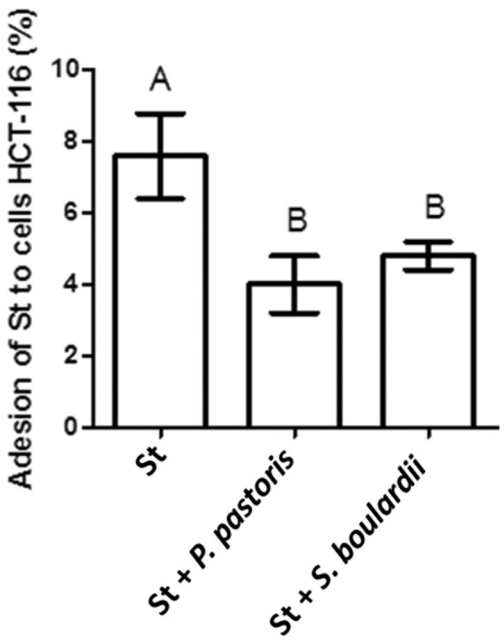

Fig. 2 Percentage of adhesion of *S.* Typhimurium to the intestinal cells HCT-116 after 1 h of incubation at 37 °C (approximately 6 log CFU per well), with *P. pastoris* X-33 or *S. boulardii* separately (both approximately 5 log CFU per well). *Different letters* represent significant differences ($p < 0.05$) by analysis of variance (ANOVA) and Tukey's test (St.: *S.* Typhimurium)

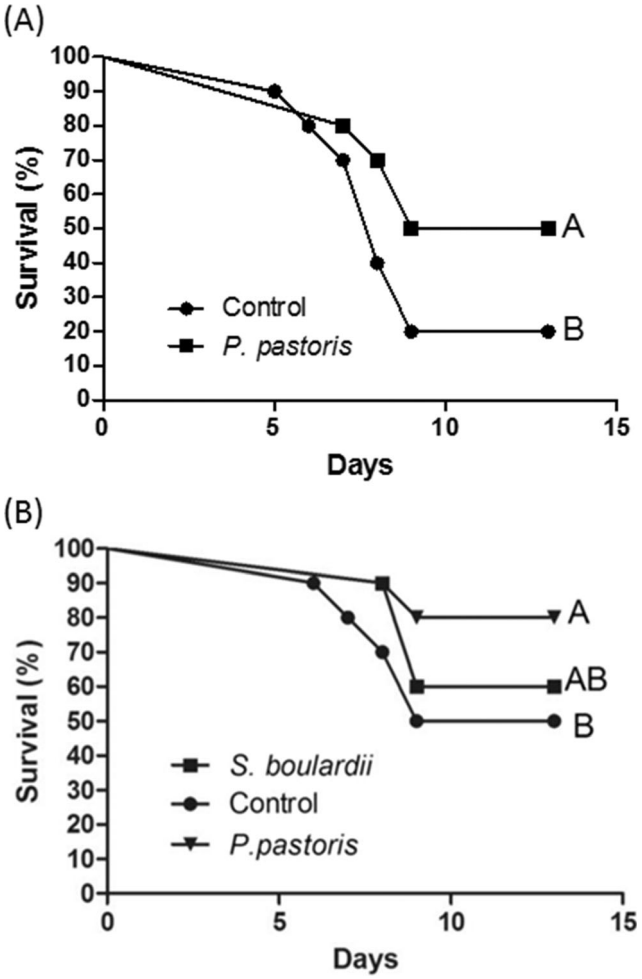

Fig. 3 Percentage of surviving mice supplemented for 20 days with 7 log CFU of *P. pastoris* X-33 by gavage (**a**) or in the diet (**b**) and challenged with 1 LD50 of *S.* Typhimurium. The animals of the negative control groups received 0.9 % saline by gavage and standard diet without probiotics, respectively. The animals in the positive control group received a diet with 7 log CFU of *S. boulardii* g^{-1} feed. Different letters represent significant differences ($p < 0.05$) by the survival test.

