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Prevalence of Virulence Genes and Antifungal Resistance in *Candida albicans* Isolated from Raw Goat Milk

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ABSTRACT

The contamination of goat milk with pathogenic fungi can cause health hazards for the consumers either they consume it raw or even in the processed form. Since there are few studies concerning yeasts in raw goat milk, the present study aimed to determine the prevalence of yeasts and isolate *Candida albicans* from raw goat milk samples. Also, this study determined the distribution of virulence genes and the antifungal susceptibility profile of *Candida albicans* isolates. A total of 30 goat milk samples (collected from free-grazing goats) were mycologically examined. The confirmed *Candida albicans* isolates were subjected to PCR assay to detect the virulence genes (SAP4, RAS1, ALS1, HWP1, and PLB1). Also, antifungal sensitivity testing was performed against the commercially available antifungal agents and probiotics (*Lactobacillus acidophilus* and *Lactobacillus plantarum*). The mycological examination revealed that 14 out of 30 (46.7%) goat milk samples were positive for yeasts and only 4 (13.3%) isolates were confirmed as *Candida albicans*. The results from the PCR assay showed that RAS1 and ALS1 were found in 4 (100%) isolates, HWP1 and SAP4 were found in 2 (50%) isolates, while PLB1 was not detected in tested *Candida albicans* isolates (0%). Antifungal sensitivity testing results showed that ketoconazole gave the best activity against *Candida albicans* isolates, followed by fluconazole, nystatin, and itraconazole. All isolates were resistant to terbinafine. Moreover, both *Lactobacillus acidophilus* and *Lactobacillus plantarum* showed antifungal effects against *Candida albicans*, but *Lactobacillus plantarum* was more effective than *Lactobacillus acidophilus*. Antifungal resistance is a major problem that can lead to failure of candidiasis treatment. Regular antifungal sensitivity testing and searching for an alternative bio-eco-friendly approach for proper control and treatment of candidiasis are strongly needed to prevent treatment failure and emergence of resistant isolates.

Keywords: Antifungal sensitivity testing, *Candida albicans*, Goat milk, Virulence genes, Probiotics.

INTRODUCTION

Yeasts are considered an important component of the microflora of dairy products and are usually found in large counts in milk because it is rich in proteins, sugars, lipids, and organic acids. Yeasts can cause biochemical deterioration in milk, leading to a serious impact on public health (Spanamberg et al., 2009). Fungi also affect milk quality and shelf life (Hasan and Yassein, 2018).

Genus *Candida* contains approximately 200 yeasts, of which *Candida albicans* is the most commonly isolated one. *Candida* species are opportunistic pathogens that are commensally found in the oral cavity, digestive system, and vagina. However, they can also cause systemic infections especially in immunocompromised and hospitalized persons (Hizlisoy et al., 2020). In *Candida albicans* pathogenesis, the adhesion and biofilm formation are assumed to be under the control of the hyphal wall protein1 (Hwp1) that is found on the surface of yeast hyphae (Sundstrom et al., 2002). Hwp1 encoding gene is part of a core of eight genes that are induced during the filamentation process of *Candida albicans*. The adhesion process of *Candida albicans* is also induced by agglutinin-like sequence 1 (ALS1) that facilitates the adherence to the endothelial cells of host and also it is important for hyphal development in addition to adherence to endothelial cells of the host (Fu et al., 2002). ALS1 and ALS3 are two genes with similar sequences and functions. Furthermore, phospholipase B (PLB1) helps in the pathogenesis of *Candida albicans* as it can mediate the systemic gastrointestinal tract manifestations (Samaranayake et al., 2005). The secreted aspartyl proteinases (Saps) help in the hydrolysis of peptide bonds of the host proteins, supposed to be a part of the virulence mechanism of *Candida albicans* (Naglik et al., 2003).

