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Inhibition of *in vitro* cell adherence of *Clostridium difficile* by *Saccharomyces boulardii*

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The influence on the adherence of *Clostridium difficile* to Vero cells of the yeast *Saccharomyces boulardii*, the yeast fractions (cytoplasm and cell wall) and the culture supernatant was investigated *in vitro*. *C. difficile* adherence was significantly inhibited when bacteria were pre-incubated with the whole yeast and the cell wall fraction; this adherence inhibition was dose-dependent. The cell wall fraction also acts upon the target cultured cells inasmuch as the level of adherence was significantly decreased when Vero cells were preincubated with it. The same experiments carried out in the presence of an inhibitor of serine proteases resulted in no inhibition of bacterial adherence. These results suggest that the yeast could inhibit adherence of *C. difficile* to cells thanks to its proteolytic activity but also through steric hindrance.

Key words: *Clostridium difficile*, *Saccharomyces boulardii*, adherence, Vero cells.

Introduction

Saccharomyces boulardii, a biotherapeutic agent, is a non pathogenic yeast originally isolated from lychee fruit and has been used successfully to prevent antibiotic-associated diarrhea and various other diarrheas [1, 2]. Recent studies have established that the yeast synthesizes proteins able to neutralize the effects of *Vibrio cholerae* [3, 4] and *Clostridium difficile* toxins A and B [5, 6]. A protective effect has also been observed for *Salmonella typhimurium* and *Shigella flexneri* infection in conventional or gnotobiotic mice [7] and *Candida albicans* in a murine model [8]. Other protective effects have been attributed to *S. boulardii* including inhibitory activity on *Entamoeba histolytica* adherence to human erythrocytes [9]. Buts *et al*. and Jahn *et al*. [10, 11] explored the positive effects of *S. boulardii* on the maturation of enterocytes of human and rat small intestine.

S. boulardii is used as a treatment in conjunction with metronidazole and/or vancomycin for relapses in patients with diarrhoea due to *C. difficile* [12, 13]. *C. difficile* is currently known as the etiological agent of 90% of cases of pseudomembranous colitis and 30% of cases of post

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antibiotic diarrhoea [14–16] linked to the release of toxins A and B. However, colonization is likely to have an important role in the infectious process with adherence playing a key role due to the presence of adhesins on *C. difficile* surface [17–21].

In this study, we wanted to assess the impact of the yeast on *C. difficile* cell adherence and modifications thereof. Therefore we conducted adherence assays using Vero cells in the presence of whole yeast and its cytoplasmic and cell wall fractions.

Results

Adherence assays

Adherence of *S. boulardii* and *C. difficile* to Vero cells was analyzed. Adherence assays performed with *S. boulardii* revealed that the yeast did not adhere to the cells (data not shown). In contrast, as it was demonstrated in our laboratory [19], *C. difficile* attaches to Vero cells after a 20-min heat shock at 60° (data non shown). The mean number of *C. difficile* adhering to cells (12 bacteria per cell) represents a positive control for adherence (relative level of adherence 100%). Pre-incubation of *C. difficile* and Vero cells with the serine protease inhibitor Phenyl-Methyl-Sulphonide-Fluoride (PMSF, Sigma) had no effect on cell adherence and on cell viability.

No bacterial adherence to the surface of yeast cells was observed by light microscopy (data not shown). Thus the yeast does not appear to possess surface receptors to allow *C. difficile* attachment.

C. difficile adherence inhibition by whole yeast and cellular fractions

S. boulardii *(whole yeast or yeast fractions) pre-incubated with* C. difficile

As shown in Figure 1, in the presence of whole yeast cells, the level of adherence reached only