Inhibition capacity of *P. pastoris* X-33 of adhesion of *S.* Typhimurium to intestinal cells HCT-116

In the experiment with HCT-116 cells, the percentage of *S.* Typhimurium adhesion to intestinal cells was reduced by approximately 47 % (7.6–4.0 %), when incubated for 1 h with *P. pastoris* X − 33, compared with bacteria incubated alone ($p < 0.05$). A similar reduction (37 %) in the adhesion percentage was observed when bacteria were incubated with *S. boulardii* (7.6–4.8 %) (Fig. 2).

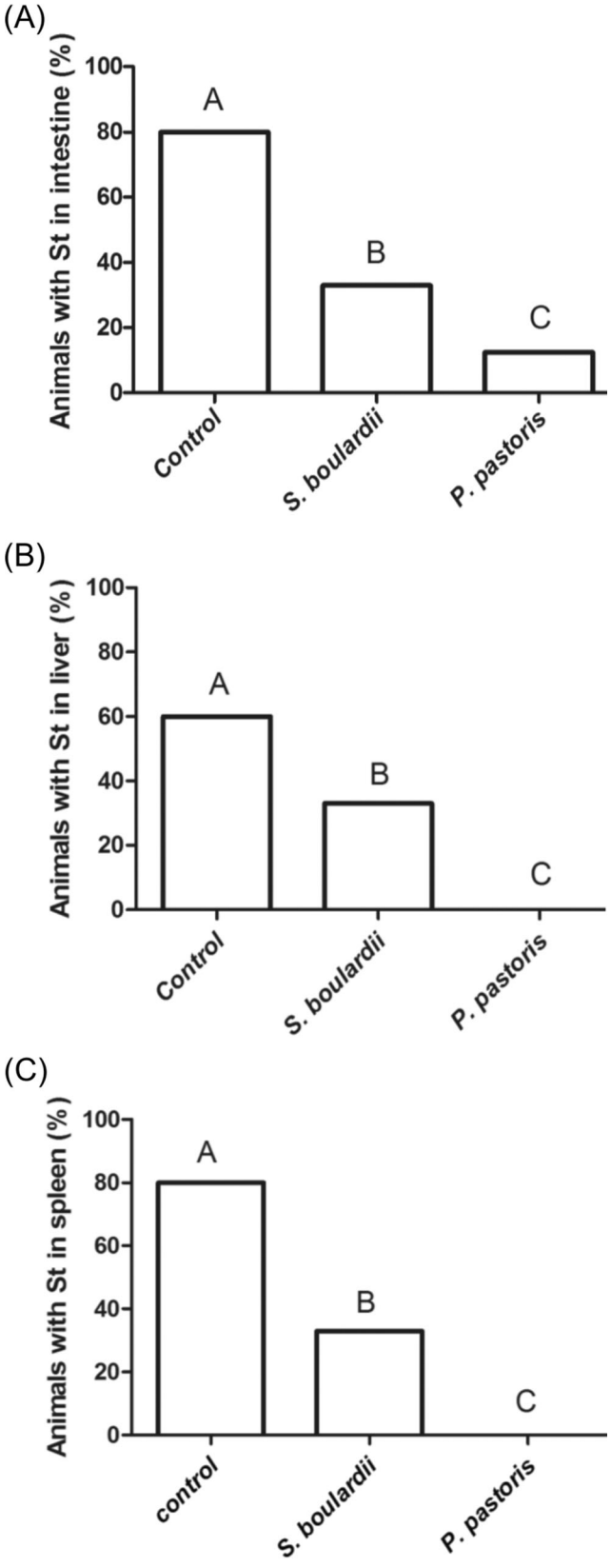

Fig. 4 Percentage of mice supplemented for 20 days with 7 log CFU of *P. pastoris* X-33 per gram of diet, which survived the challenge with 1 LD50 of *S.* Typhimurium and exhibited *S.* Typhimurium in the intestine (**a**), liver (**b**), and spleen (**c**). The animals in the negative control group received a standard diet without probiotics. The animals in the positive control group received diet with 7 log CFU of *S. boulardii*. Different letters represent significant differences ($p < 0.05$) by analysis of variance (ANOVA) and the Tukey test. *(St S. Typhimurium)*

