Characterization of kefir yeasts with antifungal capacity against Aspergillus species

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Research Article

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Abstract

Kefir is a fermented probiotic drink obtained by placing kefir granules in a suitable substrate. The kefir granules are a consortium of bacteria and yeasts embedded in a exopolysaccharide matrix. The aim of this research was the isolation and identification of yeasts from kefir of different origin, the evaluation of their antifungal capacity against *Aspergillus* spp. and the characterization of virulence related traits. Using RFLP of ITS1/ITS4 region, D1/D2 region sequencing and RAPD techniques, 20 kefir isolates were identified as *Geotrichum candidum*, *Pichia kudriavzevii*, *P. membranifaciens*, *Saccharomyces cerevisiae* and *Candida ethanolica*. Their antifungal capacity was evaluated by their conidia germination reduction, which allowed the selection of eight isolates with high to moderate conidia germination reduction against *A. flavus* and *A. parasiticus*. Furthermore, these selected isolates showed growth inhibition on contact in the dual culture assay for both *Aspergillus* species and 3 of them –belonging to *S. cerevisiae* and *P. kudriavzevii* species– generated volatile organic compounds which significantly affected the growth of both fungi. For the evaluation of virulence related traits, growth at high temperatures, enzymatic activities and the adhesion to Caco-2 cells were analyzed. The isolates did not present more than one positive virulence-related trait simultaneously. In particular, it is important to highlight that the adhesion capacity to the model of intestinal barrier was extremely low for all of them. According to the results obtained, further studies would be of interest for the possible use of these promising yeasts as biocontrol agents against fungi in food.

Introduction

Kefir is a fermented probiotic drink, which is produced by placing kefir granules into a suitable substrate for its fermentation, e.g. milk or sugary solutions. Kefir granules are composed of an exopolysaccharide matrix in which bacteria (mainly *Lactobacillus*, *Leutonostoc*, *Streptomyces*, *Lactococcus* and *Acetobacter*) and yeast (*Saccharomyces*, *Kazachstania*, *Kluyveromyces*, *Pichia*, among others) are embedded. Previous investigations have demonstrated the capacity of kefir fermented substrate to inhibit fungi. Gamba et al. (2016a) found that milk fermented with kefir granules, inhibited the growth of *Aspergillus flavus in vitro*, and when applied to corn arepas their shelf life was extended. Another study from the same authors showed that whey permeate fermented with kefir grains inhibited the growth of *A. flavus*, *A. parasiticus* and *Penicillium sumatrense*, *in vitro* and when added to bread and poultry feed, which increased their resistance to contamination by the fungi listed above (Gamba et al., 2016b). Other authors also studied the antifungal capacity of kefir fermented milk against *Fusarium* sp. and *Aspergillus* spp., finding high inhibitions (Ismaiel et al., 2011; Taheur et al., 2020).

The researches discussed above focused on kefir consortium as a whole. As regards the isolated microorganisms, lactic acid bacteria (LAB) have gained more attention and have been considerably documented in relation to their antifungal capacity against *Penicillium*, *Aspergillus*, *Fusarium* and *Botrytis* genera (Purutoğlu et al., 2020; Chen et al., 2021). Meanwhile, researches centered on kefir yeasts are scarce. In a recent study, the inhibition capacity of yeasts isolated from milk kefir – *Saccharomyces*

sp., *Meyerozyma* sp. and *Kazachstania* sp.– against *Penicillum* spp. in fruit, was evaluated and a varying degree of fungal inhibition according to the species assayed was found (Zhimo et al., 2020).

Biological control is considered a more suitable alternative for disease control due to its low impact on the environment –by the reduction of pesticides (Di Canito et al., 2021; Sellitto et al., 2021). The use of yeasts as biocontrol agents presents advantages over other microorganisms such as filamentous fungi and bacteria, since their nutritional requirements are simple, allowing the colonization of a wide range of substrates and in turn do not produce allergenic spores, mycotoxins or antibiotics (Chanchaichaovivat et al., 2007; Freimoser et al., 2019; Hernandez-Montiel et al., 2021). Several investigations demonstrated the antifungal capacity of yeasts on different food or feed matrices infected by fungi, e.g. in fruits, in vegetables and in cereals. Common contaminant fungi in food, such as *Alternaria*, *Penicillium*, *Aspergillus*, *Fusarium*, *Mucor*, *Rhizopus* and *Botrytis* genera have been inhibited by yeasts belonging to *Candida*, *Debaryomyces*, *Pichia, Saccharomyces*, *Wickerhamomyces*, *Metschnikowia* genera, among others. (Piasecka-Jozwiak and Chablowska, 2017; Freimoser et al., 2019; Hernandez-Montiel et al., 2021). Fungal inhibition may be due to different mechanisms, including: i) competition for substrates and space, ii) inhibition of fungal mycelia growth, iii) modification of fungal spore germination, iv) production of antifungal volatiles, iv) production of enzymes –mainly the ones involved in the fungal cell wall degradation, such as chitinases, glucanases and proteases– and vi) increase in the host resistance against fungi (Liu et al., 2013; Freimoser et al., 2019; Sellitto et al., 2021).

