J. Gen. Appl. Microbiol., **51**, 83–92 (2005)

Full Paper

Screening of yeasts as probiotic based on capacities to colonize the gastrointestinal tract and to protect against enteropathogen challenge in mice

Flariano S. Martins,[1,3] Regina M. D. Nardi,[1] Rosa M. E. Arantes,[2] Carlos A. Rosa,[1] Maria J. Neves,[3] and Jacques R. Nicoli[1,*]

[1] Departamento de Microbiologia and [2] Departamento de Patologia Geral, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, and [3] Laboratório de Radiobiologia, Centro de Desenvolvimento da Tecnologia Nuclear/CNEN, Belo Horizonte, MG, Brazil

(Received July 1, 2004; Accepted December 7, 2004)

Probiotics are defined as viable microorganisms that exhibit a beneficial effect on the host's health when they are ingested. Two important criteria are used for selection of probiotic microorganisms: they must be able to survive in the gastrointestinal environment and to present at least one beneficial function (colonization resistance, immunomodulation or nutritional contribution). Generally, in vitro assays demonstrating these properties were used to select probiotics but it is unclear if the data can be extrapolated to in vivo conditions. In the present work, twelve *Saccharomyces cerevisiae* strains isolated from different environments (insect association, tropical fruit, cheese and "aguardente" production) and pre-selected for in vitro resistance to simulated gastrointestinal conditions were inoculated in germ-free mice to evaluate their real capacity to colonize the mammal digestive tract. Using these data, one of the yeasts (*S. cerevisiae* 905) was selected and tested in gnotobiotic (GN) and conventional (CV) mice for its capacity to protect against oral challenge with two enteropathogenic bacteria (*Salmonella* Typhimurium and *Clostridium difficile*). The yeast reached populational levels potentially functional in the gastrointestinal portions where the enteropathogens tested act. No antagonism against either pathogenic bacterium by the yeast was observed in the digestive tract of GN mice but, after challenge with *S.* Typhimurium, mortality was lower and liver tissue was better preserved in CV animals treated with the yeast when compared with a control group ($p<0.05$). Histopathological results of intestines showed that the yeast also presented a good protective effect against oral challenge with *C. difficile* in GN mice ($p<0.05$). In conclusion, among the 12 *S. cerevisiae* tested, strain 905 showed the best characteristics to be used as a probiotic as demonstrated by survival capacity in the gastrointestinal tract and protective effect of animals during experimental infections.

Key Words——*Clostridium difficile*; germ-free and conventional mice; probiotics; *Saccharomyces cerevisiae*; *Salmonella* Typhimurium

Introduction

Probiotics are food or pharmaceutical preparations containing live non-pathogenic microorganisms which improve one of the three main beneficial functions (colonization resistance, immunomodulation or nutritional contribution) of the normal gastrointestinal microbiota

* Address reprint requests to: Dr. Jacques R. Nicoli, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, C.P. 486, Belo Horizonte, MG, Brazil.

Fax: +55–31–3499–2730

when ingested by human or animal hosts. For this reason, most of the probiotics studied or commercialized today has been selected from the digestive ecosystem. However, some of these biotherapeutics were also isolated from other non-intestinal sites. As an example of this last case, *Saccharomyces boulardii*, a non-pathogenic yeast, was isolated from lychee fruit in East Asia grows at the unusually high temperature of 37°C (McFarland and Bernasconi, 1993). Pharmacokinetic studies have shown that it achieves steady-state concentrations in the colon within 3 days when ingested and is cleared from stools 2–5 days after discontinuation (Bléhaut et al., 1989). It has been used for treatment of different types of diarrheal diseases such as antibiotic-associated diarrhea (Bartlett, 1992; McFarland et al., 1995; Surawicz, 2003; Surawicz et al., 1989a), *Clostridium difficile*-associated intestinal disease (Castagliuolo et al., 1999; Elmer et al., 1999; Kimmey et al., 1990; McFarland et al., 1994; Surawicz, 2003; Surawicz et al., 1989b, 2000), traveler's diarrhea (Scarpignato and Rampal, 1995) and diarrhea in HIV-infected patients (Born et al., 1993; Saint-Marc et al., 1991).

Various mechanisms of action have been proposed to explain *S. boulardii* protection (Czerucka and Rampal, 2002). It was demonstrated that this yeast modulates the immune system (Buts et al., 1990; Caetano et al., 1986; Rodrigues et al., 2000), degrades *C. difficile* toxins A and B and their respective receptors on colonic mucosa (Castagliuolo et al., 1996, 1999; Pothoulakis et al., 1993; Qamar et al., 2001), inhibits cholera toxin action (Brandão et al., 1998; Czerucka and Rampal, 1999; Czerucka et al., 1989, 1994; Neves et al., 2002), modulates the transduction pathway induced by enteropathogenic *Escherichia coli* (Czerucka et al., 2000), stimulates digestive enzymatic activities (Buts et al., 1986; Jahn et al., 1996) and fixes some enterobacteriaceae on its surface (Gedek, 1999).

At the moment, *S. boulardii* is practically the only yeast commercialized as probiotic in human medicine. However, other yeasts from ambiental or agroindustrial origins with similar or even better biotherapeutic properties certainly exist, particularly considering the rich biodiversity found in Brazilian microbial ecosystems.

The objective of the present study was to select yeasts as probiotic using the survival capacity in the gastrointestinal tract and the protective effect on animals during experimental infections as criteria.