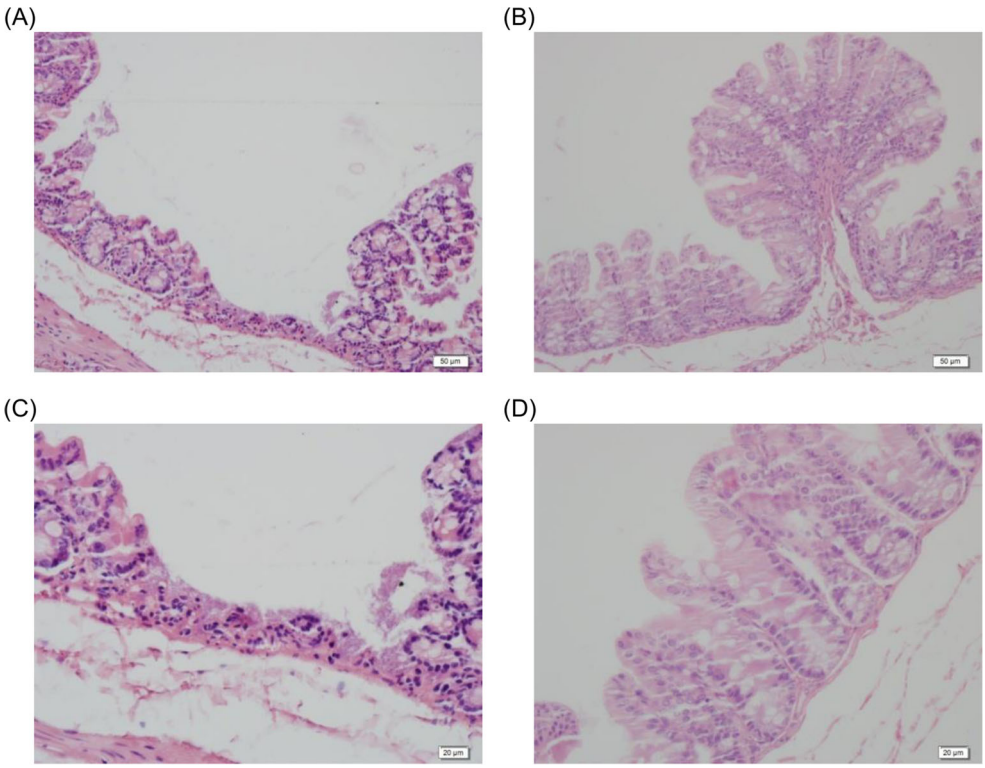

Fig. 5 Histopathological aspects of intestines of mice supplemented for 20 days with 7 log CFU of *P. pastoris* X-33 per gram of diet, which survived the challenge with 1 LD50 of *S.* Typhimurium. **a**, **c** Changes in the morphology of microvilli of the negative control group. **b**, **d** No changes in the morphology of microvilli of animals supplemented with *P. pastoris* X-33. Images **a** and **b** (×20 magnification). Images **c** and **d** (×40 magnification)

Antibacterial action of *P. pastoris* X-33 in vivo

The survival rate of the group supplemented with *P. pastoris* X-33 by gavage was 50 % (5/10) and significantly higher ($p < 0.05$) than that of the control group, where 20 % (2/10) of the animals survived (Fig. 3). In the experiment where *P. pastoris* X-33 was added to the mice diet, the survival rate of the group supplemented with yeast was 80 % (8/10) and also significantly higher ($p < 0.05$) than that of the control group, with 50 % (5/10) surviving animals. In the positive control group, supplemented with *S. boulardii*, the survival rate was 60 % (6/10) (Fig. 3b). Interestingly, the survival rates were lower in all groups in the gavage supplementation experiments than in the experiment where the yeast was added to the feed, probably due to the higher stress level caused by gavage.

