*Medical Mycology* 1999, **37**, 35–41 Accepted 20 September 1998

# *In vitro* susceptibility of *Candida* species to lactoferrin

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Lactoferrin is an antimicrobial protein present in human mucosal secretions as well as saliva. As there is no information on the relative fungicidal activity of human and bovine lactoferrin, an oral isolate of *Candida albicans* was studied for its susceptibility to these two proteins. Exposure to a concentration of 20 $\mu g\,ml^{-1}$ of either HLF or BLF at 37 °C inactivated the yeast to the same degree irrespective of the incubation time of 45, 90 or 150 min. A similar study, using 20 $\mu g\,ml^{-1}$ BLF and an incubation time of 150 min, elicited varying anticandidal activity against 35 isolates belonging to six different *Candida* species. Thus, BLF was fungicidal for the six *Candida* species in the following decreasing order, *C. tropicalis* > *C. krusei* > *C. albicans* > *C. guilliermondii* > *C. parapsilosis* > *C. glabrata*; the latter being the most resistant. These *Candida* species also demonstrated significant intra-species variation in susceptibility to the protein ($P < 0{\cdot}05$). When the yeast cells exposed to BLF were examined by cryo-scanning electron microscopy, profound cell wall changes such as cell surface blebs, swelling and cell collapse were noted. These findings suggest that lactoferrin, a constituent of saliva, may differentially modulate the carriage of *Candida* species in the oral cavity.

**Keywords** lactoferrin, *Candida* species

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## Introduction

*Candida albicans* and related *Candida* species are associated with many forms of oral candidoses frequently seen in patients with systemic diseases (e.g. diabetes, immunosuppresion, xerostomia) or those wearing full dentures [1]. The suppression of local host defence mechanisms particularly those present in saliva is thought to be of critical importance for the invasive colonization of mucosal surfaces by this organism [2].

Saliva is known to protect oral tissues in many ways. A constant flow of saliva and its non-specific antimicrobial constituents such as lactoferrin, lysozyme, secretory IgA and lactoperoxidase regulate the quality and quantity of flora on mucosal surfaces [3]. One of the more important non-specific defence factors which modulate the microbial populations in the oral cavity is lactoferrin (LF), a mammalian iron-binding, acute phase protein found in saliva [3,4], milk and other exocrine secretions [5]. It is also found in acinar epithelial cells and in secondary granules of polymorphonuclear leucocytes [6]. In mucosal secretions, the lactoferrin molecule is primarily iron-free (apo-lactoferrin), rarely exceeding 20% iron saturation [6,7]. Lactoferrin concentration in whole saliva has been reported to range from 8·5 to 24 $\mu g\,ml^{-1}$ and increases 10–15-fold in dental plaque fluid [8,9].

Many workers have independently demonstrated the microbicidal/static effect of both human lactoferrin (HLF) and bovine lactoferrin (BLF) although none have compared the relative efficacy of the latter proteins [8,10–14]. The bactericidal effect of apo-lactoferrin is thought to be due to both to iron deprivation and its direct interaction with microbial cell walls [13]. Though

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extensive information is available on the bactericidal effect of lactoferrin [12,13,15,16], relatively little is known about the antifungal effect of LF against *Candida* species [17–20].

Studies conducted by Nikawa *et al.* [19] and Samaranayake *et al.* [20] indicate significant inter- and intra-species variations in the fungicidal effect of both apo-LF and iron saturated LF against *C. albicans* and *C. krusei*. The disparate sensitivity patterns of the two *Candida* species to LF which we observed in the latter study led us to the current attempt at investigating the inter- and intra species sensitivity to LF of a battery of human pathogenic *Candida* species which included *C. albicans* (five isolates), *C. glabrata* (six isolates), *C. krusei* (six isolates), *C. parapsilosis* (five isolates), *C. tropicalis* (six isolates) and *C. guilliermondii* (six isolates). A secondary aim of the present study was to compare the fungicidal effect of BLF and HLF on *C. albicans in vitro*. Cryo-scanning electronic microscope (Cryo-SEM) was also used to observe the cell surface changes of the yeasts exposed to LF.

