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Saccharomyces cerevisiae-based probiotic as novel anti-fungal and anti-inflammatory agent for therapy of vaginal candidiasis

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Abstract

Previously we demonstrated that the treatment with live *Saccharomyces cerevisiae* exerts beneficial therapeutic effects against vaginal candidiasis. Here, we address potential mechanisms particularly examining the probiotic capacity to modulate both fungus and host-related factors. We show that the *S. cerevisiae*-based probiotic markedly affects the expression of virulence traits of *Candida albicans* such as aspartyl proteinases (SAPs) as well as hyphae-associated proteins Hwp1 and Ece1 in the vaginal cavity. On the host side, the probiotic suppression of the influx of neutrophils caused by the fungus into the vaginas of the mice is likely related to: (1) lower production of interleukin-8; and (2) inhibition of SAPs expression. However, these neutrophils displayed reactive oxygen species hyperproduction and increased killing activity as compared to the neutrophils of placebo-treated mice. There was no evidence of any cytotoxic effect by the probiotic, either when used i*n vivo* on vaginal epithelial cell and organ architecture, or in i*n vitro* in human vaginal epithelium. Inactivated yeast cells did not affect any of the factors above. In summary, the data suggest that the beneficial effect exerted by this *S. cerevisiae*-based probiotic is the result of its interference with the expression of fungus virulence factors coupled with the modulation of the inflammatory response of the host.

Keywords: yeasts, vaginal inflammation, beneficial microbes, vaginal candidiasis

1. Introduction

Probiotics are living microbial preparations able to provide health benefits to the host by affecting microbiota balance, composition and activity. It has been shown that probiotics can change the microbiota composition by increasing the populations of beneficial and protective bacteria, e.g. bifidobacteria and lactic acid bacteria, while reducing or arresting the pathogenic ones in both animal models and human studies (Looijer-Van Langen and Dieleman, 2009; Mennigen and Bruewer, 2009).

In turn, the above activities can influence the regulation of the host immune response which has become an important, potential target of probiotics. Improvements of the immune function in probiotic-treated hosts appear to be related not only to the burden of the beneficial bacterial population but also to the capacity of some of the metabolites secreted by these bacteria to modify the expression of basic functions of immune cells, such as cytokine production. This is clearly observed in the intestinal epithelium. In particular, the inhibition of pro-inflammatory cytokine expression in mesenteric lymph nodes, such as interferon-γ and interleukin (IL)-2, was observed (Cavaglieri *et al.*, 2003). Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes (Cavaglieri *et al.*, 2003) could be a consequence of direct interaction of some bacterial cell surface components such as lipoteichoic acids and lipopolysaccharides with pattern recognition receptors

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of host cells. These multiple interactions can lead to a complex cascade of cellular events fostering the regulation of immune response (Medzhitov, 2007; Vance *et al.*, 2009).

Among yeasts, *Saccharomyces cerevisiae* var. *boulardii* has been considered to be a proven probiotic since 1950; it is commercially available throughout the world, with a major indication as an antidiarrhetic agent. This probiotic is able to antagonise the inflammatory mediators that lead to intestinal tissue destruction, which may be achieved via secretion of anti-inflammatory factors (Sougioultzis *et al.*, 2006), the inhibition of tumour necrosis factor-α transcription (Dalmasso *et al.*, 2006), and the upregulation of IL-10 induction (Pontier-Bres *et al.*, 2014). Other mechanisms of immunomodulation achievable by the use of the above yeast have been described (Badia *et al.*, 2012; Martins *et al.*, 2009, 2013).

S. boulardii and *S. cerevisiae* species are genetically very close, however, there are differences, which could be related to the number of genes involved in protein synthesis and stress response (Cascio *et al.*, 2013; Edwards-Ingram *et al.*, 2007). Relative to *S. boulardii,* few studies exist about the probiotic properties of *S. cerevisiae.* In particular, studies focused on the effect of *S. cerevisiae* administration on gastrointestinal disorders (Sivignon *et al.*, 2015) and on the increase of survival and therapeutic potential of probiotic *Lactobacillus rhamnosus* (Lim *et al.*, 2015). *S. cerevisiae* CNCM I-3856 is already recognised for these probiotic properties (Cayzeele-Decherf *et al.*, 2017; Pineton de Chambrun *et al.*, 2015; Spiller *et al.*, 2016).

We previously demonstrated that local administration of *S. cerevisiae* live yeast (encoded GI) accelerates the clearance of *Candida albicans* from the vagina of experimentally-infected mice (Pericolini *et al.*, 2017). We showed that the enhanced fungal clearance was related to numerous effects directly exerted on *Candida* by the probiotic in an i*n vitro* experimental system including mechanical ones, e.g. co-aggregation and inhibition of hypha formation (Pericolini *et al.*, 2017). In this study, we have expanded on the effects of this *S. cerevisiae*-based probiotic (GI) on the expression of pathogenicity determinants by *C. albicans* i*n vivo* during vaginal candidiasis; in addition, we also examined whether the administration of *S. cerevisiae* could dampen local inflammation by affecting the number and functions of a landmark sign of inflammation, such as neutrophil (PMN) influx.

2. Materials and methods

Study products

The products studied were provided by Lesaffre Human Care (Marcq-en-Baroeul, France). GI is a proprietary, well-characterised strain of Lesaffre, registered in the French National Collection of Cultures of Microorganisms (CNCM) under the number I-3856. The *S. cerevisiae* species was determined by using phenotypic (API® ID32C, Biomerieux SAS, Marcy l'Etoile, France) and genotypic reference methods (genetic amplification and sequencing of 26S DNA) (Kurtzman and Robnett, 1997, 1998). Moreover, strain CNCM I-3856 has been characterised by PCR Interdelta typing techniques (CEN, 2009) and other genetic methods (e.g. complete genome sequencing).

The specification of the probiotic product is $\geq 5\times10^9$ cfu/g and the concentration of the batch used for these trials was 1×10^{10} cfu/g. Inactivated yeast obtained by drum-drying of live yeast *S. cerevisiae* CNCM I-3856 was used as the control strain.