In addition, it must be considered that there are general traits associated with yeasts virulence, which are important to analyzed for an antifungal agent, such as: i) adhesion to host tissue, ii) production of lipolytic and proteolytic enzymes, iii) growth at high temperatures and iv) pseudohyphae formation (Murphy and Kavanagh, 1999; Van Burik and Magee, 2001; Park et al., 2013).

Since, kefir is a promising and novel source for the isolation of yeasts with antimicrobial capacity, the aim of the present study was the identification of yeasts, from kefir granules of different origin, with inhibition capacity against *Aspergillus* spp. and the characterization of virulence related traits for their possible use as biocontrol agents in food.

Materials And Methods

Fungal strains and preparation of conidial suspensions

Aspergillus flavus and *A. parasiticus* from the culture collection of the Department of Microbiology from the National University of La Plata, both stored in soft agar at 4°C, were employed in this study. The fungi were streaked into Petri dishes containing potato dextrose agar (PDA, Biokar, Francia) and incubated at 30°C for 7 d. Then 10 ml of 0.01% (w/v) sodium lauryl sulfate (Merck, Darmstadt, Germany) and 1% (w/v) glucose were added and the conidia were scraped off with a spatula. Fungal inocula were prepared by adjusting the suspensions to 10^4 conidia/ml using a Neubauer chamber (León Peláez et al., 2012).

Kefir granules and culture conditions

Water kefir granules –CMUNLP 1, CMUNLP 2 and CMUNLP 4– and milk kefir granules –CIDCA AGK1, CMUNLP 8 and CMUNLP 9– employed in this work, were from different households of La Plata, Argentina, with the exception of CMUNLP1 which was from India and AGK1 which belongs to the collection of Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, UNLP, Argentina). The kefir granules were cultivated in bottles of 250 ml capacity with different substrates – milk (M) or whey permeate (WP) for milk kefir and muscovado (MU), molasses (MO) or chancaca (CC) for water kefir– in a granule/substrate ratio of 10% for 48 h at 30°C. Subsequently, the granules were separated from de supernatant by sieving and the granules were incorporated into the respective fresh substrate. This process was repeated three consecutive times before yeast isolation from granules and supernatant (Gamba et al., 2016a).

Yeast isolation

The yeasts isolation was carried out from the granules and supernatants obtained in the previous step, for which 10 g and 10 ml respectively, were added 90 ml of 0.85% w/v NaCl and homogenized in a BagMixer® 400 W (Interscience, France). Serial dilutions were made and 100 µl of the final dilutions were surface-spread on Petri dishes containing yeast glucose chloramphenicol agar (YGC, Biokar, Francia), in duplicate for each dilution. The incubation was carried out for 5 d at 30°C and the colonies obtained were differentiated according to their morphological characteristics, then subcultivated on Petri dishes with yeast peptone dextrose agar (YPD, 1% yeast extract, 2% peptone, 2% dextrose, 2% agar) and finally maintained in YPD slants at 4°C.

Yeast identification

Genomic DNA was extracted according to Lõoke et al. (2011). Firstly, a restriction fragment length polymorphism (RFLP) of the ITS1/ITS2 region was performed according to Esteve-Zarzoso et al. (1999). From the results obtained the yeasts were identified and grouped, then the results were confirmed by amplifying and sequencing the D1/D2 region according to (Kurtzman and Robnett, 1997). The PCR products were purified with MinElute® according to the manufacturer´s instructions and their sequencing was performed at the Genomic Unit-Valencia University (Valencia, Spain) and Macrogen (Seoul, South Korea). The identification was achieved by the comparison against the Genbank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Additionally, for the identification of *Geotrichum candidum* a randomly amplified polymorphic DNA (RAPD) analysis with the primer M13 (5'-GAGGGTGGCGGTTCT-3') was performed (Gente et al., 2006). In the case of *Saccharomyces cerevisiae*, an additional RFLP of the genes *MAG2* and *GSY1* was performed according to Pérez-Tráves et al. (2014).

