Potential anti-fungal activity of Lactobacilli Probiotic against Fluconazole Resistant Candida albicans clinical isolates

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Abstract: Fluconazole resistance by Candida albicans (C. albicans) is a growing medical issue which makes patient management more difficult. The development of new effective, safe and inexpensive alternatives becomes essential. Probiotics represent an intriguing strategy that can be applied for prevention or treatment of Candida infections. The aim of our study is to investigate the anti-fungal activity of different Lactobacillus species against fluconazole resistant C. albicans isolates and to evaluate the inhibitory effect of cell free supernatant of the most potent one. Sixty-nine C. albicans isolates were collected from different clinical specimens and subjected to antifungal susceptibility testing. Then, the anti-Candida activity of various Lactobacillus spp was investigated via different methods, spot overlay method, radial streak method and agar well diffusion method. Results showed that 33% of C. albicans isolates were resistant to fluconazole with MIC ≥ 64 μg/ml. L. rhamnosus demonstrated strong inhibitory activity against the majority of fluconazole resistant C. albicans isolates, followed by L. reuteri and L. salivarius respectively. Inhibitory activity of L. rhamnosus against C. albicans may be due to acid production. Our findings conclude that L. rhamnosus represent a promising candidate to be used in the treatment of candidiasis caused by fluconazole-resistant C. albicans strains.

Keywords: Fluconazole resistance, Candida albicans, Lactobacillus rhamnosus, Lactobacillus reuteri, Lactobacillus salivarius.

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1. INTRODUCTION

Candida species (Candida spp) causing fungal infections or candidiasis have become much more common in recent years. This is mainly attributed to the increasing population of immunocompromised patients and those hospitalized with severe underlying diseases 3. Several risk factors such as treatment by immunosuppressive and cytotoxic remedies, treatment with broad-spectrum antibiotics, acquired immune deficiency syndrome and diabetes contribute to Candida infections 1,2. Candida spp are common colonizers of the human skin, oral cavity, gut, and vagina. Being human commensals, Candida spp causes no noticeable injury or damage in healthy people; however, in certain conditions they can initiate a wide range of diseases 3.

Currently, candidiasis represents the fourth prime cause of nosocomial infections 4. Among Candida spp, Candida albicans (C. albicans) represents the most common etiological agent responsible for various types of candidiasis 5. It causes a vast range of clinical infections, ranging from superficial to potentially life-threatening systemic ones 6.

Several classes of antifungals are available for prophylaxis and treatment of candidiasis, polyenes, pyrimidines, azoles, and echinocandins 7. Azoles are the most commonly used clinical antifungal agents, with fluconazole (FLZ) is recommended as the first-line option for prevention and treatment of Candida infections 8. Fluconazole has excellent bioavailability, available in oral and parenteral formulations, and has low level of toxicity 9. Unfortunately, the ability of Candida to establish a high-level ofazole antifungal resistance have been documented, which leads to an increase in the number of treatment failures in candidiasis patients who receive long-term antifungal therapy 10.
The increase in Candida resistance to antifungals and limited variety of antifungal drugs has led to the use of ecological approaches to overcome the infections, which make probiotics a very interesting scope for further research. \(^{11,12}\)

Probiotics are live microorganisms which, when obtained in adequate amounts, confer a health benefit to the host. \(^{13}\) There are number of health benefits for probiotics comprising, improved digestion, strengthening the immune system, and encouraging vitamin production. The use of probiotics aims to reduce antibiotic use while also improving animal development and feed conversion. \(^{14}\) Lactobacillus and Bifidobacterium are the most studied probiotics for antifungal activity against Candida spp. Numerous studies have revealed that the Lactobacilli, can block the growth of C. albicans. \(^{11,12,15}\) Lactobacilli have the ability to produce several active compounds which are effective against C. albicans. \(^{16}\)

Among Lactobacilli spp that have been investigated in several in vitro studies against C. albicans are Lactobacillus rhamnosus, L. paracasei, L. reuteri, L. plantarum, L. gasseri, L. fermentum, L. jensenii, and L. acidophilus. \(^{17-19}\) The objective of our work is to investigate the anti-fungal activity of five Lactobacillus spp against fluconazole resistant C. albicans in addition to inhibitory effect of selected isolate.

