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Article

Biotherapeutic Potential of a Novel Probiotic *Kluyveromyces marxianus* Isolated from a Sourdough Starter Against Vaginal *C. albicans* Strains

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Abstract: There is an increasing interest in yeasts isolated from natural sources to be used as probiotics. *Saccharomyces*-based probiotics have been proposed as a valid alternative to the conventional drug therapy for the prevention and treatment of vulvovaginal candidiasis, also considering the resistance of some *Candida* strains to many antifungals. Here we isolated from an artisanal sourdough a new yeast strain which was identified as *Kluyveromyces marxianus* and assessed its probiotic and safety properties, which resulted comparable at all to those exhibited by the commercial probiotic *Saccharomyces boulardii*. Then, we checked the antagonistic activity of the new isolate against some clinical fluconazole resistant *C. albicans* strains, showing its ability to inhibit filamentation, biofilm formation, and adhesion of *C. albicans* to vaginal epithelial A-431 cells. Also, *K. marxianus* reduced the cell damage provoked by *C. albicans* and the expression of *SAP2* and *SAP6* genes. On the whole, our results enlarge the spectrum of the beneficial properties of the food-grade yeast *K. marxianus* showing for the first time its biotherapeutic potential as anticandidal agent.

Keywords: probiotic yeasts; *Kluyveromyces marxianus*; vulvovaginal candidiasis

1. Introduction

The Yeasts are components of the microbiota of various fermented foods and beverages and are largely employed in industry. The discovery of the antagonistic effects of yeasts, especially against other fungal species, has paved the way to focused investigations on their use as probiotics, that is of beneficial use for animal and human health [1].

Most conventional probiotics are of bacterial origin, mainly belonging to the *Lactobacillus* and *Bifidobacterium* genera [2]. More recently, several yeast strains have been reported as promising probiotic candidates as shown by the increasing number of publications over the last decades indicating the relevance of this topic [3]. Some of the differences between yeasts and bacteria, such as the larger size, which can block the attachment of pathogen and spoilage species, the presence of a cell wall containing mannans and beta-glucans, which can positively influence the intestinal microbiome and stimulate innate and acquired immunity, as well as the intrinsic resistance to antibiotics, are considered advantageous for the use of yeasts as probiotics [3].

Saccharomyces cerevisiae var. *boulardii*, first isolated in 1920, has been established as a probiotic since 1950; and now it is commercially available throughout the world, mainly used for the treatment of gastrointestinal tract disorders [4]. Later, other *Saccharomyces* and non-*Saccharomyces* yeasts isolated from vegetables, fruits, grains, and traditional fermented foods and beverages have been proposed as probiotics [5,6].

More recently, it has shown how *S. cerevisiae*-based probiotics may also be used for the prevention and treatment of vulvovaginal candidiasis (VVC), a pathology which affect about 3-

quarters of women during their reproductive age and subjected to frequent recurrences [7]. Since the vaginal microbiome plays a relevant role in VVC, a probiotic-based approach could represent a valid alternative to the conventional drug therapy, also considering the resistance of some *Candida* strains to many antifungals and their toxicity for the patients [8].

The effects of *Saccharomyces*-based treatment against VVC has been investigated both *in vitro*, and *in vivo* [9-12], definitely proving its beneficial protective and therapeutic action.

In this work we isolated from a sourdough and then characterized a new yeast strain, which resulted to be *Kluyveromyces marxianus*, and assessed its potential probiotic and safety properties, compared to those exhibited by the commercial probiotic *S. boulardii*.

K. marxianus isolates from different sources have been reported to have beneficial effects on human health. They include cholesterol reducing capability [13,14], antiproliferative effects on cancer cells [15,16], immunomodulatory action on Caco-2 cells [17], and also antimicrobial activity against various pathogen species [16,18,19]. In particular, the antifungal activity of different *K. marxianus* strains has been investigated and demonstrated against plant pathogenic fungi [20,21] and food spoilage moulds present in dairy products, so that the yeast has been proposed as a potential biocontrol agent [22]. Instead, so far, the anticandidal activity of this species has not been investigated in detail, being reported only in the case of a *K. marxianus* strain isolated from a French cheese [18].

In a previous work we isolated several *C. albicans* strains from VVC patients and characterized the clinical isolates in terms of antifungal resistance and biofilm forming ability [23], a characteristic associated to *Candida* virulence.

Therefore, here, aiming at enlarging the number and applications of available probiotic yeasts, we propose the new *K. marxianus* isolate as a novel anticandidal agent, testing its ability to antagonize fluconazole resistant *Candida albicans* vaginal strains, and presenting some insights into the mechanisms underlying its protective effect.

2. Materials and Methods

2.1 Strains and culture condition

For isolation of *K. marxianus*, 10 g of sourdough were suspended in 90 mL of PBS and homogenized with Fisherbrand 850 Homogenizer (Thermo Fisher Scientific, Waltham, MA, USA) for 20 min. The suspended sample was serially diluted in phosphate buffer saline solution (PBS) and plated onto Rosa Bengal agar (RB, Sigma-Aldrich, St. Louis, MO, USA), supplemented with chloramphenicol. The plates were incubated at 25 °C for 96 h until typical creamy yeast colonies appeared.

For most experiments, a strain of *S. boulardii* of commercial origin (Biocodex, Zambon, Italy) was used. It was obtained dissolving a capsule of the commercial product in PBS and then plating a proper diluted suspension on RB agar plates. After incubation of plates at 25 °C for 48 h, colonies appeared, and typical yeast morphology was checked by microscope observation.

Pre cultures and cultures of both *K. marxianus* and *S. boulardii* was performed in shake flasks containing YPD (yeast extract 2%, bactopectone 1%, dextrose 2%) medium, starting from one single colony, and incubated at 25 °C for 24 h at 200 rpm. The strains of *C. albicans* used were: ATCC 90028, and four clinical *C. albicans* strains, namely C7, C14, C17, and C19 isolated from VVC patients [23]. All *C. albicans* strains were cultured in YPD or RPMI (Thermo Fisher Scientific Inc., Waltham, MA, USA) medium at 37 °C.

In some experiments, to distinguish *C. albicans* from *K. marxianus* colonies, the selective chromogenic medium Brilliance Candida Agar Base (Oxoid Ltd, Wade Road, Basingstoke, Hants, RG24 8PW, UK) was used, which is properly formulated so that *C. albicans* colonies, after incubating agar plates at 37 °C, appear green, whereas *K. marxianus* colonies appear yellow/brown.

