

Evaluation of antifungal and antibiofilm effects of supernatant cell-free supernatant (CFS) of *Lactobacillus casei* alone and in combination with nystatin against *Candida albicans* strains

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ABSTRACT

Background and Objectives: *Candida albicans* (*C. albicans*) is the most common cause of candidiasis in immunocompromised patients. Biofilm-associated *Candida* can lead to treatment complications and systemic infections.

Materials and Methods: In this study, the antifungal activity of the cell-free supernatant (CFS) derived from *Lactobacillus casei* was assessed against *C. albicans* strains. To differentiate between pH- and H₂O₂-mediated effects and specific metabolite activity, all antifungal and antibiofilm assays were performed using CFS that had been neutralized to pH 7.0 and treated with catalase.

Results: The ranges of minimum inhibitory concentration (MIC) and Half-maximal inhibitory concentration (IC₅₀) for CFS were 7.81-62.5 and 95.1-159.6 µl/ml, respectively. Using corrected FICI criteria (synergy ≤ 0.5; additive > 0.5-1.0; indifference > 1.0-4.0), additive effects were observed in 50% of strains, synergy in 20% (including the standard strain PTCC 5027), and indifference in 30%.

Conclusion: The results demonstrate that the CFS of *L. casei* exhibits promising in vitro antifungal and antibiofilm activity against *C. albicans*. When used in combination with nystatin, *L. casei* CFS showed additive or synergistic effects, suggesting its potential as an adjunctive agent for further investigation. However, these findings are preliminary, and in vivo studies and clinical assessments are necessary to validate the therapeutic potential of *L. casei* CFS in the management of oral candidiasis.

Keywords: Antifungal agents; Biofilms; *Candida albicans*; Probiotics; *Lactobacillus casei*; Nystatin

INTRODUCTION

Candida albicans (*C. albicans*) is the most common cause of candidiasis in immunocompromised

patients (1, 2). Candidiasis is one of the most frequent causes of fungal infection-related mortality in patients with weakened immune systems (3, 4). The incidence of invasive candidiasis has gone up due to

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high rates of therapeutic failure caused by intrinsic and acquired resistance to several classes of antifungal agents (5). One of the novel strategies being employed to fight drug-resistant infections is the use of probiotics (6-8). Probiotics mainly belong to the *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, and *Streptococcus* and can potentially inhibit the adhesion and colonization of pathogenic microorganisms (9). *Lactobacillus* spp. (most-researched probiotics) inhibit pathogens via acidic conditions, enhance immunity, and curb *C. albicans* adhesion/colonization (10). All age groups, including infants, kids, adults, and senior citizens, can safely use probiotics (11). It is still unknown how probiotic products affect yeasts and mucosal candidiasis (12, 13). Antifungal resistance due to biofilm formation, efflux pump activity, and other mechanisms leads to treatment failures in severe fungal infections (14). Biofilms, which are communities of microorganisms attached to surfaces, play a significant role in chronic infections and infections related to medical devices. They function as protective barriers, making these infections resistant to drugs and the immune system (14, 15). *Lactobacillus* inhibits biofilms through metabolites that disrupt their structure or modify related gene expression. Probiotics effectively reduce filamentation and biofilm formation in *C. albicans*, making them a valuable strategy for controlling candidiasis (15-17). This study assessed the antifungal and antibiofilm activity of *L. casei* CFS alone and combined with nystatin against standard/resistant *C. albicans* strains. Additionally, MTT was used to assess CFS cytotoxicity on murine macrophage cells (J774-A1). As this is an in vitro study, the findings should be considered preliminary, and further in vivo and clinical investigations are required to establish therapeutic applicability.

MATERIALS AND METHODS

Ethical approval. This experimental study was approved by the Ethics Committee of the Vice-Chancellor for Research at Kerman University of Medical Sciences (Ethics Code: IR.KMU.AH.REC.1401.255).