The existence of virulence genes and the elevation in the prevalence of resistance against antifungal agents have been incriminated in the pathogenesis of *Candida albicans*. The antifungal resistance is increasing due to the use of inadequate doses of the selective therapies as well as the frequent use of antifungal agents for fungal infection prophylaxis in both humans and animals (Mendes et al., 2018). However, the relationship between virulence genes and the resistance profiles of *Candida albicans* has not been sufficiently investigated, particularly in deep *Candida albicans* infections (Shrief et al., 2019). In recent years, alternative approaches to proper control and treatment of fungal diseases have been explored.

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Probiotic bacteria are utilized in animal and human feeding to stimulate the balance of intestinal microbiota of the body. They also improve digestion, strengthen the immune system, and promote the production of vitamins. The use of probiotics can reduce the use of antibiotics and enhance animal growth (De Baets et al., 2009). *Lactobacillus acidophilus* and *Lactobacillus plantarum* are considered the commonly isolated species of *Lactobacillus* in the gastrointestinal tract and are known as probiotics (Gudadappanavar et al., 2017). *Lactobacillus* species have the potential to produce different antimicrobial compounds such as acetic acid, hydrogen peroxide, lactic acid, and different types of bacteriocins such as small heat-tolerant lantibiotics (SHSL), non-lanthionine-incorporating membrane-active peptides (MAP), larger heat-sensitive proteins (LHLP), and complex bacteriocins that include one or various chemical constituents. Due to their capability to produce variable antimicrobial compounds, these probiotics can be used for treatment and control of various manifestations (Spinler et al., 2008).

The present work aimed to isolate *Candida albicans* from raw goat milk, detect virulence genes and perform the antifungal sensitivity testing against the commercially available antifungal agents (fluconazole, itraconazole, ketoconazole, terbinafine, and nystatin) as well as investigate the ability of probiotics (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) to suppress the growth of *Candida albicans.*

MATERIALS AND METHODS

Candida isolation and identification

A total of 30 goat milk samples were collected from free-grazing goats, subcultured on Sabouraud dextrose agar plates, and incubated at 30 °C for 72 h. Pure colonies were picked to make morphological, differential biochemical identification tests and germ tube test according to (Deorukhkar and Roushani, 2018).

Virulence genes detection

DNA extraction

DNA extraction was done using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's instructions. In brief, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56 °C for 10 min. After the end of the incubation period, 200 µl of 100% ethanol was added to this mixture. Washing and centrifugation of the sample were done according to the manufacturer's recommendations. The elution of fungal nucleic acid was done with 100 µl elution buffer provided with the extraction kit.

Oligonucleotide primers

Primers (Table 1) were supplied from Metabion (Germany).

Table 1. Sequences of primers, target genes, product sizes, and conditions of PCR cycles

Target genes	Primers sequences (5'-3')	Amplicon size (base pair)	Amplification cycles (35 cycles)				Final extension	References
			Primary denaturation	Secondary denaturation	Annealing	Extension		
RAS1	(Forward) CCCAACTATTGAGGATTCTTATCGTAAA (Reverse)TCTCATGGCCAGATATTCTTCTTG	106	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	Tsang et al. (2012)
ALS1	(Forward) GAC TAG TGA ACC AAC AAA TAC CAG A (Reverse) CCA GAA GAA ACA GCA GGT GA	318	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 7 min.	Inci et al. (2013)
HWP1	(Forward) ATG ACT CCA GCT GGT TC (Reverse) TAG ATC AAG AAT GCA GC	572	94˚C 5 min.	94˚C 30 sec.	45˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	
PLB1	(Forward) ATGATTTTGCATCATTTG (Reverse) AGTATCTGGAGCTCTACC	751	94˚C 5 min.	94˚C 30 sec.	50˚C 1 min.	72˚C 1 min.	72˚C 10 min.	Mukherjee et al. (2001)
SAP4	(Forward) GCT CTT GCT ATT GCT TTA TTA (Reverse) TAG GAA CCG TTA TTC TTA CA	394	94˚C 5 min.	94˚C 30 sec.	49˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	Sikora et al. (2011)

PCR amplification

The PCR reaction was performed in a 25-µl reaction mixture containing 12.5 µl of master mix (EmeraldAmp Max, Takara, Japan), 1 µl of each primer (20 pmol concentration), 4.5 µl of distilled water, and finally 6 µl of template DNA. The reaction was done in the thermal cycler (Applied biosystem 2720, Germany).

