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Virulence related traits in yeast species associated with food; *Debaryomyces hansenii*, *Kluyveromyces marxianus*, and *Wickerhamomyces anomalus*

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ABSTRACT

There is an increase in the number of human infections associated with yeast species that are also known to be involved in processing foods such as *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Wickerhamomyces anomalus*, or *Kluyveromyces marxianus*. Clinical strains of *S. cerevisiae* have been well studied over the years and much is known regarding their virulence. However, very little is known about the virulent potential of strains of *D. hansenii*, *W. anomalus*, or *K. marxianus*. In this study, several factors related to virulence in a collection of clinical and food/environmental isolated strains of each species were determined. Physiological factors were looked at as well as parameters related to infection in an epithelial cell culture model and mortality in the invertebrate model *Galleria mellonella*. The results revealed that all of the evaluated *K. marxianus* strains (except for one strain) are positive in most physiological virulent factors, and also in the epithelial cell culture parameters. *Wickerhamomyces anomalus* strains produce 10 times higher levels of mortality in *G. mellonella* besides showed intermediate levels according to virulent factors. In the case of *D. hansenii*, all the tested strains are positive, but only in a single and different virulent parameter without a common pattern. Thus, our study reveals that some strains of *K. marxianus* and *W. anomalus*, could be a potential risk for human health and that the food/environmental strains could be the origin of infections.

1. Introduction

The history of yeasts associated with human society is synonymous and synchronous with the evolution of bread, beer and wine as global food and beverage commodities, and originated some 5000 years ago in early Neolithic times (Steensels & Verstrepen, 2014). Nowadays, the impact of yeasts on food and beverage production extends beyond the original and popular notions of bread, beer, and wine fermentations by *Saccharomyces cerevisiae*. In addition to the genus *Saccharomyces*, other yeast genera such as *Candida*, *Debaryomyces*, *Hanseniaspora*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Schizosaccharomyces* also can make a positive contribution to the manufacturing of fermented foods, dairy, meat products, cereals, coffee, and sauces (Boekhout & Robert, 2003; Romano et al., 2006, pp. 13–53).

Humans are unknowingly and inadvertently ingesting large, viable populations of a diversity of yeast species without adverse impact on their health by eating foods such as cheeses, fermented and cured meats, fruits, and fruit salads and home-brewed beer or beers enriched yeast. In terms of food safety, yeasts have a remarkably good record compared to other microorganisms like viruses, bacteria, and some filamentous fungi. On the contrary, they are also capable of causing human disease under predisposing circumstances (Puig-Asensio et al., 2014). Some well-known non-food related yeasts as *Candida albicans* and *Cryptococcus neoformans* are responsible for systemic human infections (Perez-Nadales et al., 2014). Nevertheless, the increasing frequency of individuals at risks such as people with weakened health and immune systems including cancer, AIDS and hospitalized patients, and those undergoing treatment with immunosuppressive drugs, broad-spectrum

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antibiotics, and radio-chemotherapies, has led to an increase in the reporting cases of infections by food related yeast species. These species such as. *D. hansenii* (*C. famata*), *K. marxianus* (*C. kefyr*), *Pichia membranifaciens* (*C. valida*), *Pichia kudriavzevii* (*C. krusei*), *S. cerevisiae*, *W. ickerhamomyces anomalus* (*C. pelliculosa*), and *Yarrowia lipolytica* (*C. lipolytica*) have been added to the list of opportunistic pathogens (Fleet & Balia, 2006; Muñoz et al., 2005). However, some of these species e.g. *D. hansenii*, *K. marxianus*, *S. cerevisiae*, and *W. anomalus* are included on the QPS (Qualified Presumption of Safety) list of microorganisms by the European Food Safety Authority (EFSA) (Koutsoumanis et al., 2020).

Only a limited number of studies have analyzed the potential virulence of yeast species that are present in foods and beverages, and are also related to human infections (Fleet & Balia, 2006). Previous studies have shown that some clinical isolates of *S. cerevisiae* display certain phenotypic characteristics, like growth at 42°C, hydrolytic activities, pseudo-filamentation, agar invasion, and adhesion, which have been associated with virulence in pathogenic microorganisms (Clemons et al., 1994; de Llanos et al., 2006, 2011; Llopis et al., 2014; McCusker et al., 1994; Murphy & Kavanagh, 2001). Also, opportunistic pathogenic yeasts such as *C. glabrata* and *C. albicans* can pass the intestinal barrier (Li, Redding, et al., 2007; Naglik et al., 2011) or cross the blood-brain barrier (BBB) to reach the brain (Jong et al., 2003). Some of this information is also known for *S. cerevisiae* (Pérez-Torrado et al., 2012), but limited for other species associated with human infections such as *D. hansenii*, *K. marxianus*, and *W. anomalus*.

The ability of yeast to cause spoilage of many food products, resulting in major economic losses, is well-known in numerous sectors of the food and beverage industries. However, the public health implications of yeasts in foods and beverages is a topic of emerging concern, therefore the main goal of this work is to study and compare the phenotypes related to the virulence of food-related yeasts that are also associated with human infections. During this study, the characteristics associated with virulence in a collection of clinical and food strains of the species *D. hansenii*, *K. marxianus*, and *W. anomalus* were examined. Besides the pathogenicity of representative strains using an epithelial cell culture model and Galleria mellonella as an invertebrate model hosts were also evaluated.

2. Materials and methods

2.1. Strains

In this study, 94 strains of *D. hansenii*, *K. marxianus*, and *W. anomalus* were examined. The sources of these strains, as well as the collections where they were obtained, are listed in Table 1. 31 *D. hansenii* strains were analyzed, 14 were isolated from natural habitats and foods, and 17 from patients in different hospitals. From the 37 *K. marxianus* strains, 19 were obtained from hospitals, and the remaining strains were isolated from natural habitats and foods. Finally, three strains among the 24 *W. anomalus* strains analyzed were isolated from patients. Two controls have been used, *C. albicans* SC5314 (Yagishita et al., 1981), the most common yeast pathogen, and *S. cerevisiae* D14 (Llopis et al., 2014), strain that has been described as a virulent one.

2.2. Growth at different temperatures

For viability measurements, 10 μl of appropriate dilutions (5 dilutions 1:5 from an initial OD = 0.3 culture) of the strains were plated, in triplicate, on Yeast Extract–Peptone–Dextrose (YPD) agar plates (de Llanos et al., 2006). Plates were incubated at 28, 37, 39, and 42 °C until colonies appeared for up to 3 days.

2.3. Phospholipase production

Phospholipase production was determined according to de Llanos et al. (2006) with Egg-Yolk medium used to detect lipolytic activity. This medium consists of Sabouraud Dextrose Agar (Difco, Detroit, Mich.), 11.7 g NaCl, 0.111 g CaCl2, and 10% (v/v) sterile egg yolk in 184 ml distilled water. The plates were inoculated with 10 μl of cells suspended in sterile physiological saline solution (OD 0.3) and incubated at 30 °C for 7–10 days.

The activity was visualized as an area of precipitation around each colony. Enzymatic activity was measured in terms of Pz (the ratio between the colony and precipitation area). Phospholipase production values were classified as high ($Pz \leq 0.34$), moderate ($0.55 \geq Pz \geq 0.35$), low ($0.9 \geq Pz \geq 0.56$), or negative ($Pz = 1$) (Price et al., 1982).

2.4. Pseudohyphal growth

Synthetic low ammonia dextrose media (SLAD) was used to assay pseudohyphal growth (de Llanos et al., 2006). Strains were streaked on SLAD plates and colonies features were observed and photographed after 4 days of growth at 30 °C.

2.5. Invasive growth

Strains were patched onto YPD plates to avoid scratching the agar, incubated at 30 °C for 3 days, and then left at room temperature for 2 days (de Llanos et al., 2006). A gentle stream of deionised water was then used to rinse the cells from the agar surface after which the remaining cells were observed and photographed.