Microbial strains and cell lines. Ten *C. albicans* isolates were utilized in this study, including seven sensitive clinical isolates (S1-S7) and two clinical isolates resistant to nystatin and fluconazole (R1 and

R2). All isolates were previously obtained from AIDS patients, with necessary permissions secured. The resistant clinical isolates (R1 and R2) were defined as nystatin-resistant based on their significantly elevated minimum inhibitory concentration (MIC) values (64 µg/mL) compared to the sensitive clinical isolates (16 µg/mL), representing a ≥ 4 -fold increase in MIC. We acknowledge that the Clinical and Laboratory Standards Institute (CLSI) M27 guideline does not provide official clinical breakpoints for nystatin against candida species. In the absence of standardized breakpoints, our operational definition of resistance was based on (i) a clear shift in the MIC distribution relative to susceptible isolates, consistent with approaches used in previous studies, and (ii) the previously documented reduced susceptibility of these isolates in our laboratory. The resistant clinical isolates (R1 and R2), previously characterized in our laboratory, were defined as nystatin-resistant based on their MIC values exceeding the clinical breakpoints established by the CLSI M27 guideline for yeast susceptibility testing (18). The standard strain *C. albicans* PTCC5027 was procured from the Iranian Research Organization for Science and Technology (IROST) Industrial Microorganisms Collection. Additionally, the *Lactobacillus casei* (PTCC 1608) was provided by the Pharmacy Department of Kerman University of Medical Sciences.

Preparation of *L. casei* cell-free supernatants (CFS). *L. casei* was cultured in Man-Rogosa-Sharpe (MRS) broth at 37°C under anaerobic conditions. After 48 hours, a 2 mL aliquot of the culture, adjusted to an optical density of 1 at 600 nm, was centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was collected, sterile-filtered using 0.22 µm pore-size syringe filters (TPP, Trasadingen, Switzerland), and stored at -20°C until use. For all subsequent antimicrobial and antibiofilm assays, the CFS was adjusted to pH 7.0 using sterile NaOH (1 M) and re-filtered (0.22 µm). In parallel, catalase (Sigma, final concentration 1000 U/mL) was added to neutralize hydrogen peroxide, and the mixture was incubated at 37°C for 30 min before use. Thus, all antifungal and antibiofilm activity data presented in this study were obtained using neutralized, catalase-treated CFS. Sterile MRS broth was processed identically to the CFS preparation (centrifugation, filtration, pH adjustment, catalase treatment) and served as a medium control in all assays.

Antimicrobial activity assessment. The antimicrobial activity of *L. casei* CFS against *C. albicans* was evaluated using the co-aggregation method. Briefly, *C. albicans* was cultured in Sabouraud dextrose broth at 35°C with shaking at 100 rpm. A suspension of *L. casei* (10^8 CFU/mL) was mixed with *C. albicans* (10^6 CFU/mL) and incubated at 37°C with shaking for 24 h. Absorbance was measured spectrophotometrically at 600 nm at 0, 1, 2, and 4 hours. Controls included *Lactobacillus* suspension (positive control) and Sabouraud dextrose broth (negative control). Co-aggregation was calculated using established equations (19, 20).

The antifungal activity of *L. casei* CFS and nystatin was assessed using microdilution according to the CLSI M27 guideline. Serial dilutions of nystatin (0.25-128 µg/mL) and *L. casei* CFS (0.78-100%) were prepared in a 96-well microplate, followed by the addition of *C. albicans* suspension ($1.5-2 \times 10^4$ cells/mL). After 24 hours of incubation at 37°C, the MIC and minimum fungicidal concentration (MFC) were determined (18).

Checkerboard assay for synergistic effects. The combined effects of *L. casei* CFS and nystatin were investigated using the checkerboard technique. In a 96-well microplate, nystatin (0.25-128 µg/mL) and *L. casei* CFS (0.78-100%) were serially diluted in columns and rows. *C. albicans* suspension ($1.5-2 \times 10^4$ cells/mL) was then added to each well. After 24 hours of incubation, the fractional inhibitory concentration index (FICI) was calculated. Synergy was defined as $FICI \leq 0.5$, as an additive effect between 0.5 and 1.0; values > 1.0 between 1-4 considered as indifference effect, and values > 4.0 were categorized as antagonism, in accordance with standard antifungal interaction criteria.

$$FICI = \frac{MIC_{Nys, comb}}{MIC_{Nys, alone}} + \frac{MIC_{CFS, comb}}{MIC_{CFS, alone}}$$

Biofilm formation and anti-biofilm activity. Biofilms were formed in 96-well flat-bottom microplates by inoculating *C. albicans* suspensions (10^6 cells/mL) in RPMI 1640 medium (supplemented with L-glutamine and buffered with MOPS, pH 7.0) and incubating statically at 37°C for 48 h to allow biofilm maturation. For eradication assays, the compounds (nystatin, *L. casei* CFS, or their combination) were added after 48 h of biofilm maturation and incubated for an addi-

tional 24 h at 37°C (14).

