J. Gen. Appl. Microbiol., **54**, 221–227 (2008)

Full Paper

Influence of pH conditions on the viability of *Saccharomyces boulardii* yeast

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(Received September 20, 2007; Accepted June 9, 2008)

***Saccharomyces boulardii* is a probiotic with proven health benefits. However its survival is challenged by gastrointestinal transit, and a ratio between 1 and 3% of living yeast is recovered in the feces after oral administration. The aim of the study was to determine to what extent the yeast was sensitive to gastrointestinal pH conditions. Therefore we explored the survival of different concentrations of *S. boulardii* in conditions mimicking the stomach pH (pH 1.1 0.1 N HCl) and the intestinal pH (pH 6.8 phosphate buffer) in vitro. The probiotic being commercialized as a freeze-dried powder obtained from an aqueous suspension, both forms were evaluated. In phosphate buffer pH 6.8, the viability remained stable for both forms of *S. boulardii* for 6 h. In HCl pH 1.1, viability of both forms (200 mg L^{-1}) significantly decreased from 5 min. Observation under scanning/transmission electron microscopy showed morphological damages and rupture of the yeast wall. Threshold value from which *S. boulardii* viability was unaltered was pH 4. At the highest concentration of 200 g L^{-1}, the initial pH value of 1.1 rose to 3.2, exerting a protective effect. In conclusion, although the yeast in aqueous suspension was less sensitive than the freeze-dried yeast to acidic conditions, a gastric protection for improvement of oral bioavailability of viable *S. boulardii* appears necessary.**

Key Words——acidic conditions; *Saccharomyces boulardii*; viability

Introduction

Saccharomyces boulardii yeast is considered as a probiotic according to the definition of the World Health Organization, i. e. a "live microorganism which, when administered in adequate amounts, confers a health benefit on the host" (FAO/WHO, 2001). *S. boulardii* is widely used as an anti-diarrheal agent for the prevention and treatment of infectious enteritis and *Clostridium-difficile*-associated enterocolopathies (Bergogne-Berezin, 2000; Buts et al., 1993; McFarland et al., 1994, 1995; Surawicz et al., 1989, 2000). It has also been shown to exert beneficial effects on other intestinal pathologies like Crohn's disease (Guslandi et al., 2000) and ulcerative colitis (Guslandi et al., 2003).

However, the survival of *S. boulardii* is challenged after oral administration and a ratio between 1 and 3% of the live yeast administered orally is recovered in feces (Bléhaut et al., 1989; Boddy et al., 1991).

This weak ratio could result from the destruction of the yeast during its transit within the gastrointestinal

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tract, which may be due to pH variations (Fietto et al., 2004).

Probiotics that are given orally have to firstly survive during the transit, and then persist in the gut to provide beneficial effects for the host. *S. boulardii* protects from *C. difficile* toxin A effects by the secretion of a 54-kDa serine protease which is responsible for the proteolysis of the toxin and the inhibition of toxin A binding to its brush barrier membrane receptor (Castagliuolo et al., 1996; Pothoulakis et al., 1993). In addition, the efficacy of *S. boulardii* correlates with stool concentrations of viable yeast in patients with recurrent *Clostridium difficile* disease (Elmer et al., 1999). Increasing the percentage of living yeast within the intestine would therefore potentiate the therapeutical benefit of *S. boulardii* administration. The first step toward this goal is a better knowledge of *S. boulardii* sensitivity to environmental conditions in the gastrointestinal tract. Although a number of studies have been carried out to determine the viability conditions of the *Saccharomyces cerevisiae* strains as an alimentary product (Bai et al., 2004; Bjorkqvist et al., 1997; Osorio et al., 2005; Thomsson et al., 2005), very few data are available concerning *S. boulardii* (Edwards-Ingram et al., 2007; Fietto et al., 2004). It is important to note that despite being a subtype of the *S. cerevisiae* yeast species known as *S. cerevisiae* Hansen CBS 5926, *S. boulardii* is clearly distinguishable from *S. cerevisiae* and possesses strain-specific beneficial properties (Posteraro et al., 2005).

A comparative study between *S. boulardii* and *S. cerevisiae* showed that the former's viability remained preserved down to pH 2 whereas the latter's decreased significantly at pH 6, 3 and 2. However, none of the yeast strains showed any survival at pH 1 (Edwards-Ingram et al., 2007).