2.2. Molecular characterization of the yeast isolate

Isolated yeast colonies were, was sub-cultured on Dichloran Rose Bengal Chloramphenicol agar (DRBC, Thermo Fisher Scientific Inc., Waltham, MA, USA). CTAB extraction protocol [24] was employed to extract fungal total DNA. Extracted DNA was amplified with PCR, targeting ITS-5.8S rDNA region of the fungal 18S rRNA gene (750 bp amplicon size) [25], employing a MiniAmp™ Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) TECHNE Prime Thermal Cycler, and disposing of a ITS1_f (5'-GGA AGT AAA AGT CGT AAC AAG G-3' 5'-TCC GTA GGT GAA CCTGCG G-3') and ITS4_r (5'-TCC TCC GCT TAT TG A TAT GC-3') primer set (Biofab Research, Rome, Italy). Templates were run on a 1.5% agarose gel, stained with GelRed (BIOTIUM), utilising a 100 bp DNA ladder as a reference. Sanger Sequencing reactions were performed by an external service (Biofab Research, Rome, Italy); the obtained FASTA sequences were interpreted using an editing tool, Chromas Lite v. 2.6.6 (Technelysium Pty Ltd., South Brisbane, Australia), and compared to NCBI Sequence Database sequences, using BLASTN ver. 2.2.29 (also referring to GenBank), selecting the highest percentage Identity, with a 98% cut-off and 0.0 e-value

2.3. Probiotic Evaluation of the *K. marxianus* isolate

2.3.1. Growth at 37 °C

To test the ability of the *K. marxianus* isolate and the commercial strain of *S. boulardii* to grow at the temperature of the human body, 37 °C, both strains were cultivated at this temperature in shake flasks in YPD for 24 h. Growth was assessed by OD₅₉₀ determination.

2.3.2. Acid and Bile Salt Tolerance

Acid and bile salt tolerance of *K. marxianus* and *S. boulardii* were determined as already reported [26]. Yeast cells were collected from a 18 h preculture in YPD at 37 °C, washed with PBS and resuspended in 5 mL PBS plus HCl (pH = 3.0) or PBS plus 0.3% *w/v* bile salts (Sigma Aldrich St. Louis MO, USA), so as to have an initial cell density of 108 cells mL⁻¹. After 4 h incubation at 37 °C, 150 rpm, the CFU mL⁻¹ were determined using the standard plate count method, and the percentage of residual viable cells was calculated with respect to time 0 of incubation (100%)

2.3.3. Auto-Aggregation Capacity

The auto-aggregation capacity of both *K. marxianus* isolate and *S. boulardii* was determined after 24 h according to Maione et al. [26], by determining the OD₅₉₀ of the yeast suspension before (OD₀) and after (OD_t) incubation. The auto-aggregation (A) percentage was calculated as:

$$A = \left[1 - \left(\frac{OD_t}{OD_0} \right) \right] \times 100$$

2.3.4. Hydrophobicity Assay

Cell biomass was suspended in 5 mL of PBS and absorbance at 590 nm was measured (OD₀). Then, 3 mL xylene, were added to each sample. After incubation at 37 °C for 60 min without shaking, the absorbance of the interphase was measured (OD_f) [27]. The hydrophobicity percentage was calculated as follows:

$$H = \left[1 - \left(\frac{OD_f}{OD_0} \right) \right] \times 100$$

2.3.5. Antioxidant Activity

Radical scavenging activity of *K. marxianus* and *S. boulardii* was evaluated by 2,2- diphenyl-1-picrylhydrazyl (DPPH) free radical assay [28]. The degree of discoloration of DPPH indicates the scavenging potential of the strain. A 0.2 mM DPPH solution was freshly prepared in methanol 800 µL of each strain suspension was mixed with 1 mL of 0.2 mmol L⁻¹. DPPH solution in methanol. The samples were incubated for 30 min in the dark at room temperature with agitation and then centrifuged at 2000 rpm for 2 min. The supernatant absorbance was measured at 517 nm. In parallel, ascorbic acid was used as positive control. The scavenging ability (SA) of the strain was calculated using the formula:

$$SA = [1 - \frac{OD_{517}(sample)}{OD_{517}(blank)}] \times 100$$

2.3.6. Assessment of biofilm formation

This was performed as described previously [29] and detected using the crystal violet staining method for total biofilm biomass determination, measured by OD₅₇₀ after crystal violet solubilization with acetic acid [30].

2.3.7. A-431 cells

The human epithelial A-431 cell line derived from a vaginal epithelial squamous cell carcinoma was used. The cell line was purchased from DSZM (Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich Co., St. Louis, MO, USA) supplemented with L-glutamine (2 mM) (Sigma Aldrich Co., St. Louis, MO, USA) , 1% *w/v* penicillin-streptomycin ((Sigma Aldrich Co., St. Louis, MO, USA), and heat-inactivated Fetal Bovine Serum (FBS), at 10% *w/v* (Sigma Aldrich Co., St. Louis, MO, USA); specifically, the cell line was kept in culture by-passages in fresh medium twice a week and incubated at 37 °C and 5% CO₂.

2.3.8. Adhesion to A-431 cells

To allow adhesion to human cells, *K. marxianus* and *S. boulardii* cells at a density of 10⁶ cells mL⁻¹ were added to the cell monolayer in a 48 well multiwell plate and incubated for 2 h in DMEM at 37 °C. Then, wells were washed with PBS, and 100 µL di trypsin/EDTA solution (Sigma Aldrich Co., St. Louis, MO, USA), was added. After 5-6 min incubation, 900 µL di DMEM were added for trypsin inactivation. The content of the wells was serially diluted in PBS and plated onto chloramphenicol RB agar for the yeast cell count.

Also, invasiveness of *K. marxianus* and *S. boulardii* into A-431 cells was determined, following the above described protocol, but, before trypsin treatment, the infected A-431 cells were incubated with 0,5 mg mL⁻¹ amphotericin B for 1 h, to eliminate the adhered cells on the surface and allow to count only the internalized yeast cells.

2.4. Safety Assessment of the *K. marxianus* isolate

2.4.1. Haemolytic activity

The haemolytic activity of the three isolates was carried out according to the method described by Shen et al 2022 [31], by inoculating the strains on blood agar plates (Oxoid Ltd., Basingstoke, UK) containing 5% defibrinated sheep blood for 48 h of incubation at 37 °C in order to detect patterns of haemolysis. Next, cultured plates were observed to assess a haemolytic zone, and a clear zone of hydrolysis around the colonies was considered as a positive result (β-haemolysis).

