

Oral Microbiology

Impact of lysozyme and lactoferrin on oral *Candida* isolates exposed to polyene antimycotics and fluconazole

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OBJECTIVE: To assess the antifungal activity of lysozyme and lactoferrin on 10 oral isolates each of *Candida albicans* and *Candida tropicalis* following their brief exposure (1 h) to subtherapeutic concentrations of two polyene drugs – nystatin, amphotericin B, and an azole – fluconazole.

METHODS: Yeasts were sequentially exposed to subtherapeutic concentrations of antifungals and then to either lysozyme or lactoferrin and the viability evaluated by quantifying colony-forming units.

RESULTS: The exposure of both *C. albicans* and *C. tropicalis* isolates to all three antifungal agents significantly increased their susceptibility to lysozyme ($P < 0.05$) but not to lactoferrin. Exposure to the two polyene drugs had a lesser impact on the lysozyme susceptibility of both *Candida* species compared with the azole drug. Both interspecies and intraspecies sensitivity to lysozyme was noted and *C. albicans* was less susceptible than *C. tropicalis*.

CONCLUSIONS: Lysozyme, in addition to being a potent natural antifungal agent, may act synergistically with the studied antimycotics.

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Keywords: antifungals; *Candida*; lactoferrin; lysozyme

Introduction

Oropharyngeal candidiasis is the most common opportunistic infection seen in compromised patients such as those with human immunodeficiency virus (HIV) infection (Powderly *et al.*, 1999). As the number of immunocompromised patients is increasing both in the developing and developed world

because of the pandemic of HIV as well as the medical technological advances, so does the incidence and prevalence of oral candidosis in these patient cohorts (Arendorf *et al.*, 1998; Ellepola and Samaranayake, 2000). Although *Candida albicans* is the predominant pathogen in initial and recurrent episodes of oral candidosis (Samaranayake, 1992) non-*albicans Candida* species, such as *Candida tropicalis* are infrequently but consistently isolated from these patients either in combination with *C. albicans* or as monomicrobial infections (Cartledge, Midgley and Gazzard, 1999).

In the human body, whole saliva is considered to be the principal source of a number of antimicrobial substances, such as lactoferrin and lysozyme (muramidase). These non-immune defense factors are well known for their role in regulating the quality and quantity of flora on mucosal surfaces (Tenovuo, Lumikari and Soukka, 1991). Lactoferrin is an iron-binding glycoprotein found in the specific granules of polymorphonuclear leukocytes as well as in saliva (Ellison, Giehl and LaForce, 1988; Tenovuo *et al.*, 1991). Lysozyme present in the oral cavity is derived from the major and minor salivary glands, the gingival tissue and gingival crevicular fluid (Tenovuo *et al.*, 1991). *In vitro* studies have demonstrated both bacteriostatic, bactericidal (Lassiter *et al.*, 1987) and anticandidal activity (Nikawa and Hamada, 1990; Samaranayake *et al.*, 1993; Samaranayake *et al.*, 2001) of apo-lactoferrin (i.e. iron-free lactoferrin). Lysozyme has also been shown to exert antimicrobial activity on a range of oral microorganisms *in vitro* including *Candida* species (Iacono *et al.*, 1983; Iacono *et al.*, 1985).

Oral *Candida* infection is commonly treated with the classic polyenes (nystatin and amphotericin B) and the newer triazoles such as fluconazole (Powderly *et al.*, 1999). Despite the availability of these agents for treatment of oropharyngeal candidosis, failure of therapy is not uncommon (Martin, 1990). In the mouth the diluent effect of saliva and cleansing mechanism of the oral musculature tend to reduce the availability of the agent to below that of the effective therapeutic

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concentration. Hence the organisms are only suboptimally exposed to the antimycotics and concentration is likely to vary in different niches of the mouth. Further, the emergence of drug-resistant *Candida* in general and azoles in particular also contribute to drug failure in patients with oral candidosis (White, Marr and Bowden, 1998; Cartledge *et al.*, 1999).