## Materials and methods

### Microorganisms and growth conditions

Thirty-five oral isolates of *Candida* species from the routine microbiology services of the Glasgow Dental Hospital and School (GDH) and from the Beijing Medical University (BMU), Department of Stomatology were used. The majority of the isolates were recovered from either the denture or the palate of denture stomatitis patients while the remainder were from patients with candidosis of the tongue, burning mouth syndrome, and asymptomatic carriers. All isolates were identified by sugar assimilation and fermentation tests and 'the germ tube' test [21] and reconfirmed using the API 20C system (API Products, BioMerieux, Lyon, France). Stock cultures of all the yeast isolates were maintained at 4 °C, on Sabouraud glucose agar (SGA; Oxoid Ltd, Basingstoke, UK).

To prepare inocula for the assay, a loopful of each isolate was inoculated into brain–heart infusion broth (Oxoid Ltd, Basingstoke, UK) and grown aerobically at 37 °C. After 18 h incubation, which corresponded to the stationary phase of growth, the yeasts were harvested by centrifugation at 3500 *g* for 10 min. The yeast pellet thus obtained was washed twice with ice-cold KCl buffer (0·05 mM; buffered to pH 7·0 with KOH). The yeasts were resuspended in the buffered KCl to yield a final concentration of about $5 \times 10^6$ yeasts $ml^{-1}$, using a spectrophotometer (optical density of 0·63–0·64 at 520 nm).

Because *C. albicans* is a dimorphic fungus the possible emergence of a hyphal phase was monitored in all experiments. The cells remained in the yeast (blastoconidia) phase throughout the study.

### Bovine lactoferrin (BLF) and human lactoferrin (HLF)

Bovine lactoferrin, kindly provided by Dr S. Kalfas, University of Lund, Sweden, was purified from bovine milk whey while human lactoferrin, purified from human colostrum, was a generous gift from Dr J. Tenovuo (Institute of Dentistry, University of Turku, Finland). The purity and homogeneity of the preparations were analysed with FPLC chromatography (LKB, 2138 Uvicord S, Bromma, Sweden) and SDS–PAGE (Phast Gel, Phast System, Pharmacia, Sweden) using 10–15% gradient gels. The iron-free state of apo-LF was controlled by the ferrozine method as described by Soukka *et al.* [18]. The LF was found to be practically lysozyme-free (0·5 μg $mg^{-1}$ LF) when assayed with *Micrococcus* diffusion plates (Lysozyme Kit, Kallestad Laboratories, Chaska, MN, USA) and with electrophoresis on SDS gels. The preparation was also free of IgA when tested by enzyme immunoassay [18].

Stock solutions of both HLF and BLF equivalent to a concentration of 200 μg $ml^{-1}$ were prepared with sterile distilled water, stored at 4 °C and used immediately.

### Fungicidal assay

In the first part of the study the antifungal effect of HLF and BLF, on a single isolate *Candida albicans* GDH20 was compared according to the method of Nikawa *et al.* [19]. To determine the optimal assay conditions, 100 μl of either BLF or HLF solution and 100 μl of *C. albicans* GDH20 suspension and 800 μl of phosphate-buffered KCl (0·05 mM; pH 7·0) were dispensed into a sterile incubation tube to yield a final volume of 1·0 ml and a yeast concentration of $5 \times 10^5$ yeasts $ml^{-1}$. Test samples containing three concentrations of the protein (either BLF or HLF; 5, 10 and 20 μg $ml^{-1}$) were prepared for this assay. In the control sample, either BLF or HLF was replced by an equal volume of sterile distilled water.These tubes were then incubated at 37 °C for 45, 90 and 150 min with gentle shaking. After incubation the tubes were carefully vortexed, 100 μl of each sample was diluted 1:50 and plated on SGA using a spiral plater (Spiral Systems, Cincinnati, OH, USA) and the resultant colony-forming units (cfu) quantified after 48 h incubation at 37 °C.