2.6. Adhesion assay and transepithelial electrical resistance assay

The human colon adenocarcinoma cell line Caco-2 (DSMZ, Braunschweig, Germany) was grown and maintained as in Perez-Torrado et al. (2012).

The adhesion assays were set up in 24-well polystyrene plates (PRIMOEuroclone), pre-treated with a 0.5 mg/mL collagen (Sigma) solution dissolved in 1 mM acetic acid. Treated plates were used to establish a confluent monolayer of Caco-2 cells by adding 1×10^5 cells·cm^{-2} and grow them to 95% confluency. To obtain polarized monolayers, Caco-2 cells were seeded on Millicell Hanging Cell Culture Insert (PET 8 μm pore size, 24-well, Micell) placed in 24-well plates (PRIMOEuroclone) at a density of 1×10^5 cells cm^{-2}. Experiment and transepithelial electrical resistance (TEER) measurements were done as in Perez-Torrado et al. (2012). The TEER was measured before the addition of yeast cells ($t = 0$) and then at 24 and 48 h after the inoculation of yeast cells. The TEER of monolayers with Caco-2 cell culture media without added yeast cells was used as the control for each experiment. Each assay was conducted with triplicate determinations.

2.7. Ability to cross the epithelial barrier and assessment of cytotoxicity

The ability to cross the epithelial barrier and the assessment of cytotoxicity were done according to Perez-Torrado et al. (2012). The ability of yeast cells to injure epithelial cells was assessed by measuring the release of lactate dehydrogenase (LDH) from dying cells after 48 h of exposure to the yeast. At this point, the cell culture medium of the inner chamber (apical compartment) was homogenized, transferred to 1.5 ml microcentrifuge tubes, and centrifuged at 12000 g for 2 min to remove any cell debris. 100 μl of each sample was added to the working solution (LDH-Cytox Assay Kit, BioLegend), and protected to the light. Absorbance at 490 nm was measured at 10 min. Spontaneous or 1% triton-X-100 release of LDH by uninfected cultures incubated under similar conditions was included as a negative or positive control in each experiment respectively. In both the negative and positive control the media of the outer chamber (basolateral compartment) were not removed at 24 h, and for the positive control (Triton treated cells), as Triton destroyed de monolayer, 200 μL and 1250 μL were collected for LDH assay; data were corrected according to this volume increase.

Table 1
List of *Debaryomyces hansenii*, *Kluyveromyces marxianus*, and *Wickerhamomyces anomalus* strains used in this study.

Species	Group	IdAQ	Other name	Isolation source
Debaryomyces hansenii	**FOOD\ ENVIRONMENTAL**	390	CECT 11363	Refrigerated meat
		391	CECT 11364^T	Pig sausage (France)
		399	CECT 10360	Cheese (Spain)
		978	CECT 10352	Tomato (Spain)
		3630	CECT 1071	Sausages (Germany)
		3633	CECT 10019	Frass on Philadelphus coronaries (Spain)
		3634	CECT 10126	Frass on Pinus silvestris (Spain)
		3635	CECT 10202	Fruit of Anona muricata (Spain)
		3636	CECT 10284	Frass on Cornus sanguinea (Spain)
		3639	CECT 10330	Bet-rot soil (Spain)
		3643	CECT 10380	Fig (Spain)
		3644	CECT 10386	Prune (Spain)
		3645	CECT 10414	Alpechin (Spain)
		3648	CECT 12753	Sausage
	CLINICAL	980	CECT 11370	Mycotic interdigital injury (Germany)
		2907	NCYC 8	Throat with angina
		2908	NCYC 576	Human isolation
		2909	NCYC 611	Mycosis hand injury
		2920	CBS 161	Man. Skin scales; psoriasis (Italy)
		2923	CBS 770	Man. Pus throat plates; (France)
		2924	CBS 771	Man. Periostitis (France)
		2925	CBS 788	Man. Injury (Denmark)
		2926	CBS 790	Man. Infected nail
		2927	CBS 1124	Corpse nail (Netherlands)
		2928	CBS 1125	Persistent furunculosis case (United Kingdom)
		2938	CBS 1795	Hand injury
		2939	CBS 1801	Paronychia (ingrown) (Uruguay)
		2947	CBS 5139	Man. Skin (Hungary)
		2948	CBS 5140	Man. Skin (Hungary)
		2949	CBS 5704	Infected hand (Hungary)
		2950	CBS 2653	Pig. Skin injury (United Kingdom)
Species	Group	IdAQ	Other name	Isolation source
Kluyveromyces marxianus	FOOD\ ENVIRONMENTAL	1411		Cheese, Baltasar Mayo
		1482	CECT 10357	Cheese (Spain)
		1483	CECT 10368	Yogurt (Spain)
		1486	CECT 11389	Winepress (winery) (South Africa)
		1951		Agave (Mexico)
		1968		Agave (Mexico)
		1984		Agave (Mexico)
		1480	CECT 1446	Rotting leaf of Agave arigida (South Africa)
		1485	CECT 10668	Cow back (Netherlands)
		2931	CBS 1560	Date (Netherlands)
		3631	CECT 1123	Butter (Spain)
		3632	CECT 1442	Production of food yeast from wey
		3638	CECT 10315	Date (Spain)
		3640	CECT 10367	Yogurt (Spain)
		3641	CECT 10369	Yogurt (Spain)
		3642	CECT 10379	Date (Spain)
		3646	CECT 10584	Yogurt (Netherlands)
		3647	CECT 10649	Bantú beer (South Africa)
	CLINICAL	2906	NCYC 6	Sputum of a broncomonoliasis case (Sri Lanka)
		2910	NCYC 906	Sputum
		2921	CBS 600	Sputum
		2922	CBS 607	Bronchitis patient (Sri Lanka)
		2929	CBS 1553	Lung, tuberculosis patient (Italy)
		2930	CBS 1559	Man. Amygdala injury (Italy)
		2932	CBS 1561	Human faeces
		2934	CBS 1620	Man. Broncomycosis (1910)
		2940	CBS 2173	Mastitis cow milk (United Kingdom)
		2941	CBS 2231	Lungs. Fatal tuberculosis case (Italy)
		2942	CBS 2232	Infected nail of a 2 years old girl (Austria)
		2943	CBS 3002	Sputum, asthmatic patient (Norway)
		2944	CBS 3021	Baby lung with pneumonia (Austria)
		2945	CBS 3073	Sputum (Netherlands)
		2946	CBS 3074	Man chest (Netherlands)
		3101		La Fé hospital (Spain)
		4024	CL 10118	Carlos III Health institute (Spain)
		4023	CL 7365	Carlos III Health institute (Spain)
		4022	CL 6858	Carlos III Health institute (Spain)
Species	Group	IdAQ	Other name	Isolation source
Wickerhamomyces anomalus	FOOD\ ENVIRONMENTAL	182	CECT 10320	White vine (Spain)
		201	CECT 10590	Grape juice (Spain)
		695	CECT 10014	Ffrass on Arbutus unedo (Spain)

(*continued on next page*)

Table 1 (*continued*)

	696	CECT 10021	Almond land (Spain)
	698	CECT 10083	Frass on Laurus nobilis (Spain)
	699	CECT 10111	Frass on Pinus sylvestris (Spain)
	701	CECT 10314	Lemon (Spain)
	702	CECT 10354	Lycopersicon esculentum (tomato) (Spain)
	704	CECT 10489	Alpechín (Spain)
	706	CECT 10572	White vine (Spain)
	707	CECT 10593	Grape juice (Spain)
	2180		Forest land, 30 °C (Hungary)
	2421		Pit water (Peru)
	2423		Wood that supports the house (Peru)
	2436		Crude dehuled Yucca (Peru)
	2440		Broken Camote plus sugar plus water (Peru)
	2442		Boiled Yucca after 45 min (Peru)
	2444		Boiled Yucca rested during 2 h (Peru)
	2448		Total yucca mix (chewed) (Peru)
	2449		Fermented yucca after 15 h (Peru)
	2459		Common wasp (Peru)
CLINICAL	711	CECT 11058	Lung pus of a death tuberculosis patient (Italy)
	2936	CBS 1686	Infected skin
	2937	CBS 1775	Sputum (Norway)

AQ, Amparo Querol (IATA-CSIC) collection; CECT, Spanish Type Culture Collection; CBS, Dutch Centraalbureau voor Schimmelcultures Collection; NCYC, National Collection of Yeast Cultures.