Preparation of cell free supernatants (CFS)

The identified yeasts were cultured in 50 ml of MEA broth (10 g/l malt extract, 20 g/l yeast extract) in 250 ml flasks, with two previous passages in the same medium, and incubated at 30°C for 48 h at 150 rpm. Afterwards, the fermented medium was centrifugated at 3000 rpm for 15 min and the supernatant was sterilized by filtration using 0.22 µm pore acetate membranes (Sigma-Aldrich®). MEA dishes streaked

with the CFSs and incubated at 30°C for 5 d, were employed as sterility controls. The CFSs were stored at -20°C.

Antifungal characterization

Reduction of conidia germination

The assay was performed in 96-well microplates according to Lavermicocca et al. (2003), for which 190 µl of CFS and 10 µl of a 10^4 conidia/ml suspension were added to each well. The assay was carried out in two independent repetitions, and in each assay the treatments were performed by quintuple. The control consisted of fresh sterile MEA broth with 10µl of the fungal inoculum. After incubating the microplate at 30°C for 48 h, the OD at 580 nm was measured and the percentage of germination reduction (% GR) of the conidia was calculated as % GR = $(OD_C - OD_T) \times 100 / OD_C$, where C and T correspond to the optical densities of the control and the treatment respectively. The degree of fungal inhibition was scored according to Gerez et al. (2009), where % GR greater than 20% is considered positive, between 20–40% low, 40%-70% moderate and more than 70% high inhibition.

Dual culture assay

The assay was carried out in 90 mm diameter Petri dishes with PDA, inoculating 10 µl of a 10^4 conidial/ml suspension of *A. flavus* or *A. parasiticus* 2 cm away from the border of the plate and a 2-day-old yeast culture was streaked 5 cm from the fungal inoculum. Controls consisted only of the fungal inoculum. The assay was carried out in two independent repetitions, and each treatment was carried out in triplicate. Incubation was at 30°C in the dark and the fungal growth was periodically observed for 14 d (Pantelides et al., 2015).

Volatile organic compounds

The production of antifungal VOC was determined with the doble plate method according to Vero et al. (2013). The assay consisted of facing two Petri dishes —5 cm diameter containing PDA— to form a chamber. The upper plate was surface inoculated with the corresponding yeast and the bottom plate was spotted with 10 µl of a 10^4 conidial/ml suspension of the fungus. The plates were sealed together with three turns of Parafilm®, incubated at 30°C in the dark and the fungal diameters were periodically determined. The control consisted only of the fungal inoculum. The assay was carried out in two independent repetitions, and each treatment was carried out in triplicate.

Characterization of virulence related traits

Proteolytic activity

Proteolytic activity was measure in Petri dishes according to Pailin et al. (2001) employing a solid medium composed of skim milk as the only nutrient source (5% nonfat dry milk and 2% agar). The yeast inoculum from a 2-day-old yeast culture in MEA broth, was spotted in the center of the plates and

incubated at 30°C for 5 d. The proteolytic activity was detected by the formation of a clear halo around the colonies.

Lipolytic activity

Lipolytic activity was measured in Petri dishes as reported by Singh et al. (2006). A chromogenic culture medium that consisted of 0.1% phenol red, 1% olive oil, 10 mM $CaCl_2$ and 2% agar at pH 7.3–7.4 was used. The lipolytic activity was detected as a change in color of the medium from pink to yellow.

Then, the lipolytic activity was quantified for the positive cultures in the aforementioned assay, with a titrimetric method according to Macedo et al. (1997). The measurements were made from crude extracts obtained from a broth with 10 g/l of olive oil as an enzyme inducer, inoculated with a 2-day-old yeast culture in MEA broth and incubated at 30°C for 48 h at 150 rpm. From 1ml of the supernatant, the lipolytic activity was quantified with a reaction mixture containing 25% olive oil as substrate and incubated for 1 h at 37°C. The reaction was stopped with the addition of acetone/ethanol 1:1 (v/v) and titrated with 0.05 M NaOH. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol of fatty acids in these conditions.