2.4.2. Antibiotic and antifungal resistance

Antimicrobial resistance was screened by agar disk diffusion method as described by Turchi et al 2013 [32], in accordance with the Clinical and Laboratory Standards (M44, M45). The isolated yeast strain was tested for resistance against the most commonly used antibacterial and antifungal compounds, 12 belonging to the first category and 5 to the latter, each of which has a different mode of action. The molecules tested were gentamicin (10 µg GM), ampicillin (10 µg AMP), polymyxin-B (300 U PB), amoxycillin + clavulanic acid (30 µg AMC), norfloxacin (10 µg NOR), streptomycin (10 µg STR), ceftotaxime (30 µg CTX), ciprofloxacin (10 µg CIP), azithromycin (15 µg AZ), ceftriaxone (30 µg CRO), vancomycin (20 µg VAN), rifampicin (20 µg RIF), fluconazole (10 µg FL), caspofungin (2 µg CSF), ketoconazole (30 µg KET), itraconazole (30 µg ITR), and amphotericin B (2 µg Amph B). After 24–48 h incubation at 30 °C, the diameter of the inhibition zone was measured and the strains were considered susceptible (S), intermediate (I), or resistant (R).

2.4.3. *Galleria mellonella* survival assay

To evaluate the safety of the *K. marxianus* isolate *in vivo*, larvae of *G. mellonella* were used. For each assay, 20 healthy larvae of a similar size, were selected and injected with 10 µL of the *K. marxianus* suspension in PBS through the last left pro-leg, at a density corresponding to 10⁴, 10⁵, 10⁶, 10⁷ yeast cells/larva. A similar procedure was performed for the commercial *S. boulardii*. Intact larvae or larvae injected with PBS alone were used as controls. All samples were incubated at 37 °C for 120 h. Every 24 h the number of viable larvae was registered, and the survival percentage was calculated. Larvae were considered dead when they displayed no response to touch.

2.5. Anticandidal activity of the *K. marxianus* isolate

2.5.1. Co-aggregation of *K. marxianus* with *C. albicans*

The co-aggregation of the *K. marxianus* isolate with *C. albicans*, was determined as previously described [26]. Briefly, 4 mL of a *K. marxianus* overnight culture and 4 mL of *C. albicans* overnight culture were mixed, vortexed for 10 s, and incubated for 5 and 24 h at 37 °C. Each control tube contained 4 mL of each single suspension. The absorbance (OD) of the mixed suspension was then measured at 590 nm (OD_{mix}) and compared with those of the control tubes containing *K. marxianus* (OD_{strain}) and the pathogen (OD_{pathogen}) after incubation. The percentage of co-aggregation (C) was calculated as:

$$C = \left[1 - OD_{mix} \times \frac{(OD_{strain} + OD_{pathogen})}{2} \right] \times 100$$

2.5.2. Exclusion test

The ability of pre-established *K. marxianus* biofilms to prevent the adhesion and biofilm formation of *C. albicans* on polystyrene microplates was evaluated performing an “exclusion test” according to Cisneros et al. 2021 [33], with some modifications. *K. marxianus* cells derived from overnight cultures in YPD were adjusted to 10⁶ CFU mL⁻¹. And seeded in individual wells of a 96 well multiplate. Microplates were first incubated at 37 °C for 24 h to allow cells to attach to the surface of the wells. Thereafter, supernatants were discarded, and a suspension of each *C. albicans* strain in RPMI 10⁶ CFU mL⁻¹ was added to each well containing the *K. marxianus* pre-formed biofilm.

Microplates were incubated at 37 °C for 24 h. Then, wells were carefully washed twice with PBS and then individually scraped to remove all the biofilm biomass. Cells recovered from each well were suspended, diluted in PBS, and then plated on Brilliance Candida Agar Base plates to distinguish and determine the number of viable cells of *C. albicans* and *K. marxianus*, respectively. The plates were incubated at 37 °C for 48 h. Then, for each strain, the number of colonies on the plates was determined. Results were expressed as Log₁₀ (CFU well⁻¹). Biofilm formation of *C. albicans* strains was used as control.

2.5.3. Germ tube test in the presence of *K. marxianus*

The ability of *C. albicans* to form germ tubes was assessed using the germ tube test according to Pericolini et al. [9]. Briefly, an isolated colony of *C. albicans* of each strain (ATCC90028, C7, C14, C17, and C19) taken from a 24 h YPD agar plate, was inoculated into a total volume of 0,5 mL of RPMI plus 10% Fetal Bovine Serum (Fisher Thermo Scientific, Cleveland, OH, USA) in the well of a 24-well multi plate and incubated for 4 h at 37 °C, in the presence or not of 10⁸ cells mL⁻¹ of *K. marxianus* resuspended in RPMI medium. After incubation, the wells were examined at JuLI™ Stage inverted microscope (Nanoentek) at x20 magnification.

2.5.4. Adhesion of *C. albicans* to A-431 cells in the presence of *K. marxianus*

To investigate the probiotic effects of *K. marxianus* on *Candida* adhesion to A-431 cells, we follow the protocol by Kunyet et al. [11] with some modifications. The assays of adhesion on human cells were performed under pre-inoculation, co-inoculation, and post-inoculation conditions in 48 well microplates where A-431 cells in DMEM were at 80% confluency. In all cases the density of the probiotic yeast and of each *C. albicans* strain (ATCC 90028, C7, C14, C17, and C19) was 10⁸ cells mL⁻¹ and 10⁷ cells mL⁻¹, respectively.

In the pre-inoculation condition, *K. marxianus* was inoculated into wells for 2 h at 37 °C. Then, the *Candida* strain was introduced and incubated for an additional 2 h with mild shaking (90 rpm). In the co-inoculation condition, the probiotic strain was co-inoculated with the *Candida* strain and incubated for 2 h. In the post-inoculation condition, the *Candida* strain was inoculated for 2 h before inoculation of *K. marxianus* for further 2 h.

At the end of incubation, the wells were washed with PBS, and their content was serially diluted in PBS. Dilution were plated on Brilliance Candida Agar Base to distinguish *C. albicans* from *K. marxianus* colonies, and count residual *C. albicans* colonies. Results were expressed as percentage inhibition of the adhesion, with respect to controls performed in the same condition without the inoculation of the probiotic.

