

Effects of probiotic *Saccharomyces cerevisiae* on *Candida parapsilosis* biofilm and antifungal efficacy in vitro and in a murine model in Türkiye

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Abstract

Introduction: This study investigated the effects of the probiotic *Saccharomyces cerevisiae* on *Candida parapsilosis* biofilm formation and antifungal susceptibility in vitro and evaluated its potential to enhance antifungal efficacy in a murine candidiasis model.

Methodology: *C. parapsilosis* ATCC 22019 and *S. cerevisiae* ATAFM-2021 were tested with fluconazole (FLU), caspofungin (CAS), and amphotericin B (AMB). Biofilm formation was quantified using the crystal violet assay under mono- and co-culture conditions with different inoculation timings. Antifungal susceptibility was assessed by microbroth dilution and checkerboard methods, and interactions were interpreted using the fractional inhibitory concentration index (FICI). In vivo candidiasis was induced in BALB/C mice. Antifungals were administered intraperitoneally for five days starting 24 hours after infection, while the probiotic was supplied in drinking water either 14 days before infection or 5 days after infection. Renal fungal burden was quantified as CFU/g.

Results: In vitro, *S. cerevisiae* reduced biofilm formation by 11–28%, with the strongest inhibition observed when applied before fungal inoculation. Minimum inhibitory concentration values of biofilm-embedded cells decreased, particularly for CAS and AMB. Synergistic interactions were consistently observed with CAS and AMB and were condition-dependent with FLU. In vivo, CAS demonstrated the highest efficacy. Probiotic pre-treatment enhanced the therapeutic effect of AMB but showed limited impact on CAS and FLU.

Conclusions: *S. cerevisiae* inhibited biofilm formation, reduced antifungal MIC values, and enhanced antifungal efficacy, particularly in combination with CAS and AMB. These findings support the potential role of probiotic adjunct strategies in the management of biofilm-associated candidiasis.

Key words: *Saccharomyces cerevisiae*; *Candida parapsilosis*; biofilm; synergy; antifungal; probiotic.

J Infect Dev Ctries 2026; 20(9):1382-1392. doi:10.3855/jidc.23048

(Received 12 February 2026 – Accepted 26 March 2026)

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Introduction

Candida species are the most frequently isolated fungal pathogens in intensive care units (ICUs) and other clinical settings, with *C. albicans* being the most commonly identified species. However, recent reports indicate an increasing prevalence of non-*albicans* *Candida* (NAC) species [1,2]. Among NAC species, *C. parapsilosis* is one of the most frequently encountered causative agents of invasive systemic mycoses worldwide [3], accounting for approximately 20% of *Candida* isolates from blood cultures in Asian countries and 30% in the Balkan countries [4]. Recent studies conducted in Turkey have reported *C. parapsilosis* prevalence rates ranging from 11.8% to 20% [5,6]. At our hospital, between 2017 and 2021, the isolation rate of *C. parapsilosis* was 12.7% in adult patients and 46.9% in pediatric patients [7].

Candidemia, a critical component of systemic mycoses, is predominantly observed in patients admitted to the ICU, although its incidence varies across clinical settings [8]. *C. parapsilosis*-related candidemia is particularly significant in pediatric patients and neonatal ICUs [9,10]. Due to its strong

biofilm-forming capacity, which facilitates adherence to intravascular catheters and other medical devices, it is frequently encountered in patients undergoing invasive procedures [2].

Candidemia caused by *C. parapsilosis* prolongs hospital stays, significantly increases healthcare costs, and contributes to rising mortality rates. The increasing resistance to antifungal agents used to treat candidemia has driven research toward alternative therapeutic approaches [11-13]. In recent years, probiotics have gained attention as a potential alternative treatment strategy for *Candida* infections. Several studies have demonstrated the inhibitory effects of probiotics on pathogenic microorganisms [14,15].