Biofilm quantification. After incubation, wells were gently washed twice with phosphate-buffered saline (PBS) to remove non-adherent cells. Biofilm biomass was quantified using crystal violet (CV) staining: biofilms were fixed with methanol (99.8%) for 15 min, stained with 1% (w/v) CV for 20 min, washed with distilled water, and destained with 33% acetic acid. Absorbance was measured at 570 nm. The CV assay measures total biofilm biomass, including both cells and extracellular matrix components (14).

Metabolic activity assessment. Biofilm metabolic activity (reflecting cell viability) was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After treatment, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, and plates were incubated at 37°C for 4 h. The resulting formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm. The MTT assay quantifies the metabolic activity of viable cells by measuring mitochondrial dehydrogenase activity. Absorbance values from treated wells were normalized to untreated biofilm controls (positive control, representing 100% biofilm formation/metabolic activity) and to medium-only blanks (negative control, representing background absorbance). All experiments were performed with three independent biological replicates (separate cultures on different days), each with three technical replicates (triplicate wells). Data are presented as mean $\pm$ standard deviation (SD) from the combined results of all three biological replicates ($n = 3$). The percentage inhibition was calculated as:

% Inhibition = $[(A_{\text{control}} - A_{\text{blank}}) - (A_{\text{treated}} - A_{\text{blank}})] / (A_{\text{control}} - A_{\text{blank}}) \times 100$ where A_{control} is the absorbance of untreated biofilms, A_{blank} is the absorbance of medium-only wells, and A_{treated} is the absorbance of treated biofilms (14).

IC50 determination. The concentration required to inhibit 50% of biofilm biomass or metabolic activity (IC50) was calculated by fitting normalized percentage-inhibition data to a nonlinear regression model ($\log[\text{inhibitor}]$ vs. normalized response, variable slope) using GraphPad Prism 9. IC50 values were determined for nystatin and *L. casei* CFS against all tested *C. albicans* strains.

Selectivity index (SI) and toxicity assessment. SI, defined as the ratio of toxic concentration (CC50) to effective bioactive concentration (IC50), was calculated to evaluate drug efficacy and toxicity. The IC50 for *L. casei* CFS against *C. albicans* and its toxicity toward J774.A1 macrophage cells were determined using the MTT assay.

Statistical analysis. Data were analyzed using SPSS version 20 and GraphPad Prism 9 software. Differences between groups were assessed using analysis of variance (ANOVA) and t-tests. IC50 and CC50 values were calculated using probit analysis. Results are presented as mean $\pm$ standard deviation (SD), with statistical significance set at $p < 0.05$.

RESULTS

Co-aggregation of *L. casei* and *C. albicans*. Co-aggregation percentages of *L. casei* and *C. albicans* isolates are presented in Fig. 1. The ability of *L. casei* adhere to and inhibit *C. albicans* was measured at 0, 1, 2, and 4 hours.

L. casei demonstrated significant co-aggregation with both clinical *C. albicans* isolates and reference strains. A statistically significant increase ($p < 0.05$) in co-aggregation percentage was observed after 4 hours for all tested strains. Among clinical isolates, the highest co-aggregation rates were recorded with *C. albicans* S2, S3, and S4 (64%), while the lowest co-aggregation (8%) occurred with *C. albicans* R1. No auto-aggregation was detected in any strain or isolate.

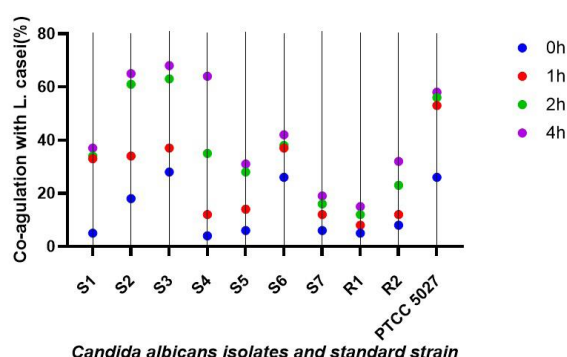


Fig. 1. Co-aggregation percentage between *L. casei* and *Candida albicans* isolated from candidiasis patients (S1, S2, S3, S4, S5, S6, S7, R1, R2) and the standard strain (PTCC5027).

The co-aggregation efficiency of *L. casei* with *C. albicans* strains was ranked as follows: *C. albicans* S3 > *C. albicans* S2 > *C. albicans* S4 > *C. albicans* PTCC 5027 > *C. albicans* S6 > *C. albicans* S1 > *C. albicans* R2 > *C. albicans* S5 > *C. albicans* S7 > *C. albicans* R1.