2.5.5. Cell damage: LDH determination

A-431 Cells were then incubated in the presence or absence of *K. marxianus* (OD₅₉₀ = 5) for 2 h at 37 °C plus 5% CO₂, extensively washed with PBS and then incubated with *C. albicans* ATCC 90028 or *C. albicans* C19 (1×10⁶ mL⁻¹) for 18 h at 37 °C plus 5% CO₂.

After incubation, the epithelial cell damage was determined by the release of lactate dehydrogenase (LDH) into the surrounding medium. Therefore, the medium was collected and LDH was measured spectrophotometrically at 492 nm using a Lactase Dehydrogenase Activity Assay kit (Sigma-Aldrich, St. Louis, MO, USA). The percent cell damage of A-431 infected with *C. albicans* was calculated as follows:

$$\text{Cell damage} = \frac{(\text{LDH activity of treated cells} - \text{spontaneous LDH activity})}{(\text{maximum LDH activity} - \text{spontaneous LDH activity})} \times 100$$

and compared to 100% *C. albicans* damage induced in each cell type [34]. The lysis buffer was used as positive control to determine the maximum LDH activity.

2.5.6. Quantitative analysis of SAP2 and SAP6 gene expression

To check the expression of *SAP2* and *SAP6* genes of *C. albicans* in the presence of *K. marxianus* , aliquots of *C. albicans* cells of ATCC 90028 and C19 strains (10^6 mL⁻¹) were incubated in YPD medium or YPD medium plus 1% of bovine serum albumin (BSA), for 24 h at 37 °C under agitation (150 rpm) in the presence or absence of *K. marxianus* cells (OD₅₉₀=5.0) [9]. Briefly, total RNA was extracted using Direct-zol™ RNA Miniprep Plus Kit (ZYMO RESEARCH). Then, 1 µg of RNA was reverse transcribed to cDNA (Bio-Rad, Milan, Italy) and analyzed by quantitative PCR run in AriaMx Real-Time PCR instrument (Agilent Technologies, Inc., Milan, Italy) according to the manufacturer’s instructions. For real-time PCR reaction 100 ng of cDNA was used. PCR System used: 95 °C for 10 min, one cycle for cDNA denaturation; 95 °C for 15 s and 60 °C for 1 min, 40 cycles for amplification; 95 °C for 15 s, one cycle for final elongation; and one cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. *C. albicans* *SAP2* and *SAP6* genes were detected by using primers previously reported [35]. The expression levels of genes were evaluated using the 2^{-ΔΔCt} method, where Ct was the average threshold cycle number from three independent experiments. Data were presented as the fold change in gene expression normalized to the *ACT1* gene as internal control [36,37].

2.6. Statistical Analyses

GraphPad Prism Software (version 8.02 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed on 14 February 2024) was used for data analysis. All data are shown as mean +/- standard deviation (SD) and were derived from two or three independent experiments. One-way or two-way analysis of variance (ANOVA) followed by Tukey’s test was used for the comparison test. The Kaplan–Meier method and log-rank (Mantel–Cox) test were used to plot *G. mellonella* survival curves.

3. Results

3.1. Isolation and molecular characterization of *K. marxianus* isolate

The yeast isolate from sourdough was subjected to molecular identification. Results of Sanger Sequencing of the yeast colonies is reported in Table 1. The isolate resulted to be *Kluyveromyces marxianus*.

Table 1. Results of Sanger Sequencing performed on yeast colonies isolated from sourdough. Obtained FASTA Sequences were compared to NCBI Sequence Database sequences, using BLASTN ver. 2.2.29.

Identified microorganism	Max score	Total score	Query Cover	e- value	% Identity	Accession N.
<i>Kluyveromyces marxianus</i> strain JYC2557	1223	1223	99%	0.0	99.55%	MK044038.1

3.2. Probiotic properties of the *K. marxianus* isolate

Some characteristics of the *K. marxianus* isolate were determined to assess the potential probiotic properties of the strain. They were: ability to grow at 37 °C, tolerance to gastric and bile acids, auto-aggregation, hydrophobicity, antioxidant power, biofilm forming capacity, and adhesion to epithelial cell lines. In parallel, the same tests were conducted with the commercial *S. boulardii* (Biocodex) strain.

On the whole, these tests are indicative of the adaptation of the isolate to the human body (growth at 37 °C, resistance to acid and bile salts), with potential benefit effect (antioxidant power) and the ability to adhere and colonize the epithelial mucosal membranes (hydrophobicity, auto-aggregation, biofilm forming ability, adhesion).

The results of these tests were reported in Table 2, where a score from 0 (minimum) to 2 (maximum) was given to the result of each test response for both the *K. marxianus* isolate and *S. boulardii*.

Table 2. Results of the tests on the probiotic properties of *K. marxianus* (Km) and *S. boulardii* (Sb), and scores attributed to each test (in italics).

	Grow th at 37 °C	HCl surviv al	Bile salt surviv al	Antioxidat ive power	Auto- aggregati on	Hydrophobi city	Biofil m	Adhesi on	Tot al scor e
	(OD ₅₉₀)	(%)	(%)	(%)	(%)	(%)	(OD ₅₇₀)	(%)	
K m	6.86	100	73	78.5	100	90	4.5	10	
	2	2	1.5	1.75	2	2	2	0.2	13.5
Sb	4.93	100	98	77	100	81	0.3	2.35	
	1.4	2	2	1.75	2	2	0.2	0.05	11.4

For a better visualization of the overall probiotic characteristics of each strain, the indicators in Table 2, were reported in radar diagrams (Figure 1).

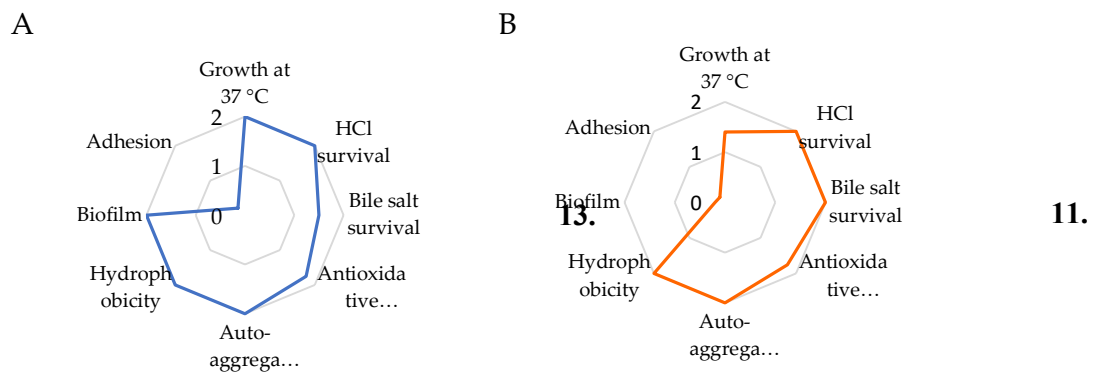


Figure 1. Radar diagrams of the probiotic properties (see Table 1) of *K. marxianus* (A) and *S. boulardii* (B). The total score of the strain is reported in the centre of the diagram.