S. boulardii is currently commercialized as a freeze-dried powder (Ultralevure®, Beauvais, France) which maintains the stability of the probiotic. But, the freeze-drying process is expensive and leads to a loss of 20 to 30% of viability compared to the initial aqueous suspension of the yeast whose viability is about 100% (Graff et al., 2008). In addition, it is not known whether the viability of the yeast within the gastrointestinal tract is influenced or not by its presentation.

In this context, the aim of our study was to explore the survival of *S. boulardii* under its freeze-dried form and in aqueous suspension in conditions mimicking gastric and intestinal pHs in vitro. In this paper, we report new data about the behavior of the yeast according to pH and about its cell structure, which could explain its weak oral bioavailability.

Materials and Methods

Materials. *S. boulardii* was supplied by Biocodex (Gentilly, France). It was presented in two forms: an aqueous suspension which corresponded to the yeast collected immediately at the end of the production process by fermentation; and a freeze-dried powder obtained from the former. The yeast cells were in stationary phase in both forms. The suspension contained 75% water (w/w) and 25% *S. boulardii* cells (w/w) and lactose (4 g L^{-1} of suspension) used as a cryoprotectant during the freeze-drying process. NaCl, $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4 were provided by Cooper (Melun, France), 0.9% NaCl solution by Fresenius (Sèvres, France), HCl by Prolabo (Fontenay sous Bois, France), and sterile water by Aguettant (Lyon, France). Pepsin was purchased from Sigma-Aldrich (Isle d'Abeau Chesnes, France). Sabouraud and chloramphenicol medium was provided by Biomérieux Laboratories (Marcy l'Etoile, France), and silicate gel by Prolabo (Fontenay sous Bois, France).

Assessment of the influence of the pH on the viability of S. boulardii. In vitro testing: A dissolution test apparatus (paddle method according to USP 1) (Prolabo, Fontenay sous Bois, France), was used for evaluating the viability of the yeast in different media. The volume of the media was 500 or 1,000 ml of pH 1.1 and pH 6.8 media at $+37 \pm 0.5^{\circ}C$ and 100 rpm. This study was divided into two parts.

The first one consisted in a comparative assay between the two forms of the yeast. Tests were carried out for 6 h with 200 mg freeze-dried yeast or 800 mg aqueous suspension of the yeast (corresponding to 200 mg of *S. boulardii* cells) in 1,000 ml of 0.1 N HCl or pH 6.8 phosphate buffer (European Pharmacopoeia, 5th edition) to mimic the pH conditions in the stomach and the colon, respectively. Samples were collected at 0, 5, 15, 30, 60, 120, 180 and 360 min in order to determine the yeast viability. Immediately after sampling of the yeast (in HCl solutions as well as in pH 6.8 phosphate buffer) the 1 ml samples were diluted in 9 ml of 0.9% NaCl solution. Subsequent serial 10-fold dilutions were carried out and the adequate dilutions were cultured. This procedure allowed stopping the exposure of the cells to acidic stress. Additional tests were

carried out for 3 h with 100 mg of freeze-dried yeast in 500 ml of HCl at pH 2, 3 and 4, respectively. Samples were collected at 0, 5, 15, 30, 60, 120 and 180 min to assess the yeast viability.

The second part of the study consisted in evaluating the viability of the freeze-dried yeast according to its concentration in acidic pH (2, 20, 200 g L^{-1} in 500 ml of 0.1 N HCl) as well as in a simulated gastric juice (USP 27th edition) at two concentrations of 2 and 20 g L^{-1}. While no pH modification could be detected with the addition of the three lowest concentrations of freeze-dried yeast in the acidic medium, when the highest concentration of 200 g L^{-1} of freeze-dried yeast was tested, the pH of 0.1 N HCl increased to 3.2. An additional dilution of HCl was therefore prepared whose initial pH of 0.6 (measured before addition of the yeast sample) rose to 1.1 in the presence of the highest concentration of *S. boulardii*. Simulated gastric juice was freshly prepared daily by suspending pepsin (3.2 g L^{-1}), and dissolving NaCl (2 g L^{-1}) in concentrated HCl (7 ml) and sufficient water to make 1,000 ml. The pH of this solution was checked using a standard pH meter (pH M210, Radiometer Analytical, Villeurbanne, France) and was 1.2. Times of sampling were the same as described above. Experiments were carried out in triplicate. Results were expressed in cfu g^{-1} of yeast.