2. METHODS

2.1. Clinical isolates and conditions for growth

The study was done on 67 C. albicans isolates from a preceding study conducted by El-Ashmony et al., 2019. \(^{20}\) Isolates were recovered from 134 different clinical samples collected from Ain-Shams University hospitals during the period from August 2018 to January 2019. Additional two C. albicans isolates recovered from seven blood samples were included in this study. C. albicans strains were grown on Sabouraud dextrose agar (Oxoid, England) for 24 h at 37°C.

C. albicans were identified by germ tube test, corn meal agar (Oxoid, England), CHROMagar Candida (Himedia, India) and VITEK-2 system. \(^{20}\)

2.2. Probiotics and growth conditions

Five lactobacilli species, including, L. rhamnosus (ATCC 7469), L. reuteri (DSM 20016), L. Salivarus (DSM 20555), L. plantarum (DSM 20174) and L. Casei (DSM 20011) were purchased from Egyptian Microbial Culture Collection (EMCC) at Cairo Microbiological Resources Centre (MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt.

All Lactobacillus strains were routinely grown in de Man, Rogosa, and Sharpe (MRS) broth or agar (Hi media, India) and incubated under microaerophilic conditions for 24 h at 37°C.

2.3. Antifungal susceptibility testing

Testing C. albicans isolates for susceptibility to different antifungals was performed using disk diffusion method. \(^{21}\) Then, minimum inhibitory concentration (MIC) of fluconazole (FLZ) was evaluated by broth micro-dilution (BMD) method.

2.3.1. Disc diffusion (DD) method

Disc diffusion (DD) method was done according to CLSI M44-ED3 method using Mueller Hinton agar with additional 2% glucose. Methylene blue at concentration of 0.5μg/ml was added. Antifungal discs used in this study were fluconazole (25 μg), nystatin (100IU), amphotericin B (100IU) and voriconazole (1 μg), (100IU) which were purchased from Oxoid, England. The zones of inhibition of FLZ and voriconazole were interpreted according to CLSI M60-ED2 interpretive break points, while nystatin and amphotericin B, the interpretive break points were taken from published studies as shown in Table (1).

<table>
<thead>
<tr>
<th>Table 1. Inhibition Zone diameters of antifungal disc for C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antifungal agents</strong></td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td><strong>Fluconazole</strong></td>
</tr>
<tr>
<td><strong>Voriconazole</strong></td>
</tr>
<tr>
<td><strong>Amphotericin p</strong></td>
</tr>
<tr>
<td><strong>Nystatin</strong></td>
</tr>
</tbody>
</table>

S: Sensitive; I: Intermediate; SDD: Susceptible dose dependent; R: Resistant.

SDD means that susceptibility is dependent on achieving the maximal possible blood level.

2.3.2. Broth micro-dilution (BMD) Method

The MIC of FLZ was evaluated by broth micro-dilution (BMD) method referring to CLSI protocol. \(^{22}\) The test was done using Roswell Park Memorial Institute (RPMI) 1640 broth medium with L-glutamine and the pH indicator, phenol red but lacking bicarbonate. The glucose was added to a final concentration of 2% (RPMI 2% G). The recommended buffer to be used for RPMI 1640 medium was 3- (N-morpholino) propane-sulfonic acid (MOPS) at a final concentration of 0.165 mol/L and pH 7.0. Each experiment contained the growth control and sterilized media control wells. The results were interpreted according to CLSI M60-ED2 interpretive break points as shown in Table 2.
Table 2. Interpretive break points of fluconazole (FLZ) antifungal drug for C. albicans according to CLSI M60-ED2

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Minimum Inhibitory Concentration Breakpoint (mg/l) (µg/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>S  ≤ 2</td>
<td>SDD  4</td>
</tr>
</tbody>
</table>

S: Sensitive; SDD: Susceptible dose dependent; R: Resistant.

The categorical agreement between the DD method and BMD methods was determined by calculating the kappa value.

