

In Vivo and *In Vitro* Studies on Virulence and Host Responses to *Saccharomyces cerevisiae* Clinical and Non-Clinical Isolates

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Abstract: We have studied the virulence and host responses to several clinical and non-clinical *Saccharomyces cerevisiae* isolates: two vaginal isolates (60, 61), one isolate from faeces (20), a brewer's yeast isolate used in dietetics (D14), one *S. boulardii* isolate from a commercial probiotic product, and a reference natural wine yeast (CECT 10431). Hematogenously disseminated infection in a mouse model demonstrated that four isolates (all, except 20 and 10431) were able to colonize preferentially the brain, as well as kidney and spleen, to a lesser extent, of immunocompetent mice. *In vitro* adhesion assays to epithelial and endothelial cell lines also showed an increased adherence ability of strains 60, 61, D14 and *S. boulardii*. *In vitro* cytokine production assays by RAW 264.7 murine macrophages challenged with yeasts showed a relative increased production of TNF- α in response to the 20 and 10431 strains; viability of RAW cells after coculture was similar in all cases (2-5% non-viable cells) except for 60 strain (11% non-viable cells). *In vitro* phagocytosis assays of yeasts by RAW cells showed that two isolates (D14 and particularly *S. boulardii*) were engulfed less efficiently. These results point out that *S. cerevisiae* isolates, from both clinical and non-clinical (dietetic and probiotic) origin, may vary in the expression of putative virulence factors contributing to their ability to develop the infectious process.

Keywords: *Saccharomyces cerevisiae*, virulence, adhesion, proinflammatory cytokines, phagocytosis.

INTRODUCTION

Saccharomyces cerevisiae strains are the most widely used yeast in industrial food and beverage production, traditionally regarded as absolutely safe, and even *S. boulardii*, considered as a subtype of *S. cerevisiae*, has been described as a biotherapeutic agent in the prevention and treatment of antibiotic-associated diarrhoea and colitis in humans [1-3]. Moreover, *S. cerevisiae* strains can colonize the respiratory, gastrointestinal and genitourinary tracts in a saprophytic way without causing disease; however, in the last decades there is increasing evidence indicating that *S. cerevisiae* is responsible for superficial diseases, that are not life-threatening, although in severely immunocompromised individuals serious systemic infection may occur [4-7]. Improvement of the identification at the species level, the increasing population of immunocompromised individuals, the widespread use of immunosuppressive therapies and broad-spectrum antibacterial chemotherapy, as well as the use of permanent intravascular catheters are factors contributing to this phenomenon [7]. Most nosocomial cases of *S. cerevisiae* fungemia in immunocompromised or critically-ill patients appear to arise from the use of *S. boulardii* as a biotherapeutic agent, although baker's yeasts

have been also isolated from blood [8-10]. The main routes for bloodstream infections are enteral translocation of ingested yeasts and direct contamination of the central venous catheter insertion site [11-14]. In addition, severe infections by *S. cerevisiae* have been occasionally reported in patients with no obvious predisposing factors [15, 16]. Using molecular techniques to characterize *S. cerevisiae* clinical isolates, both *S. boulardii* and baker's yeasts were identified [10, 17]; the link between a subset of clinical isolates and probiotic or baking *S. cerevisiae* strains has been confirmed by phylogenetics and metabolomics [18]. The association between clinical isolates and non-clinical strains points out to food and probiotic/biotherapeutic strains from *Saccharomyces* species as a possible origin of human systemic infections. Therefore, as the reports of *S. cerevisiae* infections are increasing steadily, this yeast species is now considered as an emerging low virulence opportunistic pathogen rather than non-pathogenic yeast [4-7].

Similarly to other opportunistic fungal pathogens, such as its close relative *Candida* species, the ability to colonize and cause disease in the host may depend on a delicate balance between the immune status of the host and the expression of microorganism-related putative virulence factors [19, 20]. These factors are still poorly understood, as the interest in *S. cerevisiae* as an opportunistic pathogen is recent and there are few studies about the origin and mechanisms of infection [21-23]. These reports describe physiological traits with no direct evidence of their role during infection; the majority of

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the clinical isolates secrete higher levels of protease and phospholipase, grow better at 42 °C, and show strong pseudohyphal growth relative to industrial yeasts. Similarly, the information concerning the interactions between *S. cerevisiae* and the host defences is scant, and murine models have been studied with varying results [22, 24-29]. Comparison of *S. cerevisiae* clinical and non-clinical strains by molecular typing and determination of putative virulence traits has revealed that no specific virulence factor clearly separates the strains of clinical origin from the strains of non-clinical origin [30]. Adhesion to host tissues and recognition of fungal ligands by pattern recognition receptors (PRRs) that trigger innate immune responses (such as phagocytosis and cytokine production) are key mechanisms for initiating colonization and determining the fate of the infectious process [19, 20, 31]. It is well known in pathogenic fungi that the cell wall, as the outermost structure of the cell, plays a major role in the interactions with the host [32]. In this context, the *S. cerevisiae*-derived cell wall particle Zymosan has been widely used to study host responses such as phagocytosis and cytokine production, through recognition by PRRs [33-37].

In this work we have studied the virulence in a mouse model and the *in vitro* host responses to different clinical and non-clinical *S. cerevisiae* isolates (two vaginal isolates, one isolate from faeces, a brewer's yeast isolate used in dietetics, one *S. boulardii* isolate used as a biotherapeutic agent, and a reference wine strain) in order to define yeast traits related to virulence and their association with clinical and/or non-clinical isolates.

MATERIALS AND METHODOLOGY

Yeast Strains and Culture Conditions. Six *S. cerevisiae* strains (three clinical and three non-clinical) were used in this study (Table 1): two closely related commensal isolates from vagina (strains 60 and 61), and a commensal isolate from faeces (strain 20), collected by the Microbiology Service of the Vall d'Hebron Hospital (Barcelona, Spain) and characterized by molecular techniques [17]; a brewer's yeast isolate used in dietetics (*S. cerevisiae* D14 strain), obtained from a commercial dietetic product at the Instituto de Agroquímica y Tecnología de Alimentos (CSIC, Burjassot, Spain); a strain of *S. cerevisiae var boulardii* (*S. boulardii*) isolated from a commercial probiotic preparation [17], and the reference CECT (Spanish Type Culture Collection) 10431, a natural wine strain [23]. All

selected yeast strains, except *S. cerevisiae* CECT 10431, exhibit potential virulence factors such as growth at 42 °C, secretion of protease and phospholipase activities and pseudohyphal growth [23] (Table 1). Also two *C. albicans* strains were used in some assays: the high virulence ATCC 26555 strain and the PCA2 strain, a non-germinative low-virulence strain, widely used in host-fungus interaction studies [38-41]. Yeast strains were routinely grown on YPD (1% yeast extract, 2% peptone, 2% glucose) plates cultured at 28 °C for 24 h. Exponentially growing cultures were performed in liquid YPD at 28 °C for 16-20 h.

Endotoxin-free starved viable and fixed (inactivated) yeasts were obtained following standard procedures as previously described [42, 43]. All the assays were performed under conditions to minimize endotoxin contamination. Endotoxin-free water and phosphate buffered saline (PBS) were used; liquid culture media were passed through a endotoxin removing gel (Pierce, Rockford, IL, USA) and tested for the absence of endotoxin by the E-toxate assay (Sigma-Aldrich, Madrid, Spain).

Infection Model in Mouse. Female mice (8-10 weeks old) of three different inbred strains (DBA/2, C3H/HeN and C57BL/6) were used (Harlan Ibérica, Barcelona, Spain). All assays involving mice were approved by the Institutional Animal Care and Use Committee.