Determination of viable yeasts: Yeast viability has been defined as the proportion of the cells that are capable of multiplication after incubation in standard microbiological conditions. It was evaluated after serial 10-fold dilutions (described above) by a plate culture method using Sabouraud and chloramphenicol agar at +30°C for 72 h. Following this time, colonies on plates were enumerated and expressed as cfu g^{-1} of yeast cells. The detection limit was 10^2 cfu g^{-1}.

Electron microscopy: For electron microscopic analysis, the following samples were examined using standard techniques: the freeze-dried yeast (200 mg L^{-1} and 200 g L^{-1}) and the aqueous suspension of the yeast (200 mg L^{-1} of yeast cells) were exposed for 5 and 120 min in 0.1 N HCl. Control samples of each form of *S. boulardii* (freeze-dried and aqueous suspension), not submitted to any stress condition, were also examined. Briefly, each sample was fixed with 2.5% glutaraldehyde in 0.01 M PBS buffer (pH 7.4) overnight at +4°C. Post fixation was carried out using a 2% osmium tetroxide solution for 2 h at room temperature, and followed by dehydration through graded ethanol series. For scanning electron microscopy, samples were gold covered (1–2 nm) by cathodic spraying (Fine coat ion sputter JFC-1100, Jeol S.A., Tokyo, Japan) and examined at 17 kV using Jeol electron microscope (JSM-35CF, Jeol S.A.). For transmission electron microscopy study, each sample was embedded in Epon epoxy resin. Ultrathin sections (80–100 nm) were performed, stained with uranyl acetate and lead citrate and examined at 80 kV using Jeol electron microscope (JEM-100S, Jeol S.A.). At least 300 cells were examined in each sample and their general aspect expressed as "dense," when the intracellular medium was dark and no organit could be seen, — or "light," when under any stress, the intracellular medium was less dark and vacuoles could be observed. The presence of vacuoles, the aspect of bud scars and existence of membrane alterations (deformation or rupture) were enumerated and expressed as percentages.

Statistical analysis. Results are presented as mean ± SD. Statistical analysis was performed using the *F* test followed by the *t* test, except for comparison between viability of freeze-dried yeast at different concentrations or at different acidic pH for which an analysis of variance followed by the *t* test were used. Differences between means were considered significant at $p<0.01$ or 0.05.

Results

Effect of 6.8 and acidic pH on the yeast in aqueous suspension and freeze-dried yeast

At the concentration of 200 mg of *S. boulardii* cells per liter in pH 6.8 phosphate buffer solution, the viable yeast number at time 0 was significantly less important for the freeze-dried yeast than for the yeast in the aqueous suspension ($3.4\pm0.3\times10^{10}$ vs. $7.7\pm1.4\times10^{10}$ cfu g^{-1}, $p<0.01$). At 360 min, in this medium, the viability remained unchanged for both forms with $2.9\pm0.4\times10^{10}$ cfu g^{-1} for the freeze-dried yeast and $7.7\pm1.8\times10^{10}$ cfu g^{-1} for the aqueous suspension. The survival of the yeast in 0.1 N HCl is shown in Fig. 1. At time 0, the initial viability was significantly lower for the freeze-dried yeast than for the yeast in aqueous suspension ($2.6\pm0.2\times10^{10}$ vs. $6.5\pm0.9\times10^{10}$ cfu g^{-1}, $p<0.01$). The viability started to decrease significantly from 5 min for both forms of the yeast in this medium. Although the viability of the yeast in aqueous suspension decreased more slowly than that of the

freeze-dried yeast (10.7±0.1 vs. 9.9±0.2 log cfu g^{-1} at 5 min, $p<0.01$; 8.3±0.4 vs. 5.9±1.3 log cfu g^{-1} at 60 min, $p<0.05$; 7.6±0.3 vs. 5.3±0.8 log cfu g^{-1} at 120 min, $p<0.01$; 6.7±0.6 vs. 5.4±0.7 log cfu g^{-1} at 180 min, $p<0.05$), no significant difference of viability was observed between the two forms at 360 min. The observation under scanning electron microscopy showed that both forms of yeast exposed for 120 min at pH 1.1 presented morphological damage with the wall of some yeasts being consistently deformed ("budding" phenomenon) (Figs. 2B–3B) compared to the control sample (Figs. 2A–3A). Under transmission electron microscopy, the clarifying of the cells was obvious: the ratio of 80% / 20% of dense / light cytoplasm in the control sample of the freeze-dried yeast (Fig. 3C) was reversed and only 20% of the cells remained dense while 80% were light and contained numerous vacuoles (Fig. 3D). This ratio was 15%/85% for the yeast in aqueous suspension (vs. 98%/2% in the control sample (Fig. 2C)) and an increase of the number of vacuoles was observed in 95% of the cells (Fig. 2D). "Budding," observed under scanning electron microscopy, corresponded to wall deformation and rupture (Figs. 2D–3D). The higher the pH, the better the viability of the freeze-dried yeast with a threshold value at pH 4 in which the viability was totally preserved for 180 min (viability at pH 4 vs. viability at pH 1, 2 and 3, $p<0.01$) (Fig. 4).

