Impact of Brief Exposure to Drugs with Antifungal Properties on the Susceptibility of Oral *Candida dubliniensis* Isolates to Lysozyme and Lactoferrin

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Significance of the Study

- Lysozyme and lactoferrin are salivary constituents with anti-candidal activity. *Candida* infections are managed with antimycotic drugs; there is no information on the impact of these drugs (i.e., synergism or antagonism) on the susceptibility of *Candida dubliniensis* to lysozyme and lactoferrin. This study reveals that exposure to antifungal drugs increases the susceptibility of *C. dubliniensis* to lysozyme and lactoferrin.

Keywords

Antifungal agents · *Candida dubliniensis* · Lactoferrin · Lysozyme

Abstract

Objective: Lysozyme and lactoferrin have anti-candidal activity. *Candida dubliniensis* is associated with oral candidiasis. *Candida* infections are managed with nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine. *Candida* species undergo a brief exposure to therapeutic agents in the mouth. There is no data on the influence of limited exposure to antimycotics on the sensitivity of *C. dubliniensis* to lactoferrin and lysozyme. Hence, this study observed the changes in the sensitivity of *C. dubliniensis* to anti-candidal action of lactoferrin and lysozyme after transitory exposure to sub-lethal concentrations of antifungals. **Materials and Methods:** After determination of the minimum inhibitory concentration (MIC), 20 *C. dubliniensis* isolates were

exposed to twice the concentration of MIC of nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine for 1 h. Drugs were removed by dilution and thereafter the susceptibility of these isolates to lysozyme and lactoferrin was determined by colony-forming unit quantification assay. **Results:** Exposure of *C. dubliniensis* to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine resulted in an increase in susceptibility to lysozyme by 9.45, 30.82, 30.04, 50.64, 55.60, and 50.18%, respectively ($p < 0.05$ to $p < 0.001$). Exposure of *C. dubliniensis* to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine resulted in an increase in susceptibility to lactoferrin by 13.54, 16.43, 17.58, 19.60, 21.32, and 18.73, respectively ($p < 0.05$ to $p < 0.001$). **Conclusion:** Brief exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine enhances the antifungal effect of lysozyme and lactoferrin on *C. dubliniensis* isolates in vitro.

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Introduction

Candida dubliniensis is currently widely acknowledged as an opportunistic pathogen allied with recurrent oral candidiasis in patients with acquired immunodeficiency syndrome (AIDS). It has also been isolated from the niches of the oral milieu of diabetic and cystic fibrosis patients. *C. dubliniensis* has been isolated from upper respiratory tract specimens and blood as well as from a case of endocarditis involving a prosthetic aortic valve, which indicates that it can disseminate to other sites [1–4]. Moreover, cases of spondylodiscitis, multifocal osteomyelitis, and leptomenigeal disease have been shown to be caused by *C. dubliniensis* [5–7]. Additionally, resistance to fluconazole has been observed in *C. dubliniensis* isolates obtained from AIDS patients and stable resistance to this triazole can be readily produced in *C. dubliniensis* following exposure to this drug in vitro [8]. Likewise, a breakthrough in *C. dubliniensis* fungemia was made in a patient during sustained exposure to voriconazole [9]. Longitudinal genotyping of *C. dubliniensis* isolates from patients with HIV infection has shown resistance to itraconazole, even in the absence of prior azole therapy [10]. *C. dubliniensis* strains resistant to 5-fluorocytosine have also been isolated in Kuwait and in the Middle East region [11, 12].

Human whole saliva is accepted as a principal source of numerous antimicrobial substances, such as lactoferrin and lysozyme. These nonimmune protective determinants are well known for their functionality in regulating the quantity and quality of microflora on mucosal surfaces [13]. Lactoferrin, an iron-binding glycoprotein, is found in granules of polymorphonuclear leukocytes and in saliva [13]. Lysozyme, which is present in the oral environment, arises from the salivary glands, gingival tissue, and crevicular fluid [13]. In vitro investigations have validated bacteriostatic, bactericidal, and anticandidal activity of apo-lactoferrin, which is an iron-free lactoferrin [14, 15]. Lysozyme has also been shown to exert antimicrobial activity on a range of oral microorganisms in vitro including *Candida* species [15].