No *S.* Typhimurium was detected in the feces of surviving animals supplemented with *P. pastoris* X-33 13 days after challenge. In animals supplemented with *S. boulardii*, the result was the same. However, in the group not supplemented with the yeasts, approximately 8.78 log CFU of *S.* Typhimurium were recovered per gram of feces (not shown).

In the intestine of mice surviving the challenge, results were similar to those in the feces. Low levels of *S.* Typhimurium were observed in the intestine of animals fed with *P. pastoris* X-33 (0.78 log CFU) and *S. boulardii* (1.36 log CFU), and higher levels in the intestines of animals that received diet without probiotics (3.17 log CFU) (results not shown). The percentage of surviving animals with *S.* Typhimurium in the intestine also differed significantly: 12.5 % in the *P. pastoris* X-33 group, 33 % in the *S. boulardii* group, and 80 % in the control group (Fig. 4a).

In the assessment of bacterial translocation 13 days after challenge, no *S.* Typhimurium was detected in the liver and spleen of animals supplemented with *P. pastoris* X-33, while in the control group, 3.3 log CFU were detected in both organs. In the group supplemented with *S. boulardii*, 0.3 and 0.84 log CFU were observed, respectively (results not shown). The percentage of surviving animals with *S.* Typhimurium in the liver and spleen supplemented with *P. pastoris* X-33 in the diet (0 % in both organs) was significantly lower ($p < 0.05$) in the control group (60 and 80 %, respectively) and in the group supplemented with *S. boulardii* (33 % in both organs) (Fig. 4b, c).

The percentage of surviving animals in which *S.* Typhimurium was detected in the intestine, liver, and spleen, which had been supplemented with *S. boulardii*, was significantly lower ($p < 0.05$) than in the control group (80, 60, and 80 %, respectively), but higher than in the group supplemented with *P. pastoris* X-33 (12.5, 0, and 0 %, respectively) (Fig. 4a–c).

With regard to the intestinal injuries, mild to moderate lesions were observed in the intestinal microvilli of the surviving animals of all groups (Fig. 5). The percentage of surviving animals with moderate as well as light intestinal lesions, 13 days after the challenge, and supplemented with *P. pastoris* X-33 in diet (25 and 12.5 %, respectively) was also lower ($P < 0.05$) in the control group (60 and 40 %, respectively)