The *K. marxianus* isolate showed very good probiotic properties (total score 13,5), even better than the commercial *S. boulardii* strain (total score 11,4). Most characteristics result similar between

the two yeasts, but the *K. marxianus* isolate showed a better adaptation to the body temperature (37 °C), a much better ability to form biofilm *in vitro*, and a higher adhesion to A-431 cells than *S. boulardii* (Table 2 and Figure 1). Though adhesion to A-431 cells was not so high, it is worth of note that invasiveness of both the strains into the A-431 cells was absent (data not shown).

3.3. Safety assessment

Safety of the *K. marxianus* isolate was assessed *in vitro* by testing both its haemolytic activity on blood agar plates and resistance to antibiotics and antifungals.

As shown in Figure 2, no haemolytic activity was shown by the *K. marxianus* isolate, as well as by the *S. boulardii* strain.

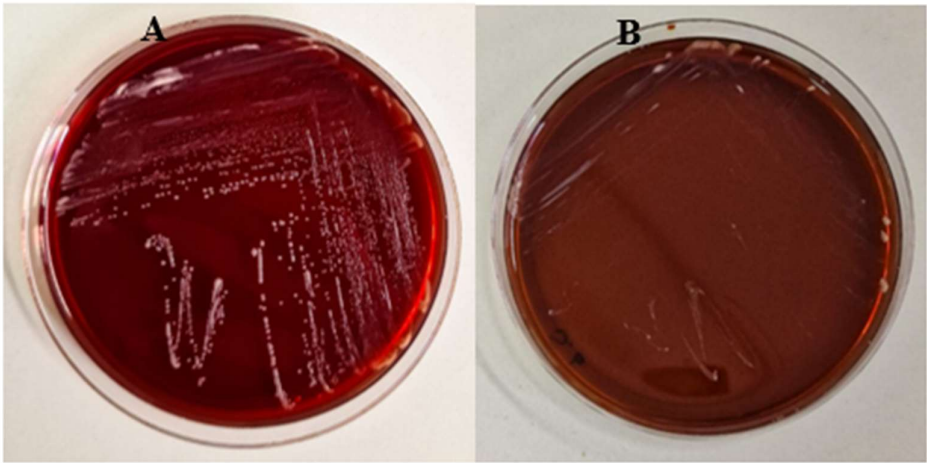


Figure 2. Blood agar plates showing the absence of haemolytic activity for *K. marxianus* (A) and *S. boulardii* (B).

As regards resistance to antimicrobials, the yeast isolate was resistant to all the most common antibiotics, and sensitive to the tested antifungals, except for itraconazole (ITR). A similar response was obtained by *S. boulardii* (Table 3).

Table 3. Antimicrobial resistance of *K. marxianus* isolate (Km) and the commercial *S. boulardii* (Sb). The strains were considered susceptible (S), intermediate (I) or resistant (R).

	G M	AM P	P B	AM C	NO R	ST R	CT X	CI P	A Z	CR O	VA N	RI F	FL C	CS F	KE T	IT R	Amp hB
Km	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R	S
Sb	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R	S

Due to their simplicity of use and low cost, the larvae of *G. mellonella* represent an optimal model to evaluate the virulence of fungi and other microbial species [38], so we used them to assess the safety *in vivo* of the *K. marxianus* isolate. Larvae survival after injection of the yeast strain at different final cell densities (from 10⁴ to 10⁷ yeast cells per larva) was determined. In parallel the same test was carried out with the *S. boulardii* strain. No effect of the yeast injection was observed in the case of the *K. marxianus* isolate, since larvae survival remained 100% up to 120 h incubation, likewise to what observed in the case of the commercial probiotic *S. boulardii* (Figure 3).

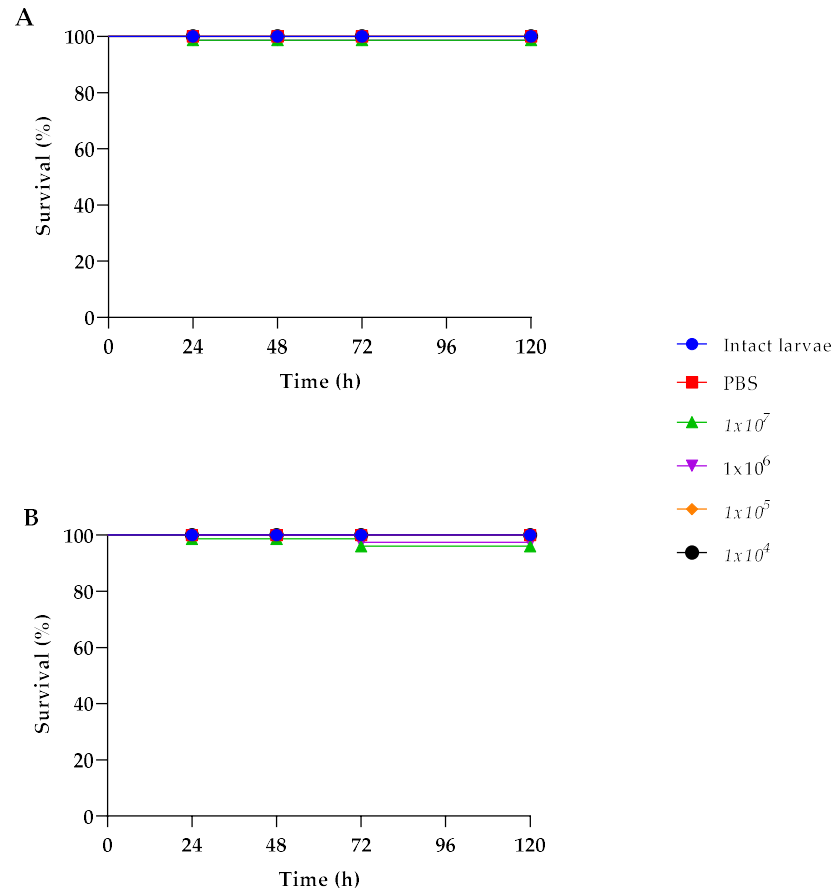


Figure 3. Survival curves of *G. mellonella* larvae injected with *K. marxianus* (A) and *S. boulardii* (B) at density ranging from 10^4 to 10^7 CFU per larva.