Probiotics, as defined by the World Health Organization (WHO), are live microorganisms that confer a health benefit on the host [16]. While probiotics primarily consist of bacteria, they also include yeasts that naturally occur in fermented foods, can be added to other food products, and are available as dietary supplements. The seven major genera commonly used in probiotic formulations include *Lactobacillus*, *Bifidobacterium*, *Streptococcus*,

Enterococcus, *Escherichia*, and *Bacillus* [16]. The Turkish Probiotic Guide also recommends the *S. cerevisiae* strain for various conditions and it is commercially available [17]. Probiotics exhibit health benefits that may be non-specific, species-specific, and/or strain-specific [16]. These effects include inhibiting the proliferation of pathogenic microorganisms in the gastrointestinal tract by promoting colonization resistance, improving gut transit, or restoring a disrupted microbiota, producing bioactive metabolites (e.g., short-chain fatty acids), and reducing luminal content [18].

The treatment of invasive *Candida* infections relies on a limited number of antifungal classes, including polyenes, echinocandins, and azoles. In recent years, the development of resistance to these agents has posed a significant threat to public health. Factors such as prolonged antibiotic use, extended hospital stays, and host-related conditions can disrupt the microbiota and lead to *Candida* overgrowth [19].

Several studies have demonstrated the potential of probiotics in managing gastrointestinal disorders caused by *Escherichia coli* (traveler's diarrhea), *Clostridium difficile*, and *Helicobacter pylori* infections. In addition, research has suggested that probiotic yeasts may mitigate complications associated with *Candida* infections [20-22]. However, data on the interactions between probiotic yeasts and *Candida* species in vivo remains limited.

Kunyeit et al. [23] investigated the therapeutic potential of *S. cerevisiae* against *C. albicans* and NAC species, reporting a 34% reduction in *C. parapsilosis* adhesion. The authors concluded that food-derived yeasts act as effective probiotics against pathogenic *Candida* species by inhibiting adhesion, reducing biofilm metabolic activity, and consequently controlling colonization [23].

There remains a pressing need for further research into the effects of probiotic yeasts, particularly in biofilm-associated invasive *Candida* infections. Existing studies suggest that probiotic metabolites play a crucial role in human health and disease, yet their precise origins and clinical implications in *Candida*-related infections remain incompletely understood.

This study aimed to investigate the effects of *S. cerevisiae*, a probiotic yeast, on *C. parapsilosis* candidemia using an in vivo experimental mouse model, as well as its impact on *C. parapsilosis* biofilm formation and antifungal susceptibility through in vitro assays.

Methodology

Ethical Approval

This study was reviewed for its rationale, objectives, approach, and methodology in accordance with the Kocaeli University Animal Experiments Local Ethics Committee Directive and was approved for ethical compliance with decision number KOU HADYEK 5/2-2022 on October 26, 2022.

Microorganisms

Standard *C. parapsilosis* ATCC 22019 strain from the American Type Culture Collection (ATCC) and *S. cerevisiae* ATAFM-2021 as a probiotic strain were used. This strain of *C. parapsilosis* was sourced from the Culture Collection of Kocaeli University, while *S. cerevisiae* was obtained from Sakarya Training and Research Hospital, Türkiye.

Antifungal Susceptibility Testing

C. parapsilosis (ATCC 22019) was used as the quality control strain. Antifungal susceptibility test was performed with amphotericin B (AMB), fluconazole (FLU), and caspofungin (CAS) using 96-well U-bottom microplates (Lp Italiana, Italy) following the CLSI M27-A3 microdilution method guidelines [24]. Microplates were incubated at 37 °C. Minimum inhibitory concentration (MIC) values were evaluated after 48 h for AMB and FLU and after 24 h for CAS. For FLU and CAS MIC endpoints were determined visually as the lowest concentration that inhibited visible growth, and for AMB, MIC was defined as the well where 100% inhibition was observed according to CLSI criteria. MIC for planktonic cells were determined, with MIC₅₀ defined as the concentration reducing growth by 50% compared to the control well [24]. Testing was performed in three independent replicates.

Strain Purification and Enrichment

After thawing from -80°C storage, *C. parapsilosis* and *S. cerevisiae* strains were subcultured onto Sabouraud dextrose agar (SDA) for purification. To promote robust biofilm formation, *C. parapsilosis* colonies were subsequently transferred into yeast peptone broth (YPB) for enrichment.