The data from the dose, and time-response study described above was used to compare the relative potency of BLF on the six isolates each of *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. guilliermondii* and five isolates each of



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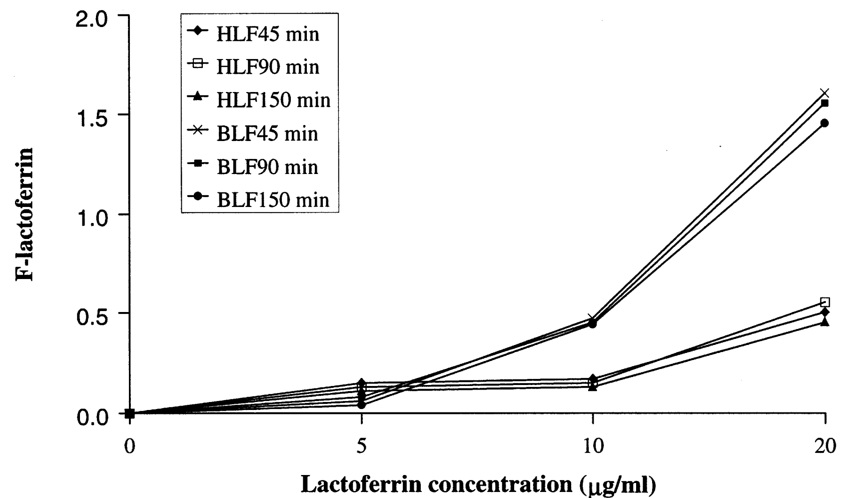


**Fig. 1** The effect of different concentrations of bovine and human lactoferrin (5, 10 and 20 μg ml$^{-1}$ on *Candida albicans* GDH20, incubated for 45, 90 and 150 min at 37 °C.

*C. albicans* and *C. parapsilosis*. This part of the study was performed using a standard BLF concentration (a final concentration of 20 μg ml$^{-1}$) and a standard exposure time (150 min).

All experiments were carried out on two separate occasions, with quadruplicate determinations on each occasion. When the assays were conducted with different *Candida* species, *C. albicans* GDH18 was used as the positive control throughout.

The fungicidal activity of lactoferrin denoted by F-lact was determined by computing the negative logarithmic ratio of the number of cfu in the test suspension and the number of cfu in the control suspension as follows:

F-lact = − log (cfu test suspension/
cfu control suspension)

The F-lact value, therefore, indicates the number of lactoferrin resistant yeast cells as a positive number. For example an F-lact value of 1 would indicate a 10% resistant population and therefore the cytotoxicity of the enzyme would be 90%. Thus, the higher the value of the F-lact value the higher the fungicidal activity.

### Cryo-SEM observation

To visualize the topographical features of the yeasts some of the residual yeast suspensions (after the assay procedure) were used. Thus, immediately after the assay, the test and the control tubes were carefully vortexed and fixed with 1% osmium tetroxide for 4 h. Then the *Candida* suspensions were filtered using a 1 μm diameter Millipore filter (Millipore Corporation, Bedford, MA, USA) secured in a filter holder. The filter with the retained yeasts was then washed with sterile distilled water and fitted onto a SEM stub for cryo-fixation in liquid-$N_2$ for 30 s. The whole assembly was introduced into the cryo-chamber of the SEM and allowed to sublime for 20 min at − 80 °C, sputter coated with gold for 60 s at − 160 °C and observed under the SEM at − 190 °C [22].

### Statistical analysis

The numerical data obtained were analysed by Student's *t*-test, Analysis of Variance (ANOVA) at 5% and 1% levels, and the Tukey–Kramer Multiple Comparisons (post hoc) Test.