A plethora of drugs with antimycotic pharmacodynamics are available for the treatment of oral candidiasis. These include the polyene antifungals such as nystatin and amphotericin B, echinocandins such as caspofungin, the azoles such as ketoconazole (an imidazole) and fluconazole (a triazole), and the widely used oral mouth wash chlorhexidine gluconate [16, 17]. However, the diluting impact of saliva and the cleansing outcome of the oral musculature in the oral environment tend to reduce the

availability of these pharmaceuticals to levels below their effective therapeutic concentrations, thus reducing their therapeutic efficacy [16, 17]. Hence, the opportunistic *Candida* may undergo a brief exposure to antifungal pharmaceuticals during therapy, a scenario all too familiar in the niches of the oral cavity [16, 17].

Recent research has demonstrated that brief exposure to sub-therapeutic concentrations of drugs with antimycotic pharmacodynamics such as nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate could modulate the pathogenic features of oral *Candida*, ranging from adhesion to denture acrylic and buccal epithelial cells, cell surface hydrophobicity, germ tube formation, and production of pathogenic enzymes such as candidal hemolysin and phospholipase [18–23]. However, only one study has investigated the impact of lysozyme and lactoferrin on oral *Candida* (i.e., *Candida albicans* and *Candida tropicalis*) pre-exposed to three antimycotic agents such as nystatin, amphotericin B, and fluconazole, for a brief period [24]. Such an impact on the clinically important *C. dubliniensis* isolates, with a wide array of different drugs with antifungal properties, has not been investigated so far. Hence, based on these considerations, supported by the findings of a recent prevalence study which showed that oral *C. dubliniensis* isolates had the highest prevalence among non-*albicans* oral *Candida* species in Kuwait [25], the main aim of this study was to investigate the changes in susceptibility to lactoferrin and lysozyme of 20 oral isolates of *C. dubliniensis* after brief exposure (1 h) to sub-therapeutic concentrations of nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate. In addition, as *C. dubliniensis* and *C. albicans* are phenotypically similar *Candida* species, *C. albicans* oral isolates obtained from Kuwait were also studied for the purpose of comparison of results between these two *Candida* species.

Materials and Methods

Organisms

Twenty oral isolates each of *C. dubliniensis* and *C. albicans* maintained as stock cultures and stored in the Oral Microbiology Laboratory, Faculty of Dentistry and in the Mycology Unit, Faculty of Medicine, Kuwait University were used. The stock cultures of *Candida* were from the oral rinse samples obtained in a previous investigation, from patients attending the Kuwait University Dental Clinic for dental treatment [25]. These patients were healthy adults and none of the patients from whom the isolates were obtained had oral candidiasis. Identification and confirmation of the isolates were carried out as per our previous investigations [18–

23]. Initially, all *Candida* isolates were tested for germ tube formation. Their colony characteristics were also determined by means of CHROMagar *Candida* medium (Becton Dickinson and Company, Sparks, MD, USA) and carbohydrate assimilation profiles were obtained using the VITEK 2 *Candida* identification system (BioMérieux, France). Subsequently, identification of these isolates was confirmed further by using semi-nested PCR amplification of the internal transcribed spacer 2 region of rDNA followed by direct DNA sequencing of the internal transcribed spacer region of rDNA. The semi-nested PCR amplification was performed as described previously [18–23]. Briefly, amplification with universal fungal primers was carried out in 50 µL containing 1 × AmpliTaq PCR buffer I, 1 U AmpliTaq DNA polymerase, deionized water, 10 pmol each of CTSF and CTSR primers, 1 µL of DNA and 0.1 mM of each dNTP. After amplification in the first step, 1 µL of the product was further amplified using CTSR and *C. dubliniensis*-specific CDDT primer yielding an amplicon of 105 bp as described previously [18–23]. Thereafter, the amplified products (20 µL) were resolved by electrophoresis in agarose gels.