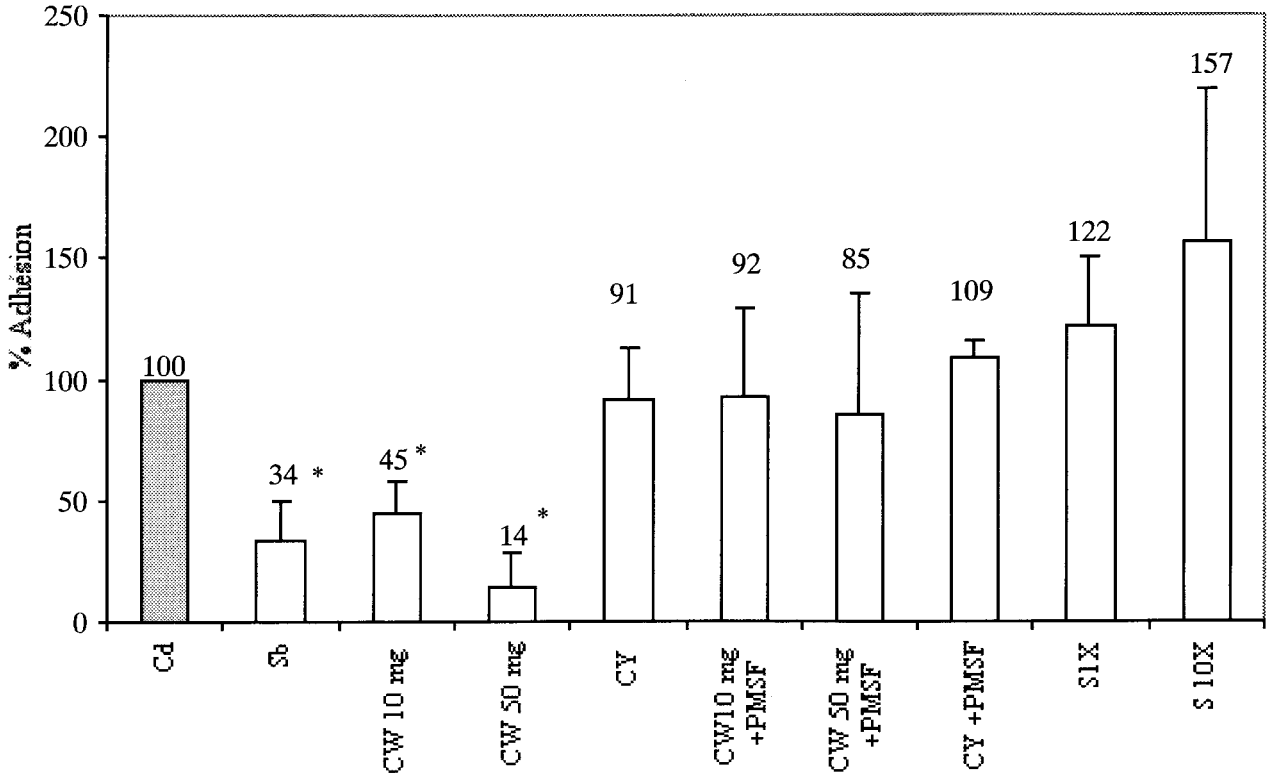

Figure 1. Adherence of *C. difficile* to Vero cells after incubation with whole yeast *S. boulardii* and yeast fractions. Cd = *C. difficile* without incubation with the yeast as a positive control for adherence (relative level of adherence 100%); Sb = whole yeast; CW = yeast cell wall fraction; CW + PMSF = yeast cell wall fraction with PMSF; CY = yeast cytoplasmic fraction; CY + PMSF = yeast cytoplasmic fraction with PMSF; S1 × = yeast culture supernatant; S10 × = concentrated (10 ×) culture supernatant. Student's *t*-test was used for statistical analysis. (*) significant difference as compared to control Cd.