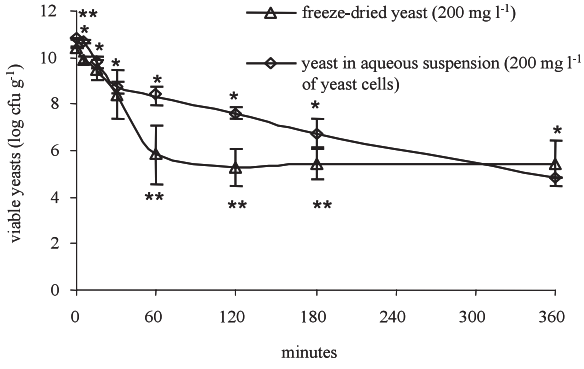

Fig. 1. Viability of *S. boulardii* in freeze-dried form and aqueous suspension (200 mg *S. boulardii* cells L^{-1}) in 0.1 N HCl ($n=6$ for each assay).

*$p<0.05$ vs. time 0 for both forms of the yeast, **$p<0.05$ vs. the yeast in aqueous suspension at the same time.

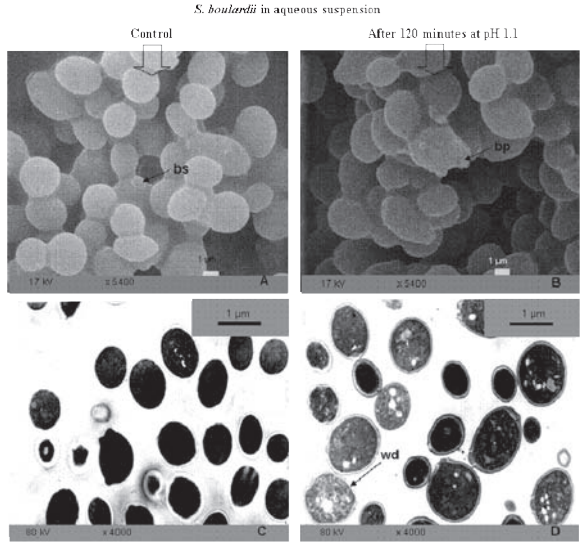

Fig. 2. Electron microscopic analysis of control samples of *S. boulardii* (untreated) and *S. boulardii* exposed to 0.1 N HCl at pH 1.1.

Observation of the yeast in aqueous suspension, untreated, under scanning (A×5,400) and transmission (C×4,000) electron microscopy and after 120 min at a concentration of 200 mg *S. boulardii* cells L^{-1} under scanning (B×5,400) and transmission (D×4,000) electron microscopy. (bs: bud scar, bp: "budding" phenomenon, wd: wall deformation).

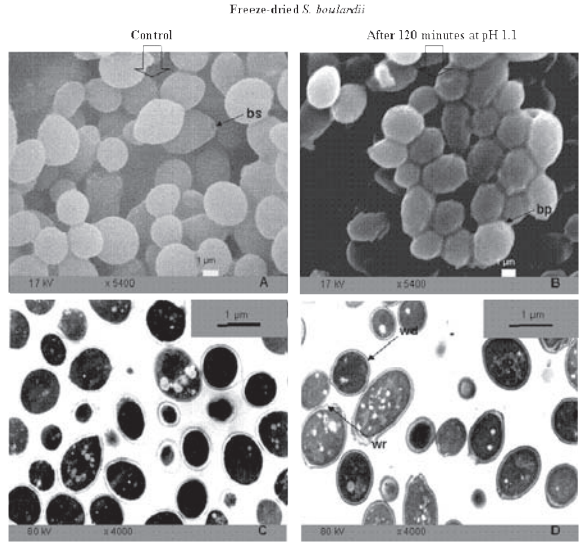

Fig. 3. Electron microscopic analysis of control samples of *S. boulardii* (untreated) and *S. boulardii* exposed to 0.1 N HCl at pH 1.1.