## Results

In the case of *C. albicans* GDH20, the fungicidal effect of both HLF and BLF was dose dependent ($P < 0{\cdot}05$, two-way ANOVA) but not time dependent (Fig. 1). For instance, exposure to either BLF or HLF concentration of 20 μg ml$^{-1}$ reduced the viability of the yeasts to the same degree irrespective of whether the incubation period was 45 or 150 min. Exposure to a 20 μg ml$^{-1}$ concentration of BLF for 150 min at 37 °C was chosen as the standard experimental condition for further studies with six different *Candida* species.

The results of the next series of experiments to assess the relative potency of BLF in killing the six isolates each of *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. guilliermondii* and five isolates each of *C. albicans* and *C. parapsilosis* are shown in Figs. 2 and 3 and Tables 1 and 2. The results demonstrated that *C. glabrata* and *C. parapsilosis* were the least sensitive to BLF while the other species such as *C. krusei* and *C. tropicalis* were relatively more



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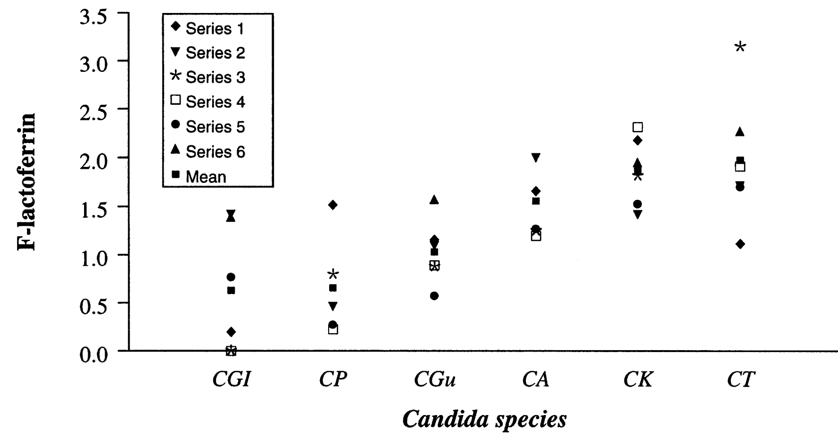


**Fig. 2** The fungicidal effect of bovine lactoferrin (20 μg ml$^{-1}$ for 150 min) on six different species of *Candida*. The test assay for each *Candida* isolate was carried out on two separate occasions with quadruplicate samples on each occasion.

susceptible. There was also evidence of differences in susceptibility to the enzyme among the isolates within a given species as exemplified by the wide range of F-lact values ($P < 0{\cdot}05$). The *Candida* species examined were susceptible to BLF in the following decreasing order: *C. tropicalis* > *C. krusei* > *C. albicans* > *C. guilliermondii* > *C. parapsilosis* > *C. glabrata*.

There was a significant difference between the F-lact values of *C. glabrata* and the following *Candida* species: *C. albicans*, *C. krusei* and *C. tropicalis* ($P < 0{\cdot}02$). Similarly F-lact values between *C. tropicalis* and *C. parapsilosis* and *C. glabrata* were significant at $P < 0{\cdot}01$.

Cryo-SEM was performed on an isolate from each of the more sensitive *Candida* species. Namely, *C. albicans* (GDH20), *C. parapsilosis* (GDH3) and *C. krusei* (GDH2) (Fig. 3). When compared with the control, cell surface changes were observed in the *Candida* species exposed to BLF. Such topographic abnormalities included, ballooning, deflated cells and surface irregularities with pits and fissures. Degenerated and dead cells were also observed in test isolates in comparison with the control isolates.

## Discussion

Biological fluids such as saliva contain a number of specific and non-specific defence factors [23]. Amongst these, lactoferrin, lysozyme and secretory IgA are known to be highly effective in regulating oral microbial populations including *Candida* [3]. A number of investigators have documented the effects of some of these components on oral *Candida* species [12,17–20,24–27] although none have compared the fungicidal effect of HLF and BLF. Further, the relative susceptibility of various *Candida* species to HLF is not well-known.