Materials and Methods

Mice. Germ-free 21-day-old NIH mice (Taconic, Germantown, NY, USA) were used in this work. The animals were housed in flexible plastic isolators (Standard Safety Equipment Co., McHenry, IL, USA) and handled according to established procedures (Pleasants, 1974). Experiments with gnotobiotic mice were carried out in micro-isolators (UNO Roestvaststaal B.V., Zevenaar, The Netherlands). Conventional NIH mice were derived from the germ-free colony and only used after at least two generations following the conventionalization. Water and commercial autoclavable diet (Nuvital, Curitiba, PR, Brazil) were sterilized by steam and administered ad libitum to all the animals. Conventional mice were maintained in an open animal house and controlled lighting (12 h light, 12 h dark) was used for all the animals. All experimental procedures were carried out according to the standards set forth in the "Guide for the Care and Use of Laboratory Animals" of the National Research Council (1996).

Microorganisms. The yeasts used in this work (Table 1) belong to the Yeasts Bank maintained by Dr. C. A. Rosa from the Laboratory of Ecology and Biotechnology of Yeasts (Department of Microbiology, Federal University of Minas Gerais, MG, Brazil). The yeasts were characterized phenotypically by methods currently used in yeast taxonomy (Yarrow, 1998). Identities were verified using the keys described by Kurtzman and Fell (1998), and also using the computer program YEASTCOMPARE (Ciriello and Lachance, 2001) which compares the nutritional characteristics of any yeast with those of known species. The *Salmonella enterica* subsp. *enterica* serovar Typhimurium of human origin belongs to our laboratory and *Clostridium difficile* (American Type Culture Collection—ATCC 9689) was obtained from Fundação Oswaldo Cruz (Fiocruz, Rio de Janeiro, RJ, Brazil).

Treatments. A single dose of 10^8 viable cells of each *S. cerevisiae* strain was administered to germ-free mice by intragastric intubation for colonization experiments or 10 days before the experimental challenge with pathogenic bacteria. The same dose was administered daily to conventional mice, 10 days before the challenge and during the entire experimental period. The control conventional and gnotobiotic groups were treated with 0.9% saline according to the same schedule as the corresponding experimental groups.

Table 1. Yeast strains used in the present study.

	Saccharomyces cerevisiae strain[a]	Micro-habitat
01	UFMG 20	*Drosophila*[b]
02	UFMG 21	*Drosophila*[c]
03	UFMG 22	*Drosophila*[b]
04	UFMG 23	Tropical fruit[d]
05	UFMG 24	Estuary[e]
06	UFMG 829	"Aguardente" production[f]
07	UFMG 905	"Aguardente" production[f]
08	UFMG 2105	"Aguardente" production[f]
09	UFMG 2439	"Aguardente" production[f]
10	UFMG 2469	"Aguardente" production[f]
11	UFMG B99.32.9	Canastra cheese[g]
12	UFMG CH4	Chironomide[h]

[a] All the strains were purified and characterized according to standard methods (Yarrow, 1998) and identified by the keys as described by Kurtzman and Fell (1998) and Barnett et al. (1990). [b] Isolated from Tropical Forest, Brazil (Morais et al., 1992). [c] Isolated from Tijuca Forest, Brazil (Morais et al., 1992). [d] Isolated from a small tropical fruit from Atlantic Rain Forest, Rio de Janeiro, Brazil. [e] Isolated from estuary in Rio de Janeiro, Brazil. [f] Isolated from "cachaça" production in Minas Gerais, Brazil. [g] Isolated from cheese production in Serra da Canastra, Brazil. [h] Isolated from fly larvae.

Experimental infections. *Salmonella* Typhimurium and *C. difficile* were grown in liquid Brain Heart Infusion medium (Difco, Detroit, MI, USA) at 37°C during 24–48 h under aerobe or anaerobe (anaerobe chamber Forma Scientific, Marietta, OH, USA, containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2) conditions, respectively. Mice were inoculated by the orogastric route with 0.1 ml of the bacterial suspension containing 10^2 or 10^4 viable cells for gnotobiotic or conventional mice, respectively.

Microbial counts. For colonization experiments, feces freshly collected or contents from stomach, proximal, median and distal small intestine, cecum and colon of gnotobiotic and/or conventional NIH mice were diluted 100-fold in saline and vortexed. Serial 10-fold dilutions were made and 1 ml poured onto Sabouraud Dextrose agar (Difco) supplemented with 100 mg/L chloramphenicol. For experiments with pathogenic challenge, 0.1 ml from serial dilutions was plated onto McConkey agar (Difco) for *S.* Typhimurium, Brain Heart Infusion supplemented with yeast extract (0.5%), hemine (0.1%) and menadione (0.1%) for *C. difficile* and Sabouraud Dextrose agar (Difco) for *S. cerevisiae* and incubated at 37°C for 24–48 h for bacterial counts (under aerobe or anaerobe conditions depending on the bacteria) and 48–72 h for yeast counts. For bacterial or yeast counts, media were supplemented with 100 mg/L cycloheximide or 100 mg/L chloramphenicol, respectively.

Experimental design. For experiments of colonization and enteropathogenic challenge, each animal group consisted of 5 and 10 mice, respectively. Population levels of the pathogenic bacteria and of the yeast in the feces and cumulative mortality were noted during the challenge experiments. At the end of the experiments, all remaining mice were sacrificed by cervical dislocation.

Histopathological and morphometrical examinations. Tissue samples from intestines and liver of mice sacrificed at the end of the experiments were fixed in buffered 4% formaldehyde and processed for paraffin embedding. The histopathological sections (3–5 μm) were stained with hematoxylin-eosin. The slides were coded and examined by a single pathologist, who was unaware of the experimental conditions of each group. 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

Table 2. Yeasts counts (log_{10} cfu/g of feces±SD) for 10 days in the feces of gnotobiotic mice after a single inoculum of 10^8 cells.