Average ± sd of triplicate experiments is represented as a released percentage of the total (triton-X-100) LDH activity.

2.8. Evaluation of the pathogenicity using Galleria mellonella

Six instar larvae of the greater wax-moth *Galleria mellonella* (UK Waxworms Ltd., Sheffield, England) were stored in the dark at 20 °C to prevent pupation. Larvae weighing 0.22 ± 0.03 g were selected and used within 5 days of receipt. Twenty healthy larvae were randomly chosen for each assay and placed in sterile 9 cm Petri dishes lined with Whatman filter paper and containing some wood shavings. To prepare the inoculum to infect *Galleria*, a single colony from each yeast strain was inoculated into 1 L flasks containing 200 ml of SDA broth (Oxoid Ltd., Basingstoke, England) and grown overnight at 30 °C, under shaking at 180 rpm. Yeast cells were harvested by centrifugation at 4000 rpm, washed twice in PBS, and enumerated with a haemocytometer to determine the cell density of each culture.

Wax moth larvae killing assays were carried out as described previously (Cotter et al., 2000). Briefly, groups of 20 larvae were each inoculated with 20 μL of either a 5×10^9, 5×10^8, or 5×10^7 yeast suspension (final inoculum concentration of 10^8, 10^7, and 10^6 yeast cells/larval) in PBS buffer. As a control, 20 larvae were injected with PBS alone. Each larva was inoculated through the last left pro-leg into the haemocoel with a Myjector U-100 insulin syringe (Terumo Europe N·V., Belgium). Larvae were acclimatized at 30 °C for 1 h before undertaking all experiments and incubated at 30 °C in the dark post challenge. Survival was recorded at 24, 48, and 72 h. Larvae survival was confirmed visually and by physical stimulation with a sterile pipette tip.

Fungal burden (CFU/ml) in larvae was determined at 24, 48, and 72 h post challenge after inoculation with a final dose of 1×10^8 yeast cells/larvae for *K. marxianus* and 1×10^7 yeast cells/larvae for *W. anomalus*. Three inoculated larvae from each treatment group were homogenized in 5 mL of sterile PBS using a stomacher digester (60 s on max) (Micro-BIOMASTER; Seward Stomacher 80, Lab Systems). Ten-fold dilutions of the resulting homogenate were made in PBS and 100 μL from each was plated onto SDA plates containing chloramphenicol (50 mg/ml) (Sigma Aldrich) to inhibit bacterial growth. Plates were incubated at 30 °C for 48 h and the CFU/ml determined for each strain. Results were expressed as the average and standard error.

2.9. Statistical procedure

All experiments were performed at least in triplicate. Statistical analyses were done using STATISTICA 7 (StatSoft, Tulsa, OK, USA). To determine statistically significant data comparing phenotypes of the different species and comparing clinical and environmental/food strains in cell culture experiments, one-way ANOVA (α 0.05) were performed. X^2-tests were performed to confirm significant differences in the other virulence factors between clinical and environmental/food strains. A p-value < 0.05 was considered to be significant.

Experiments with *G. mellonella* were performed on three independent occasions and results are expressed as the mean and the SE. Analysis of changes in haemocyte density and yeast density were performed by one-way analysis of variance (ANOVA) followed, where appropriate, by Tukey's multiple comparison test. If heterogeneity of variances existed, robust tests were applied. Welch's test was used to check differences between treatments and Games-Howell's test to establish differences among groups. Differences were considered significant at $p < 0.05$. All statistical analyses listed were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Differences in virulence factors in clinical and no clinical strains

Certain phenotypic characteristics have been associated with virulence in pathogenic microorganisms. These phenotypic traits include growth at 37 °C, 39 °C, and 42 °C associated with the ability to survive febrile states. Pseudohyphal growth was associated with tissue penetration capacity and adherence capacity with the ability to invade host cells. The hydrolytic-enzyme secretion capacity was an important property of pathogenic fungi and was related to the cell damage. These properties were studied within a collection of clinical and food yeasts of the species *D. hansenii*, *K. marxianus*, and *W. anomalus*. The results for each species are summarized in Fig. 1 and Tables 1S, 2S, and 3S. A statistical analysis has been done to compare clinical and non-clinical strains (Table 4S), although this analysis has certain limitations due to the limited number of clinical strains in some species.

Only four strains of *D. hansenii* grew at 37 °C and no strains grew at 39 °C and 42 °C (Fig. 1 and Table 1S). In the cases of phospholipase activity, 57% of non-clinical and 35% of clinical strains were able to produce low levels of this enzyme (Table 1S) and no significant differences were found between clinical and non-clinical strains (Table 4S). Fig. 2A shows differences in the pseudohyphal formation length among *D. hansenii* strains, however, no significant differences between clinical and non-clinical strains were found (Table 4S) and pseudohyphal