The results of the first series of experiments evaluating the relative fungicidal effect of BLF and HLF on a single isolate of *C. albicans* suggest that the potency of the human and the bovine variant of lactoferrin in killing the yeast is dose dependent although the latter is more potent than the former, especially at higher concentrations. For example at physiological concentrations seen in mucosal secretions (*c.* 20 μg ml$^{-1}$), the activity of BLF was approximately 3-fold higher than that of HLF. Despite this relative difference in the fungicidal activity of HLF and BLF the exposure period to either derivative of lactoferrin (45, 90, 150 min) did not appear to affect the total number of organisms killed to a significant extent (Fig. 1). Although Soukka *et al.* [18] in a study with one isolate of *C. albicans* (ATCC 28366) also reported that the candidacidal activity of HLF is dose dependent (at pH 7·0) they did not compare the two derivatives of LF. Indeed, to our knowledge, the current study is the first documented investigation comparing the relative fungicidal effect of bovine and human lactoferrin. The fact that BLF was more potent than HLF makes the former a more powerful tool for eliciting inter- and intra-species differences in lactoferrin susceptibility of *Candida* species, if any. Therefore in the second series of experiments which attempted to determine the differential susceptibility of *Candida* species to lactoferrin, BLF was used instead of HLF.

The observation that the candidacidal activity of both BLF and HLF, was relatively constant irrespective of the exposure time (Fig. 1), implies that once the total lactoferrin in the supernatant is exhausted -due possibly to interactions with fungal cell walls, the residual ‘resistant’ cells in the suspension may be protected from the action of LF. The other possibility of continued exposure to lactoferrin after plating out (i.e. a ‘carry over’ effect) can



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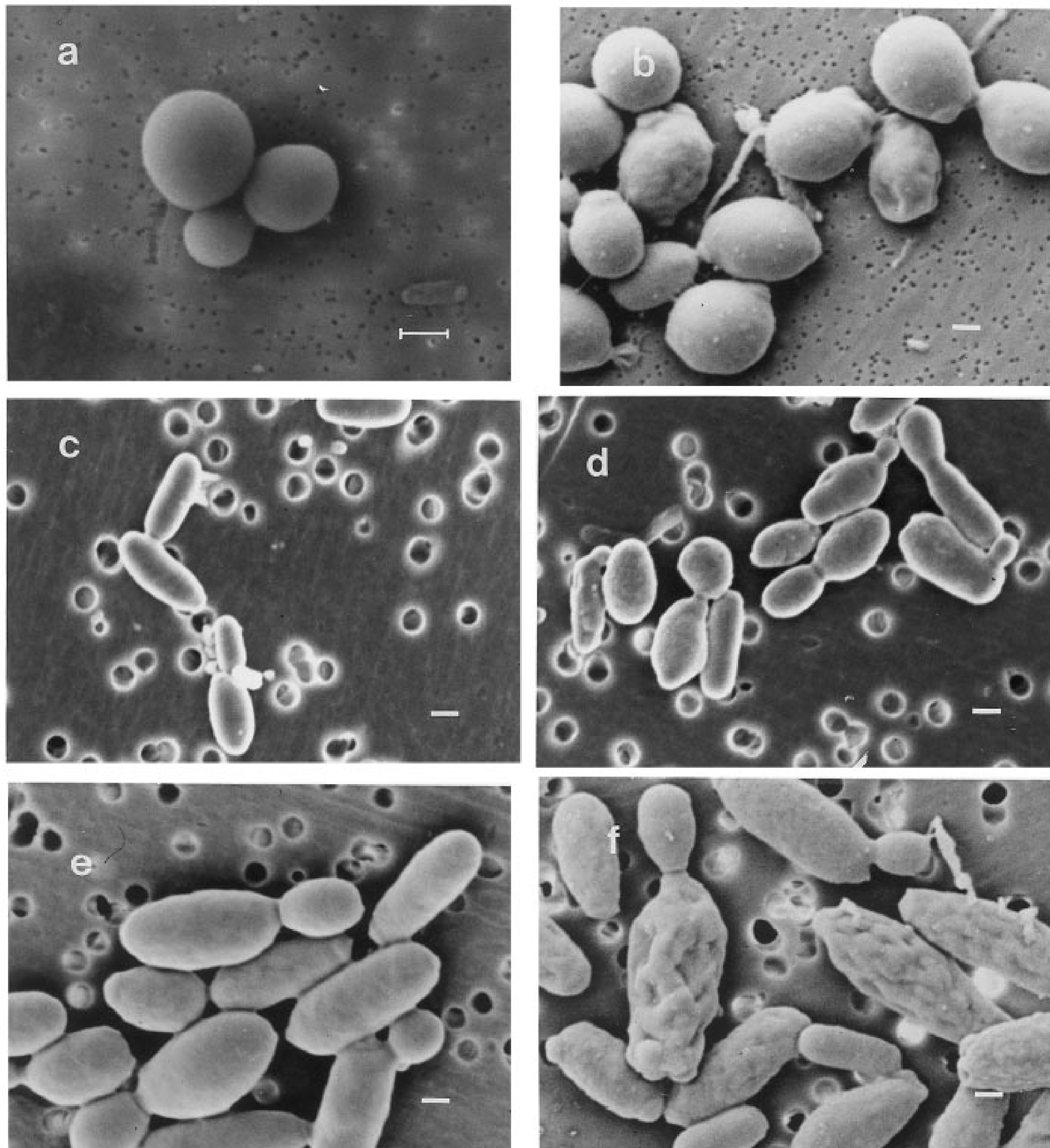