Strain	Time (days)			
	1	4	7	10
UFMG 20	0	0	0	0
UFMG 21	5.83±0.56	5.29±0.94	5.29±0.94	5.18±0.34
UFMG 22	0	0	0	0
UFMG 23	6.76±0.29	4.83±0.60	5.33±0.33	5.33±0.33
UFMG 24	6.92±0.14	5.26±0.37	6.11±0.41	5.86±0.09
UFMG 829	7.11±0.12	5.40±0.30	6.08±0.42	5.13±0.38
UFMG 905	7.62±0.13	7.20±0.13	6.15±0.12	6.17±0.79
UFMG 2105	7.05±0.44	5.10±0.48	5.98±0.20	5.79±0.10
UFMG 2439	7.18±0.23	5.15±0.19	5.59±0.08	5.82±0.11
UFMG 2469	7.48±0.28	5.83±0.62	5.99±0.11	5.58±1.07
UFMG CH4	7.39±0.22	5.75±0.81	5.69±0.19	6.08±0.33
UFMG B.99.32.9	6.44±0.11	5.12±0.15	5.12±0.15	6.07±0.56

N=5.

(10×).

Statistical analysis. The results shown are from one representative of at least three independently performed. Statistical significance of the results was evaluated by Student's *t* test and analysis of variance (ANOVA) for all data, except survival, for which the Mann-Whitney Rank test was used. The level of significance was set at $p<0.05$. Statistical analyses were performed using the programme Sigma Stat (Jandel Scientific Software, version 1.0, San Rafael, CA, USA).

Results

Results showed that there was some discrepancy between in vitro assays and in vivo colonization of the mammal gastrointestinal tract. The yeasts UFMG 20 and UFMG 22 presented a good resistance to simulated exposure to bile salts and gastric and intestinal environments (data not shown), but they were not capable of colonizing the digestive tract of germ-free mice (Table 2). On the other hand, *S. cerevisiae* 905, from "aguardente" (cachaça) production, presented the best data in terms of viable intestinal populations (Table 2) with fecal levels which never fell below 10^6 cfu/g, contrarily to all other tested yeasts. At the end of the colonization experiment, mice monoassociated with *S. cerevisiae* 905 showed normal aspects at histological examination of intestines and liver when compared to control animals. For this reason, this yeast strain was selected for the next experimental steps.

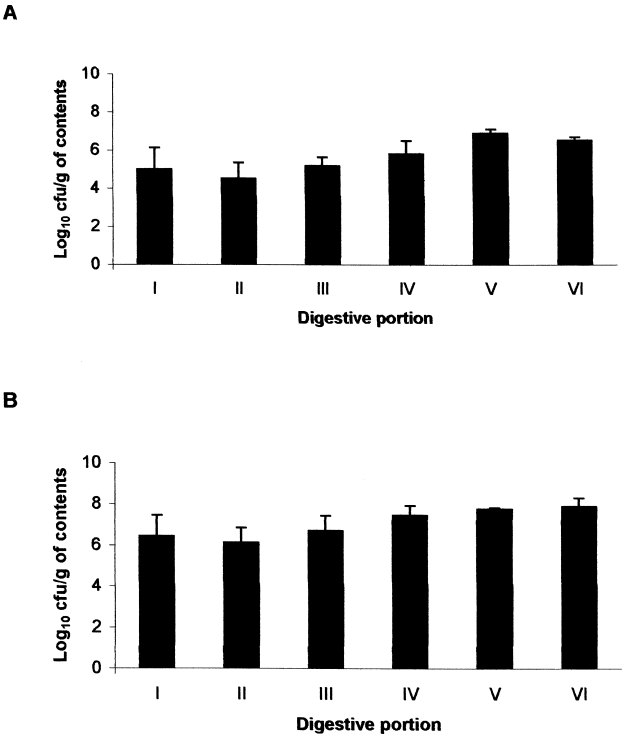

Fig. 1. *Saccharomyces cerevisiae* UFMG 905 population levels in the stomach (I), proximal (II), median (III) and distal (IV) small intestine, cecum (V) and colon (VI) of gnotobiotic (A) and conventional (B) NIH mice.

Figure 1 shows the yeast population levels in the contents of different portions of the gastrointestinal