Analysis of PCR products

Electrophoresis of the PCR products was performed on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at ambient temperature using gradients of 5 V/cm. For gel electrophoresis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the product size. Photographing the agarose gel was performed using a gel documentation system (Alpha Innotech, Biometra, Germany) and the data was analyzed through computer software.

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Antifungal susceptibility testing

Cultures preparation

The suspension of each *Candida albicans* isolate was prepared in sterile distilled water and was adjusted to 0.5 McFarland turbidity standard to obtain a concentration of 10^6 CFU/ml. *Lactobacillus acidophilus* DSMZ 20079 and *Lactobacillus plantarum* DSM 20179 were purchased from MIRCEN (Microbiological Resource Center), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Both strains were activated on de Man, Rogosa, and Sharp (MRS) broth (Biolife, Italy) at 37 °C for 24 h. Serial dilutions were prepared to obtain a concentration of 10^6 CFU/ml.

Well diffusion method

The antifungal sensitivity testing was performed on *Candida albicans* isolates. The adjusted spore suspensions were evenly spread on the surface of Sabouraud dextrose agar plates. Holes were made on the medium by using a 6 mm cork borer. The commercial antifungal drugs, including fluconazole (25 µg), itraconazole (8 µg), ketoconazole (15 µg), terbinafine (1 µg), nystatin (100 IU). A volume of 100 µl of the antifungal agent was placed into each well. Also, *Lactobacillus acidophilus* and *Lactobacillus plantarum* (100 µl/well of 10^6 CFU/ml suspension) were placed into the wells. The plates were then incubated at 37 °C for 24 h. Zones of inhibition were measured in millimeters; the inhibition zone is the area surrounding the hole where there is no growth of inoculated fungus. All assays were carried out in triplicates to calculate the mean results (Abdullah et al., 2013).

RESULTS AND DISCUSSION

In the last decades, *Candida* species especially *Candida albicans* have been considered an important healthcare-associated infection (Cox, 1993). The discrimination of various *Candida* species mostly relies on different biochemical identifications, germ tube test, and the unique culture characteristics on specific media (Vijayalakshmi et al., 2016).

In the present study, 14 out of 30 (46.7%) goat milk samples were positive for yeasts. Dos Santos and Marin (2005) isolated fungi from 32% of tested bovine milk specimens, of which 17.3% were *Candida* species. Dworecka-Kaszak et al. (2012) isolated fungi from only 14% of the tested samples also Krukowski et al. (2001) detected fungi in 9.6% of the tested milk specimens in the Lublin district located in Poland. Dos Santos and Marin (2005) declared that the percentage of fungal isolation in surveys performed in many countries varies significantly, with 6.1% rates as described in Egypt by Awad et al. (1980), 1.3% in Denmark (Aalbek et al., 1994), and 12.07% in Brazil (Costa et al., 1993).

