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Does Probiotic Yeast Act as Antigenotoxin?**

Malda Maija Toma, Jekabs Raipulis, Inta Kalnina and Reinis Rutkis*

Institute of Microbiology and Biotechnology, University of Latvia, Kronvalda boulv. 4, LV-1586, Riga, Latvia

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Summary

The effect of probiotic yeast *Saccharomyces boulardii* on genotoxicity induced by the well-known mutagen 4-nitroquinoline-N-oxide (4-NQO), as well as antibacterial (furazolidone) and antibiotic (nalidixic acid) drugs, has been studied using the short-term bacterial assay, SOS chromotest, with *Escherichia coli* PQ 37 as the test organism. It has been shown that *S. boulardii* possesses antigenotoxic activity, revealed by SOS chromotest, when coincubated with these genotoxins. A weaker antigenotoxic activity against the same compounds was observed with *S. carlsbergensis*, too.

Key words: *Saccharomyces boulardii*, genotoxins, SOS chromotest, antigenotoxicity

Introduction

It has been almost a century ago since Eli Metchnikoff proposed the, then revolutionary, idea to consume viable bacteria to promote health. Since that time, the area of what is now known as »probiotics« has made tremendous progress, particularly during the past two decades. It was Vergin (*1*) who first introduced the term »probiotic«, when he compared in his paper »Anti- and probiotika« the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population with factors »probiotika« favourable to the gut microflora. However, these agents are now being reconsidered as alternatives to antibiotics because of the increase of antibiotic-resistant strains of bacteria (*2*). Now probiotics are defined as non-pathogenic microorganisms that, when ingested, exert a positive influence on host health or physiology (*3*). Microorganisms that are probiotic in humans include yeasts (*4*), bacilli (*5*), *Escherichia coli* (*6*), enterococci (*7*), and the more commonly used bifidobacteria and lactic acid bacteria, such as lactobacilli, lactococci and streptococci (*8,9*).

Non-pathogenic yeasts present some characteristics that differentiate them from other probiotics. The natural resistance they have to antibiotics has constituted an interesting basis for their application as probiotics. In this regard, *Saccharomyces boulardii* was the most widely tested in experimental and clinical assays, showing very important results.

This microorganism was initially isolated from Indochinese fruits (lychee). Fruits contaminated with *Saccharomyces boulardii* were used in the local popular medicine for the treatment of diarrhea. A product containing this microorganism was introduced in France with the same objective in 1950, and in the present days (Ultralevure, Biocodex, Monterouge, France) it is widely commercialized in European, African, and South American countries. It is generally administered in lyophilized powder, corresponding to approximately $3 \cdot 10^{10}$ cells/g. Unless exogenously administered, *S. boulardii* is not found as part of the intestinal microflora of laboratory animals or of humans, and under normal conditions, it does not permanently colonize the gastrointestinal tract when orally administred, and is eliminated within 24–72 hours if not reinoculated (*10*).

The pharmacodynamics of *S. boulardii* involves three different aspects: (*i*) a direct antagonistic effect on enterobacteria and other yeasts, (*ii*) an antisecretory effect by acting specifically on the binding of toxins to intesti-

*Corresponding author; Phone: ++371 703 48 87; Fax: ++371 703 48 85; E-mail: toma@lanet.lv
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nal receptors, and (*iii*) a trophic effect by stimulating enzymatic activities and intestinal defense mechanisms (*11*). Besides, *S. boulardii* can also destroy the receptor site for *Clostridium difficile* toxin A and B by producing protease (*12*), it is thermotolerant and grows at the unusually high temperature of 37 °C. The taxonomy of the genus *Saccharomyces* based on phenotypic and genotypic methods has been revised several times over the years and now, using microsatellite polymorphism typing, allows to discriminate *S. boulardii* from other strains of *S. cerevisiae* (*13*). In contrast to lactic acid and bifidobacteria, *S. boulardii* is naturally resistant to antibiotics (*14*).

Only a few reports have evidenced inhibition of xenobiotics in the gastrointestinal tract by foods containing probiotics. The prevalent hypothesis of antigenotoxicity is related to the binding of mutagens to live cells or their metabolites (*15*). Nevertheless, the molecular basis of these interactions is not yet clear. No information is available about possible antigenotoxicity of *S. boulardii*.

The aim of this paper was to examine the effect of probiotic yeast on the genotoxic activity of 4-nitroquinoline-N-oxide (4-NQO), furazolidone and nalidixic acid. We chose 4-NQO as a well-known mutagen, a direct-acting agent, which produces strand scission and formation of charge transfer adducts on DNA. The activity of this mutagen on microorganisms has also been demonstrated (*16*). Other substances, furazolidone and nalidixic acid, were chosen as broad spectra antibacterial and antibiotic representatives, respectively.

