

Conditions influencing the in vitro antifungal activity of lactoferrin combined with antimycotics against clinical isolates of *Candida*

Impact on the development of buccal preparations of lactoferrin

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Lactoferrin, an iron-binding glycoprotein, is a potential agent for the treatment of oropharyngeal Candidiasis. The aim of the present study was to test the capability of lactoferrin, combined or not combined with conventional antifungal agents, to inhibit the growth of different *Candida* species under various experimental conditions to be of guidance in the development of a suitable pharmaceutical formulation containing lactoferrin. The anti-*Candida* activities of lactoferrin were considerably higher using RPMI instead of SLM as assay medium. They were moreover increased by raising the medium pH from 5.6 to 7.5. With the 'standard' antifungal agent fluconazole similar results were found as for lactoferrin, but the medium type and pH did not affect MIC values of amphotericin B. The addition of saliva to medium did not reduce the antifungal activities of the individual compounds. Synergistic inhibitory effects on *Candida* growth were found for combinations of lactoferrin and fluconazole or amphotericin B, irrespective of the medium type and pH, or the addition of saliva. This indicates that for treatment of oral Candidiasis a formulation containing lactoferrin seems appropriate; results may be optimized if the formulation is provided with buffer capacity to attain pH 7.5 in the mucosal fluid. The synergistic effects between lactoferrin and 'standard' antifungals indicate that combinations should be considered in such a formulation.

Key words: Lactoferrin; *Candida*; pH; saliva; antifungal agents; fluconazole.

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Candida infections are widely distributed among patients with lowered resistance, such as patients infected with human immunodeficiency virus (HIV) or patients receiving radio- or chemotherapy. Nowadays, these patients are ex-

tensively treated with amphotericin B or fluconazole. The widespread use of these antimycotic agents, however, poses major problem in the long run due to toxicity and the emergence of drug-resistant *Candida* species (1, 2).

Therefore, the development of alternative therapeutic compounds is required in order to control such yeast infections. Potential candi-

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dates are antimycotic proteins naturally present in saliva, such as histatins, lysozyme or lactoferrin, because of their broad-spectrum activity and lack of toxicity (3–5). Lactoferrin is an iron-binding glycoprotein that reaches significant concentrations in body fluids, varying from $\pm 0.2 \mu\text{g/ml}$ in plasma to $\pm 7 \text{ mg/ml}$ in colostrum (6). It has antimicrobial properties against bacteria, yeasts and viruses. In addition, it plays an important role in the host defense against infections on mucosal surfaces and in colostrum and milk (6–9).

Previously, we demonstrated that lactoferrin alone (0.5–100 mg/ml, depending on the *Candida* species) or combined with standard antimycotic agents (fluconazole, amphotericin B or 5-fluorocytosine) inhibited *Candida* growth (8). The pronounced cooperative anti-*Candida* activities of the combinations were reflected in significant reductions in minimum inhibitory concentrations (MIC) of the antimycotic drugs if combined with relatively small concentrations of lactoferrin (e.g. 1 mg/ml), even in the case of drug-resistant *Candida* strains.

An in vitro activity is, however, not always correlated with an effective therapeutic activity in vivo. Medium composition and pH are reported to significantly influence the activities of antimycotic drugs (10–12), and thus these factors endanger extrapolation of in vitro data to in vivo situations. Furthermore, the anti-*Candida* activity of drugs administered in the oral cavity may be highly influenced by the presence of saliva components (13). In the present study we, therefore, examined the influence of assay medium composition, pH, saliva, and other antifungal drugs on the activity of lactoferrin in more detail. These results will provide essential information for the development of a tablet formulation containing lactoferrin that can be used for the treatment of oropharyngeal Candidiasis.

MATERIALS AND METHODS

Microorganisms.

Three *Candida albicans* strains (isolate numbers C70, Y106 and Y127), two *Candida glabrata* strains (isolate numbers Y110 and Y111) and one *Candida tropicalis* strain (isolate number C71) were obtained from the routine microbiology laboratory (University Hospital Groningen, The Netherlands). They were isolated from the oral cavity and displayed different susceptibility to the antifungal agents amphotericin

B, fluconazole and 5-fluorocytosine. *C. albicans* ATCC 10231 was used as control. All strains were stored on Sabouraud dextrose agar slopes (Oxoid, Unipath Ltd., Basingstoke, UK) at 4°C.

