International Journal of Food Microbiology 144 (2011) 393–399

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

In vivo virulence of commercial *Saccharomyces cerevisiae* strains with pathogenicity-associated phenotypical traits

R. de Llanos [a], S. Llopis [a], G. Molero [b], A. Querol [a], C. Gil [b], M.T. Fernández-Espinar [a,*]

[a] Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos CSIC, PO Box 73, 46100 Burjassot, Valencia, Spain
[b] Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 21 May 2010
Received in revised form 14 October 2010
Accepted 21 October 2010

Keywords:
S. cerevisiae
Clinical strains
Industrial strains
In vivo virulence
Baker's strain
S. cerevisiae var. *boulardii*

ABSTRACT

Two commercial *Saccharomyces cerevisiae* strains, a baker's strain and the bio-therapeutic agent Ultralevure, have been proposed as a possible exogenous source of human colonization (de Llanos et al., 2004, 2006a). Moreover, these strains express phenotypical traits associated to pathogenicity (de Llanos et al., 2006b). Taking into account that both commercial preparations represent an important source of living *S. cerevisiae* cells we have performed an *in vivo* study to evaluate whether there is a potential safety risk to humans. Their virulence was compared with that of other commercial strains with less virulent traits, and with clinical isolates, using two murine models (BALB/c and DBA/2N mice). Burden determination in the brain and kidneys showed that the ability to disseminate, colonize and persist was manifested not only by clinical isolates but also by commercial strains. Among these, the baker's strain and Ultralevure were able to cause the death of BALB/c mice at rates similar to those shown by two of the clinical isolates. These results highlight the pathogenic potential of these strains and show that four-week-old BALB/c mice are an appropriate murine model to study the virulence of yeasts with low or moderate pathogenicity. Furthermore, we have shown the positive effect of an immunosuppressive therapy with cyclophosphamide in the virulence of the baker's strains and Ultralevure but not in the rest of the commercial strains under study. The data suggest that although *S. cerevisiae* has always been considered a GRAS microorganism, commercial preparations should include only those strains shown to be safe in order to minimize complications in risk groups.

1. Introduction

Saccharomyces cerevisiae has traditionally been used in industrial fermentative processes, such as beer and wine production or baking, and as a nutritional supplement or even as a bio-therapeutic agent. The latter example concerns the probiotic strain of this species, *S. cerevisiae* var. *boulardii*, which has been widely used in Europe to treat several types of diarrhoea. Although *S. cerevisiae* and *S. cerevisiae* var. *boulardii* have commonly been considered as safe microorganisms, this concept is currently changing due to an increased number of human infections associated with this yeast species. In fact, *S. cerevisiae* is now considered to be an emerging opportunistic pathogen, which has been addressed on several occasions (Hazen, 1995; Herbrecht and Nivoix, 2005; Murphy and Kavanagh, 1999; Pontón et al., 2000). *S. cerevisiae* can cause clinically relevant infections in a variety of immunocompromised patients and affect different body sites (Enache-Angoulvant and Hennequin, 2005; Lolis et al., 2008; Montineri et al., 2008; Muñoz et al., 2005; Swinne et al., 2009; Williams et al., 2007); furthermore, it can sometimes even affect patients showing no obvious predisposition (Jensen et al., 1976; Smith et al., 2002; Sobel et al., 1993).

Since the concern shown in *S. cerevisiae* as an opportunistic pathogen is very recent, and considering its long beneficial association with food and beverage production, few studies have tackled the virulence potential of this yeast species. Some studies have assessed virulent traits *in vitro* (de Llanos et al., 2006b; Klingberg et al., 2008; McCusker et al., 1994; Yáñez et al., 2009) while others have used murine models to study virulence *in vivo* (Byron et al., 1995; Clemons et al., 1994; McCullough et al., 1998; Yáñez et al., 2009). These reports provide evidence of the potential of some *S. cerevisiae* strains to cause disease regardless of host immune status. Most of these strains were isolated from clinical samples but, interestingly, some non-clinical isolates also displayed this ability.