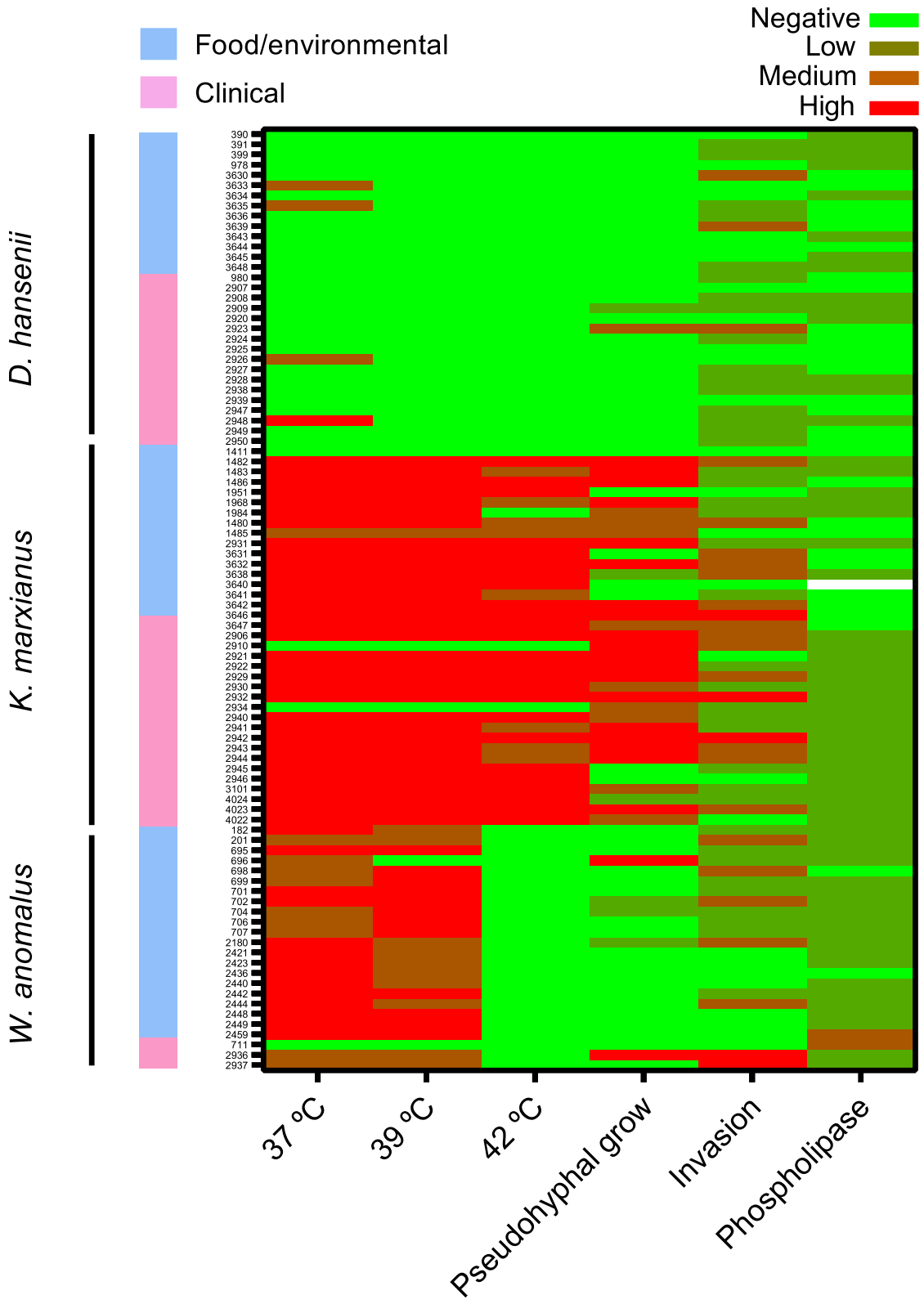


Fig. 1. A heatmap representing virulent phenotypes levels obtained with all the 94 strains from *D. hansenii*, *K. marxianus*, and *W. anomalus* species. Each phenotype was evaluated visually and a negative, low, medium, or high level was assigned. The origin of the strains, clinical or food/environmental, are also represented. Absent data are represented as a white cell.

growth was only observed in two clinical strains. According to the results, no significant differences were found between food and clinical isolates (Table 4S), and in a few strains were identified a few characteristics associates with virulence.

The results obtained with the 37 strains of *K. marxianus* are presented in Fig. 1 and Table 2S. The majority of strains can grow at high temperatures, even at 42 °C except two clinical and two non-clinical strains. Interestingly the clinical strains of *K. marxianus* showed significantly

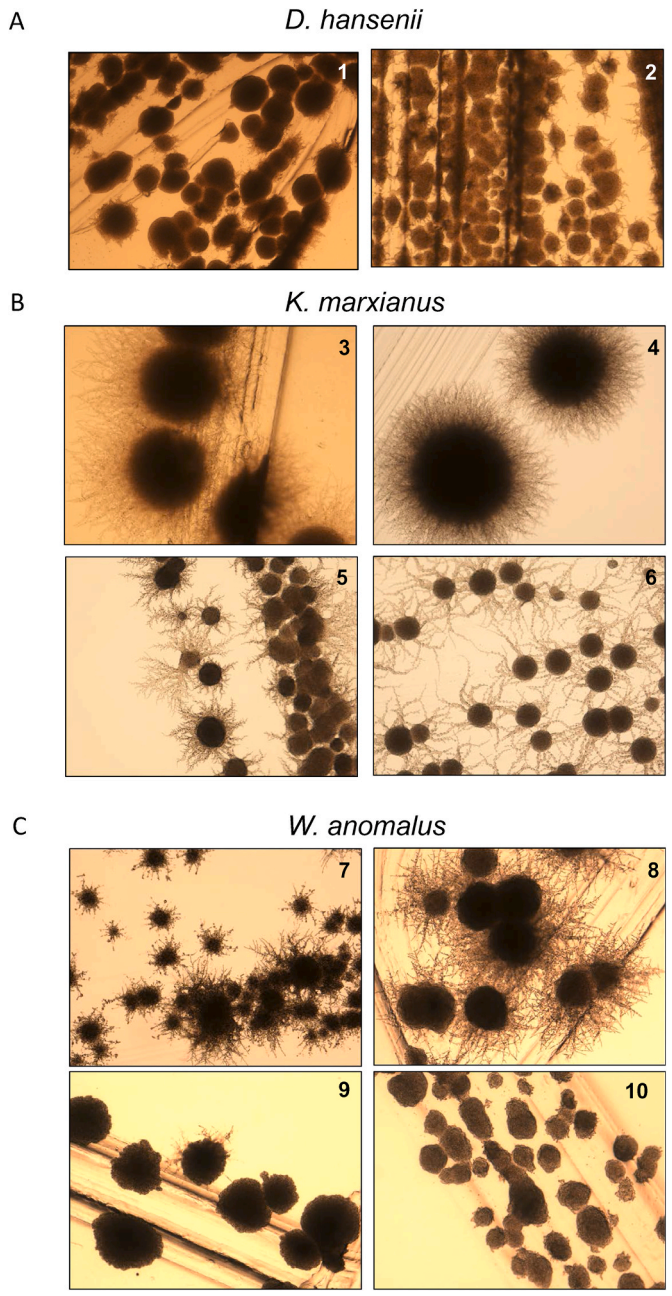


Fig. 2. Examples of pseudohyphal growth in the species. A) *D. hansenii*: 1, Ph± (AQ2909); 2, Ph+(AQ2923). B) *K. marxianus*: 3, Ph++ (AQ1482); 4, Ph++ (AQ1486); 5, Ph++ (AQ2906), 6, Ph++ (AQ2943). C) *W. anomalus*: 7 Ph++ (AQ696); 8 Ph++ (AQ2936); 9 Ph± (AQ702); 10 Ph± (AQ704). Ph++ All pseudohyphae with long chains; Ph + All pseudohyphae with short chains; Ph ± Mixture of colonies able and unable to pseudohyphae. Ph– Absence of pseudohyphal growing.

higher phospholipase activity compared to the non-clinical strains (Table 4S). 89% of the clinical strains were able to exhibit pseudohyphal growth (Fig. 2B) and no significant differences were found between the clinical and non-clinical groups regarding the capability of agar invasion (Table 4S). It is interesting to highlight that all the *K. marxianus* strains showed two or more factors associated with virulence. The majority of the strains could grow at 42 °C, showed high pseudohyphal growth, and showed invasive growth. The combined data suggest that strains of this species could be virulent independently of the isolation source.

The results obtained for the phenotypic characterisation of *W. anomalus* strains are shown in Fig. 1 and Table 3S. No strain could grow at 42 °C but the majority of strains grew at 39 °C. *W. anomalus* strains were able to produce phospholipase, mainly with low activity and no significant differences were found between clinical and no clinical strains (Table 4S). Only one clinical strain and four non-clinical strains were able to produce pseudohyphae (Fig. 2C) with no significant differences among the two groups (Table 4S). Regarding the invasion data, two clinical strains, AQ2936 and AQ2937 were unable to be washed and the non-clinical strains, when invaded, only remained poorly in the agar. In this case, significant differences were found between both groups (Table 4S), being the clinical strains significantly more invasive than the other strains, although only 3 clinical strains were tested.

3.2. Differences in virulence aspects related to epithelial cell culture model

As epithelia are the first barrier against infection, it is important to study the relationship between the probable pathogens and the epithelia. Adhesion to epithelial cells is the first step in the pathogenesis of several species. The ability of these strains to cross the human epithelial barrier and the ability to damage human epithelia are other important characteristics of infection. Therefore the adherence level to human cells was tested as well as the transcytosis ability, the epithelial integrity by measuring the *trans*-epithelial electrical resistance or TEER and the cellular damage by measuring the LDH levels, a compound released by dead cells, using a Caco-2 monolayer. Due to the complexity to perform these experiments with a high number of strains, we selected 14 strains. A summary of the obtained results is shown in Table 2.