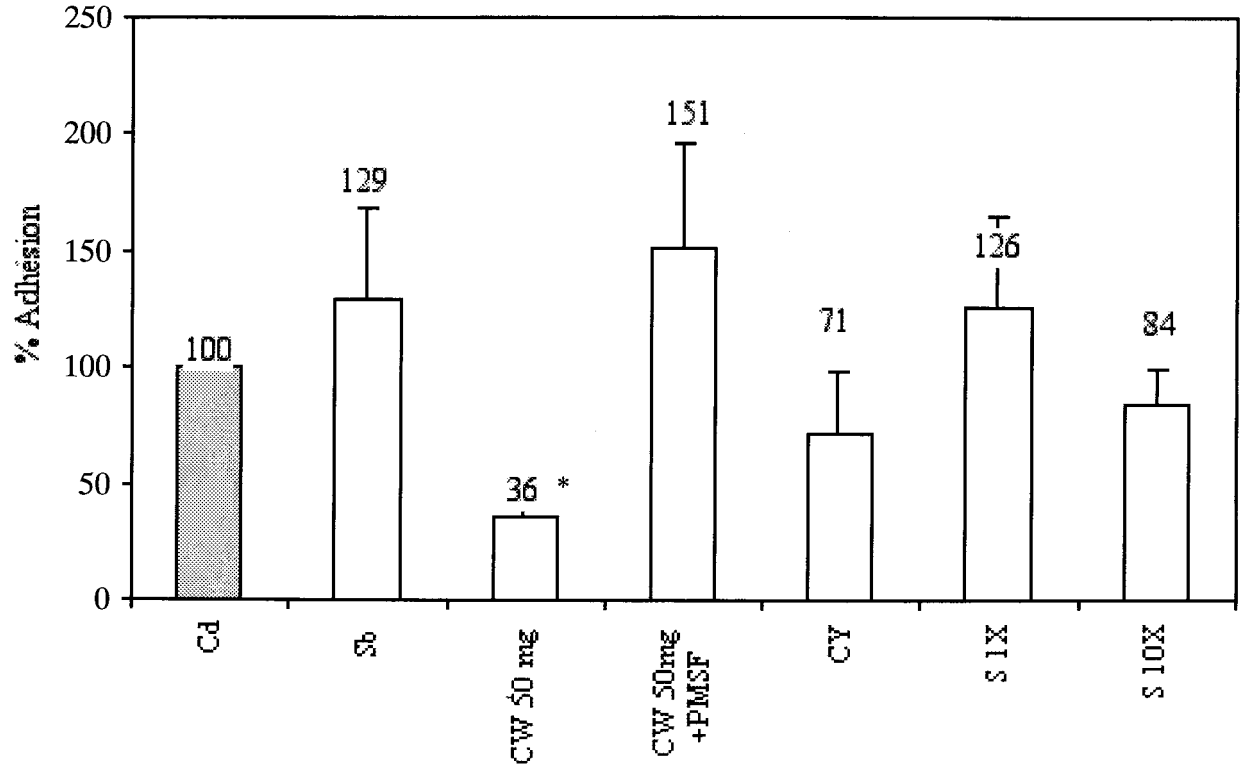

Figure 2. *C. difficile* cell adherence after preincubation of whole yeast *S. boulardii* and yeast fractions with Vero cells. Cd = *C. difficile* without incubation as a positive control for adherence (relative level of adherence 100%); Sb = whole yeast; CW = yeast cell wall fraction; CW + PMSF = yeast cell wall fraction with PMSF; CY = yeast cytoplasmic fraction; S1 × = yeast culture supernatant; S10 × = concentrated (10 ×) culture supernatant. Student's *t*-test was used for statistical analysis. (*) significant difference as compared to control Cd.

34% as compared with control ($P<0.001$). The cell wall fraction of *S. boulardii* also reduced *C. difficile* adherence to Vero cells. When bacteria were pre-incubated with increasing quantities of the cell wall fractions (10 and 50 mg), the level of adherence reached 45% ($P=0.009$) and 14% ($P=0.004$), respectively, compared with the control. Furthermore, with PMSF in the yeast preparations, levels of adherence were close to that of the control: 92% and 85% with 10 mg and 50 mg of membrane extracts, respectively. The yeast culture supernatant, whether not concentrated or 10-fold concentrated, did not interfere with *C. difficile* adherence to tissue culture cells. In the presence of the cytoplasmic contents of *S. boulardii* prepared with or without PMSF, the level of adherence was close to that of the control (91% and 109%, respectively).

S. boulardii *(whole yeast or yeast fractions) pre-incubated with Vero cells*

We determined whether *S. boulardii* and its cellular fractions act on the Vero cell receptors mediating *C. difficile* adherence.

Pre-incubation of target cells with whole yeast cells did not interfere significantly with *C. difficile* adherence (Fig. 2). When Vero cells were pre-incubated with 50 mg of *S. boulardii* cell wall fraction, a pronounced reduction of *C. difficile* adherence was evident (36% of control level, $P<0.001$). As in the previous experiment, the level of adherence did not decrease in the presence of PMSF. Incubation of target cells with the yeast culture supernatant, whether concentrated or not, had no influence on levels of *C. difficile* adherence (126% and 84% with the non concentrated and concentrated supernatant, respectively). When Vero cells were preincubated with the intracellular contents of the yeast, the level of bacterial adherence was 71% as compared with the positive control (not significantly different).