Adhesion assay

The adhesion of the yeast isolates to Caco-2 cells —an *in vitro* model of intestinal barrier cells— was performed according to Pérez-Torrado et al. (2012). The Caco-2 cells were set up in a 24-well plate as describe by the authors until a confluent monolayer was established, then they were washed with pre-warmed (37°C) phosphate-buffered saline (PBS, pH 7.4) and 250 µl of fresh medium was added along with 50 µl of each respective yeast suspension of 6 x 10^4 cells/ml to each well. The plates were incubated at 37°C under 5% CO_2 for 1 h. Afterwards, the monolayer was washed three times with PBS, and 300 µl of PBS were added to recover the adhered cells with a cell scraper. The adhere and non-adhere yeasts cells were counted in Petri dishes with YPD agar and the percentage of adhesion was calculated with respect to the total number of yeasts cells. *Candida albicans* SC5314 and *Wickerhamomyces anomalus* 2937 strains were used as high and medium adhesion controls respectively, which belong to the collection of Amparo Querol (IATA-CSIC, Spain). The assay was carried out in two independent repetitions, and each treatment was carried out in triplicate.

Statistics

The statistical analysis of the data was carried out by STATISTICA 7 software (StatSoft, OK, USA). Linear regression of the exponential phase and ANOVA and LSD test were the tests performed.

Results And Discussion

Presence and identification of yeasts from kefir

Twenty yeast isolates were obtained from kefir grains of different origins, fermented on various substrates and identified by phenotypic and molecular techniques in the following species: *Geotrichum candidum*, *Saccharomyces cerevisiae*; *Pichia membranifaciens*; *Pichia kudriavzevii* and *Candida ethanolica* (Table 1). The RFLP of the ITS1/ITS2 region allowed to identify and group in the five different species listed above, which was confirmed by amplifying and sequencing the D1/D2 region. In the case of *G. candidum* an additional RAPD analysis with the primer M13 was performed and the presence of three bands with an intense one at 860 ppb, confirmed its identification (Gente et al., 2006). When RFLP of specific genes was performed to differentiate between *Saccharomyces* spp., the bands obtained concurred with *S. cerevisiae* (Pérez-Través et al., 2014).

As shown in Table 1 the presence of the species varied with the origin of the kefir granule, as well as with the substrate used for its fermentation. The results obtained coincide with those reported in different studies, where the kefir consortium was highly variable depending on its origin, storage and fermentation conditions (Garrote et al., 1998; Hsieh et al., 2012; Nielsen et al., 2014).

Table 1
Identification of yeasts from kefir granules of different origin and culture conditions

Isolate	Species	Kefir Grain	Type of kefir*	Fermentation substrate**	Fraction of isolation
Gc G	*Geotrichum candidum*	CIDCA AGK1	MK	M	Granule
Gc 2		CIDCA AGK1	MK	M	Fermented substrate
Gc 30		CMUNLP 9	MK	M	Fermented substrate
Gc 31		CMUNLP 8	MK	M	Granule
Pk 14	*Pichia kudriavzevii*	CIDCA AGK1	MK	WP	Fermented substrate
Pk 20		CIDCA AGK1	MK	WP	Granule
Pk 22		CMUNLP 1	WK	MU	Granule
Pk 23		CMUNLP 1	WK	MU	Granule
Pk 25		CMUNLP 1	WK	CC	Granule
Pk 27		CMUNLP 1	WK	MU	Fermented substrate
Pm 5	*Pichia membranifaciens*	CMUNLP 1	WK	MU	Fermented substrate
Pm 9A		CMUNLP 1	WK	MO	Fermented substrate
Pm 11		CMUNLP 1	WK	CC	Fermented substrate
Pm 16		CMUNLP 1	WK	MU	Fermented substrate
Pm 17		CMUNLP 1	WK	MU	Fermented substrate
Pm 21		CMUNLP 2	WK	MU	Granule

* MK: milk kefir; WK: water kefir

** M: milk; WP: whey permeate; MU: muscovado; MO: molasses and CC: chancaca.

Isolate	Species	Kefir Grain	Type of kefir*	Fermentation substrate**	Fraction of isolation
Pm 24		CMUNLP 1	WK	MO	Granule
Pm 29		CMUNLP 4	WK	MU	Granule
Sc 9B	*Saccharomyces cerevisiae*	CMUNLP 1	WK	MO	Fermented substrate
Ce 26	*Candida ethanolica*	CMUNLP 1	WK	MU	Fermented substrate
* MK: milk kefir; WK: water kefir					
** M: milk; WP: whey permeate; MU: muscovado; MO: molasses and CC: chancaca.					