Microbial strains and growth conditions

The origin and the characteristics of the highly virulent *C. albicans* strain (CA-6) have previously been described (Bistoni *et al.*, 1986). The cultures were maintained by serial passages on YPD agar (yeast extract, peptone and dextrose anhydrous. All from Sigma-Aldrich, St. Louis, MO, USA). The yeast cells were harvested by suspending a single colony in saline, washed twice, counted in a haemocytometer and adjusted to the desired concentration. *Staphylococcus aureus* was isolated from an Italian patient and obtained from the laboratory of Bacteriology of Santa Maria della Misericordia Hospital, Perugia, Italy. *S. aureus* was grown in mannitol salt agar (MSA; Oxoid, Basingstoke, UK).

Ethics statement

The procedures involving the animals and their care were conducted in conformity with national and international laws and policies. All animal experiments were performed in agreement with the EU Directive 2010/63, the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the National Law 116/92. The protocol was approved by Perugia University Ethics Committee for animal care and use (Comitato Universitario di Bioetica, permit number 149/2009-B). All the animals were housed in the animal facility of the University of Perugia (Authorisation number 34/2003A). Mice were acclimatised for a week before starting the experiments. 5-8 mice were housed in each cage and were provided with food and water *ad libitum*. All efforts were made to minimise suffering during experiments.

Mice

Female CD1 mice obtained from Charles River (Calco, Italy) were used at 4 to 6 weeks of age. Animals were used under specific-pathogen free conditions that included testing sentinels for unwanted infections; according to

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the Federation of European Laboratory Animal Science Association standards, no infections were detected.

Infection and treatment

Mice were maintained under pseudoestrus condition by subcutaneous injection at the base of a fold of loose skin (area at the neck) (Shimizu, 2004) of 0.2 mg estradiol valerate in 100 µl of sesame oil (Sigma-Aldrich) 5 days prior to infection and then weekly until the completion of the study. Mice anaesthetised with 2.5-3.5 (v/v) isoflurane gas were injected intravaginally with or without saline, GI (10 or 100 mg/ml, 10 µl/mouse) or inactivated whole yeast used as control strain (100 mg/ml, 10 µl/mouse) every day starting from day +1 post-infection with 10 µl of 2×10^9 cells/ml of *C. albicans.* In selected experiments, uninfected control mice were daily injected intravaginally with saline, GI or inactivated yeast (both 100 mg/ml, 10 µl/mouse). *Candida* cell suspensions were administered with a mechanical pipette into the vaginal lumen, close to the cervix. To favour vaginal contact and adsorption of fungal cells, mice were held head down for 1 min following inoculation. Mice were then allowed to recover for 24-48 h, during which the *C. albicans* infection was established.

Vaginal washes

At day +3, +8 and +14 post-infection, vaginal washes were conducted using 250 µl of saline, given in five separate 50 µl volumes, with repeated aspiration for 10 to 20 times. Vaginal washes were centrifuged and the supernatants were collected and tested for cytokine and beta-defensins production. The total cellular fraction was used for flow cytometry analysis, killing activity and gene expression analysis.

Cell lines and vaginal epithelium

The human A-431 vaginal epithelial cell line and the human HeLa epithelial cervical cell line, obtained from ATCC (Manassas, VA, USA), were grown in Dulbecco's Modified Eagle Medium (DMEM; EuroClone S.p.A., Pero, Italy) plus 10% of fetal calf serum (FCS). Human vaginal epithelium was obtained by the differentiation of A-431 cell line as previously described (Ridge *et al.*, 1991).

Flow cytometry analysis

Total cellular fractions obtained from vaginal washes were fixed with 1.5% formalin for 10 min, washed, allowed to react with a phycoerythrin-conjugated mAb to mouse Ly-6G (PMN; 10 µl/10^6 cells; Rat IgG_{2b}; AbD Serotec, Kidlington, UK) for 20 min at room temperature (RT) in the dark. After incubation, cells were washed twice with fluorescent buffer (FB), resuspended in 0.5 ml of FB and then analysed by flow cytometry using a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ, USA). Data are expressed as the percentage of positive cells (PMN) (Pericolini *et al.*, 2015). Autofluorescence was assessed by using untreated cells. Control staining of cells with irrelevant Abs was used to obtain background fluorescence values.

Cytokine and beta-defensins production

The supernatants from vaginal washes were collected and tested for IL-8 and beta-defensins levels by specific ELISA assays (both from MyBioSource, San Diego, CA, USA). Cytokine and beta-defensins titres were calculated relative to standard curves.

Peritoneal neutrophils

After 14 days post vaginal infection, peritoneal neutrophils (pPMN) from uninfected mice, were collected 18 h after the intraperitoneal injection of 0.5 ml endotoxin-free 10% thioglycolate solution (Difco, Becton Dickinson) and used as a control of killing activity.

Antimicrobial activity

100 µl of cellular fraction (1×10^4 cells/ml), obtained from vaginal washes of mice treated for 14 days as described above, or pPMNs (1×10^4 cells/ml) were incubated in flat bottom 96-well microtiter tissue culture plates with 100 µl of *S. aureus* (2×10^2 cells/ml) in RPMI-1640 + 5% FCS for 2 h at 37 °C plus 5% CO_2. After incubation, plates were vigorously shaken and cells were lysed by adding Triton X-100 (0.1% in distilled water; final concentration in the well 0.01%). Serial dilutions were prepared in distilled water from each well. The samples were then spread on MSA in triplicate and cfu values were evaluated after 24 h of incubation at 37 °C. Killing activity was expressed as the percentage of cfu inhibition (Mosci *et al.*, 2014). In selected experiments, the total cellular fraction or pPMNs (both 4×10^6 cells/ml) were incubated with PE-conjugated mAb to mouse Ly-6G (PMN; 10 µl/10^6 cells; Rat IgG_{2b}; AbD Serotec) for 20 min at RT in the dark. After incubation, cells were washed and incubated with 1 µM of 2',7'-dichlorofluoresceindiacetate for 30 min at RT and then incubated in the presence of phorbol myristate acetate (100 ng/ml) (Sigma-Aldrich) for 1 h at 37 °C plus 5% CO_2. After incubation, cells were analysed by flow cytometry using a FACSCalibur system. Data are expressed as the mean of fluorescence intensity of 2',7'-dichlorofluorescein (DCFH) on gated PMN positive cells. Autofluorescence was assessed by using untreated cells.