C. parapsilosis colonies were transferred to 20 mL of yeast peptone dextrose (YPD) medium in glass flasks and incubated at 37°C with orbital shaking at 175 rpm for 24 hours. After incubation, cultures were transferred to 50 mL Falcon tubes and centrifuged (Eppendorf, Germany) at 3000 rpm for 5 minutes at 4°C. The supernatant was discarded, and the pellet was

resuspended in 20 mL of phosphate-buffered saline (PBS), vortexed (Ika, Germany), and washed as described by Pierce *et al.* [25].

Biofilm Formation

The biofilm formation experiment was structured and modified from the work of Kunyeyt *et al.* (2019) as follows:

- Control 1: Biofilm formation of *C. parapsilosis* only.
- Control 2: Biofilm formation of *S. cerevisiae* only.
- Group 1 (Simultaneous): *C. parapsilosis* and *S. cerevisiae* were co-inoculated and incubated together.
- Group 2 (*C. parapsilosis* First): *C. parapsilosis* was incubated for 60 minutes before adding *S. cerevisiae*.
- Group 3 (*S. cerevisiae* First): *S. cerevisiae* was incubated for 60 minutes before adding *C. parapsilosis*.

Inoculum suspensions were prepared in Roswell Park Memorial Institute Medium (RPMI) medium at 1×10^6 CFU/mL for *C. parapsilosis* and 1×10^8 CFU/mL for *S. cerevisiae* [26]. Biofilms were formed using 96-well flat-bottom ELISA plates (Corning, USA). Each well was inoculated with 100 μ L of the prepared suspensions and incubated at 37°C with orbital shaking at 175 rpm for 24 hours. Before quantitative analysis, wells were washed three times with 200 μ L of sterile PBS using a multichannel pipette, then blotted dry on paper towels to remove residual liquid.

Biofilm quantification was performed using the crystal violet (CV) staining method. Wells were stained with 100 μ L of 1% CV solution [27] and incubated at room temperature for 15 minutes. Excess dye was removed by washing wells three times with 200 μ L of distilled water, followed by drying. To solubilize the retained dye, 100 μ L of 95% ethanol was added to each well and incubated for 15 minutes at room temperature. Optical density (OD) was measured at 590 nm using an ELISA plate reader (Corning, ABD). Each experiment was conducted in triplicate, and the mean OD values were used for statistical analysis. OD values were normalized against negative control wells prior to statistical analysis.

The checkerboard method

The effect of *S. cerevisiae* on the antifungal susceptibility of *C. parapsilosis* biofilms to AMB, FLU, and CAS was evaluated using the checkerboard microdilution method. The fractional inhibitory

concentration index (FICI) was calculated as the ratio of MIC in the combination of antifungal plus probiotic over the MIC of antifungal alone:

$$FICI = (MIC \text{ of drug A in combination} / MIC \text{ of drug A alone}) + (MIC \text{ of drug B in combination} / MIC \text{ of drug B alone}).$$

Interpretation: FICI ≤ 0.5 , Synergistic; FICI = 0.5 – 1, Additive; FICI = 1 – 4, Indifferent (No effect); FICI > 4 , Antagonistic [28].

Biofilm Inhibition Calculation

Biofilm inhibition was calculated using the following formula [29]:

$$(1 - A/B) \times 100, \text{ where } A = OD_{590 \text{ nm of the test well}} \text{ and } B = OD_{590 \text{ nm of the positive control well}}.$$

In vivo methods

This study involved 72 male BALB/C mice, aged 6 to 8 weeks, weighing between 22 and 32 grams. The mice were sourced from the production laboratory of the Kocaeli University Animal Research and Application Unit. Throughout the experiment, the mice were housed in automatically regulated rooms with a 12-hour light/dark cycle, temperature ($22 \pm 2^\circ\text{C}$), and humidity (45-50%). They were kept in transparent polycarbonate cages and provided with food and clean water *ad libitum*. No food or water restrictions were applied to any of the mice during the experiment.

No special adaptation procedures were implemented for the laboratory environment where the studies on experimental animals were conducted. Cages were set up with groups of two randomly selected mice, and the planned treatments were given once daily. Mice exhibiting hair loss, fever, movement disorders, or poor physical condition were excluded from the study.

