



Effect of *Saccharomyces boulardii* Extract on *SAP2* Gene Expression and Antifungal Susceptibility of *Candida albicans*

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Received 2017 August 26; Revised 2017 December 23; Accepted 2018 January 09.

Abstract

Background: *Saccharomyces boulardii*, a non-pathogenic yeast, is effective on prevention and treatment of intestinal infections caused by bacterial pathogens and also affects the virulence factors of *Candida albicans*. *SAP2*, an important potential virulence factor of *C. albicans* is a suitable target for a new research.

Objectives: The current study aimed at investigating the effect of *S. boulardii* extract on the expression of *SAP2* gene and antifungal susceptibility of *C. albicans* isolates.

Methods: The current study investigated *SAP2* relative expression and antifungal susceptibility patterns of *C. albicans* species isolated from oral lesions of HIV⁺ patients and the standard strain ATCC 10231 after exposure to *S. boulardii* extract. Susceptibility of *C. albicans* isolates to antifungal drugs was assessed according to those proposed in the clinical and laboratory standards institute (CLSI) M44-A2 reference method. The real-time polymerase chain reaction (PCR) was performed to evaluate *SAP2* gene expression.

Results: *Saccharomyces boulardii* extract 0.48 mg/mL changed the antifungal susceptibility pattern of *C. albicans* isolates to ketoconazole and itraconazole. Before exposure, 53.3% of the isolates were sensitive to ketoconazole, while it increased to 73.3% after exposure to *S. boulardii* extract. The sensitivity to itraconazole increased from 6.7% to 26.7% after exposure to *S. boulardii* extract. Although susceptibility of *C. albicans* isolates to other antibiotics (fluconazole, clotrimazole, nystatin, and amphotericin-B) did not change after exposure to *S. boulardii* extract, there was a significant difference in the average inhibitory zones of the isolates after exposure to *S. boulardii* extract.

Conclusions: The level of *SAP2* expression significantly reduced in the isolates treated with 0.48 mg/mL *S. boulardii* extract. Comparison of *SAP2* gene expression between ketoconazole-resistant and -intermediate isolates, and the ketoconazole-sensitive ones demonstrated the relative higher gene expression in resistant and intermediate isolates. *Saccharomyces boulardii* extract can be considered as a suitable probiotic candidate to prevent and treat *C. albicans* infections.

Keywords: Gene Expression, *Saccharomyces boulardii*, *SAP2*, *Candida albicans*

1. Background

Candida albicans, a normal flora in most human, is predominantly isolated from the skin, mouth, vagina, and gastrointestinal tract. Under certain conditions such as indiscriminate consumption of antifungal drugs, immunosuppressive treatments, long-term catheterization, and longer survival of immunocompromised individuals, *C. albicans* changes from commensal microflora to opportunistic pathogens causing life-threatening infections (1). Resistance of *albicans* and non-*albicans* *Candida* spp. to antifungal drugs, especially azoles, is dramatically increasing, and therefore, further researches are needed to inves-

tigate the effect of other agents on their virulence properties. Oropharyngeal candidiasis (OPC) is the major HIV related oral lesion and about 90% of HIV⁺ patients develop oropharyngeal or esophagol candidiasis (2). Oropharyngeal candidiasis due to antimicrobial resistance of *Candida* species is a major problem for HIV⁺ patients (3).

The most important virulence factors of *C. albicans* are adhesion, dimorphism, phenotypic switching, cell wall components (beta-glucans and chitin), phospholipase production, biofilm formation, and aspartyl proteinase secretion (4). Among these factors, 10 proteins (*SAP1- SAP10*) usually involve in tissue invasions. Several studies confirmed

that hyphal formation, adhesion, and phenotypic switching are attributed to the production of such proteins (5, 6). *SAP2* is the most effective factor on the hydrolysis of many proteins, including albumin, haemoglobin, keratin, and immunoglobulins (7). An animal model study showed that *SAP2* could remove yeasts and there was no sign of infection in the studies mice. Tissue analysis of the mice infected with *SAP2* indicated the significant removal of *C. albicans* and decreased ability of yeasts for colonization and adhesion to cell surface (8).

Saccharomyces boulardii, non-pathogenic yeast, are used as probiotics in prevention and treatment of diarrhoea (9). The yeast emerges its pathogenicity through several ways such as stimulation of enzymatic activity and immune responses in the intestinal mucosa, regulation of host cell signals, and pro-inflammatory gene expression (10). *Saccharomyces boulardii* stimulates the intestinal mucosa by the secretion of nutritional factors and polyamines, which enhance host immune defence (11). Oral administration of live *S. boulardii* prevents *C. albicans* infection in mesenteric lymph nodes, blood, and some organs such as the liver and kidneys (12). *Saccharomyces boulardii* reduces inflammation and intestinal colonization of *C. albicans* in mice (13).