The species *G. candidum* was isolated from milk kefir (both kefir granules and the fermented milk), but it was not found in water kefir. The presence of this species in milk kefir has been previously reported by several authors (Garrote et al., 1997; Witthuhn et al., 2005; Timar, 2010), but not in water kefir. Meanwhile, *P. kudriavzevii* was isolated from both types of kefir, except when fermented in milk; although, Gao et al. (2012) and Basavaiah et al. (2019) reported its presence in milk fermented with milk kefir. In this research, *P. membranifaciens* was the most variously found species, it was present in the different water kefir grains and in the different fermentation conditions; however, it was not found in milk kefir. Similar results were reported for water kefir by Miguel et al. (2011), Gamba et al. (2019) and Gonda et al. (2019), while *P. membranifaciens* was isolated in milk kefir by Kalamaki et al. (2017). Lastly, *S. cerevisiae* and *C. ethanolica* were isolated from the fermented substrate of water kefir CMUNLP 1. Sarikkha et al. (2015) were the first ones to isolate *C. ethanolica* from water kefir, while Azi et al. (2020) reported that it was the predominant species in water kefir beverage grown in soy whey. No reports were found regarding *C. ethanolica* isolated from milk kefir. Contrarily, *S. cerevisiae* was extensively found in water and milk kefir alike (Gulitz et al., 2011; Magalhães et al., 2011; Laureys and De Vuyst, 2014).

Antifungal characterization

Reduction in conidia germination

The antifungal activity was firstly analyzed by coculturing CFSs with conidia suspensions of *A. flavus* and *A. parasiticus* separately and measuring the reduction in conidia germination for the identified isolates. These assays showed that most of the yeasts had inhibition capacity towards both *Aspergillus* species, which varied according to the yeast species analyzed (Fig. 1). *A. flavus* showed the highest values of inhibition when coculture with the CFSs of *G. candidum* isolates and one isolate of *P. membranifaciens*. The CFSs from Pm 29, Pm 24 and Ce 26, exerted null inhibition, while the rest of the CFSs presented inhibitions ranging from moderate to low (see Materials and Methods). Regarding *A.*

parasiticus, its conidia germination was inhibited by eight of the twenty CFSs, while the rest had null inhibition. Therefore, *A. parasiticus* in relation to *A. flavus*, was less inhibited by the yeasts under the established experimental conditions. Nevertheless, the isolates with higher inhibition capacity coincided for both fungi, being *G. candidum* the species with the highest values. A similar behavior was obtained by Kumar et al. (2017) for the CFSs of *G. candidum*.

From the results obtained, eight yeast isolates (Gc G, Gc 2, Gc 30, Gc 31, Pm 9A, Sc 9B, Pk 20 and Pk 27) with the highest inhibition capacity for both *Aspergillus* species were selected to further analyze their antifungal capacity by the following assays.

Dual culture assay

In the dual culture assay, none of the yeast could completely inhibit the fungal growth and there was no inhibition at a distance, however mutual inhibition on contact was observed for all the yeast isolates studied (data not shown). The mycelium of both fungi, in the proximity of the antagonistic yeast, was weak and did not present conidia. This behavior has also been observed by Pantelides et al. (2015) for *A. tubigensis* grown with *Candida* spp., *Cryptococcus magnus* and *Aureobasidium pullulans* and by Masih et al. (2002) for *Botrytis cinerea* grown with *P. membranifaciens.*

During the 14-day assay, on day 12 *A. flavus* and *A. parasiticus* grew over the yeast

Pm 9A, while the rest of the isolates withheld the inhibition. Similar results have been obtained by Kumar et al. (2017), who found a clear inhibition zone for *A. parasiticus* NRRL299 when grown with *G. candidum* isolated from cereal crops and by Choińska et al. (2020), who found that *P. kudriavzevii* isolated from rye grains could inhibit the growth of *Fusarim* sp., *Mucor* sp., *Botrytis cinerea*, *Penicillium* sp. and *A. fumigatus* at great extent.

Volatile organic compounds

The effect of the VOCs produced by the previously selected yeast isolates, measured by the double Petri dish method, is shown in Table 2. As can be seen, there were statistically significant differences between the growth rate of the control and the treatments for *A. parasiticus* and *A. flavus*. In particular, *A. parasiticus* did not present significant differences among the treatments, while *A. flavus*did.