Recent studies have revealed that a brief exposure to subtherapeutic concentrations of antimycotics modulates the pathogenic features of *Candida*, ranging from adhesion to denture acrylic (Ellepola and Samaranyake, 1998b) and buccal epithelial cells (Ellepola and Samaranyake, 1998a), cell surface hydrophobicity (Egusa *et al.*, 2000), and germ tube formation (Ellepola and Samaranyake, 1998c). However, there is no information on the impact of lysozyme and lactoferrin on *Candida* species pre-exposed to antimycotics for a brief period. Hence, the main aim of this study was to compare the impact of lactoferrin and lysozyme on 10 oral isolates each of *C. albicans* and *C. tropicalis* after brief exposure (1 h) to subtherapeutic concentrations of nystatin, amphotericin B and fluconazole.

Materials and methods

Fungi and culture conditions

A total of 10 oral isolates each of *C. albicans* and *C. tropicalis* from HIV-infected subjects with oral candidiasis were used in the study. All isolates were from patients attending the Queen Elizabeth Hospital, Hong Kong and were obtained by the oral rinse technique (Samaranyake *et al.*, 1986). The procedure involves the patient holding and rinsing 10 ml of sterile phosphate-buffered saline (PBS) (0.01 M, pH 7.2) in the mouth for 60 s. The solution is then expectorated into a chilled container, immediately transported to the laboratory and 50 μ l of concentrate is then inoculated onto Sabouraud's agar medium (Oxoid Limited, Basingstoke, England) using a spiral plating system (Spiral Systems, Cincinnati, OH, USA). After 24–48 h incubation at 37°C, the growth is subcultured for purity and identified using the germ-tube test and the commercially available API-20C AUX system (bio-Mérieux, Basingstoke, UK) (Buesching, Kurek and Roberts, 1979). Stock cultures were maintained at –20°C. After recovery these were maintained on Sabouraud's dextrose agar and stored at 4–6°C, during the experimental period.

Antifungal agents

Three antifungal agents obtained as reagent grade powders were tested: nystatin and amphotericin B (Sigma Chemicals Co. St Louis, MO, USA) were dissolved in dimethylsulfoxide and absolute ethanol (3:2 ratio), respectively. Fluconazole (Pfizer Inc. New York, USA) was dissolved in absolute methanol as per protocol described previously (Ellepola and Samaranyake, 1998a, b; Egusa *et al.*, 2000). All agents were prepared initially as 10 000 μ g ml⁻¹ solutions and stored at –20°C until used.

Minimum inhibitory concentrations (MIC) determinations

Broth microdilution method. Antifungal susceptibility to nystatin was determined as per the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 1997). The inoculum was prepared from 24 h cultures of the yeasts. Cell suspensions were prepared in RPMI-1640 medium (Life technologies, New York, USA) and adjusted to a final inoculum of 10⁵ cfu ml⁻¹. Testing was performed in 96-well round-bottomed microtiter plates. The plates were incubated at 37°C and read at 24 h. The MIC of nystatin was defined, as the lowest concentration at which there was 100% inhibition of yeast growth (concentration range 0.39–3.12 μ g ml⁻¹).

E-test method. The MIC determinations of amphotericin-B and fluconazole were performed using the E-test. The E-test (AB Biodisk, Solna, Sweden) is a patented commercial method for the quantitative determination of MICs of antimicrobial drugs. Comparisons of the E-test method with the NCCLS broth dilution method have demonstrated high levels of agreement (Warnock, Johnson and Rogers, 1998).

The inoculum for the E-test was prepared from a 24-h culture of *Candida* species. Cell suspensions were prepared in sterile distilled water and adjusted to a concentration corresponding to a 0.5 McFarland standard using a spectrophotometer, at 520 nm (Beckman, DU 530 spectrophotometer, Life Science, CA, USA). The medium used was RPMI-1640 agar (1.5%) with 2% glucose buffered with morphopropane sulfonic acid (pH 7.0). The plates were inoculated by dipping a sterile swab into the appropriate cell suspension and streaking it across the entire surface of the agar in three directions. They were then dried at room temperature for 15 min before the E-test strips were applied. Afterwards, the plates were incubated at 37°C and read at 24 h. The E-test MIC was read as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip (NCCLS, 1997).