2.4. Screening of Lactobacilli for antifungal activity against C. albicans isolates

Five lactobacillus strains were primarily screened for antifungal activity using spot overlay method and radial streak method.

2.4.1. Spot overlay method

In spot overlay method, each of the five lactobacillus isolates was cultivated on MRS broth at 37°C for 24 h. The cultures were diluted to an optical density of 1 at wavelength 600 nm. Ten microliters of each diluted culture were spotted on the surface of MRS agar plate and incubated at 37°C for 48 h under microaerophilic conditions. After incubation, the grown Lactobacilli colonies on MRS agar plate were overlaid with 10 ml of 0.5-0.7% Yeast extract peptone dextrose soft agar containing 10^7 CFU/mL C. albicans. After solidification of soft agar, plates were aerobically incubated at 37°C for another 24 h to allow the growth of candida isolates. Anti-Candida activity was indicated by inhibition zones above Lactobacillus spot. The inhibition zone diameter was measured.

2.4.2. Radial streak method

Each probiotic bacteria was prepared to 0.5 McFarland standard and inoculated on a sterile MRS agar plate in the form of a circle in the center of the plate. The plate was incubated at 37°C for 48 h. Following incubation, C. albicans strains equivalent to 0.5 McFarland standard were added to the plates by making radial lines of inoculum from the boundary to the center of the plate. The plate was further incubated aerobically for 24 h at 37°C. The anti-Candida activity was observed as an inhibitory zone around Lactobacillus spot and the inhibition zone diameter was measured.

2.5. Cell-free supernatant (CFS) preparation

We prepared CFS from the most potent Lactobacillus strain that displayed strong inhibitory activity against resistant C. albicans isolates. Overnight culture of bacterial strain in MRS broth was modified to OD 600 nm of 1.0. Then, two mL of the diluted culture was used for inoculation of 100 mL of MRS broth and incubated under microaerophilic conditions at 37°C for 48 h. The CFS was obtained by centrifugation at 11,000 x g at 4°C for 10 min, then, filter-sterilized through sterile 0.22 µM membrane filter and tested for its anti-Candida activity as previously described. Also, the pH of CFS was determined by pH meter.

2.6. Determination of anti-Candida activity of cell-free supernatant (CFS)

The inhibitory effect of the prepared CFS against FLZ resistant C. albicans isolates was determined using a well diffusion assay as described previously. The isolates of C. albicans were sub-cultured in SDA for 24 h at 37°C. Twenty milliliters molten SDA was allowed to cool to 45°C and seeded with 100 µL of 0.5 McFarland Candida suspension, then, poured in petri dish (9 cm). After solidification of the agar plate, six-millimeter diameter wells were made in the agar plate by the use of a sterile cork borer and filled with 0.1 ml of CFS. The plates were left for 2 h at 4°C for diffusion and then incubated aerobically at 37°C for 24 h. Following incubation, inhibitions zone around the wells were manually measured.

2.7. Preparation of concentrated cell-free supernatant (cCFS)

Concentrated cell-free supernatant (cCFS) was prepared by freeze drying of sterile CFS in a freeze-drier (Model PDF 0350) and concentrated to 33 times its original volume in sterile distilled water to yield cCFS which is 33x strength. The anti-Candida activity of cCFS was also determined by well diffusion assay.

2.8. Effect of pH adjustment of concentrated cell-free supernatant (cCFS)

To investigate if the acids in cCFS are responsible for the anti-Candida activity, the pH of cCFS was neutralized to the value of 7 and then,
tested for inhibitory activity by agar well diffusion method and compared with untreated CFS as control.  

2.9. Effect of heat on concentrated CFS

Concentrated CFS was independently treated at different temperature, 40°C, 60°C, 80°C and 100°C for half an hour and 121°C for fifteen minutes. Anti-Candida activity of the treated CFS was investigated by agar well diffusion method. and compared with untreated CFS as control.

2.9. Effect of heat on concentrated CFS

Concentrated CFS was independently treated at different temperature, 40°C, 60°C, 80°C and 100°C for half an hour and 121°C for fifteen minutes. Anti-Candida activity of the treated CFS was investigated by agar well diffusion method. and compared with untreated CFS as control.