Effect of *S. boulardii* on *C. difficile* surface protein profile

To explore the activity of *S. boulardii* on *C. difficile* membrane proteins, the adherence of radio-

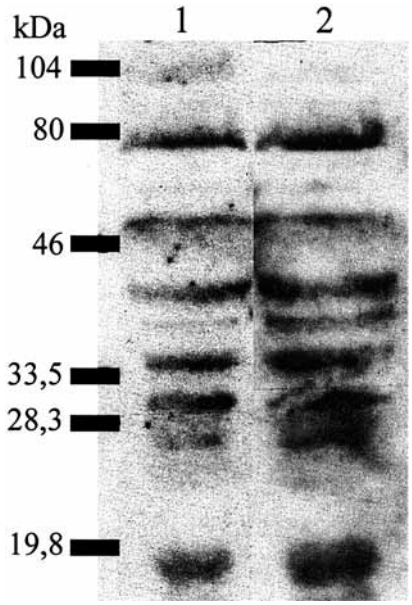

Figure 3. Adherence of radiolabelled Vero cells to surface proteins of *C. difficile* transferred to a membrane. Lane 1 = membrane not incubated with whole yeast *S. boulardii*; lane 2 = membrane incubated with whole yeast *S. boulardii*.

labelled Vero cells to membrane-transferred *C. difficile* surface proteins after preincubation or not with the yeast was investigated. The analysis of the autoradiogram revealed eight major bands after autoradiography in the assay and the control without any difference between the two profiles (Fig. 3).

Discussion

The pathogenicity of *C. difficile*, the causative agent of pseudomembranous colitis and post-antibiotic diarrhoea, is linked to the release of toxins A and B in the digestive tract. The yeast *S. boulardii* has been extensively used in the treatment or prevention of *C. difficile* diarrhoea and its action seems to be multifactorial. In addition to toxins, however, *C. difficile* colonization is likely to have an important role in the infectious process with adherence playing a key role.

First, the capacity of adherence of *S. boulardii* to Vero cells was determined with preliminary experiments. *S. boulardii* does not adhere to Vero cells. We have previously demonstrated [16, 18] that the adherence of *C. difficile* to Vero cells (monkey kidney epithelial cells) and intestinal cell lines, e.g. Caco-2, is equivalent; thus receptors for this bacterium seem to be present on both cell lines. Because of the easier manipulation of Vero cells, they were chosen for our studies.

Different mechanisms could be responsible for the activity of *S. boulardii* against *C. difficile* in the gut, for example adherence to *S. boulardii*. To examine this hypothesis we assessed the adhesive properties of *C. difficile* to *S. boulardii*. The results demonstrated that the yeast does not appear to possess surface receptors that would allow *C. difficile* attachment. This is in contrast to the results of Gedek [22] who observed adherence of enteropathogenic isolates of *Escherichia coli* and *Salmonella typhimurium* to the yeast by means of lectin receptors of the yeast.

In order to determine the impact of the yeast and its cell fractions on the adherence of *C. difficile* to Vero cells, adherence inhibition by whole yeast and cellular fractions was conducted. Only the cell wall fraction of *S. boulardii* displayed inhibition similar to that of whole cells. Moreover, the inhibitory effect of the membrane fraction was dose-dependent. These results suggest that one or several proteins of the cell wall of the yeast could be responsible for the inhibition of adherence of *C. difficile* to Vero cells. These proteins could be proteases, because with the serine protease inhibitor PMSF the effect was eliminated. However, we cannot exclude the hypothesis of steric hindrance as the mixture of *C. difficile* and whole yeast or membrane extract was incubated with the cultured cells during the assay. This mechanism of steric hindrance of *C. difficile* adherence was reported for a strain of *Lactobacillus* in the urinary tract [23].

With pre-incubation of *S. boulardii* with Vero cells the results suggested that the yeast cell wall appears to be able to modify the surface receptors involved in the adherence of *C. difficile*. Again, in presence of PMSF, the inhibitory effect of the cell wall is abolished, suggesting a role for proteases. Moreover, the hypothesis of competition between the yeast and the bacteria can be excluded because *S. boulardii* does not adhere to Vero cells. Thus, the proteolytic enzyme(s) could act both on the receptors of the bacteria and the Vero cells, thereby inhibiting the adherence. Castagliuolo *et al*. [5, 6] characterized a serine protease of 54 kDa secreted by *S. boulardii* which is able to degrade the toxins A and B of *C. difficile* and also the toxin A intestinal receptor

in rats. It would be interesting to establish its role in *C. difficile* cell attachment.