Candida is mostly known as an opportunistic mycotic infection, and the origin of infection may come from the surface of the udder, milking machines, milker's palms, animal feed, straw, ground, sanitary agents, remedies, and other utensils. In immunosuppressive circumstances, the balance of pathogens population size may be altered, and the mycotic elements along with the other pathogens are capable of defeating the udder defensive techniques. Although the distribution of *Candida* species exhibits large diversity in different localities, it is crucial to give attention toward the increased cases of mammary gland infections caused by *Candida* species in the last decade (Dworecka-Kaszak et al., 2012). In the present work, only 4 (13.3%) isolates were confirmed as *Candida albicans.* Spanamberg et al. (2014) isolated *Candida albicans, Candida glabrata, Candida tropicalis*, and *Pichia guilliermondii* from normal and mastitic ewe milk specimens. *Candida albicans* was the most frequently isolated species in a study performed by Costa et al. (1993) but Dworecka-Kaszak et al. (2012) isolated mostly *Candida parapsilosis*. Krukowski et al. (2001) stated that *Candida kefyr*, *Candida cirferi*, and *Candida krusei* were more frequently isolated from cow milk in Poland.

In the present work, the virulence genes were detected in the four isolates of *Candida albicans* (Table 2 and Figures 1-5). The results revealed that RAS1 and ALS1 were found in 4 (100%) isolates, HWP1 and SAP4 were found in 2 (50%) isolates but PLB1 was not detected in any of the four isolates (0%). Similar results were obtained by Vijayalakshmi et al. (2016) who detected the virulence genes of HWP1 in 77%, INT1 in 72%, ALS1 in 65%, SAP1 in 65%, and PLB1 in 52% of multi-drug resistant *Candida albicans*. Also, Abdul-Lateef et al. (2015) detected the virulence genes of INT1, ALS1 in a higher frequency (100%), HWP1 in 90.9%, SAP1 in 59.09%, and PLB1 in 13.63% of tested *Candida albicans* isolates. However, Inci et al. (2013) detected the ALS1 gene in 53.9% of tested *Candida albicans* isolates, while the HWP1 gene was found in only 5.3% of tested isolates. The diversity in the prevalence percentages of virulence genes may attribute to various issues including the number of specimens under investigation and the difference in the isolation origins of *Candida albicans* (Vijayalakshmi et al., 2016).

Table 2. The distribution of the virulence genes in *Candida albicans* isolates recovered from goat milk

Isolates	RAS1	ALS1	HWP1	PLB1	SAP4
1	+	+	-	-	-
2	+	+	+	-	+
3	+	+	+	-	+
4	+	+	-	-	-

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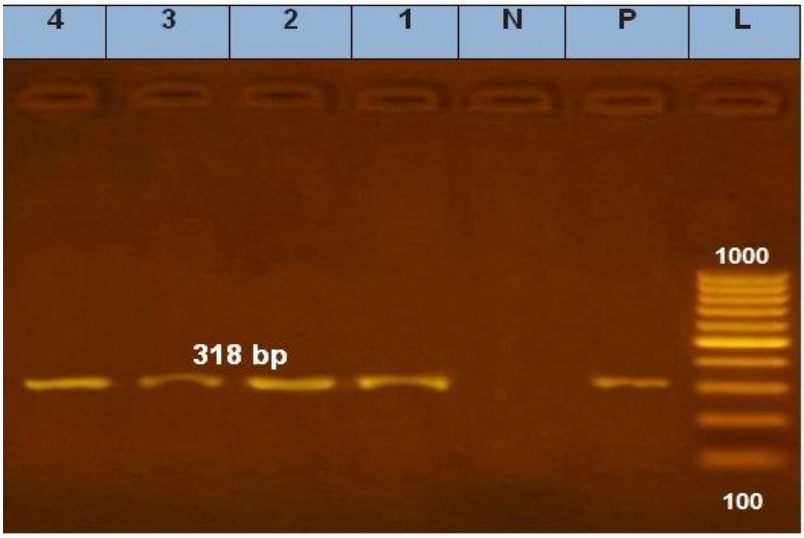

Figure 1. Agarose gel electrophoresis showing PCR-amplified product of ALS1 gene of *Candida albicans* derived from goat milk. (Lane L: 100-bp DNA ladder, Lane P: positive control, Lane N: negative control, Lanes 1-4: tested *Candida albicans* isolates).

