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# 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. salivarus* 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 salivarus*.



## 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 [1]. 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 superfacial 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 of azole 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].



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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 [22].

#### *2.3.1. Disc diffusion (DD) method*

Disc diffusion (DD) method was done according to CLSI M44-ED3 method [21] 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 [23] interpretive break points, while nystatin and amphotericin B, the interpretive break points were taken from published studies [24,25] as shown in Table (1).

**Table 1. Inhibition Zone diameters of antifungal disc for *C. albicans***

| Antifungal agents | Disc content | Zone diameter (mm) | | | | References |
|---|---|---|---|---|---|---|
| | | S | I | SDD | R | |
| **Fluconazole** | 25 µg | ≥ 17 | -- | 14 – 16 | ≤13 | **CLSI M60-ED2** |
| **Voriconazole** | 1 µg | ≥ 17 | 15–16 | ----- | ≤14 | **CLSI M60-ED2** |
| **Amphotericin p** | 100 IU | >10 | ------- | ----- | ≤10 | **30, 31** |
| **Nystatine** | 100 IU | >10 | ------ | ---- | ≤10 | **30, 31** |

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 [23] 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**

| Antifungal agents | Minimum Inhibitory Concentration Breakpoint (mg/l) (µg/ml) | | | Reference |
|---|---|---|---|---|
| | S | SDD | R | |
| **Fluconazole** | ≤ 2 | 4 | ≥ 8 | **CLSI M60-ED2** |

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 overlayed 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 [18,19].

*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 [26].

*The calculation of the inhibitory effect results:*

The growth inhibitory activity (GIA) for both methods was computed by subtracting the colony diameter (CD) of the spot of Lactobacillus from the inhibition zone diameter observed (IZD) and dividing by two as following, GIA = (IZD-CD)/2. The grading system of GIA was used, where GIA less than 0.5 mm was documented as negative (–), (0.5-2) mm as weak positive (+), while (2- 3.5) mm as intermediate positive (++), and ≥ 3.5 mm as strong positive (+++) [18,26].

### 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 [27,28]. 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 [26,29]. 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 [30].

### 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 cCFS as control [31].

### 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 cCFS was investigated by agar well diffusion method. and compared with untreated cCFS as control [18,30,32].

### Statistical analysis

All measurements were carried out in triplicate. The results were presented as mean ± standard deviation (SD). Analysis of data was performed by using one-way analysis of variance (ANOVA) and compared means by using student t test and Chi square test. A P-value < 0.05 was considered statistically significant.

## 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

| Antifungal | Susceptible | | Susceptible dose-dependent | | Resistant | | Total | |
|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % |
| **Fluconazole** | 42 | 60.9 | 4 | 5.8 | 23 | 33.3 | 69 | 100.0 |
| **Voriconazole** | 67 | 97.1 | 0 | 0.0 | 2 | 2.9 | 69 | 100.0 |
| **Amphotrcine B** | 69 | 100.0 | 0 | 0.0 | 0 | 0.0 | 69 | 100.0 |
| **Nystatine** | 69 | 100.0 | 0 | 0.0 | 0 | 0.0 | 69 | 100.0 |

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**

| Fluconazole (FLZ) | DD | | BMD | |
|---|---|---|---|---|
| | No. | (%) | No. | (%) |
| **Susceptible** | 42 | 60.9 | 40 | 58.0 |
| **Susceptible dose dependent** | 4 | 5.8 | 6 | 8.7 |
| **Resistant** | 23 | 33.3 | 23 | 33.3 |
| **Total** | **69** | **100** | **69** | **100** |
| **κ (p)** | **0.945* (<0.001*)** | | | |
| **Agreement** | **Very good** | | | |
| **% of Agreement** | **97.1%** | | | |

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 *lactobacillus* 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 depicated in Table (5).