Comparative analysis of CFS activity before and after neutralization and catalase treatment. The pH of the original (untreated) *L. casei* CFS was 3.8 ± 0.2 . To differentiate between pH-mediated effects, hydrogen peroxide activity, and specific metabolite activity, we conducted comparative assays using untreated CFS, neutralized CFS (pH 7.0), and neutralized CFS treated with catalase (1000 U/mL). Untreated CFS exhibited the strongest activity (MIC range: 0.98-7.81 μ l/mL). Neutralization to pH 7.0 reduced activity (MIC range: 3.9-31.25 μ l/mL), and subsequent catalase treatment further reduced but did not eliminate activity (MIC range: 7.81-62.5 μ l/mL). In addition, untreated CFS showed MIC ranged 12.5-45.3 μ l/mL while neutralized CFS to pH 7.0 showed MICs of 31.2-95.1; the neutralized, catalase-treated CFS (MIC range: 95.1-159.6 μ l/mL) retained significant antifungal and antibiofilm activity against all tested *C. albicans* strains, confirming that *L. casei* produces specific metabolites with antifungal properties beyond pH- and H₂O₂-mediated effects. All subsequent experiments described in this study were performed using neutralized, catalase-treated CFS.

Growth inhibition of *C. albicans* isolates by *L. casei*, nystatin, and their combination. *L. casei* CFS inhibited the growth of all tested *C. albicans* strains. The strongest inhibitory effect was observed against *C. albicans* S2, with a MIC of 7.81 μ l/ml. For sensitive *C. albicans* isolates, the MIC50 of *L. casei* CFS was 31.25 μ l/ml, while resistant isolates and standard strains exhibited a higher MIC50 of 62.5 μ l/ml.

Nystatin showed MIC values of 16 μ g/ml against sensitive *C. albicans* strains and 64 μ g/ml against resistant and standard strains. The MIC and MFC values for *L. casei* CFS and nystatin, alone and in combination, are detailed in Table 1.

Combining nystatin and *L. casei* CFS demonstrated synergistic effects against the *C. albicans* standard strain PTCC 5027 (FICI = 0.37) and one sensitive isolate, S5 (FICI = 0.24). Additive effects (FICI > 0.5 to ≤ 1.0) were observed in 50% of strains (S1, S3, S7,

Table 1. Interactions of *L. casei* CFS with nystatin against *C. albicans* isolates and standard strains

Isolates	MIC and MFC				FICI	Interpretation
	Alone		Combined			
	Nystatin MIC/	CFS MIC/MFC	Nystatin MIC	CFS MIC		
	MFC (µg/ml)	(µl/ml)	(µg/ml)	(µl/ml)		
<i>C. albicans</i> S1	16/32	31.25/31.25	8	15.62	0.99	Additive
<i>C. albicans</i> S2	16/32	7.81/31.25	16	15.62	3	Indifference
<i>C. albicans</i> S3	16/32	31.25/31.25	8	15.62	0.74	Additive
<i>C. albicans</i> S4	16/32	31.25/31.25	16	31.25	2	Indifference
<i>C. albicans</i> S5	16/32	31.25/31.25	2	3.9	0.24	Synergism
<i>C. albicans</i> S6	16/32	31.25/ 31.25	16	31.25	2	Indifference
<i>C. albicans</i> S7	16/32	31.25/125	8	15.62	0.99	Additive
<i>C. albicans</i> R1	64/128	62.5/62.5	16	31.25	0.75	Additive
<i>C. albicans</i> R2	64/128	62.5/ 62.5	16	31.25	0.75	Additive
<i>C. albicans</i> PTCC 5027	64/128	62.5/250	8	15.62	0.37	Synergism

MIC values are reported as follows: nystatin in µg/mL, CFS in µL/mL. FICI was calculated using the formula above. FICI interpretation: ≤ 0.5 = synergy; > 0.5 to ≤ 1.0 = additive; > 1.0 to ≤ 4.0 = indifference; > 4.0 = antagonism

R1, and R2), while indifference (FICI > 1.0 to ≤ 4.0) was noted in the remaining 30% (S2, S4, and S6). These findings indicate that *L. casei* CFS enhances the susceptibility of *C. albicans* isolates to nystatin, predominantly through additive effects, with true synergy observed in a subset of strains. Interactions between the two agents were analyzed using the checkerboard method (Table 1).