Genotoxicity and antigenotoxicity evaluations were obtained by SOS chromotest, a standardised short-term assay for detecting DNA-damaging agents. The method is highly sensitive and specific in discriminating between carcinogens and anticarcinogens, and its performance in identifying direct acting genotoxins is proved (*17,18*).

Materials and Methods

Strains of yeasts *Saccharomyces boulardii* (probiotic yeast), synonym *S. cerevisiae* Hansen CBS 5926, and *S. carlsbergensis* PS-1 (non-probiotic yeast) from Culture Collection of Institute of Microbiology and Biotechnology were used for the study. Cultures were grown overnight in the YEGPB medium, containing glucose 20 g/L, peptone 10 g/L and yeast extract 5 g/L. *S. boulardii* and *S. carlsbergensis* were grown at 37 °C and 30 °C, respectively.

4-NQO, furazolidone and nalidixic acid were purchased from Sigma-Aldrich (Sigma, USA). Stock solutions of 4-NQO and furazolidone (1 mg/mL) were diluted in 50 % dimethyl sulphoxide, stock solution of nalidixic acid (1 mg/mL) in 0.1 M KOH, and aliquots were stored at –20 °C until testing. Working solutions were obtained by suitable dilution in saline. The substrates *o*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl phosphate for colorimetric evaluation of β-galactosidase (BG; E.C. 3.2.1.23) and alkaline phosphatase (AP; E.C. 3.1.3.1) respectively, were purchased from Sigma-Aldrich (Germany, Taufkirchen). Cultures of yeasts were grown overnight and harvested by centrifugation (6000 × *g* for 15 min), then washed and resuspended in physiological saline until 10^6–10^7 cells/mL were obtained. Genotoxins were added at final concentrations: 4-NQO 0.01 mM, nalidixic acid 0.1 mM, and furazolidone 0.05 mM, respectively, and coincubation was carried out at 37 °C for 150 min with shaking. After coincubation the residual genotoxic activity was determinated in supernatants (obtained after centrifugation at 6000 × *g* for 15 min). In the coincubation experiments the absorbance of cell suspensions at 570 nm was close to 1.00. Genotoxins were also incubated with cell-free culture media from stationary growth phase. Experiments were also done with heat-treated yeast cells killed by immersing test tubes in a water bath at 100 °C for 15 min.

Genotoxicity assays of 4-NQO, furazolidone and nalidixic acid were carried out after cell coincubation, using SOS chromotest. This test was based on the use of genetically modified *Escherichia coli* PQ37, in wich the *lacZ* is under the control of the *sfiA* gene (*17*). Briefly, the test consisted of colorimetric assays of enzymatic activities after incubation of the test strain PQ37 (exponential phase) in the presence of the tested compounds. After two hours of incubation at 37 °C with shaking, the mixtures were divided into two series, one for BG activity measurement (induction assay) and the other for AP (control of protein synthesis). The colorimetric assay for BG and AP was performed according to Quillardet *et al.* (*19*). The activity was calculated in arbitrary enzyme activity units: $A_{420nm} \cdot 1000/t$ (A_{420nm}=absorbance at 420 nm; t=substrate conversion time in minutes). The absorbance at 420 nm was measured using spectrophotometer model Specol 11. The induction factor of *sfi*A::*lacZ* expression was taken to be the ratio of BG activity and AP activity at the respective concentration of the test compound, divided by its control value (in the absence of genotoxins). In this study experiments were performed without metabolic activation. The experiments were repeated five times and determinations were done in triplicate. All results are presented as mean values with the standard error (±SE) calculated with 95 % confidence.

To determine whether certain genotoxin had fluorescent activity, we used ordinary principle that maximum of absorbance spectrum coincided with maximum of fluorescence excitation spectrum for each genotoxin. Absorbance spectrum was recorded with Shimadzu UV-Visible recording spectrophotometer UV-260. Fluorescence spectra were recorded with Jobin Yvon fluorescence spectrophotometer JY3 CS. Intensity of fluorescence was measured during incubation period in cell suspensions after the detection of maximum of excitation and emission spectra for each genotoxin.