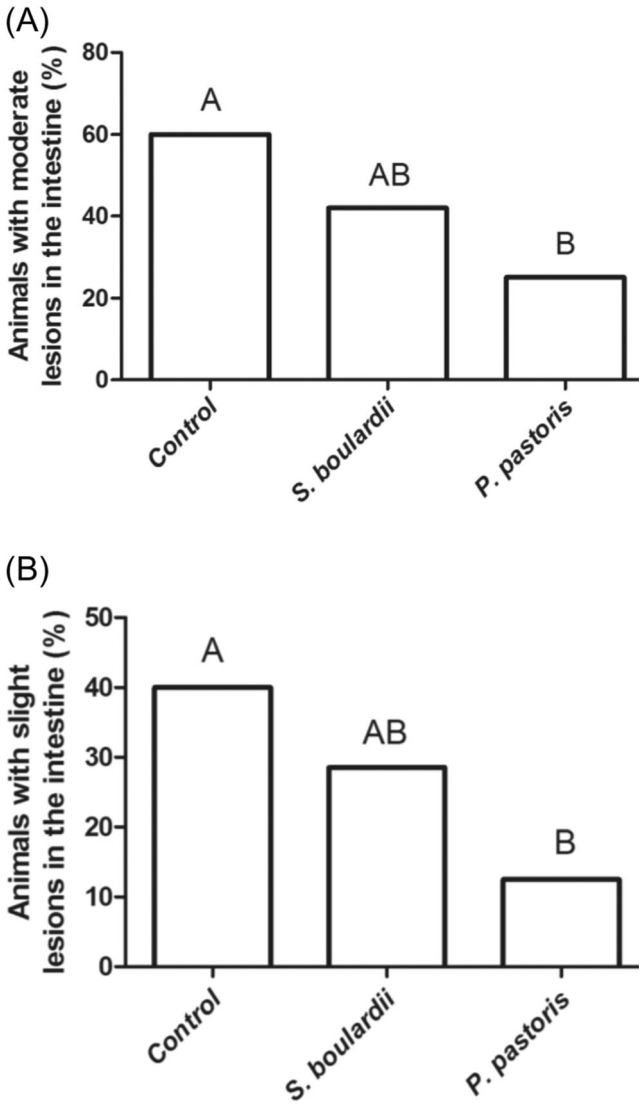

Fig. 6 Percentage of mice supplemented for 20 days with 7 log CFU of *P. pastoris* X-33 per gram of diet, which survived the challenge with 1 LD50 of *S.* Typhimurium and had moderate (**a**) and light intestinal lesions (**b**). The animals in the negative control group received a standard diet without probiotics. The animals in the positive control group received diet with 7 log CFU of *S. boulardii*. Different letters represent significant differences ($p < 0.05$), by analysis of variance (ANOVA) and the Tukey test.

and the group treated with *S. boulardii* (42 and 28 %, respectively). In relation to the animals supplemented with *S. boulardii*, no significant difference was observed in the percentage of mice with injuries, compared with the control group (Fig. 6a, b).

Discussion

This study shows for the first time that the yeast *P. pastoris* X-33, widely used as a heterologous protein production platform, has probiotic properties with outstanding antibacterial activity against *S.* Typhimurium. The yeast remained viable and at appropriate concentration in the diet for at least 2 months, survived the stresses of the gastrointestinal tract in vitro and in vivo, caused no behavioral changes or lesions when administered to mice, inhibited the growth of *S.* Typhimurium in culture media, reduced adhesion of the bacteria to intestinal cells HCT-116, and decreased the mortality of experimentally challenged animals. These results complement studies on broiler chicken, showing that *P. pastoris* supplementation is safe and beneficial, increasing the weight gain of these animals (Gil de los Santos et al. 2012).

Probiotics are challenged by chemical barriers until they reach their site of action, barriers as the gastric juice, digestive enzymes, bile salts, and the alkaline pH of the intestine (Conway 1996; Erkkila and Petaja 2000). In this study, the yeast *P. pastoris* X-33 withstood the simulated conditions of the GI tract, with a survival rate of approximately 77 %, a lower result than by the bacterium *Rhodopseudomonas palustris* (Fang et al. 2012) and the yeast *Wicherhamomyces anomalus* (Garcia-Hernandez et al. 2012), by which survival rates exceeding 90 % were obtained. However, these microorganisms were not challenged with such adverse and reality-near conditions as those in this study, since the GI tract simulation experiments were not performed in sequence, to simulate gastric conditions, and thereafter, intestinal conditions, nor were they challenged with the digestive enzymes pepsin and pancreatic in the experiments. *P. pastoris* X-33 also survived the TGI conditions of mice. Twenty-four hours after administration (7 log), a population of approximately 6 $\log_{10}$ yeast per gram of fresh feces was isolated. This concentration dropped drastically after 48 h, indicating that the supply to the animal must be performed every 24 h, to ensure appropriate concentrations in the host intestine, since the probiotic effect is dose dependent (FAO/WHO 2002). Yeasts are generally eliminated by the host due to competition with the GI tract microbiota. Therefore, they have to be administered regularly (Fuller 1989). Two to 5 days after intake stop of *S. boulardii*, the yeast was no longer found in the feces of the host (Blehaut et al. 1989). Among the 103 candidate yeasts, the probiotics evaluated by Tiago et al. (2009), including 48 of the genus *Pichia*, only three (*Pichia kluyveri*, *Metschnikowia reukaufii*, and *Zygosaccharomyces fermentati*) were found in the feces 4 days after interrupting the supply (Vieira et al. 1998).