Since no clinical strain of *D. hansenii* showed virulent characteristics in the previous analysis, four strains with different characteristics were selected; AQ2926 and AQ2948 that can grow at 37 °C, AQ2909 that can form pseudohyphae, and AQ2947 that can invade agar plates (Table 3). Any of these strains showed adherence to Caco-2 monolayers, presenting lower levels than 1.4% and no differences were found with *S. cerevisiae* strain D14, while *C. albicans* adhered to more than 50% (Table 2). Also, no differences with D14 were found either in cytotoxicity, translocation, or in TERR values.

We selected two clinical (AQ2934 and AQ2942) and two non-clinical (AQ1444 and AQ1482) strains of *K. marxianus* with different virulent characteristics (Table 3). The clinical strain AQ2942 showed significantly higher levels in adherence compared to the control *S. cerevisiae* strain D14 (5.3% vs 0.51%). and also presented higher washing resistance than the other strains. Analyses of transcytosis ability and the monolayer integrity showed that most *K. marxianus* strains show very low values, comparable to *S. cerevisiae*, althought the AQ1482 strain presented higher values but not significatively different from the others due to the elevated variability of the transcitosis assays. Also, *K. marxianus* strains neither affect monolayer integrity during the 48 h of the assay. The monolayers in contact with strains AQ1482, AQ2934, and AQ2942 released higher levels of LDH activity (27.8–29.1%) (Table 2), producing significantly higher cytotoxicity levels than the strain D14 (13.2%) which suggests that cytotoxicity can be related to the pathogenicity of this species, although with statistically lower levels than pathogenic *C. albicans* SC5314 (91.85%). However, no clear differences were found between clinical and no-clinical strains.

Six strains of *W. anomalus*, with different virulence phenotypes, were selected, two clinical (AQ936 and AQ2937), and four non-clinical clinical strains (AQ2421, AQ2436, AQ2444, and AQ2459 (Table 3). The results showed that two groups can be observed among these strains (Table 2). In the first group, strains AQ2436, AQ2444, and AQ2936 showed adhesion levels between 0.7 and 2.7%. The second group includes strains AQ2421, AQ2459, and AQ2937 presented significantly higher adherence levels (12–17.5%). As observed for *K. marxianus*, *W. anomalus* strains mainly show very low values, comparable to *S. cerevisiae*, although the strains AQ2421 and AQ2936 at the 24 h time point and AQ2436 at 48 h time point presented higher values but not significatively different from the others due to the elevated variability of

Table 2
Cell culture assays with food/environmental and clinical strains.

Species	Strain	% adhered cells	Translocated cells		TERR		Cytotoxicity % LDH activity
			24 h	48 h	24 h	48 h	
C. albicans	**SC5314**	50.12 ± 5.41[a]	3.88E4±7.23E3[a]	5.58E7±8.66E6[a]	0.15 ± 0.04[a]	0.04 ± 0.01[a]	91.85 ± 20.5[a]
S. cerevisiae	**D14**	0.51 ± 0.01[b]	0.00 ± 0.00[b]	97.33 ± 78.01[b]	0.87 ± 0.03[b]	0.71 ± 0.02[b]	13.27 ± 1.10[b]
D. hansenii	**AQ2909**	0.34 ± 0.23[b]	1.33 ± 2.31[b]	42.67 ± 26.63[b]	0.83 ± 0.08[b]	0.91 ± 0.14[b,c]	8.70 ± 4.22[b,c]
	AQ2926	0.71 ± 0.84[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.96 ± 0.05[b]	0.91 ± 0.05[b,c]	1.15 ± 1.46[c]
	AQ2947	1.34 ± 0.41[b]	2.67 ± 2.31[b]	6.00 ± 8.48[b]	0.99 ± 0.19[b]	1.16 ± 0.13[c]	1.90 ± 2.85[b,c]
	AQ2948	0.29 ± 0.42[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.79 ± 0.03[b]	0.78 ± 0.03[b,c]	7.14 ± 7.63[b,c]
K. marxianus	**AQ1411**	3.27 ± 0.31[b,c]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	1.01 ± 0.05[b]	1.00 ± 0.14[b]	6.16 ± 2.16[d]
	AQ1482	3.53 ± 0.20[b,c]	7.81E3±1.34E4[b]	1.44E2±1.90E2[b]	0.99 ± 0.2[b]	0.91 ± 0.16[b]	28.28 ± 2.55[b]
	AQ2934	2.33 ± 0.49[c]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	1.00 ± 0.01[b]	0.99 ± 0.08[b]	27.85 ± 0.77[b]
	AQ2942	5.30 ± 0.42[b]	2.67 ± 4.62[b]	0.00 ± 0.00[b]	0.94 ± 0.23[b]	0.96 ± 0.28[b]	27.85 ± 0.77[b]
W. anomalus	**AQ2421**	12.29 ± 1.79[b]	1.52E3±2.43E3[b]	1.64E4±2.83E4[b]	0.92 ± 0.27[b]	0.84 ± 0.15[b]	26.04 ± 0.92[b]
	AQ2436	0.73 ± 0.24[c]	280.00 ± 367.00[b]	1.48E3±1.28E3[b]	0.97 ± 0.09[b]	0.88 ± 0.10[b]	4.12 ± 3.49[d]
	AQ2444	1.47 ± 0.63[c]	73.33 ± 91.59[b]	157.33 ± 206.78[b]	1.21 ± 0.05[b]	1.11 ± 0.06[b]	3.01 ± 2.93[d]
	AQ2459	17.49 ± 1.57[b]	0.00 ± 0.00[b]	385.33 ± 455.48[b]	1.11 ± 0.02[b]	0.98 ± 0.02[b]	25.90 ± 0.76[b]
	AQ2936	2.78 ± 0.74[c]	6.97E3±1.15E4[b]	125.00 ± 217.00[b]	0.99 ± 0.10[b]	0.88 ± 0.29[b]	27.86 ± 2.80[b]
	AQ2937	12.42 ± 4.97[b]	1.33 ± 3.27[b]	88.00 ± 7.28E3[b]	1.11 ± 0.10[b]	0.93 ± 0.27[b]	26.13 ± 0.64[b]

Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, $\alpha = 0.05$, $n = 3$).
Clinical strains names are underlined.

Table 3
Virulence factors of the selected strains for cell culture assays and in the virulence evaluation using G. mellonella.

IdAQ	Temperature of growing&				Pseudohyphal growing[Δ]	Invasion	Phospholypase
	28 °C	37 °C	39 °C	42 °C			
D. hansenii							
2909	++	-	-	-	±	±	+ (Low)
2926	++	+	-	-	-	-	- (Negative)
2947	++	-	-	-	-	±	- (Negative)
2948	++	++	-	-	-	±	+ (Low)
K. marxianus							
1411[a]	++	-	-	-	-	-	- (Negative)
1482[a]	++	++	++	++	++	+	+ (Low)
2934[a]	++	-	-	-	+	±	+ (Low)
2942[a]	++	++	++	++	++	++	+ (Low)
W. anomalus							
2421	++	++	+	-	-	-	+ (Low)
2436[a]	++	++	+	-	-	-	- (Negative)
2444[a]	++	++	+	-	-	+	+ (Low)
2459	++	++	++	-	-	-	++ (Moderate)
2936[a]	++	+	+	-	++	++	+ (Low)
2937	++	+	+	-	-	++	+ (Low)

[&] ++ Dense growth at all dilutions; + Dense growth in the direct and 1st dilution or poor growth in all dilutions; ± Poor growth in 1st dilution. – No growth.
Δ ++ All pseudohyphae with long chains; + All pseudohyphae with short chains; ± Mixture of colonies able and unable to pseudohyphae. – Absence of pseudohyphal growing.
[$] ++ The plate cannot be washed; + The plate is washed but there are many cells attached to the edges; ± The plate is washed but there are few cells attached to the edges; - The plate is completely washed.
[#] ++ All cells penetrate intensely into agar; + All cells penetrate little into agar; ± Few cells penetrate into agar; No cells penetrate into agar.
Clinical strains names are underlined.
[a] , strain used in the evaluation study using the insect larvae G. mellonella.