Finally the whole yeast does not modify the surface protein profile in *C. difficile*, suggesting that the inhibitory effect of whole yeast was not due to modifications in surface protein composition; the mechanism seems rather imply steric hindrance.

In conclusion, *S. boulardii* could act in different ways to protect the host against microbial pathogens. This protective effect has been observed against several pathogens, but the mechanisms involved have not always been elucidated. One mechanism could be the degradation of bacterial toxins by proteolytic enzymes of the yeast [3–6]. The results of our in vitro experiments using cultured cells with different yeast extracts show that *S. boulardii* could inhibit the adherence of *C. difficile* to cells and suggest that a cell wall associated proteolytic enzyme could be responsible for destroying the adhesins in *C. difficile* and also the cell surface receptors. However, the hypothesis of steric hindrance by the yeast on the adherence of *C. difficile* to Vero cells could not be eliminated.

Materials and Methods

Microorganisms and growth conditions

The *S. boulardii* yeast, kindly supplied by Biocodex, Montrouge, France, was cultured on YPD agar (yeast peptone dextrose; Difco) or in YM broth (yeast maltose; Difco) at 30°C for 24 h with shaking.

The highly toxigenic and adherent *C. difficile* strain 79-685, a clinical isolate from a pseudomembranous colitis (Institut de Bactériologie, Strasbourg, France) was used in all experiments. It was grown in anaerobic conditions (Oxoid Ltd, Basingstoke, Hampshire, U.K.) in prereduced TGY broth (tryptone glucose yeast broth; Difco) at 37°C for 24 h.Bacteria and yeast were washed twice in sterile phosphate buffer saline, pH 6.8 (PBS) and diluted to give a final dilution of approximately 4×10^8 cells/ml. In all experiments bacteria were treated at 60°C for 20 min in aerobic conditions to increase adherence of *C. difficile* prior to contact with cultured cells, as has been shown in our laboratory [17, 19].

Cell cultures and adherence assays

The cells were suspended in minimum essential medium (MEM) with Earle's salts (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies), 1% non essential amino acids (Life Technologies), 200 mM L-glutamine (Life Technologies), 200 units/ml penicillin (Sigma) and 40 μg/ml streptomycin (Sigma). Cells were seeded in culture dishes in aliquots of 2 ml containing approximately 8×10^5 cells. After 24 h of growth in 5% CO_2 atmosphere at 37°C, confluent cells were washed twice with PBS and added with 1 ml of MEM without fetal calf serum.

Adherence of S. boulardii *and* C. difficile *to Vero cells*

One ml of yeast and or bacteria both grown in broth were prepared as described above and incubated with the cell monolayer at 37°C for 60 min. Non adherent microorganisms were eliminated by 5 washings with PBS. Cell monolayers were fixed with methanol and stained with May–Grünwald–Giemsa (Sigma); adherent microorganisms were enumerated by light microscopy at 1000 × magnification.

The adherence index is given as the average number of adhering bacteria per cell from at least three different assays and represents a positive control (relative level of adherence 100%).

Adherence of C. difficile *to* S. boulardii

Bacteria and yeast were cultured in liquid medium, then washed in PBS and diluted to 4×10^8 cells/ml as described above. One ml of each suspension was mixed and incubated at 37°C for 1 h under anaerobic conditions. This suspension was directly observed by light microscopy at 1000 × magnification.

C. difficile adherence inhibition by whole yeast and cellular fractions

Cytoplasmic and cell wall fractions of *S. boulardii*, kindly supplied by Biocodex, were obtained as follows: a 24-h yeast culture in broth was washed three times in 0.9% NaCl and diluted up to 10^9 cells/ml. One part of this suspension was supplemented with 1 mM PMSF (Phenyl Methyl Sulfonide Fluoride-Sigma). Two cellular fractions, prepared with or without PMSF, were

extracted by crushing yeast cells with a 0.5 volume of glass beads (0.45–0.5 mM) in an MKS cell homogenizer (B. Braun, Melsungen, Germany) (3 pulses of 1 min). The intact cells were eliminated from the supernatant with three successive centrifugations at $1150 \times g$ at 4°C. The cytoplasmic and cell wall fractions were separated by centrifugation at $20\,000 \times g$ at 4°C for 30 min. The pellet containing the cell wall fraction was washed five times in phosphate buffer 0.1 M, pH 8.5, then four times in distilled water. The supernatant fraction was recovered from a 24-h yeast culture. The yeast cells were removed by centrifugation at $3500 \times g$ for 10 min and filtered with a 0.22 μM syringe filter. The supernatant was concentrated 10-fold with Ultrafree-15 Centrifugal Filter Device (Millipore).