Animals were randomly allocated to the experimental groups before infection and treatment procedures. The experimental groups and treatment protocols were modified from the source by Leao *et al.* [30], and the experimental groups are defined in Table 1. In the candidiasis infection model induced by *C. parapsilosis*, each mouse was administered 0.2 mL of a suspension containing 10^8 CFU/mL of *C. parapsilosis* via the tail vein under brief ether anesthesia [31]. The mice were monitored daily for their activity, hair loss, and physical condition.

The treatment for the *Candida* infection model induced by *C. parapsilosis* commenced 24 hours post-infection. In the groups receiving treatment, the antifungal agents were administered intraperitoneally once daily for 5 days at the following doses: CAS (1 mg/kg), AMB (1 mg/kg), and FLU (5 mg/kg), each

Table 1. In vivo Experimental Groups and Treatment Protocols.

Group Code	Group Name	FLU	CAS	AMB	Probiotic	Infection
K	Control Group (Non-Infected, No Treatment)	-	-	-	-	-
F	Control Group (Infected, No Treatment)	-	-	-	-	Yes
KP	Probiotic-Only Control (Non-Infected)	-	-	-	Yes	-
FP	Probiotic-Only (Infected, No Treatment)	-	-	-	Yes	Yes
FP-CAS	Caspofungin + Probiotic (Post-Infection)	-	Yes	-	Yes	Yes
FP-AMB	Amphotericin-B + Probiotic (Post-Infection)	-	-	Yes	Yes	Yes
FP-FLU	Fluconazole + Probiotic (Post-Infection)	Yes	-	-	Yes	Yes
FPP	Probiotic (14 Days Pre + 5 Days Post)	-	-	-	Yes	Yes
KPP	Probiotic-Only (19 Days, Non-Infected)	-	-	-	Yes	-
FPP-CAS	Probiotic + Caspofungin (14 Days Pre + Post)	-	Yes	-	Yes	Yes
FPP-AMB	Probiotic + Amphotericin-B (14 Days Pre + Post)	-	-	Yes	Yes	Yes
FPP-FLU	Probiotic + Fluconazole (14 Days Pre + Post)	Yes	-	-	Yes	Yes

FLU: fluconazole; CAS: caspofungin; AMB: amphotericin B. K: non-infected untreated control; F: infected untreated control; KP: probiotic-only control (non-infected); FP: infected + probiotic (post-infection); FPP: probiotic administered 14 days before infection and continued 5 days post-infection; FP-CAS/AMB/FLU: infected + probiotic (post-infection) + antifungal; FPP-CAS/AMB/FLU: infected + probiotic (pre- and post-infection) + antifungal; KPP: probiotic-only (19 days: non-infected).

administered in a volume of 0.2 mL. In the control groups that did not receive antifungal treatment, 0.2 mL of sterile water was administered intraperitoneally.

For probiotic applications, a suspension containing 10^8 CFU/mL was prepared from a fresh culture of *S. cerevisiae*. This suspension was added to the drinking water 24 hours post-infection [30]. The drinking water was not changed during the probiotic application period, and the remaining drinking water in the cage was measured in milliliters at the end of the study. At the end of the 5-day antifungal treatments, the mice were sacrificed after intraperitoneal administration of ketamine hydrochloride (0.1 mL/100 g) (Richterpharma, Austria) and xylazine hydrochloride (0.1 mL/100 g) (Richterpharma, Austria). After sacrifice, both kidneys were collected and individually weighed before homogenization. From each homogenized sample, 100 µL was inoculated onto SDA plates and incubated at 37°C for 72–96 hours before colony counting. Post-incubation, the colonies were examined using Gram staining. For microscopic examination, isolates were inoculated onto Cornmeal-Tween 80 agar using the Dalmau plate technique and

incubated at 25°C for 72 hours before evaluation under light microscopy. The following methods were used to identify *C. parapsilosis* from yeast colonies: specific microscopic morphology on Cornmeal-Tween 80 Agar and the VITEK 2 YST ID Card (bioMérieux, France).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 14.0). Continuous variables are expressed as mean ± standard deviation. Differences between groups were evaluated using the Student's t-test. A $p < 0.05$ was considered statistically significant.

Results

The MIC values for *C. parapsilosis* and *S. cerevisiae* strains indicated susceptibility against AMB, FLU, and CAS according to CLSI reference guidelines. The MIC values for *C. parapsilosis* and *S. cerevisiae* strains against tested antifungals are presented in Table 2.