Growth inhibitory effects of *Lactobacillus casei* CFS and nystatin on *C. albicans* biofilm formation. The half-maximal inhibitory concentration (IC₅₀), defined as the minimum concentration required to inhibit 50% of biofilm metabolic activity (as measured by MTT assay). It is important to note that nystatin IC₅₀ values are expressed in µg/mL, while CFS IC₅₀ values are expressed in µL/mL, reflecting the distinct nature of these preparations (a purified compound versus a complex supernatant) (Table 2).

Determination of the selectivity index of *L. casei* CFS and nystatin against *C. albicans*. The results showed that the IC₅₀ of nystatin and *L. casei* CFS on mouse macrophage cells were 208.6 ± 12.6 µg/ml and 385.2 ± 28.4 µl/ml, respectively. Therefore, it shows that the toxicity of *L. casei* CFS for mammalian cells by murine macrophages is lower compared to the nystatin drug.

The SI was used to measure antifungal specificity

Table 2. IC₅₀ values of *L. casei* CFS and nystatin against *C. albicans* biofilm

Strains	Nystatin IC50 (mean±SD) (µg/ml)	CFS IC50 (mean±SD) (µl/ml)	P Value *
<i>C. albicans</i> S1	56.8 ± 3.5	132.3 ± 21.3	P<0.001
<i>C. albicans</i> S2	61.3 ± 13.2	121.6 ± 22.0	P<0.001
<i>C. albicans</i> S3	52.3 ± 13.8	110.0 ± 21.3	P<0.001
<i>C. albicans</i> S4	63.2 ± 27.3	112.7 ± 11.3	P<0.001
<i>C. albicans</i> S5	49.2 ± 12.9	108.3 ± 13.0	P<0.001
<i>C. albicans</i> S6	71.6 ± 18.3	111.7 ± 13.1	P<0.001
<i>C. albicans</i> S7	44.9 ± 15.3	102.3 ± 11.8	P<0.001
<i>C. albicans</i> R1	134.3 ± 18.6	189.6 ± 20.0	P<0.001
<i>C. albicans</i> R2	122.8 ± 14.8	173.3 ± 19.0	P<0.001
<i>C. albicans</i> PTCC 5027	136.9 ± 23.3	180.3 ± 18.6	P<0.001

IC₅₀ values were determined by MTT assay and represent the concentration required to inhibit 50% of biofilm metabolic activity. CFS: *L. casei* cell-free supernatant. * P value between IC₅₀ of nystatin and IC₅₀ of CFS.

ty over mammalian cell toxicity, with higher values correlating to a wider therapeutic window. SI values close to 1 generally indicate poor selectivity between antifungal efficacy and host cell toxicity, reflecting a narrow therapeutic window. As shown in Table 3, the SI of nystatin ranged from 1.5 to 4.2, with the lowest SI (1.5) observed against nystatin-resistant *C.*

Table 3. Selectivity Index of nystatin, *L. casei* CFS, and combination therapy against *C. albicans* clinical isolates and standard strains.

Isolates	Nystatin SI (µg/ml)	CFS SI (µl/ml)	Nystatin/ CFS
<i>C. albicans</i> S1	3.7	2.9	7.5
<i>C. albicans</i> S2	3.4	3.2	6.0
<i>C. albicans</i> S3	4.0	3.5	7.5
<i>C. albicans</i> S4	3.3	3.4	6.4
<i>C. albicans</i> S5	4.2	3.6	7.8
<i>C. albicans</i> S6	2.9	3.4	5.2
<i>C. albicans</i> S7	4.6	3.8	8.0
<i>C. albicans</i> R1	1.6	2.0	3.9
<i>C. albicans</i> R2	1.7	2.2	3.4
<i>C. albicans</i> PTCC 5027	1.5	2.1	3.5

CFS: *L. casei* cell-free supernatant. SI: selective index. SI = CC_{50} / IC_{50} . Higher SI values reflect reduced cytotoxicity and enhanced antifungal efficacy. Nystatin/ CFS: the combination of nystatin and *L. casei* CFS.

albicans isolates. Similarly, the SI of *L. casei* CFS ranged from 2.0 to 3.8, with the minimum value (2.0) corresponding to resistant strains. These SI values indicate modest to moderate selectivity, suggesting that while the tested agents exhibit some preference for fungal cells over mammalian cells, their therapeutic window is relatively narrow. The combination of nystatin and *L. casei* CFS showed improved SI values (3.4 to 8.0), suggesting that combining agents may enhance the therapeutic index.