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Quantitative analysis of *SAP2*, *SAP6*, *HWP1* and *ECE1* genes expression

Vaginal washes of mice, intravaginally infected and treated as described above with GI (10 or 100 mg/ml, 10 µl/mouse) or inactivated yeast (100 mg/ml, 10 µl/mouse), were obtained 3, 8 and 14 days post-infection with 10 µl of 2×10^9 cells/ml of *C. albicans.* Vaginal washes were centrifuged at 3,000 rpm for 5 min, then cellular fractions were lysed using Trizol reagent (Life Technology, Waltham, MA, USA).

Total RNA was extracted and retro-transcribed by using the Moloney murine leukaemia virus reverse transcriptase reaction (M-MLV RT), as described in the manufacturer's instructions. cDNA concentration was determined using a spectrophotometer. *C. albicans ACT1, SAP2, SAP6, HWP1* and *ECE1* genes were detected using primers reported elsewhere (Moyes *et al.*, 2016; Naglik *et al.*, 2006, 2008). Real-time PCR (quantitative PCR) was performed in 96-well PCR plates using SYBR green (all from BioRad, Hercules, CA, USA). For real-time PCR reactions 100 ng of cDNA was used. All samples were measured in triplicate. The relative level of *Candida* genes expression at different time-points post-infection was reported as $2^{-\Delta\Delta CT}$ relative to transcripts of *C. albicans* inoculum (Pericolini *et al.*, 2017). Amplification conditions used were the same for *ACT1*, *SAP2, SAP6, HWP1* and *ECE1*: 3 min at 95 °C, 40 cycles of 10 s at 95 °C and 30 s at primer specific annealing temperature. The experiments were performed using the Eppendorf Mastercycler (Hamburg, Germany).

Cytotoxicity assay

A-431 and HeLa cells (both 1×10^6/ml) were grown for 24 h in 96-well microtiter plates (100 µl/well). Vaginal epithelium was obtained by differentiation of A-431 cell line (1×10^6/ml) for 5 days in 96-well microtiter plates (100 µl/well) (Ridge *et al.*, 1991). Before stimulation, cells were incubated for 2 h in DMEM medium without FCS. Cells were then incubated in the presence or absence of 100 µl of inactivated yeast (control strain) or GI (both 100 mg/ml) for 1 h at 37 °C plus 5% CO_2, extensively washed five times with phosphate buffered saline (PBS) and then incubated with 100 µl of *C. albicans* (1×10^6/ml) for 18 h at 37 °C plus 5% CO_2. After co-incubation, the epithelial cell damage was determined by the release of lactate dehydrogenase (LDH) into the surrounding medium. LDH was measured spectrophotometrically at 492 nm using a Cytotoxicity Detection kit (LDH) from Pierce (Thermo Scientific, Waltham, MA, USA). The percent cytotoxicity of epithelial cells infected with *C. albicans* was calculated as follows: ((LDH activity of treated cells – spontaneous LDH activity)/(maximum LDH activity – spontaneous LDH activity)) × 100 and compared to 100% *C. albicans* damage induced in each cell type (Wachtler *et al.*, 2011).

In selected experiments, A-431, HeLa cells and vaginal epithelium, were incubated in the presence or absence of 100 µl of different doses of inactivated yeast or GI (both 2, 10 or 100 mg/ml) for 1 h at 37 °C plus 5% CO_2, then extensively washed five times with PBS. After washing, 100 µl of medium or 100 µl of *C. albicans* (1×10^6/ml), as positive control, were added. After 18 h of incubation at 37 °C plus 5% CO_2, the epithelial cell damage was determined by the release of LDH into the surrounding medium as above described. The percentage cytotoxicity of epithelial cells treated with different doses of inactivated yeast, GI or with *C. albicans*, was expressed as % of LDH release. The lysis buffer was used as positive control.

Histology

Mice, treated as above described, were sacrificed and vaginas were removed and immediately fixed in 10% (v/v) neutral buffered formalin for 24 h. They were then dehydrated, embedded in paraffin, sectioned into 3- to 4-µm thick sections, and stained with haematoxylin/eosin.

Statistical analysis

The results reported are the means ± standard error of the mean (SEM) of 6-9 different mice from 2-3 experiments. For LDH assay the results reported are the means ± SEM of triplicate samples of four different experiments. Data were evaluated using ANOVA. Post-hoc comparisons were done with Bonferroni's test and with Dunnet's test for LDH assay. A value of $P<0.05$ was considered significant.

3. Results

As reported in detail in the Materials and Methods section, in our experimental model, mice were kept under pseudoestrus and infected with *C. albicans*. The probiotic was administered intravaginally daily while monitoring the infection; vaginal cells or cell-free vaginal washes were examined to determine the effects of GI compound on fungus and host determinants of vaginal candidiasis.

Effects of *Saccharomyces cerevisiae* on *Candida albicans* virulence expression in the mouse vagina

Among the virulence factors of *C. albicans*, the aspartyl proteases (SAPs) have been shown to play a role in both experimental and clinical vaginitis (Naglik *et al.*, 2008; Pericolini *et al.*, 2015). Specifically, in this study, we focused on Sap2 and Sap6 that are strictly associated to immunopathogenic process of vaginal candidiasis. In particular, investigations by our research group showed that Sap2 and Sap6 induce an inflammatory response by monocytes *in vitro* (Gabrielli *et al.*, 2015; Pietrella *et al.*, 2013) and vaginal inflammation *in vivo* (Gabrielli *et al.*, 2016; Pericolini *et al.*, 2015). We therefore assessed the

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expression of two representative *SAP* genes (*SAP2* and *SAP6*) in the infected mouse vagina at various times after challenge of animals, treated or not, with two doses of the probiotic. The results reported in Figure 1A show that both doses of GI were able to produce a marked suppression of *SAP2* expression both at its maximal expression (day 3) and during its declining expression (day 8). On day 14 post-challenge, low levels of *SAP2* gene expression were detected, with minimal or negligible effects of the probiotic. A similar trend of GI inhibitory effect was also noticed on *SAP6* gene expression, although this gene maintained an appreciable level of expression on day 14 (Figure 1B). No inhibition of *SAP* expression was observed with inactivated yeast used as control strain at the same concentration as the probiotic (100 mg/ml, 10 μl/mouse) (data not shown).