Antifungal Agents and Media

Antifungal agents were prepared as done previously [18–23]. Briefly, nystatin, amphotericin B, ketoconazole, and fluconazole (Sigma, St. Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO). Caspofungin (Merck and Company Inc., Waterhouse Station, NJ, USA) was dissolved in sterile distilled water. These antimycotic agents were prepared initially as stocks of 10,000 µg/mL and stored at –20 °C before use. They were suspended/diluted in the following medium during the exposure period (1 h). RPMI-1640 medium buffered with 0.165 M MOPS (3-(*N*-morpholino)propanesulfonic acid) containing L-glutamine and lacking sodium bicarbonate (Sigma, USA) was dissolved in 1 L of sterile distilled water and adjusted to a pH of 7.2 and filter-sterilized. This medium was stored at 2–8 °C for 2–3 months. The stock solution was used to obtain drug concentrations (i.e., 2 × minimum inhibitory concentration [MIC]). Chlorhexidine gluconate 0.2% (GlaxoSmith-Kline, Brentford, UK) was dissolved in sterile phosphate-buffered saline (PBS) at pH 7.2 and was diluted to obtain a concentration of 0.005% prior to each experiment as previously described [18].

Determination of MIC

MIC values for nystatin and chlorhexidine were obtained by the broth dilution technique by performing twofold serial dilutions of the drugs in microtiter plates using an inoculum of $1-5 \times 10^5$ colony-forming units (CFU)/mL [18, 22]. The MIC values were obtained visually and spectrophotometrically at 595 nm following 24 h of incubation at 37 °C. The MIC was defined as the lowest concentration of the drug that inhibited growth of *Candida*, as indicated by the absence of turbidity (optically clear). *C. albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 were used as reference strains.

The MIC values for amphotericin B, caspofungin, ketoconazole, and fluconazole were determined by E tests as described previously according to the manufacturer's recommendations (AB BIODISK, Solna, Sweden) [18–23]. Each test isolate was freshly subcultured. Five isolated colonies were uniformly suspended in sterile saline, and turbidity was adjusted to 0.5 McFarland standard ($1-5 \times 10^6$ cells/mL). This inoculum was swabbed onto the agar plates of 150 mm diameter and allowed to dry for 10–15 min. Thereafter, the E test strips were placed. RPMI-1640 agar supple-

mented with 2% glucose and buffered with MOPS (0.165 M; pH 7.0) was used for susceptibility testing according to the method recommended by the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards; M27-A2). The plates were incubated at 35 °C, and MIC was read after 24–48 h of incubation. The point where inhibition ellipses intercepted the scale on the antifungal strip was taken as the MIC for each test isolate. Reference strains of *C. albicans*, ATCC 90028, and *C. parapsilosis*, ATCC 22019, were used for quality control of the susceptibility testing. Interpretive susceptibility breakpoints for fluconazole were those recommended by Clinical and Laboratory Standards Institute document M27-A2. Due to the lack of defined susceptibility breakpoints for amphotericin B and ketoconazole, an isolate was considered susceptible with an MIC breakpoint of ≤ 1.0 µg/mL for amphotericin B and 0.125 µg/mL for ketoconazole as determined in previous studies [18–22, 25]. Isolates with MIC of ≤ 2.0 µg/mL for caspofungin were considered as susceptible [23, 25].

Preparation of Yeast Cell Suspension and Exposure to Antimycotic Agents

A previously described method was used for this purpose [18–23]. Briefly, yeast cells maintained on Sabouraud's dextrose agar were inoculated onto fresh plates and incubated overnight at 37 °C for 24 h prior to use. The organisms were harvested and a cell suspension prepared in sterile PBS of pH 7.4 to an optical density of 1.5 at 520 nm. From this cell suspension, 1 mL was added to tubes containing 4 mL of RPMI broth (control) and 4 mL of RPMI/drug solution (test), in which the drug concentrations were twice the MIC values. This yielded a cell suspension of 10^6 cells mL^{–1} in each assay tube. The tubes were then incubated at 37 °C for 1 h in a rotary incubator. Following this limited exposure, the drugs were removed by two cycles of dilution with sterile PBS and centrifugation for 10 min at 3,000 g. Afterwards the supernatant was completely decanted, and the pellets were resuspended in 5 mL of sterile PBS. This procedure for drug removal has shown to reduce the concentration of the drug as much as 10,000-fold, thereby minimizing any carry-over effect of the drug [18–23]. Viable counts of the control and the test were performed after each procedure of drug removal. As the procedure of drug removal effectively eliminated any carry-over effect, there was virtually no difference in the viable counts of the control and the tests following exposure to already diluted sub-therapeutic concentrations of the drug as observed in previous studies [18–23].