3. RESULTS

A total sixty-nine C. albicans isolates obtained from 141 different clinical specimens were included in the current study. C. albicans was determined the most widespread species isolated with incidence rate 48.9%.

3.1. Antifungal susceptibility of C. albicans clinical isolates

All C. albicans isolates were examined for their susceptibility to several antifungal agents (amphotercine B, nystatine, voriconazole and FLZ) via disc diffusion (DD) method. We noticed that all isolates (100%) were sensitive to amphotercine B and nystatine. While for FLZ, 42 isolates (60.7%) were susceptible (S), 4 isolates (5.8%) were susceptible dose dependent (SDD) and 23 isolates (33.33%) were resistant (R). Out of 23 FLZ resistant isolates, only two isolates showed also resistance to voriconazole as depicted in Table 3.

Table 3. Antifungal susceptibility pattern of C. albicans isolates by disc diffusion method

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Susceptible</th>
<th>Susceptible dose-dependent</th>
<th>Resistant</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>42</td>
<td>60.9</td>
<td>4</td>
<td>5.8</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>67</td>
<td>97.1</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Amphotrcine B</td>
<td>69</td>
<td>100.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Nystatine</td>
<td>69</td>
<td>100.0</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Broth micro-dilution (BMD) method showed that 23 isolate (33.3%) were FLZ resistant with MIC ≥ 64 μg/ml in 15 isolate and ≥ 32 μg/ml in 8 isolates.

Six isolates (8.7%) were FLZ susceptible dose dependent with MIC= 4. The remaining isolates, 40 (57.97%) were FLZ sensitive with MIC < 2.

There was a very good agreement between DD method and BMD in determining the susceptibility profile of C. albicans to FCZ as shown in Table 4.

Table 4. Degree of agreement between DD method and BMD method by Kappa value

<table>
<thead>
<tr>
<th>Fluconazole (FLZ)</th>
<th>DD</th>
<th>BMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>Susceptible dose dependent</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Resistant</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>κ (p)</td>
<td>0.945* (&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Agreement</td>
<td>Very good</td>
<td></td>
</tr>
</tbody>
</table>

DD: disc diffusion; BMD: broth micro-dilution. κ: kappa test. *: Statistically significant at p ≤ 0.05

3.3. Screening anti-Candida activity of lactobacilli strains

We tested five lactobacilli spp for anti-Candida activity using the agar overlay method and in order to verify the experimental data, radial streak method was done to obtain mutual agreement. The results of both methods were similar and showed that all Lactobacillus strains had variable inhibition activities against C. albicans growth Figure (1). The level of the inhibition activity was statistically significantly different relying on lactobacillus strain (supplementary table (1s)). Among which L. rhamnosus ATCC 7469 was the best one with high inhibition activity against majority of C. albicans isolates (82.6%), followed by L. reuteri. L. salivarus which showed intermediate to strong growth inhibition activity. While L. plantarum and L. Casei growth inhibition activity was found to be weak or even abolished as depicted in Table (5).
Figure 1: Growth inhibition of *C. albicans* by Lactobacillus strains. A: Spot overlay method, B: Radial streak method, 1-6: *C. albicans* strains LB: Lactobacillus strains.

Table 5. Growth inhibition degree/activity of *Lactobacillus* spp on fluconazole resistant *C. albicans* isolates

<table>
<thead>
<tr>
<th><em>C. albicans</em> isolates no</th>
<th>Growth inhibition activity of <em>Lactobacillus</em> spp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>+++</td>
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<tr>
<td>6</td>
<td>+++</td>
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<td>+++</td>
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<td>22</td>
<td>+++</td>
</tr>
<tr>
<td>23</td>
<td>+++</td>
</tr>
</tbody>
</table>