Starved viable yeasts were diluted in endotoxin-free PBS to the appropriate cell concentration. Mice (n = 14) were challenged intravenously (i.v.) with 2×10^7 yeast cells (as determined by microscopic counting) in a volume of 0.2 ml PBS, and survival was checked daily for three weeks. To assess the tissue outgrowth of the microorganism, at the indicated days post-infection the colony-forming units (cfu) were determined in homogenized organs (spleen, kidney and brain) from three infected animals following standard procedures.

Cell Lines, Growth Conditions and Adhesion Assays. The human umbilical vein endothelial cell line ECV304 (ECACC-92091712) was grown in M-199 cell culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin stock solution (Gibco, Barcelona, Spain), at 37 °C in 5% CO₂. The human epithelial cell line HeLa S3 (ATCC CCL-2.2) was grown in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1% non-

Table 1. *S. cerevisiae* Strains Used in this Study

| Isolate | Reference | Source |
|---|-------------------------------------|--|
| <i>S. cerevisiae</i> 60 ^(a, b) | D07.10GE/99 | Clinical isolate (vagina) |
| <i>S. cerevisiae</i> 61 ^(a, b) | D14.18GE/99 | Clinical isolate (vagina) |
| <i>S. cerevisiae</i> 20 ^(a, b) | G18.19CG/98 | Clinical isolate (faeces) |
| <i>S. cerevisiae</i> 10431 ^(b) | CECT 10431 | Spanish natural wine |
| <i>S. cerevisiae</i> D14 ^(c) | Phytodepur ^(d) Lot 41 | Commercial dietetic product (INTERSA, S.A.) |
| <i>S. cerevisiae var. boulardii</i> 108 ^(a, b) | Ultralevura ^(d) Lot R-08 | Commercial probiotic preparation (UPSAMEDICA S.L., Biocodex) |

^(a) Isolates previously collected by the Microbiology Service of the Vall d'Hebron Hospital (Barcelona, Spain) and characterized by De Llanos *et al.* (2004) [17].

^(b) Isolates previously characterized on the basis of potential virulence factors by De Llanos *et al.* (2006) [23].

^(c) Isolate obtained at the Instituto de Agroquímica y Tecnología de Alimentos (CSIC, Spain) (unpublished results).

^(d) Commercial name.

essential amino acids, and 1% penicillin/streptomycin stock solution (Gibco, Barcelona, Spain) at 37 °C in 5% CO₂.

For adherence assays, cells were grown up to the formation of a confluent monolayer on a six-well tissue culture plate. After removal of the culture medium, 100 starved yeast cells in 1 ml HBSS (Hanks' balanced salt solution, Gibco, Barcelona, Spain) were added to each well. Yeast cells were previously quantified by microscopic counting and properly diluted in HBSS. Following incubation of cocultures for 45 min at 37 °C in 5% CO₂, the non-adhered yeast cells were removed by aspiration of the supernatant, and each well was rinsed twice with 5 ml HBSS. Each well was then overlaid with molten Sabouraud-dextrose agar at 45 °C, and the plate incubated 24-48 h at 28 °C for colony development. The number of cfu was determined and adherence is expressed as the percentage (%) of the adhered cells. Total, adhered and non-adhered, cells (100 %), were determined as cfu in control plates: 1 ml HBSS containing the yeast suspension used for the adherence assays was plated, overlaid with molten Sabouraud-dextrose agar at 45 °C, and incubated 24-48 h at 28 °C for colony development. Quadruplicate determinations were performed for each assay.

Measurement of *In Vitro* Cytokine Production. The murine macrophage cell line RAW 264.7 (ATCC TIB-71) was used for *in vitro* assays of TNF- α production in response to both viable and inactivated yeast cells. RAW cells were routinely cultured in RPMI cell culture medium supplemented with 5% FBS and 1% penicillin/streptomycin stock solution (Gibco, Barcelona, Spain) at 37 °C in 5% CO₂. For coculture assays, macrophages were resuspended in the same medium and plated in a 96-well tissue culture plate (1 x 10⁵ cells in 200 μ l each well) and incubated for 2 h at 37 °C in 5% CO₂, prior to the challenge. Starved viable yeast cells (1 x 10⁵) or inactivated yeasts (2 x 10⁵) were resuspended in the same cell culture medium and added (10 μ l each well) to macrophages to give a 1/1 or 1/2 ratio (macrophage/yeast) respectively. Cocultures were incubated for 6 h at 37 °C in 5% CO₂. Assays in the absence of exogenous stimuli were used as negative controls to check background activation. Supernatants were then harvested and tested for TNF- α using a commercial enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, USA) as previously described [35, 41, 42]. Triplicate determinations were performed in each assay.

Proliferation of yeast cells and viability of macrophages were also determined after cocultures. Viable yeasts were determined as cfu by plating serial dilutions from each well (before and after coculture) on YPD plates; result is expressed as the percentage (%) of viable yeasts after 6 h of coculture. Viability of macrophages was determined by flow cytometry; briefly, after 2 h of coculture, macrophages (1 x 10⁵) were removed from wells, resuspended in 100 μ l PBS and labelled with 0.5 μ g/ml fluorescein isothiocyanate (FITC)-anti F4/80 mAb (clone BMB8, eBioscience, San Diego, USA) for 20 min at 4 °C, and then washed with PBS and added with propidium iodide (10 μ g/ml) (Sigma-Aldrich, Madrid, Spain); non-viable macrophages (labelled with both FITC-anti F4/80 and propidium iodide) were determined by flow cytometry analysis with an EPICS XL-MCL flow cytometer (Beckman Coulter).

Phagocytosis Assays. Viable yeast cells were labelled with FITC according to standard procedures [35]. Briefly, yeast were resuspended in carbonate buffer pH 10 (5 x 10⁶ cells/ml) containing 100 μ g of FITC per ml, and incubated at room temperature for 2 h. Soluble FITC was removed by extensive washing with PBS, and homogeneous distribution of FITC labelling was checked by flow cytometry. Macrophages (RAW 264.7 cells) resuspended in supplemented RPMI medium with antibiotics (see above) were plated in a 96-well tissue culture plate (1 x 10⁵ cells in 200 μ l each well), added with FITC-labelled yeast cells (5 x 10⁵ cells in 20 μ l of RPMI medium each well) and incubated for 30 min at 37 °C in 5% CO₂. Macrophages were labelled with 0.25 μ g/ml phycoerythrin (PE)-anti F4/80 mAb (clone BM8, eBioscience, San Diego, USA) for 20 min at 4 °C and then washed with PBS, as previously described [44]. The extent of phagocytosis was assessed as the percentage of cells both red- and green-labelled by flow cytometry analysis with an EPICS XL-MCL flow cytometer (Beckman Coulter). To differentiate cell surface-adherent yeasts from the internalised ones, trypan blue (0.2 %) was added to quench extracellular fluorescence. Triplicate determinations were performed in each assay.

Statistical Analysis. Survival curves were analysed by Kaplan-Meier log rank test. The two tailed *t*-test was used to analyse results of fungal burden in organs, cytokine production, adhesion and phagocytosis between individual yeast strains by pair comparisons. Significance was accepted at *P* < 0.05 (*) and *P* < 0.01 (**).