The *Candida* pellets obtained were washed twice by suspending in ice-cold 0.05 mM potassium chloride (KCl), buffered to pH 7.0 with potassium hydroxide (KOH) and thereafter harvested by centrifugation at 3,500 g for 5 min. The yeasts were resuspended in the buffered KCl to give a final concentration 5×10^6 cells mL^{–1} [24].

Lactoferrin and Lysozyme

Human apo-lactoferrin (Sigma Chemical Co., Poole, UK) was used for the study. A stock solution of apo-lactoferrin (0.016 g of the protein in 2 mL sterile distilled water) was prepared at a dilution of 200 µg mL^{–1} with sterile distilled water as previously performed [24]. Hen egg-white lysozyme (Sigma Chemical Co.) was similarly used for all the experiments. A stock solution of lysozyme (0.016 g of the protein in 2 mL sterile distilled water) was prepared at a dilution of 300 µg mL^{–1} as done previously [24].

Lactoferrin- and Lysozyme-Induced Antifungal Assays

The antifungal effect of either lactoferrin (F_{LF}) or lysozyme (F_{LZ}) on the isolates was determined with minor modifications [24, 26]. Briefly, suspensions of either 200 $\mu\text{g mL}^{-1}$ of apo-lactoferrin or 100 $\mu\text{g mL}^{-1}$ of lysozyme, and 100 μL of the *Candida* suspension were dispensed into sterile tubes containing 800 μL of 0.05 mM phosphate-buffered KCl (0.05 mM; pH 7.0) to yield a cell concentration of $5 \times 10^5 \text{ mL}^{-1}$ yeasts. Thus, the final concentrations of apo-lactoferrin and lysozyme in the suspensions were 20 and 30 $\mu\text{g mL}^{-1}$, respectively. In the control sample, 100 μL sterile distilled water was substituted for the protein. Both the lactoferrin- or lysozyme-containing tubes and the control tubes were then incubated at 37°C for 1 h with gentle shaking. After incubation, the tubes were carefully vortexed; 100- μL samples were diluted 1:50 and plated on Sabouraud's dextrose agar and the resultant CFU were quantified after 48 h of incubation at 37°C.

The antifungal activity of lactoferrin (F_{LF}) or lysozyme (F_{LZ}) was computed using the formula for both drug-exposed (control [i.e., without lactoferrin or lysozyme] and with lactoferrin or lysozyme) and unexposed isolates (control [i.e., without lactoferrin or lysozyme] and with lactoferrin or lysozyme):

$$F_{LF} \text{ or } F_{LZ} = (\text{CFU mL}^{-1} \text{ of control suspension} - \text{CFU mL}^{-1} \text{ of lactoferrin or lysozyme suspension}) / \text{CFU mL}^{-1} \text{ of control suspension.}$$

Thus, the higher the F_{LF} or F_{LZ} value, the higher the sensitivity of the isolates to either protein.

All experiments were repeated on three separate occasions with duplicate determinations on each occasion. *C. dubliniensis* reference strain CD36 and *C. albicans* reference strain ATCC 90028 were also used in the above assays.

Statistical Analysis

The data obtained from all three assays in duplicate were analyzed as done previously [18–23] using the ANOVA Dunnett *t* test, which treats one group as a control (unexposed to drugs) and compares all other groups (exposed to drugs) against it. A *p* value of <0.05 was considered statistically significant.

Results

The MIC ($\mu\text{g/mL}$) values of 20 isolates of *C. dubliniensis* and *C. albicans* to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate were: nystatin: 0.09–0.78; amphotericin B: 0.002–0.125; caspofungin: 0.003–0.19; ketoconazole: 0.002–0.012; fluconazole: 0.016–0.38; and chlorhexidine gluconate: 0.00125%. These values were within the normal MIC range for the *Candida* species for each of the drugs tested as seen in previous studies as well [18–23]. Thus, none of the isolates was resistant to the tested drugs.