GIA = (IZD - CD)/2. GIA < 0.5 mm was recorded as negative (−), (0.5, 2) mm as weak positive (+), (2, 3.5) mm as intermediate positive (++), and ≥ 3.5 mm as strong positive (+++). GIA: Growth inhibition activity; IZD: Inhibition zone diameter; CD: Colony diameter
3.4. Anti-Candida activity of the *L. rhamnosus* ATCC 7469 cell-free supernatant

When testing the impact of *L. rhamnosus* ATCC 7469 cell free supernatant (CFS) with pH of 4±2 on the growth of FLZ resistant *C. albicans* isolates by agar well diffusion method, no growth inhibition was observed so, Candida antifungal action was not detected. Yet, concentrated CFS (cCFS) was statistically significant difference (P < 0.05) for growth inhibition of all tested isolates Table (6).

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFS</td>
<td>10.17 ± 0.29</td>
</tr>
<tr>
<td>cCFS</td>
<td>17.17 ± 0.29</td>
</tr>
<tr>
<td>t (p)</td>
<td>29.698 (&lt;0.001*)</td>
</tr>
</tbody>
</table>

CFS: cell free supernatant; cCFS: concentrated cell free supernatant, t: Student t-test, p: p value for comparing between the studied groups, *: Statistically significant at p ≤ 0.05, Growth inhibition zone diameter was measured in millimeter after 24 h.

3.5. Effect of pH adjustment on concentrated cell free supernatant

When the cCFS was neutralized to pH 7, the inhibitory zone was significantly reduced compared to the untreated cCFS (Figure (2)) and supplementary figure (1s).

3.6. Effect of heat treatment on concentrated cell free supernatant

After heat treatment of cCFS at different temperatures, there was no significant difference in the size of the inhibition zone when compared to untreated cCFS (P > 0.05), Figure (3). The result showed that the anti-Candida compound existent in the CFS was extremely stable throughout a wide range of temperatures showing that the antifungal substance(s) are thermally stable.

![Figure 2](https://aijpms.journals.ekb.eg/)  
*Figure 2. Effect of pH adjustment on cCFS; A: neutralized cCFS to pH 7; B: untreated cCFS.*

![Figure 3](https://aijpms.journals.ekb.eg/)  
*Figure 3. Thermal stability of *L. rhamnosus* cCFS on FLZ resistant *C. albicans* isolates.*
4. DISCUSSION

*Candida* species are the most abundant microbial pathogen causing fungal infections. The number fungal infection with candida have increased in recent year in recent years 33. *C. albicans* is the most prevalent etiological agent that causes candidiasis of various forms 3.

The current study was carried out on 69 *C. albicans* isolates from 141 different clinical specimens. The incidence of *C. albicans* was high representing 48.9% which was similar to two recent studies in Egypt by Farhan et al and El-Gniny et al 34,39. They reported 59.6% and 57.4% prevalence rate of *C. albicans* isolates respectively. Also, Mohammed et al stated that *C. albicans* was the predominant isolated species (51%) 36.

Fluconazole (FLZ) which is a member of azole antifungal drugs is the most commonly prescribed drug for treatment of *C. albicans* infections. It has many advantages represented in being inexpensive, effective, relatively safe and available in different forms 9,37. However, the ability of Candida to show an elevated level of azole resistance have been documented 10. FLZ resistance of *C. albicans* is a growing medical issue which makes patient management more difficult 38.

In the current study, 69 *C. albicans* clinical isolates was subjected to susceptibility testing against several antifungal agents. The level of FLZ resistance among *C. albicans* isolates was high reaching 33.3%. Several investigations reported approximately the same level of FLZ sensitivity against *C. albicans*. Kaur et al found that 36.3% of *C. albicans* isolated from different samples were FLZ resistant 39. Mohamed et al reported 23% FLZ resistance in *C. albicans* isolates 36.

However, recent studies reported extremely high FLZ resistance rate of 86.2% and 94% among *C. albicans* isolates 34,40. This high level of FLZ resistance might be due to extensive and prolonged use of this antifungal drug especially as prophylactic in cancer patients and in intensive care units 40. In our scenario, the use of FLZ as an empirical therapy in the treatment of different forms of candidiasis contributes to FLZ resistance.