Table 4 S
p-value of the Pearson Chi-square analysis comparing clinical and food/environmental groups.

Virulence factors		*D. hansenii*	*K. marxianus*	*W. anomalus*
Temperature of growing	37°C	0.499	0.512	0.007
	39°C	No growth	0.512	0.091
	42°C	No growth	0.659	No growth
Pseudohyphal growing		0.415	0.605	0.216
Invasion		0.266	0.846	0.001
Phospholypase		0.224	0.0005	0.226

Underlined values, samples with significative differences (p-value < 0.05).

the transcitosis assays. Also, *W. anomalus* strains did not affect monolayer integrity during the 48 h of the assay (Table 2). Cytotoxicity results showed that strains AQ2421, AQ2459, AQ2936, and AQ2937 produced higher levels of LDH activity (26–28%), a factor associated with virulence, although significantly lower than *C. albicans* SC5314 (91.85%). As in the other species, although there are statistical differences between individual strains, no clear differences were found between the group, clinical and non-clinical strains.

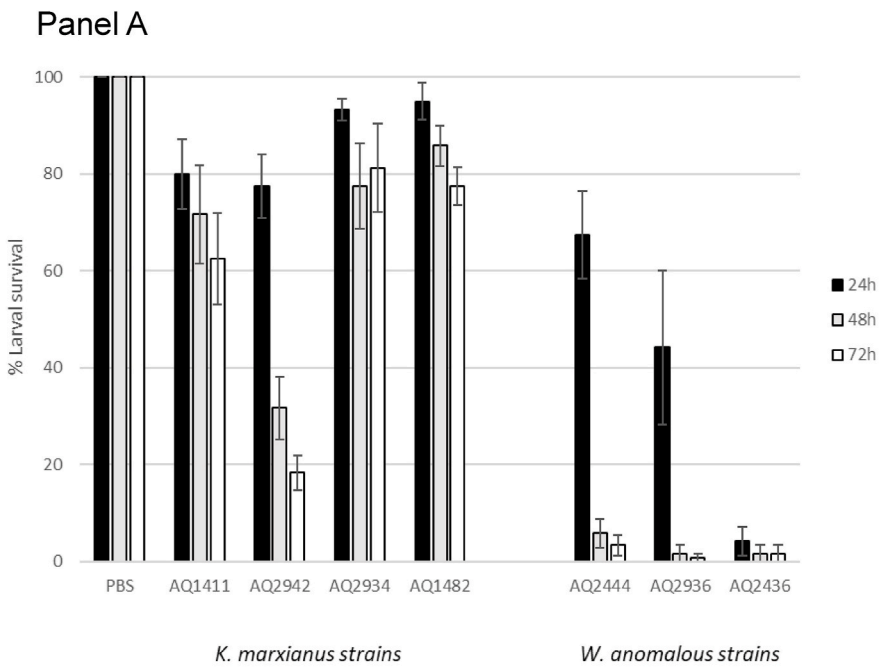
3.3. Virulence evaluation of *K. marxianus* and *W. anomalus* strains using invertebrate model *G. mellonella*

As *D. hansenii* didn't show clear virulence characteristics in the previous sections, we decided not to include in *G. mellonella* experiments. For *K. marxianus* and *W. anomalus*, the tested strains are listed in

Table 3.

To evaluate the virulence of these strains in the insect larvae *G. mellonella*, the survival rate in larvae challenged with an inoculum of infection of 10^8 cells/larvae were analyzed at 30 °C as the temperature of infection. A reduction in survival over the 72 h incubation period was observed for all strains. *K. marxianus* showed high survival, 80% for AQ2934 and AQ1482 and 60% for AQ1411, except for AQ2942 with a survival rate of 20% (Fig. 3, panel A). It is important to highlight that *W. anomalous* strains were able to significantly reduce *G. mellonella* survival during the first 24 h. Specifically, AQ 2436 showed a reduction of 96% of larval survival whereas AQ 2444 and AQ2936 showed a reduction of 30% and 56% of larval survival, respectively (Fig. 3, panel A). Since the infective dose of 10^8 cells/larvae reduced significantly larval population after infection with *W. anomalus* strains, a lower inoculation dose of 10^6 and 10^7 cells/larvae were tested. The results indicated that inoculation doses of 10^6 yeast cells of *W. anomalus* strains had 100% survival at 72 h. When the dose was increased to 10^7 yeast cells/larva, the survival rate is strain dependent. A survival rate of 20%, 30%, and 50% was observed after 72 h of infection for strain AQ2936; AQ2444, and AQ2436 respectively (Fig. 4, panel B). According to this data, the recommendation is an inoculum of 10^7 cells/larvae at 72 h. For the *K. marxianus* strains it was observed that an inoculum of 10^8 cells/larva is the optimal doses for a 72 h experiment (Fig. 3, panel A).

Since the fungal burden is considered an important marker for virulence in *G. mellonella*, the proliferation of *K. marxianus* and *W. anomalus* strains with subsequent insect mortality was also studied (Fig. 4). Larvae were inoculated by intra-haemocoel injection either with 10^8 *K. marxianus* yeast cells or 10^7 *W. anomalus* yeast cells according to the previous data. The four *K. marxianus* strains were able to maintain high levels of fungal cells in the larvae during 3 days of



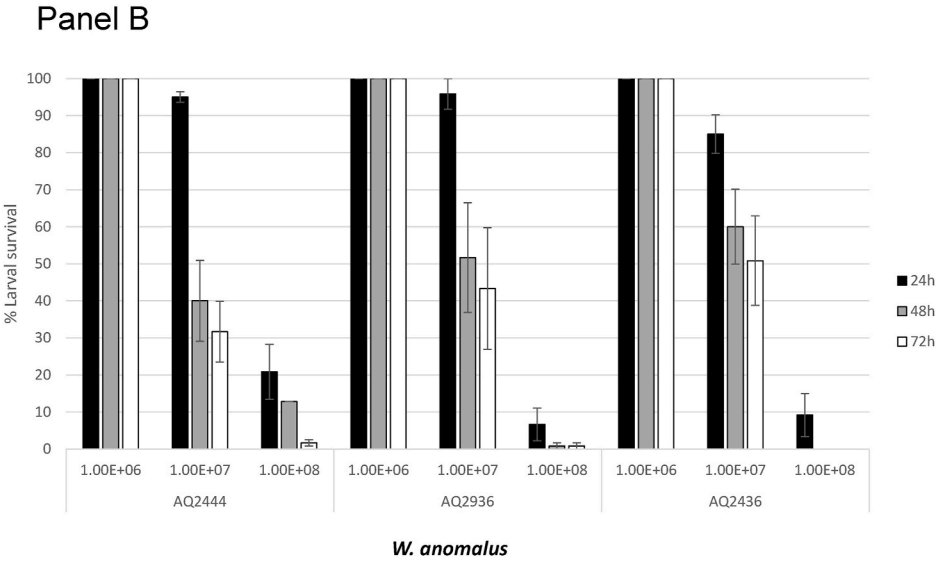


Fig. 3. Percentage of survival of *G. mellonella* larvae infected by different strains of *K. marxianus* and *W. anomalus*, larvae were inoculated with a dose of 10^8 yeast cells/larva and incubated at 30°Cf or 72 h (Panel A). Percentage of survival of larvae infected by different strains of *W. anomalus* inoculated with 10^6, $10^{7,}$ or 10^8 yeast cells/larva and incubated at 30 °C for 72 h (Panel B). All values are the mean ± SE of three independent replicates. The survival data for PBS infected larva is not included in panel B for simplification (100% survival rate was observed at all time points).