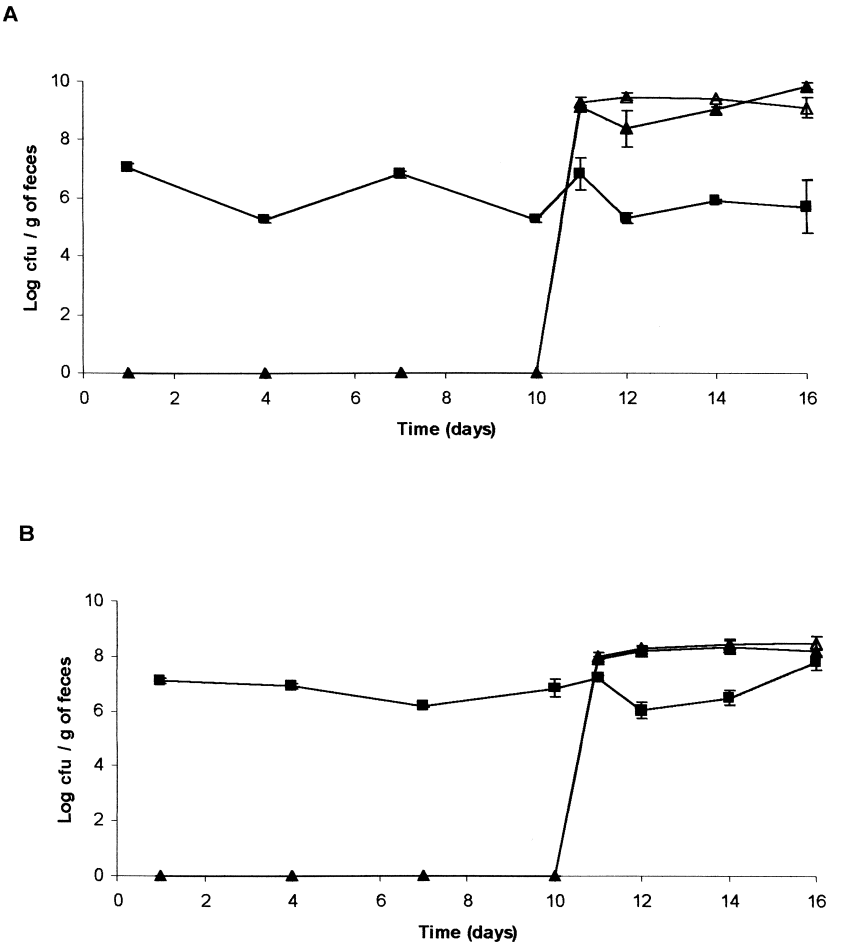

Fig. 2. Fecal populations of *Salmonella* Typhimurium (A) and *Clostridium difficile* (B) in gnotobiotic NIH mice treated (▲) or not (△) with *Saccharomyces cerevisiae* UFMG 905 for 10 days before the challenge with the bacteria.

Fecal population numbers of *Saccharomyces cerevisiae* UFMG 905 (■). After challenge, the yeast was inoculated at 2 days intervals.

tract of gnotobiotic (Fig. 1A) and conventional (Fig. 1B) mice. Yeast populations increased all along the small intestine and reached their highest levels in the final portions of the digestive tract. These levels were higher in conventional than in monoassociated mice.

Figure 2 shows that *S. cerevisiae* 905 became established in the digestive tract of gnotobiotic mice 24 h after the oral inoculation and the number of viable cells ranged around 10^6 to 10^7 cfu/g of feces. The kinetics of the establishment of *S.* Typhimurium and *C. difficile* in the experimental and control gnotobiotic mice are also shown in Fig. 2,A and B, respectively. In experimental gnotobiotic mice harboring the yeast, the two bacteria became established at levels of about 10^8–10^9 viable cells/g of feces and remained at these high levels until the animals died or were sacrificed. These levels were equivalent to those observed in gnotobiotic mice harboring the bacteria alone.

Figure 3 shows survival of conventional NIH mice treated or not with the yeast for 10 days and then challenged with *S.* Typhimurium. After 28 days of infection, 55% of the animals in the experimental group survived versus 15% in the control group. This difference in survival was statistically significant (p=0.003). Histological examination of liver from these groups corroborates the survival data. In conventional mice treated with the yeast and challenged with *S.* Typhimurium a better preservation of liver tissue was observed when compared with mice not treated (Fig. 4). Additionally, inflammatory infiltrate with higher numbers (p=0.0014) of foci containing polymorphonuclear and mononuclear cells was observed in control mice when compared to experimental animals (Table 3). Under gnotobiotic conditions, there was no difference in survival between

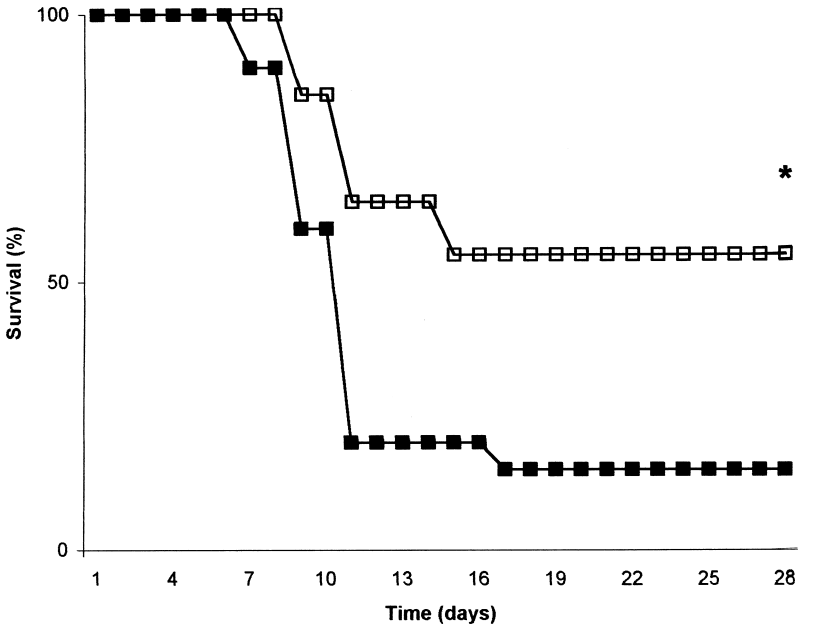

Fig. 3. Survival of conventional NIH mice treated (□) or not (■) with *Saccharomyces cerevisiae* UFMG 905 10 days before the challenge with *Salmonella* Typhimurium.
* $p=0.003$.

control and experimental mice challenged with *S.* Typhimurium and all the animals died in 6 to 7 days (data not shown).

Protective effect of the yeast against experimental infection with *C. difficile* was observed in the colon and cecum of gnotobiotic mice (Fig. 5). Experimental animals showed less intense lesions such as edema, inflammatory cell infiltrate and hyperemia. In conventional animals, experimental challenge with *C. difficile* is not possible due to the normal microbiota and its strong colonization resistance effect.