3.4. Anticandidal activity of *K. marxianus*

Co-aggregation of a probiotic with a pathogen is indicative of its capacity to prevent subsequent adhesion and colonization, therefore we first tested the ability of the *K. marxianus* isolate to co-aggregate with *C. albicans*. The percentages of co-aggregation were significant, being about 50% with both ATCC 90028 and C19 strain after 5 h incubation and increased up to 75% for both strains after 24 h.

Then, we wanted to assess the effect of the putative probiotic *K. marxianus* on filamentation of *C. albicans* strains, considering that filamentation is a key virulence factor for *Candida* species and positively correlated with adhesion and biofilm formation. In Figure 4 the results of a germ test of *C. albicans* ATCC and *C. albicans* C19 in the presence of *K. marxianus* are shown. A clear full inhibition of the germ tube formation was observed for both the strains. Similar results were obtained with all the other *C. albicans* vaginal strains (data not shown).

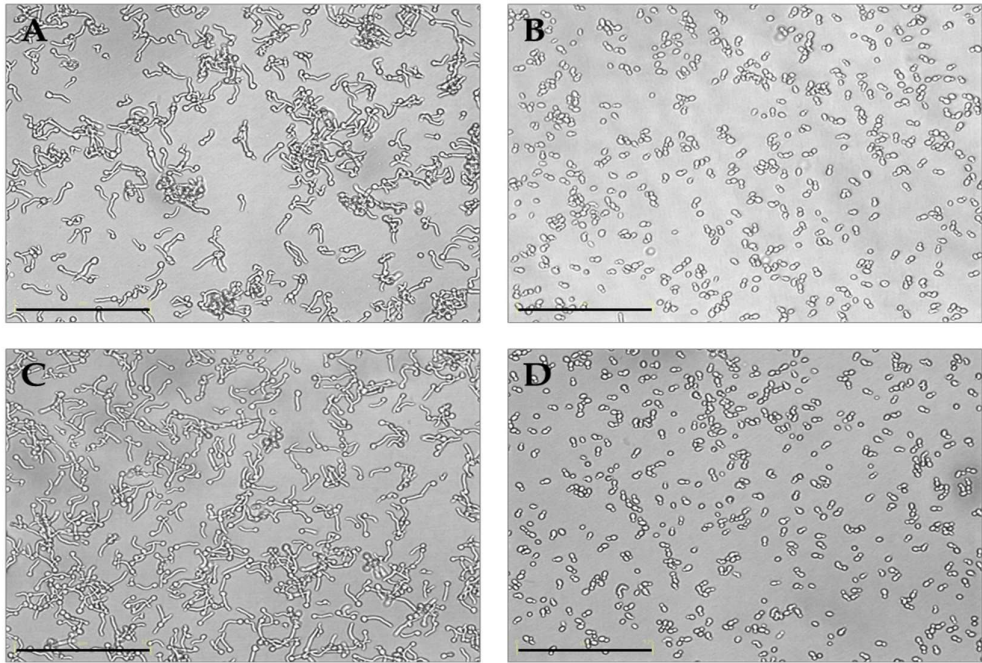


Figure 4. Inhibition of filamentation of *C. albicans* ATCC 90028 (A, B) and *C. albicans* C19 (C, D) in the presence of *K. marxianus* (B, D). In panels A and C controls are shown. Bar corresponds to 125 μm.

As previously reported [23], all the *C. albicans* strains used in this work were able to form biofilms *in vitro* on polystyrene microplates. Here, we wanted to assess the ability of a pre-established biofilm of the *K. marxianus* isolate to prevent such biofilm formation. So, the formation of biofilms of *C. albicans* ATCC 90028 and the clinical strains were evaluated in the so called “exclusion test”. The results clearly indicated that surface colonization and ability to form biofilms was significantly reduced in the presence of a pre-formed *K. marxianus* biofilm for all the *C. albicans* strains examined (Figure 5).

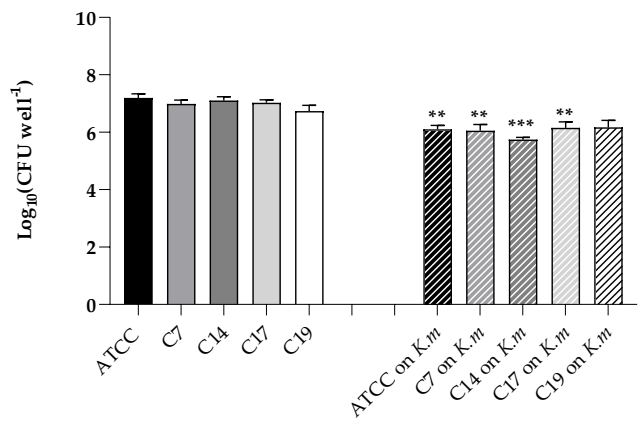


Figure 5. Reduction of biofilm formation (Log₁₀CFU well⁻¹) in polystyrene microwells of *C. albicans* ATCC 90028, C7, C14, C17 and C19 strains in the presence of a preformed *K. marxianus* biofilm. Data reported are the means of three independent experiments ± SDs; statistical significant is indicated by ** $p < 0.01$; *** $p < 0.001$ (Tukey’s test).

Also , we tested the effect of the putative probiotic on the adhesion of *Candida* strains on A-431 cells, investigating different conditions of treatment: pre-inoculation, where the probiotic yeast was added to the cell monolayer prior to application of the *Candida* strains; co-inoculation, where the live probiotic yeast and *Candida* strains were simultaneously applied; and post-inoculation, where

probiotic yeast treatment was performed after the application of *Candida* strains. In the three conditions and for all the strains examined, the reduction of adhesion was clear (Figure 6), and resulted very high (90%) for the strains C7, C14 and C19 in the condition of co-inoculation.

