**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 in *vivo* on vaginal epithelial cell and organ architecture, or in *in 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-\(\gamma\) 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.
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 antiinfective agent. This pro-biotic is able to antagonise the inflammatory mediators that lead to intestinal tissue destruction, which may be achieved via secretion of anti-inflammatory factors (Souggioulitzis 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 *in 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 in 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-Barœul, 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 \( \geq 5 \times 10^9 \) cfu/g and the concentration of the batch used for these trials was \( 1 \times 10^{10} \) 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
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 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 2×10⁶ 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, closed 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/10⁶ cells; Rat IgG2b; 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 FACS Calibur 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 (1×10⁶ cells/ml), obtained from vaginal washes of mice treated for 14 days as described above, or pPMNs (1×10⁶ cells/ml) were incubated in flat bottom 96-well microtiter tissue culture plates with 100 μl of *S. aureus* (2×10⁵ cells/ml) in RPMI-1640 + 5% FCS for 2 h at 37° C plus 5% CO₂. 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 4×10⁶ cells/ml) were incubated with PE-conjugated mAb to mouse Ly-6G (PMN; 10 μl/10⁶ cells; Rat IgG2b; 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% CO₂. After incubation, cells were analysed by flow cytometry using a FACS Calibur 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.
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 2x10⁶ 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 leukemia 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^-ΔΔCT 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 1x10⁶/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 (1x10⁶/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% CO₂, extensively washed five times with phosphate buffered saline (PBS) and then incubated with 100 µl of C. albicans (1x10⁶/ml) for 18 h at 37 °C plus 5% CO₂. After co-inoculation, 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% CO₂, then extensively washed five times with PBS. After washing, 100 µl of medium or 100 µl of C. albicans (1x10⁶/ml), as positive control, were added. After 18 h of incubation at 37 °C plus 5% CO₂, 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
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 in 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](image-url)
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**

**Neutrophil 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.
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.
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 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.
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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 microbialic 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 *in 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

![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.](http://www.wageningenacademic.com/doi/pdf/10.3920/BM2017.0099)
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Ece1, a source of Candida toxic active peptides (Moyses et al., 2016), are indeed inhibited in 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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