Assay media.

Two antifungal agent-free media were used: Sabouraud Liquid Media (SLM pH 5.6; obtained from Oxoid) and RPMI 1640 (with L-glutamine, w/o NaHCO_3 supplemented with 2% glucose; obtained from Gibco BRL, Paisley, Scotland). In some experiments, saliva, collected from human volunteers, was added 1:1 v/v to the assay medium, which was twice the regular concentration resulting in “normal” end concentrations. The saliva was sterilized by filtering through 0.2 μm filters.

Antifungal agents.

Bovine lactoferrin (bLF, 93% purity, iron saturation of $\pm 10\%$; DMV International, Veghel, The Netherlands) was dissolved in medium and the pH was set to the value used in the particular experiments. For some experiments, also human lactoferrin (hLF, purity $>95\%$, iron saturation $\pm 6\%$; Numico Research B.V., Wageningen, The Netherlands) was used, which was isolated from human milk by cation exchange chromatography. Fluconazole (Diflucan® I.V.; Pfizer B.V., Cappellet a/d IJssel, The Netherlands) and 5-fluorocytosine (Ancotil®, Roche Nederland B.V., Mijdrecht, The Netherlands) were also directly dissolved in medium, while amphotericin B (Fungizone®, Bristol-Myers Squibb Company, Woerden, The Netherlands) was prepared to a stock concentration of 5 mg/ml in sterile water, and further diluted in medium.

Growth curves.

We automatically recorded growth curves of the *Candida* isolates using a Bioscreen incubator/reader (LabSystems, Helsinki, Finland). The test inoculum (1.10^5 CFU/ml) was added to Bioscreen 100-well plates, and medium and compounds to be tested were added in appropriate amounts. The plate was incubated for 24 h at 37°C in air in the Bioscreen incubator/reader; OD 600 nm was read every 15 min (preceded by shaking). Sets of OD measurements were plotted to obtain growth curves. The antimycotic activity of lactoferrin was assessed by relating the area under the curve (AUC) in the presence of lactoferrin to that in the control situation.

Determination of MIC values.

50 μl of the test inoculum (1.10^4 – 5.10^4 CFU/ml), prepared as described (8), was added to 96-well plates (Corning Costar, Cambridge, UK). Subsequently, bLF (0.001–10 mg/ml), fluconazole or amphotericin B (both 0.0001–330 $\mu\text{g/ml}$) was added to a final volume of 200 $\mu\text{l/well}$. Controls were included for the determination of growth characteristics of each *Can-*

didia species in the absence of antifungal agent. After inoculation, plates were incubated for 24 h at 35°C in air without agitation. Turbidity measurements were performed (after resuspension of the culture) at 630 nm in an automated microplate reader (EL_x800, Bio-Tek Instruments, Inc., Winooski, VT) at t=0, hourly at 18–24 h (includes the exponential phase of the growth curve), and at 48 h (8). The absence of growth was confirmed by inoculating 20 µl onto Sabouraud agar and subsequent incubation for 5 days at 35°C in air. MIC was defined as the lowest concentration antifungal agent that substantially inhibited the growth of *Candida* after 24 h (14).

Synergy experiments.

The combined effects of bLF and fluconazole or amphotericin B against the *Candida* growth were assessed as described previously (8). The experimental conditions were similar to the ones used for determination of MIC. We used a *C. glabrata* (Y110) and a *C. albicans* (Y127) isolate, which differed in susceptibility to amphotericin B, fluconazole or lactoferrin. A dilution matrix, with 8-fold drug dilutions which also included the drugs used individually, was prepared and the results of the turbidity measurements were used to calculate the inhibitory effects of the drug combinations on *Candida* growth. Maximum *Candida* growth was set at 0% and complete growth inhibition at 100%. These results were presented as growth inhibition curves.

