LACTOFERRIN RESEARCH

Vaginal Health

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Activated Lactoferrin and Fluconazole Synergism Against *Candida albicans* and *Candida glabrata* Vaginal Isolates

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OBJECTIVE: To evaluate the fungistatic activity of activated lactoferrin (ALF), fluconazole (FCN) individually and in combination against Candida vaginal isolates as well as to measure the time to recovery from the fungistatic effects after exposure in vitro to threshold minimal inhibitory concentrations (MIC).

STUDY DESIGN: Fungistasis patterns for ALF (2.5 mg/mL) and FCN (0.25 mg/mL) were tested at threshold MIC against vaginal isolates of C albicans (n = 5) and C glabrata (n = 5) grown in Sabouraud's dextrose broth against 10^5 yeast inoculum at 37°C for 48 hours by microscale optical density (OD) assay according to the following criteria: "Total stasis" indicates that an agent elicited no change or a change in turbidity <0.1 OD unit for >48 hours (complete growth inhibition), "stasis recovery" (SR) is the time point at which turbidity of a previous stasis system shows an upward growth trend for >0.1 OD unit (recovery from growth inhibition), and "partial stasis"

(PS) is proliferation after stasis recovery, measured as a percentage relative to growth control at any time (incomplete growth inhibition).

RESULTS: For ALF (2.5 mg/mL), the mean SR time was 15.6 ± 2 hours for C albicans (n = 5) and 27.5 ± 2 hours for C glabrata (n = 5). The SR patterns for FCN were strain dependent and showed a wide range of deviation for both Candida species; accordingly, the values were 15.8 ± 9 hours for C albicans and 25.5 ± 12 hours for C glabrata. After 48 hours exposure to C albicans, ALF and FCN elicited a mean PS of 27.5 ± 2% and 24.8 ± 7%, respectively. The PS values at 48 hours showed a marked variation between C glabrata isolates, 29.1 ± 24% for ALF and 21.5 ± 38% for FCN. However, a combination of ALF and FCN at their threshold MIC showed significant drug synergism, causing total stasis of both species of Candida isolates. Thus, no SR for any Candida isolate was detected at or beyond 48 hours. Conversely, native lactoferrin failed to demonstrate such

The ALF/FCN combination is a novel antifungal potentiation system that could be effective for clinical management of VVC.

potent synergism with FCN against either Candida *species.*
CONCLUSION: *The combination of ALF and FCN at the threshold MIC elicited potent synergism, leading to total fungistasis of* C albicans *and* C glabrata *vaginal pathogens. ALF is a new class of fungistatic agent with a mode of action distinct from that of azoles.* (J Reprod Med 2004;49:800–807)

Vulvovaginal candidiasis (VVC) is a common fungal infection of the female genital tract. Conventional treatment consists primarily of repeated, often monthly, short courses of topical vaginal therapy prescribed with the first appearance of symptoms.[1] Fluconazole (FCN) is a frequently used therapeutic in the clinical management of VVC due to its high water solubility and wide tissue distribution after oral administration.[2] The cellular target for FCN activity is identified as cytochrome P450-dependent C14α-demethylase, an active component of the ergosterol biosynthetic pathway.[3] Accordingly, FCN causes ergosterol depletion and accumulation of 14α-methyl-sterols in the plasma membrane of *Candida* species.[4] However, long-term use of FCN often leads to drug resistance in *Candida* strains.[5] FCN resistance has been linked to (1) up-regulation of the *ERG11* gene, which encodes the drug target enzyme C14α-demethylase; (2) reduced affinity of azoles to cellular targets; (3) accumulation of a less toxic ergosterol intermediate, C14α-methylfecosterol; and (4) active efflux of FCN away from *Candida* spp, at least 2 types of efflux pumps, the ATP binding cassette transporter superfamily and the major facilitators, are known to contribute to drug resistance.[5-12]

Lactoferrin (LF) is a major innate defense factor in exocrine secretions and neutrophil granules.[13] The ability of LF to bind 2 Fe^{+3} ions with high affinity in cooperation with 2 bicarbonate ions is an essential characteristic that contributes to its major biologic properties, including antimicrobial activity. Accordingly, induction of iron-deprivation stasis is the mechanism by which LF protects mucosal surfaces, including vaginal epithelia from pathogens.[14] LF also binds with high affinity to specific pore-forming channel proteins on the microbial cell surface and alters the membrane diffusion of antimicrobial agents.[15-17] Fungistasis and membrane drug diffusion effects of native LF against *Candida* spp

with limited efficacy have been reported.[18-22] Recently, a molecular activation process to enhance the antimicrobial activity of native LF has been developed.[23] This resulting, activated LF (ALF), is a microbial blocking agent severalfold more potent than native LF, with specific activity against

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The cumulative effect of fungistasis and adhesion blockade activity... makes ALF a promising antimicrobial agent for protection of the vaginal mucosa.

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pathogens without affecting commensal flora, such as vaginal lactobacilli.

In a previous study we reported potent fungistasis activity for ALF against vaginal isolates of *C albicans* and *C glabrata* for an extended period, 96 hours.[24] Furthermore, ALF has effectively blocked the attachment of *Candida* hyphae and blastospores to vaginal epithelia. This study was undertaken to further elucidate the potential of ALF to elicit antifungal synergism with FCN against *C albicans* and *C glabrata* vaginal isolates.

Materials and Methods
LF

LF (lot 10099751) purified from cow's milk was provided by DMV International Nutritionals, Veghel, the Netherlands. A 2% (wt/vol) native solution was prepared by dissolving LF (2.0 g) in 100 mL sterile PBS (pH 7.2). A 2% (wt/vol) ALF solution was prepared in accordance with Naidu using deionized water.[23] Native LF (2.0 g) was dissolved in 100 mL sterile buffer solution containing 1 mM EDTA (Versene NA™, Dow Chemicals, Freeport, Texas), 10 mM NaHCO3 (Fisher, Fairlawn, New Jersey) and 100 mM NaCl (Sigma Chemicals, St. Louis, Missouri). After adjusting the pH to 8.2 (with $NaHCO_3$), food grade pectin (0.02 g; CU 201, Herbstreith & Fox, Nürenbürg, Germany) was added to this solution with gentle stirring for partial immobilization of the dissolved LF. Formation of immobilized LF was confirmed by gel filtration chromatography using a Sephacryl S-200 high-resolution column (Amersham Biosciences, Piscataway, New Jersey).

FCN

FCN (Diflucan® intravenous infusion glass bottles, Pfizer Roerig, New York, New York) in 0.9% saline was obtained as 2 mg/mL stock solution and stored at 26°C until used. FCN was diluted in glass vials to required working concentrations in 0.9% saline prior to testing.

Vaginal Candida Isolates

Clinical strains of *Candida* spp were isolated from VVC patients at the Microbiology Laboratory, Mayo Clinic, Scottsdale, Arizona. Isolates were identified and speciated to *C albicans* and *C glabrata* according to MicroScan (Dade Behring, Dearfield, Illinois, and Vitek Microbial ID system. Bio-Merieux, Durham, North Carolina). *C albicans* (n = 5) and *C glabrata* (n = 5) were received as slant cultures on Sabouraud's dextrose (SD) agar. After revival, pure yeast cultures were stored on Microbank™ beads (Pro-Lab Diagnostics, Austin, Texas) at –80°C. Yeast were grown in SD broth overnight at 35°C for all experimental purposes.