2. Objectives

The present study aimed at evaluating the impact of *S. boulardii* extract on *SAP2* gene expression and its antifungal susceptibility on *C. albicans* isolates.

3. Methods

3.1. Fungal Isolates and Growth Conditions

Fourteen clinical species of *C. albicans* isolated from oral lesions of HIV⁺ patients and the standard strain ATCC 10231 were provided by mycology research center, faculty of veterinary Medicine, University of Tehran, Iran. Lyophilized *S. boulardii* (Biocodex laboratories) was kindly donated by Dr. Mahzonieh (Shahrekord University). *Candida albicans* isolates were grown on Sabouraud dextrose agar (SDA, Himedia labs, India) and *S. boulardii* isolates on potato dextrose agar (PDA, Himedia labs, India) both at 30°C for 48 hours.

3.2. Preparation of *S. boulardii* Extract

Saccharomyces boulardii extract was prepared as described in the authors' previous paper (14) with some modifications. For this purpose, a single colony of *S. boulardii* was inoculated into 5 mL of 2 media cultures: 1-Yeast nitrogen base (YNB, Sigma, Germany) (pH 5.5) plus 2% D-glucose,

2- Potato dextrose broth (PDB, Ibresco, Iran); both incubated for 18 hours at 30°C. The pre-cultures were then inoculated into 250 mL YNB and PDB media and reincubated for 24 hours. One liter of the 24-h *S. boulardii* culture was centrifuged at 3000 × g for 10 minutes; supernatant was separated and passed through 0.22-mm filter (Sartorius, Germany) for sterilization. Then, the supernatant was extracted with one-fifth (v/v) of the solvent (ethylacetate) for 3 hours, with changing the solvent every 30 minutes. Solvent was removed by the rotary evaporator. Finally, 20- and 50-mg dry masses were obtained from *S. boulardii* YNB and PDB cultures. Extracted residues were normalized with methanol to the final concentration of 48 mg/mL and used as stock samples. Methanol concentration in the samples was less than 1%. Dry mass obtained from *S. boulardii* PDB culture was used in the experiments.

3.3. Antifungal Assay

Antifungal activity of *S. boulardii* extract against *C. albicans* isolates was assessed by those proposed in the clinical and laboratory standards institute (CLSI) M27-A3, the micro broth dilution reference method (15). Serial dilutions of the stock solution were prepared in RPMI 1640 with MOPS buffer (Sigma, St Louis, MO, USA) to get the final serial concentrations of *S. boulardii* extract ranging from 0.375 to 48 mg/mL. One millilitre of fungal suspension ($1 - 8 \times 10^3$ cell/mL) was distributed in a 96-well plate and incubated at 30°C for 48 hours and fungal growth was then evaluated visually. Positive control comprised of broth media and fungal suspension and the negative control contained *S. boulardii* extract inoculated into broth media; all cultures were incubated under the same conditions. The lowest concentration that did not permit any visible fungal growth was considered as the minimum inhibitory concentration (MIC). The minimum fungicidal concentration (MFC) was the lowest concentration that did not permit any growth on the plate. The procedure was repeated three times for all the isolates.

3.4. Antifungal Susceptibility

Susceptibility of *C. albicans* species to antifungal drugs was assessed according to M44-A2 CLSI (16). Mueller-Hinton agar (Himedia labs, India) containing 2% glucose was used for this purpose; accordingly, a fungal suspension (10^6 cell/mL) was prepared. After 48 hours incubation at 30°C, the diameter of growth-inhibition zone was measured in millimetres. This process was performed before and after the treatment with *S. boulardii* extract. Standard antifungal disks (Rosco, Denmark) used in this experiment were: fluconazole 25 µg, ketoconazole 15 µg, itraconazole 10 µg, clotrimazole 10 µg, nystatin 50 µg, and amphotericin-B 10 µg.

3.5. Gene Expression Assay

To induce *SAP2* expression, the protocol described by Wu et al., was used (17) with some modifications. *Candida albicans* was grown in yeast carbon base (YCB, Sigma, USA) plus 2% D-glucose and 2% bovine serum albumin (BSA, Sigma, USA) in a shaker incubator for 18 hours at 30°C; then, the culture was washed with PBS (phosphate buffer saline) to get a final concentration of 10⁵ cell/mL. Each sample included 10 mL of *C. albicans* culture and 0.48 mg/mL of *S. boulardii* extract. All samples centrifuged at 3000 × g for 10 minutes. Total RNA was extracted from the obtained pellets using total RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. The quantity of the extracted RNA was determined measuring the optical density (OD) at 260 and 280 nm wavelengths using a UV/VIS spectrophotometer (Ultrospec 2000, Pharmacia). The cDNA was synthesized using QuantiTect Reverse (Qiagen, Germany), according to the manufacturer's instructions for 1 µg of total RNA.