The mean F_{LZ} value of *C. dubliniensis* isolates unexposed to antimycotics was 0.550. Brief exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate significantly (*p* <

0.05 to *p* < 0.001) increased the susceptibility of the isolates to lysozyme, yielding mean F_{LZ} values of 0.602, 0.714, 0.710, 0.821, 0.848, and 0.820, respectively (Table 1). Thus, exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate provided mean percentage increase in susceptibility to lysozyme by 9.45, 30.82, 30.04, 50.64, 55.60, and 50.18%, respectively (Table 1). The mean F_{LZ} value of *C. albicans* isolates unexposed to antimycotics was 0.562. Brief exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate significantly (*p* < 0.05 to *p* < 0.001) increased the susceptibility of the isolates to lysozyme, yielding mean F_{LZ} values of 0.612, 0.712, 0.711, 0.821, 0.853, and 0.817, respectively (Table 1). Thus, exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate provided mean percentage increase of susceptibility to lysozyme by 8.90, 26.69, 26.51, 46.08, 51.77, and 45.37%, respectively (Table 1).

The mean F_{LF} value of *C. dubliniensis* isolates unexposed to antimycotics was 0.347. Brief exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate significantly (*p* < 0.05 to *p* < 0.001) increased the susceptibility of the isolates to lactoferrin, yielding mean F_{LF} values of 0.394, 0.404, 0.408, 0.415, 0.421, and 0.412, respectively (Table 2). Thus, exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate provided mean percentage increase of susceptibility to lactoferrin by 13.54, 16.43, 17.58, 19.60, 21.32, and 18.73%, respectively (Table 2). The mean F_{LF} value of *C. albicans* isolates unexposed to antimycotics was 0.357. Brief exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate significantly (*p* < 0.05 to *p* < 0.001) increased the susceptibility of the isolates to lactoferrin, yielding mean F_{LF} values of 0.400, 0.409, 0.413, 0.423, 0.429, and 0.420, respectively (Table 2). Thus, exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine gluconate provided mean percent increase in susceptibility to lactoferrin by 12.04, 14.56, 15.68, 18.48, 20.16, and 17.65%, respectively (Table 2).

Discussion

As the frequency of oral as well as systemic candidiasis-associated *C. dubliniensis* increases, there is a concomitant need for improvement in the pharmacodynamics of drugs with antifungal properties to manage such

Table 1. The antifungal activity of lysozyme (F_{LZ}) on oral *Candida dubliniensis* and *Candida albicans* isolates (*n* = 20 each) following brief exposure to nystatin (NYS), amphotericin B (AMP-B), caspofungin (CASPO), ketoconazole (KETO), fluconazole (FLU), and chlorhexidine (CHX)

Isolates	Control	NYS	AMP-B	CASPO	KETO	FLU	CHX
<i>C. dubliniensis</i>							
Mean	0.550	0.602	0.714	0.710	0.821	0.848	0.820
SEM	0.007	0.005	0.005	0.004	0.006	0.008	0.007
SD	0.029	0.020	0.021	0.019	0.028	0.037	0.030
Increase in sensitivity		9.45	30.82	30.04	50.64	55.60	50.18
<i>p</i> value		<0.05	<0.001	<0.001	<0.001	<0.001	<0.001
<i>C. albicans</i>							
Mean	0.562	0.612	0.712	0.711	0.821	0.853	0.817
SEM	0.005	0.004	0.004	0.004	0.005	0.005	0.006
SD	0.021	0.020	0.020	0.017	0.023	0.024	0.028
Increase in sensitivity		8.90	26.69	26.51	46.08	51.77	45.37
<i>p</i> value		<0.05	<0.001	<0.001	<0.001	<0.001	<0.001

Percent increase in sensitivity to lysozyme (%) following exposure to these drugs is given at the bottom of the table. All values indicate the average of three experiments done in duplicate.