Microscale Optical Density (OD) Assay (MODA)

Antifungal activity of ALF, native LF and FCN against *C albicans* and *C glabrata* was evaluated in vitro. A 100-μL volume of sterile SD broth (2 × concentration) was added to all 96 wells of a sterile microtiter plate (Costar® 3596, Corning, New York). ALF, native LF, FCN or sterile EDTA/bicarbonate buffer (control), each 50 μL in volume, was added to wells and inoculated with 50 μL yeast suspension containing $\sim 10^5$ yeast/mL (diluted from a precalibrated [OD 1.0 at 600 nm] solution of 10^8 yeast/mL). Yeast growth was monitored at 35°C as turbidity change in broth at different time points by measuring OD at 600 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, California). Prior to OD measurement, the contents of each well were mixed with a sterile pipette to obtain a uniform yeast suspension. Wells containing SD broth without yeast inoculum served as the sterility control. Wells containing SD broth inoculated with yeast but without antifungal (i.e., ALF, LF or FCN) served as the positive growth control. Based on the above working controls of sterility as well as growth, when a test strain proliferates typically under defined conditions of inoculation and incubation, the turbidity (OD) changes in the microbial growth media, as measured at 600 nm with the following criteria for MODA: "Total stasis" indicates that an agent elicited no change or a change in tur-

bidity ≤0.1 OD unit for ≥48 hours (complete growth inhibition), "stasis recovery" is the time point when turbidity of a previous stasis system showed an upward growth trend for ≥0.1 OD unit; and "Partial stasis" is proliferation after stasis recovery, measured as a percentage relative to growth control at any time (incomplete growth inhibition).

Measurement of Yeast Generation Time

From the MODA experiment, the total number of viable yeast cells was enumerated at 0, 24 and 48 hours and compared with the viable counts of inocula to calculate generation time for each *Candida* species under a given test condition. A 200-μL *Candida* cell suspension from the microtiter plate was centrifuged, and the treatment solutions were aspirated to remove antifungal exposure. Yeast cells were resuspended in 0.5 mL normal saline and serially diluted 10-fold in normal saline. Selected dilutions were plated in duplicate on SD agar for total yeast counts using an Autoplate® 4000 device (Spiral Biotech, Norwood, Massachusetts). Plating was performed in an exponential log dilution setting on the spiral autoplater. Agar plates were incubated at 35°C for 24–48 hours, and the total colony counts were estimated using an automated infrared Q-count device (Spiral Biotech). Data were expressed as colony-forming units per milliliter of growth medium.

Statistical Analysis

Interaction between ALF and FCN was assessed at each time by using a 2-way analysis-of-variance model. Differences < 0.05 were considered significant. All p values and confidence intervals are 2 sided.

Results
Native LF and ALF Effects with FCN

Native LF, ALF at 2.5 mg/mL concentration and FCN at 0.25 mg/mL dosage were tested for fungistatic activity either alone or in combination against vaginal *Candida* isolates (Figure 1). Stasis recovery time points for *C albicans* strain NTRL809A were estimated at 13.8 and 15.4 hours for ALF and FCN, respectively. Native LF (2.5 mg/mL) under similar test conditions (inoculum 10^5 yeast) failed to elicit fungistasis. Partial stasis values were 86% and 69% at 24 hours and 28% and 34% at 48 hours for ALF and FCN, respectively. A combination of ALF and FCN, however, resulted in total stasis of *C albicans* growth for up to 48 hours, significantly higher
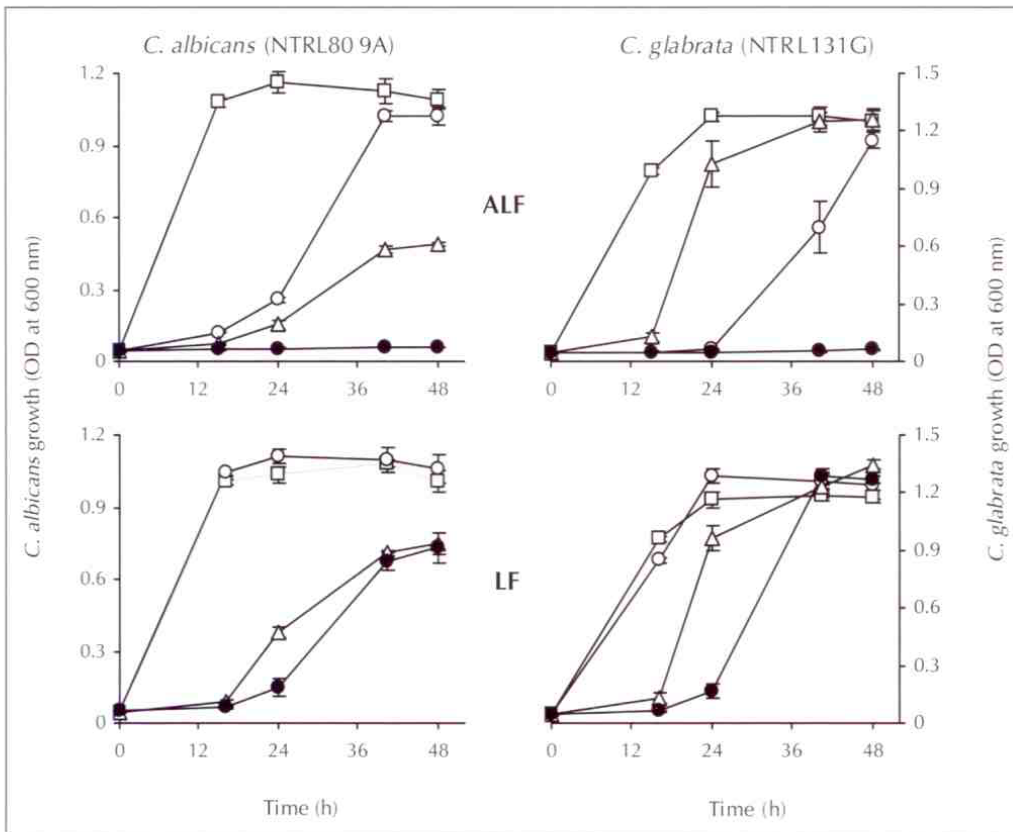
Figure 1 Effects of native LF, ALF and FCN treatment on growth-multiplication of *C albicans* strain NTRL809A and *C glabrata* strain NTRL131G. An inoculation of 10^5 yeast in SD broth incubated at 35°C containing a 2.5 mg/mL dosage of native LF or ALF (○), 0.25 mg/mL dosage of FCN (△) or combination dosages of ALF + FCN (●) was used. Growth of yeast inoculum in SD broth without an antifungal was the control (□). Each data point represents an average of a quadruplicate reading.

(p < 0.0001) than ALF or FCN, individually. Native LF in combination with FCN showed an additive effect at 24 hours that progressively diminished within 48 hours.

C glabrata strain NTRL131G in the presence of ALF and FCN showed stasis recovery at 29.6 and 9.3 hours, respectively. Native LF was ineffective against *C glabrata* under similar test conditions. ALF elicited total stasis at >24 hours, and 55% partial stasis was observed after 48 hours of exposure. Partial stasis values for FCN were 21% at 24 hours and 4% at 48 hours. Finally, a combination of these 2 agents resulted in fungistasis of *C glabrata*, significantly more potent (p < 0.0001) than ALF and FCN alone measured at their individual dosages at 48 hours. The native LF and FCN mixture demonstrated a drug potentiation effect at 24 hours that progressively diminished within 48 hours.