Results and Discussion

The genotoxic activity of nalidixic acid was investigated using the *E. coli* PQ37 genotoxicity assay. The results are shown in Fig. 1. It was detected that nalidixic acid produced a marked and progressive increase in the activity of BG and the induction factor, without a remarkable inhibitory effect on constitutive activity of AP. The genotoxicity of 4-NQO is well known, and this mutagen is widely used as a reference genotoxin for positive control in SOS chromotest (*20*). The genotoxic property of

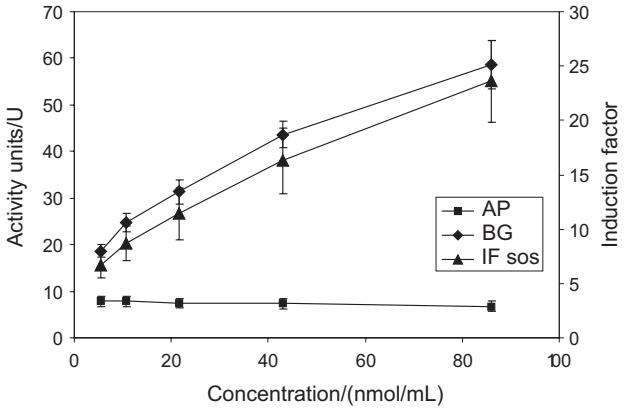

Fig. 1. Dose response in the SOS chromotest for nalidixic acid: dependence of the induction factor (IF) and the activities of enzymes β-galactosidase (BG) and alkaline phosphatase (AP) on nalidixic acid concentration

furazolidone and its dose response were demonstrated in our previous work (*21*).

Inhibition of SOS induction factor was used to assay antigenotoxic effect of *S. boulardii* against 4-NQO, furazolidone and nalidixic acid. Results were expressed as percentage of remaining genotoxic activity relative to the positive control for each compound (Fig. 2). These data showed that remaining genotoxic activity after coincubation with *S. boulardii* was the lowest for 4-NQO. For the other two genotoxins the effect was less pronounced. The antigenotoxic activity of *S. boulardii* did not depend on the mode of overnight cultivation (aerated *versus* non-aerated). It was also important to investigate antigenotoxic activity of supernatant of live or killed cells (heat treated) and cell-free culture media after coincubation with genotoxins 4-NQO, furazolidone and nalidixic acid. Fig. 3A shows that, in general, the supernatant of live yeast cells is more efficient against the genotoxins studied. As shown, yeast cells exhibited different levels of antigenotoxicity against three genotoxins. It is also evident that cell-free spent media possess some antigenotoxicity. The observation that activity of genotoxins was inhibited, even to a minor extent, by cell-free culture media obtained by centrifugation from stationary phase yeast cultures indicates that some metabolites could also have a role in the described phenomenon. Antimutagenic activity of acetic, butyric, lactic, and pyruvic acids against some mutagens and promutagens, including 4-NQO, had been thoroughly studied previously (*22*).

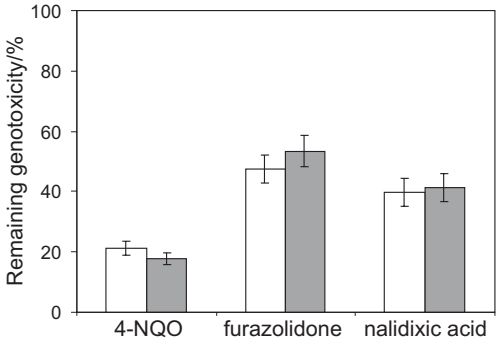

Fig. 2. Genotoxicity of some mutagens remaining after coincubation with *S. boulardii* (empty bars – aerated culture, grey bars – non-aerated culture). 100 % of genotoxicity – induction factors of compounds determined without coincubation

Similar experiments were also done with *S. carlsbergensis* to find out whether this property is common for the *Saccharomyces* genera. Results suggested (Fig. 3B) that *S. carlsbergensis* also possesses antigenotoxic properties, but in a minor extent. In both cases higher antigenotoxicity was observed after coincubation with live yeast cells in comparison with cell-free spent media. In general, supernatants from the live cells always showed significantly higher activity against the genotoxins studied.

To determine whether changes in genotoxicity during coincubation were caused by changes in chemical structure of genotoxins or by other mechanisms, such as genotoxin accumulation in cellular compartments, fluorimetrical analysis was applied. We presumed that if genotoxins are fluorescent, then changes of their molecular structure would induce changes in fluorescence. All three genotoxins showed fluorescent activity, with following excitation and emission maximum spectra (λ_{ex} and λ_{em}): 4-NQO 365 and 435 nm, furazolidone 362 and 442 nm, and nalidixic acid 315 and 360 nm.

Addition of 4-NQO and furazolidone to cell suspensions did not cause modification of fluorescence. This could be explained by the fact that concentrations of these genotoxins in the coincubation medium were not enough by far to detect such an increase. That is a reason why this approach was not applicable in this case.