RESULTS

Virulence of *S. cerevisiae* Strains in a Mouse Model of Infection

As susceptibility to fungal infections may vary among mouse strains, we performed the study of the virulence of *S. cerevisiae* strains using three different inbred strains: DBA/2, C3H/HeN and C57BL/6. Survival curves of animals (n = 8) i.v. injected with 2 x 10⁷ yeasts were similar in all cases (not shown). C57BL/6 mice were resistant to all yeast strains tested, as no animal died during the assay. The other two mouse strains (DBA/2 and C3H/HeN) showed an identical behaviour, as only one mouse died when animals were injected with the *S. cerevisiae* D14 strain. Therefore, no statistical differences were found in survival concerning both mouse and yeast strains. Interestingly, all three mouse strains showed temporary but clear symptoms of infection (lethargy, ruffled appearance), particularly in response to *S. cerevisiae* strains 60, 61 and D14, suggesting differences of virulence among yeast strains. Such differences were demonstrated when fungal burden in organs was determined in DBA/2 mice. As shown in Fig. (1), at day 7 post-infection the faecal isolate 20 and the laboratory wine yeast CECT 10431 showed very low levels of fungal burden in the organs analysed. *S. cerevisiae* strains 60, 61 and D14 showed significant levels of fungal burden in brain, whereas *S. boulardii* showed an intermediate colonization (Fig. 1A). Strain D14 of *S. cerevisiae* showed detectable capability to invade the kidney, whereas *S. cerevisiae* 60, 61 and *S. boulardii* showed a more limited ability to colonize kidneys, with no statistical difference as compared to the *S. cerevisiae* CECT 10431 strain and the faecal isolate 20 (Fig. 1B). *S.*

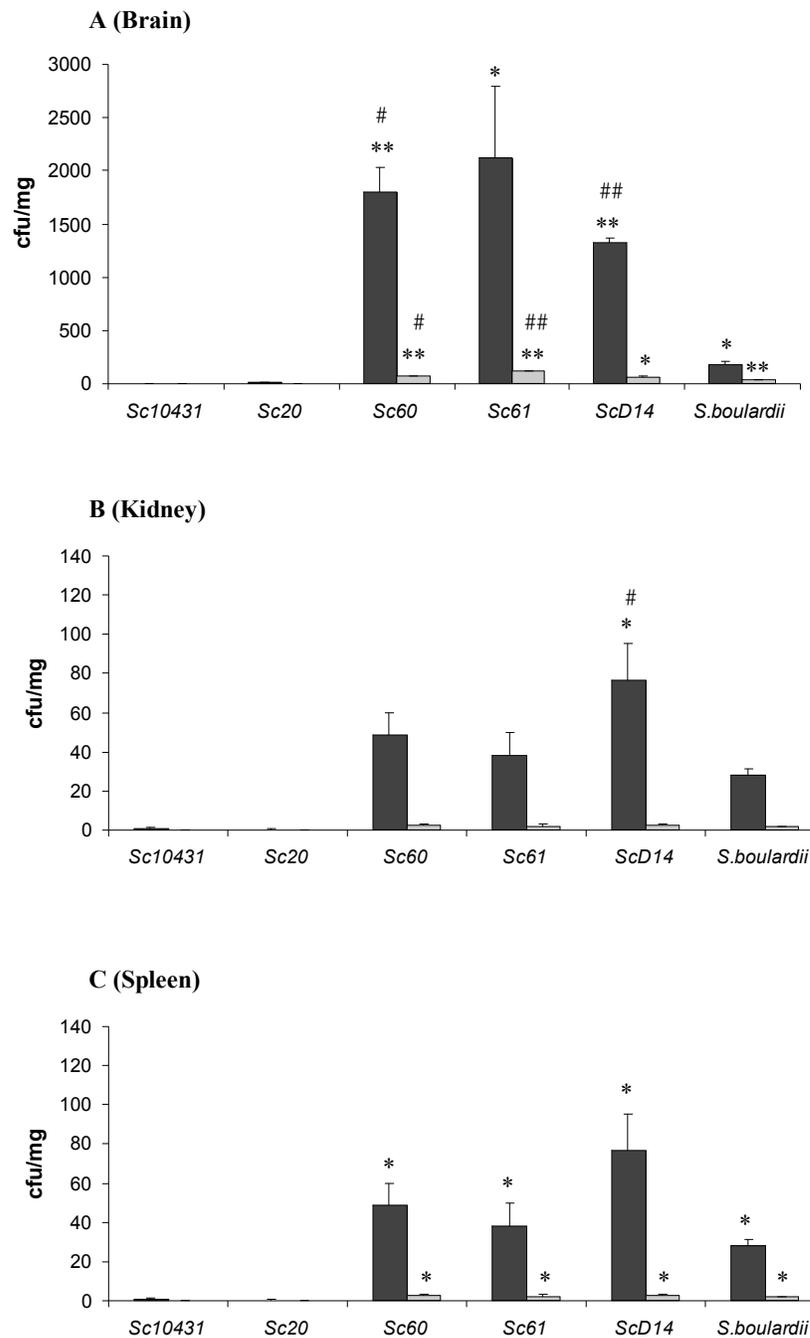


Fig. (1). Fungal burden in organs from mice experimentally infected with yeast isolates. DBA/2 mice ($n = 14$) were i.v. injected with 2×10^7 yeast cells of *S. cerevisiae* CECT 10431 (Sc10431), *S. cerevisiae* clinical isolates 20 (faecal), 60 and 61 (vaginal) (Sc20, Sc60 and Sc61, respectively), *S. cerevisiae* D14 strain (ScD14), and *S. boulardii*. At day 7 (black bars) and 14 (grey bars) post-infection the tissue outgrowth of yeasts was determined in homogenized organs from three animals: brain (A), kidney (B) and spleen (C). Results are expressed as mean values of cfu/mg \pm standard deviation from one representative assay of two. * $P < 0.05$, ** $P < 0.01$ when strains are individually compared to either *S. cerevisiae* CECT 10431 strain or the faecal isolate 20 (statistical significance are identical for both comparisons). # $P < 0.05$, ## $P < 0.01$ when 60, 61 and D14 strains are individually compared to *S. boulardii*. All other comparisons between yeast strains are statistically not significant.

cerevisiae 60, 61, D14 and *S. boulardii* strains showed a limited, but significant ability to colonize the spleen when compared to the faecal isolate 20 and the CECT 10431 *S. cerevisiae* strains (Fig. 1C). In agreement with the lack of mortality, at day 14 post-infection fungal burden diminished in all cases, indicating that the clearance of the microorganisms occurs in immunocompetent mice. Similar

results were obtained when fungal burden in organs was studied in C3H/HeN mice (not shown). These results point out that some *Saccharomyces* strains, from both clinical and non-clinical (dietetic and probiotic/biotherapeutic) origin, possess the ability to infect organs, with special affinity for the brain.

Adhesion of *S. cerevisiae* Strains to Epithelial and Endothelial Cells

As adhesion to host tissues is a key step to establish the infectious process and therefore it is considered as a fungal virulence attribute [19, 32], we investigated the ability of yeast strains to adhere *in vitro* to epithelial and endothelial cell monolayers. In these assays two *C. albicans* strains were

used as controls: the high virulence ATCC 26555 strain, and the low virulence PCA2 strain.