To perform real-time polymerase chain reaction (PCR), primers were designed using the NCBI Primer-Blast (Table 1). The *Act1* gene was used as the housekeeping gene. The real-time PCR was performed as described in the authors' previous paper (8) on a real-time thermal cycler (Rotor-Gene Q; Qiagen, Germany). The reaction conditions were 95°C for 15 minutes, followed by 45 cycles at 94°C for 15 seconds and 60°C for 1 minute. The mRNA expression level of *SAP2* and *Act-1* was determined using QuantiTect SYBR Green PCR Kit (Qiagen). Real-time PCR was carried out in a total volume of 10 µL containing: 1 µL of cDNA, 5 µL QuantiTect SYBR Green PCR Kit (Qiagen), and 10 pM of each forward and reverse specific primer. Amplicons specificity was checked by verifying a single peak on the melting curves. All samples and controls were normalized against the reference gene. No control template and reverse transcriptase control were included in each PCR run. All assays were carried out 3 times as independent PCR runs for each cDNA sample. The $\Delta\Delta CT$ method (18) was used to assess the quantification.

Table 1. Primer Sequences Used for Real-Time PCR to Detect *Candida albicans* *SAP2* Gene

Primer	Primer Sequence
<i>SAP2</i> (FW)	TGCTCTTGCTATTGCTTTATTAGTC
<i>SAP2</i> (REV)	TAGTAACTGGGAATGCTTTAGGAG
<i>Act1</i> (FW)	CTTCACTGCCATTCTAATTGTCTCA
<i>Act1</i> (REV)	GCTGTCCAACCTTTGATGAGTTCC

3.6. Statistical Analysis

Statistical analysis was performed using SSPS version 19; P < 0.05. Paired and one-sample Student t tests were used to examine significant differences between the groups.

4. Results

Saccharomyces boulardii extract was prepared on 2 media cultures: YNB plus 2% D-glucose, and PDB. The total dry mass obtained from 1 litre of YNB culture was 20 mg, whereas it was 50 mg from PDB culture. *Saccharomyces boulardii* extract did not show any fungicidal and inhibitory effects against *C. albicans* isolates. Antifungal susceptibility of *C. albicans* to ketoconazole and itraconazole changed after treatment with *S. boulardii* extract. Before the exposure of *C. albicans* isolates to *S. boulardii* extract, %53.3 of the isolates were sensitive to ketoconazole, whereas it increased to %73.3 after the exposure to ketoconazole. The rate of sensitivity to itraconazole increased from %6.7 to %26.7 of after treatment (Table 2).

Table 2. Ketoconazole and Itraconazole Susceptibility Pattern of *Candida albicans* Before and After Treatment with *Saccharomyces boulardii* Extract

Susceptibility Pattern	Before Exposure (%)	After Exposure (%)
Ketoconazole		
Sensitive	53.3	73.3
Intermediate	33.3	26.7
Resistant	13.3	0
Itraconazole		
Sensitive	6.7	26.7
Intermediate	86.7	73.3
Resistant	6.7	0

Although susceptibility pattern of *C. albicans* isolates to other antibiotics (fluconazole, clotrimazole, nystatin, and amphotericin-B) did not change after exposure to *S. boulardii* extract, their antifungal inhibitory zones showed a significant difference before and after the exposure to the extract. The mean size of the growth-inhibitory zone in the treated isolates was significantly higher than that of the untreated isolates (Figure 1 and Table 3).