In addition, drug-drug interactions were characterized with a three-dimensional analytical method (8, 15). Theoretical additive effects of two antifungals were calculated from the individual dose-response values. Subsequently, these theoretical data were compared with the actual experimental dose-response curves. For an additive interaction of two compounds, the experimental dose-response curves coincide with the theoretical ones, but any peaks above or below these baseline values (0% synergy) are indicative of synergistic or antagonistic interactions. In other words, baseline values do not imply a lack of effect, only a lack of synergism.

RESULTS

The effect of medium pH and composition on the anti-Candida activities

The growth of *Candida* was influenced by the pH and composition of the medium, as can be seen in Fig. 1. In general, the *Candida* isolates incubated in RPMI grew more slowly at pH 7.5 as compared to pH 5.6. In particular, the growth of the *C. glabrata* isolates was delayed in RPMI pH 7.5. Also differences in the growth characteristics were observed between the two

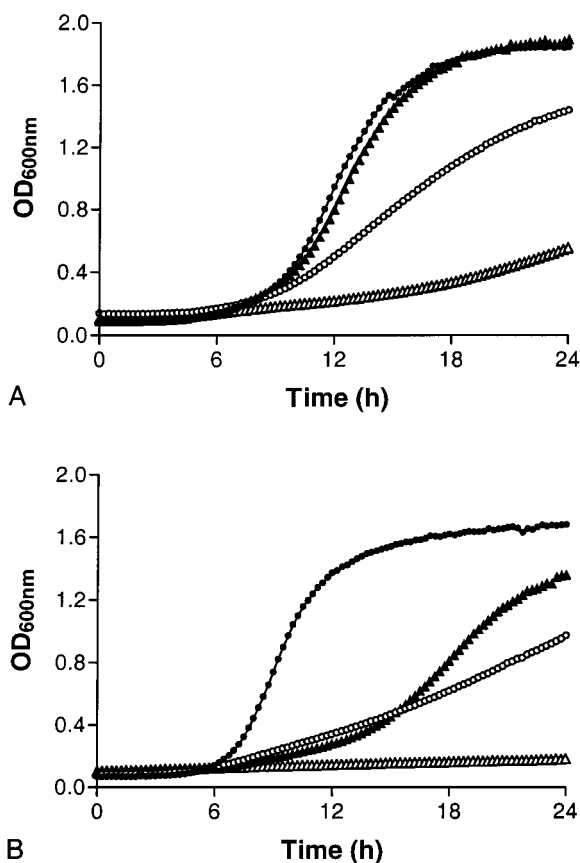


Fig. 1. The inhibitory effects of 2.5 mg/ml bovine lactoferrin (open symbols) on the growth of *C. glabrata* as compared to control growth (closed symbols). These effects were measured in SLM (Fig. A) or RPMI (Fig. B) of pH 5.5 (circles) and pH 7.5 (triangles).

media tested, of which RPMI pH 5.6 displayed a more rapid growth of *Candida*. Only minor pH-dependent effects were found in SLM incubations.

Fig. 1 shows a typical example of the growth curve of a *Candida* isolate (*C. glabrata*) in the presence of lactoferrin. A strong inhibition of the *Candida* growth was observed for lactoferrin in both RPMI and SLM at various pH values. This inhibition by lactoferrin was reflected in a delayed onset of growth and/or a reduced maximal OD after 24 h of incubation. In the case of *C. glabrata* little or no growth was found in RPMI pH 7.5 containing 2.5 mg/ml lactoferrin during the 24 h incubation period. Both bovine and human lactoferrin were tested for their ability to inhibit *Candida* growth and their anti-*Candida* activities were found to be very similar.

Comparable results were obtained for *C. albicans* (results not shown).

The inhibitory effects of lactoferrin on *Candida* growth during 24 h were reflected in a reduced AUC as compared to the control situation. We therefore calculated an activity index of lactoferrin by relating the AUC in the presence of lactoferrin to the AUC in the control situation. The results were shown in Fig. 2. A higher activity index value indicates a stronger growth inhibition by lactoferrin. Lactoferrin displayed the highest activity against the *C. glabrata* strains, a moderate effect against *C. tropicalis*, and lowest activity against *C. albicans*. The effects of lactoferrin against all *Candida* species were increased by using RPMI pH 7.5 instead of pH 5.5. No clear pH effect was observed for SLM.