During biofilm quantification, in the first phase of the experiment, *C. parapsilosis* biofilm was formed, followed by the investigation of biofilms co-cultured with *S. cerevisiae*. The absorbance value of the biofilm formed solely by *C. parapsilosis* was measured at 2.80, whereas the absorbance value of the biofilm formed by *S. cerevisiae* alone was 0.29. The negative control absorbance was recorded as 0.12. The absorbance values of the biofilm formed in Group 1 and Group 2

Table 2. MIC values of antifungal agents against the tested strains.

Antifungal	<i>C. parapsilosis</i> MIC (µg/mL)	<i>S. cerevisiae</i> MIC (µg/mL)
Amphotericin B	0.25	0.06
Fluconazole	1	0.125
Caspofungin	1	0.003

Table 3. The results of 50% SMICs, *S. cerevisiae*, and antifungal agents in *C. parapsilosis* Biofilm.

	SMIC ₅₀ values (µg/mL)		
	Amphotericin-B	Fluconazole	Caspofungin
<i>C. parapsilosis</i> (Control)	2	2	0.030
Group 1	1	0.5	0.015
Group 2	0.5	0.5	0.015
Group 3	0.5	0.125	0.015

SMIC₅₀, sessile minimum inhibitory concentration resulting in 50% biofilm reduction compared with the positive control well. Group 1: *S. cerevisiae* and *C. parapsilosis* were co-inoculated simultaneously. Group 2: *C. parapsilosis* biofilm was established prior to the addition of *S. cerevisiae*. Group 3: *S. cerevisiae* was pre-incubated before introduction of *C. parapsilosis*.

were measured as 2.50 and 2.39, respectively, and these values were not significantly different from the biofilm absorbance value of *C. parapsilosis* alone ($p = 0.700$ and $p = 0.331$, respectively; $p > 0.05$). However, in Group 3, the biofilm absorbance was measured as 2.03, which was significantly reduced ($p = 0.0173$, $p < 0.05$) (Figure 1).

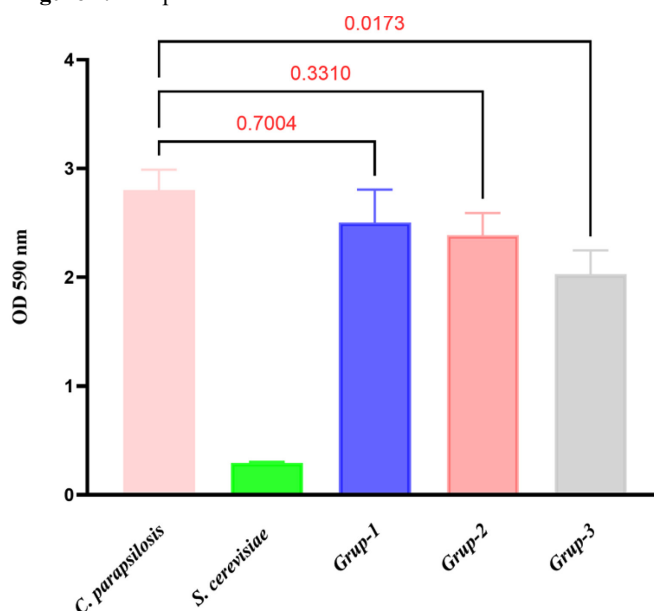
The lowest concentration that resulted in a 50% reduction in biofilm inhibition, compared to the positive control well (containing medium and *C. parapsilosis*), was determined as the sessile MIC (SMIC) value. The biofilm-containing well without antifungal agents was designated as the positive control (OD = 2.80), whereas the biofilm-free well was designated as the negative control (OD = 0.04). After *C. parapsilosis* biofilm formation in the microplates, antifungal susceptibility testing and SMIC calculations were performed following standard procedures [25]. The results of these experiments, which evaluated antifungal susceptibility in the presence of probiotics, are presented in Table 3.