The CC_{50} values (208.6 ± 12.6 µg/mL for nystatin, 385.2 ± 28.4 µg/mL for *L. casei* CFS, and 308.6 ± 22.8 µg/mL for the combination) indicate that *L. casei* CFS is less toxic to murine macrophages than nystatin alone. However, the SI values approaching 1.0 for resistant isolates highlight the narrow therapeutic margin and the need for caution in clinical application.

DISCUSSION

The increasing challenge of candidiasis in immunocompromised hosts, driven partly by antifungal-resistant *Candida* species, necessitates exploring prophylactic options like probiotics for infection prevention (21, 22). This study demonstrated that *Lactoba-*

cillus casei significantly co-aggregates with diverse *C. albicans* strains (clinical isolates and references). The co-aggregation increased significantly over time, showing a statistically stronger association after 4 hours of co-incubation. Crucially, co-aggregation efficiency was highly strain-dependent. Clinical isolates varied widely, with peaks of 64% (S2, S3, S4) contrasting sharply with lows like 8% (R1). The overall ranking of strains by efficiency was: *C. albicans* PTCC 5027 > S6 > S1 > R2 > S5 > S7 > R. In a study conducted by Jørgen et al., both probiotic strains *Lactobacillus reuteri* ATCC PTA 5289 and *Lactobacillus reuteri* DSM 17938 demonstrated the ability to co-aggregate with clinical and reference strains of *Candida*. Co-aggregation increased significantly over time (at 1, 2, and 4 hours) for all combinations of *Candida* and probiotic strains ($p < 0.05$) (19). Chew et al. reported that *L. reuteri* co-aggregated most effectively with *C. tropicalis* and *C. krusei*, as measured by the co-aggregation ratio (23). Prior research highlights variable *Lactobacillus-Candida* co-aggregation, influenced by strain specificity, lower than the 64% observed here. Ahire et al. similarly demonstrated co-aggregation of vaginal *Lactobacillus* spp. with *C. albicans*, *E. coli*, *G. vaginalis*, and *P. mirabilis* (24). A key consideration in studies evaluating probiotic-derived antifungal activity is distinguishing between non-specific inhibition due to acidic pH or hydrogen peroxide and the activity of specific bioactive metabolites. In the present study, we addressed this by performing comparative assays with untreated CFS (pH 3.8), neutralized CFS (pH 7.0), and neutralized CFS treated with catalase. While acidic pH and H_2O_2 contributed substantially to the observed inhibitory effects, neutralized, catalase-treated CFS retained significant and consistent activity against all tested *C. albicans* strains. This finding is in agreement with previous studies demonstrating that *Lactobacillus* spp. produce a range of antifungal compounds, including bacteriocins, fatty acids, and other organic molecules, independent of pH or H_2O_2 (25-27). The retention of activity after neutralization and catalase treatment strongly supports the conclusion that *L. casei* produces specific antifungal metabolites capable of inhibiting *C. albicans* growth and biofilm formation. According to the findings of this study, *L. casei* CFS inhibited the growth of all tested *C. albicans* strains. The strongest inhibitory effect was observed against *C. albicans* S2 (MIC = 7.81 µl/ml). For susceptible isolates, the MIC

of *L. casei* CFS was 31.25 µl/ml, whereas resistant isolates and standard strains showed a higher MIC of 62.5 µl/ml. Nystatin exhibited MIC values of 16 µg/ml and 64 µg/ml against susceptible and resistant/standard strains, respectively. Findings suggest overlapping resistance mechanisms affecting both agents. Salari et al. (2020) reported MICs of 100-200 µl/ml for *L. acidophilus* CFS against *Candida* spp., with *C. albicans* more susceptible than *C. parapsilosis* (18).

Checkerboard analysis revealed that the combination of *L. casei* CFS and nystatin produced a range of interactions depending on the *C. albicans* strain. True synergy (FICI ≤ 0.5) was observed in 20% of strains (PTCC 5027 and S5), while additive effects (FICI > 0.5 to ≤ 1.0) were the most frequent interaction, occurring in 50% of strains (S1, S3, S7, R1, and R2). The remaining 30% of strains exhibited indifference (FICI > 1.0 to ≤ 4.0). No antagonism was observed. These findings indicate that *L. casei* CFS enhances nystatin susceptibility in *C. albicans*, predominantly through additive interactions, with true synergy observed in a subset of strains, including the standard strain PTCC 5027.