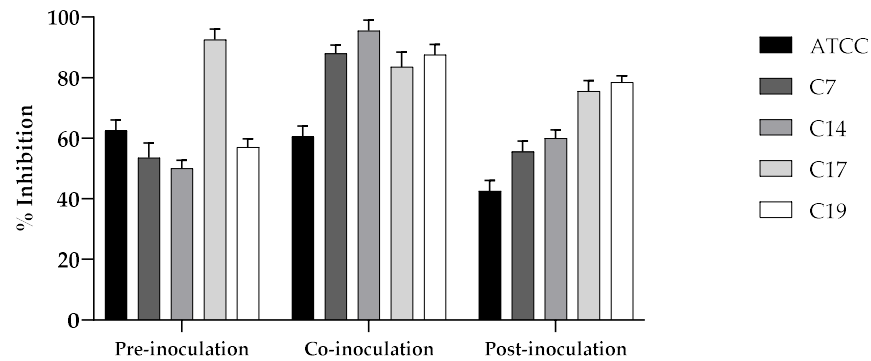


Figure 6. Inhibition of adhesion of *C. albicans* ATCC 90028, C7, C14, C17 and C19 strains on A-431 cells in conditions of pre-inoculation, co-inoculation, and post-inoculation with the *K. marxianus* isolate. Data reported are the means of three independent experiments $\pm$ SDs.

The possible protective effect of *K. marxianus* against the damage provoked by *C. albicans* infection in A-431 cells was also ascertained, determining the LDH release from the cell line preincubated with the *K. marxianus* isolate and then infected with *C. albicans* ATCC 90028 or *C. albicans* C19 for 18 h. The results showed a clear prevention of the cell damage provoked by the *C. albicans* strains in the presence of the probiotic yeast (Figure 7). It is worth noting that the *K. marxianus* isolate did not exert any significant cytotoxic effect on A-431 cells, since the LDH released from cells treated with the probiotic alone was comparable with the spontaneous release of LDH (data not shown).

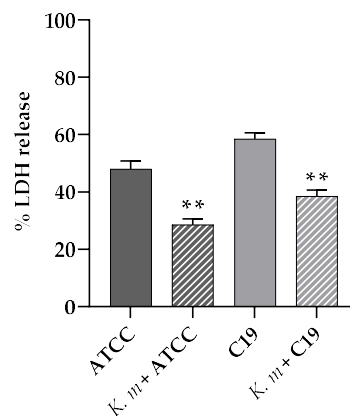


Figure 7. Cell damage evaluated by LDH release from A-431 cells infected with *C. albicans* ATCC 90028 and C19 strain and effect of the probiotic *K. marxianus*. Data reported are the means of three independent experiments $\pm$ SDs; statistical significant is indicated by ** $p < 0.01$ (Sidak's test).

Since aspartyl proteases exert a main contribution to virulence and invasiveness of *C. albicans*, we checked the expression of two of the genes (*SAP2* and *SAP6*) encoding for such proteins when *C. albicans* was co-incubated with the putative probiotic *K. marxianus* isolate. The results of the RTqPCR reported in Figure 8 clearly show that in the presence of the isolate the expression of both *SAP2* and *SAP6* resulted greatly reduced both in the ATCC and the clinical C19 strain.

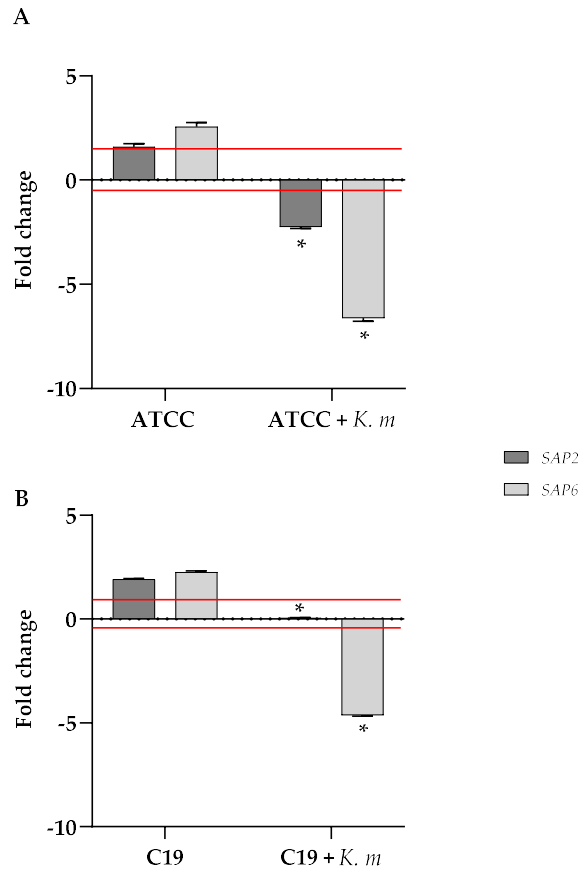


Figure 8. Quantitative analysis of *SAP2* and *SAP6* genes expression in *C. albicans* ATCC 90028 (A) and *C. albicans* C19 (B) strains, incubated in YPD medium plus 1% BSA with or without *K. marxianus*. cDNA quantities were reported as fold changes relative to the *Candida* strain alone. Data reported are the means of three independent experiments $\pm$ SDs. Statistical significant is indicated by * $p < 0.1$ (Sidak's test).

4. Discussion

In the recent years the interest towards the probiotic potential of non-conventional yeasts has greatly increased. Non-*Saccharomyces* yeasts mainly belonging to the genera of *Pichia*, *Debaryomyces*, *Wickerhamomyces*, *Zygosaccharomyces* and *Kluyveromyces* have been isolated from different sources and tested as probiotics, in alternative to the well-established commercial species *S. cerevisiae* var. *boulardii* [6,39].

The food-grade yeast *K. marxianus* is characterized by rapid growth rate, easy cultivation, thermotolerance, versatile metabolism, and ability to secrete lytic enzymes, which make this species particularly suitable for many food and biotechnological applications [40,41].

As specifically regards the probiotic properties of *K. marxianus*, they were ascertained since 2011, when it was shown that the yeast strain isolated from a dairy product [17] was able to adhere to human enterocyte-like Caco-2 cells and modulate colon microbiota, increasing the bifidobacterial concentration, and inducing the formation of higher amounts of the short-chain carboxylic acids acetate and propionate. The strain was *K. marxianus* B0399, which was subsequently introduced in the market as a new generation of probiotic lactic yeast (Turval B0399®).