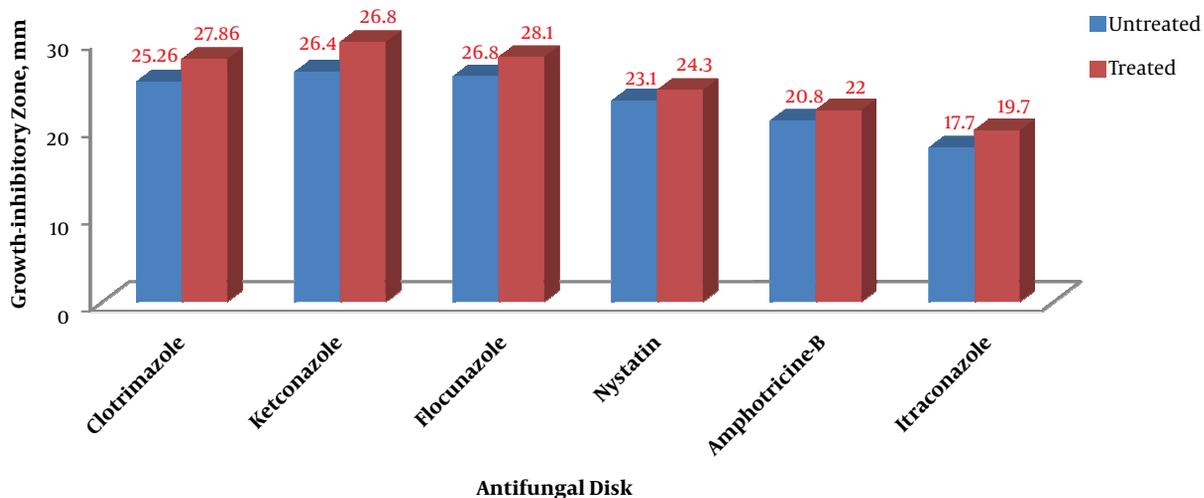
To determine the MIC of the extract influenced by *SAP2* gene expression, 0.48 - 0.24 and 0.16 mg/mL concentrations were examined. *SAP2* gene expression did not show any changes in 0.16 mg/mL. *SAP2* gene expression also showed no significant reduction in the treated isolates compared with the untreated ones in 0.24 mg/mL of the

Table 3. Comparison of the Mean Size of Growth Inhibitory Zone of *Candida albicans* Isolates Before and After Treatment with *Saccharomyces boulardii* Extract^a

Pair (Before and After Treatment with <i>S. boulardii</i> Extract)	Mean (mm)	Std. Error Mean	Sig.
Clotrimazole		0.60000	0.001
Before	25.2		
After	27.86		
Ketoconazole		1.09022	0.008
Before	26.4		
After	29.8		
Itraconazole		0.60945	0.005
Before	17.7		
After	19.7		
Fluconazole		0.65611	0.005
Before	25.9		
After	28.1		
Nystatin		0.36775	0.006
Before	23.1		
After	24.3		
Amphotericin-B		0.36775	0.006
Before	20.8		
After	22		

^a A significant difference was observed between the mean sizes of growth inhibitory zone in treated and untreated *C. albicans* isolates. Statistical analysis was performed using the paired samples t test. P values < 0.05 were considered significant.

Figure 1. Mean Size of the Growth-Inhibitory Zone (Untreated/Treated)

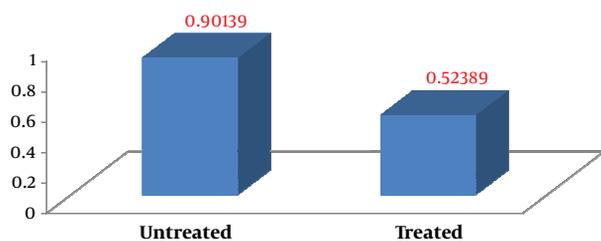


The mean size of growth-inhibitory zone in the treated *C. albicans* isolates is significantly higher than that of the untreated isolates.

extract. In 0.48 mg/mL *S. boulardii* extract, the *SAP2* expression level was significantly downregulated ($P < 0.009$) (Figure 2).

Independent samples t test used to compare the mean values of *SAP2* gene expression in ketoconazole-resistant, -intermediate, and -sensitive *C. albicans* isolates demon-

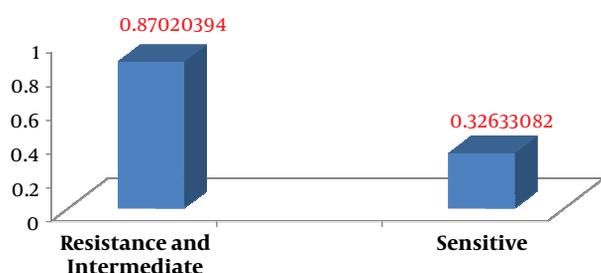
Figure 2. SAP2 Relative Expression Level in Untreated/Treated *Candida albicans* Isolates in 0.48 mg/mL of *S. boulardii* Extract



Statistical analysis was performed using independent samples t test. P values < 0.05 were considered significant.

stated that the SAP2 gene expression level in resistant and intermediate isolates was significantly higher than that of sensitive isolates ($P < 0.02$) (Figure 3 and Table 4).

Figure 3. SAP2 Relative Expression Level in Ketoconazole-Resistant,-Intermediate, and -Sensitive *C. Albicans* Isolates



Statistical analysis was performed using independent samples t test. P values < 0.05 were considered significant.