The formation of *C. albicans* biofilms exacerbates candidiasis, complicating treatment and creating a pressing need for innovative solutions (28). Probiotics offer a potential strategy by rebalancing the microbiome. Biofilm formation underlies the primary mechanism of antifungal resistance and pathogenesis in *Candida* spp., imparting intrinsic resistance (29). Probiotics disrupt *Candida* biofilm development through multiple mechanisms: immunomodulation (innate/adaptive responses, IgA enhancement), antioxidant effects, and pathogen-inhibiting exopolysaccharides. Notably, select *Lactobacillus* species block early colonization by suppressing fungal adhesion (29-31). *Lactobacillus* CFS likely disrupts biofilms via pH reduction, antimicrobial peptides, and metabolites (32). Rather et al. noted *L. fermentum* KAU0021 CFS IC50 values of 101.5 µl/ml and 203 µl/ml against forming and mature *C. albicans* biofilms, respectively (33). Our antibiofilm assays employed two complementary quantification methods: crystal violet (CV) staining to assess total biofilm biomass and MTT reduction to measure metabolic activity, which correlates with cell viability (34, 35). The observed reduction in both CV-stained biomass and MTT activity suggests that *L. casei* CFSF, not only disrupts biofilm architecture but also compromises the metabolic activity of biofilm-embedded

cells. However, we acknowledge that while CV and MTT assays provide valuable quantitative data, they do not offer detailed structural or ultrastructural information. Techniques such as confocal laser scanning microscopy (CLSM) with live/dead staining or scanning electron microscopy would be valuable in future studies to visualize biofilm architecture, matrix integrity, and morphological changes (e.g., inhibition of yeast-to-hyphae transition) induced by *L. casei* CFS (36, 37). Such microscopic evaluations would further elucidate the mechanisms underlying the antibiofilm activity observed in this study.

The SI values obtained in this study indicate the therapeutic potential of *L. casei* CFS and nystatin. The SI values recorded for *L. casei* CFS (2.0–3.8) demonstrate moderate preferential activity against fungal cells relative to mammalian macrophages, reflecting a detectable but relatively narrow therapeutic window. Crucially, an SI value of 1.0, observed for the combination against mammalian cells, does not indicate "negligible cytotoxicity"; in pharmacological terms, SI values close to 1 indicate a narrow therapeutic window and poor selectivity between antifungal activity and host cell toxicity. A compound with SI=1.0 would be equally toxic to host cells as to the pathogen, rendering it unsuitable for therapeutic use. The modest SI values observed for both nystatin and *L. casei* CFS are not unexpected. Nystatin is known for its narrow therapeutic index and significant toxicity, which limits its systemic use. For *L. casei* CFS, which contains a complex mixture of metabolites, moderate selectivity is reasonable. This does not diminish the potential value of probiotic-derived compounds as adjunctive therapies rather than standalone antifungal agents (26, 27). Importantly, the combination of *L. casei* CFS with nystatin showed improved SI values (3.4-8.0) compared to either agent alone, suggesting that combination therapy may enhance the therapeutic index. This observation aligns with the additive and synergistic effects observed in our checkerboard assays. It supports the potential of *L. casei* CFS as an adjunctive therapy to reduce the effective dose of conventional antifungals, thereby potentially mitigating toxicity. However, the SI values obtained in this study (particularly those approaching 1.0 for resistant isolates) underscore the need for caution and highlight that these agents, especially when used alone, have a narrow safety margin. Further studies, including in vivo efficacy and toxicity assessments, are essential to fully evaluate the

clinical applicability of *L. casei* CFS. The observed selectivity of *L. casei* CFS is particularly significant. Probiotic-derived compounds, such as organic acids, bacteriocins, and fatty acids present in CFS, often target fungal membranes or metabolic pathways distinct from conventional drugs, potentially offering a lower propensity for inducing resistance and reduced host cell damage compared to some fungicidal agents (25). While the SI of *L. casei* CFS appears comparable to, or sometimes exceeds, that of nystatin against resistant isolates in this study, further investigation into its precise antifungal components and mechanisms is warranted to fully understand its therapeutic potential and optimize its application, potentially as an adjunctive therapy.

Recommendation. Based on the findings of this study and the current challenges in managing candidiasis, particularly in immunocompromised hosts and against resistant strains, the following recommendations are proposed:

Further mechanistic investigation: Conduct in-depth studies to identify and characterize the specific bioactive compounds within *L. casei* CFS responsible for co-aggregation, growth inhibition, biofilm disruption, and synergy with nystatin. This should include analysis of organic acids, bacteriocins, fatty acids, exopolysaccharides, and other metabolites. Understanding the precise modes of action is crucial for optimizing therapeutic applications.