ALF and FCN Effects on Yeast Generation Time

The generation (doubling) time for *C albicans* was estimated as 110 minutes in SD broth with 10^5 yeast inoculum (Table I). A total of 13 generations were required for *C albicans* strain NTRL809A to reach

stationary growth phase in the controls. Treatment with ALF or FCN reduced yeast multiplication to 4 replicates by prolonging the generation time to 360 minutes. The ALF/FCN combination treatment, however, significantly eliminated (p < 0.0001) the doubling of *C albicans*; therefore, no generation time was detected.

C glabrata strain NTRL131G at 10^5 inoculum in SD broth replicated after 110 minutes and reached the stationary growth phase after 16 generations under control conditions. The presence of ALF and FCN in growth medium retarded the yeast replication to 2 and 10 generations, respectively. Accordingly, treatments with ALF and FCN extended the doubling time of *C glabrata* to 720 minutes (2 doublings) and 144 minutes (10 doublings), respectively. Accordingly, FCN was less effective against *C glabrata*. Finally, ALF/FCN treatment, in combination, resulted in significant antifungal synergism (p < 0.0001), therefore, no generation time was observed.

These data suggested that ALF (2.5 mg/mL) and FCN (0.25 mg/mL) as individual treatments were partially effective against VVC pathogens. Howev-

Table I *Effects of ALF, Native LF, FCN Alone and Combinations on Yeast Cell Replication Rates of* C albicans *(NTRL 809A) and* C glabrata *(NTRL 131G) Vaginal Isolates*

Test	*C albicans* inoculum (3.0×10^5)			*C glabrata* inoculum (2.7×10^5)		
	Yeast count	Generations	2× time (min)	Yeast count	Generations	2× time (min)
Control	1.0×10^9	13	110	1.2×10^9	13	110
LF	2.2×10^9	13	110	9.6×10^8	12	120
ALF	4.0×10^6	4	360	8.3×10^5	2	720
FCN	2.8×10^6	4	360	1.7×10^8	10	144
LF+FCN	1.5×10^6	3	480	3.7×10^6	4	360
ALF+FCN	1.2×10^5	0	0	2.1×10^5	0	0

ALF and native LF were tested at 2.5 mg/mL and FCN at 0.25 mg/mL against yeast grown at 35°C in SD broth and measured by MODA.
Viable yeast counts were performed on SD agar with growth solutions from MODA at 0 (inoculum) and 24 hours, using an automated spiral plating system.

er, a combined dose of their threshold minimal inhibitory concentrations (MIC) resulted into a potent synergism and elicited stasis against both vaginal isolates of *C albicans* and *C glabrata* strains.

ALF/FCN Synergism Against Candida *Isolates* (n = 10)

Based on the above data, the spectrum of fungistatic synergism between ALF and FCN was further evaluated in different vaginal isolates of *C albicans* (n = 5) and *C glabrata* (n = 5) strains. Stasis recovery and partial stasis values for individual *Candida* isolates following treatment with ALF (2.5 mg/mL) and FCN (0.25 mg/mL) either alone or in combination are shown in Table II. The data were further elaborated as percentage growth pattern for each strain, with average values for each group of *Candida* species at defined time points following treatment with both antifungal agents (Figure 2).

The average stasis recovery for *C albicans* isolates was estimated as 15.6 ± 2 hours and 15.8 ± 9 hours for ALF and FCN, respectively. The average partial stasis values after 48 hours drug exposure were $27.5 \pm 2\%$ for ALF and $24.8 \pm 7\%$ for FCN. Unlike their individual doses, the combination of ALF and FCN resulted in significant synergism ($p < 0.0001$), causing total fungistasis with all 5 *C albicans* test strains.

ALF and FCN susceptibility patterns showed a high degree of deviation between the 5 test isolates of *C glabrata*. The average value for stasis recovery was 27.5 ± 2 hours, and partial stasis after 48 hours exposure was $29.1 \pm 24\%$ with ALF treatment. After FCN exposure, an average stasis recovery was observed at 25.5 ± 12 hours, and partial stasis after 48 hours was estimated at $21.5 \pm 38\%$. Despite the marked drug susceptibility deviations among individual strains, ALF and FCN, when combined,

Table II *Stasis Recovery (SR) and Partial Stasis Values for Vaginal* Candida *Isolates Following Treatment with ALF and FCN Either Alone or in Combination*

Test strain	ALF (2.5 mg/mL)		FCN (0.25 mg/mL)		ALF + FCN	
	SR time (h)	PS% (48 h)	SR time (h)	PS% (48 h)	SR time	PS% (48 h)
C albicans						
NTRL809A	13.8	27.9	15.4	33.5	ND	TS
NTRL358A	15.6	6.9	13.0	23.1	ND	TS
NTRL931A	17.7	19.7	9.4	29.4	ND	TS
NTRL128A	16.2	57.6	8.4	22.8	ND	TS
NTRL224A	14.8	7.0	16.1	15.4	ND	TS
C glabrata						
NTRL131G	29.6	55.1	9.3	4.3	ND	TS
NTRL807G	25.3	5.6	43.1	89.5	ND	TS
NTRL814G	25.2	5.6	25.6	4.0	ND	TS
NTRL127G	30.3	50.9	24.9	4.7	ND	TS
NTRL224G	27.3	28.2	24.6	5.1	ND	TS

TS = total stasis, ND = nondetectable.
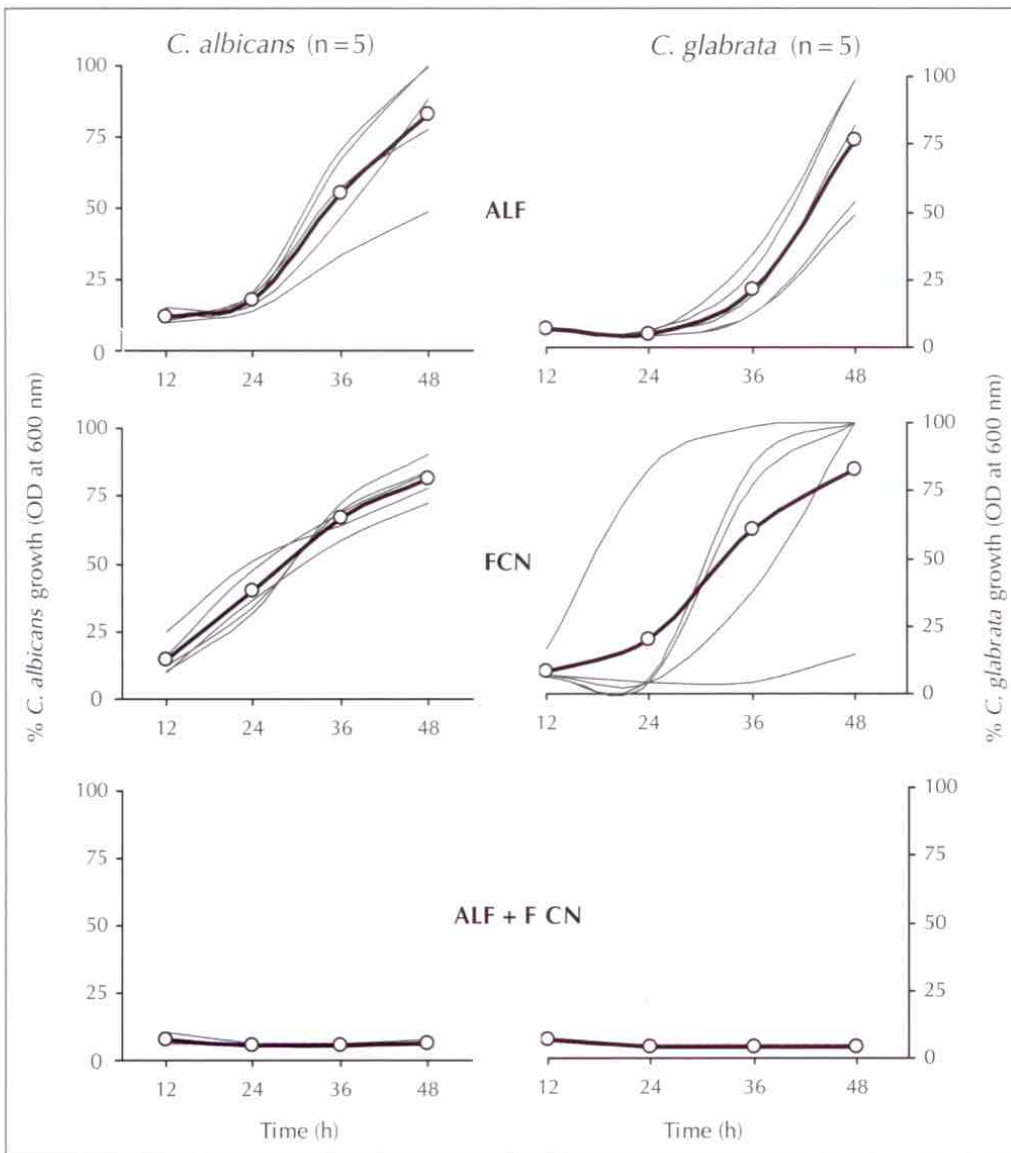
Figure 2 Percentage growth pattern for each strain of *C albicans* (n = 5) and *C glabrata* (n = 5) isolates, with average values (solid line) for each group of species at defined time intervals following treatment with ALF (2.5 mg/mL), FCN (0.25 mg/mL) and their combination dosages.