Observation of the freeze-dried yeast, untreated, under scanning (A×5,400) and transmission (C×4,000) electron microscopy and after 120 min at a concentration of 200 mg L^{-1} under scanning (B×5,400) and transmission (D×4,000) electron microscopy. (bs: bud scar, bp: "budding" phenomenon, wd: wall deformation, wr: wall rupture).

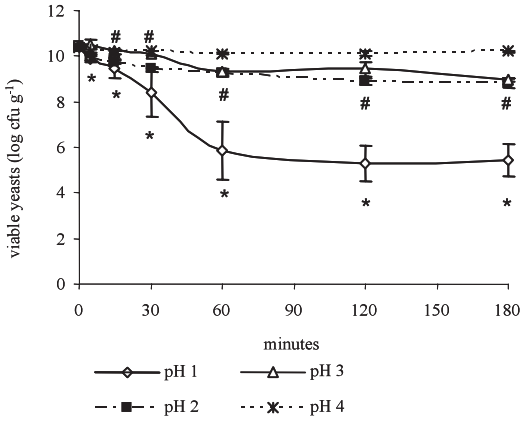

Fig. 4. Viability of freeze-dried *S. boulardii* at 200 mg L^{-1} in HCl at pH 1, 2, 3 and 4 ($n=3$ for each assay).

*$p<0.01$ from 5 min vs. time 0 at pH 1 and 2 ; #$p<0.01$ from 15 min vs. time 0 at pH 3.

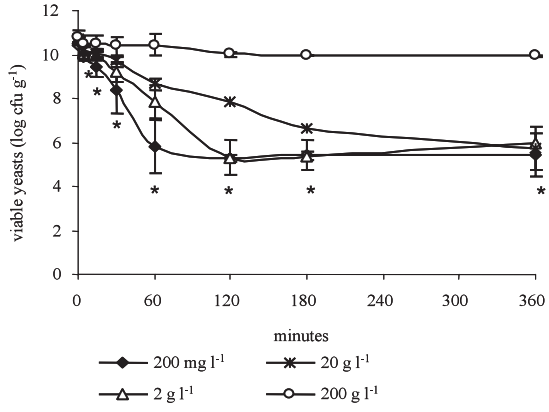

Fig. 5. Viability of increasing concentrations of freeze-dried *S. boulardii* in 0.1 N HCl ($n=3$ for each assay).

*$p<0.05$ at 200 mg L^{-1}, 2 and 20 g L^{-1} vs. time 0.

Survival of increasing concentrations of the freeze-dried yeast in acidic pH

The higher the concentration of the freeze-dried yeast was, the higher the survival in acidic conditions (Fig. 5). The viability started to decrease significantly from 5 min at 2 and 20 g L^{-1} ($p<0.05$ vs. time 0). No significant difference was observed between concentrations of 2 and 20 g L^{-1} at 30 min with about 1.5 and 1 log decrease compared with time 0. At the concentration of 200 g L^{-1}, viability remained stable for 360 min while pH rose to 3.2. The observation under scanning electron microscopy of the freeze-dried yeast (200 g L^{-1}) exposed for 120 min to 0.1 N HCl showed few alterations confirmed under transmission electron microscopy (data not shown). The same assay, carried out in a more concentrated HCl solution (initial pH

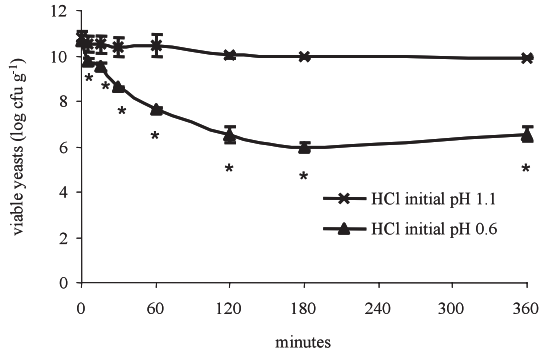

Fig. 6. Effect of the initial pH of the medium (measured before addition of the yeast sample) on the viability of freeze-dried *S. boulardii* at 200 g L^{-1} ($n=3$ for each assay).