Strain-specific screening and selection: Given the critical role of strain specificity observed in co-aggregation efficiency, antifungal susceptibility, and biofilm synergy, future research and potential therapeutic development must prioritize rigorous screening and selection of both probiotic (*L. casei*) strains and their derived CFS, as well as characterization of target *C. albicans* strains.

This study can help with the preclinical and clinical development of combination therapy. The therapeutic potential of *L. casei* CFS as an adjunctive therapy alongside conventional antifungals like nystatin can be explored for treating biofilm-associated candidiasis, increasing the efficacy of nystatin against nystatin-resistant *C. albicans* isolates (where additive effects were demonstrated), and possibly lowering the required dosage of conventional antifungals, thereby decreasing potential toxicity.

In vivo safety and efficacy studies: Progress to well-designed in vivo models (e.g., immunosup-

pressed animal models of invasive or mucosal candidiasis) to confirm the promising safety profile (indicated by the favorable Selectivity Index) and the therapeutic efficacy of *L. casei* CFS, both alone and in combination with nystatin, observed in vitro.

CONCLUSION

In this study, both *L. casei* cells and CFS demonstrated inhibitory activity against the tested *C. albicans*. However, the response was strain-dependent, with nystatin-resistant and reference strains generally showing lower susceptibility than nystatin-sensitive isolates. Importantly, *L. casei* CFS retained significant antifungal and antibiofilm activity even after neutralization and catalase treatment, suggesting that its activity was not attributable solely to acidic pH or hydrogen peroxide.

The observed activity of *L. casei* CFS, which was comparable to that of nystatin under the conditions evaluated, highlights the potential of this complex biological preparation as a source of antifungal and antibiofilm agents. These findings support the potential role of *L. casei*-derived preparations as complementary candidates for the management of *C. albicans* infections, particularly in the context of increasing antifungal resistance and the need for alternative or adjunctive antimicrobial strategies. However, the present findings are based on in vitro experiments and should not be interpreted as evidence of clinical efficacy or as a basis for recommending probiotics or CFS as a treatment for patients with immunodeficiency.

Further studies are required to identify and characterize the bioactive compounds responsible for the activity of *L. casei* CFS, elucidate their mechanisms of action, and evaluate their safety, pharmacological properties, and efficacy in appropriate in vivo models and, ultimately, well-designed clinical studies. Such investigations are necessary to determine whether *L. casei*-derived preparations can be safely and effectively developed as complementary approaches to conventional antifungal therapy.

Study limitations. The sample size in this study was limited to ten *C. albicans* isolates, including only two nystatin-resistant strains. While the results provide preliminary evidence of the antifungal and antibiofilm potential of *L. casei* CFS, the small

number of resistant isolates constrains the generalizability of findings regarding resistance mechanisms and combination efficacy. Future studies with larger and more diverse clinical collections are warranted to validate these observations. While we conducted comparative assays to evaluate the contribution of acidic pH and hydrogen peroxide to the observed antifungal activity, further characterization of the specific bioactive metabolites present in *L. casei* CFS is warranted. Identification and purification of these compounds would facilitate a more complete understanding of the mechanisms underlying the antifungal and antibiofilm effects observed in this study. Additionally, while CV and MTT assays provided reliable quantitative measures of biofilm biomass and metabolic activity, respectively, the absence of microscopic evaluation (e.g., confocal microscopy or scanning electron microscopy) limits the mechanistic interpretation of our antibiofilm findings. Future studies incorporating such imaging techniques would provide valuable insights into biofilm architecture, matrix disruption, and morphological changes induced by *L. casei* CFS.

Furthermore, the SI values obtained in this study (ranging from 1.5 to 8.0) indicate only modest to moderate selectivity, particularly against resistant isolates where SI values approached 1.0. While SI > 1.0 indicates some degree of selectivity, substantially higher SI values (typically ≥ 10) are generally considered desirable for antifungal drug development. The narrow therapeutic window suggested by our SI data underscores that *L. casei* CFS and nystatin, particularly when used alone, may have limited clinical applicability as standalone therapies. However, the improved SI values observed with combination therapy (up to 8.0) are encouraging and warrant further investigation. Future studies should include comprehensive in vivo toxicity and pharmacokinetic evaluations to better assess the safety and therapeutic potential of these agents.

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