Salmonellosis is one of the major foodborne illnesses, causing considerable losses in animal production and being considered one of the world's major zoonoses, affecting the public health, with high endemicity and morbidity and particularly difficult to control. The WHO recommends the search for alternative treatments for foodborne infections, and probiotics have attracted attention in this sense (Jain et al. 2008). Although the effectiveness of probiotics against salmonellosis in humans has not yet been confirmed, results have been promising in experimental models (Fang et al. 2012;

Martins et al. 2010; Santos et al. 2001; Truusalu et al. 2008). In this study, *P. pastoris* X-33 inhibited the growth and increased the growth time of *S.* Typhimurium in vitro, being more effective than a blend of *Lactobacillus acidophilus* and *Lactobacillus casei* (Milette et al. 2007). In the test with intestinal cells HCT-116, *P. pastoris* X-33 reduced the adhesion of *S.* Typhimurium by 47 %, comparable to the result with *S. boulardii* (control). Similar results were reported by Martins et al. (2010) in an evaluation of the ability of *S. boulardii* to inhibit the adhesion and invasion of *S.* Typhimurium in intestinal T84 cells. Finally, the protective potential of this strain was observed in the challenge experiments with *S.* Typhimurium in animals supplemented with *P. pastoris* X-33 by gavage or in the diet, since the survival rate increased by 30 %, whereas *Salmonella* concentration and lesions in the surviving animals were reduced. These results corroborate findings of other studies, which showed a protective effect of *S. boulardii* and other yeasts against infection caused by *S.* Typhimurium in mice (Martins et al. 2009; Martins et al. 2010; Martins et al. 2013; Rodrigues et al. 1996). The mechanisms proposed to explain the antibacterial activity of *S. boulardii* against *S.* Typhimurium are immunomodulation, competition for nutrients, bacteria adsorption, and modulation of signaling pathways involved in the activation of inflammation (Martins et al. 2005; Martins et al. 2011; Martins et al. 2013). Although these mechanisms may be associated with *P. pastoris* X-33, further studies are required to determine the mechanisms of action of this yeast against *S.* Typhimurium.

Thus, *P. pastoris* X-33, a yeast with the GRAS (generally recognized as safe) status, is widely used in the production of heterologous proteins, it can grow from industrial waste and effluents (Celik et al. 2008, Gil de los Santos et al. 2012), remain viable in animal feed for at least 2 months, and has probiotic properties, making it a probiotic option with excellent antibacterial activity against *Salmonella* Typhimurium. The ease of genetic manipulation and the existence of several vectors for the expression of heterologous proteins indicate this yeast as a carrier of biofunctional molecules in animals feed.

Acknowledgments The authors thank professors Odir A. Dellagostin and Vinicius F. Campos for their contribution. They are also indebted to the Brazilian Council for Scientific and Technological Development (CNPq) and Brazilian Federal Agency for Support and Evaluation of Graduate Education (Capes) for funding the project.

Conflict of interest The authors declare that they have no competing interests.

References

Blehaut H, Massot J, Elmer GW, Levy RH (1989) Disposition kinetics of *Saccharomyces boulardii* in man and rat. Biopharm Drug Dispos 10:353–364

Boddy AV, Elmer GW, McFarland LV, Levy RH (1991) Influence of antibiotics on the recovery and kinetics of *Saccharomyces boulardii* in rats. Pharm Res 8:796–800

Brandao RL, Castro IM, Bambirra EA, Amaral SC, Fietto LG, Tropia MJ, Neves MJ, Dos Santos RG, Gomes NC, Nicoli JR (1998) Intracellular signal triggered by cholera toxin in *Saccharomyces boulardii* and *Saccharomyces cerevisiae*. Appl Environ Microbiol 64:564–568