In addition, MIC values of bLF against *C. albicans* and *C. glabrata* isolates were determined in either SLM or RPMI at various pH levels (Table 1). The MIC of lactoferrin differed considerably between the species tested and also between the two assay media used. The MIC values of lactoferrin in RPMI were considerably

lower than in SLM. In addition, pH-dependent effects were measured, although they were not as clear as in Fig. 2.

Comparable to the activities observed with lactoferrin, we noticed that in general fluconazole was also able to inhibit *Candida* growth more efficiently if the assay was performed in RPMI medium as compared to SLM. For example, the MIC of fluconazole measured in SLM pH 6.5 was >330 µg/ml and 0.07 µg/ml in RPMI pH 6.5 (*C. albicans* Y106) and for the *C. glabrata* isolate Y110 MIC values of 100 µg/ml were measured in SLM pH 6.5 and 0.1 µg/ml in RPMI pH 6.5. A trend towards a pH-dependent effect could also be demonstrated for fluconazole in SLM medium, that is MIC values were lower in SLM at pH 7.5. The MIC values measured at pH 6.5 and at pH 7.5 were, respectively, for *C. glabrata* (Y110) 100 and 0.2 µg/ml and for *C. albicans* (Y106) >330 and 0.08 µg/ml. In contrast, anti-*Candida* activities of the antifungal agents amphotericin B and 5-fluorocytosine showed no medium or pH dependence.

The effect of saliva on the anti-Candida activities

The addition of saliva to SLM resulted in a minor delay in growth rate of *Candida*, but after 24 h *Candida* titers were identical to medium alone. Interestingly, addition of saliva to RPMI (>5%) resulted in a decreased *Candida* growth. Therefore, we were unable to assess the anti-*Candida* effects of the antifungal agents in RPMI-containing saliva.

The MIC of lactoferrin and fluconazole were slightly affected by addition of saliva, whereas no differences in MIC were measured for amphotericin B (Table 2).

The effects of pH, medium composition and saliva on the anti-Candida effects of lactoferrin in combination with other antifungal compounds

Previously, an improved antifungal activity was found for lactoferrin combined with other 'standard' anti-*Candida* agents (8). We now assessed whether pH, medium composition or saliva had any effect on the anti-*Candida* activity obtained with the different antifungal drug combinations.

pH. First, the influence of medium pH on the inhibition of *Candida* growth with combinations of lactoferrin and amphotericin B was

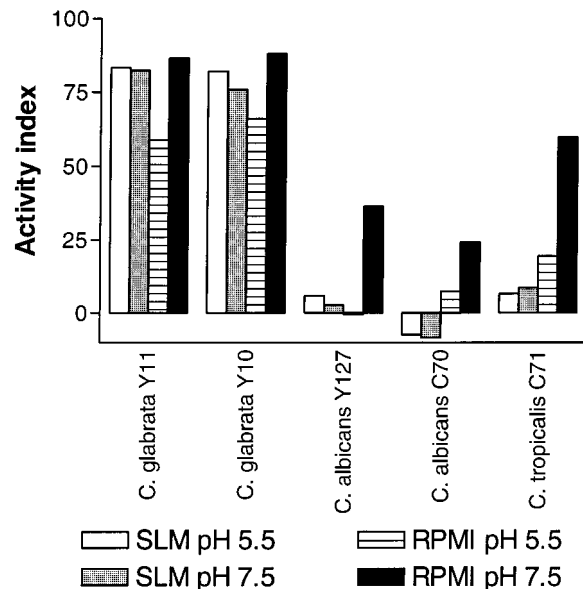


Fig. 2. The effect of bovine lactoferrin (2.5 mg/ml) on the growth of different *Candida* species in SLM or RPMI of various pH. The activity index is calculated by dividing the AUC of the *Candida* growth curve during 24 h incubation in the presence of lactoferrin by the AUC of the control situation. Note that increasing the pH of RPMI resulted in an improved activity of lactoferrin.