To gain further insight into the mechanism of the probiotic mediated inhibition of *C. albicans* hyphal growth observed i*n vitro* (Pericolini *et al.*, 2017), we analysed the expression of two important hyphal growth-associated genes, e.g. *HWP1* (hyphal wall protein 1) and *ECE1* (extent of cell elongation 1), in fungal cells recovered from vaginal washes at various time after challenge. In particular, Ece1 protein is essential for hypha initiation and elongation (Finkel and Mitchell, 2011; Sudbery, 2011). The *HWP1* gene of *C. albicans*, encoding for a fungal cell wall protein required for hyphal development (Hofs *et al.*, 2016; Naglik *et al.*, 2011), was also tested in cells from vaginal washes. The results (Figure 2) show that both genes are appreciably expressed early (day 3) during *C. albicans* infection, and only negligibly later on. Importantly, the treatment with both doses of GI completely inhibited their expression. Conversely, inactivated yeast did not produce any inhibition of *HWP1* and *ECE1* gene expression at any time investigated (data not shown). Moreover, we have already showed that the daily intravaginal administration of GI significantly reduced *C. albicans* burden until 12 days post-infection (Pericolini *et al.*, 2017).

Based on mechanical effects exerted on *Candida* by compounds, as shown by Pericolini *et al.* (2017), and considering that hyphal cells of *C. albicans* can directly damage host epithelial cells, and that some of the peptides derived from Ece1 processing have *Candida*-toxic activity

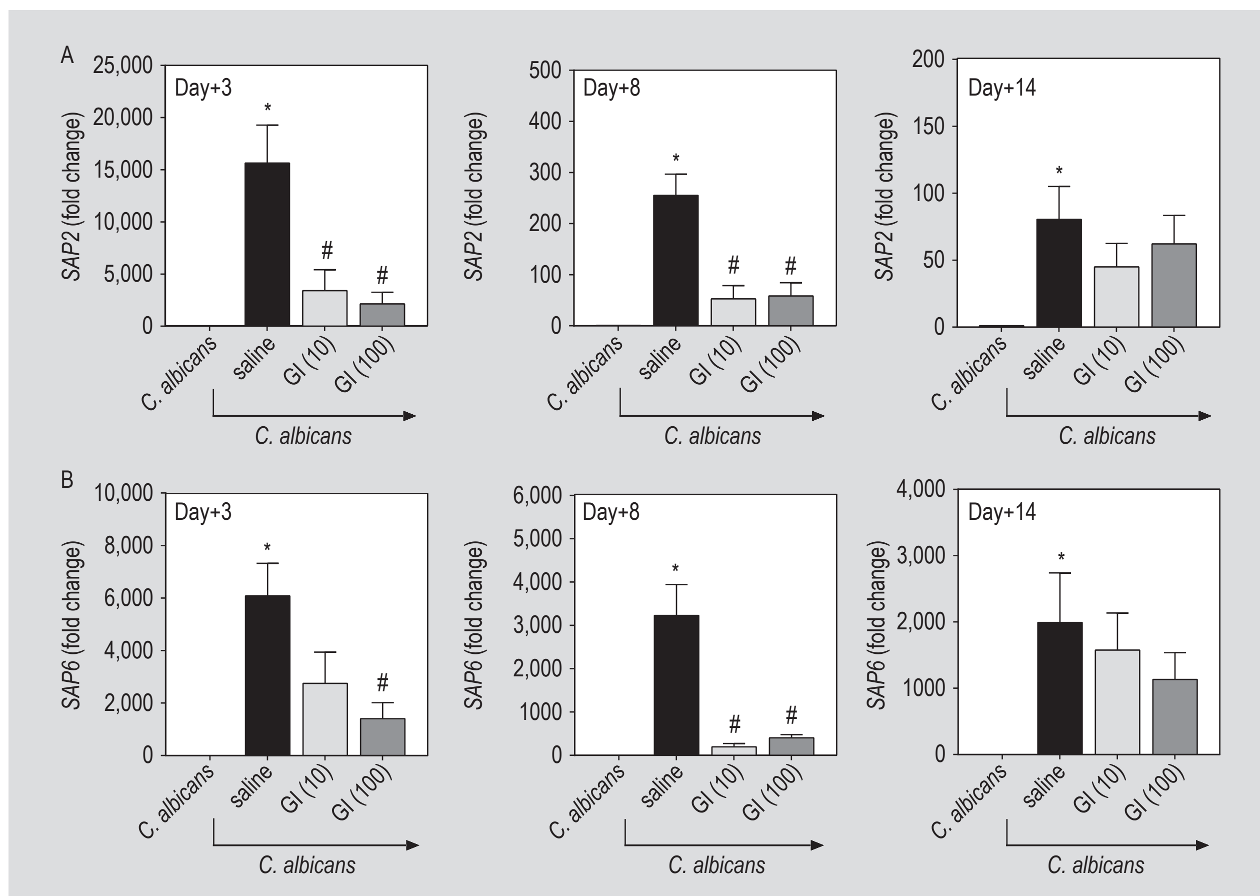

Figure 1. Effect of *Saccharomyces cerevisiae* live yeast (GI) on *Candida albicans* (A) *SAP2* and (B) *SAP6* expression during vulvovaginal candidiasis. cDNA quantities were reported as $2^{-\Delta\Delta CT}$ relative to transcripts expressed in the *C. albicans* inoculum (leftmost column in all graphs). Data show mean ± standard error of the mean of triplicates samples of 6-8 different mice. * *P*<0.05 saline-treated infected mice vs fungal challenge. # *P*<0.05 GI-treated infected mice vs saline-treated infected mice.