showed significant synergism ($p < 0.0001$) causing total fungistasis of all 5 test strains of *C glabrata*.

Discussion

Resistance to FCN is highly prevalent among *Candida* pathogens; therefore, any development of a new antifungal agent and/or synergist to overcome such drug resistance is urgent.[5] Since VVC is a recurrent infection in the immunocompromised host, wherein a *Candida* pathogen proliferates with slow-steady kinetics, such factors as the infectious dose of blastospores and antifungal activity for an extended period need to be balanced in the development of any therapeutic regimen. In a previous study we estimated that ~10^5 blastospores of *Candida* are required to infect a 1-cm^2 surface of the vagi-

nal epithelium and that the yeast cells replicated at a rate of ~110/min under optimal conditions.[24] Furthermore, we have shown that ALF (5 mg/mL) elicited fungistasis of *C albicans* and *C glabrata* at 10^5 inoculum for 96 hours and significantly blocked both hyphal and blastospore interactions with vaginal epithelia. In the present study we demonstrated the synergistic potential of ALF with FCN at threshold MIC to inhibit vaginal *Candida* pathogens.

In 1994, our laboratory first reported the ability of LF to bind specific receptors on pathogens and potentiate diffusion of antibiotics across microbial membranes.[15,17] Furthermore, LF interactions with the pathogen surface seem to enhance an array of antimicrobial effects, including stasis, adhesion blockade, altered membrane diffusion rates, opson-

ic activity, intracellular damage and plasmid removal.[13-16,25] The development of ALF technology is essentially to capture and enhance the multiple antimicrobial spectrum of native LF.[23] Thus, apart from stasis and adhesion blockade effects, ALF seems to enhance membrane diffusion of FCN by binding to *Candida* surfaces; to limit the iron-dependent cytochrome P450, the FCN cellular target, as well as other components of the electron transport system; and thus to cause synergistic potentiation of FCN. In this study, ALF/FCN synergism demonstrated potent fungistatic efficacy against both *C albicans* and FCN-resistant *C glabrata*.

Synergism between ≥2 drugs results from the combination of different antimicrobial mechanisms. FCN synergism with terbinafine, ibuprofen, sodium salicylate and propyl paraben against *C albicans* has been reported.[26,27] Synergism of LF and LF-derived peptides with FCN, itraconazole, amphotericin B and 5-fluorocytosine against *Candida* spp has also been reported.[18-22] A cationic peptide derived from the N-terminus region of LF (LFcin) has been reported to disrupt the fungal cell membrane and elicit fungicidal activity against *C albicans*.[18,19] That group further reported cidal synergism between FCN and LFcin B (0.1 mg/mL) against an azole-resistant *C albicans* strain when tested at a low yeast inoculum of 5×10^2 yeast in low-ionic RPMI growth medium, for 15 hours.[19] Cationic peptides cause cytolysis by inducing membrane perturbations of microbial and eucaryotic cells in low ionic environments.[28] Thus, most cationic peptides, including LFcins, fail to function in physiologic saline.[29,30] Unlike LFcin B, ALF demonstrated anti-*Candida* efficacy and FCN synergism in high-ionic, iron-rich, regular SD broth against 10^5 blastospore inocula over 48 hours of stasis. Moreover, ALF/FCN synergism resulted in stasis, independent of the species, strain or FCN-resistance characteristics of vaginal *Candida*. The cidal lytic activity of cationic peptides could leave intracellular yeast debris, including mycotoxins, on a diseased, compromised vaginal mucosal surface; that is highly undesirable. Based on viable yeast recovery data, we clearly showed that ALF/FCN synergism is a stasis phenomenon. Furthermore, the microbial blocking activity of ALF reported in our earlier study could detach and exclude *Candida* from the vaginal mucosa to complement fungistasis.

Lupetti et al also reported potentiation of FCN with noncandidacidal concentrations of an N-terminal peptide (1-11) of human LF.[22] In their study, a higher inoculum, 6 logs (10^6 yeast), of FCN-resistant *C albicans*, when treated with hLF peptide (8 μM) and FCN (0.2 mg/mL), showed 0.64- and 0.76-log viable yeast reduction, respectively, which aggregated to 1.4-log inhibition. A combination of both treatments caused a 1.86-log reduction, indicating that the reported phenomenon was an additive, not synergistic. Furthermore, survival of $> 10^4$ cells of *C albicans* from hLF peptide/FCN treatment suggested limited antifungal activity of the combination. In our study the yeast generation time data suggested that ALF/FCN mixtures eliminated any budding or replication of *C albicans* and *C glabrata* at 5-log inocula with no detectable stasis recovery over 48 hours. Also, the ALF/FCN mixtures demonstrated true synergism to the extent of about 70% and 560% significantly greater efficacy ($p < 0.0001$) than ALF and FCN individual and combined treatment for *C albicans* and *C glabrata*, respectively.

In conclusion the ALF/FCN combination is a novel antifungal potentiation system that could be effective for clinical management of VVC. The cumulative effect of fungistasis and adhesion blockade activity, besides clearing the infection load, makes ALF a promising antimicrobial agent for protection of the vaginal mucosa.