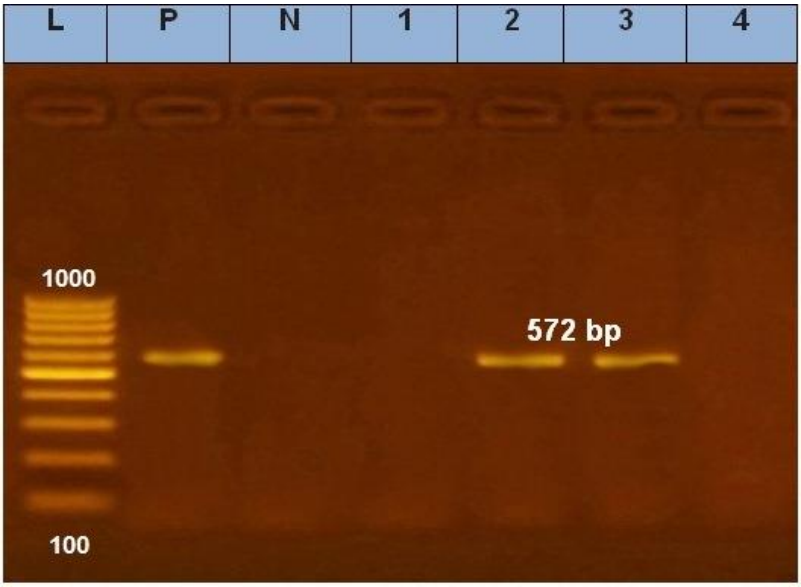

Figure 2. Agarose gel electrophoresis showing PCR-amplified product of HWP1 gene of *Candida albicans* derived from goat milk. (Lane L: 100-bp DNA ladder, Lane P: positive control, Lane N: negative control, Lanes 1-4: tested *Candida albicans* isolates).

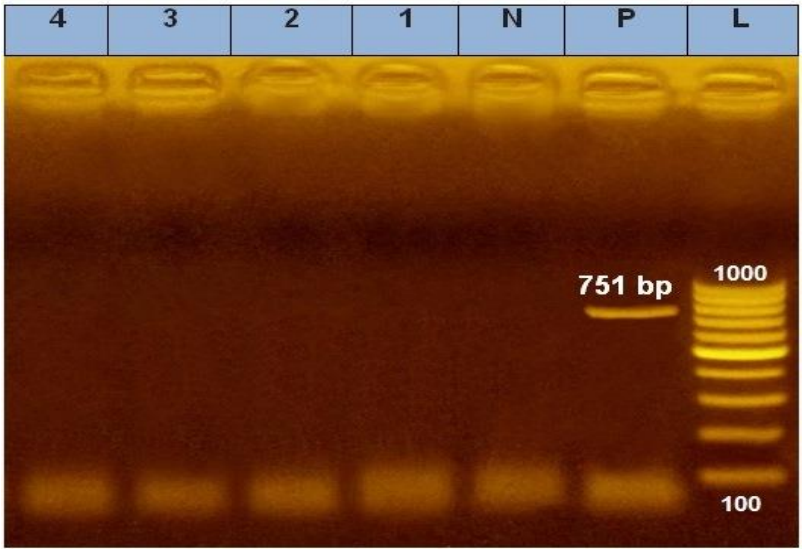

Figure 3 Agarose gel electrophoresis showing PCR-amplified product of PLB1 gene of *Candida albicans* derived from goat milk. (Lane L: 100-bp DNA ladder, Lane P: positive control, Lane N: negative control, Lanes 1-4: tested *Candida albicans* isolates).