Preparation of the yeast suspension and exposure to antifungal agents. Yeast cells, maintained on Sabouraud's dextrose agar, were inoculated onto fresh plates and incubated overnight for 24 h prior to use. The organisms were harvested and a cell suspension prepared in sterile PBS of pH 7.4, at 520 nm corresponding to an optical density 0.5 McFarland standard. 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. This gave a cell suspension of 10⁶–10⁷ cells ml⁻¹ in each assay tube.

The tubes were then incubated at 37°C for a period of 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 3000g. Afterwards, the supernatant was completely decanted and the pellets were resuspended in 5 ml of sterile PBS. It has been found by previous investigators that removal of 90% of the supernatant with two

washings reduces antimicrobial concentration 100-fold, while complete decanting of the supernatant with two washings (as carried out in the current study) reduces the concentration by 10 000-fold (McDonald, Craig and Kunin, 1977). Hence this method virtually eliminates any 'carry-over effect' of the drug following its removal. This washing procedure was then repeated and the pellets resuspended in 2.5 ml of sterile PBS. Viable counts of the control and test were done by spiral plating after drug removal and control suspensions were reconstituted as needed to obtain a cell concentration comparable to the test.

The yeast pellet thus obtained was washed twice by suspending in ice-cold 0.05 mM KCl (which was buffered to pH 7.0 with KOH) and harvested by centrifugation at 3500 g for 5 min (Soukka, Tenovuo and Lenander-Lumikari, 1992). The yeasts were resuspended in the buffered KCl to give a final concentration 5×10^6 yeasts ml⁻¹.

Iron-free lactoferrin (apo-lactoferrin)

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.

Lysozyme

Hen-egg-white lysozyme (Sigma Chemical Co., Poole, UK) was 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.

Apo-lactoferrin and lysozyme induced fungicidal assays

The fungicidal effect of either apo-lactoferrin or lysozyme on drug-exposed test isolates and control isolates was determined by the method of Soukka *et al* (1992) with minor modifications. Suspensions of either 200 µg ml⁻¹ of apo-lactoferrin or 100 µg ml⁻¹ of lysozyme, and 100 µl of the yeast were dispensed into sterile incubation tubes containing 800 µl of 0.05 mM phosphate-buffered KCl (0.05 mM; pH 7.0) to yield a yeast cell concentration of 5×10^5 yeasts ml⁻¹. Thus, the final

concentrations of apo-lactoferrin and lysozyme in the test suspensions were 20 and 30 µg ml⁻¹, respectively. In the control sample 100 µl sterile distilled water was substituted for the protein. Both the test and control tubes were then incubated at 37°C for 1 h with gentle shaking. After incubation, the test and control tubes were carefully vortexed; 100 µl samples were diluted 1:50 and plated on Sabouraud's dextrose agar using a spiral plater (Spiral Systems, Cincinnati, OH, USA), and the resultant cfu were quantified after 48 h incubation at 37°C.

The fungicidal activity of apo-lactoferrin or lysozyme was computed using the formula:

$$F_{LF} \text{ or } F_{LZ} = (\text{cfu ml}^{-1} \text{ of control suspension} - \text{cfu ml}^{-1} \text{ of test suspension}) / (\text{cfu ml}^{-1} \text{ of control suspension})$$

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

All the experiments described above were conducted on two separate occasions each with triplicate samples.

Statistical analysis

The intra- and inter-species differences of the fungicidal effect of the two proteins were analyzed by ANOVA. Multiple comparisons between isolates exposed to the three drugs and control were analyzed using the Tukey-Kramer multiple comparisons test using GraphPad InStat version 3.00 (GraphPad Software Inc., San Diego, CA, USA).

Results

MIC

The MIC values of the 10 isolates of *C. albicans* to nystatin, amphotericin B and fluconazole, were 0.78–1.56, 0.19–0.38 and 0.12–0.38 µg ml⁻¹, respectively, whereas the MIC values of the 10 isolates of *C. tropicalis* to nystatin, amphotericin B and fluconazole, were 0.78, 0.25–0.38 and 0.25–0.50 µg ml⁻¹, respectively. These values were within the normal MIC range for the

Table 1 The F_{LZ} values of *Candida albicans* isolates exposed to nystatin, amphotericin B and fluconazole