Bron PA, Van Baarlen P, Kleerebezem M (2012) Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. Nat Rev Microbiol 10:66–78. doi:10.1038/nrmicro2690

Çelik E, Ozbay NE, Çalik P (2008) Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*. Ind Eng Chem Res 47:2985–2990. doi:10.1021/ie071613o

Conway P (1996) Selection criteria for probiotic microorganisms. Asia Pac J Clin Nutr 5:10–14

Cregg JM, Barringer KJ, Hessler AY, Madden KR (1985) *Pichia pastoris* as a host system for transformations. Mol Cell Biol 5:3376–3385

Cregg JM, Cereghino JL, Shi J, Higgins DR (2000) Recombinant protein expression in *Pichia pastoris*. Mol Biotechnol 16:23–52

Czerucka D, Roux I, Rampal P (1994) *Saccharomyces boulardii* inhibits secretagogue-mediated adenosine 3′,5′-cyclic monophosphate induction in intestinal cells. Gastroenterology 106:65–72

Czerucka D, Dahan S, Mograbi B, Rossi B, Rampal P (2000) *Saccharomyces boulardii* preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic *Escherichia coli*-infected T84 cells. Infect Immun 68:5998–6004

Czerucka D, Piche T, Rampal P (2007) Review article: yeast as probiotics—*Saccharomyces boulardii*. Aliment Pharmacol Ther 26:767–778

Drago L, Gismondo MR, Lombardi A, de HC, Gozzini L (1997) Inhibition of in vitro growth of enteropathogens by new *Lactobacillus* isolates of human intestinal origin. FEMS Microbiol Lett 153: 455–463

Duc lH, Hong HA, Barbosa TM, Henriques AO, Cutting SM (2004) Characterization of *Bacillus* probiotics available for human use. Appl Environ Microbiol 70:2161-2171. doi:10.1128/AEM.70.4.2161-2171.2004

Erkkila S, Petaja E (2000) Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci 55:297–300

Fang LC, Li Y, Cheng P, Deng J, Jiang LL, Huang H, Zheng JS, Wei H (2012) Characterization of *Rhodopseudomonas palustris* strain 2C as a potential probiotic. Acta Pathol Microbiol Immunol Scand *120*: 743–749. doi:10.1111/j.1600-0463.2012.02902.x

FAO/WHO. Guidelines for the evaluation of probiotics in food. FAO/WHO.Food and Agriculture Organization/World Health Organization. [Food and Agriculture Organization/World Health Organization.], 1–11. 2002. London

Fuller R (1989) Probiotics in man and animals. J Appl Bacteriol *66*(5): 365–378

Garcia-Hernandez Y, Rodriguez Z, Brandao LR, Rosa CA, Nicoli JR, Iglesias AE, Perez-Sanchez T, Salabarria RB, Halaihel N (2012) Identification and in vitro screening of avian yeasts for use as probiotic. Res Vet Sci 93:798–802. doi:10.1016/j.rvsc.2011.09.005

Gil de Los Santos, JR, Storch, OB, Fernandes, CG, Gil-Turnes, C (2012) Evaluation in broilers of the probiotic properties of *Pichia pastoris* and a recombinant *P. pastoris* containing the *Clostridium perfringens* alpha toxin gene. Vet. Microbiology. 156(3–4):448-451. doi:10.1016/j.vetmic.2011.11.019

Jain S, Yadav H, Sinha PR, Naito Y, Marotta F (2008) Dahi containing probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* has a protective effect against *Salmonella enteritidis* infection in mice. Int J Immunopathol Pharmacol 21:1021–1029

Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S, Arvilommi H (1992) Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus strain*. Pediatr Res 32:141–144

Le Blanc AM, Castillo NA, Perdigon G (2010) Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar Typhimurium infection. Int J Food Microbiol *138*(3):223–231. doi:10.1016/j.ijfoodmicro.2010.01.020