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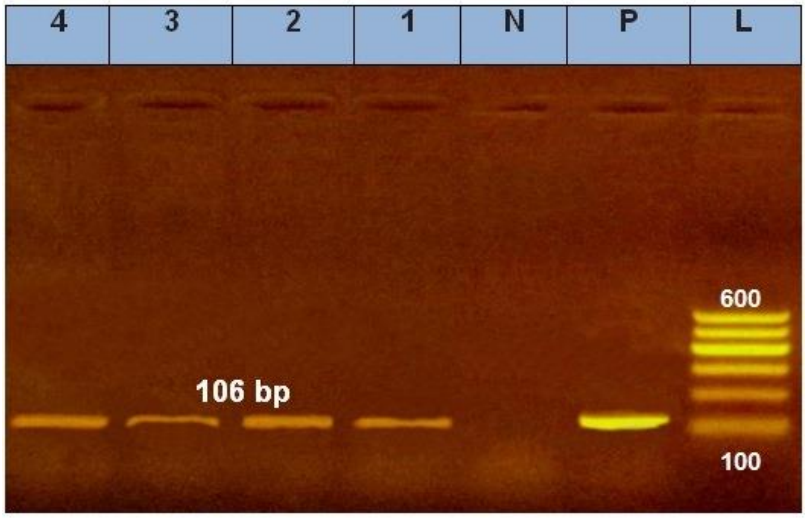

Figure 4. Agarose gel electrophoresis showing PCR-amplified product of RAS1 gene of *Candida albicans* derived from goat milk. (Lane L: 100-bp DNA ladder, Lane P: positive control, Lane N: negative control, Lanes 1-4: tested *Candida albicans* isolates).

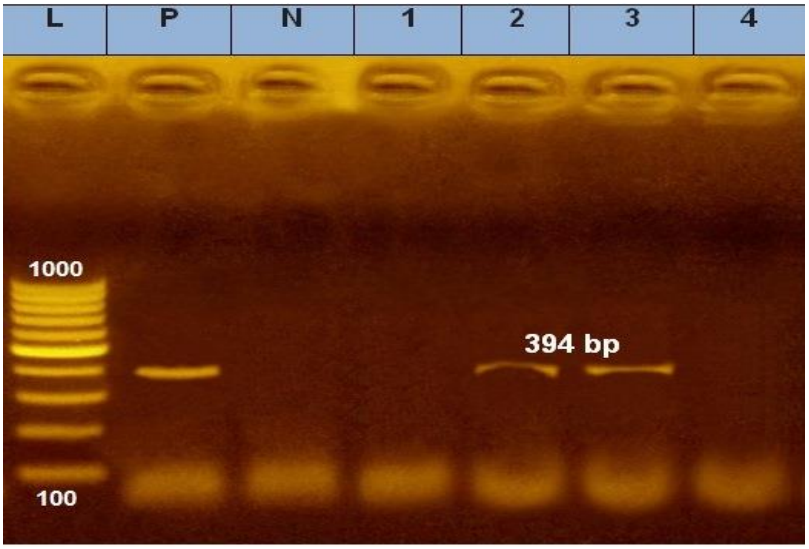

Figure 5. PCR results for the amplification of SAp4 gene of *Candida albicans* derived from goat milk. (Lane L: 100-bp DNA ladder, Lane P: positive control, Lane N: negative control, Lanes 1-4: tested *Candida albicans* isolates).

In recent years, antimycotic agents have shown an increase in usage as the fungal infections have also increased, leading to the suppression of endogenous fungal microflora, and the emergence of more resistant isolates due to the inhibition of susceptible ones (Koç, 2003). Some *Candida* species are naturally resistant to some antimycotics and it was revealed that many *Candida* isolates possess resistance against amphotericin B and others developed resistance against ketoconazole (Gunes et al., 2001). In the present study, ketoconazole showed the best activity against *Candida albicans*, followed by fluconazole. While nystatin and itraconazole showed lower activity against *Candida albicans*. All isolates were resistant to terbinafine (Table 3 and Figure 6).