Table 2
Effect of yeasts volatile organic compounds on *Aspergillus* spp. growth

Yeast Isolate*	*Aspergillus flavus*		*Aspergillus parasiticus*	
	Growth rate ($mm\ h^{-1}$)	Lag phase (h)	Growth rate ($mm\ h^{-1}$)	Lag phase (h)
Control	0.581^{a}	18.0^{c}	0.479^{a}	14.7^{b}
Gc G	$0.463^{b,c}$	20.5^{c}	0.207^{b}	11.2^{b}
Gc 2	$0.460^{b,c}$	19.0^{c}	0.162^{b}	10.3^{b}
Gc 30	$0.412^{b,c}$	20.7^{c}	0.200^{b}	13.5^{b}
Gc 31	0.480^{b}	22.7^{c}	0.146^{b}	12.7^{b}
Pm 9A	$0.401^{b,c}$	18.3^{c}	0.260^{b}	15.7^{b}
Sc 9B	0.361^{c}	**36.5^{b}**	0.253^{b}	$26.3^{a,b}$
Pk 20	$0.397^{b,c}$	**$46.1^{a,b}$**	0.183^{b}	**97.5^{a}**
Pk 27	$0.428^{b,c}$	**46.7^{b}**	0.183^{b}	**90.8^{a}**

*Control: *Aspergillus* spp. without yeast, Gc: *Geotrichum candidum*, Pk: *Pichia kudriavzevii*; Pm: *Pichia membranifaciens*; Sc: *Saccharomyces cerevisiae.* Different letters in each column mean statistical differences $p < 0.05$ within the column. The significantly largest increment in the lag time are in bold.

As observed in the results, the VOC had a larger influence on the lag phase than on the growth rate of both *Aspergillus* species. Pk 20 and Pk 27 were the isolates which extended the lag phase for both fungi; they more than doubled the lag phase for *A. flavus* and more than quintuplicated it for *A. parasiticus*, in comparison with the corresponding control. These results highlight the greater susceptibility of *A. parasiticus* to the volatile compounds produced by *P. kudriavzevii*. The isolate of *S. cerevisiae* also exerted a significant effect over *A. flavus* lag phase, but not as marked as *P. kudriavzevii* isolates. The rest of the isolates did not present statistical differences with the control.

In summary, the yeast isolates selected presented fungal inhibition evidenced by the three methodologies used for their evaluation. In particular, the values obtained in the germination reduction assay where CFS are used, might indicate that the isolates —specially *G. candidum* strains— released antifungal metabolites to the medium. Kawtharani et al. (2020) attributed phenillactic acid (PLA) produced by *G. candidum* in liquid cultures, with the capacity to reduce *Fusarium* spp. growth. In the same manner, Abdel-Kareem et al. (2019) observed for *S. cerevisiae* a similar behavior, a high growth reduction and no sporulation of *A. flavus* in coculture with the yeast. The authors associated this inhibitory capacity to 4-hydroxyphenethyl alcohol, 4,4-dimethyloxazole and 1,2-benzenedicarboxylic acid dioctyl ester.

Furthermore, VOC generated by *P. kudriavzevii* isolates had the highest effect over *A. flavus* and *A. parasiticus* lag phase, which could mean an additional mode of action for these isolates. Choińska et al. (2020) found that VOC produced by *P. kudriavzevii* caused inhibition against species of *Penicillium, Fusarium, Aspergillus* and *Mucor*. Among the VOC determined, they found phenylethyl alcohol and its acetates which have proven antifungal activity (Hua et al., 2014).

The evaluation of the isolates capacity to inhibit *Aspergillus* growth, evidenced that the eight selected yeasts showed high values of inhibition in at least one of the assays described above, in addition to the reduction in the conidia production –which could be related to a lower dispersion of the contaminating fungus. Hence, the selected yeasts were then characterized in terms of virulence traits, a relevant aspect for their possible use as biocontrol agents in food.

Characterization of virulence related traits

Growth at different temperatures, enzymatic activity and adhesion capacity

To analyze the virulence related traits of the selected yeast isolates, the growth at high temperatures, the production of proteolytic and lipolytic activities and the adhesion to Caco-2 cells –a model of intestinal barrier cells– were evaluated.

As shown in Table 3, all the selected isolates grew at 28°C (temperature used as control), while at 37°C only the *S. cerevisiae* isolate and *P. kudriavzevii* isolates (Pk 20 and Pk 27) were able to grow. These last two isolates were the only ones that could grow at 42°C. The ability to grow at high temperatures (37–42°C) is an important attribute associated with fungal pathogenesis (Van Burik and Magee, 2001). Although Llanos et al. (2006) suggested that growth at high temperature does not seem to be the sole factor of the isolate's virulence, they also proposed that the growth at 42°C should be used as one of the preventive criteria for industrial strains (Llanos et al., 2006). Hence, caution should be taken with *P. kudriavzevii*isolates, which were the only ones that could grow at this temperature.