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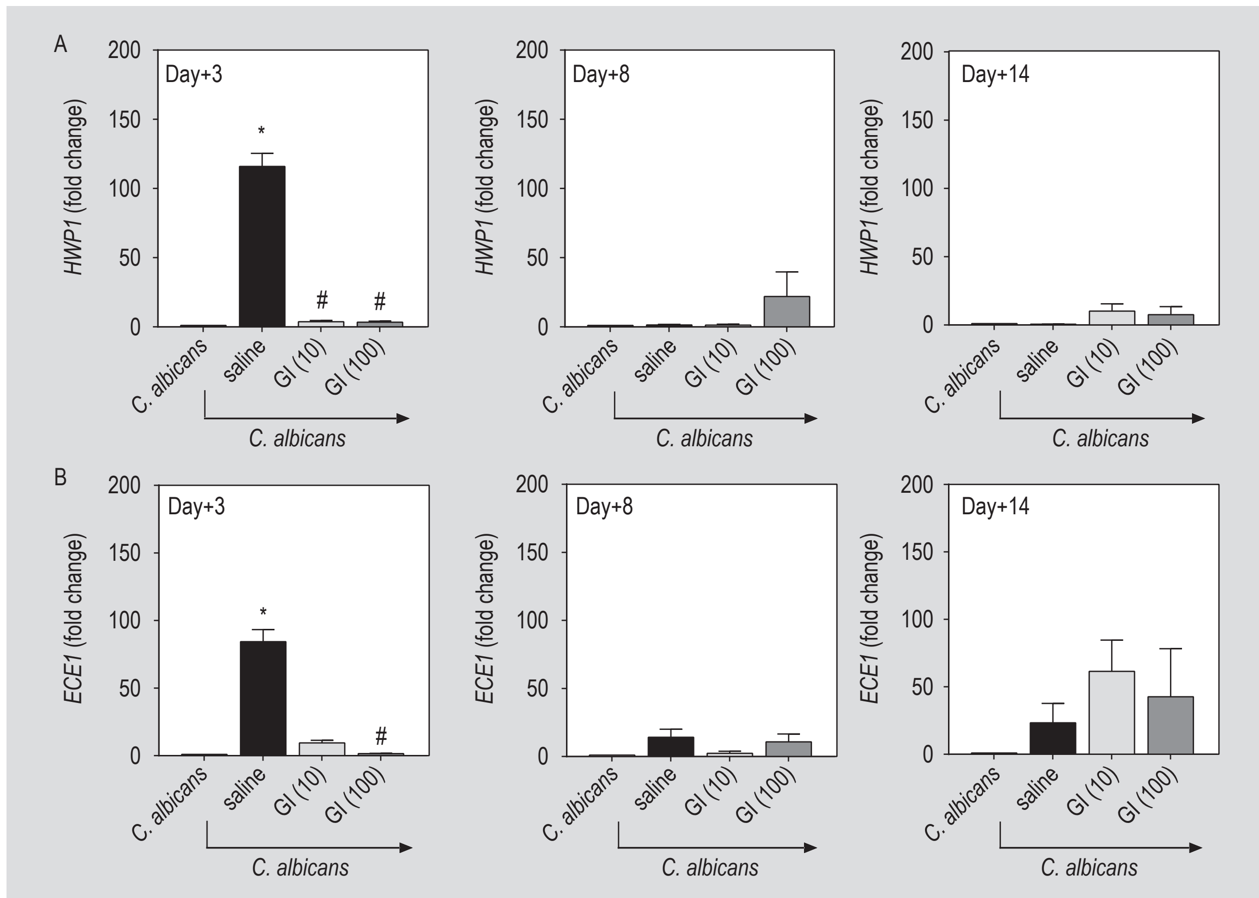

Figure 2. Effect of *Saccharomyces cerevisiae* live yeast (GI) on (A) *HWP1* and (B) *ECE1* expression during vulvovaginal candidiasis. cDNA quantities were reported as $2^{-\Delta\Delta CT}$ relative to transcripts expressed in the *C. albicans* inoculum (leftmost column of all graphs). Data show mean ± standard error of the mean of triplicates samples of 6-7 different mice. * *P*<0.05 saline-treated infected mice vs fungal challenge. # *P*<0.05 GI-treated infected mice vs saline-treated infected mice.

(Moyes *et al.*, 2016), we also tested the possibility that GI exerts a protective role on vaginal and cervico-vaginal epithelial cells exposed to the fungus *in vitro*. This was done by analysing LDH release using the human vaginal epithelial cell line A-431, the human epithelial cervical cell line HeLa and human vaginal epithelium. The higher dose of GI produced a clearly protective effect on *C. albicans*-induced damage in all cell lines tested (Figure 3A). Importantly, the same dose of GI did not exert, by itself, any damage to all above-mentioned cells (Figure 3B).

Effects of *Saccharomyces cerevisiae* on vaginal inflammation by *Candida albicans*

Neutrofil influx and activity

PMN cells recruited to the vagina during *C. albicans* infection are considered a marker of inflammatory response and are heavily involved in the phlogistic process induced in mice by vaginal challenge with *C. albicans*. This process, uncoupled from the fungus infectious burden, is long-lasting and can be related to the inflammation-promoting activity of some fungal products (Pericolini *et al.*, 2015). As previously reported, the influx of PMN was massive in an early phase of vaginal infection with *Candida* and maintained a high level until day 30 post infection (Pietrella *et al.*, 2011). Therefore, it was important to assess whether the probiotic was also capable of modulating PMN influx during the infection. To this aim, we first analysed the influx of PMNs into the mouse vagina 14 days post challenge and the effect of GI administration on it. As shown in Figure 4, the percentage of PMNs recruited to the mouse vagina is substantially reduced on treatment with high dose of GI (Figure 4A). In a second series of experiments, we asked whether some functional activities of PMN cells were affected by GI compound. Thus, we determined reactive oxygen species (ROS) production by vaginal PMNs and β-defensins presence in the vaginal washes. Figure 4B shows that PMNs from infected mice treated with both doses of GI produced more ROS than PMNs from saline-treated infected mice. In contrast, no effect on β-defensins production was shown by treatment with GI (Figure 4C). Furthermore, we analysed whether the increase of ROS production could influence antimicrobial capacity of cells