TABLE 1. The minimum inhibitory concentrations (MIC) of lactoferrin ($\text{mg}\cdot\text{ml}^{-1}$) against various *Candida* isolates assessed in SLM and RPMI both at different pH

Isolate	Species	SLM ^a pH 5.6	SLM			RPMI		
			pH 6.5	pH 7.0	pH 7.5	pH 6.5	pH 7.0	pH 7.5
10231 ^c	<i>C. alb.</i>	97	>10 ^b	>10	3.3	>10	>10	0.01
Y106 ^d	<i>C. alb.</i>	0.5	2.1	8.6	>10	0.03	0.03	0.4
Y127	<i>C. alb.</i>	98	>10	>10	>10	>10	0.08	>10
Y110	<i>C. glab.</i>	31	>10	>10	1	0.02	0.01	0.01
Y111	<i>C. glab.</i>	6	1.0	1.0	0.6	0.04	0.02	0.01

^a The MIC values in SLM pH 5.6 were determined in an earlier study (8)

^b The highest concentration tested at pH 6.5, 7.0 and 7.5 was $10 \text{ mg}\cdot\text{ml}^{-1}$

^c Isolate 10231 is an ATCC strain

^d All Y-isolates are clinical *Candida* isolates.

TABLE 2. The minimum inhibitory concentrations (MIC) of antimycotica in the absence and presence of saliva (1:1) using SLM pH 7.5

Isolate	Species	Lactoferrin (mg/ml)		Fluconazole ($\mu\text{g/ml}$)		Amphotericin B ($\mu\text{g/ml}$)	
		SLM	SLM +saliva	SLM	SLM +saliva	SLM	SLM +saliva
10231	<i>C. alb.</i>	3.3	>10	>330	>330	0.1	0.1
Y127	<i>C. alb.</i>	>10	9.2	0.1	>330	0.1	0.3
Y110	<i>C. glab.</i>	1	10.8	40	134	0.2	0.4

assessed using SLM at pH 5.6 and pH 7.5 (Fig. 3). If one of the antifungals was added at its MIC value, a complete inhibition of the yeast was achieved at pH 7.5. In SLM pH 7.5, combinations of lactoferrin and amphotericin B were able to inhibit the growth of *C. albicans* at con-

centrations below their individual MIC value. For instance, using only 3.3 mg/ml lactoferrin in combination with $0.01 \mu\text{g/ml}$ amphotericin B, a complete inhibition of the *Candida* growth was observed, while the individual MIC values were $>10 \text{ mg/ml}$ for lactoferrin and $0.1 \mu\text{g/ml}$