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ISSVD PROCEEDINGS

Activated Lactoferrin's Ability to Inhibit *Candida* Growth and Block Yeast Adhesion to the Vaginal Epithelial Monolayer

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OBJECTIVE: To study in vitro *growth-inhibitory effects of activated lactoferrin (ALF) against vaginal isolates of* Candida *species and to measure the ability of ALF to block interactions of* Candida albicans *and* Candida glabrata *to the vaginal epithelial (VE) monolayer.*

STUDY DESIGN: In vitro *effects of ALF on growth of* C albicans *and* C glabrata *in Sabouraud dextrose (SD) broth were measured as change in broth turbidity by microscale optical density assay. ALF was tested at 5 and 2.5 mg/mL concentrations against* 10^5 *yeast cell inoculum at 37°C for 96 hours and compared with native lactoferrin and control (growth in broth without ALF). VE cells were isolated from human vaginal tissue biopsies to establish a functional monolayer for yeast interaction studies. ALF effects on* Candida *interactions with the VE monolayer were tested using* ³H-thymidine–*labeled yeast. Prophylactic (treatment prior to yeast inoculation onto VE) and therapeutic (treatment to detach*

> **ALF [is] a potent natural antifungal agent that inhibits growth, blocks adhesion and detaches C albicans and C glabrata from vaginal epithelial cells.**

VE-adherent yeast) potential of ALF (5 mg/mL) was evaluated against vaginal isolates of C albicans *strain NTRL809A and* C glabrata *strain NTRL131G.*

RESULTS: *Growth of* Candida *species indicated that a* 10^5 *yeast inoculum in SD broth proliferated to a stationary growth equilibrium (~10^9 yeast cell density) in 18 hours (~2 hours of generation time). ALF (5 mg/mL) elicited > 96 hours of total stasis (100% growth inhibition) and was significantly effective against both* Candida *species (p < 0.0001). At 2.5 mg/mL dilution, ALF sustained total stasis activity to an average of 18 hours and 24 hours for* C albicans *(n = 5) and* C glabrata *(n = 5), respectively. Interaction studies indicated avid binding of* C albicans *(70–140 ×10^3 yeast) and* C glabrata *(50–75 ×10^3 yeast) per square centimeter of VE monolayer. ALF-treated VE showed significant blockade (p < 0.05) of yeast adhesion by 33% and 58% with* C albicans *and* C glabrata, *respectively. ALF treatment of yeast-VE complexes resulted in signif-*

icant detachment (p < 0.05) of C albicans *and* C glabrata, *by 58% and 51%, respectively.*
CONCLUSION: ALF is a natural fungistatic agent with potent yeast adhesion-blocking and detachment properties and is effective against the vaginal pathogens C albicans *and* C glabrata. *(J Reprod Med 2004;49: 859–866)*

Keywords: lactoferrin; *Candida;* vaginal diseases; candidiasis, vulvovaginal.

Vulvovaginal candidiasis (VVC) is commonly associated with immunocompromised host populations. Alteration or dysfunction of innate host defense in vaginal mucosa is a major predisposing factor for several infections, including vaginal yeast colonization.[1] Accordingly, VVC is more prevalent during pregnancy, when high hormone levels lead to an increase in vaginal glycogen, providing a favorable environment for growth of *Candida*.[2] It is estimated that 75% of women experience at least one episode of VVC during the childbearing period. Of these women, 40–50% experience a second attack, and a small subpopulation (<5%) experiences recurrent, often intractable episodes.[3] VVC is prevalent among diabetics and women taking oral contraceptives, antibiotics and adrenal steroids.[4-8]

Lactoferrin (LF), an iron-binding glycoprotein, is a primary innate defense factor present in milk, saliva, tears and other exocrine secretions that bathe tissue surfaces, including vaginal mucosa.[9] Furthermore, as a major granular component of polymorphonuclear lymphocytes, LF has a protective role in inflammation and cell mediated immunity.[10] Anti-*Candida* activity of LF, first reported by Kirkpatrick et al, is a fungistatic effect attributed to its ability to bind and sequester iron from the milieu.[11] Fungicidal activity for LF against *Candida albicans* and *Candida krusei*, by mechanisms related to altered cell surface permeability, has also been reported.[12,13]

Activated lactoferrin (ALF) is a novel formulation of native LF with enhanced function due to a specific molecular-milieu optimization process.[14] ALF is a natural microbial-blocking agent that detaches or prevents attachment of yeast and other microbes to mucosal surfaces and selectively inhibits proliferation of pathogens without affecting commensal flora.[15]

This study assessed the fungistatic activity of ALF for extended periods of time against vaginal isolates of *Candida* species at yeast cell densities relevant to infection and also evaluated the effects of

ALF in blocking interactions of *C albicans* and *Candida glabrata* within the vaginal epithelial (VE) monolayer.

Materials and Methods

LF

LF (lot 10099751) purified from cow's milk was provided by DMV International Nutritionals, Veghel, the Netherlands. A 2% (wt/vol) native solution was prepared by dissolving LF (2.0 g) in 100 mL sterile phosphate-buffered saline (PBS) (pH 7.2). A 2% (wt/vol) activated LF (ALF) solution was prepared using deionized water.[14] LF (2.0 g) was dissolved in a 100 mL sterile buffer solution containing 1 mM EDTA (Versene NA™, Dow Chemicals, Freeport, Texas), 10 mM NaHCO$_3$ (Fisher, Fairlawn, New Jersey) and 100 mM NaCl (Sigma Chemicals, St. Louis, Missouri). After adjusting the pH to 8.2 (with NaHCO$_3$), food grade pectin (0.02 g; CU 201, Herbstreith and Fox, Nürenburg, Germany) was added to this solution with gentle stirring for partial immobilization of the dissolved LF. The formation of immobilized LF was confirmed by gel filtration chromatography using a Sephacryl S-200 HR column (Amersham Bioscience Corp., Piscataway, New Jersey).

Candida *Isolates*

Clinical strains of *Candida* species were isolated from VVC patients at the Microbiology Laboratory, Mayo Clinic, Scottsdale, Arizona. Isolates were identified and speciated to *C albicans* and *C glabrata* with the MicroScan (Dade Behring, Deerfield, Illinois) and Vitek Microbial ID system (BioMerieux, Durham, North Carolina). *C albicans* (n = 5) and *C glabrata* (n = 5) were received as slant cultures on Sabouraud dextrose (SD) agar. After revival, pure yeast cultures were stored on Microbank™ beads (Pro-Lab Diagnostics, Austin, Texas) at −80°C. Yeast was grown in SD broth overnight at 35°C for all experimental purposes, unless stated otherwise.

Microscale Optical Density (OD) Assay (MODA)

Antifungal activity of ALF and native LF on growth of *C albicans* and *C glabrata* was evaluated *in vitro*. Sterile SD broth (2× concentration), 100 μL, was added to 96 wells of a sterile microtiter plate (Costar® 3596, Corning, New York). A 50-μL volume of ALF, native LF or sterile EDTA/bicarbonate buffer was added to designated wells followed by inoculation with 50 μL yeast suspension containing ∼10^5 yeast per milliliter (diluted from an optically

After overnight VE cell proliferation, KSF medium was removed from the tissue culture flask, replaced with different dilutions of ALF, native LF or EB buffer and further incubated in 6.5% CO_2 at 37°C. A commercial LDH assay kit was used according to the product protocol (Sigma) to quantitate the LDH leakage into the medium at various time points. Briefly, 0.1 mL of growth medium from each flask was removed and placed in a 96-well microplate (Costar® 3596, Corning). The assay was carried out immediately after each time point. This assay is based on the reduction of nicotinamide adenine dinucleotide by LDH activity, and the resulting reactant is utilized in the stoichiometric conversion of a tetrazolium dye. This derived, colored compound is measured at 490 nm in a microplate reader (VersaMax, Molecular Devices).