Discussion

Theoretically, any non-pathogenic bacterium, fungus, yeast or protozoan is a possible candidate for probiotic use. However, the number and the diversity of these microorganisms make the screening of biotherapeutic agents among them a very hard task. A simple and efficient method for this selection is necessary to process a very high number of microorganisms. In vivo assays are time consuming and involve a large number of animal groups and for these reasons are used only after the selection of a limited number of probiotic candidates. One procedure generally described in literature is the in vitro screening based on the capacity of a microorganism to survive to simulated conditions found in the digestive tract since this is indispensable for a probiotic to act. However, it is not very clear if results obtained in in vitro experiments can be always extrapolated to in vivo environments.

Very few yeasts have been studied as possible biotherapeutics and *S. boulardii* is one of the first and currently the only one commercialized in human medicine. Other *Saccharomyces* spp. or members of other yeast genera probably have probiotic activity similar to that of *S. boulardii* or even better. Actually, some authors have reported the use of some strains of *S. cerevisiae* but in very few experimental and clinical trials (Chia et al., 1995; Izadnia et al., 1998; Kovacs and Berk, 2000; Schellenberg et al., 1994). As one of the largest and most biodiverse countries in the world, Brazil may provide a rich source of microorganisms for potential probiotic use. In the present study, ambiental and agroindustrial yeast strains isolated in Brazil were tested as possible biotherapeutics for intestinal infections. Yeasts identified as *S. cerevisiae* were selected because they are already known to be relatively safe microorganisms that are genetically very close to *S. boulardii*.

The microorganisms used as probiotics must confront a variety of simultaneous or sequential adverse conditions such as mild heat shock (internal body temperature), acidic gastric juice, basic pancreatic juice

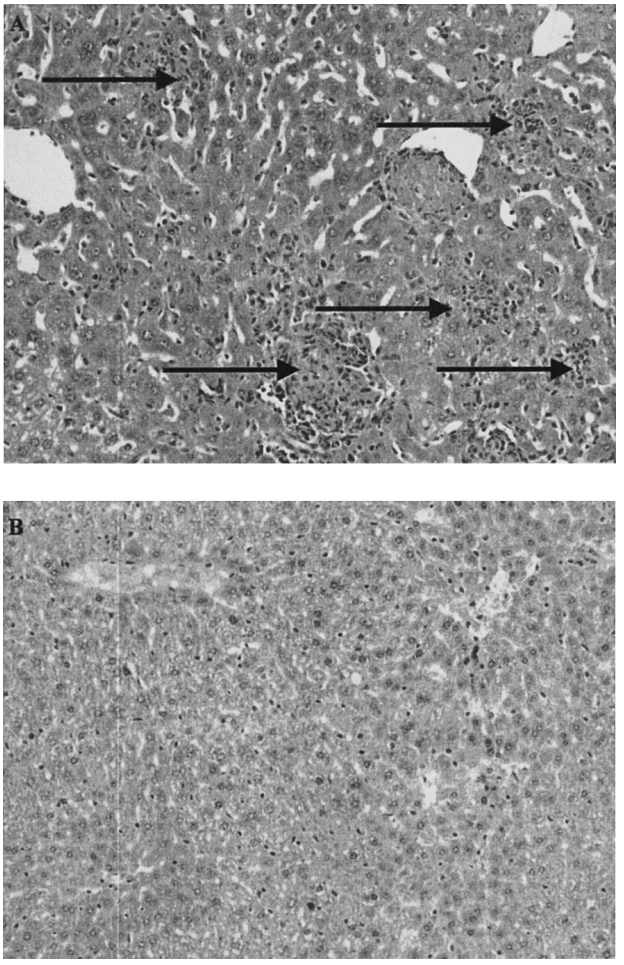

Fig. 4. Histopathological aspect of liver from conventional NIH mice not treated (A) or treated (B) with the yeast *Saccharomyces cerevisiae* 905 for 10 days and then challenged with *Salmonella* Typhimurium.

Hematoxylin and eosin (100×). Arrows indicate inflammatory focus.

and the presence of lysozyme and bile salts. This problem is particularly important when the probiotics are not originally from the digestive tracts of mammals, as is the case for *Saccharomyces* strains. In this context, in vitro selection of probiotic microorganisms is done by the exposure of microbial candidates to conditions simulating the gastrointestinal conditions and determination of their survival. In the present study, twelve *S. cerevisiae* previously selected for their survival capacities under these in vitro conditions were used for the in vivo steps.

Among the selected strains, those isolated from an agroindustrial environment ("cachaça" production, a typical "aguardente" from Brazil and cheese) showed slightly better capacity to colonize and maintain high population levels in the digestive tract of germ-free mice, particularly *S. cerevisiae* 905 (Table 2). This fact can be due to the higher resistance ability of these microorganisms developed in the more competitive conditions found during the fermentation process. Using these data, *S. cerevisiae* 905 was selected and tested in gnotobiotic and conventional mice for its capacity to colonize the various portions of the digestive tract and to protect against oral challenge with enteropathogen bacteria (*S.* Typhimurium and *C. difficile*).

The *S.* Typhimurium strain used in the present study induces enteroinvasive disease both in germ-free and conventional mice (Rodrigues et al., 2000). On the other hand, *C. difficile* fails to multiply in the conventional mouse intestine but establishes itself in the germ-free animals. Yeasts are also drastically eliminated or repressed from the digestive tract of mammal host harboring a normal complex intestinal microbiota,

Table 3. Number of inflammatory foci versus units of area in the liver of conventional NIH mice treated or not with the yeast *Saccharomyces cerevisiae* 905 for 10 days and then challenged with 10^4 cells of *Salmonella* Typhimurium for 28 days.