Table 3
Enzymatic activity and the effect of temperature on yeasts growth

Yeast Isolate*	Growth temperature			Enzymatic activity	
	28°C	37°C	42°C	Proteolytic	Lipolytic ($\mu mol\ h^{-1}\ ml^{-1}$)**
Gc G	+	-	-	-	32.5^{c}
Gc 2	+	-	-	-	$83.75^{b,a}$
Gc 30	+	-	-	-	73.75^{b}
Gc 31	+	-	-	-	96.25^{a}
Pm 9A	+	-	-	-	-
Sc 9B	+	+	-	-	-
Pk 20	+	+	+	-	-
Pk 27	+	+	+	-	-

*Gc: *Geotrichum candidum*, Pk: *Pichia kudriavzevii*; Pm: *Pichia membranifaciens*; Sc: *Saccharomyces cerevisiae.* " + " : positive measurement; " – " : negative measurement.

**Different letters mean statistical differences $p < 0.05$

Regarding the enzymatic activities, the proteolytic activity was not detected for the isolates under study and only *G. candidum* isolates presented lipolytic activity. Among *G. candidum* isolates, Gc 31 and Gc 2 had the highest lipolytic activity, with values of 96.25 and 83.75 µmol of fatty acid/h.ml respectively. These values are comparable to the ones obtained for the same species by Sacristán et al. (2012). These enzymatic activities can be considered as virulence factors, since the proteolytic activity has been associated with an active mechanism of pathogens to enter the host tissue (Mayer et al., 2013), while the lipolytic activity has been related to growth, morphology, adherence and dissemination in the host by human pathogenic yeasts (Park et al., 2013).

Lastly, the adhesion of the yeast isolates to Caco-2 cells, was analyzed employing two isolates as control, Ca SC5314 and Wa 2937, which had high and medium adhesions respectively according to Perez-Través et al. (2021). As shown in Table 4, the adhesion values for the treatments, which range between 0.4 and 2.2% and present significant differences, are considerably low compared to the controls.

Table 4
Yeasts adhesion to human Caco-2 epithelial monolayers

Yeast Isolate*	**Adhesion (%)****
Gc G	2.2[c]
Gc 2	0.7[d]
Gc 30	0.7[d]
Gc 31	0.6[d,e]
Pm 9A	0.8[c,d]
Sc 9B	0.4[e]
Pk 20	0.9[c,d]
Pk 27	1.0[c,d]
Ca SC5314 Control	62.1[a]
Wa 2937 Control	13.7[b]

*Gc: *Geotrichum candidum*, Pk: *Pichia kudriavzevii*, Pm: *Pichia membranifaciens*; Sc: *Saccharomyces cerevisiae*, Ca SC5314: *Candida albicans* SC5314, Wa 2937: *Wickerhamomyces anomalus* 2937. **** Different letters mean statistical differences $p < 0.05$

These values are similar to the ones obtained for yeasts isolated from milk kefir by Diosma et al. (2014), who also found low adhesion values to Caco-2/TC7 cells for *Kluyveromyces. marxianus* CIDCA 8154 (3.0 ± 0.9%) and *S. cerevisiae* CIDCA 8112 (0.5 ± 0.1%). Pérez-Torrado et al. (2012) found that all the *S. cerevisiae* isolates studied, clinical and non-clinical, presented low adhesions when compared with the controls (*C. albicans* and *C. glabrata*). The authors suggested that the low adhesions observed could be due to the fact that the yeasts did not present active mechanisms and their ability to cross the epithelial barrier would be opportunistic due to barrier integrity loss.

The tests carried out showed that the yeast isolates under study did not present more than one positive trait related to virulence at the same time. In particular, their adhesion capacity, which is an essential attribute since is the first step for yeasts to invade and infect a host, was extremely low (Table 4). Pérez-Través et al. (2021) recommended for *Debaryomyces hansenii* and *Kluyveromyces marxianus* strains not to be used in the food industry if they presented two or more virulence traits.

In conclusion, the yeasts species isolated varied with the origin of the kefir granule and the substrate used for its fermentation. From twenty identified isolates, eight presented significant antifungal capacities against *A. parasiticus* and *A. flavus*. These yeasts reduced the mycelial growth as well as the generation of conidia, which has an impact on the fungal dispersion capacity. In addition, three of them generated antifungal VOC with a pronounced effect on the fungi lag phase. Furthermore, the isolates did not present more than one positive virulence related traits and, in particular, their adhesion capacity to Caco-2 cells was extremely low. According to the results obtained, further studies would be of interest for the possible use of these promising yeasts as biocontrol agents against fungi in food.