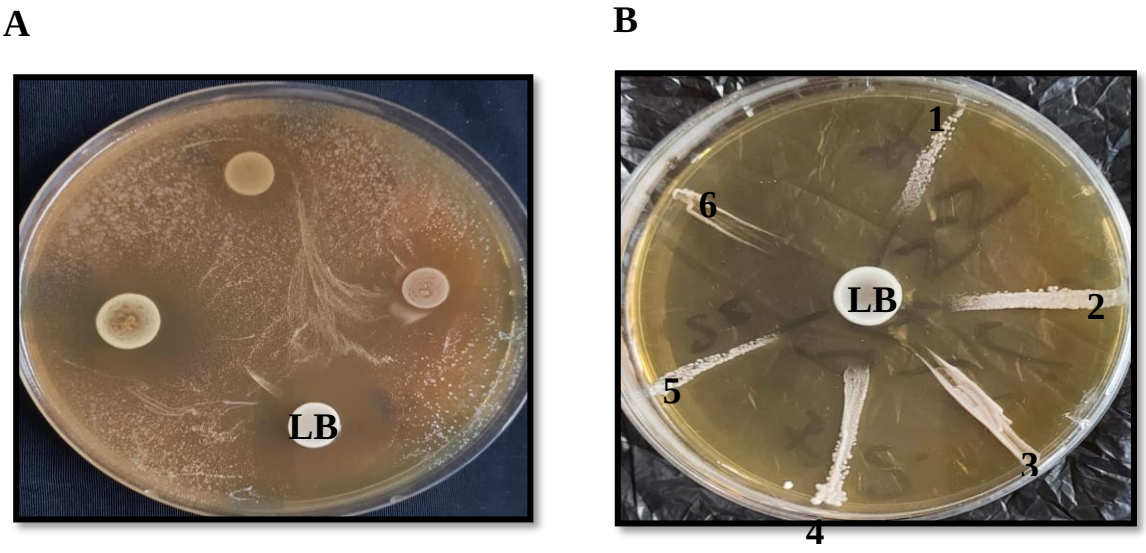


**Figure 1: Growth inhibition of *C. albicans* by Lactobacillus strains.**
A: Spot overlay method, B: Radial streak method, 1-6: *C. albicans* strains LB: Lactobacillus strains.

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

| ***C. albicans* isolates no** | **Growth inhibition activity of *Lactobacillus* spp** | | | | |
|---|---|---|---|---|---|
| | *L. rhamnosus* (ATCC 7469) | *L. reuteri* (DSM 20016) | *L. Salivarus* (DSM 20555) | *L. plantarum* (DSM 20174) | *L. Casei* (DSM 20011) |
| **1** | +++ | +++ | +++ | ++ | ++ |
| **2** | +++ | ++ | ++ | - | + |
| **3** | +++ | ++ | ++ | + | - |
| **4** | ++ | +++ | ++ | + | + |
| **5** | +++ | ++ | +++ | - | - |
| **6** | +++ | ++ | ++ | + | - |
| **7** | ++ | ++ | +++ | + | - |
| **8** | +++ | ++ | ++ | - | - |
| **9** | +++ | +++ | ++ | + | - |
| **10** | +++ | +++ | ++ | + | ++ |
| **11** | +++ | ++ | + | + | - |
| **12** | ++ | ++ | ++ | + | + |
| **13** | + ++ | +++ | ++ | + | + |
| **14** | +++ | +++ | +++ | + | - |
| **15** | ++ | +++ | ++ | - | + |
| **16** | + ++ | + | ++ | - | - |
| **17** | +++ | +++ | ++ | + | + |
| **18** | +++ | +++ | ++ | + | + |
| **19** | +++ | ++ | +++ | + | - |
| **20** | +++ | +++ | +++ | - | - |
| **21** | +++ | +++ | ++ | - | + |
| **22** | +++ | ++ | + | + | - |
| **23** | +++ | ++ | ++ | - | - |

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 6. Inhibition zone diameter of CFS and cCFS**

| Supernatant | Inhibition zone (mm) |
|---|---|
| CFS | 10.17 ± 0.29 |
| Ccfs | 17.17 ± 0.29 |
| **t (p)** | **29.698* (<0.001*)** |

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 $p \leq 0.05$, 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).