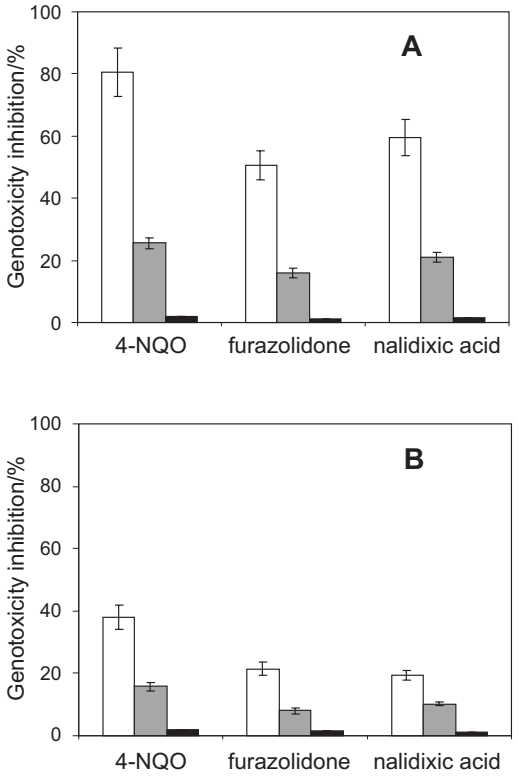

Fig. 3. Antigenotoxicity against 4-NQO, furazolidone and nalidixic acid of supernatant of live cells (empty bars), killed cells (black bars) and cell-free culture media from stationary growth phase (grey bars) of *Saccharomyces* (**A** – *S. boulardii*, **B** – *S. carlsbergensis*) cultures. Relative % inhibition was calculated from residual activity evaluated on yeasts in relation to that of genotoxins

Yet, the fact that 4-NQO and furazolidone showed fluorescent activity provides a chance to use this method in other applications. Nalidixic acid, after its addition to yeast cell suspensions, rapidly modified fluorescence at λ_{ex}=315 nm and λ_{em}=360 nm more than five times, hence it was possible to use fluorescent approach to monitor nalidixic acid during coincubation period. In *S. boulardii* culture after two hours of coincubation fluorescence intensity decreased for more than (16±1.0) %. On the contrary, in *S. carlsbergensis* fluorescence decrease was negligible, less than (4±0.3) %. The fluorescence data were in line with SOS chromotest, confirming that antigenotoxicity of *S. carlsbergensis* was much lower. Modification of fluorescence presumably shows changes of chemical structure of nalidixic acid during coincubation, but the fluorescent approach needs further investigations.

Antimutagenic and anticarcinogenic features of other probiotic microorganisms, especially bacteria, are well known, and it has been shown that some species of *Lactobacillus* and bifidobacteria *in vitro* can inactivate various mutagens, including 4-NQO (*23*). We conclude that also the probiotic yeast *S. boulardii* possesses a strong antigenotoxic activity against 4-NQO and furazolidone, revealed by the SOS chromotest, as well as against nalidixic acid, as demonstrated both by SOS chromotest and fluorometry. Unexpectedly, at the same time, the non-probiotic representative of the same genera, *S. carlsbergensis*, possesses detectable, although much weaker, antigenotoxic properties. Our experimental results about the inherent *S. boulardii* antigenotoxicity seem to give additional credit to this functional property of probiotic yeast. Antigenotoxicity together with resistance to temperature and acidic stresses (*24)* and antibiotics (*14*) makes it especially promising among other probiotics.

In conclusion, we hypothesize that *S. boulardii* could be implicated in the conversion of different genotoxic compounds to unreactive products also *in vivo* in such a complicated microecosystem as the intestinal tract of humans.

Acknowledgements

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Ima li probiotički kvasac antigenotoksično djelovanje?

Sažetak

Proučen je utjecaj probiotičkog kvasca *Saccharomyces boulardii* na genotoksičnost induciranu poznatim mutagenom 4-nitrokinolin-N-oksidom (4-NQO), kao i na antibakterijske (furazolidon) i antibiotičke (nalidiksinska kiselina) spojeve. Procjena genotoksičnosti ispitana je SOS kromotestom koristeći genetički modificiranu bakteriju *Escherichia coli* PQ 37 kao test-organizam. Ovim je testom utvrđeno da kvasac *S. boulardii* ima antigenotoksično djelovanje kada su stanice kvasca inkubirane zajedno s navedenim genotoksinima. Opaženo je da i kvasac *S. carlsbergensis* prema istim spojevima ima vidljivo, ali kudikamo slabije antigenotoksično djelovanje.