S. boulardii *(whole yeast or yeast fractions) pre-incubated with* C. difficile

C. difficile cells were pre-incubated with different fractions of the yeast prepared with or without PMSF before contact with Vero cells. Bacteria (4×10^8/ml) were incubated at 37°C for 1 h. with (a) 1 ml of whole yeast (4×10^8/ml); (b) 1 ml of the cell wall fraction (10 or 50 mg/ml); (c) the same volume of the cytoplasmic fraction; (d) One- ($1\times$) or ten-fold ($10\times$) concentrated yeast culture supernatant. Cells were recovered by centrifugation ($5000 \times g$ at 4°C for 10 min) and resuspended in 1 ml of PBS prior to contact with Vero cells. *C. difficile* adherence after preincubation with the different yeast cellular fractions and culture supernatant was compared to control (100% adherence).

S. boulardii *(whole yeast or yeast fractions) pre-incubated with Vero cells*

Target cells were pre-incubated with whole yeast or its cell fractions prepared with or without of PMSF prior to contact with *C. difficile*. Vero cells were pre-incubated, at 37°C for 1 h with (a) 1 ml of whole yeast (4×10^8 /ml); (b) 1 ml of the cell wall fraction (50 mg/ml); (c) the same volume of the cytoplasmic fraction; (d) $1\times$ or $10\times$ concentrated yeast culture supernatant. After incubation, Vero cells were washed twice with PBS and 1 ml of MEM without fetal calf serum was added. Bacteria were subsequently deposited on the cells and incubated at 37°C for 1 h in anaerobiosis. After preincubation of the Vero cells with the different yeast cellular fractions and supernatant, *C. difficile* adherence was compared to control (100% adherence).

Effect of *S. boulardii* on *C. difficile* surface protein profile

Extraction and separation of surface proteins of *C. difficile* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described by Wexler [24]. Subsequently, proteins were electrically transferred to polyvinylidene fluoride membranes (Millipore). One membrane was subsequently incubated with whole yeast cells (4×10^8/ml) at 37°C for 2 h, whereas the other one was incubated with PBS and used as a negative control. The two membranes were washed with PBS and incubated for 90 min at 37°C under 5% CO_2 with Vero cells metabolically labeled with L-[^{35}S]methionine (NEN, Inc. Boston, MA, U.S.A.) for 4 h (10^5 cells/cm^2 of membrane). After five washings with PBS, Vero cells bound to *C. difficile* proteins were revealed by exposure of the membranes to Kodak Biomax photographic film (Sigma).

Statistical analysis

All the results are expressed as arithmetic means with a minimum of three adherence assays. Student's test was used to compare the inhibition of *C. difficile* adherence with the positive control and the different yeast cellular fractions tested.

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References

1 Elmer GW, Surawicz CM, Mcfarland LV. Biotherapeutic agents. A neglected modality for the treatment and prevention of selected intestinal and vaginal infections. *JAMA* 1996; **275**: 870–6.
2 McFarland LV, Bernasconi P. A review of an innovative biotherapeutic agent. *Microbial Ecol Health Dis* 1993; **6**: 157–71.
3 Czerucka D, Rampal P. Effect of *Saccharomyces boulardii* on cAMP- and Ca_2^+-dependent Cl-secretion in T84 cells. *Dig Dis Sci* 1999; **44**: 2359–68.
4 Czerucka D, Roux I, Rampal P. *Saccharomyces boulardii* inhibits secretagogue-mediated adenosine 3',5'-cyclic monophosphate induction in intestinal cells. *Gastroenterology* 1994; **106**: 65–72.