Table 3. Results of antifungal sensitivity of *Candida albicans* isolates derived from goat milk against different antifungal agents and probiotics

***Candida albicans* isolates**	**Inhibition zone diameter (mm)**						
	Fluconazole (25 µg)	**Itraconazole (8 µg)**	**Ketoconazole (15 µg)**	**Terbinafine (1 µg)**	**Nystatin (100 IU)**	***Lactobacillus acidophilus* (10^6)**	***Lactobacillus plantarum* (10^6)**
1	22.7±1.5	19. 3±0. 7	33.3±0.9	Resistant	22.3±0.3	14. 7±0.3	16.3±0.9
2	37.3±1.5	21. 7±0.9	32.3±1.5	Resistant	22±1	12.3±0.3	14.7±0.3
3	36.3±0.9	21.3±0.9	35. 7±0.3	Resistant	22.7±1.5	13±0.6	15±1.6
4	29±2.1	18.3±0.9	31. 7±0.9	Resistant	21±0.6	11. 7±0.9	13.3±0.9

Data are expressed as Mean ± Standard Error

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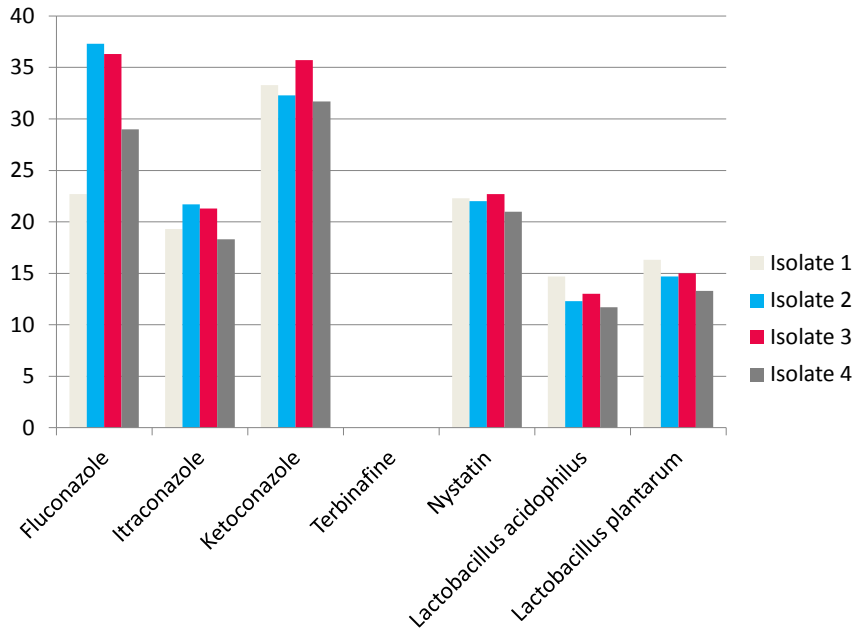

Figure 6. Comparison of the antifungal sensitivity results of *Candida albicans* isolates against commercially available antifungal agents and probiotics.

Sonmez and Erbas (2017) found high resistance rate (100%) to fluconazole, miconazole, amphotericin B, and flucytosine while high susceptibility to ketoconazole. Two (20%) *Candida albicans* isolates were found to be highly susceptible to nystatin while others were of medium susceptibility hence Sonmez and Erbas (2017) concluded that ketoconazole is the drug of choice in treating *Candida* infections. Lyon et al. (2010) found that the susceptibility of fluconazole significantly increased from 87.5% in 2005 to 97.4% in 2007. Furthermore, Monroy-Pérez et al. (2016) stated that all strains of *Candida albicans* were sensitive to nystatin, and 37 (94.9%) and 38 (97.4%) strains were resistant to fluconazole and ketoconazole, respectively. Shrief et al. (2019) declared that the resistance against antifungal drugs including itraconazole, fluconazole, and caspofungin was 8% for each one and 9% for amphotericin B. Moreover, Dos Santos Abrantes et al. (2014) reported that more than 50% of *Candida albicans* isolates obtained from South Africa and Cameroon showed resistance against fluconazole. However, an earlier study conducted in South Africa revealed 100% susceptibility of *Candida albicans* to fluconazole because this study was performed before the introduction of fluconazole to HIV-AIDS patients as the fluconazole resistance was not developed yet (Blignaut et al., 2002). In the last years, the increase in clinical manifestations due to *Candida* species and the different sensitivity patterns against the used antifungal agents highlight the importance of *in vitro* susceptibility testing in selecting appropriate antifungal agents (Sonmez and Erbas, 2017). Antifungal susceptibility testing is a great tool that may specify clinical response, help in the efficient selection of antifungal agents, and predict antifungal treatment failure. Antifungal susceptibility testing of *Candida* species and tracing the emergence of resistant isolates is of great importance in order to make information available to the clinicians for a proper therapeutic outcome (Khan et al., 2018).