**Fig. 3** Ultrastructural features of *Candida albicans* (a, b), *C. parapsilosis* (c, d) and *C. krusei* (e, f) exposed to 20 μg ml$^{-1}$ of bovine lactoferrin for 150 min. Note the 'bleb-like' and 'basket weave' surface features after exposure to lactoferrin (b, d, f) compared with the controls (a, c, e).

be ruled out as the yeast suspension was diluted 50-fold prior to spiral plating onto SGA plates to quantify the resistant yeast cells.

There are no studies in the literature on the differential susceptibility to lactoferrin amongst the common pathogenic *Candida* species as most have compared *C. albicans* with another species. One study which investigated the effect of LF on two species of *Candida* reported that *C. krusei* is more sensitive to lactoferrin than *C. albicans* [19]. Soukka *et al.* [18] also documented similar results in a subsequent study. These results were further confirmed by a very recent investigation where we studied 20 mucosal isolates of *C. krusei* and five isolates of *C. albicans* and observed that the former on average were 1·4 times more susceptible to apo-lactoferrin ($P < 0{\cdot}05$) [20]. However, intra-species differences to the protein was not revealed in either species. These data tend to agree with the current findings although we were unable to show a



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**Table 1** The effect of bovine lactoferrin on yeast isolates belonging to six different *Candida* species (the higher the F-lactoferrin value, the higher the fungicidal activity)

| *Candida* species | Number of isolates | F-lactoferrin value | |
|---|---|---|---|
| | | Mean ± SD | Range |
| *C. glabrata* | 6 | 0·63 ± 0·63 | 0·08–1·43 |
| *C. parapsilosis* | 5 | 0·66 ± 0·53 | 0·22–1·51 |
| *C. guilliermondii* | 6 | 1·03 ± 0·33 | 0·57–1·56 |
| *C. albicans* | 5 | 1·55 ± 0·37 | 1·19–2·01 |
| *C. krusei* | 6 | 1·86 ± 0·35 | 1·43–2·17 |
| *C. tropicalis* | 6 | 1·97 ± 0·68 | 1·11–3·14 |

SD, standard deviation.

significant difference in LF sensitivity between *C. albicans* and *C. krusei*.