On the other hand, several studies reported low FLZ resistance by *C. albicans* isolates. In 2019, Sardari et al found that *C. albicans* has a low rate of FLZ resistance about 5% 41. They suggested that this low level of resistance might be attributed to the use of an adequate dosage of antifungal drugs or completion of the treatment course. Also, Amr et al has reported 8.5% FLZ resistance rate 42.

Variation in FLZ resistance rate among different studies may be due to different sources of specimens, previous FLZ exposure, differences in the patient population and use of FLZ as prophylactic in some immunocompromised individuals.

In the current study, both disc diffusion (DD) method and broth micro-dilution (BMD) method were used to assess the *C. albicans* isolates' antifungal susceptibility to FLZ. The results showed a very good agreement rate between both methods (97.1%). These findings are in conformity with those conducted by Jeon et al., 202143 and Kumar et al., 2015 44. They reported 97%, 100% categorical agreement between DD method and the BMD method for assessing FLZ susceptibility against *C. albicans* isolates 33,44. Kumar et al concluded that DD methods can be used as substitute for the reference BMD method for Candida spp susceptibility testing of FLZ 44.

On the basis of what we have found, it seems that the disc diffusion is beneficial or advantageous method for testing the FLZ activity against *C. albicans*.

Susceptibility testing for Candida isolates is critical as it provides significant input about the pattern of resistance and aids in the selection of the best antifungal agent for treatment. The DD method is simple to use and may be interpreted in as little as 24 hours while the BMD approach is somewhat tedious and requires more technical skills 45.

Regarding voriconazole, we noted that only 2 isolates (2.9%) were resistant. This could be as a result of limited use of this drug in the current scenario. However, other studies reported elevated level of resistance 46,47.

All *C. albicans* isolates exhibited 100% sensitivity to amphotericin B. This result is analogous to studies conducted by other authors 35,36. Despite the fact that polyenes have been used for a long time, resistance to amphotericin B is rare, because it has fungicidal activity as it binds to ergosterol that present in the fungal membrane, resulting in pore formation and hence disrupting membrane structure and function 46. However, it may cause side effects such as nephrotoxicity and hypokalemia which limit its use 47.

In a similar way, All *C. albicans* isolates were completely sensitive to nystatin. Nenoff et al stated that *C. albicans* in vitro resistance to nystatin has not been evidenced 48.

Because of the rapid increase in fungal resistance to antifungal drugs, limited number of available antifungals and even more so, the rate of production of new antifungals is insufficient to fulfil current demanded, the development of new treatment strategy or improving those that presently exist to overcome these infections is needed 48.

Probiotics represent an intriguing strategy that can be used for prevention or treatment of Candida
infections. Lactobacilli have sparked a lot of attention as a way to fight Candida infections. The anti-Candida activity of probiotic Lactobacilli has been proved in many studies. In the current work, we primarily screened five Lactobacillus spp, L. rhamnosus (ATCC 7469), L. reuteri (DSM 20016), L. Salivarus (DSM 20555), L. plantarum (DSM 20174) and L. Casei (DSM 20011) for anti-Candida activity via spot overlay method. This method allows numerous lactobacillus strains to be screened simultaneously on a single plate. In order to verify the experimental data, radial streak method was done to obtain mutual agreement. This method allow testing of several organisms against one lactobacillus strain on one plate. The results of both methods are quite similar. The results revealed various anti-Candida activity among the five different strains as defined by the size of the inhibition zone and growth inhibition activity (GIA). The highest activity was detected by L. rhamnosus ATCC 7469 followed by L. reuteri (DSM 20016) and L. Salivarus (DSM 20555) respectively.

De Gregorio et al., 2019 tested various lactobacillus strains against Candida spp including C. albicans. They found that strains of L. rhamnosus, L. reuteri and L. salivarius showed better anti-Candida activity which is similar to our study.

In our study, L. plantarum (DSM 20174) and L. Casei showed a weak and/or no anti-Candida activity against C. albicans isolates tested. However, other studies have been established the anti-Candida activity of both species. Our results can be explained by antimicrobial activity of lactobacilli is strain dependent.