Assays of adherence to endothelial ECV304 cells showed that *Candida* strains possess a much higher adhesion ability than *Saccharomyces* isolates: about 65% and 30% for *C. albicans* ATCC 26555 and PCA2 strains, respectively (Fig. 2A). Adherence of *Saccharomyces* isolates was hetero-

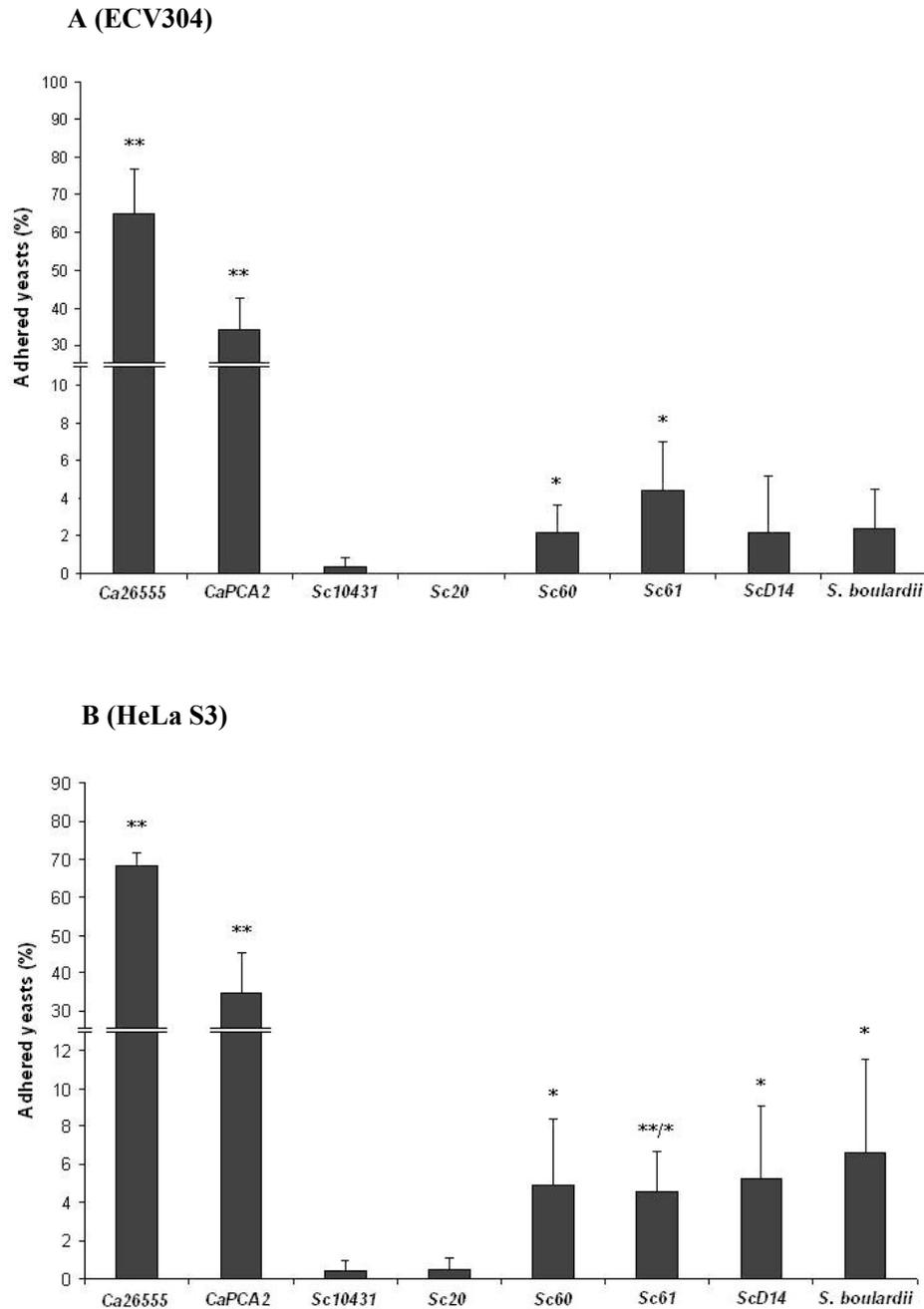


Fig. (2). Adhesion of yeasts to cell culture monolayers. Yeast cells (100 cfu) of tested strains (*S. cerevisiae* CECT 10431, *S. cerevisiae* clinical isolates 20, 60 and 61, *S. cerevisiae* D14 strain, *S. boulardii*, named as in Fig. (1) legend, and *C. albicans* ATCC 26555 and PCA2 strains) were added to plates containing monolayer cultures of the endothelial ECV304 cell line (A) and the epithelial HeLa S3 cell line (B). After 45 min of incubation at 37 °C in 5% CO₂ and extensive washing, molten Sabouraud-dextrose agar was added and incubated at 37 °C for 24-48 h for colony development of adhered yeasts. Results are expressed as the percentage of adhered yeast cells and data represent mean values ± standard deviation of quadruplicates from one representative assay of two. * $P < 0.05$, ** $P < 0.01$ when strains are individually compared to either *S. cerevisiae* CECT 10431 strain or the faecal isolate 20 (differences in statistical significance for both comparisons are indicated: Sc10431/Sc20).

geneous among isolates: vaginal isolate 61 showed the highest adherence (4 %), vaginal isolate 60, dietetic strain D14 and *S. boulardii* showed a minor adherence ability (2 %), whereas the laboratory CECT 10431 and the faecal 20 strains showed the lowest adherence levels (0.5 % and non-detectable, respectively). Differences between vaginal isolates (60 and 61) and strains CECT 10431 and 20 were statistically significant. As expected, differences between all *S. cerevisiae* strains and *C. albicans* strains were also statistically significant (not shown). These results indicate that *Saccharomyces* isolates, both clinical and non-clinical, may possess a limited but significant adherence ability to endothelial cells.

Results of yeast adherence to epithelial HeLa S3 cells were more homogeneous (Fig. 2B). Again *Candida* strains showed a good ability of adhesion (70 % and 35 % for *C. albicans* ATCC 26555 and PCA2 strains, respectively). Vaginal isolates 60 and 61 and non-clinical D14 and *S. boulardii* strains showed similar adherence ability (5-6 %), whereas strains 20 and CECT 10431 showed only a residual adherence (< 0.5 %), and these differences were statistically significant. As expected, differences between all *S. cerevisiae* isolates and *C. albicans* strains were also statistically significant (not shown). These results confirm that clinical and non-clinical *Saccharomyces* isolates possess detectable adherence properties that can be envisaged as a potential virulence factor.

In vitro Cytokine Production by Macrophages in Response to *S. cerevisiae* Strains

Production of proinflammatory cytokines, such as TNF- α , upon recognition of microbial ligands by immune cells plays a key role in host protection against infection, and modulation of this response by the pathogen may favour the progress of the infectious process, as described for the fungal species *C. albicans* [20, 31, 45]. Therefore, we studied the ability of yeast cells to induce *in vitro* the production of TNF- α by the macrophage cell line RAW 264.7. Macrophages were challenged with viable yeast cells for 6 h to allow production of detectable levels of cytokine. Results (Fig. 3A) showed that total production of cytokine was similar in response to all yeast stimuli used, including *C. albicans* PCA2 strain, except for *S. cerevisiae* CECT 10431 which induces lower levels of TNF- α production. Negative controls without exogenous stimuli resulted in the production of non-detectable cytokine levels.

As viable yeast cells were used as stimuli, several factors may influence cytokine production, such as proliferation of yeast population during the assay and viability of macrophages after challenging. Therefore, we determined the ability of yeast isolates to proliferate during coculture with macrophages. As shown in Fig. 3B), the total *C. albicans* PCA2 population increased 5-fold, whereas the number of yeast cells strongly decreased in the case of the *S. cerevisiae* CECT 10431 and remained almost unchanged for *S. cerevisiae* 20; the yeast population of the other four *S. cerevisiae* isolates increased about 2- to 4-fold. Differences between strains were statistically significant when individually compared to both *S. cerevisiae* CECT 10431 and 20 strains (Fig. 3B). This indicates that yeast strains possess a different ability to proliferate under the

conditions of the assay, therefore modifying the amount of fungal challenge during coculture assays. Fig. 3C) shows the relative production of TNF- α standardized according to the final yeast population, although these data may overestimate the effect of fungal growth. *C. albicans* PCA2 strain showed the lower levels of relative cytokine production, whereas strains *S. cerevisiae* CECT 10431 and 20 induced the highest levels of cytokine production, and the other four *Saccharomyces* isolates showed intermediate levels. Differences between strains were statistically significant when individually compared to both *S. cerevisiae* CECT 10431 and 20 strains (Fig. 3C). These differences among *Saccharomyces* isolates were also statistically significant when macrophages were challenged with inactivated yeast stimuli (Fig. 4); interestingly differences between *S. cerevisiae* 60, 61, D14 and *S. boulardii* strains individually compared to *C. albicans* PCA2 were statistically significant (not shown).