Declarations

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Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare no competing interests.

Consent for publication

Not applicable.

Availability of data and material

Please contact author for data requests.

Authors' contributions

All the authors were involved in the design, data collection, data analysis, data interpretation, elaboration and discussion of the manuscript.

Authors' information

Not applicable.

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Figures

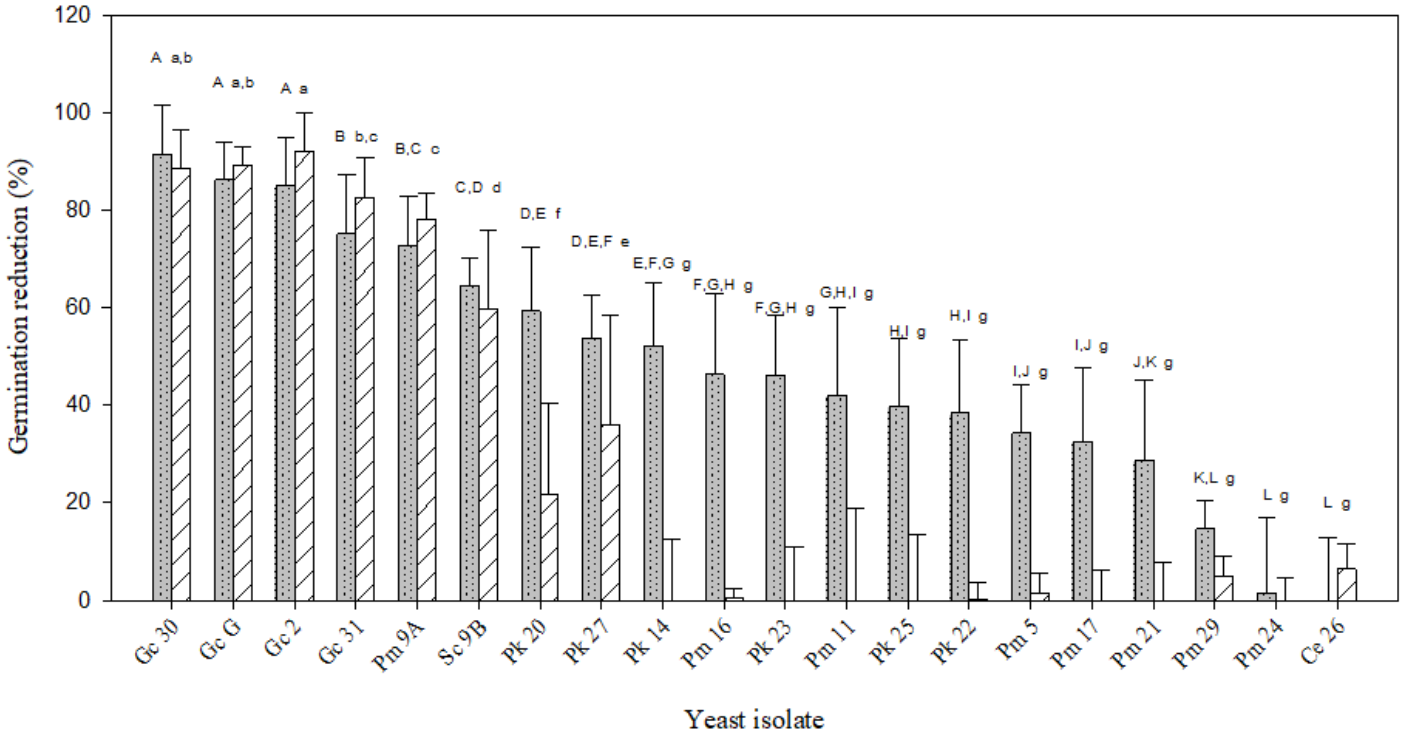

Fig. 1 Germination reduction of conidia of (▭) *Aspergillus flavus* and (▨) *Aspergillus parasiticus* against cell-free supernatants of Gc: *Geotrichum candidum*, Pk: *Pichia kudriavzevii*; Pm: *Pichia membranifaciens*; Sc: *Saccharomyces cerevisiae*; Ce: *Candida ethanolica*. Different letters in each bar mean statistical differences $p < 0.05$

Figure 1

See image above for figure legend