Love KR, Politano TJ, Panagiotou V, Jiang B, Stadheim TA, Love JC (2012) Systematic single-cell analysis of *Pichia pastoris* reveals secretory capacity limits productivity. PLoS One *7*. doi:10.1371/journal.pone.0037915

Martins FS, Barbosa FHF, Penna FJ, Rosa CA, Nardi RMD, Neves MJ, Nicoli JR (2005) Estudo do potencial probiótico de linhagens de *Saccharomyces cerevisiae* através de testes in vitro. Rev BioCie Terra ISSN 1519–5228 5(2):1–13

Martins FS, Veloso LC, Arantes RM, Nicoli JR (2009) 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*(6):738–744. doi:10.1111/j.1472-765X.2009.02732.x

Martins FS, Dalmasso G, Arantes RM, Doye A, Lemichez E, Lagadec P, Imbert V, Peyron JF, Rampal P, Nicoli JR, Czerucka D (2010) Interaction of *Saccharomyces boulardii* with *Salmonella enterica* serovar Typhimurium protects mice and modifies T84 cell response to the infection. PLoS One 5. doi:10.1371/journal.pone.0008925

Martins FS, Elian SD, Vieira AT, Tiago FC, Martins AK, Silva FC, Souza EL, Sousa LP, Araujo HR, Pimenta PF, Bonjardim CA, Arantes RM, Teixeira MM, Nicoli JR (2011) 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 301:359–364. doi:10.1016/j.ijmm.2010.11.002

Martins FS, Vieira AT, Elian SD, Arantes RM, Tiago FC, Sousa LP, Araujo HR, Pimenta PF, Bonjardim CA, Nicoli JR, Teixeira MM (2013) Inhibition of tissue inflammation and bacterial translocation as one of the protective mechanisms of *Saccharomyces boulardii* against *Salmonella* infection in mice. Microbes Infect 15(4):270–279. doi:10.1016/j.micinf.2012.12.007

Milette M, Luquet FM, Lacroix M (2007) In vitro growth control of selected pathogens by *Lactobacillus acidophilus* and *Lactobacillus* case in-fermented milk. Lett Appl Microbiol 44:314–319

Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. Am J Hyg 27:493–497

Rodrigues AC, Nardi RM, Bambirra EA, Vieira EC, Nicoli JR (1996) Effect of *Saccharomyces boulardii* against experimental oral infection with *Salmonella* Typhimurium and *Shigella flexneri* in conventional and gnotobiotic mice. J Appl Bacteriol 81:251–256

Saad SMI (2006) Probióticos e prebióticos: o estado da arte. Rev Bras Ciênc Farm 42:1–16

Santos DG, Gil-Turnes C, Conceição FR (2012) Bioremediation of parboiled rice effluent supplemented with biodiesel-derived glycerol using *Pichia pastoris* X-33. Sci World J. doi:10.1100/2012/492925

Santos RL, Zhang S, Tsolis RM, Kingsley RA, Adams LG, Baumler AJ (2001) Animal models of *Salmonella* infections: enteritis versus typhoid fever. Microbes Infect 3:1335–1344

Tiago FC, Martins FS, Rosa CA, Nard RMD, Cara DC, Nicoli JR (2009) Physiological characterization of non-saccharomyces yeasts from agro-industrial and environmental origins with possible probiotic function. World J Microbiol Biotechnol 25:657–666

Torres FAG, Moraes LMP (2000) Proteínas recombinantes produzidas em leveduras. Biotechnol Cien Desenv 12:20–22

Truusalu K, Mikelsaar RH, Naaber P, Karki T, Kullisaar T, Zilmer M, Mikelsaar M (2008) Eradication of *Salmonella* typhimurium infection in a murine model of typhoid fever with the combination of probiotic *Lactobacillus fermentum* ME-3 and ofloxacin. BMC Microbiol 8:132. doi:10.1186/1471-2180-8-132

Vieira LQ, Oliveira MR, Neumann E, Nicoli JR, Vieira EC (1998) Parasitic infections in germfree animals. Braz J Med Biol Res 31:105–110