<i>C. albicans</i>	Lysozyme (Control)	Nystatin	Increase in sensitivity (%)	Amphotericin B	Increase in sensitivity (%)	Fluconazole	Increase in sensitivity (%)
HK1KD	0.52 ± 0.11	0.53 ± 0.10	2	0.66 ± 0.05	26	0.81 ± 0.02	55
HK2OB	0.46 ± 0.06	0.50 ± 0.05	8	0.59 ± 0.04	28	0.80 ± 0.02	73
HK4RB	0.42 ± 0.13	0.64 ± 0.10	53	0.57 ± 0.11	36	0.80 ± 0.06	92
HK5SD	0.42 ± 0.06	0.62 ± 0.06	49	0.74 ± 0.02	77	0.79 ± 0.01	89
HK6SC	0.41 ± 0.05	0.47 ± 0.06	15	0.67 ± 0.04	63	0.79 ± 0.02	93
HK8CA	0.49 ± 0.04	0.56 ± 0.09	14	0.67 ± 0.04	37	0.80 ± 0.03	63
HK9TB	0.47 ± 0.03	0.53 ± 0.03	13	0.70 ± 0.04	50	0.81 ± 0.02	73
HK10OD	0.37 ± 0.04	0.38 ± 0.03	9	0.39 ± 0.04	54	0.39 ± 0.05	78
HK36SC	0.54 ± 0.04	0.51 ± 0.03	6	0.68 ± 0.03	24	0.81 ± 0.02	50
HK39RE	0.49 ± 0.02	0.58 ± 0.02	19	0.66 ± 0.04	35	0.81 ± 0.01	66
Mean	0.47 ± 0.06	0.54 ± 0.06	17	0.66 ± 0.04	42	0.80 ± 0.02	72

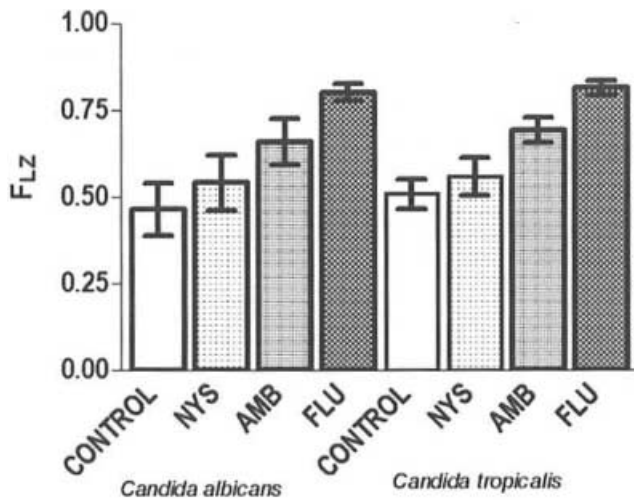


Figure 1 The F_{LZ} values of *Candida albicans* and *Candida tropicalis* isolates exposed to nystatin (NYS), amphotericin B (AMB) and fluconazole (FLU)

respective *Candida* species for each of the drugs tested. Thus, none of the isolates was resistant to the tested drugs.

Impact of lysozyme on C. albicans isolates exposed to antimycotics

The mean F_{LZ} value of *C. albicans* isolates unexposed to antimycotics was 0.47. Brief exposure to nystatin,

amphotericin B and fluconazole significantly increased the susceptibility of the isolates to lysozyme, yielding mean F_{LZ} values of 0.54 ($P < 0.001$), 0.66 ($P < 0.001$) and 0.80 ($P < 0.001$), respectively (Table 1; Figure 1).

There were significant intra-species variations in lysozyme susceptibility of the two *Candida* species exposed to all three drugs. These ranged from -6 to 53% for nystatin; 24-77% for amphotericin B and 50-93% for fluconazole. In general, the fluconazole exposed yeasts were more susceptible to lysozyme than either the nystatin or amphotericin B exposed yeasts ($P < 0.001$). Similarly, amphotericin B exposed yeasts were significantly more susceptible to lysozyme than nystatin exposed yeasts ($P < 0.001$). Interestingly, one *C. albicans* isolate (HK36SC) exposed to nystatin demonstrated marginal increased resistance (6%) to lysozyme – an aberrant finding which was not significant.