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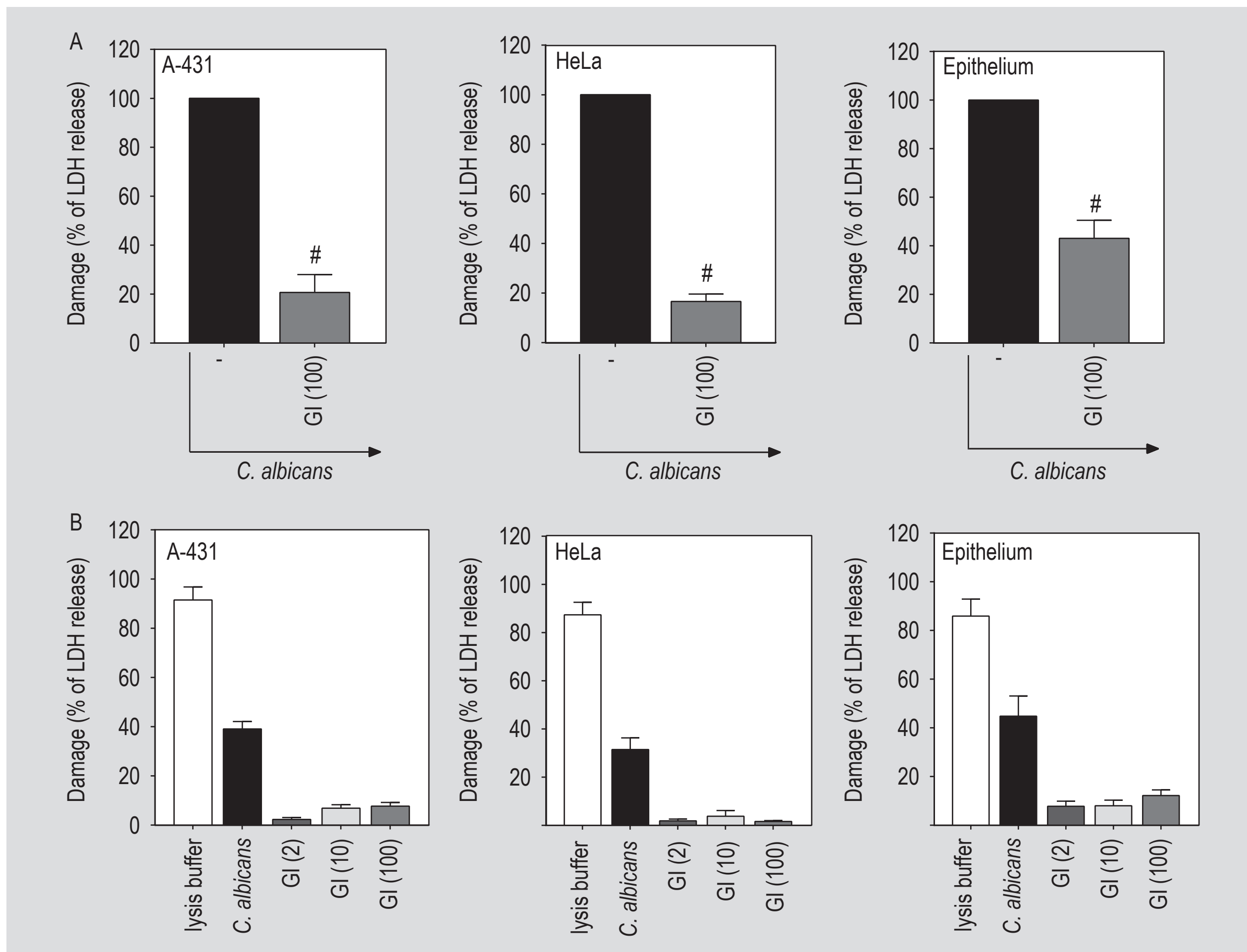

Figure 3. Effect of *Saccharomyces cerevisiae* live yeast (GI) on cellular damage in A-431 cells, HeLa cells, or vaginal epithelium. Cellular damage was expressed as percentage of lactate dehydrogenase release (LDH) (A and B) and compared to 100% *Candida* damage induced in each cell type (A). Data show the mean ± standard error of the mean of triplicate samples of 4 different experiments. # *P*<0.05 GI-treated vs untreated.

from vaginal washes (70% PMN). Given that *C. albicans* is present in vagina of infected mice we tested the PMN activity against an unrelated microorganism, such as *S. aureus.* A significant enhancement of killing activity against *S. aureus* in the cellular fraction from GI-treated infected mice was observed (Figure 4D). The inactivated whole yeast used as control at dose of 100 mg/ml (10 µl/mouse) did not influence PMN infiltration nor ROS and nor β-defensins release and killing activity (data not shown).

IL-8 is a key cytokine associated with inflammatory responses and plays a major role in PMN recruitment and degranulation. Given that recruitment of PMNs into the vaginal environment is an essential factor for inflammation, IL-8 was tested in vaginal washes of mice treated with GI compound. The results show that 100 mg/ml GI strongly reduced IL-8 production at each time point tested after *Candida* challenge, whereas a lower dose of GI only resulted in a reduction after 14 days (Figure 5). Inactivated whole yeast did not modulate IL-8 production (data not shown).

Altogether, these data show that PMNs, i.e. a landmark sign of vaginal inflammation, are not only reduced numerically, possibly due to decreased IL-8 secretion, but also positively stimulated for anti-microbial activity by the probiotic treatment.

Histology

Infiltration of PMNs in histological preparations from *C. albicans* infected mice, untreated or treated with a low dose of GI, was manifested 14 days after challenge (Figure 6A,B). This infiltration disappeared with the high dose of GI (Figure 6C). Histological examination of vaginal tissue of uninfected animals treated with saline (Figure 6D), or GI (Figure 6E) showed complete integrity of vaginal tissue, as well as no PMN recruitment confirming the absence of cytotoxicity for both compounds.