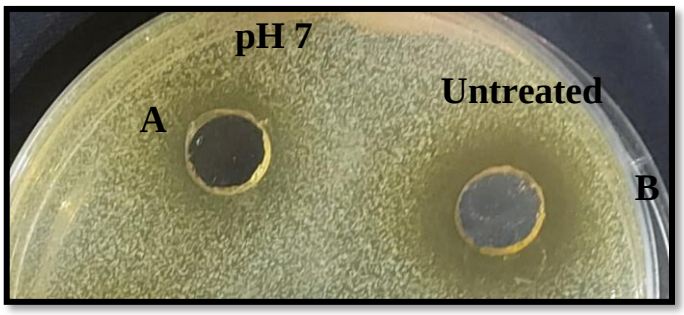


**Figure 2. Effect of pH adjustment on cCFS;** A: neutralized cCFS to pH 7; B: untreated cCFS.

### 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.

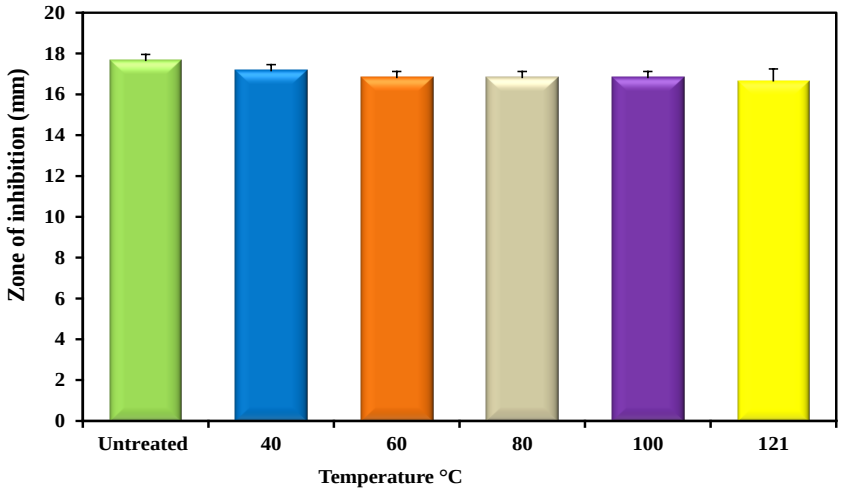


**Figure 3.** Thermal stability of *L. rhamnosus* cCFS on FLZ resistant *C. albicans* isolates.

## 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 [5].

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* [34,35]. 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*., 2021[43] 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 [43,44]. 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 [40,42].

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 [38].

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 [16]. The anti-Candida activity of probiotic Lactobacilli has been proved in many studies [16,19,26,49,50.]

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 [19] 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 [50,51] Our results can be explained by antimicrobial activity of lactobacilli is strain dependent [52].

We choosed *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 [52].

Several studies have highlighted on the anti-Candida activity of *L. rhamnosus* strains and it's potential use against *C. albicans* infections [53-55]. 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 [26,29]. 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 [19]. 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$\pm$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 [56].

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* [19] 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 [52].

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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## REFERENCES

1- Sardi JC, Scorzoni L, Bernardi T, Fusco-Almeida AM, Giannini MM. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. Journal of medical microbiology. 2013 Jan 1;62(1):10-24.

2- Szweda P, Gucwa K, Romanowska E, Dzierz K, Naumiuk Ł, Brillowska-Da A, Wojciechowska-Koszko I, Milewski S. Mechanisms of azole resistance among clinical isolates of Candida glabrata in Poland. Journal of medical microbiology. 2015 Jun 1;64(6):610-9.

3- Singh DK, Tóth R, Gácser A. Mechanisms of pathogenic Candida species to evade the host complement attack. Frontiers in cellular and infection microbiology. 2020 Mar 12;10:94.

4- Sikora A, Zahra F. Nosocomial infections. InStatPearls [Internet] 2021 Feb 10. StatPearls Publishing.

5- Li Y, Sun L, Lu C, Gong Y, Li M, Sun S. Promising antifungal targets against Candida albicans based on ion homeostasis. Frontiers in Cellular and Infection Microbiology. 2018:286.

6- Mane A, Vidhate P, Kusro C, Waman V, Saxena V, Kulkarni-Kale U, Risbud A. Molecular mechanisms associated with fluconazole resistance in clinical Candida albicans isolates from India. Mycoses. 2016 Feb;59(2):93-100.

7- Bhattacharya S, Sae-Tia S, Fries BC. Candidiasis and mechanisms of antifungal resistance. Antibiotics. 2020 Jun;9(6):312.

8- Nishimoto AT, Sharma C, Rogers PD. Molecular and genetic basis of azole antifungal resistance in the opportunistic pathogenic fungus Candida albicans. Journal of Antimicrobial Chemotherapy. 2020 Feb;75(2):257-70.