Table 2. The antifungal activity of lactoferrin (F_{LF}) on oral *Candida dubliniensis* and *Candida albicans* isolates following brief exposure to nystatin (NYS), amphotericin B (AMP-B), caspofungin (CASPO), ketoconazole (KETO), fluconazole (FLU), and chlorhexidine (CHX)

Isolates	Control	NYS	AMP-B	CASPO	KETO	FLU	CHX
<i>C. dubliniensis</i>							
Mean	0.347	0.394	0.404	0.408	0.415	0.421	0.412
SEM	0.012	0.011	0.012	0.012	0.012	0.012	0.012
SD	0.053	0.050	0.052	0.053	0.055	0.054	0.054
Increase in sensitivity		13.54	16.43	17.58	19.60	21.32	18.73
<i>p</i> value		<0.05	<0.05	<0.01	<0.01	<0.001	<0.01
<i>C. albicans</i>							
Mean	0.357	0.400	0.409	0.413	0.423	0.429	0.420
SEM	0.009	0.008	0.008	0.010	0.010	0.010	0.007
SD	0.041	0.036	0.035	0.045	0.044	0.043	0.031
Increase in sensitivity		12.04	14.56	15.68	18.48	20.16	17.65
<i>p</i> value		<0.05	<0.05	<0.05	<0.01	<0.001	<0.01

Percent increase in sensitivity to lactoferrin (%) following exposure to these drugs is given at the bottom of the table. All values indicate the average of three experiments done in duplicate.

infections. In this study, brief exposure of *C. dubliniensis* oral isolates to a sub-MIC dose of the drugs was attempted, so as to mimic the oral conditions in vitro as done previously [18–24]. Thereafter, the changes in susceptibility to the antifungal effect of two salivary constituents, lysozyme and lactoferrin, were evaluated on this *Candida* species (in addition, as *C. dubliniensis* and *C. albicans* are phenotypically similar, *C. albicans* oral isolates were also exposed to the same conditions for the purpose of comparison of results between these two *Candida* species).

In our study, the MIC values of all tested antifungal agents were within the range of the reference strain, which implies that the *Candida* isolates tested were susceptible to the drugs used. Furthermore, these values were within the MIC range obtained in previous investigations of *C. dubliniensis* and *C. albicans* isolates [18–20, 23].

In the present study, it was noted that *C. dubliniensis* and *C. albicans* isolates significantly exhibited increased susceptibility to both lysozyme and lactoferrin when pre-exposed to sub-MIC concentration of 6 different agents

with antifungal properties for a brief period. In a previous study, too, though not significant for some of the tested isolates, an overall increase in susceptibility to both these salivary constituents were reported following brief exposure of *C. albicans* and *C. tropicalis* isolates to nystatin, amphotericin B, and fluconazole [24].

The precise mechanism by which lysozyme interrelates with the fungal cell wall is not clear. However, two distinctive complementary mechanisms have been suggested. Firstly, the enzymatic hydrolysis of N-glycosidic bonds that link polysaccharides and structural proteins of the candidal cell wall may result in consequent injury to the cytoplasmic membrane [27]. Secondly, impairment of the plasma lemma of the yeast cell may be followed by activation of mannan synthetase, chitin synthetase, and probably other enzymes that code for the yeast cell wall constituents [27]. In addition, the fungicidal effect of lactoferrin and apo-lactoferrin-mediated killing of *Candida* has been shown to depend on factors such as pH, temperature, and incubation period [26]. Again, the accurate mode of action of lactoferrin on *Candida* is not clear although it is speculated that sequestration of elemental iron and its deprivation may play a role [24]. It has also been noted that lactoferrin interacts with the candidal cell surface causing extracellular leakage of proteins and the formation of surface blebs [24].

As the precise mechanism by which lysozyme and lactoferrin interact with the candidal cell wall is not yet clear, it is not possible to definitively explain the higher sensitivity of *C. dubliniensis* and *C. albicans* isolates to both lysozyme and lactoferrin pre-exposed to drugs with antifungal properties. However, we speculate that it may be partially related to the mechanism of action of these drugs on *Candida* contributing to a synergistic effect with these two salivary constituents. For instance, polyenes (nystatin and amphotericin B) bind to sterol components in the cell wall of *Candida* and make it more permeable [16, 17]. Also, it has been advocated that the creation of sterols or their precursors may be repressed by the polyenes [16, 17]. Furthermore, studies have revealed internally misshapen cells with an intact cell wall leaving “ghost-like” cells and collapsed *Candida* cells following exposure to sub-fungicidal concentrations of nystatin [16, 17]. In addition, microbial appendices such as the outer membrane protein, lipoprotein, phospholipid, lipopolysaccharide, and fimbriae may be altered by polyenes [16, 17]. Similarly, caspofungin affects the enzyme responsible for biosynthesis of β -1,3 glucan ensuing in disrupting the integrity of the yeast cell wall [16, 17, 23] and azoles (ketoconazole and fluconazole) act by altering the fungal cell