Later on, other authors have reported several other beneficial effects of *K. marxianus* mainly isolated from dairy products [42]. Very recently Nag et al. [43] observed *in vitro* that this yeast improved insulin sensitivity and reduced fat storage in fat cells, suggesting benefits for type 2 diabetes and obesity. Moreover, *K. marxianus* produce high value-added bioingredients (oligo-

saccharides, -nucleotides, -peptides) that, used as prebiotics, have revealed to increase the intestinal microbiota or added to foods, enhance flavours, stabilize food emulsions, act as immunopotentiators, have hypocholesterolemic action, and promote protection against bacterial infections [44].

In this work, we isolated a *K. marxianus* strain from a sourdough used in an artisanal bakery as starter for the leavening of bread. This practice, which has recently undergone a revival also in industrial bakeries, exploits the spontaneous fermentation of yeasts and lactobacilli present in the sourdough, and confers the final product a distinctive taste and aroma.

Aiming at proposing the new *K. marxianus* isolate as a potential probiotic, we performed an initial *in vitro* screening of the properties necessary to adapt to the human body with beneficial effects. The tests were done in parallel with a reference probiotic strain, the commercial *S. boulardii* (Biocodex). To comprehensively evaluate and visualize the results, radar charts were used [31], which clearly indicate that the new strain has excellent probiotic properties, a result which is in agreement with those reported for other *K. marxianus* isolates [22]. The comparison showed that *K. marxianus* better adapts to the body temperature and forms thicker biofilm *in vitro*. A good biofilm ability on abiotic surfaces has been reported in the case of several *K. marxianus* strains from different dairy products, and it is considered an essential trait for the outcome of the final product [45]. As regards the adhesion to vaginal epithelial cells, it resulted higher than that of *S. boulardii* on the same cells, but not so high (10%). Many different values of adhesion to Caco-2 cells have been reported for *K. marxianus*, varying from 8 to 19 % depending on the strain [46].

Adhesion of probiotics to epithelial cells is generally viewed as a prerequisite for their colonization, which may constitute a physical barrier against the adhesion of pathogens to the mucosa. However, also low-adhering *S. cerevisiae* strains have shown to display protective effects towards bacterial infections, *via* reduction of the intestinal pro-inflammatory response [16]. For some authors [47,48] a good adhesion of a probiotic strain is even considered an undesirable virulence factor, because preceding the possible invasion of the strain into the human cells. In our case, it is worth of note that invasion of *K. marxianus* into A-431 cells was totally absent.

Safety evaluation was conducted to confirm the non-pathogenic nature of the *K. marxianus* isolate: the yeast resulted non-haemolytic, resistant to all the most common antibiotics, and sensitive to the tested common antifungals. Then, to assess the safety of the *K. marxianus* isolate also *in vivo*, we used the *G. mellonella* model. Indeed, *G. mellonella* larvae have been established as an animal model alternative to mammals to test the pathogenicity of fungal species since 2000 [49], and more recently used for the study of the safety of probiotic putative strains *in vivo* [26,50]. Our results clearly showed that the survival of the *Galleria* larvae was not affected at all by the injection with *K. marxianus*, even at the highest density tested, similarly to what observed with *S. boulardii*. So, the *in vivo* tests unequivocally assessed the safety of the new *K. marxianus* isolate.

The opportunistic pathogen yeast *C. albicans* is the agent of the vast majority of VVC, often associated to strains resistant to conventional antifungals. In the perspective of a possible use of *K. marxianus* as a novel probiotic for both prevention and therapy of VVC, we analysed the antagonistic activity of the yeast isolate against some vaginal strains of *C. albicans*.

Our results clearly showed the co-aggregation ability of *K. marxianus* with *C. albicans* and a full inhibition of the germ tube formation upon treatment with the live yeast, a result in agreement with other authors who found the same effects with *Saccharomyces* strains [9,10]. It is well known that the polymorphic *C. albicans* may shift from the yeast-like morphology to germ tube, pseudo-hyphae and hyphae. Such filamentation is pivotal for biofilm formation and epithelial invasion, representing a well-known virulence factor for *Candida* [51].

Further, and in accordance with this result, we demonstrated a reduction of biofilm formation *in vitro* for all the tested *C. albicans* strains in the presence of *K. marxianus* preformed biofilm. Such "exclusion effect" [33] is indicative of a clear antagonism between the putative probiotic and *Candida*, due to a physical block for the pathogen colonization on the abiotic surface, without excluding the contribution of other mechanisms of action.

The adhesion of *Candida* to epithelial vaginal cells is certainly mandatory for invasiveness and pathogenesis, so we also investigated the effect of *K. marxianus* on *Candida* adhesion to A-431 cells. It resulted significantly impaired by the presence of the live putative probiotic, which showed its efficacy in both inhibition of adhesion and displacement of *Candida* cells, suggesting its potential use in both preventing and ameliorating *Candida* infections. These results are in agreement with those obtained by Kunyet et al. [11] with *Saccharomyces* strains able to hinder *Candida* adhesion both on polystyrene surfaces and A-431 cell lines.

Indeed, the presence of *K. marxianus* isolate protected the epithelial cells from the damage induced by *C. albicans*, as shown by the less amount of LDH released from A-431 cells pretreated with the probiotic strain and then infected with the pathogen.

The secretion of aspartyl proteases encoded by *SAP* genes plays an essential role in the development of pathogenesis of *C. albicans*, being involved in the shift of the yeast to hyphal morphology which is determinant for invasiveness [52]. Consistently with the observed inhibition of the germ tube formation, we also demonstrated the ability of the probiotic isolate to reduce the expression of *SAP2* and *SAP6* genes in *Candida* under conditions of serum-induced hyphal differentiation. This result is in agreement to what already observed for other yeasts proposed as anticandidal probiotic agents [9,10].

Apparently, *K. marxianus* is able not only to protect the epithelial cells from adhesion *via* a mechanical hindrance to *Candida* colonization, but also to affect some virulence factors such as hyphal differentiation and secretion of aspartyl proteases, presumably due to products of its metabolism and/or competition for those serum components inducing *Candida* hyphal morphogenesis. Further investigations are necessary to shed light in the details of *Kluyveromyces/Candida* interactions.

In conclusion, our results enlarge the spectrum of the beneficial properties of the food-grade yeast *K. marxianus* showing for the first time its biotherapeutic potential in the prevention and treatment of VVC.

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