Radioadhesion Assay to Measure Yeast-VE Monolayer Interactions

A 0.1-mL inoculum of overnight culture of *Candida* species grown in SDB was reinoculated in 10 mL of SDB containing ³H-thymidine (20 µCi). *Candida* vaginal isolates were grown at 37°C to exponential phase (~ 10 hours) to allow optimum uptake and incorporation of ³H-thymidine into the yeast cell DNA. ³H-thymidine–labeled yeast was harvested by centrifugation (7,500 g), washed and resuspended in KSF medium. The final density of *Candida* suspension was optically adjusted to 0.2 OD at 600 nm (corresponding to ~ 10^8 yeast per milliliter). A correlation between the radioactivity of ³H-thymidine (measured as disintegrations per minute [DPM]) and viable yeast count on SD agar was established.

Interaction studies were performed with plastic inserts (Thermanox 174950, Nunc, Roskilde, Denmark) placed into each tissue culture plate well (Costar® 3524, 24-well plate). One milliliter of *Candida* suspension (~ 10^8 yeast) was added to each insert (area = 2 cm²) containing ~ 10^6 VE cells. The ratio between VE and yeast was maintained at about 1:200. After 2 hours of interaction at 37°C, nonadherent yeast suspension was aspirated, and wells were washed twice with PBS. Inserts were removed from the well and transferred to scintillation vials containing 1 mL sterile distilled water. After incubation at 55°C for 30 minutes in water bath, the vials were cooled, and a 10-mL Scintisafe® gel (Fisher Scientific, Chicago, Illinois) was added. Finally, the ³H-thymidine radioactivity bound to the inserts was measured as DPM using a liquid scintillation analyzer (Tri-Carb 2300 TR, Packard Instrument

Co., Meriden, Connecticut).

For yeast-VE adhesion-blocking studies, ALF and other treatments were performed for 2 hours prior to yeast interaction with VE. For yeast detachment studies, ALF and other treatments were performed for 2 hours with yeast-VE complexes.

Statistical Analysis

Mean levels of yeast growth were compared among treatment groups at each time, and the statistical significance was calculated by using a 1-way analysis-of-variance model. p Values < 0.05 were considered significant, and these values as well as confidence intervals were 2 sided. Differences between test groups of yeast-VE interactions were analyzed by *t* test using the SigmaStat 3.0 program (SPSS Inc.,
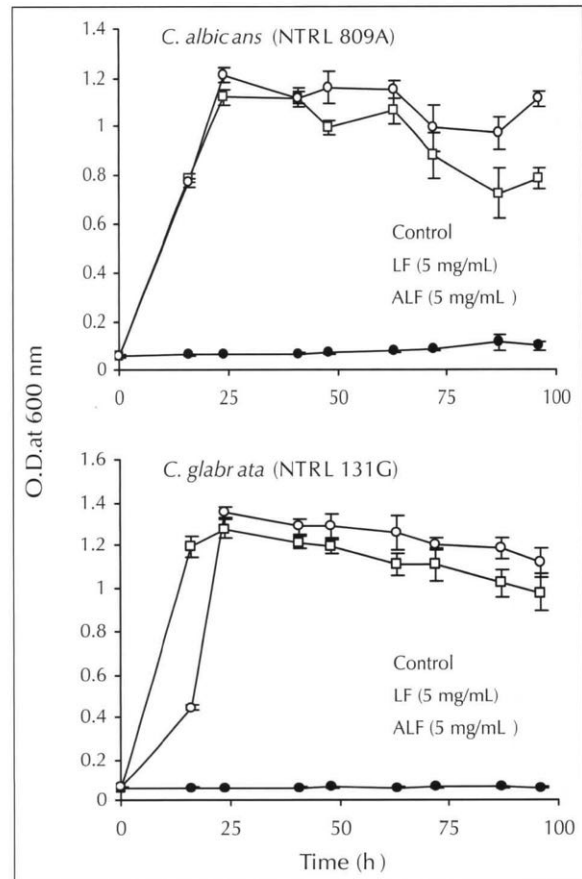
Figure 1 Effects of ALF (5 mg/mL) and native LF (5 mg/mL) on *C albicans* and *C glabrata* (10^5 blastospore inoculum) growth in SD broth at 37°C. ALF elicited total fungistasis, which was more effective (p < 0.0001) than native LF or control.

precalibrated (OD 1.0 at 600 nm) solution of 10^8 yeast per milliliter). After inoculation, the microplate was incubated at 35°C, and the yeast growth was monitored at different time points as turbidity change in culture medium by measuring OD at 600 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, California). Prior to OD measurement, the contents of each microplate well were mixed with a sterile pipette to uniformly suspend the yeast in the medium. Wells containing broth without yeast inoculum served as a sterility control. Wells containing broth inoculated with yeast but without any antifungal served as a positive growth control. Based on the above working controls of sterility as well as growth, when a test strain proliferated typically under defined conditions of inoculation and incubation, the turbidity (OD) changes in the microbial growth medium were measured at 600 nm with the following criteria for MODA: *Total stasis* is an agent's eliciting no change or a change in turbidity ≤ 0.1 OD unit for ≥ 48 hours, *stasis recovery* is the time point at which the turbidity of a previous stasis system shows an upward growth trend for ≥ 0.1 unit, and *partial stasis* is proliferation after stasis recovery, measured as the percentage value relative to the growth control at any time.

Establishment of a Primary VE Cell Line

Vaginal tissue scrapings and biopsies were collected from 7 volunteers at the Mayo Clinic, Scottsdale, Arizona, and its institutional review board approved the sampling protocol. Specimens were transported under refrigerated conditions in Eagle's Minimum Essential Media (EMEM) (BioWhittaker, Walkersville, Maryland) to the tissue culture laboratory for processing. Vaginal tissue specimens were centrifuged at 400 g in a Beckman GS-15 centrifuge (Beckman Coulter Inc., Fullerton, California) for 5 minutes, the transport medium was decanted, and specimens were resuspended in EMEM containing a $4\times$ concentration of antibiotics (penicillin-G, 400 U/mL; streptomycin SO_4, 400 µg/mL; and fungizone, 10 µg/mL; all obtained from Sigma). After incubation at 37°C for 3 hours, the antibiotic medium was removed by centrifugation, and cells from both specimens were resuspended in 2 mL of keratinocyte serum-free (KSF) medium (Gibco BRL/Invitrogen, Grand Island, New York) supplemented with 50 µg/mL bovine pituitary extract and 5 ng/mL human recombinant epidermal growth factor 1-53 (Gibco

BRL/Invitrogen) supplemented with penicillin-G (100 U/mL), streptomycin SO_4 (100 µg/mL) and L-glutamine (117 µg/mL). The resuspended cells from tissue scrapings were plated onto a cell culture dish (35 mm) (Corning) and incubated at 37°C in a 5% CO_2 incubator (Sheldon Labs, Cornelius, Oregon).