For AMB, the SMIC₅₀ value for *C. parapsilosis* alone was determined as 2 µg/mL, while in Group 1, the SMIC₅₀ was 1 µg/mL, and in Groups 2 and 3, it was reduced to 0.5 µg/mL. For FLU, the SMIC₅₀ value for *C. parapsilosis* alone was 2 µg/mL, whereas in Group 1, it was 0.5 µg/mL, and in Groups 2 and 3, it was further reduced to 0.125 µg/mL. For CAS, the SMIC₅₀ value for *C. parapsilosis* alone was determined as 0.03 µg/mL, while in all groups, the SMIC₅₀ was reduced to 0.015 µg/mL (Table 3).

After calculating the biofilm inhibition rates, an increase in inhibition was observed in all groups with increasing AMB concentrations. While *C. parapsilosis* exhibited a 50% biofilm inhibition rate at an AMB concentration of 2 µg/mL, the inhibition rates at the same concentration were determined as 55%, 58%, and 54.5% in Group 1, Group 2, and Group 3, respectively. The anti-biofilm effects of different FLU concentrations during different stages of probiotic

exposure on *C. parapsilosis* biofilm were evaluated. An increase in inhibition was observed in all groups with increasing FLU concentrations. While *C. parapsilosis* exhibited a 50% biofilm inhibition rate at a FLU concentration of 2 µg/mL, the inhibition rates at the same concentration were determined as 59.2%, 59.1% and 61.7% in Group 1, Group 2, and Group 3, respectively. Similarly, the anti-biofilm effects of different CAS concentrations were evaluated, and an increase in inhibition was observed in all groups with

Figure 1. Comparison of Biofilm Absorbance Values.



Biofilm biomass was quantified by crystal violet staining and measured at OD590 nm under mono- and co-culture conditions. *C. parapsilosis* alone served as the biofilm control, and *S. cerevisiae* alone was included as a probiotic control. In Group 1, both microorganisms were co-inoculated simultaneously; in Group 2, *C. parapsilosis* was allowed to adhere for 60 min before addition of *S. cerevisiae*; in Group 3, *S. cerevisiae* was pre-incubated for 60 min prior to the introduction of *C. parapsilosis*. Data represent mean $\pm$ SD of three independent experiments performed in triplicate. A significant reduction in biofilm biomass was observed when *S. cerevisiae* was pre-applied (Group 3) compared to *C. parapsilosis* alone ($p < 0.05$).

Table 4. Interaction Types Observed in *C. parapsilosis* Biofilm Against Antifungal and Probiotic Combinations.

Antifungal	Group	MIC	FICI	Interaction Type
Fluconazole	<i>C. parapsilosis</i> (Control)	4	-	-
	Group 1	4	1	No Difference
	Group 2	2	0.5	Synergistic
	Group 3	2	0.5	Synergistic
Amphotericin-B	<i>C. parapsilosis</i> (Control)	4	-	-
	Group 1	2	0.5	Synergistic
	Group 2	2	0.5	Synergistic
	Group 3	2	0.5	Synergistic
Caspofungin	<i>C. parapsilosis</i> (Control)	4	-	-
	Group 1	2	0.5	Synergistic
	Group 2	1	0.25	Synergistic
	Group 3	1	0.25	Synergistic

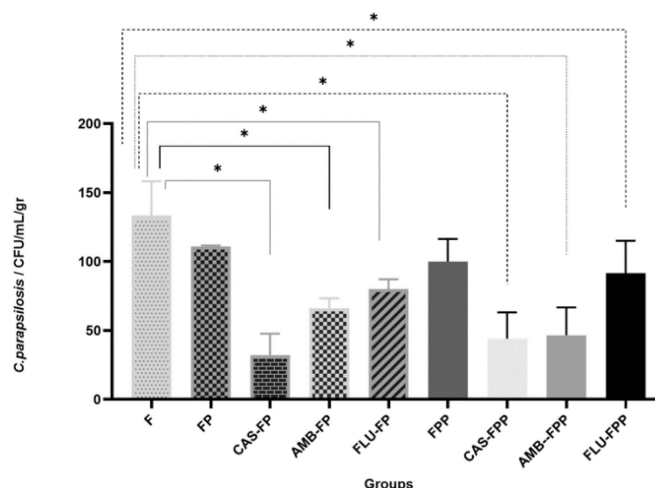
Group 1: simultaneous co-inoculation of *S. cerevisiae* and *C. parapsilosis*; Group 2: *C. parapsilosis* biofilm established prior to the addition of *S. cerevisiae*; Group 3: *S. cerevisiae* pre-incubated before introduction of *C. parapsilosis*.

increasing CAS concentrations. While *C. parapsilosis* displayed a 50% biofilm inhibition rate at a CAS concentration of 0.03 µg/mL, the inhibition rates at the same concentration were found to be 62.5%, 61% and 72.7% in Group 1, Group 2, and Group 3, respectively.