membranes blocking the 14 α -demethylation step in the biosynthesis of ergosterol. The consequential exhaustion of ergosterol and accretion of 14 α -methyl sterols leads to alterations in a number of membrane-associated functions [16, 17]. Scanning and transmission electron microscopic studies have exhibited that the antifungal effect of chlorhexidine is most likely due to the loss of cytoplasmic components and coagulation of nucleoproteins and associated morphological changes in the cell wall structure [16, 17]. Hence, taken together, the increased susceptibility to both lysozyme and lactoferrin following exposure to polyenes, echinocandins, azoles, and chlorhexidine may be related to the pharmacodynamics of these drugs on the cell wall of *Candida*.

One previous report has described the influence of nystatin, amphotericin B, and fluconazole on the susceptibility of *C. albicans* and *C. tropicalis* isolates to lysozyme and lactoferrin [24], but the current study is first to evaluate the effect of lactoferrin and lysozyme on a large number of *C. dubliniensis* and *C. albicans* isolates ($n = 40$) obtained from a single geographic location, following exposure to drugs with a wide array of antifungal pharmacodynamics, ranging from polyenes, echinocandins, azoles, and chlorhexidine. However, we studied only two putative salivary antifungal defenses; therefore, these findings cannot be interpreted as fully expressive of the oral milieu because other nonspecific salivary defense factors such as histatins, cystatins, lactoperoxidase, chromogranin A as well as specific defenses such as secretory IgA are likely to operate in vivo [28]. However, our data indicate that exposure to drugs such as polyenes, echinocandins, azoles as well as chlorhexidine gluconate may enhance the susceptibility of *Candida* to lysozyme and lactoferrin, thereby increasing the defense capability of these salivary constituents towards this yeast. Interestingly, resistance of *C. dubliniensis* to fluconazole, voriconazole, and itraconazole has been documented previously [8–10]. Furthermore, resistance of *C. dubliniensis* to 5-fluorocytosine in isolates obtained from Kuwait and the Middle East region has also been documented [11, 12]. Occurrence of such resistance indicates the need for possible alternative therapeutic modalities which may facilitate the management of candidiasis. In this context, the current study suggests that exposure to polyene, echinocandin, and azole antifungal agents and chlorhexidine may induce a synergistic effect by enhancing the susceptibility of *C. dubliniensis* to naturally occurring salivary constituents such as lysozyme and lactoferrin, which may provide additional information about pharmacodynamics of these drugs on *C. dubliniensis* isolates (as well as

C. albicans isolates). In addition, in our study there was no significant difference in the impact of antifungal drugs on the susceptibility of the two *Candida* species towards lysozyme and lactoferrin. This is not surprising as these two *Candida* species are considered phenotypically almost identical *Candida* species. However, the overall lower susceptibility of *C. albicans* species to lysozyme and lactoferrin compared to *C. dubliniensis* species indicates that *C. albicans* species are more resilient to the impact of both these salivary constituents even following exposure to antifungal drugs. This further adds credence to the fact that *C. albicans* is more virulent and pervasive than *C. dubliniensis*.

Conclusion

Brief exposure to nystatin, amphotericin B, caspofungin, ketoconazole, fluconazole, and chlorhexidine enhances the antifungal effect of lysozyme and lactoferrin

on both *C. dubliniensis* and *C. albicans* isolates in vitro. However, more investigations with sessile *Candida* isolates encased in a biofilm, as opposed to the planktonic isolates used in the current study, are warranted to further augment the current evidence.

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Disclosure Statement

The authors declare no conflict of interest.

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