Impact of lysozyme on C. tropicalis isolates exposed to antimycotics

The mean F_{LZ} value for *C. tropicalis* isolates unexposed to antimycotics was 0.51 (Table 2). As in the case of *C. albicans* brief exposure to nystatin, amphotericin B and fluconazole significantly increased the susceptibility levels of *C. tropicalis* with F_{LZ} values of 0.56 ($P < 0.001$), 0.69 ($P < 0.001$) and 0.81 ($P < 0.001$), respectively. The percentage increase in sensitivity of lysozyme ranged from 1 to 25% for nystatin; 27-48% for amphotericin B; and 45-76% for fluconazole. As in the case of *C. albicans*, fluconazole exposed yeasts were

Table 2 The F_{LZ} values of *Candida tropicalis* isolates exposed to nystatin, amphotericin B and fluconazole

<i>C. tropicalis</i>	Lysozyme (Control)	Nystatin	Increase in sensitivity (%)	Amphotericin B	Increase in sensitivity (%)	Fluconazole	Increase in sensitivity (%)
HK1KA	0.54 ± 0.04	0.56 ± 0.03	3	0.69 ± 0.05	27	0.83 ± 0.01	53
HK1KE	0.54 ± 0.03	0.55 ± 0.02	1	0.71 ± 0.01	32	0.83 ± 0.01	53
HK4LA	0.47 ± 0.03	0.58 ± 0.06	25	0.67 ± 0.01	42	0.82 ± 0.01	76
HK5LG	0.49 ± 0.03	0.52 ± 0.02	6	0.69 ± 0.03	42	0.81 ± 0.01	66
JK5LF	0.49 ± 0.02	0.55 ± 0.04	12	0.73 ± 0.02	48	0.80 ± 0.01	63
HK9LF	0.56 ± 0.03	0.59 ± 0.02	6	0.72 ± 0.02	29	0.81 ± 0.02	45
HK9LG	0.51 ± 0.04	0.55 ± 0.04	7	0.63 ± 0.03	24	0.81 ± 0.01	60
HK36LA	0.52 ± 0.04	0.65 ± 0.06	25	0.68 ± 0.02	30	0.83 ± 0.01	59
HK44LF	0.48 ± 0.04	0.51 ± 0.04	6	0.68 ± 0.04	42	0.78 ± 0.04	63
HK44TD	0.50 ± 0.04	0.55 ± 0.05	10	0.73 ± 0.02	45	0.82 ± 0.01	63
Mean	0.51 ± 0.04	0.56 ± 0.04	10	0.69 ± 0.02	36	0.81 ± 0.01	60

Table 3 The F_{LZ} values of *Candida albicans* isolates exposed to nystatin, amphotericin B and fluconazole

<i>C. albicans</i>	Lysozyme (Control)	Nystatin	Increase in sensitivity (%)	Amphotericin B	Increase in sensitivity (%)	Fluconazole	Increase in sensitivity (%)
HK1KD	0.32 ± 0.06	0.34 ± 0.05	6	0.36 ± 0.04	14	0.37 ± 0.03	16
HK2OB	0.39 ± 0.03	0.41 ± 0.03	5	0.42 ± 0.03	7	0.43 ± 0.04	9
HK4RB	0.32 ± 0.05	0.34 ± 0.04	5	0.35 ± 0.06	8	0.35 ± 0.05	10
HK5SD	0.25 ± 0.07	0.29 ± 0.07	16	0.29 ± 0.08	15	0.30 ± 0.06	20
HK6SC	0.43 ± 0.03	0.43 ± 0.03	0	0.43 ± 0.04	-1	0.39 ± 0.05	-1
HK8CA	0.37 ± 0.04	0.38 ± 0.03	2	0.39 ± 0.04	6	0.39 ± 0.05	7
HK9TB	0.26 ± 0.04	0.26 ± 0.04	-3	0.27 ± 0.03	2	0.27 ± 0.02	2
HK10OD	0.40 ± 0.04	0.42 ± 0.06	5	0.44 ± 0.04	8	0.45 ± 0.06	11
HK36SC	0.23 ± 0.06	0.24 ± 0.04	7	0.23 ± 0.05	4	0.24 ± 0.04	8
HK39RE	0.35 ± 0.04	0.37 ± 0.03	7	0.39 ± 0.05	12	0.39 ± 0.04	13
Mean	0.33 ± 0.05	0.35 ± 0.04	5	0.36 ± 0.04	7	0.36 ± 0.04	9

more susceptible to lysozyme than nystatin and amphotericin B exposed yeasts (Table 2). Similarly amphotericin B exposed yeasts were significantly more susceptible to lysozyme than nystatin exposed yeasts ($P < 0.001$).