5 Castagliuolo I, LaMont JT, Nikulasson ST, Pothoulakis C. *Saccharomyces boulardii* protease inhibits *Clostridium difficile* toxin A effects in the rat ileum. *Infect Immun* 1996; **64**: 5225–32.
6 Castagliuolo I, Riegler MF, Valenick L, LaMont JT, Pothoulakis C. *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins A and B in human colonic mucosa. *Infect Immun* 1996; **67**: 302–7.
7 Rodrigues AC, Nardi RM, Bambirra EA, Vieira EC, Nicoli JR. Effect of *Saccharomyces boulardii* against experimental oral infection with *Salmonella typhimurium* and *Shigella flexneri* in conventional and gnotobiotic mice. *J Appl Bacteriol* 1996; **81**: 251–6.
8 Ducluzeau R, Bensaada M. Comparative effect of a single or continuous administration of *Saccharomyces boulardii* on the establishment of various strains of *Candida* in the digestive tract of gnotobiotic mice. *Ann Microbiol (Paris)* 1982; **133**: 491–501.
9 Rigothier MC, Maccario J, Gayral P. Inhibitory activity of *Saccharomyces* yeasts on the adhesion of *Entamoeba histolytica* trophozoites to human erythrocytes *in vitro. Parasitol Res* 1994; **80**: 10–15.
10 Buts JP, De Keyser N, Marandi S *et al. Saccharomyces boulardii* upgrades cellular adaptation after proximal enterectomy in rats. *Gut* 1999; **45**: 89–96.
11 Jahn HU, Ullrich R, Schneider T *et al.* Immunological and trophical effects of *Saccharomyces boulardii* on the small intestine in healthy human volunteers. *Digestion* 1996; **57**: 95–104.
12 McFarland LV, Surawicz CM, Greenberg RN *et al.* A randomized placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. *JAMA* 1994; **271**: 1913–8.
13 Surawicz CM, McFarland LV, Greenberg RN *et al.* The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with *Saccharomyces boulardii. Clin Inf Dis* 2000; **31**: 1012–7.
14 Bartlett JG, Chang TW, Gurwiyh M, Gorbach SL, Onderdonk AM. Antibiotic associated pseudomembranous colitis due to toxin producing *Clostridia. N Eng J Med* 1978; **298**: 531–4.
15 Larson HE, Honour P, Price AB, Borriello SP. *Clostridium difficile* and aetiology of pseudomembranous colitis. *Lancet* 1978; **1**: 1063–6.
16 Lyerly DMH, Krivan HC, Wilkins TD. *Clostridium difficile*: its disease and toxins. *Clin Microbiol Rev* 1988; **1**: 1–12.
17 Eveillard M, Fourel V, Barc MC *et al.* Identification and characterization of adhesive factors of *Clostridium difficile* involved in adhesion to human colonic enterocyte-like Caco-2 and mucus-secreting HT29 cells in culture. *Mol Microbiol* 1993; **7**: 371–81.
18 Hennequin C, Porcheray F, Waligora-Dupriet AJ *et al.* GroEL (Hsp60) of *Clostridium difficile* is involved in cell adherence. *Microbiology* 2001; **147**: 87–96.
19 Karjalainen T, Barc MC, Collignon A *et al.* Cloning of a genetic determinant from *Clostridium difficile* involved in adherence to tissue culture cells and mucus. *Infect Immun* 1994; **62**: 4347–55.
20 Waligora AJ, Barc MC, Bourlioux P, Collignon A, Karjalainen T. *Clostridium difficile* cell attachment is modified by environmental factors. *Appl Environ Microbiol* 1999; **65**: 4234–8.
21 Waligora AJ, Hennequin C, Mullany P, Bourlioux P, Collignon A, Karjalainen T. Characterization of a cell surface protein of *Clostridium difficile* with adhesive properties. *Inf Immun* 2001; **69**: 2144–53.
22 Gedek BR. Adherence of *Escherichia coli* serogroup O 157 and the *Salmonella typhimurium* mutant DT 104 to the surface of *Saccharomyces boulardii. Mycoses* 1999; **42**: 261–4.
23 Reid G. The scientific basis for probiotic strains of *Lactobacillus. Appl Environ Microbiol* 1999; **65**: 3763–6.
24 Wexler H, Mulligan ME, Finegold SM. Polyacrylamide gel electrophoresis patterns produced by *Clostridium difficile. Rev Infect Dis* 1984; **6 Suppl 1**: S229–34.