Due to the elevated prevalence of candidiasis in immunodeficient individuals, the emergence of resistance in *Candida* species to current antimycotics, the treatment failures and the frequent relapse of candidiasis, the use of some beneficial and harmless compounds such as probiotics for the treatment and control of this disease can be recommended as an interesting safe medicinal way (Silva et al., 2016). Probiotics can be used to effectively combat pathogens with no adverse effect on normal microbiota. In this concern, the use of probiotics can be a substitute in the food and pharmaceutical industries (Abdhul et al., 2015). In the current study, both *Lactobacillus plantarum* and *Lactobacillus acidophilus* showed antifungal activity against *Candida albicans* but *Lactobacillus plantarum* showed higher activity in comparison to *Lactobacillus acidophilus*. These results were similar to those obtained by Hasslöf et al. (2010) who revealed that *Lactobacillus acidophilus* had weaker inhibition activity in comparison with the other probiotic strains. Also, Strus et al. (2005) assured that tested probiotics, including *Lactobacillus plantarum* and *Lactobacillus acidophilus*, suppressed the growth of *Candida albicans* to a certain degree. Kovachev and Vatcheva-Dobrevska (2015) revealed that local application of probiotics such as *Lactobacillus acidophilus* may improve the effectiveness of conventional antimycotics and prevent recurrent infection in women with *Candida albicans* vaginal manifestation. Jiang et al. (2014) revealed that *Candida albicans* was the most susceptible yeast to lactobacilli but another study conducted by Salari and Almani (2020) found that cell concentrations of 10^2 to 10^{10} CFU/ml for both *Lactobacillus acidophilus* and *Lactobacillus plantarum* were able to suppress the growth of most of the tested *Candida* species, except for *Candida albicans*, however, *Candida albicans* displayed very high susceptibility to cell-free supernatants of two *Lactobacillus* species. The differences in the results of various studies may attribute to differences in the clinical isolates of *Candida*

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albicans, the examined *Lactobacillus* strains, the investigations used for testing the antifungal susceptibility, tested *Candida* species, the initial counts of *Lactobacillus* species, the length of the incubation period, and the source of the *Candida* species isolation.

CONCLUSION

In the current study, both probiotics *Lactobacillus acidophilus* and *Lactobacillus plantarum* exhibited antifungal effects against *Candida albicans*. However, *Lactobacillus plantarum* was higher in the activity than *Lactobacillus acidophilus*. Fungal mastitis cases due to *Candida* species are spreading and candidiasis cases in immunocompromised individuals are also increasing along with the development of resistant strains that can lead to treatment failures. Plus the synthetic antimycotics have serious side effects. Therefore, further studies on experimentally infected animals are strongly recommended to evaluate the antifungal activity of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and other *Lactobacillus* species and determine their precise mode of action.

DECLARATIONS

Authors' contribution

Mona MH Soliman designed the plan of the study, shared in performing the experiments and writing of the manuscript. Mai M Kandil shared in performing the experiments and manuscript writing. Elnemr SA shared in performing the experiments and shared in analyzing the data. Azza SM Abuelnaga shared in performing the experiments and analyzing the data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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