9- Ben-Ami R. Treatment of invasive candidiasis: A narrative review. Journal of Fungi. 2018 Sep;4(3):97.

10- Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. Azole antifungal resistance in Candida albicans and emerging non-albicans Candida species. Frontiers in microbiology. 2017 Jan 12;7:2173.

11- Matsubara VH, Bandara HM, Mayer MP, Samaranayake LP. Probiotics as antifungals in mucosal candidiasis. Clinical Infectious Diseases. 2016 May 1;62(9):1143-53.

12- Jørgensen MR, Kragelund C, Jensen PØ, Keller MK, Twetman S. Probiotic Lactobacillus reuteri has antifungal effects on oral Candida species in vitro. Journal of oral microbiology. 2017 Jan 1;9(1):1274582.

13- FAO/WHO. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food, London Ontario, Canada, April 30–May 1, 2002.

14- Silva MP, Rossoni RD, Junqueira JC, Jorge AO. Probiotics for prevention and treatment of candidiasis and other infectious diseases: Lactobacillus spp. and other potential bacterial species. Probiot. Prebiot. Hum. Nutr. Health. 2016 Jul 13.

15- Bulgasem B Y, Lani MN, Hassan Z, Yusoff WMW, Fnaish SG. Antifungal activity of lactic acid bacteria strains isolated from natural honey against pathogenic Candida species. Mycobiology. 2016; 44(4), 302-309.

16- Vazquez-Munoz R, Dongari-Bagtzoglou A. Anticandidal activities by Lactobacillus species: an update on mechanisms of action. Frontiers in Oral Health. 2021:47.

17- Köhler GA, Assefa S, Reid G. Probiotic interference of Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 with the opportunistic fungal pathogen Candida albicans. Infectious diseases in obstetrics and gynecology. 2012 Oct;2012.

18- Wang S, Wang Q, Yang E, Yan L, Li T, Zhuang H. Antimicrobial compounds produced by vaginal Lactobacillus crispatus are able to strongly inhibit Candida albicans growth, hyphal formation and regulate virulence-related gene expressions. Frontiers in microbiology. 2017 Apr 4; 8:564.

19- De Gregorio PR, Silva JA, Marchesi A, Nader-Macías ME. Anti-Candida activity of beneficial vaginal lactobacilli in in vitro assays and in a murine experimental model. FEMS yeast research. 2019 Mar;19(2): foz008.

20- El-Ashmony MM, Hafez HM, Abd-El-Hamid DH, Abd El-Rahman OA, and Rasslan F. Evaluation of Different Phenotypic Methods for Identification of Candida Species Isolated from Clinical Samples. Egypt. J. Med. Lab. Sci. 2019 August; 28 (2): ISSN 1110-5593.

21- Clinical and Laboratory Standards Institute (CLSI). Method for antifungal disk diffusion susceptibility testing of yeasts. 3rd ed. (M44-Ed3). Wayne, PA: Clinical and Laboratory Standards Institute, 2018.

22- Clinical and Laboratory Standards Institute (CLSI). Reference method for broth dilution antifungal susceptibility testing of yeasts. 4th ed. (M27-Ed4). Wayne, PA: Clinical and Laboratory Standards Institute, 2017.

23- Clinical and Laboratory Standards Institute (CLSI). Performance standards for antifungal susceptibility testing of yeasts. 2nd ed. (M60-Ed2). Wayne, PA: Clinical and Laboratory Standards Institute, 2020.

24- Dota KF, Freitas AR, Consolaro ME, Svidzinski TI. A challenge for clinical laboratories: detection of antifungal resistance in Candida species causing vulvovaginal candidiasis. Laboratory Medicine. 2011 Feb 1;42(2):87-93.

25- ElFeky DS, Gohar NM, El-Seidi EA, Ezzat MM, AboElew SH. Species identification and antifungal susceptibility pattern of Candida isolates in cases of vulvovaginal candidiasis. Alexandria Journal of Medicine. 2016 Aug 16;52(3):269-77.

26- Coman MM, Verdenelli MC, Cecchini C, Silvi S, Orpianesi C, Boyko N, Cresci A. In vitro evaluation of antimicrobial activity of L actobacillus rhamnosus IMC 501®, L actobacillus paracasei IMC 502® and SYNBIO® against pathogens. Journal of applied microbiology. 2014 Aug;117(2):518-27.