Tissue biopsies were rinsed twice with PBS, aseptically sliced with forceps and a scalpel to remove any fat or necrotic cells and transferred to a sterile petri dish containing 2 mL EMEM and 2 mL trypsin-versene reagent (BioWhittaker). The immersed tissue was subjected to finer dissection and incubated at 37°C for 30 minutes, and the enzymatic hydrolysis with trypsin was stopped with complete medium (EMEM containing 10% fetal bovine serum [FBS], BioWhittaker). Cells were separated from the reagent mixture by centrifugation, resuspended in 5 mL complete medium, and seeded in tissue culture plates. After incubation (in CO_2) at 37°C for 24 hours, complete medium was decanted and replaced with KSF medium. Tissue culture plates were periodically examined for VE cell attachment and proliferation to >50% of the growth surface. The KSF medium was decanted, and VE cells were subcultured after a 3-mL PBS rinse. After an additional trypsin hydrolysis step, as described above, a primary VE cell line was established and maintained in T-75 vented tissue culture flasks (Becton Dickinson, Franklin Lakes, New Jersey) containing ~25 mL KSF medium with 10% FBS and grown to the confluent VE monolayer for experimental purposes.
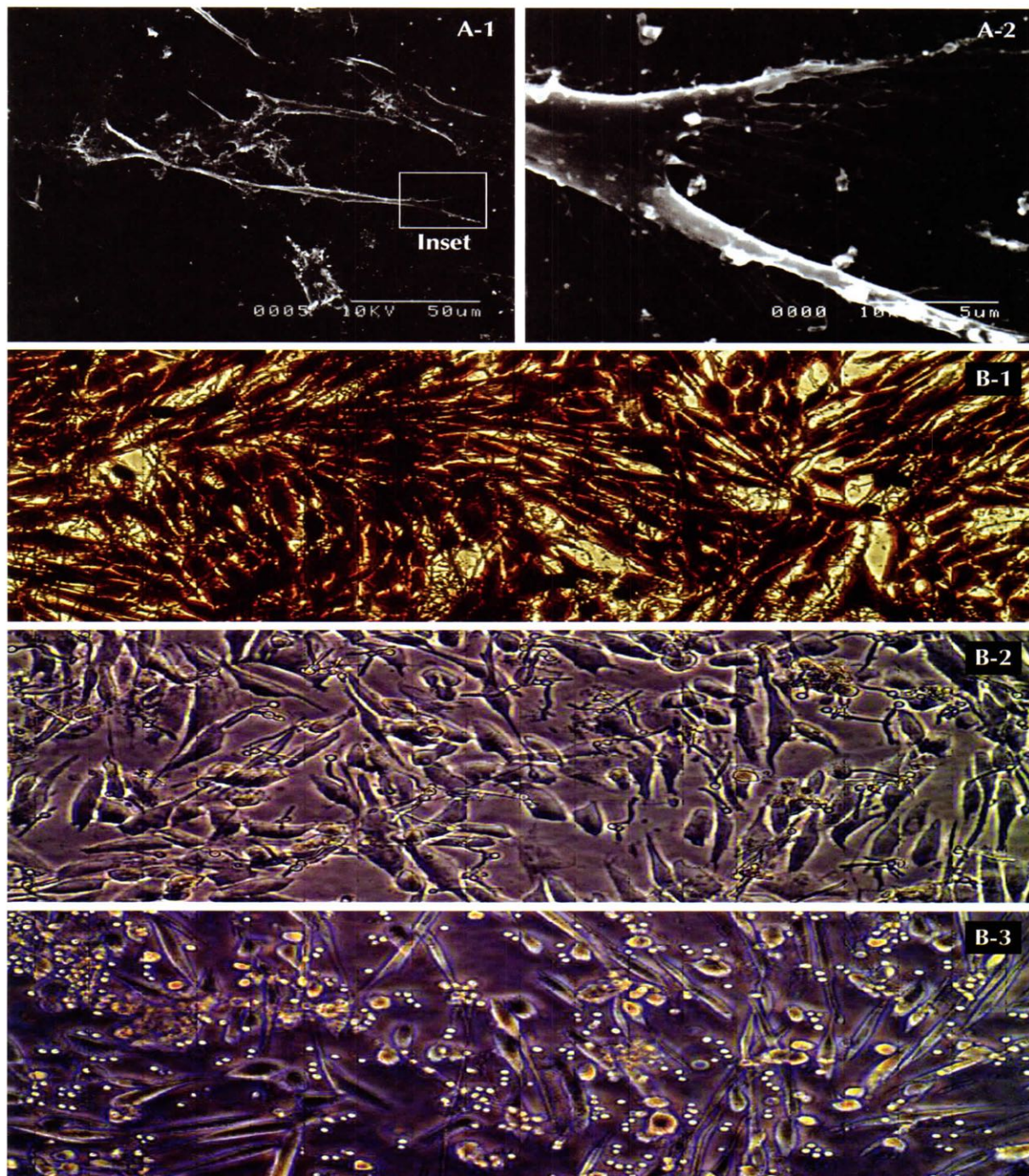
Scanning Electron Microscopy

VE cell monolayer on plastic coverslips was fixed with glutaraldehyde (2.5% vol/vol) at room temperature for 24 hours and washed twice with PBS, pH 7.2; that was followed by dehydration with multiple increments (i.e., 10–95%) of ethanol and absolute ethanol for 15 minutes each. After critical point drying the dehydrated samples were sputter coated with gold palladium using an Anatech Hummer V sputter coater (San Diego, California). Samples were visualized under a Hitachi S-530 scanning electron microscope (Rexdale, Ontario, Canada).

Lactate Dehydrogenase (LDH) Assay

Viability (membrane integrity) of VE cells was measured as a function of LDH leakage into the medium in the presence or absence of test substance.

Figure 2 Scanning electron microscopy of (A-1) elongated VE cells at 600× magnification. (A-2) Inset: morphologic characteristics of adherent VE cells with distinct cellular attachment structures at 6,000× magnification. (B-1) Effects of ALF on VE-yeast interactions visualized under light microscopy at 200× magnification. VE stained with 0.5% crystal violet shows typical nucleated fibroblastic epithelial cells with prominent cell-cell interactions via transmembrane proteoglycan matrix. (B-2) *C albicans* strain NTRL809A bound to VE with typical yeast germination and hypha formation on the epithelial surface. (B-3) *C glabrata* strain NTRL131G with firm attachment of blastospores to the VE monolayer.

Chicago, Illinois). Any differences between mean values of the compared groups when $p < 0.05$ was considered statistically significant.

Results

ALF Effects on Yeast Growth

Fungistatic activity of ALF was tested against *C albicans* strain NTRL809A and *C glabrata* strain NTRL131G, and the effects were compared with those of native LF (Figure 1). An inoculum of $\sim 10^5$ blastospores in SD broth at 37°C exponentially proliferated to reach a stationary growth equilibrium ($\sim 10^9$ yeast cell density) in 18 hours (~ 2 hours of generation time) for both *Candida* species. ALF at a 5 mg/mL concentration elicited total stasis (100% growth inhibition) for 96 hours and was significantly effective ($p < 0.0001$) against both *Candida* species as compared to native LF. ALF at 2.5 mg/mL further sustained total stasis activity up to an average time point of 18 and 24 hours for *C albicans* (n = 5) and *C glabrata* (n=5), respectively (data not shown). In contrast, under similar inoculation and incubation conditions, native LF (5 and 2.5 mg/mL concentrations) failed to elicit fungistasis against either *Candida* species.

VE Monolayer and ALF Interactions

SEM analysis showed elongated VE cells with typical cytomorphologic characteristics. The adherent VE cells demonstrated distinct attachment structures that are essential to cellular binding and proliferation (Figure 2A-1 and A-2). VE cells stained with 0.5% crystal violet (Fisher Diagnostics, Middletown, Virginia) were also examined by phase-contrast microscopy (TE300, Nikon, Melville, New York). VE monolayer demonstrated typical nucleated epithelium with a defined membrane lining. Prominent cell-cell interactions via transmembrane proteoglycan matrix constituents were evident throughout the monolayer (Figure 2B-1). Viability and tolerance of VE cells in the presence of ALF was determined by LDH assay. ALF (5 mg/mL) exposure for >10 hours did not affect VE cell viability. After 48 hours of exposure, however, VE cell viability was reduced to 40% with ALF as compared to the control. This loss of viability was common to the aging of the VE (sloughed "lifted," nonadherent) cell population as compared to the dividing progeny cells. The activation process significantly reduced ($p < 0.001$) VE cell death caused by native LF, 10% and 20% after 48-hour and 72-hour exposures, respectively (data not shown). Based on these data,

ALF was tested at a 5 mg/mL concentration for ~ 2-hour exposure for VE interaction studies.