Viability of RAW cells was also determined after 2 h of coculture with viable yeast stimuli (Fig. 5). All *Saccharomyces* strains caused similar mortality rates (2-5 % of non-viable RAW cells), except strain 60 which caused an increased mortality rate (11 %). Differences between strain 60 and both CECT 10431 and 20 strains were statistically significant. The low virulence *C. albicans* PCA2 strain induced intermediate levels of non-viable macrophages (6-7 %), whereas the high virulence *C. albicans* ATCC 26555 strain caused an increased mortality rate (19 %), statistically significant when compared to all other strains, including the 60 isolate.

Phagocytosis of *S. cerevisiae* Strains by Macrophages

The above described results point out that phagocytosis of viable yeast cells may also vary among strains. The ability of RAW cells to phagocytose yeast cells was determined by flow cytometry analysis after 30 min of coculture with FITC-labelled yeast. Results (Fig. 6) indicated that the laboratory reference *S. cerevisiae* CECT 10431 strain is the best phagocytosed yeast (about 60 % of macrophages were able to engulf yeast cells). Commensal *S. cerevisiae* 20, 60 and 61 isolates showed similar phagocytose rates (33-46 %) as well as *C. albicans* PCA2 (39 %) and ATCC 26555 (29 %) strains. Interestingly, dietetic and biotherapeutic yeast strains (D14 and *S. boulardii*) were poorly phagocytosed (19 % and 9 % respectively), and these differences were statistically significant when *S. boulardii* was compared to all commensal and *C. albicans* strains (Fig. 6), and when ScD14 was compared to *C. albicans* PCA2 and *S. cerevisiae* 60 and 61 strains (not shown).

DISCUSSION

Molecular characterization of *S. cerevisiae* clinical isolates has demonstrated their association with non-clinical strains, and therefore exposure due to the widespread handling by consumers of probiotic and baker's yeast may originate human colonization [10, 17, 18]. Potential virulence factors, such as secretion of higher levels of hydrolytic enzymes (proteases and phospholipases), the ability to grow at 42 °C and to develop pseudohyphal growth are frequently associated with clinical isolates and have been proposed as preventive criteria in the selection for industrial strains [23]. However, no specific virulence factor has been found that

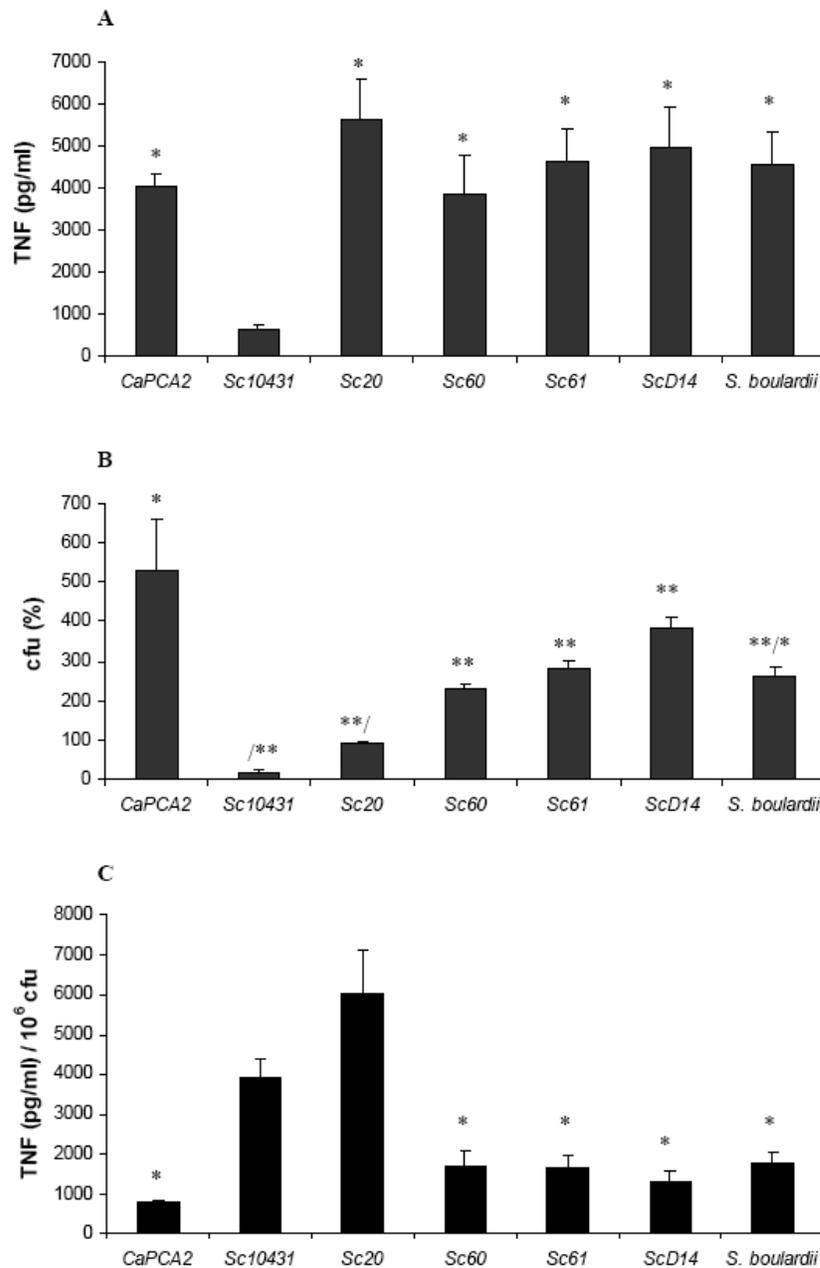


Fig. (3). Production of TNF- α by macrophages in response to viable yeasts. RAW 264.7 macrophages (1×10^5 cells) were cocultured with viable yeast (1×10^5 cells) of the tested strains (*S. cerevisiae* CECT 10431, *S. cerevisiae* clinical isolates 20, 60, and 61, *S. cerevisiae* D14 strain, *S. boulardii*, and *C. albicans* PCA2 strain), and incubated for 6 h at 37 °C in 5% CO₂. (A) The concentration of TNF- α in the culture supernatants was measured by ELISA. Results are shown as means \pm standard deviation of triplicates from one representative assay of two. (B) Proliferation of yeasts after coculture. Viable yeasts were determined as cfu by plating serial dilutions on YPD plates (before and after coculture), and results are expressed as the percentage (%) of viable cells \pm standard deviation of triplicates from one representative assay of two. (C) Relative production of TNF- α by macrophages in response to viable yeast. Total production of TNF- α , shown in panel A, was standardized according to the final yeast population. Results are expressed as the ratio TNF- α production/ 10^6 cfu \pm standard deviation of triplicates from one representative assay of two. * $P < 0.05$, ** $P < 0.01$ when strains are individually compared to *S. cerevisiae* CECT 10431 (A), and compared to either *S. cerevisiae* CECT 10431 strain or the faecal isolate 20 (Sc10431/Sc20) (B, C).

clearly separates the strains from clinical and non-clinical origin [30]. Since *in vivo* and *in vitro* experimental approaches to study interaction of fungal cell with the host have been a very useful tool to elucidate molecular mechanism leading to infection by *C. albicans* [20, 31, 35, 36, 45], in this work we have studied the interactions of six *S. cerevisiae* (three clinical and three non-clinical) isolates

from different origins (two commensal isolates from vagina, one commensal isolate from faeces, two strains from dietetic and probiotic products, and one reference wine strain) with the host, in order to gain information about virulence-related factors contributing to the infectious process and their association with clinical and/or non-clinical isolates.