Group	Number of inflammatory foci[a]	Area (units of area)[b]	Focus/units of area
Control	25	$8.7\times10^6\,\mu m^2$	25
	20	$8.7\times10^6\,\mu m^2$	20
	31	$8.7\times10^6\,\mu m^2$	31
Media			25.3*
Experimental	9	$8.7\times10^6\,\mu m^2$	9
	17	$8.7\times10^6\,\mu m^2$	12
	2	$8.7\times10^6\,\mu m^2$	3
Media			8.0*

[a] Inflammatory focus is defined as accumulation of inflammatory cells in number higher than 10 cells, accompanied by necrotic alterations of the associated parenchyma. [b] The unit of area measured in all animals is the sum of ten camps for 10×. N=3 animals. * p=0.014.

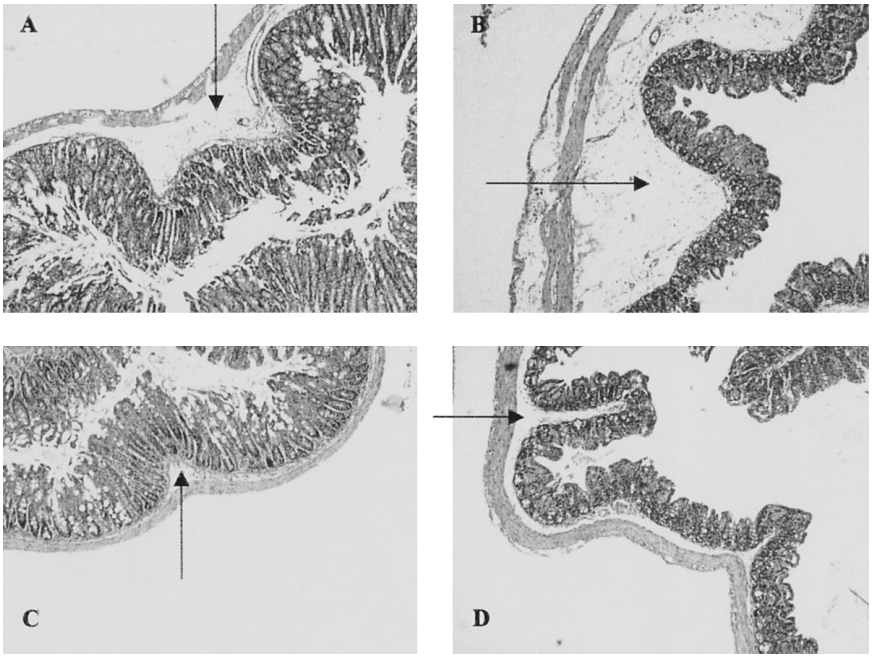

Fig. 5. Histopathological aspect of colon (A and C) and cecum (B and D) from gnotobiotic NIH mice monoassociated (C and D) or not (A and B) with the yeast *Saccharomyces cerevisiae* 905 for 10 days and then challenged with *Clostridium difficile*.

Hematoxylin and eosin (40×). Arrows indicate edema areas.

but its implantation is possible in germ-free animals. For these reasons, the gnotobiotic mouse provides a simplified in vivo system which allows the observation of ecological interactions in the gastrointestinal tract between microbial strains inoculated in this ecosystem.

The terminal ileum is the primary site of *S.* Typhimurium invasion while infection by *C. difficile* occurs in the more lower portions of the digestive tract (cecum and colon). To be efficient for protection, a probiotic must be present viably and in high population levels at the site of infection by enteropathogens. In gnotobiotic as well as in conventional mice, *S. cerevisiae* 905 reached populational levels potentially functional in the gastrointestinal portions where each enteropathogen tested acts (Fig. 1). As expected, in both gnotobiotic and conventional animals higher populations of the yeast were observed in the cecum and colon when compared to the small intestine where peristalsis is faster. Curiously, the yeast populations were higher in all portions of conventional animals when compared to gnotobiotic mice. This was probably due to the daily dose administered to conventional mice instead of a unique initial dose used in gnotobiotic animals.

The yeast was not capable of protecting the mice against the oral challenge with *S.* Typhimurium when the normal microbiota was absent. Nevertheless, it appears that it may contribute to a complementary protection (in hosts with perturbed or impaired intestinal microbiota, for example) as can be seen observed by the lower mortality (Fig. 3) and the good preservation of liver tissue (Table 3) of conventional mice treated with the yeast and challenged with *S.* Typhimurium. These results were similar to those obtained by Rodrigues et al. (2000) under the same experimental conditions. The data suggest that the yeast may inhibit or reduce translocation by this pathogen and the comparison of the number of inflammation foci in the liver reinforces this hypothesis (Table 3).

Better preservation of the lower intestinal tract was observed after oral infection with *C. difficile* in gnotobiotic mice treated with the *S. cerevisiae* 905 when compared with the control group (Fig. 5). This protective effect against *C. difficile* has already been observed by many authors utilizing *S. boulardii* (Castagliuolo et al., 1996; Castex et al., 1990; Corthier et al., 1986, 1992; Czerucka et al., 1991; Elmer and Corthier, 1991; Pothoulakis et al., 1993) and a strain of *S. cerevisiae* (Izadnia et al., 1998).

Antagonism by inhibitory compound production is one of the hypothesis more frequently used to explain probiotic acts against enteropathogenic microorganisms. A growth inhibition of several pathogenic bacteria by *S. boulardii* has been described, only in vitro (Brugier and Patte, 1975). The protection against *S.* Typhimurium and *C. difficile* obtained in mice previously associated or treated with the yeast is not due to the reduction of the bacterial populations in the intestines (Fig. 2). This result was similar to that previously observed in vivo with *S. boulardii* (Rodrigues et al., 2000). Some other properties could explain the protective effect against the enteropathogenic bacteria such as immunomodulation, modulation of toxin production or action and competition for adhesion sites or nutrients in the presence of the yeast. Experiments based on these hypothesis are currently being carried out in our laboratory to explain the protective phenomenon observed with *S. cerevisiae* 905.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Centro do Desenvolvimento Tecnológico Nuclear/Comissão Nacional de Energia Nuclear (CDTN/CNEN). We thank Maria Gorete Barbosa Ribas and Antônio Mesquita Vaz for valuable technical assistance.