We chose L. rhamnosus ATCC 7469 for subsequent experiment as it exhibited potent activity against majority of C. albicans isolates. Recent study by Rose Jørgensen et al reported that L. rhamnosus strains exhibited the best growth inhibition activity among tested strains against candida spp.

Several studies have highlighted on the anti-Candida activity of L. rhamnosus strains and it’s potential use against C. albicans infections. In the present study, L. rhamnosus ATCC 7469 was tested for it’s ability to produce inhibitory compounds in cell free supernatant (CFS) that inhibited C. albicans growth.

So, we assessed the anti-candida activity of L. rhamnosus CFS via agar well diffusion method but no growth inhibition activity was observed against all tested C. albicans isolates despite the demonstrated potent activity by spot overlay and radial streak methods. Other studies have discovered the same phenomenon. For instance, Coman et al and verdinelli et al who demonstrated that Lactobacillus stains exhibited growth inhibition activity against Candida strains in radial streak method. While, the well diffusion testing revealed no inhibition against any Candida spp tested. They stated that radial streak method was superior to well diffusion method in studying the antimicrobial activity due to good diffusion of inhibitory metabolites from Lactobacillus strains in the agar medium.

Also, De Gregorio et al assessed the effect of CFS of Lactobacillus on Candida spp growth via multiple antimicrobial assay as solid medium assay and liquid medium assay. De Gregorio and his colleagues did not detect anti-Candida activity in agar well diffusion method in contrast to liquid medium assay. All tested Lactobacillus CFS significantly inhibited the growth of Candida strains tested in liquid medium assay. They suggested that it might be caused by the media's physical state, and the environment where the inhibitory compounds do their effects. Also, the concentration of anti-Candida substances released by lactobacilli into solid or liquid media.

Although the anti-Candida activity of CFS with pH 4±2 has not been detected, concentrated CFS (cCFS) revealed growth inhibition activity against all tested isolates. We suggest that the concentration of anti-Candida compounds released in the CFS is not high enough to confer the anti-candida activity. In addition, concentration of lactobacilli CFS have been done in several studies to improve the antifungal activity.

To find out the nature of CFS, the cCFS was neutralized to pH 7 and tested for activity. We found that the anti-Candida activity of neutralized cCFS against C. albicans was significantly decreased compared to the untreated cCFS indicating that activity may be attributed to acid production or other anti-candida compound that act synergistically with acids. Our findings point to the contribution of an acidic compound(s) in anti-Candida activity, which is similar to previous study by De Gregorio et al who found that NaOH-neutralized CFS abolished the anti-Candida activity of lactobacillus CFS and suggested that organic acids released in CFS were responsible for the antagonistic effect. Also Rose Jørgensen et al suggested that the acid production by L. rhamnosus DSM 32991 and L. rhamnosus DSM 32992 has an essential role in Candida growth suppression.

The heat stability of the L. rhamnosus cCFS was tested using five different thermal treatments: 40°C, 60°C, 80°C, 100°C for half an hour, and 121°C for 15 minutes. We found that the anti-Candida activity of cCFS not affected by various heat treatment indicating that the anti-Candida substance(s) in the cCFS are resistant to heat. Crowly et al established that the antifungal activity of cCFS from lactic acid bacteria remained stable at 121°C for 15 minutes revealing that the antifungal
compound(s) are resistant to heat and hence, non-volatile. 30

5. CONCLUSIONS

The current study substantiated that the incidence of fluconazole resistance by C. albicans isolates has been increasing. Disc diffusion method can be used as alternatives to the reference broth micro-dilution method for testing the susceptibility of C. albicans to fluconazole. Increased fluconazole resistance has created a need for new strategy to be developed. L. rhamnosus exhibited potent activity against majority of C. albicans resistant isolates. So, it represents a promising probiotic candidate to be used in the treatment of candidiasis caused by fluconazole-resistant C. albicans strains.

The anti-Candida activity produced by L. rhamnosus might be driven by acid or acid dependent compounds. Further investigation is needed to purify and identify the compound that exactly responsible for the anti-Candida activity.

Supplementary Materials: Supplementary file 1

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