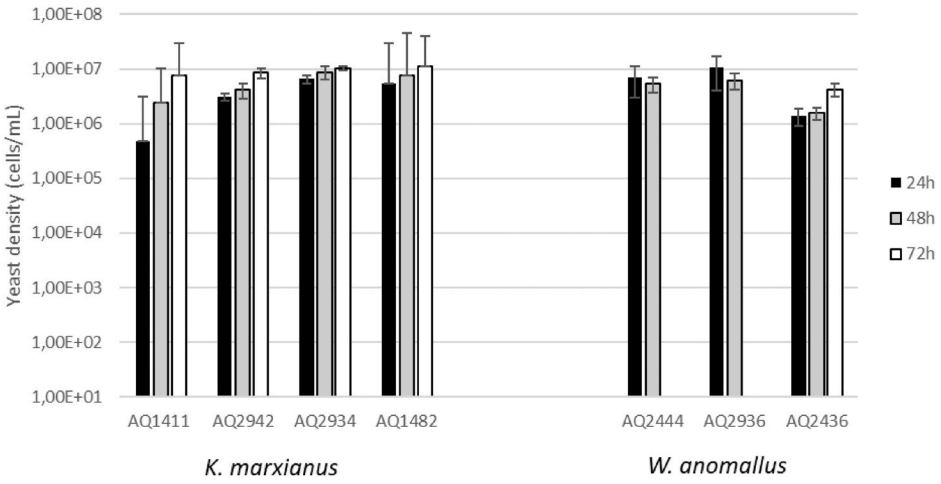


Fig. 4. Proliferation in *G. mellonella* larvae of *K. marxianus* and *W. anomalous*. Larvae were inoculated by intra-haemocoel injection with 10^8 *K. marxianus* yeast cells and 10^7 *W. anomalus* yeast cells and incubated at 30 °C for 72 h. All values are the mean ± SE of three independent replicates.

incubation, but only strain AQ1411 demonstrated some degree of proliferation in the larvae over the 72 h (Fig. 4). These results indicate a correlation between yeast proliferation in the host with the subsequent insect mortality for the *K. marxianus* strains tested. For *W. anomalous*, it is worth mentioning that despite the yeast density for the three strains was studied throughout the 72 h experiment period, data could not be determined for strains AQ2936 and AQ2444 due to the bad health status that the larvae showed. Nevertheless, data for strains AQ2436 demonstrated some degree of proliferation over the 72 h (Fig. 4).

These results indicate a correlation between yeast proliferation in the host with the subsequent insect mortality for the *K. marxianus* and *W. anomalous* strains tested.

4. Discussion

Few studies are related to the safety or virulence aspects of food yeast species except in the case of *S. cerevisiae* (for a revision see Pérez-Torrado & Querol, 2016) and very little is known about the virulent potential of other yeast frequent isolated on foods like *D. hansenii*, *W. anomalus*, or *K. marxianus*. In the present study, we observed that some *D. hansenii* strains, independently of the origin, were able to grow at 37 °C, produce pseudohyphae, and invade agar plates and show characteristics associates with virulence in pathogenic yeast species like *C. albicans* (Höfs et al., 2016; Mayer et al., 2013). *K. marxianus* strains showed even more factors associated with virulence, growth at 42 °C, pseudohyphal and invasive growth. This could suggest that the presence of a high number of viable cells of these strains in food could be a potential risk for immunosuppressed people (Fleet & Balia, 2006). A good recommendation for the food industry is to avoid the use of *D. hansenii* and *K. marxianus* strains that are positive in two or more virulence factors.

Wickerhamomyces anomalus strains present lower levels of virulence factors compared to *K. marxianus*, but a higher number of the tested strains can grow at 39 °C and present a higher capability of adhesion to the agar, thus adherence may be a mechanism of infection of these strains as has been described in *C. albicans* (Höfs et al., 2016; Mayer et al., 2013; Nobile & Johnson, 2015).

Epithelia are the first barrier against infection, and a good system to study the virulence of yeasts (Höfs et al., 2016). *D. hansenii* strains did not show a strong character associate with virulence using a Caco-2 monolayer model. However, 60% of the tested strains of *W. anomalus* produced higher cytotoxicity levels on epithelial monolayers comparing to the rest of the species tested. The *W. anomalus* data suggest that the mechanism could be less related to the damaging potential of pseudohyphal growth, as in the case of *C. glabrata* and *S. cerevisiae*, which showed very low cytotoxicity and damaging potential compared with *C. albicans* (Li, Kashleva, et al., 2007; Pérez-Torrado et al., 2012). However, many strains of *K. marxianus* showed higher levels of adhesion and cytotoxicity levels on epithelial monolayers, indicating again that some strains of this species are a potential risk for immunosuppressed patients and the mechanism of infection could be similar to *C. albicans*, that grow in pseudohyphae form, generating invasion and cytotoxicity in epithelial tissues (Naglik et al., 2011).

As has been observed, some yeasts could be risks to the population. For this purpose, we need a simple test to evaluate yeast pathogenicity before their use in foods. During this study, it was demonstrated that *G. mellonella* can be used to evaluate the pathogenicity of *K. marxianus*. The virulence of *K. marxianus* in *G. mellonella* correlates well with the physiological and epithelial cell model factors studied, which suggests a potential risk of these strains in the food industry. This risk is also supported by Koutsoumanis et al. (2020) data, where *K. marxianus* strains were isolated from humans suffering from fungal infections. Interestingly, in the present work, *W. anomalus* demonstrated to be the most lethal species in *G. mellonella*. The mortality rate along with yeast proliferation ability should be included as makers of *W. anomalus* risk, as was also demonstrated in the case of other yeast species like *S. cerevisiae* and *C. albicans* (Bergin et al., 2003).

According to the obtained data, it was not possible to differentiate the clinical strains and food/environmental isolated. This suggests that the strains isolated from patients could have the origin in food products and be able to disseminate and produce the infections, as has been suggested for *S. cerevisiae* (de Llanos et al., 2006) and *K. marxianus* (Seth-Smith et al., 2020). Therefore, future genomic studies including more strains of *D. hansenii*, *K. marxianus*, and *W. anomalus* from non-clinical and clinical environments even strains from patients with systemic infections, should be performed to unravel the origin of those strains. On the other hand, it is not clear at this point whether the ingestion of yeast fermented food products with an elevated content of living cells or the food contamination and yeast growth are the main origin of these infections. Thus, much more research on the genetic and phenotypic characteristics of these strains is needed to shed light on this subject.

CRediT authorship contribution statement

Laura Peréz-Través: obtained the virulence factors data. **Rosa de Llanos:** obtained the data using the invertebrate. **Allen Flockhart:** obtained the data using the invertebrate. **Lydia García-Domingo:** obtained the virulence factors data. **Marizeth Groenewald:** mellonella and designed this part of the experiments, mellonella, support the study with yeast strains and reviwed the different versions of the manuscript.

Roberto Pérez-Torrado: obtained the epithelial cell culture data and designed this part of the experiments. **Amparo Querol:** designed the study, wrote the first versions and the final version of the article, and obtained the financial support.

Declaration of competing interest

As the corresponding author, I warrant that: all the authors have seen and approved the present manuscript, they have contributed significantly to different parts of the work, and this manuscript has not been published elsewhere and is not being considered for publication in any other journal and not.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.107901.