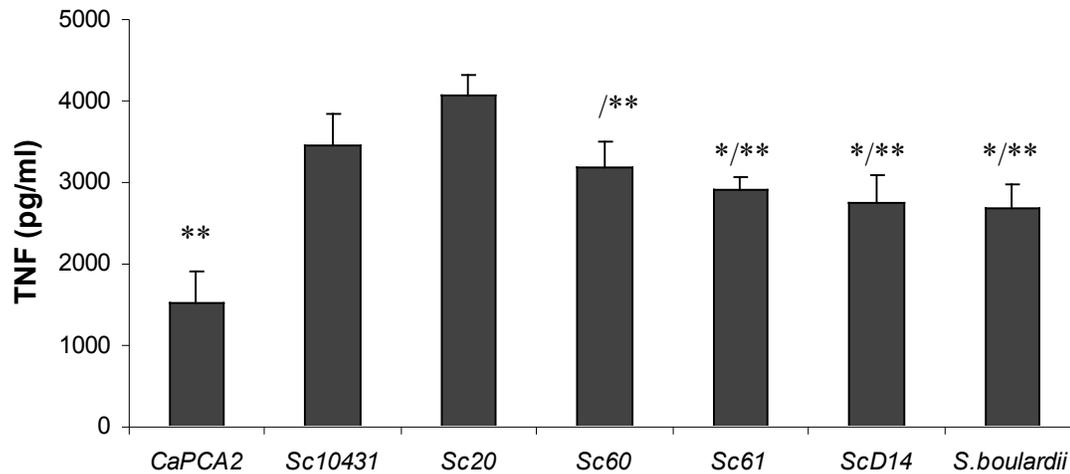


Fig. (4). Production of TNF- α by macrophages in response to inactivated yeasts. RAW 264.7 macrophages (1×10^5 cells) were cocultured with inactivated yeasts (2×10^5 cells) of the tested strains (*S. cerevisiae* CECT 10431, *S. cerevisiae* clinical isolates 20, 60, and 61, *S. cerevisiae* D14 strain, *S. boulardii* and *C. albicans* PCA2 strain), and incubated for 6 h at 37 °C in 5% CO₂. The concentration of TNF- α in the culture supernatants was measured by ELISA. Results are shown as means \pm standard deviation of triplicates from two representative assays. * $P < 0.05$, ** $P < 0.01$ when strains are individually compared to either *S. cerevisiae* CECT 10431 strain or the faecal isolate 20 (Sc10431/Sc20).

In vivo models of systemic infection in mouse strains demonstrate that all *S. cerevisiae* isolates possess a very limited virulence potential, as a high i.v. dose of yeast (20×10^7) did not result in significant mortality of immunocompetent animals in all three mouse strains tested. It should be noted that i.v. administration of one million of *C. albicans* yeast usually results in a significant mortality of animals in a few days [35, 36, 39]. Therefore, pathogenicity of *S. cerevisiae* isolates is far too low to be comparable to *C. albicans* pathogenic potential. Nevertheless, analysis of fungal burden during infection revealed some interesting aspects. Clinical (vaginal) and non-clinical (dietetic and biotherapeutic) isolates showed a clear ability to invade organs, preferentially the brain, of immunocompetent mice, which may account for the symptoms observed in infected animals. By contrast, both the faecal isolate and the reference wine strain, which differ in the expression of potential

virulence factors, were unable to invade these organs, pointing out that virulence and expression of potential virulence traits do not correlate necessarily. The clearance of fungal burden during infection explains the absence of mortality of animals and further suggests a limited pathogenicity of isolates in immunocompetent mice. It is remarkable that one dietetic isolate (D14) showed a high ability to invade organs (including kidney) and was responsible for the only two deaths observed in animals, suggesting a higher virulence potential.

We also determined the adherence properties of the isolates to cell cultures, as adhesion to host tissues allows colonization, a key step to establish the infectious process [19, 32]. Results showed that both clinical (vaginal) and non-clinical (dietetic and biotherapeutic) isolates possess a limited, but detectable ability to adhere to endothelial, and particularly to epithelial cell lines, as compared to the faecal

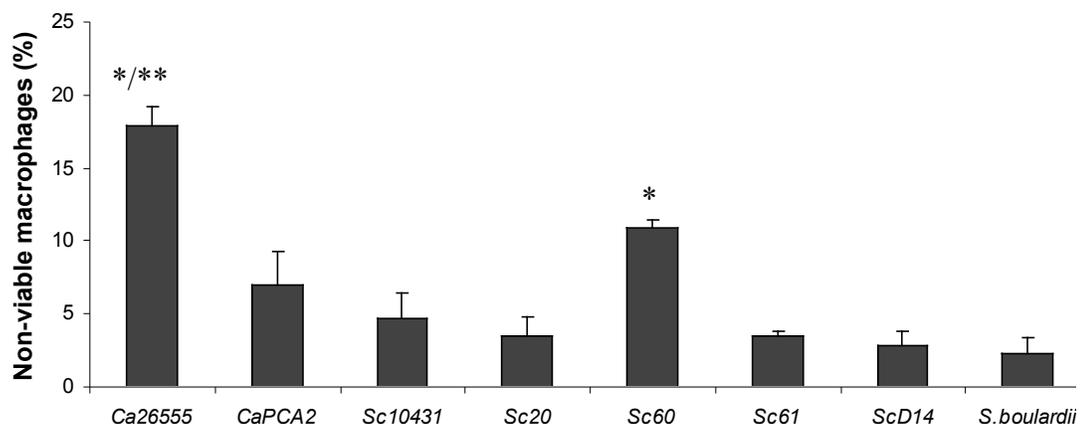


Fig. (5). Viability of macrophages after coculture with viable yeasts. RAW 264.7 macrophages were cocultured with viable yeast cells (1:1 ratio) for 2 h at 37 °C in 5% CO₂. Macrophages were labelled with FITC anti-F4/80 mAb, and non-viable macrophages were stained with propidium iodide and quantified by flow cytometry analysis. Strains assayed are named as in Fig. (2). Data represent the percentage of non-viable cells \pm standard deviation of triplicates from one representative assay of two. * $P < 0.05$, ** $P < 0.01$ when strains are individually compared to either *S. cerevisiae* CECT 10431 strain or the faecal isolate 20 (Sc10431/Sc20).