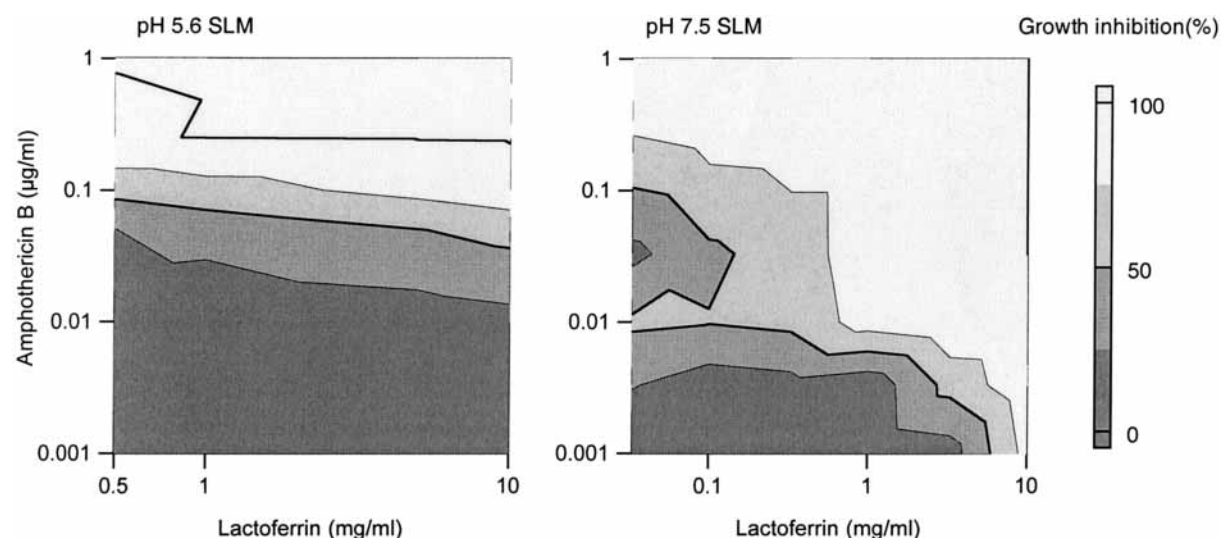


Fig. 3. Influence of pH on the combined inhibitory effects of lactoferrin and amphotericin B on the growth on *C. albicans* (Y127). Left: inhibitory effects determined in SLM pH 5.6. Right: inhibitory effects determined in SLM pH 7.5. The top elevation of a three-dimensional dose response graph (concentration amphotericin B versus concentration lactoferrin versus percentage of *Candida* growth inhibition) is presented. Extent of inhibition of the *Candida* growth is indicated by the right positioned gray color bar.

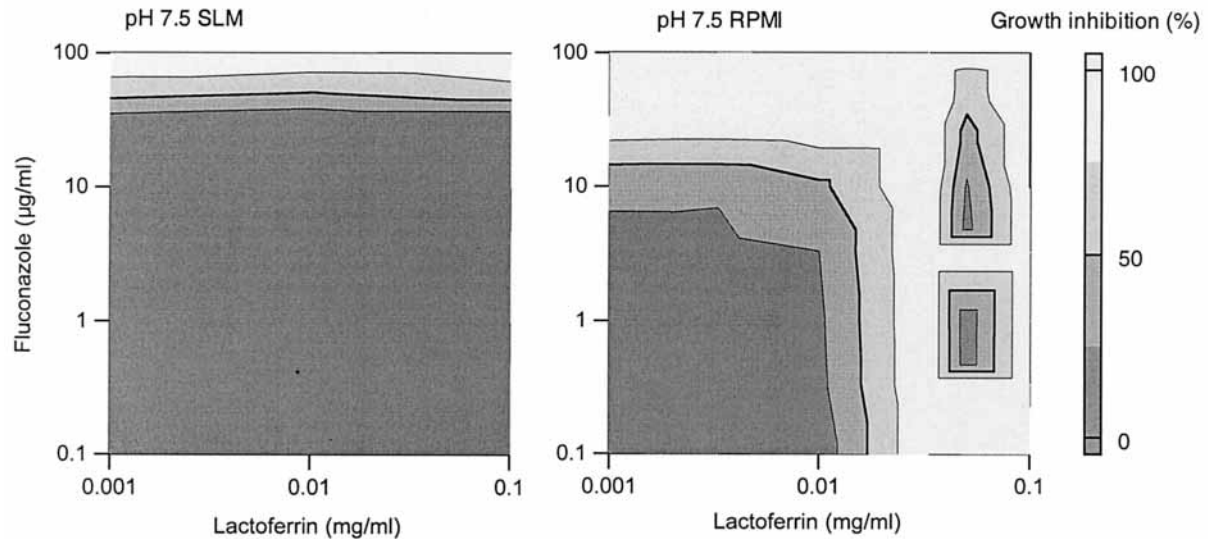


Fig. 4. Influence of assay medium on the combined inhibitory effects of lactoferrin and fluconazole on the growth on *C. glabrata* (Y110). Left: inhibitory effects determined in SLM pH 7.5. Right: inhibitory effects determined in RPMI pH 7.5. The top elevation of a three-dimensional dose response graph (concentration fluconazole versus concentration lactoferrin versus percentage of growth inhibition) is presented. Extent of inhibition of the *Candida* growth is indicated by the right positioned gray color bar.

for amphotericin B (Fig. 3). Combinations of lactoferrin and amphotericin B tested at a pH of 7.5 also resulted in synergistic effects against the other *Candida* strain (*C. glabrata*) tested.