Such results are worrying because humans can ingest a large number of viable cells of commercial and food-related *S. cerevisiae* strains daily. Among the main sources of living cells are the dietary supplements and the bio-therapeutic agent marketed as Ultralevure (*S. cerevisiae* var. *boulardii*). Both types of preparations are consumed during long periods of time and in high doses; an average of 2.1×10^7 and 8×10^9 yeast cells per day are recommended on the respective labelling. Viable cells are also ingested, albeit inadvertently, through

* Corresponding author. Present address: Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos (CSIC), PO Box 73, E-46100 Burjassot, Valencia, Spain. Tel.: +34 963 900 022; fax: +34 963 636 301.
E-mail address: tfer@iata.csic.es (M.T. Fernández-Espinar).

0168-1605/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2010.10.025

food and beverage products with significant populations of viable yeast cells [a compilation of yeast species found in different types of foods can be found in Deak and Beuchat (1996), Romano et al. (2006) and Tudor and Board (1993)]. In the case of some cheese, where *S. cerevisiae* and other yeast species play an essential role in the ripening process, the number of yeast cells can be as high as 10^9 CFU/g (Fröhlich-Wyder, 2003; Jacques and Casaregola, 2008). Another important source of living cells is fermented products (wine, beer and baked foods) where *S. cerevisiae* is naturally present or is added as a starter culture to trigger fermentation. Among these products beer of the Weissbier type, very popular in Central Europe, contains a large number of living *S. cerevisiae* cells since it is not filtered before bottling. Given the elaboration process of wine, beer and bakery products, living yeast cells are absent in the final product; however, contamination of products by commercial strains of *S. cerevisiae* due to process malfunctioning or after manufacturing cannot be ruled out. If that happens, yeasts can grow uncontrollably during the storage period thereby constituting a source of living cells at the time of consumption (Deak and Beuchat, 1996; Loureiro and Malfeito-Ferreira, 2003; Loureiro and Querol, 1999). The same occurs in all those foods susceptible to alteration by *S. cerevisiae* such as fresh fruits, fruit juices, soft drinks, high-sugar products, fermented vegetables, acid-preserved foods, bakery products and dairy products (Deak and Beuchat, 1996).

Furthermore, yeast handlers are at risk since an oral entry can arise from a hand-to-mouth contact after hand contamination. These include winemakers, bakers, yeast producers, pharmaceutical industry workers, researchers, the hospital staff responsible for handling packets or capsules *S. cerevisiae* var. *boulardii* (Ultralevure) and consumers at home.

It is noteworthy that among the strains ingested via the aforementioned means there are some with ability to pass through the intestinal barrier, reach the blood stream and develop systemic infection. There is clinical evidence of this mechanism, a case of fever following the prolonged ingestion of a dietary supplement containing between 10^7 and 10^8 of *S. cerevisiae* per gram (Jensen et al., 1976) and those cases of fungemia thought to be a consequence of the digestive translocation of *S. cerevisiae* var. *boulardii* (Lherm et al., 2002; Enache-Angoulvant and Hennequin, 2005; Muñoz et al., 2005).

S. cerevisiae can also enter the blood stream without the need to cross the intestinal wall. In the case of *S. cerevisiae* var. *boulardii*, in most cases the origin of fungemia has been associated with a contamination of the central venous line by the colonized hands of health-care workers (Enache-Angoulvant and Hennequin, 2005; Muñoz et al., 2005). Other authors have addressed the possibility that other strains, introduced in the hospital environment through foods with substantial yeast loads, could reach the catheters (Clemons et al., 2010; Fleet and Balia, 2006).