Impact of lactoferrin on C. albicans isolates exposed to antimycotics

The mean F_{LF} value of *C. albicans* isolates unexposed to antifungals was 0.33 (Table 3). Pre-exposure of the isolates to any of the three agents tested did not have a significant impact on the susceptibility of the yeasts to lactoferrin. The F_{LF} values noted were 0.35, 0.36 and 0.36 for nystatin, amphotericin B and fluconazole, respectively. Although there was a marginal increase in the sensitivity of *C. albicans* isolates, after exposure to antimycotics this was not significant.

Impact of lactoferrin on C. tropicalis isolates exposed to antimycotics

The mean F_{LF} value of all 10 *C. tropicalis* isolates was 0.39 prior to drug exposure (Table 4). As in the case of *C. albicans*, exposure of the isolates to any of the antifungal agents did not significantly affect their susceptibility to lactoferrin.

Inter-species variation in lysozyme and lactoferrin susceptibility

Although *C. tropicalis* isolates were significantly more sensitive to lysozyme than *C. albicans* this marginal

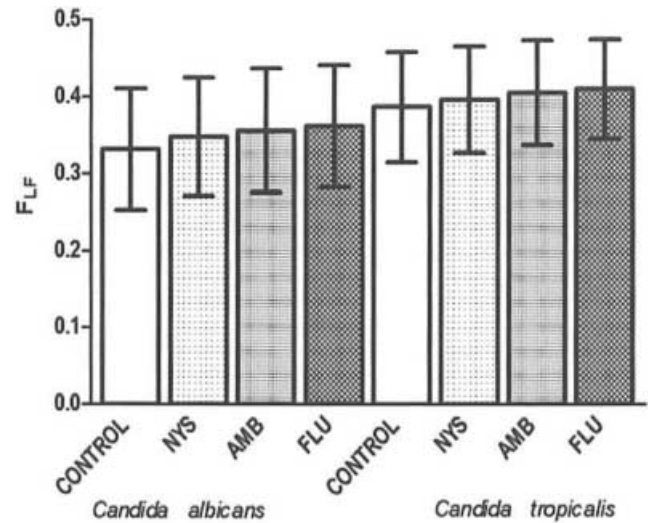


Figure 2 The F_{LF} values of *Candida albicans* and *Candida tropicalis* isolates exposed to nystatin (NYS), amphotericin B (AMB) and fluconazole (FLU)

difference in sensitivity tended to disappear when the organisms were exposed to nystatin and fluconazole, but not amphotericin B (Table 5). There was also a significant inter-species variation in the susceptibility to lactoferrin with the F_{LF} values of 0.33 and 0.39 for *C. albicans* and *C. tropicalis*, respectively. The higher sensitivity of *C. tropicalis* isolates to lactoferrin persisted

Table 4 The F_{LF} values of *Candida tropicalis* isolates exposed to nystatin, amphotericin B and fluconazole