When lactoferrin was combined with flu-

conazole, the inhibitory effects on the *Candida* growth (*C. glabrata* Y110 and *C. albicans* Y127) occurred at lower concentrations in SLM pH 7.5 than at pH 5.6. Again the anti-fungal effects were measured at concentrations

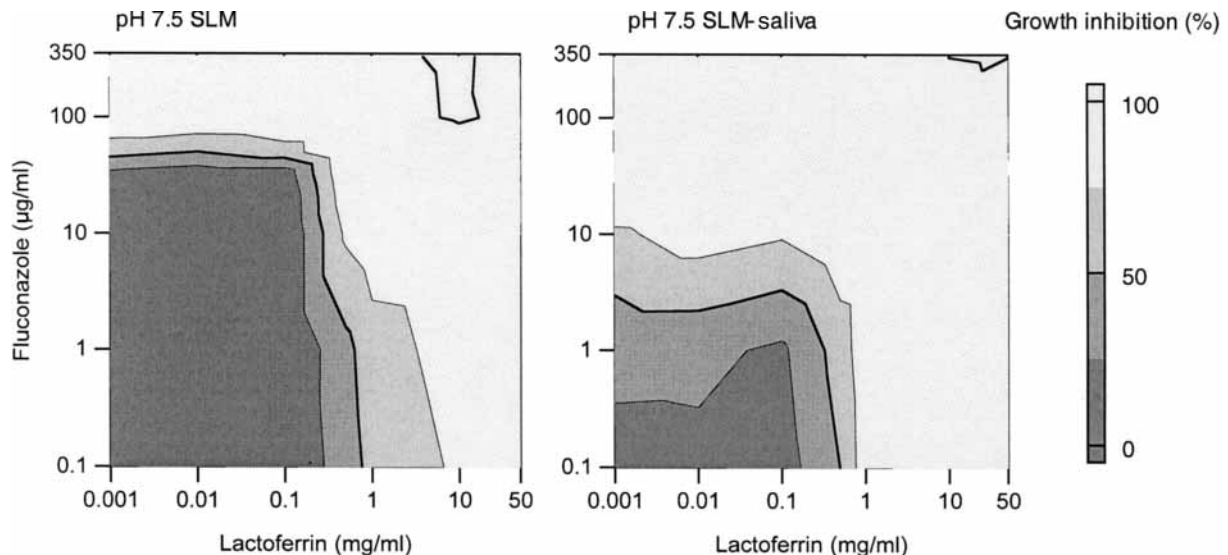


Fig. 5. Influence of saliva on the combined inhibitory effects of lactoferrin and fluconazole on the growth on *C. glabrata* (Y110). Left: inhibitory effects determined in SLM pH 7.5. Right: inhibitory effects determined in SLM-saliva pH 7.5. The top elevation of a three-dimensional dose response graph (concentration fluconazole versus concentration lactoferrin versus percentage of growth inhibition) is presented. Extent of inhibition of the *Candida* growth is indicated by the right positioned gray color bar.

lower than their individual MIC values. Synergistic anti-*Candida* effects were measured at SLM pH 5.6 (maximal 20% synergy), but these were most pronounced (up to 80% synergy) using SLM pH 7.5. 80% synergy was observed with a combination of 3.3 mg/ml lactoferrin and 0.1 µg/ml fluconazole against *C. albicans* Y127 in SLM pH 7.5. However, this combination also showed some antagonistic activity in SLM pH 7.5, whereas no antagonism was observed at pH 5.6.

Medium

The influence of medium composition on the synergistic effects of the combination of lactoferrin with fluconazole or amphotericin B was assessed by comparing incubation in SLM pH 7.5 with RPMI pH 7.5. When lactoferrin and fluconazole or amphotericin B were combined, a complete inhibition of yeast growth was achieved at pH 7.5 using one of the antifungals at its MIC value (see Table 1), independent of the medium composition. Interestingly, using RPMI instead of SLM considerably lower concentrations of lactoferrin and fluconazole could be used to obtain complete growth inhibition (Fig. 4). 33 µg/ml fluconazole and 0.03 µg/ml lactoferrin concentrations completely inhibit yeast growth in RPMI, whereas in SLM concentrations of at least 100 µg/ml fluconazole or 1 mg/ml lactoferrin must be present.

Saliva. The addition of saliva to the assay affected the MIC values of the individual compounds to a minor extent. Meanwhile, for the combination of lactoferrin with fluconazole or amphotericin B the addition of saliva to SLM pH 7.5 significantly improved the inhibitory effects of the individual compounds. Fig. 5 shows this for the combination of lactoferrin and fluconazole. A lowering of concentrations necessary to obtain complete growth inhibition was achieved, i.e. 100% growth inhibition of *C. glabrata* Y110 occurred using only 0.001 mg/ml lactoferrin in combination with 20 µg/ml fluconazole, which was not observed in the absence of saliva.