27- Chew SY, Cheah YK, Seow HF, Sandai D, Than LT. Probiotic L actobacillus rhamnosus GR-1 and L actobacillus reuteri RC-14 exhibit strong antifungal effects against vulvovaginal candidiasis-causing C andida glabrata isolates. Journal of Applied Microbiology. 2015 May;118(5):1180-90.

28- Muhialdin BJ, Hassan Z, Saari N. In vitro antifungal activity of lactic acid bacteria low molecular peptides against spoilage fungi of bakery products. Annals of Microbiology. 2018 Sep;68(9):557-67.

29- Verdenelli MC, Coman MM, Cecchini C, Silvi S, Orpianesi C, Cresci A. Evaluation of antipathogenic activity and adherence properties of human L actobacillus strains for vaginal formulations. Journal of applied microbiology. 2014 May;116(5):1297-307.

30- Crowley S, Mahony J, van Sinderen D. Broad-spectrum antifungal-producing lactic acid bacteria and their application in fruit models. Folia Microbiologica. 2013 Jul;58(4):291-9.

31- Arrioja-Bretón D, Mani-López E, Palou E, López-Malo A. Antimicrobial activity and storage stability of cell-free supernatants from lactic acid bacteria and their applications with fresh beef. Food Control. 2020 Sep 1;115:107286.

32- Baharudin MM, Ngalimat MS, Mohd Shariff F, Balia Yusof ZN, Karim M, Baharum SN, Sabri S. Antimicrobial activities of Bacillus velezensis strains isolated from stingless bee products against methicillin-resistant Staphylococcus aureus. PLoS ONE. 2021; 16(5): e0251514.

33- Alshaikh NA, Perveen K. Susceptibility of Fluconazole-Resistant Candida albicans to Thyme Essential Oil. Microorganisms. 2021 Dec;9(12):2454.

34- Farhan MA, Moharram AM, Salah T, Shaaban OM. Relation between antifungal resistance and enzymatic activity of yeast causing oral and vaginal mycosis. Assiut Univ J Bot Microbiol. 2019; 48(1):1–16.

35- El-Ganiny AM, Yossef NE, Kamel HA. Prevalence and antifungal drug resistance of nosocomial Candida species isolated from two university hospitals in Egypt. Current medical mycology. 2021 Mar;7(1):31.

36- Mohamed AO, Mohamed MS, Hussain MA, Ahmed IF. Detection of antifungal drug-resistant and ERG11 gene mutations among clinical isolates of Candida species isolated from Khartoum, Sudan. F1000Research. 2020;9.

37- Costa-de-Oliveira S, Rodrigues AG. Candida albicans antifungal resistance and tolerance in bloodstream infections: The triad yeast-host-antifungal. Microorganisms. 2020 Feb;8(2):154.

38- Keereedach P, Hrimpeng K, Boonbumrung K. Antifungal activity of Thai cajuput oil and its effect on efflux-pump gene expression in fluconazole-resistant Candida albicans clinical isolates. International Journal of Microbiology. 2020 Nov 4;2020.

39- Kaur R, Dhakad MS, Goyal R, Kumar R. Emergence of non-albicans Candida species and antifungal resistance in intensive care unit patients. Asian Pacific Journal of Tropical BiomSayededicine. 2016 May 1;6(5):455-60.

40- Sayed SA, Hassan EA, Hameed MR, Agban MN, Saleh MF, Mohammed HH, Abdel-Aal AB, Elgendy SG. Ketorolac-fluconazole: A New Combination Reverting Resistance in Candida albicans from Acute Myeloid Leukemia Patients on Induction Chemotherapy: In vitro Study. Journal of Blood Medicine. 2021;12:465.

41- Sardari A, Zarrinfar H, Mohammadi R. Detection of ERG11 point mutations in Iranian fluconazole-resistant Candida albicans isolates. Current Medical Mycology. 2019 Mar;5(1):7.

42- Amr GE, Atef DM, Salah AM. Identification and Anti-Fungal Resistance Profile of Different Candida Species Isolated from Patients in ICUs. Int J Curr Microbiol App Sci. 2019;8(6):564-73.