We believe that the current investigation with 35 isolates of *Candida* belonging to six species is the most extensive undertaken thus far and the results indicate a hierarchy of sensitivity to LF amongst *Candida* species. Thus, *C. glabrata* was the most resistant to LF and *C. tropicalis* together with *C. krusei* the most sensitive. Interestingly, the most common human pathogen, *C. albicans* occupied a central position in the hierarchy of susceptibility implying that factors other than LF may play a role in fostering their presence on mucosal surfaces. It is however interesting that *C. glabrata* is the second most common *Candida* species isolated from the oral cavity [28] and its resistance to LF may contribute to this phenomenon. On the other hand the exquisite sensitivity of *C. krusei* to lactoferrin may be a contributory factor for their relative low prevalence *in vivo*. Studies by Tobgi *et al*. [29] have also shown significant differences in the susceptibility to lysozyme (another important antimicrobial agent operating in the mouth) between six *Candida* species. They reported *C. albicans* to be the most resistant to lysozyme while *C. krusei* was the most sensitive. Thus, it is likely that antimicrobial proteins such as lactoferrin and lysozyme acting in tandem may regulate yeast-cell populations on human mucosal surfaces.

A number of workers have attempted to elucidate the microbicidal mechanisms of lactoferrin [30–32] and some have employed ultrastructural investigations using electron microscopy [19,33]. In our previous studies, we confirmed these findings in *C. albicans* and *C. krusei* using SEM. These clearly indicated cell-surface alterations and the formation of bleb-like structures on both species of *Candida* exposed to apo-LF. We have extended these investigations in the present study, using cryo-scanning electron microscopy to identify the cell surface changes in six species of *Candida* treated with BLF. These confirmed our previous findings in addition to demonstrating topographic changes on all the species of *Candida* examined irrespective of the exposure time (15, 45, 90 and 150 min). These results support the theory that BLF is capable of destabilizing the outer cell membrane of pathogenic *Candida* species altering the cell membrane permeability and causing the eventual death of the organism. Perhaps the subtle variations in the composition of the cell walls of different *Candida* species may contribute to this difference, and this is an area which warrants further investigations.

**Table 2** A table showing the inter-species variations in the sensitivity of *Candida* to bovine lactoferrin

| *Candida* species | CT | CK | CA | CGu | CP | CGl |
|---|---|---|---|---|---|---|
| CT | – | $P > 0{\cdot}05$ NS | $P > 0{\cdot}05$ NS | $P < 0{\cdot}05$ + | $P < 0{\cdot}001$ + | $P < 0{\cdot}001$ + |
| CK | | – | $P > 0{\cdot}05$ NS | $P < 0{\cdot}05$ + | $P < 0{\cdot}001$ + | $P < 0{\cdot}001$ + |
| CA | | | – | $P > 0{\cdot}05$ NS | $P < 0{\cdot}05$ + | $P < 0{\cdot}05$ + |
| Cgu | | | | – | $P > 0{\cdot}05$ NS | $P > 0{\cdot}05$ NS |
| CP | | | | | – | $P > 0{\cdot}05$ NS |
| Cgl | | | | | | – |

*P* indicates significance level of the F-lact values.
CT, *Candida tropicalis*; CK, *C. krusei*; CA, *C. albicans*; Cgu, *C. guilliermondi*; CP, *C. parapsilosis*; Cgl, *C. glabrata*.
NS, not significant.
+, significant.



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## Acknowledgements

We are grateful to Professor Jorma Tenovuo (University of Turku, Turky, Finland) and Dr S. Kalfas (Department of Oral Microbiology, University of Lund, Sweden) for the generous gift of human and bovine lactoferrin, respectively, and the Committee of Research and Conference Grants of the University of Hong Kong for funding this project.

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