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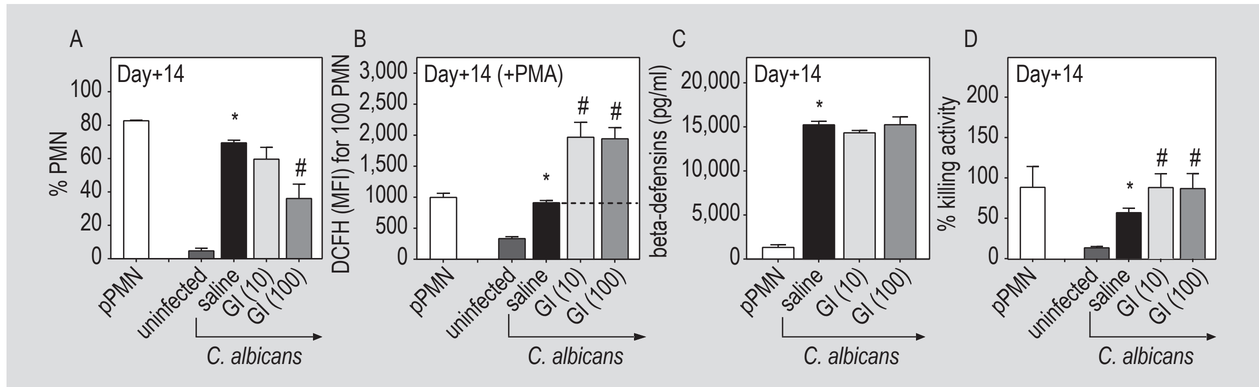

Figure 4. Effect of *Saccharomyces cerevisiae* live yeast (GI) on *Candida albicans*-induced neutrophil (PMN) vaginal influx. (A) Percentage of PMN in cellular fractions from vaginal washes. (B) Reactive oxygen species production as DCFH (mean of fluorescence intensity (MFI)).. (C) Total beta-defensins production. (D) Killing activity of vaginal cellular fractions or pPMNs against *Staphylococcus aureus.* * *P*<0.05 saline-treated infected mice vs saline-treated uninfected mice. # *P*<0.05 GI-treated infected mice vs saline-treated infected mice.

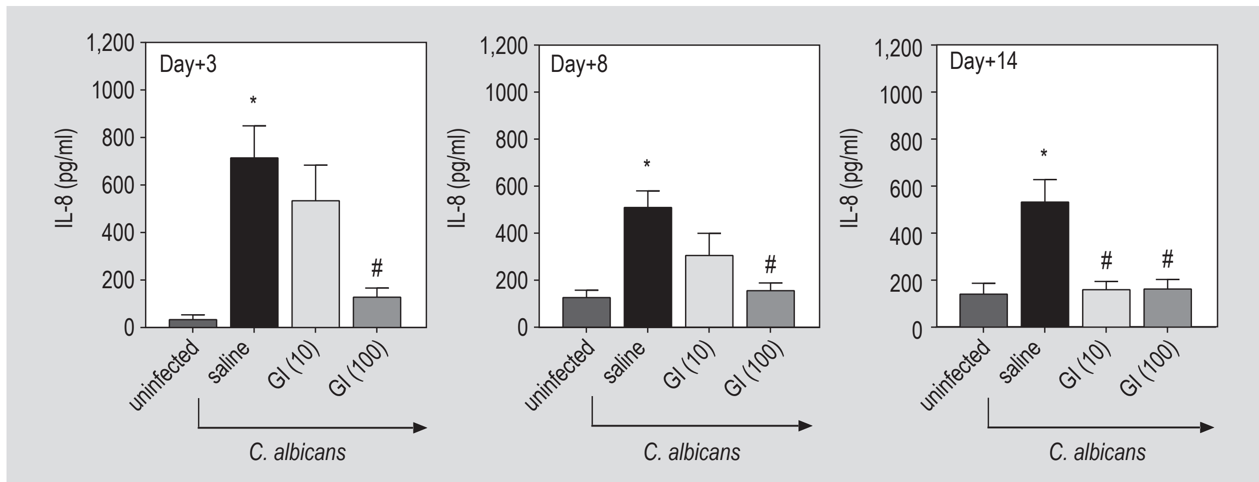

Figure 5. Effect of *Saccharomyces cerevisiae* live yeast (GI) on *Candida albicans*-induced interleukin 8 (IL-8) production. * *P*<0.05 saline-treated infected mice vs saline-treated uninfected mice. # *P*<0.05 GI-treated infected mice vs saline-treated infected mice.

4. Discussion

In recent years, there has been an increasing interest on the modulation of intestinal microbiota and host immune responses by probiotics. Probiotics are live microorganisms that when administered in appropriate amounts provide an health benefit to the host (FAO/WHO, 2002). Various microorganisms have been considered as probiotic including bacteria, such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus* species, and yeasts, such as *S. cerevisiae* (Cheung *et al.*, 2009; Jubran *et al.*, 2013; Nicholson *et al.*, 2012; Tremaroli and Backhed, 2012). These microorganisms have been used extensively for both prevention and treatment of various inflammatory and intestinal disorders of infectious and non-infectious nature, such as inflammatory bowel disease (Sartor, 2005) and infectious diarrhoea (Van Niel *et al.*, 2002). Several findings further support the hypothesis that probiotics could provide an approach for the management of stress response that contributes to intestinal dysfunction (Kennedy *et al.*, 2014).

Expanding on our previous *in vitro* studies, we show here that daily intravaginal administration of *S. cerevisiae* CNCM I-3856 leads to a remarkable inhibition of the expression of several fungal components that play critical roles in fungal virulence at the vaginal level. Some of these components, such as secretory aspartyl proteinases, contribute to vaginal inflammation that is typical of candidal vaginitis. In addition, we show that recruited PMNs – a landmark inflammatory sign – are reduced in number, but not in their potential antimicrobial activity following treatment with the *S. cerevisiae*-based probiotic. Altogether, these effects can underlie the observed beneficial activity of GI on the clearance of the vaginal infection. Finally, we observed that the vagina and the vaginal tissue were totally preserved in architectural integrity in mice treated with GI. Treatment with the probiotic alone at doses used in our experimental