DISCUSSION

Twenty to forty percent of the human population harbor *Candida* species in the oral cavity,

but mainly immunocompromised or nutritionally deprived individuals are prone to develop oral Candidiasis. For example, patients infected with HIV undergo several episodes of oral thrush. The difficulty in coping with opportunistic infections may be partly due to the failure of various intrinsic components of the host defense system (e.g. immunoglobulins or lactoferrin) to kill the *Candida* species. We here show that lactoferrin may be used in a therapeutic formulation to deal with *Candida* infections. Previously, we demonstrated a negative correlation between the prevalence of *Candida* and the concentration of lactoferrin in saliva of HIV-1-infected persons. It was argued that prophylactic treatment of these patients with additional amounts of lactoferrin might be worth considering (16). In the present study we tested the capability of lactoferrin to inhibit *Candida* growth under experimental conditions (pH, medium composition, presence of saliva), that represented different aspects of the oral environment.

Increasing the pH of the assay medium from 5.6 to 7.5 resulted in more efficient inhibition of the *Candida* growth by lactoferrin and fluconazole. This increased anti-*Candida* activity is in accordance with previous studies showing that the effect of various antifungal compounds was increased at higher pH (17–20). Yet fluconazole activity testing at non-alkaline pH values was recommended by others (21). At a more alkaline pH, carboxylic acid groups of surface proteins of *Candida* will be deprotonized to anionic groups, which may provide more interaction sites for cationic antifungal agents (22). This would also apply to lactoferrin, a glycoprotein with cationic domains (pI 8.0). Indeed, this study demonstrated a better anti-*Candida* activity of the protein at pH 7.5 as compared to 5.6. Previous studies with lactoferricin, a supposed active part of lactoferrin, showed an optimal antifungal activity at a pH of 6.0 (23), while for apo-lactoferrin this occurred at pH 7.0 (13). Furthermore, the lower antifungal activity of lactoferrin at acidic pH values may be explained by increased activity of the *Candida* proteinases resulting in a less active protein (24). Thus, the antifungal effect of lactoferrin can be increased if its formulation induces a proper pH of the mucosal fluid in the oral cavity.

All compounds displayed a higher anti-*Can-*

did activity when RPMI was used as assay medium instead of SLM, indicating that different environments may influence the extent of the antifungal effect. In the oral cavity, a difference in environmental conditions between persons may be the result of for instance nutrition or drug intake. This was previously observed for fluconazole (25). In the present study, this effect was particularly pronounced for lactoferrin, whose activity increased by a factor of 1000 in RPMI. It is unlikely that the lactoferrin effects were related to the iron content of the media, since at least RPMI does not contain iron salts. Also, active proteinases in the SLM peptic meat digest might be able to degrade lactoferrin or other components in the medium may directly bind to the active domain of lactoferrin.

Our results show that the presence of saliva is not harmful for the anti-*Candida* activities of lactoferrin. This is an encouraging observation for the development of a local application of lactoferrin in cases of oral Candidiasis. Interestingly, combinations of lactoferrin with other antifungals were clearly more effective in inhibiting *Candida* growth in the presence of saliva. Among other factors this may be due to the presence of lactoferrin or other cationic proteins in saliva. For practical purposes our present study indicates that a proper formulation containing lactoferrin may be able to provide strong antifungal effects if locally applied in the oropharyngeal cavity and that the presence of saliva is unlikely to mask its anti-*Candida* activity. The latter comprises a problem for other antimicrobial mouth washes, for instance those containing chlorhexidine (26).

Encouraged by these findings we are developing a pH-buffered oral formulation containing lactoferrin for the treatment of oral *Candida* infections, and have shown in healthy volunteers that sufficiently high concentrations of lactoferrin in saliva can be obtained at a proper local pH (9). Studies of pH-buffered lactoferrin formulations in HIV-infected patients suffering from oral Candidiasis are underway to demonstrate the beneficial effects of lactoferrin on *Candida* infections.

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