*$p<0.05$ vs. time 0.

measured before addition of the yeast sample was 0.6), whose final pH reached 1.1 after addition of 200 g L^{-1} of the freeze-dried yeast, showed that the viability of *S. boulardii* decreased in the same manner as that of lower concentrations from 5 min up to 360 min ($p<0.05$ vs. time 0) (Fig. 6). There were 2 and 3 log of decrease at 30 and 60 min, respectively.

The viability of the freeze-dried yeast evaluated up to 360 min in simulated gastric fluid containing pepsin to mimic the stomach condition (pH=1.2) was not different from that observed in 0.1 N HCl (data not shown).

Discussion

In the present study, we investigated the effects of pH on the viability of *S. boulardii* under its freeze-dried form and in aqueous suspension in order to determine to what extent the sensitivity of *S. boulardii* to gastrointestinal conditions could explain the weak oral bioavailability of the viable yeast.

Because of its administration by the oral route, *S. boulardii* viability is challenged during its transit through the gastrointestinal tract, especially by pH variations. We therefore studied in vitro the effect of two extreme pHs corresponding to the stomach pH (0.1 N HCl=1.1) and the colon pH (phosphate buffer solution=6.8). We tested the freeze-dried form of *S. boulardii* which is the commercially available one and the aqueous suspension of the yeast from which it was obtained.

The lowest and highest concentrations of *S. boulardii* to be tested were chosen according i) to the European Pharmacopoeia (5th edition) dissolution method

which prescribes to evaluate one dosage form—i.e. 200 mg of freeze-dried yeast corresponding to one sachet—in 1 L of dissolution medium, ii) to practical considerations, *S. boulardii* being usually administered to the animal as a suspension of the freeze-dried yeast at a concentration of 200 g L^{-1} for in vivo evaluation (Bléhaut et al., 1989; Elmer and Corthier, 1991). Intermediate concentrations of 2 and 20 g L^{-1} were subsequently tested owing to apparent differences in results first observed with extreme concentrations of *S. boulardii* tested in 0.1 N HCl. The freeze-dried yeast appeared to be sensitive to acidic conditions, except for the highest concentration, i.e. 200 g L^{-1}. However, checking the pH of the HCl medium containing 200 g L^{-1} of *S. boulardii* revealed an unexpected increase of up to 3.2. In these conditions, the pH increased quickly, reaching 2.95 at 15 min and seemed to protect the yeast from death observed at lower concentrations of *S. boulardii* at pH 1.1. The repetition of the experiment with 200 g L^{-1} of *S. boulardii* in HCl, whose dilution was adjusted in order to obtain a final pH of 1.1 in the medium, confirms the loss of viability of the yeast in acidic conditions. The sensitivity of *S. boulardii* to acidic environment is in accordance with data obtained by Fietto et al. (2004). Our data regarding the morphological alterations subsequent to exposure of *S. boulardii* to 0.1 N HCl are close to those of other authors with the *S. cerevisiae* yeast. Indeed, Ludovico et al. (2001) showed that *S. cerevisiae* exposure for 200 min to acetic acid at pH 3.0 resulted in cell death accompanied by ultrastructural alterations typical of necrosis. In our study, the threshold pH value appears to be 4, from which the viability of the yeast is unaltered.

On the other hand, at pH 6.8 in phosphate buffer solution, the viability of the yeast (freeze-dried and in aqueous suspension) is unchanged for 360 min compared with time 0. These data are in accordance with previous studies showing the unaltered viability of *S. boulardii* and *S. cerevisiae* (Fietto et al., 2004) exposed to a pH close to neutral. Given the sensitivity of *S. boulardii* to acidic pH, it seems necessary to protect the yeast from destruction in the stomach in order to increase its intestinal survival after oral administration.

Conclusion

In conclusion, despite a degradation slower than that of the freeze-dried yeast, *S. boulardii* in aqueous suspension remains sensitive to acidic pH up to a value of 3 and a gastric protection for improvement of oral bioavailability of viable *S. boulardii* appears necessary.

Acknowledgments

This work was supported by Biocodex, France. The authors acknowledge Pr. V. Tricottet, Dr. Samir Nakib, C. Dampier and C. Vialle for their contribution to this project as well as Mrs. G. Arnaud-Vincent for the English language corrections.

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