<i>C. tropicalis</i>	Lysozyme (Control)	Nystatin	Increase in sensitivity (%)	Amphotericin B	Increase in sensitivity (%)	Fluconazole	Increase in sensitivity (%)
HK1KA	0.25 ± 0.06	0.28 ± 0.06	12	0.29 ± 0.06	14	0.30 ± 0.06	21
HK1KE	0.37 ± 0.02	0.39 ± 0.05	6	0.40 ± 0.07	10	0.39 ± 0.02	8
HK4LA	0.35 ± 0.04	0.36 ± 0.04	3	0.36 ± 0.05	3	0.37 ± 0.04	3
HK5LG	0.38 ± 0.03	0.38 ± 0.04	-2	0.39 ± 0.03	2	0.39 ± 0.03	1
HK5LF	0.46 ± 0.04	0.47 ± 0.04	2	0.47 ± 0.04	3	0.48 ± 0.04	4
HK9LF	0.44 ± 0.04	0.45 ± 0.04	2	0.45 ± 0.05	4	0.46 ± 0.03	6
HK9LG	0.45 ± 0.06	0.45 ± 0.06	1	0.45 ± 0.04	2	0.46 ± 0.04	3
HK36LA	0.36 ± 0.04	0.36 ± 0.06	1	0.38 ± 0.04	6	0.39 ± 0.05	9
HK44LF	0.41 ± 0.07	0.42 ± 0.06	2	0.43 ± 0.06	4	0.43 ± 0.04	5
HK44TD	0.40 ± 0.04	0.41 ± 0.03	3	0.42 ± 0.03	5	0.42 ± 0.06	7
Mean	0.39 ± 0.04	0.40 ± 0.05	2	0.40 ± 0.05	5	0.41 ± 0.04	6

Table 5 A comparison of the mean F_{LZ} and F_{LF} values of *C. albicans* and *C. tropicalis* isolates exposed to three different antifungal agents

	Control	Nystatin	Amphotericin B	Fluconazole
Lysozyme sensitivity (F_{LZ})				
<i>C. albicans</i> (mean ± s.d.)	0.47 ± 0.08	0.54 ± 0.08	0.66 ± 0.07	0.80 ± 0.02
Range	0.20–0.64	0.36–0.70	0.34–0.77	0.69–0.85
<i>C. tropicalis</i> (mean ± s.d.)	0.51 ± 0.04	0.56 ± 0.05	0.69 ± 0.04	0.81 ± 0.02
Range	0.41–0.61	0.46–0.72	0.60–0.75	0.72–0.85
P-Level	$P < 0.001$	NS	$P < 0.05$	NS
Lactoferrin sensitivity (F_{LF})				
<i>C. albicans</i> (mean ± s.d.)	0.33 ± 0.08	0.35 ± 0.08	0.36 ± 0.08	0.36 ± 0.08
Range	0.14–0.49	0.19–0.52	0.18–0.51	0.19–0.52
<i>C. tropicalis</i> (mean ± s.d.)	0.39 ± 0.07	0.40 ± 0.07	0.40 ± 0.07	0.41 ± 0.06
Range	0.14–0.52	0.18–0.52	0.18–0.52	0.20–0.53
P-Level	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.05$

even after their exposure to nystatin, amphotericin B and fluconazole ($P < 0.05$) (Figure 2).

Discussion

As the frequency of fungal infections increases, there is a concomitant need for increased understanding of the pharmacodynamic properties of the antifungals available and those recently introduced. A number of effective antifungal agents are currently used for the treatment of oropharyngeal candidiasis. However, their efficacy is dependent on many factors. In the mouth, *Candida* is constantly exposed to an environmental milieu replete with saliva and its many antimicrobial constituents. The constant salivary flushing action also works in favor of the organism when antifungal agents are delivered topically by reducing the drug concentration below therapeutic levels. Thus the organisms undergo only a limited exposure to the antifungal agents during treatment as the concentration of the drug tends to vary in different niches of the oral cavity. In this study, exposure of the yeast to a sub-MIC dose of the drugs was attempted, so as to mimic these oral conditions *in vitro* and then the effect of two salivary antifungal constituents lysozyme and lactoferrin were evaluated on two, commonly isolated *Candida* species.