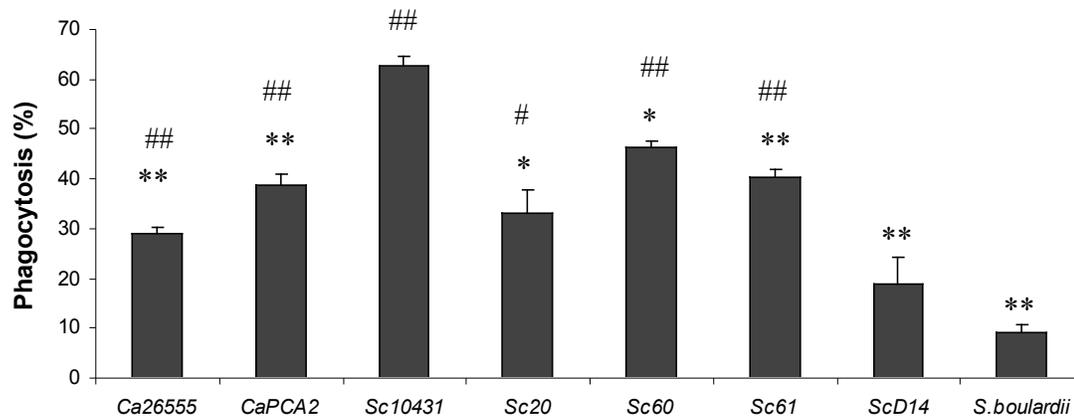


Fig. (6). Phagocytosis of yeast isolates by macrophages. RAW 264.7 macrophages were cocultured with FITC-labelled viable yeasts (ratio 1:5) for 30 min at 37 °C in 5% CO₂. Macrophages were labelled with PE-anti F4/80 mAb and the extent of phagocytosis was assessed by flow cytometry analysis. Strains assayed are named as in Fig. (2). Data represent the percentage of macrophages containing internalised yeast \pm standard deviation of triplicates from one representative assay of two. * $P < 0.05$, ** $P < 0.01$ when strains are individually compared to *S. cerevisiae* CECT 10431 strain. # $P < 0.05$, ## $P < 0.01$ when individual strains are individually compared to *S. boulardii*.

isolate and the reference wine strain, which were much less adherent, and to *C. albicans*, which possess a higher adherence potential. A similar result was reported for the adhesion of *S. cerevisiae* clinical and non-clinical isolates to Caco-2 and buccal epithelial cells [30, 46]. This ability to adhere to host cells can be considered as a virulence factor contributing to the pathogenicity of *S. cerevisiae* isolates.

Proinflammatory response to invading pathogens plays an essential role in host protection, and modulation of this response by the pathogen may contribute to development of disease, as demonstrated to occur in response to *C. albicans*; production of proinflammatory cytokines, such as TNF- α , is triggered upon recognition of well conserved fungal ligands by PRRs on immune cells, and expression and accessibility of these ligands may vary among strains [20, 31, 35, 36, 45]. *In vitro* production of TNF- α by macrophages in response to inactivated *S. cerevisiae* isolates showed significant differences among strains, with the highest production of cytokine observed in response to the faecal isolate and the reference *S. cerevisiae* wine strain. Results of TNF- α production in response to viable yeasts confirm this observation, although they are more difficult to interpret since several factors should be considered, as already mentioned in the Results section. Our data indicate that the relative production of TNF- α is lower in clinical (vaginal) and non-clinical (dietetic and biotherapeutic) *S. cerevisiae* strains as compared to the faecal isolate and the reference strain, although higher than cytokine production in response to *C. albicans* PCA2. As TNF- α is an essential cytokine for host protection against fungal infections [20, 31], it can be suggested that the lower relative levels of cytokine production observed in those strains may probably contribute to their increased virulence. In addition, viable yeasts may possess properties able to modulate interaction with macrophages. This is supported by the observed ability of clinical (vaginal) and non-clinical (dietetic and biotherapeutic) isolates of *S. cerevisiae* to proliferate in the presence of macrophages, although viability of RAW cells was only significantly decreased in response to the clinical isolate 60. Moreover, non-clinical (dietetic and biotherapeutic) strains showed a decreased phagocytosis rate as compared to

clinical and reference isolates; this observation is of interest as destruction of microbes by phagocytes is the main defence against primary infection by pathogenic yeast species, such as *C. albicans* [20, 31]. Therefore, an increased resistance to phagocytosis may also contribute to the pathogenesis of dietetic and probiotic strains. Our results point out that yeast strains, such as *S. boulardii* and D14, present in probiotic and dietetic commercial products, are able to express *in vitro* some traits that can be considered as putative virulence factors, and therefore the widespread handling of these products by the consumers must be cautiously regarded, as it may cause human colonization and/or transmission of infection by touch contamination in immunocompromised patients.

As above mentioned, the ability of *S. cerevisiae* strains to invade the host does not necessarily correlate with the expression of potential virulence traits *in vitro*. The differences observed among *S. cerevisiae* strains concerning interaction with host (adhesion, cytokine production and phagocytosis) may be partly due to differential expression of surface molecules which specifically interact with host receptors. Composition and organization of *S. cerevisiae* cell wall is similar to that of *C. albicans*, made basically of chitin, glucan, and mannoproteins, and therefore it is assumable that both species may share some ligands involved in host interaction [32, 47-49]. In fact, the yeast cell wall particle Zymosan is recognized by host PRRs, such as dectin-1 and TLR2, which also recognize *C. albicans*, and play a key role in host defences against candidiasis [20, 31-37, 45]. It should be noted that the expression of these ligands, as well as the expression of virulence factors in *S. cerevisiae*, may be strain dependent and also environmentally regulated, as does occur in *C. albicans* [19,32], and therefore one single isolate may express putative virulence factors depending on growth conditions. Further studies are required to identify particular virulence factors in *S. cerevisiae* isolates and to determine their expression and role in virulence.

In conclusion, our results supports the notion that clinical and non-clinical (dietetic and probiotic) *S. cerevisiae* isolates

may share some properties in their relationship with the host (such as adhesion to host tissues, resistance to phagocytosis and the ability to modulate the proinflammatory response) that contribute to the infectious process. These properties can be considered as new putative virulence factors that should be added to the already described (growth at 42 °C, pseudohyphal development, and production of proteases and phospholipases). Therefore, we propose that selection criteria for industrial strains should also include studies concerning host-pathogen interactions, such as adhesion to cell lines, *in vitro* cytokine production and phagocytosis, in order to select safe strains.