5. Discussion

Resistance of *C. albicans* against antifungal agents is dramatically increasing in recent decades due to indiscriminate consumption of antimicrobial agents in order to prevent and treat infections. SAP2, an important potential virulence factor of *C. albicans*, is a suitable target for a new research. The current study aimed at investigating SAP2 gene expression and antifungal susceptibility patterns of *C. albicans* species isolated from oral lesion of HIV⁺ patients and *C. albicans* standard strain ATCC10231 before and after treatment with *S. boulardii* extract. Dry mass obtained from 1 litre of YNB culture was 20 mg, whereas it was 50 g for the PDB culture. The current study achievements were higher than previous reports by 25 - 35 mg dry mass from 2 litres of YNB culture (14). *Saccharomyces boulardii*

extract did not show any fungicidal and inhibitory effects against *C. albicans* isolates.

Results of the current study were similar to those of the previous ones; confirming the potential ability of *S. boulardii* extract on growth restriction and removal of *C. albicans* (14, 19). Dry masses obtained from PDB and YNB cultures and used in the current study experiments showed similar results. Antifungal susceptibility pattern of *C. albicans* to ketoconazole and itraconazole changed after treatment with *S. boulardii* extract. The SAP2 relative expression level was significantly downregulated after the exposure to 0.48 mg/mL of *S. boulardii* extract. Also, SAP2 gene expression level in resistance and intermediate isolates was significantly higher than that of the sensitive isolates. The results are similar to those of previous studies.

Wenli Feng et al., found that SAP2 activity of itraconazole-resistant *C. albicans* strains was higher than that of itraconazole-sensitive strains. They measured relative expression levels of *CDR1*, *CDR2*, *MDR1*, and *SAP2* using real-time PCR. Positive correlation between relative expression of *SAP2* and *MDR1* was demonstrated (20). Anna Murzyn et al., reported that the expression level of *HWP1*, *INO1*, and *CSH1* (genes associated with *C. albicans* virulence) decreased in the isolates treated with *S. boulardii* extract (21). Anna Krasowska et al., demonstrated the inhibitory effect of live *S. boulardii* and its extract on the hyphal and pseudohyphal formation (14). Results obtained from the current and previous researches indicated that *S. boulardii* extract can inhibit some virulence factors such as SAP2, and thereby, change antifungal susceptibility of *C. albicans*.

6. Conclusion

In conclusion, since *S. boulardii* extract did not show any fungicidal and inhibitory effects against *C. albicans*, it can be considered as a suitable probiotic candidate to control and treat *C. albicans* infections.

Acknowledgments

The authors are grateful to Prof Mahzonieh (faculty of veterinary Medicine, Shahrekord University, Iran) for the kindly donation of *S. boulardii* (Biocodex laboratories). They also thank Prof Khosravi and Dr Ashrafi Tamai (faculty of veterinary Medicine, Tehran University) for the kindly donation of *C. albicans* isolates.

Footnotes

Authors' Contribution: Study concept and design, Reza Mohammad Salehi, Parviz Owlia, Mansour Bayat, Seyed

Table 4. Comparison of the Mean SAP2 Relative Expression in Ketoconazole-Resistant, -Intermediate, and -Sensitive *Candida albicans* Isolates^a

Susceptibility Pattern	No.	Std. Error Mean	Mean	Sig.
Resistance and intermediate species	7	0.177166733	0.87020394	0.020
Sensitive species	8	0.71790599	0.32633082	

^aStatistical analysis was performed using independent samples t test. P values < 0.05 were considered significant.

Latif Mousavi Gargari, and Seyed Jamal Hashemi; acquisition of data, Reza Mohammad Salehi, Parviz Owlia, and Seyed Latif Mousavi Gargari; analysis and interpretation of data, Reza Mohammad Salehi, Parviz Owlia, and Seyed Latif Mousavi Gargari; drafting of the manuscript, Reza Mohammad Salehi, Parviz Owlia, and Seyed Latif Mousavi Gargari; critical revision of the manuscript for important intellectual content, Parviz Owlia and Seyed Latif Mousavi Gargari; statistical analysis, Reza Mohammad Salehi, Parviz Owlia, and Seyed Latif Mousavi Gargari; administrative technical and material support, Reza Mohammad Salehi, Parviz Owlia, and Seyed Latif Mousavi Gargari; study supervision, Reza Mohammad Salehi, Parviz Owlia, Mansour Bayat, Seyed Latif Mousavi Gargari, and Seyed Jamal Hashemi.

Conflict of Interests: The authors declared no conflict of interest.

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