References

Barnett, J. A., Payne, R. W., and Yarrow, D. (1990) Yeast: Characteristics and Identification, University Press Cambridge, Cambridge.

Bartlett, J. G. (1992) Antibiotic-associated diarrhea. *Clin. Infect. Dis.*, **15**, 573–581.

Bléhaut, H., Massot, J., Elmer, G. W., and Levy, R. H. (1989) Disposition kinetics of *Saccharomyces boulardii* in man and rat. *Biopharm. Drug Dispos.*, **10**, 353–364.

Born, P., Lersch, C., Zimmerhackl, B., and Claassen, M. (1993) The *Saccharomyces boulardii* therapy of HIV-associated diarrhea (letter). *Dtsch. Med. Wochenschr.*, **118**, 765.

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

Brugier, S. and Patte, F. (1975) Antagonisme in vitro entre l'ultra-levure et différents germes bactériens. *Med. Paris*, **45**, 61–66.

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

Buts, J. P., Bernasconi, P., Van Craynest, M. P., Maldague, P., and De Meyer, R. (1986) Response of human and rat small intestinal mucosa to oral administration of *Saccharomyces boulardii*. *Pediatr. Res.*, **20**, 192–196.

Caetano, J. A. M., Paramés, M. T., Babo, M. J., Santos, A., Ferreira, A. B., Freitas, A. A., Coelho, M. R. C., and Mateus, A. M. (1986) Immunopharmacological effects of *Saccharomyces boulardii* in healthy volunteers. *Int. J. Immunopharmacol.*, **8**, 245–259.

Castagliuolo, I., Lamont, J. T., Nikulasson, S. T., and Pothoulakis, C. (1996) *Saccharomyces boulardii* protease inhibits *Clostridium difficile* toxin A effects in the rat ileum. *Infect. Immun.*, **64**, 5225–5232.

Castagliuolo, I., Riegler, M. F., Valenick, L., Lamont, J. T., and Pothoulakis, C. (1999) *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins A and B in human colonic mucosa. *Infect. Immun.*, **67**, 302–307.

Castex, F., Corthier, G., Jouvert, S., Elmer, G. W., Lucas, F., and Bastide, M. (1990) Prevention of *Clostridium difficile*-induced experimental pseudomembranous colitis by *Saccharomyces boulardii*: A scanning electron microscopic and microbiological study. *J. Gen. Microbiol.*, **136**: 1085–1089.

Chia, J. K. S., Chan, S. M., and Goldstein, H. (1995) Baker's yeast as adjunctive therapy for relapses of *Clostridium difficile* diarrhea. *Clin. Infect. Dis.*, **20**, 1581.

Ciriello, C. J. and Lachance, M. A. (2001) YEASTCOMPARE, University of Western Ontario, London, ON, Canada.

Corthier, G., Dubos, F., and Ducluzeau, R. (1986) Prevention of *Clostridium difficile* induced mortality in gnotobiotic mice by *Saccharomyces boulardii*. *Can. J. Microbiol.*, **32**, 894–896.

Corthier, G., Lucas, F., Jouvert, S., and Castex, F. (1992) Effect of oral *Saccharomyces boulardii* treatment on the activity of *Clostridium difficile* toxins in mouse digestive tract. *Toxicon*, **30**, 1583–1589.

Czerucka, D. and Rampal, P. (1999) Effect of *Saccharomyces boulardii* on cAMP- and Ca^{2+}-dependent Cl^- secretion in T84 cells. *Dig. Dis. Sci.*, **44**, 2359–2368.

Czerucka, D. and Rampal, P. (2002) Experimental effects of *Saccharomyces boulardii* on diarrheal pathogens. *Microbes Infect.*, **4**, 733–739.

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

Czerucka, D., Nano, J. L., Bernasconi, P., and Rampal, P. (1989) Effect of *Saccharomyces boulardii* on cholera toxin-induced cAMP levels in rat epithelial intestinal cell lines. *Gastroenterol. Clin. Biol.*, **3**, 383–384.

Czerucka, D., Nano, J. L., Bernasconi, P., and Rampal, P. (1991) Response of the IRD intestinal epithelial cell line to *Clostridium difficile* toxins A and B in rats. Effect of *Saccharomyces boulardii*. *Gastroenterol. Clin. Biol.*, **15**, 22–27.

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

Elmer, G. W. and Corthier, G. (1991) Modulation of *Clostridium difficile* induced mortality as a function of the dose and the viability of the *Saccharomyces boulardii* used as a preventative agent in gnotobiotic mice. *Can. J. Microbiol.*, **37**, 315–317.

Elmer, G. W., Mcfarland, L. V., Surawicz, C. M., Danko, L., and Greenberg, R. N. (1999) Behaviour of *Saccharomyces boulardii* in recurrent *Clostridium difficile* disease patients. *Aliment. Pharmacol. Ther.*, **13**, 1663–1668.

Gedek, B. R. (1999) Adherence of *Escherichia coli* serogroup O 157 and the *Salmonella typhimurium* mutant DT 104 to the surface of *Saccharomyces boulardii*. *Mycoses*, **42**, 261–264.