43-Jeon S, Shin JH, Lim, HJ, Choi MJ, Byun SA, Lee D, Lee SY, Won EJ, Kim SH, Shin MG. Disk Diffusion Susceptibility Testing for the Rapid Detection of Fluconazole Resistance in Candida Isolates. Annals of laboratory medicine. 2021 Nov; 41(6), 559-67.

44- Kumar D, Bhattacharyya S, Gupta P, Banerjee G, Singh M. Comparative analysis of disc diffusion and E-test with broth micro-dilution for susceptibility testing of clinical Candida isolates against amphotericin B, fluconazole, voriconazole and caspofungin. Journal of clinical and diagnostic research: JCDR. 2015; 9(11), DC01.

45-Jayachandran AL, Katragadda R, Ravinder T, Vajravelu L, Manorajan L, Hemalatha S, & Shanmugam K. Antifungal susceptibility pattern among candida species: an evaluation of disc diffusion and micro broth dilution method.Journal of Microbiology and Infectious Diseases. 2018; 8(03), 97-102.

46- Terças AL, Marques SG, Moffa EB, Alves MB, de Azevedo CM, Siqueira WL, Monteiro CA. Antifungal drug susceptibility of Candida species isolated from HIV-positive patients recruited at a public hospital in São Luís, Maranhão, Brazil. Frontiers in microbiology. 2017 Mar 2; 8:298.

47- Karimzadeh I, Heydari M, Ramzi M, Sagheb MM. Frequency and associated factors of amphotericin b nephrotoxicity in hospitalized patients in Hematology-Oncology Wards in the Southwest of Iran. Nephro-urology monthly. 2016 Sep;8(5).

48- Nenoff P, Krüger C, Neumeister C, Schwantes U, Koch D. In vitro susceptibility testing of yeasts to nystatin–low minimum inhibitory concentrations suggest no indication of in vitro resistance of Candida albicans, Candida species or non-Candida yeast species to nystatin. Clin. Med. Investig. 2016 Nov 22; 1:71-6.

49- Hefzy EM, Khalil MA, Amin AA, Ashour HM, Abdelaliem YF. Bacteriocin-like inhibitory substances from probiotics as therapeutic agents for Candida vulvovaginitis. Antibiotics. 2021 Mar;10(3):306.

50- Parolin C, Croatti V, Laghi L, Giordani B, Tondi MR, De Gregorio PR, Foschi C, Vitali B. Lactobacillus Biofilms Influence Anti-Candida Activity. Frontiers in microbiology. 2021;12.

51- Song YG, Lee SH. Inhibitory effects of Lactobacillus rhamnosus and Lactobacillus casei on Candida biofilm of denture surface. Archives of oral biology. 2017 Apr 1; 76:1-6.

52- Rose Jørgensen M, Thestrup Rikvold P, Lichtenberg M, Østrup Jensen P, Kragelund C, Twetman S. Lactobacillus rhamnosus strains of oral and vaginal origin show strong antifungal activity in vitro. Journal of oral microbiology. 2020 Jan 1;12(1):1832832.

53- Ribeiro FC, De Barros PP, Rossoni RD, Junqueira JC, Jorge AO. Lactobacillus rhamnosus inhibits Candida albicans virulence factors in vitro and modulates immune system in Galleria mellonella. Journal of applied microbiology. 2017 Jan;122(1):201-11.

54- Mailänder-Sánchez D, Braunsdorf C, Grumaz C, Müller C, Lorenz S, Stevens P, Wagener J, Hebecker B, Hube B, Bracher F, Sohn K. Antifungal defense of probiotic Lactobacillus rhamnosus GG is mediated by blocking adhesion and nutrient depletion. PLoS One. 2017 Oct 12;12(10): e0184438.

55- Ribeiro FC, Iglesias MC, Barros P P, Santos SSF, Jorge AOC, and Leão MV. P. Lactobacillus rhamnosus Interferes with Candida albicans Adherence and Biofilm Formation: A Potential Alternative Treatment of Candidiasis. Austin J Pharmacol Ther. 2021, 9(2).1133.

56- Mani-López E, Arrioja-Bretón D, López-Malo A. The impacts of antimicrobial and antifungal activity of cell-free supernatants from lactic acid bacteria in vitro and foods. Comprehensive Reviews in Food Science and Food Safety. 2022 Jan;21(1):604-41.