VE Monolayer and Yeast Interactions

Based on the DNA uptake of ^{3}H-thymidine, a correlation for each radioactive DPM was established at 125 and 1,200 viable yeast count for *C albicans* and *C glabrata*, respectively. Interaction studies indicated avid binding of *C albicans* (70–140 $\times 10^3$ yeast) and *C glabrata* (50–75 $\times 10^3$ yeast) per square centimeter of a confluent VE surface. *C albicans* strain NTRL809A bound to VE with typical yeast germination and hypha formation on the monolayer (Figure 2B-2). *C glabrata* strain NTRL131G showed firm attachment of blastospores to a confluent VE surface (Figure 2B-3). These data indicate that *in vitro* cultured VE retained the cell surface characteristics (receptors) essential to yeast binding, germination and hypha formation, therefore suitable for interaction studies.

ALF Blocking of Yeast Adhesion to VE Monolayer

Pretreatment of VE with ALF (5 mg/mL) for 2 hours, followed by pathogen challenge ($\sim 10^8$ yeast) for 2 hours, resulted in significant blocking ($p < 0.05$) of yeast adhesion, by 33% and 58%, as compared to controls, for *C albicans* and *C glabrata*, respectively (Table I). Under similar conditions, native LF elicited 10% ($p = 0.242$) and 16% ($p = 0.264$) VE adhesion blocking of *C albicans* and *C glabrata*, respectively.

ALF Detachment of Yeast-VE Complexes

ALF treatment for 2 hours caused significant detachment ($p < 0.05$) of preformed yeast-VE complexes by 58% and 51%, for *C albicans* and *C glabrata*, respectively, as compared to all test groups (Table I). Under similar conditions, native LF elicited 10% ($p = 0.522$) and 15% ($p = 0.361$) detachment of VE-bound *C albicans* and *C glabrata*, respectively.

Discussion

Candida species are resident microflora on skin, mucosal surfaces of the intestinal, and respiratory and genital tracts of normal, healthy individuals. Equilibrium between the host and yeast microflora ensures the avirulent, commensal status of this microorganism. This equilibrium is attained both by specific immune responses and nonspecific factors released by mucosal secretions, such as IgA, lysozyme and LF.[1,16,17] Immunocompromised conditions, such as neutropenia, and reduced flow

Table I *ALF Effects on Interactions of ^{3}H-Thymidine–Labeled Candida Species with the VE Monolayer*

Test parameter	Total ^{3}H-thymidine labeled yeast bound to VE ($\times$1,000)			
	Adhesion blocking (% effect)		Detachment (% effect)	
	C albicans	C glabrata	C albicans	C glabrata
Control	135 ± 12 (0%)	69 ± 9 (0%)	74 ± 4 (0%)	59 ± 4 (0%)
E/B buffer	137 ± 11 (−2%)	59 ± 5 (14%)	70 ± 4 (5%)	55 ± 3 (7%)
Normal LF	121 ± 8 (10%)	58 ± 5 (16%)	67 ± 4 (10%)	50 ± 3 (15%)
ALF	90 ± 4 (33%)[a]	29 ± 3 (58%)[a]	31 ± 3 (58%)[a]	29 ± 2 (51%)[a]

[a]Results in the ALF-treated group were significantly different ($p < 0.05$) than in the native LF, EDTA/bicarbonate buffer or control group in each column.

rates of exocrine secretions could result in LF deficiency or dysfunction and predispose to opportunistic pathogenesis by commensal flora, such as *Candida* species.[18,19]

Fungistasis for an extended period of time against a highly dense yeast population is critical to the clinical management of VVC. In this study, ALF (5 mg/mL) elicited total fungistasis (100% growth inhibition) of *C albicans* and *C glabrata* at 10^5 (5-log) yeast inoculum in SD broth for 96 hours. In contrast, native LF failed to elicit total or partial fungistasis under similar inoculation conditions. This blastospore density (inoculum) was chosen based on the observation in this study that ~10^5 yeast bound per square centimeter surface of VEM *in vitro*. Kuipers and coworkers tested anti-*Candida* activity of apo-LF (iron-free form) and native LF at 2.5 mg/mL concentrations against 4-log yeast inoculum for 24 hours and reported partial fungistasis, ~25% and ~75% activity against *C albicans* (n = 2) and *C glabrata* (n = 2), respectively.[20] In our study, ALF at 2.5 mg/mL concentration elicited total fungistasis of 5-log yeast inoculum for an average of 18 and 24 hours for *C albicans* (n = 5) and *C glabrata* (n = 5), respectively. This suggests that ALF elicited enhanced fungistatic activity as compared to native LF and other LF forms.

Adherence of *Candida* to vaginal epithelium is an important first step in persistent yeast colonization, resulting in symptomatic or asymptomatic infection. Human exfoliated vaginal epithelial cells have been commonly used for yeast adherence studies. The physiologic state of the donor (i.e., diabetes, pregnancy, resident microflora) constitutes variability in VE cell interactions with *Candida*.[21] To overcome this limitation, we processed human vaginal tissue biopsies and established a novel VE cell monolayer, fully functional for *Candida* adhesion studies. The safety and tolerance of VE cells to ALF were established at 5 mg/mL concentration.

The activation process markedly and significantly reduced ($p < 0.001$) the cytotoxicity of native LF by 10–20%, depending on its VE cell exposure time.

Pathogenesis of VVC is a 3-stage mechanism consisting of blastospore adhesion, germination (mycelium or hypha development) and epithelial invasion of *C albicans*.[22] Adhesion is critical to blastospore survival; accordingly, *C albicans* is more adherent than *C glabrata* and other non-*albicans* pathogens; that explains its higher frequency in clinical settings.[23] Our data also show that the adherence of *C albicans* to VE was significantly higher ($p < 0.05$) than that of *C glabrata*.

Mannoproteins on the candidal surface bind to specific phospholipid- and fibronectin-containing receptors in the VE cell membrane during yeast adhesion.[24] Any ligands that competitively bind to these specific VE membrane receptors with high affinity could interfere with yeast infection. In this study, pretreatment of VE cells with ALF caused a potent blockade of yeast adhesion, suggesting binding of ALF to the VE membrane. Furthermore, ALF treatment effectively detached the preformed yeast-VE cell complexes; that indicated competitive displacement, resulting from a high-affinity interaction of ALF with the VE membrane. Further studies are under way to elucidate ALF interactions with the VE monolayer.

The hyphal form of *C albicans* is more capable of adhering to mucosal cells than the blastospore form and thus is more likely to invade host tissues to initiate clinical disease.[25] In our study, ALF demonstrated a potent blocking effect against both hyphal forms of *C albicans* and blastospore forms of *C glabrata* adherent to VE cells.

Conventional methods for clinical management of VVC rely upon the use of antifungal drugs designed to kill the yeast or inhibit growth. Fungicidal agents cause cytolysis that release intracellular mycotoxins and proinflammatory yeast debris into the

mucosal milieu; that could further compromise the vaginal host defense. An alternative approach, aimed at disrupting yeast adherence to vaginal mucosa, might have potential in controlling VVC, particularly when combined with a fungistatic effect. In summary, ALF fits this new category as a potent natural antifungal agent that inhibits growth, blocks adhesion and detaches *C albicans* and *C glabrata* from vaginal epithelial cells.

Acknowledgments

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