Based on these considerations, it is essential to study the negative impact of commercial and food-related *S. cerevisiae* strains on public health. There is increasing concern in this respect as reflected in a recent review about this topic (Fleet and Balia, 2006). The present work is an effort to delve into the safety risk posed by two specific strains, the baker's strain "Cinta Roja" and the bio-therapeutic agent Ultralevure, because (1) we have previous data based on genetic typing (de Llanos et al., 2004, 2006a) that indicate the possible exogenous origin of human colonization by these strains, (2) we showed that they possess varying degrees of pathogenicity-associated phenotypical traits (de Llanos et al., 2006b) and (3) they are widely handled.

Their potential ability to develop systemic infection has been evaluated and compared with other commercial strains displaying less virulent traits and some clinical isolates. An *in vivo* system of intravenous inoculation has been used as a screening method before broaching the complex system of gastrointestinal translocation. BALB/c and DBA/2N mice were chosen for the study following the recommendation of using multiple models before assigning a degree of virulence to a strain (Byron et al., 1995). In addition, the relationship between drug-induced immunosuppression and strain virulence was explored.

2. Materials and methods

2.1. S. cerevisiae isolates

Nine isolates of *S. cerevisiae* were used for this study, in accordance with previous *in vitro* studies of virulence (de Llanos et al., 2006b). Of these, four were commercial strains displaying different levels of positivity for several phenotypical traits associated to pathogenicity and four were clinical isolates of different origin. The natural wine strain CECT 10431 was used as negative control because it displays negative virulent traits (de Llanos et al., 2006b) and is unable to proliferate in any organ after its intravenous inoculation in murine systems (Yáñez et al., 2009).

The sources and characterization of these isolates, together with their virulent traits, are summarized in Table 1.

2.2. Mice

BALB/c (inbred, immune competent) and DBA/2N (inbred, C5-deficient) mouse strains were used for the study of comparative virulence. Cyclophosphamide (CY) treated ICR/Swiss (outbred, immunocompetent) mice were used for the murine model of immunosuppression. Non-treated ICR/Swiss mouse strains were included as a control of immunosuppression. The three murine models used four or five-week-old female animals (Harlan Ibérica, Barcelona, Spain). In the case of BALB/c mice, an additional study was done with animals aged between 6 and 7 weeks. After arrival, animals were left for between 4 and 5 days to acclimatize and they were allowed access to food and water *ad libitum*. CY-treated ICR/Swiss mice were housed in micro-isolator boxes provided with sterilized corncob bedding and water.

Animal studies were carried out at the Animal Facilities of the School of Medicine, Universidad Complutense de Madrid and at the School of Pharmacy, University of Valencia, Spain. All the assays involving mice were approved by the Institutional Animal Care and Use Committee.

2.3. Neutropenia

Neutropenia was induced in ICR/ Swiss mice by intraperitoneal (i.p.) injection of 200 mg/kg of cyclophosphamide (CY) (Sigma-Aldrich) one day before inoculation with each *S. cerevisiae* strain (day −1 with respect to inoculation). Five days later (day +4), all animals received a second dose of CY (200 mg/kg) to maintain neutropenia until the end of the assay. Similar treatments using lower doses have shown severe neutropenia in ICR mice can be achieved and maintained (Zuluaga et al., 2006).

2.4. Experimental infection

S. cerevisiae strains were grown at 30 °C on GPYA agar plates (0.5% w/v yeast extract (Pronadisa), 0.5% w/v peptone (Oxoid LTD, Basingstoke, England), 4% w/v glucose (Panreac, Barcelona, Spain), and 2% w/v agar (Panreac, Barcelona, Spain). After 24 h, cells were harvested, washed twice with sterile phosphate-buffered saline (PBS) and diluted to the desired density in the same buffer for injection. Mice were infected by intravenous (i.v.) inoculation into the tail vein with 2×10^7 viable CFU of yeast in a volume of 0.2 ml of PBS, as previously described (Clemons et al., 1994) and were sacrificed by cervical dislocation to determine burdens in the brain and kidneys at different time points post-infection.

Ninety BALB/c and DBA/2N mice, aged between 4 and 5 weeks, were used respectively (10 mice per *S. cerevisiae* strain). On day +7,