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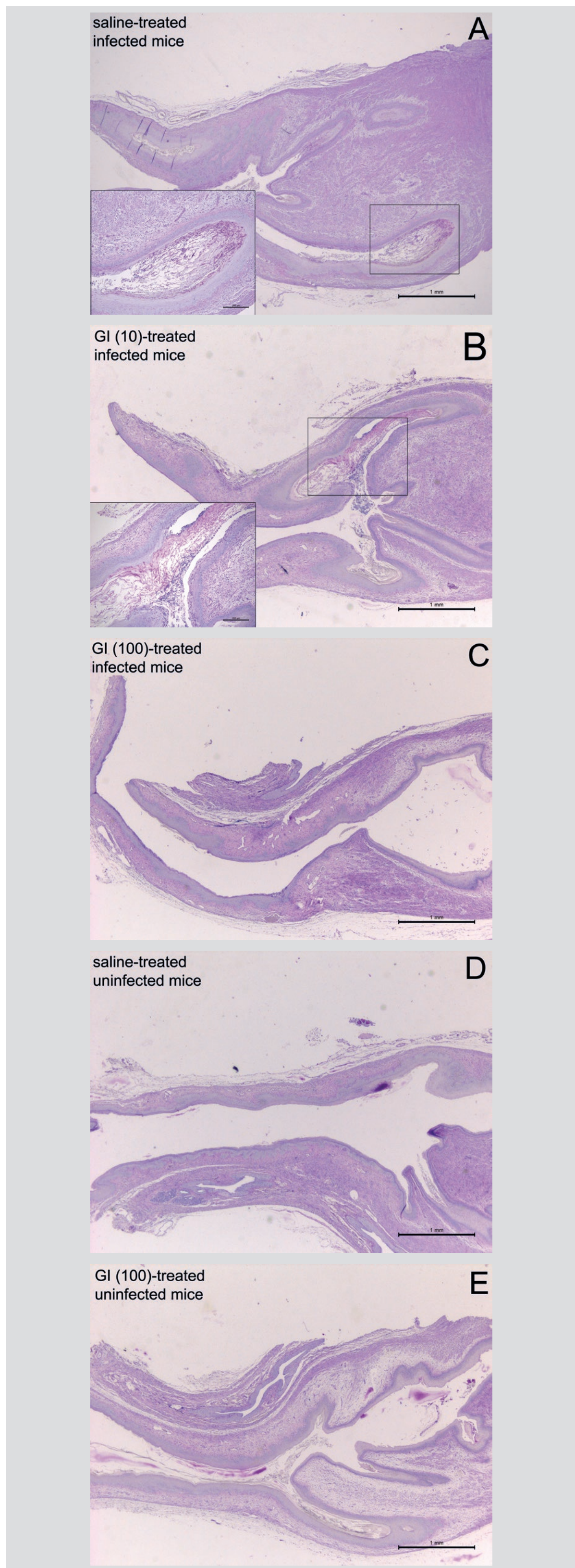

Figure 6. Histological analysis of vaginal tissues from infected mice (14 days post-infection) treated daily with saline (A), *Saccharomyces cerevisiae* live yeast (GI) (10 or 100 mg/ml, 10 µl/mouse) (B and C, respectively). Uninfected mice treated daily with saline (D) or GI (100 mg/ml, 10 µl/mouse) (E) served as controls.

system did not show any toxicity, here evaluated as damage to a vaginal epithelial cell line, a cervical cell line, and a human vaginal epithelium. More importantly, when these cells were infected with *C. albicans*, protection from fungus-induced damage was conferred by *S. cerevisiae.*

Previously we demonstrated that Saps can mediate inflammasome activation both directly, and via their secretion from *C. albicans* cells during vaginal infection (Pericolini *et al.*, 2015). This activation participates greatly in inflammatory processes that characterise this pathology (Pericolini *et al.*, 2015). Given that Sap2 also exerts a chemotactic activity (Gabrielli *et al.*, 2016), the reduction by the probiotic of *C. albicans*-induced PMN influx into vagina could be ascribed, at least in part, to the marked inhibition of *SAP2* expression, to which the noted inhibition of the *SAP6* expression could also participate.

Furthermore, during vaginal candidiasis there is a consistent infiltration of PMNs, which represent about 70% of the inflammatory cellular exudates of the infected vagina (Figure 4A); therefore, the significant decrease of PMNs could be also accounted for the observed decrease in IL-8 in the vaginal washes of GI-treated infected animals. Of importance, this is the first observation that the treatment with this *S. cerevisiae*-based probiotic not only does not alter the microbicidal potential of PMNs, but actually appears to be able to enhance this potential since ROS and killing activity are increased, and β-defensins are not decreased by the probiotic. This suggests that PMNs could be active candidacidal cells in the presence of GI while they are not without treatment (Naglik *et al.*, 2014; Williams, 2010).

Several anti-inflammatory effects have been reported for probiotics such as *S. boulardii*. Results from *in vitro* and *in vivo* experiments show that *S. boulardii* modulates the host's gastrointestinal immune system including IgA secretion as well as IL-10 production (Martins *et al.*, 2009). In addition, anti-inflammatory ability was observed in a mouse model of *Salmonella* infection (Martins *et al.*, 2013). Although several reports deal with *S. boulardii*, very few reports have studied effectiveness of *S. cerevisiae* in preventive or therapeutic treatment in infectious diseases. In a recent paper, we demonstrated that the treatment with live *S. cerevisiae* was therapeutic in vaginal candidiasis by accelerating the clearance of the fungus. This effect was due to multiple interactions of *S. cerevisiae* with *C. albicans* including promotion of coaggregation, inhibition of adherence to epithelial cells, and inhibition of *Candida* virulence factors (Pericolini *et al.*, 2017). All these latter effects were only demonstrated in i*n vitro* systems. In this report, we demonstrate that this *S. cerevisiae*-based probiotic markedly reduces the inflammatory response that is a key player in vaginal candidiasis (Peters *et al.*, 2014; Vecchiarelli *et al.*, 2015). In addition, several virulence factors of the fungus, including the recently described

Ece1, a source of *Candida* toxic active peptides (Moyes *et al.*, 2016), are indeed inhibited i*n vivo* during vaginal candidiasis. Indeed, the acceleration of *C. albicans* clearance and attenuation of inflammatory response following treatment with *S. cerevisiae* probiotic is likely due to direct inhibition of aspartyl proteases production and yeast-hypha transition, two factors considered of utmost importance in the pathogenesis of vaginal candidiasis (Cassone *et al.*, 2016). These effects, in addition to those shown *in vitro*, and coupled with the absence of obvious cytotoxicity of the product, demonstrate that the probiotic activity of *S. cerevisiae* studied here is of high complexity, with multiple and possibly interacting factors. We suggest that at least some of the above *in vitro* and *in vivo* effects of GI can provide a reasonable basis for explaining the reported benefits of using this probiotic in vaginal candidiasis. Our data invite the consideration of this *S. cerevisiae*-based probiotic for initial clinical trials in humans.

Conflict of interest

This work was realised in the frame of a service agreement supported by Lesaffre International.

The authors declared no conflict of interest.

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