Izadnia, F., Wong, C. T., and Kocoshis, S. A. (1998) Brewer's yeast and *Saccharomyces boulardii* both attenuate *Clostridium difficile*-induced colonic secretion in the rat. *Dig. Dis. Sci.*, **43**, 2055–2060.

Jahn, H. U., Ullrich, R., Schneider, T., Liehr, R. M., Schieferdecker, H. L., Holst, H., and Zeitz, M. (1996) Immunological and tropical effects of *Saccharomyces boulardii* on the small intestine in healthy human volunteers. *Digestion*, **57**, 95–104.

Kimmey, M. B., Elmer, G. W., Surawicz, C. M., and Mcfarland, L. V. (1990) Prevention of further recurrences of *Clostridium difficile* colitis with *Saccharomyces boulardii*. *Dig. Dis. Sci.*, **35**, 897–901.

Kovacs, D. J. and Berk, T. (2000) Recurrent *Clostridium difficile*-associated diarrhea and colitis treated with *Saccharomyces cerevisiae* (baker's yeast) in combination with antibiotic therapy: A case report. *J. Am. Board Farm. Pract.*, **13**, 138–140.

Kurtzman, C. P. and Fell, J. W. (1998) The Yeasts: A Taxonomic Study, 4th ed., Elsevier Science Publishers, Amsterdam.

McFarland, L. V. and Bernasconi, P. (1993) *Saccharomyces boulardii*: A review of an innovative biotherapeutic agent. *Microb. Ecol. Health Dis.*, **6**, 157–171.

McFarland, L. V., Surawicz, C. M., Greenberg, R. N., Fekety, R., Elmer, G. W., Moyer, K. A., Melcher, S. A., Bowen, K. E., Cox, J. L., and Noorani, Z. (1994) A randomized placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. *J. Am. Med. Assoc.*, **271**, 1913–1918.

McFarland, L. V., Surawicz, C. M., Greenberg, R. N., Elmer, G. W., Moyer, K. A., Melcher, S. A., Bowen, K. E., and Cox, J. L. (1995) Prevention of beta-lactam-associated diarrhea by *Saccharomyces boulardii* compared with placebo. *Am. J. Gastroenterol.*, **90**, 439–448.

Morais, P. B., Hagler, A. N., Rosa, C. A., Mendonça-Hagler, L. C., and Klaczko, L. B. (1992) Yeasts associated with *Drosophila* in tropical forests of Rio de Janeiro, Brazil. *Can. J. Microbiol.*, **38**, 1150–1155.

National Research Council (1996) Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington.

Neves, M. J., Etchebehere, L., Brandão, R. L., Castro, I. M., Lima, M. E., and Nicoli, J. R. (2002) Partial characterization of cholera toxin binding on membranes of *Saccharomyces boulardii*. *Microecol. Ther.*, **29**, 185–190.

Pleasants, J. R. (1974) Gnotobiotics. *In* Handbook of Laboratory Animal Science, ed. by Melby, E. C., Jr. and Altmann, N. H., CRC Press, Cleveland, pp. 119–174.

Pothoulakis, C., Kelly, C. P., Joshi, M. A., Gao, N., O'Keane, C. J., Castagliuolo, I., and Lamont, J. T. (1993) *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin A binding and enterotoxicity in rat ileum. *Gastroenterology*, **104**, 1108–1115.

Qamar, A., Aboudola, S., Warny, M., Michetti, P., Pothoulakis, C., Lamont, J. T., and Kelly, C. P. (2001) *Saccharomyces boulardii* stimulates intestinal immunoglobulin A immune response to *Clostridium difficile* toxin A in mice. *Infect. Immun.*, **69**, 2762–2765.

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

Saint-Marc, T., Rossello-Prats, L., and Touraine, J. L. (1991) Efficacy of *Saccharomyces boulardii* in the treatment of diarrhea in AIDS (letter). *Ann. Med. Intern.*, **142**, 64–65.

Scarpignato, C. and Rampal, P. (1995) Prevention and treatment of traveler's diarrhea: A clinical pharmacological approach. *Chemotherapy*, **41**, 48–81.

Schellenberg, D., Bonington, A., Champion, M., Lancaster, R., Webb, S., and Main, J. (1994) Treatment of *Clostridium difficile* diarrhea with brewer's yeast. *Lancet*, **343**, 171–172.

Surawicz, C. M. (2003) Probiotics, antibiotic-associated diarrhoea and *Clostridium difficile* diarrhoea in humans. *Best Pract. Res. Clin. Gastroenterol.*, **17**, 775–783.

Surawicz, C. M., Elmer, G. W., Speelman, P., McFarland, L. V., Chinn, J., and Van Bell, G. (1989a) Prevention of antibiotic-associated diarrhea by *Saccharomyces boulardii*. *Gastroenterology*, **96**, 981–988.

Surawicz, C. M., Mcfarland, L. V., Elmer, G., and Chinn, J. (1989b) Treatment of recurrent *Clostridium difficile* colitis with vancomycin and *Saccharomyces boulardii*. *Am. J. Gastroenterol.*, **84**, 1285–1287.

Surawicz, C. M., McFarland, L. V., Greenberg, R. N., Rubin, M., Fekety, R., Mulligan, M. E., Garcia, R. J., Brandmarker, S., Bowen, K., Borjal, D., and Elmer, G. W. (2000) The search for a better treatment for recurrent *Clostridium difficile* disease: Use of high-dose vancomycin combined with *Saccharomyces boulardii*. *Clin. Infect. Dis.*, **31**, 1012–1017.

Yarrow, D. (1998) Methods for the isolation, maintenance and identification of yeasts. *In* The Yeasts, A Taxonomic Study, 4th ed., ed. by Kurtzman, C. P. and Fell, J. W., Elsevier, Amsterdam, pp. 77–100.