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REFERENCES

- [1] McFarland LV, Surawicz CM, Greenberg RN, *et al.* A randomised placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. *J Med Am Assoc* 1994; 271: 1913-9.
- [2] Doron SI, Hibberd PL, Gorbach SL. Probiotics for prevention of antibiotic-associated diarrhoea. *J Clin Gastroenterol* 2008; 42 (Suppl 2): 58-63.
- [3] Goldin BR, Gorbach SL. Clinical indications for probiotics: an overview. *Clin Infect Dis* 2008; 46 (Suppl 2): 96-100.
- [4] Hazen KC. New and emerging yeast pathogens. *Clin Microbiol Rev* 1995; 8: 462-78.
- [5] Murphy A, Kavanagh K. Emergence of *Saccharomyces cerevisiae* as a human pathogen: implications for biotechnology. *Enzyme Microb Technol* 1999; 25: 551-7.
- [6] Pontón J, Rùchel R, Clemons KV, *et al.* Emerging pathogens. *Med Mycol* 2000; 38 (Suppl 1): 225-36.
- [7] Enache-Angoulvant A, Hennequin C. Invasive *Saccharomyces* infection: a comprehensive review. *Clin Infect Dis* 2005; 41: 1559-68.
- [8] Herbrecht R, Nivoix Y. *Saccharomyces cerevisiae* fungemia: an adverse effect of *Saccharomyces boulardii* probiotic administration. *Clin Infect Dis* 2005; 40: 1635-7.
- [9] Muñoz P, Bouza E, Cuenca-Estrella M, *et al.* *Saccharomyces cerevisiae* fungemia: an emerging infectious disease. *Clin Infect Dis* 2005; 40: 1625-34.
- [10] De Llanos R, Querol A, Pemán J, *et al.* Food and probiotic strains of *Saccharomyces cerevisiae* species as a possible origin of human systemic infections. *Int J Food Microbiol* 2006; 110: 286-9.
- [11] Hennequin C, Kaufmann-Lacroix C, Jobert A, *et al.* Possible role of catheters in *Saccharomyces boulardii* fungemia. *Eur J Clin Microbiol Infect Dis* 2000; 19: 16-20.
- [12] Lherm T, Monet C, Nougère B, *et al.* Seven cases of fungemia with *Saccharomyces boulardii* in critically ill patients. *Intensive Care Med* 2002; 28: 797-801.
- [13] Cassone M, Serra P, Mondello F, *et al.* Outbreak of *Saccharomyces cerevisiae* subtype *boulardii* fungemia in patients neighboring those treated with a probiotic preparation of the organism. *J Clin Microbiol* 2003; 41: 5340-3.
- [14] Graf C, Gavazzi G. *Saccharomyces cerevisiae* fungemia in an immunocompromised patient not treated with *Saccharomyces boulardii* preparation. *J Infect* 2007; 54: 310-1.
- [15] Jensen DP, David MD, Smith MD. Fever of unknown origin secondary to brewer's yeast ingestion. *Arch Intern Med* 1976; 136: 332-3.
- [16] Smith D, Metzgar D, Wills C, Fierer J. Fatal *Saccharomyces* aortic graft infection. *J Clin Microbiol* 2002; 40: 2691-2.
- [17] De Llanos R, Querol A, Planes MP, Fernández-Espinar MT. Molecular characterization of clinical *Saccharomyces cerevisiae* isolates and their association with non-clinical strains. *Syst Appl Microbiol* 2004; 27: 427-35.
- [18] MacKenzie DA, Defernez M, Dunn WB, *et al.* Relatedness of medically important strains of *Saccharomyces cerevisiae* as revealed by phylogenetics and metabolomics. *Yeast* 2008; 25: 501-12.
- [19] Calderone RA. *Candida* and candidiasis. 2nd ed. New York: American Society for Microbiology 2002.
- [20] Zelante T, Montagnoli C, Bozza S, *et al.* Receptors and pathways in innate antifungal immunity: the implications for tolerance and immunity to fungi. *Adv Exp Med Biol* 2007; 590: 209-21.
- [21] McCusker JH, Clemons KV, Stevens DA, Davis RW. *Saccharomyces cerevisiae* virulence phenotype as determined with CD-1 mice is associated with the ability to grow at 42 °C and form pseudohyphae. *Infect Immun* 1994; 62: 5447-55.
- [22] McCullough MJ, Clemons KV, McCusker JH, Stevens DA. Species identification and virulence attributes of *Saccharomyces boulardii*. *J Clin Microbiol* 1998; 36: 2613-7.
- [23] De Llanos R, Fernández-Espinar MT, Querol A. A comparison of clinical and food *Saccharomyces cerevisiae* isolates on the basis of potential virulence factors. *Antonie Van Leeuwenhoek* 2006; 90: 221-31.
- [24] Holzschu DL, Chandler FW, Ajello L, Ahern DG. Evaluation of industrial yeast for pathogenicity. *Sabouradia* 1979; 17: 71-8.
- [25] Maejima K, Shimoda K, Morita C, *et al.* Colonization and pathogenicity of *Saccharomyces cerevisiae* MC16 in mice and cynomolgus monkeys after oral and intravenous administration. *Jpn J Med Sci Biol* 1980; 33: 271-6.
- [26] Clemons KV, McCusker JH, Davis RW, Stevens DA. Comparative pathogenesis of clinical and nonclinical isolates of *Saccharomyces cerevisiae*. *J Infect Dis* 1994; 169: 859-67.
- [27] Byron JK, Clemons KV, McCusker JH, *et al.* Pathogenicity of *Saccharomyces cerevisiae* in complement factor five-deficient mice. *Infect Immun* 1995; 63: 478-85.
- [28] Okawa Y, Yamada Y. Lethality of yeast with low pathogenicity in mice immunocompromised by cyclophosphamide treatment. *Biol Pharm Bull* 2002; 25: 940-2.
- [29] Wheeler RT, Kupiec M, Magnelli P, *et al.* A *Saccharomyces cerevisiae* mutant with increased virulence. *Proc Natl Acad Sci USA* 2002; 100: 2766-70.
- [30] Klingberg TD, Lesnik U, Arneborg N, *et al.* Comparison of *Saccharomyces cerevisiae* strains of clinical and nonclinical origin by molecular typing and determination of putative virulence traits. *FEMS Yeast Res* 2008; 8: 631-40.
- [31] Romani L. Immunity to fungal infections. *Nat Rev Immunol* 2004; 4: 1-23.
- [32] Gozalbo D, Roig P, Villamón E, Gil ML. *Candida* and candidiasis: the cell wall as a potential molecular target for antifungal therapy. *Curr Drug Targets Infect Disord* 2004; 4: 117-35.
- [33] Underhill DM. Macrophage recognition of zymosan particles. *J Endotoxin Res* 2003; 9: 176-80.
- [34] Gantner BN, Simmons RM, Canavera SJ, *et al.* Collaborative induction of inflammatory response by dectin-1 and Toll-like receptor 2. *J Exp Med* 2003; 197: 1107-17.
- [35] Villamón E, Gozalbo D, Roig P, *et al.* Toll-like receptor 2 is essential in murine defenses against *Candida albicans* infections. *Microb Infect* 2004; 6: 1-7.
- [36] Gil ML, Gozalbo D, TLR2, but not TLR4, triggers cytokine production by murine cells in response to *Candida albicans* yeasts and hyphae. *Microb Infect* 2006; 8: 2229-34.
- [37] Goodridge HS, Simmons RM, Underhill DM. Dectin-1 stimulation by *Candida albicans* or zymosan triggers NFAT activation in macrophages and dendritic cells. *J Immunol* 2007; 178: 3107-15.
- [38] De Bernardis F, Adriani FD, Lorenzini R, *et al.* Filamentous growth and elevated vaginopathic potential of a non-germinative variant of *Candida albicans* expressing low virulence in systemic infection. *Infect Immun* 1993; 61: 1500-8.
- [39] Villamón E, Gozalbo D, Roig P, *et al.* Myeloid differentiation factor 88 (MyD88) is required for murine resistance to *Candida albicans* and is critically involved in *Candida*-induced production of cytokines. *Eur Cytokine Netw* 2004; 15: 263-71.
- [40] Villamón E, Gozalbo D, Roig P, *et al.* Toll-like receptor 2 is dispensable for acquired host immune resistance to *Candida albicans* in a murine model of disseminated candidiasis. *Microb Infect* 2004; 6: 542-8.
- [41] Murciano C, Villamón E, Yáñez A, *et al.* Impaired immune response to *Candida albicans* in aged mice. *J Med Microbiol* 2006; 55: 1649-56.
- [42] Murciano C, Yáñez A, Gil ML, Gozalbo D. Both viable and killed *Candida albicans* cells induce *in vitro* production of TNF- α and

- IFN- γ in murine cells through a TLR2-dependent signalling. Eur Cytokine Netw 2007; 18: 1-5.
- [43] Murciano C, Villamón E, O'Connor JE, *et al.* Killed *Candida albicans* yeasts and hyphae inhibit gamma interferon release by murine natural killer cells. Infect Immun 2006; 74: 1403-6.
- [44] Murciano C, Yáñez A, O'Connor JE, *et al.* Influence of aging on murine neutrophil and macrophage function against *Candida albicans*. FEMS Immunol Med Microbiol 2008; 53: 214-21.
- [45] Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol 2008; 6: 67-78.
- [46] Murphy AR, Kavanagh KA. Adherence of clinical isolates of *Saccharomyces cerevisiae* to buccal epithelial cells. Med Mycol 2001; 39: 123-7.
- [47] Klis FM, Mol P, Hellingwerf K, Brul S. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 2002; 26: 239-56.
- [48] Klis FM, de Groot P, Hellingwerf K. Molecular organization of the cell wall of *Candida albicans*. Med Mycol 2001; 39 (Suppl 1): 1-8.
- [49] Ruiz-Herrera J, Elorza MV, Valentin E, Sentandreu R. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. FEMS Yeast Res 2006; 6: 14-29.

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