References

Bergin, D., Brennan, M., & Kavanagh, K. (2003). Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes and Infection, 5*(15), 1389–1395. https://doi.org/10.1016/j.micinf.2003.09.019

Boekhout, T., & Robert, V. (2003). Yeasts in food: Benefical and detrimental aspects. In *Woodhead Publishing Limited. CRC Press*. https://doi.org/10.1533/9781845698485.451

Clemons, K. V., McCusker, J. H., Davis, R. W., & Stevens, D. A. (1994). Comparative pathogenesis of clinical and nonclinical isolates of *Saccharomyces cerevisiae*. *The Journal of Infectious Diseases, 169*(4), 859–867. https://doi.org/10.1093/infdis/169.4.859

Cotter, G., Doyle, S., & Kavanagh, K. (2000). Development of an insect model for the in vivo pathogenicity testing of yeasts. *FEMS Immunology and Medical Microbiology, 27*(2), 163–169. https://doi.org/10.1016/S0928-8244(99)00185-6

12 Fleet, G. H., & Balia, R. (2006). The public health and probiotic significance of yeasts in foods and beverages. In A. Querol, & G. H. Fleet (Eds.). *Yeasts in Food and Beverages, 1st* pp. 381–398) (Springer-Verlag).

Höfs, S., Mogavero, S., & Hube, B. (2016). Interaction of *Candida albicans* with host cells: Virulence factors, host defense, escape strategies, and the microbiota. *Journal of Microbiology, 54*(3), 149–169. https://doi.org/10.1007/s12275-016-5514-0. Microbiological Society of Korea.

Jong, A. Y., Chen, S. H. M., Stins, M. F., Kim, K. S., Tuan, T. L., & Huang, S. H. (2003). Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. *Journal of Medical Microbiology, 52*(8), 615–622. https://doi.org/10.1099/jmm.0.05060-0

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Fernández Escámez, P. S., Maradona, M. P., … Herman, L. (2020). Scientific opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). *EFSA Journal, 18*(2). https://doi.org/10.2903/j.efsa.2020.5966

Li, L., Kashleva, H., & Dongari-Bagtzoglou, A. (2007a). Cytotoxic and cytokine-inducing properties of *Candida glabrata* in single and mixed oral infection models. *Microbial Pathogenesis, 42*(4), 138–147. https://doi.org/10.1016/j.micpath.2006.12.003

Li, L., Redding, S., & Dongari-Bagtzoglou, A. (2007b). *Candida glabrata*, an emerging oral opportunistic pathogen. *Journal of Dental Research, 86*(3), 204–215. https://doi.org/10.1177/154405910708600304. J Dent Res.

de Llanos, R., Fernández-Espinar, M. T., & Querol, A. (2006). A comparison of clinical and food *Saccharomyces cerevisiae* isolates on the basis of potential virulence factors. Antonie van Leeuwenhoek. *International Journal of General and Molecular Microbiology, 90*(3), 221–231. https://doi.org/10.1007/s10482-006-9077-7

de Llanos, R., Llopis, S., Molero, G., Querol, A., Gil, C., & Fernández-Espinar, M. T. (2011). In vivo virulence of commercial *Saccharomyces cerevisiae* strains with pathogenicity-associated phenotypical traits. *International Journal of Food Microbiology, 144*(3), 393–399. https://doi.org/10.1016/j.ijfoodmicro.2010.10.025

Llopis, S., Hernández-Haro, C., Monteoliva, L., Querol, A., Molina, M., & Fernández-Espinar, M. T. (2014). Pathogenic potential of *Saccharomyces* strains isolated from dietary supplements. *PloS One, 9*(5). https://doi.org/10.1371/journal.pone.0098094

Mayer, F. L., Wilson, D., & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence, 4*(2), 119–128. https://doi.org/10.4161/viru.22913. Taylor and Francis Inc.

McCusker, J. H., Clemons, K. V., Stevens, D. A., & Davis, R. W. (1994). *Saccharomyces cerevisiae* virulence phenotype as determined with CD-1 mice is associated with the ability to grow at 42°C and form pseudohyphae. *Infection and Immunity, 62*(12), 5447–5455. https://doi.org/10.1128/iai.62.12.5447-5455.1994

Muñoz, P., Bouza, E., Cuenca-Estrella, M., Eiros, J. M., Perez, M. J., Sanchez-Somolinos, M., … Pelaez, T. (2005). *Saccharomyces cerevisiae* fungemia: An emerging infectious disease. *Clinical Infectious Diseases, 40*(11), 1625–1634. https://doi.org/10.1086/429916

Murphy, A. R., & Kavanagh, K. A. (2001). Adherence of clinical isolates of *Saccharomyces cerevisiae* to buccal epithelial cells. *Medical Mycology, 39*(1), 123–127. https://doi.org/10.1080/mmy.39.1.123.127

Naglik, J. R., Moyes, D. L., Wächtler, B., & Hube, B. (2011). *Candida albicans* interactions with epithelial cells and mucosal immunity. *Microbes and Infection, 13*(12–13), 963–976. https://doi.org/10.1016/j.micinf.2011.06.009. Elsevier Masson.

Nobile, C. J., & Johnson, A. D. (2015). *Candida albicans* biofilms and human disease. *Annual Review of Microbiology, 69*(1), 71–92. https://doi.org/10.1146/annurev-micro-091014-104330

Perez-Nadales, E., Almeida Nogueira, M. F., Baldin, C., Castanheira, S., El Ghalid, M., Grund, E., Lengeler, K., Marchegiani, E., Mehrotra, P. V., Moretti, M., Naik, V., Oses-Ruiz, M., Oskarsson, T., Schäfer, K., Wasserstrom, L., Brakhage, A. A., Gow, N. A. R., Kahmann, R., Lebrun, M. H., & Wendland, J. (2014). Fungal model systems and the elucidation of pathogenicity determinants. *Fungal Genetics and Biology, 70*(100), 42–67. https://doi.org/10.1016/j.fgb.2014.06.011. Academic Press Inc.

Pérez-Torrado, R., Llopis, S., Jespersen, L., Fernández-espinar, T., & Querol, A. (2012). Clinical *Saccharomyces cerevisiae* isolates cannot cross the epithelial barrier in vitro. *International Journal of Food Microbiology, 157*(1), 59–64. https://doi.org/10.1016/j.ijfoodmicro.2012.04.012

Pérez-Torrado, R., & Querol, A. (2016). Opportunistic strains of *Saccharomyces cerevisiae*: A potential risk sold in food products. In *Frontiers in microbiology, 6*. https://doi.org/10.3389/fmicb.2015.01522. Issue JAN). Frontiers Media S.A.

Price, M. F., Wilkinson, I. D., & Gentry, L. O. (1982). Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia, 20*, 7–14. https://doi: 10.1007/s10482-006-9077-7.

Puig-Asensio, M., Padilla, B., Garnacho-Montero, J., Zaragoza, O., Aguado, J. M., Zaragoza, R., … Mularoni, A. (2014). Epidemiology and predictive factors for early and late mortality in *Candida* bloodstream infections: A population-based surveillance in Spain. *Clinical Microbiology and Infections, 20*(4), O245–O254. https://doi.org/10.1111/1469-0691.12380

Romano, P., Capece, A., & Jespersen, L. (2006). Taxonomic and ecological diversity of food and beverage yeasts. In *Yeasts in Food and Beverages*. https://doi.org/10.1007/978-3-540-28398-0_2. Springer Berlin Heidelberg.

Seth-Smith, H. M. B., Büchler, A. C., Hinic, V., Medinger, M., Widmer, A. F., & Egli, A. (2020). Bloodstream infection with *Candida kefyr/kluyveromyces marxianus*: Case report and draft genome, 4. In *Clinical Microbiology and Infection, 26* pp. 522–524). https://doi.org/10.1016/j.cmi.2019.11.014. Elsevier B.V.

Steensels, J., & Verstrepen, K. J. (2014). Taming wild yeast: Potential of conventional and nonconventional yeasts in industrial fermentations. *Annual Review of Microbiology, 68*(1), 61–80. https://doi.org/10.1146/annurev-micro-091213-113025

Yagishita, K., Jinnouchi, H., & Yamamoto, H. (1981). Studies on the mechanism of synergistic action with synergisidin and imidazole antimycotics. *Japanese Journal of Antibiotics, 34*(11), 1485–1495. http://www.ncbi.nlm.nih.gov/pubmed/7038186.