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 10⁵ 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 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 10⁵ yeast inoculum in SD broth proliferated to a stationary growth equilibrium (~10⁶ 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 × 10³ yeast) and C glabrata (50–75 × 10³ 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 signifi-

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

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candidiasis, vulvovaginal.

Vulvovaginal candidiasis (VVC) is commonly asso-
icted with immunocompromised host popula-
tions. Alteration or dysfunction of innate host de-
fense in vaginal mucosa is a major predisposing
factor for several infections, including vaginal yeast
colonization. Accordingly, VVC is more prevalent
during pregnancy when high hormone levels lead
to an increase in vaginal glycogen, providing a fa-
orable environment for growth of Candida. It is es-
imated 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 re-
current, often intractable episodes. VVC is preva-
ent among diabetics and women taking oral con-
traceptives, antibiotics and adrenal steroids.

Lactoferrin (LF), an iron-binding glycoprotein, is
a primary innate defense factor present in milk, sali-
va, tears and other exocrine secretions that bathe
tissue surfaces, including vaginal mucosa. Furthermore,
as a major granular component of polymor-
phonuclear lymphocytes, LF has a protective role in
inflammation and cell mediated immunity. 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. Func-
gidal activity for LF against Candida albicans and Can-
dida krusei, by mechanisms related to altered cell
surface permeability, has also been reported.

Activated lactoferrin (ALF) is a novel formula-
tion of native LF with enhanced function due to a
specific molecular-milieu optimization process. ALF is a natural microbial-blocking agent that de-
taches or prevents attachment of yeast and other
microbes to mucosal surfaces and selectively in-
hibits proliferation of pathogens without affecting
commensal flora.

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

**Materials and Methods**

**LF**

LF (lot 10099751) purified from cow’s milk was pro-
vided by DMV International Nutritional, 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. 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 NaHCO₃ (Fisher, Fairlawn, New Jer-
sy) and 100 mM NaCl (Sigma Chemicals, St. Louis,
Missouri). After adjusting the pH to 8.2 (with
NaHCO₃), food grade pectin (0.02 g; CU 201, Herb-
streith and Fox, Nürenburg, Germany) was added
to this solution with gentle stirring for partial im-
mobilization of the dissolved LF. The formation of
immobilized LF was confirmed by gel filtration
chromatography using a Sephacryl S-200 HR col-
umn (Amersham Bioscience Corp., Piscataway,
New Jersey).

**Candida Isolates**

Clinical strains of Candida species were isolated
from VVC patients at the Microbiology Labo-
atory, Mayo Clinic, Scottsdale, Arizona. Isolates were
identified and speciated to C albicans and C glabrata
with the MicroScan (Dade Behring, Deerfield, Illi-
nois) 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 vol-
ume of ALF, native LF or sterile EDTA/bicarbonate
buffer was added to designated wells followed by
inoculation with 50 μL yeast suspension containing
10⁵ yeast per milliliter (diluted from an optically
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 4x 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) 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.
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% CO₂ 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 ~10⁸ 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 774950, Nunc, Roskilde, Denmark) placed into each tissue culture plate well (Costar® 3524, 24-well plate). One milliliter of Candida suspension (~10⁸ yeast) was added to each insert (area = 2 cm²) containing ~10⁶ 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., Chicago, Illinois).

![Graph](image-url)

**Figure 1** Effects of ALF (5 mg/mL) and native LF (5 mg/mL) on *C. albicans* and *C. glabrata* (10⁵ 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.
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 ~10^5 blastospores in SD broth at 37°C exponentially proliferated to reach a stationary growth equilibrium (~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, Midletown, 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 ^3H^-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 × 10^3 yeast) and *C. glabrata* (50-75 × 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 (~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 1  ALF Effects on Interactions of \(^{3}\)H-Thymidine-Labeled Candida Species with the VE Monolayer

<table>
<thead>
<tr>
<th>Test parameter</th>
<th>(C.) albicans</th>
<th>(C.) glabrata</th>
<th>(C.) albicans</th>
<th>(C.) glabrata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>133 ± 12 (0%)</td>
<td>69 ± 9 (0%)</td>
<td>74 ± 4 (0%)</td>
<td>59 ± 4 (0%)</td>
</tr>
<tr>
<td>E/B buffer</td>
<td>137 ± 11 (-2%)</td>
<td>59 ± 5 (14%)</td>
<td>70 ± 4 (5%)</td>
<td>55 ± 3 (7%)</td>
</tr>
<tr>
<td>Normal LF</td>
<td>121 ± 8 (10%)</td>
<td>58 ± 5 (16%)</td>
<td>67 ± 4 (10%)</td>
<td>50 ± 3 (15%)</td>
</tr>
<tr>
<td>ALF</td>
<td>90 ± 4 (33%)</td>
<td>29 ± 3 (58%)</td>
<td>31 ± 3 (58%)</td>
<td>29 ± 2 (51%)</td>
</tr>
</tbody>
</table>

*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 fungistic 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 fungistic 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.

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References