We have earlier established that the exposure of *Candida* to sub-MIC concentrations of antifungal agents induces a postantifungal effect on the isolates (Anil, Ellepola and Samaranyake, 2001). In the present study it was noted that both *C. albicans* and *C. tropicalis* isolates exhibited increased susceptibility to lysozyme when pre-exposed to sub-MIC concentration of three different antifungal agents for 60 min. The fungicidal effect of lysozyme was maximal for fluconazole-exposed yeasts followed by amphotericin B and nystatin. The precise mechanism by which lysozyme interacts with the fungal cell wall is not clear. However, two distinct complementary mechanisms have been suggested: first, the enzymatic hydrolysis of N-glycosidic bonds that link polysaccharides and structural proteins of the yeast cell wall may result in subsequent injury to the cytoplasmic membrane; and second, damage to the plasmalemma of *C. albicans* cell is followed by activation of mannan synthetase, chitin synthetase and probably other enzymes that code for the yeast cell wall constituents (Kamaya, 1970; Marquis *et al*, 1991). We are unable to offer a precise explanation for the higher sensitivity of the fluconazole exposed yeasts – although the mechanisms of action on the yeast plasma membrane of the azole and polyene group of drugs may partly explain this observation. We believe that the current study is the first to evaluate the synergistic effect of naturally occurring salivary constituents and antifungal agents belonging to both polyene and azole groups. However, Nishiyama *et al* (2001) have recently reported the synergistic action of lysozyme and another novel azole-lanocanazole on the cell wall of *Candida*. Ultrastructural studies by the latter group found that a combination of an azole and lysozyme affects *Candida*

cell wall formation by interrupting the normal assembly and integration of cell wall components.

The drug exposed *C. tropicalis* isolates were significantly more sensitive to lysozyme than similarly treated *C. albicans* indicating the hierarchy of virulence between these species. Significant variations in sensitivity of *Candida* species to lysozyme have been reported earlier (Tobgi, Samaranyake and MacFarlane, 1988; Nikawa *et al*, 1993). For instance, in a pioneering study Tobgi *et al*, (1988) demonstrated difference in the susceptibility of *Candida* species to lysozyme and found *C. krusei* to be the most susceptible species followed by *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. albicans* and *C. glabrata*. Our findings on susceptibility to lysozyme are compatible with those of the latter group. Interestingly we found that the significantly high susceptibility of *C. tropicalis* compared with *C. albicans* disappeared once they were exposed to the antifungals implying that even subinhibitory concentrations of antifungals found intraorally may have potent effects and, act in partnership with the natural salivary defenses to kill the invading *Candida* irrespective of the species differences.

In addition to lysozyme, saliva contains a potent antifungal in the form of lactoferrin. Relatively recently, Soukka *et al* (1992) demonstrated the fungicidal effect of various forms of lactoferrin and reported that apolactoferrin-mediated killing of *Candida* was dependent on factors such as pH, temperature and incubation period. Similarly Nikawa *et al* (1993) investigated the fungicidal effect of human lactoferrin on five oral isolates each of *C. krusei* and *C. albicans* and noted highly significant intra- and inter-species variations in fungicidal activity and that *C. krusei* was significantly more susceptible to lactoferrin than *C. albicans*. The precise action of lactoferrin on *Candida* is not clear although sequestration of elemental iron and its deprivation may play a role. In addition, Nikawa *et al* (1993) have noted that lactoferrin interacts with the yeast cell surface causing extracellular leakage of proteins and the formation of surface blebs.

The current results reconfirm that lactoferrin has a suppressive effect on both *Candida* species tested although *C. tropicalis* was significantly more sensitive than *C. albicans*. However, as opposed to lysozyme, pre-exposure of the isolates to polyenes or the azole did not have an impact on the susceptibility of the yeasts to lactoferrin (Tables 3 and 4). The latter finding was surprising as some have shown a synergistic activity of lactoferrin with azole antifungal agents (Nikawa *et al*, 1993; Wakabayashi *et al*, 1996). Furthermore, our experiments conducted with only two putative salivary antifungal defenses cannot be construed as fully representative of the oral environment as other non-specific salivary defenses such as histatins, cystatins, lactoperoxidase, chromogranin A and, specific defenses including secretory IgA are likely to operate *in vivo* (Nieuw Amerongen and Veerman, 2002).

To conclude, our data indicate that the salivary lysozyme has a synergistic antimycotic effect on the

polyene and azole antifungals that are topically delivered. As compared with the two polyene drugs, exposure to the azole drug sensitized the organisms to both salivary proteins to a much greater extent. These observations tend to agree with the superior clinical efficacy of fluconazole compared with polyenes. Finally, the current study which is the first to evaluate the effect of lactoferrin and lysozyme *per se* on a large number of *C. albicans* and *C. tropicalis* isolates substantiate previous data on the higher position occupied by the former in the hierarchy of virulence of *Candida* species. Whether these could synergize the lysozyme mediated anticandidal activity reported here need to be studied further.

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