The interaction types observed in *C. parapsilosis* biofilms in response to the combination of *S. cerevisiae* and antifungals are presented in Table 4. Following FIC_i calculations, the interaction type for AMB and CAS combinations was "synergistic" in all groups. In contrast, for FLU, the interaction type was "no difference" in Group 1, while it was classified as "synergistic" in Groups 2 and 3. The probiotic application was carried out in two different time frames: one group received probiotics for 14 days before infection and continued for an additional 5 days post-infection. In the uninfected control group (K), which did not receive probiotics or antifungals, no yeast colonies were isolated. Similarly, in the groups that received only probiotics (P and PP), *C. parapsilosis* was not detected. Following infection, the control group (F), which did not receive probiotics or antifungals, had an average count of *C. parapsilosis* at 133.3 CFU/mL/g. In the group that received only probiotic treatment post-infection (FP), the average *C. parapsilosis* count was 110.8 CFU/mL/g, which did not show a statistically significant difference compared with the F group ($p > 0.05$). In the group that received CAS together with probiotics (CAS-FP), and similarly in AMB-FP and FLU-FP the colony count was reduced to 32 CFU/mL/g, 66 CFU/mL/g, and 80 CFU/mL/g, respectively. When comparing the mean *C. parapsilosis* counts in the CAS-FP, AMB-FP, and FLU-FP groups to the F group, the differences were statistically significant (Table 5).

In the group that received probiotics 14 days before infection and continued with probiotic and antifungal treatment post-infection, the mean *C. parapsilosis* count in the experiment group treated with only probiotics (FPP) was 115 CFU/mL/g. These values for cultures treated with CAS (CAS-FPP), AMB (AMB-FPP) and FLU (FLU-FPP), the mean colony count was 48.5 CFU/mL/g, 46.5 CFU/mL/g and 91.5 CFU/mL/g,

Figure 2. Average *C. parapsilosis* Count in In-Vivo Experimental Groups (CFU/mL/g).



Mean *C. parapsilosis* colony counts recovered from kidney tissues of BALB/C mice after experimental candidiasis and treatment. Group codes are as follows: K, non-infected, no treatment; F, infected, no treatment; KP, probiotic-only control (non-infected); FP, infected + probiotic only (post-infection); CAS-FP, infected + caspofungin + probiotic (post-infection); AMB-FP, infected + amphotericin B + probiotic (post-infection); FLU-FP, infected + fluconazole + probiotic (post-infection); FPP, infected + probiotic (14 days pre-infection + 5 days post-infection); KPP, probiotic-only control (19 days, non-infected); CAS-FPP, infected + probiotic (14 days pre-infection + 5 days post-infection) + caspofungin; AMB-FPP, same regimen + amphotericin B; FLU-FPP, same regimen + fluconazole. Bars represent mean $\pm$ SD; fungal burden is expressed as CFU/mL/g of kidney tissue.

Table 5. *C. parapsilosis* Colony Counts in In-Vivo Experimental Groups.

Main Group	Groups	Minimum	Maximum	Mean $\pm$ Standard Deviation
Control	K	0	0	0
	F	100	160	133.3 $\pm$ 24.94
Post-Infection Probiotic + Antifungal Treatment Groups	P	0	0	0
	FP	110	112	110.8 $\pm$ 0.96
	FP-CAS	12	50	32.0 $\pm$ 15.58
	FP-AMB	58	74	66.0 $\pm$ 7.30
	FP-FLU	74	90	80.0 $\pm$ 7.12
Probiotic 14 days Pre-Infection + Antifungal Treatment Groups	PP	0	0	0
	FPP	80	120	115.0 $\pm$ 16.33
	FPP-CAS	30	72	48.5 $\pm$ 19.11
	FPP-AMB	30	76	46.5 $\pm$ 20.22
	FPP-FLU	60	116	91.5 $\pm$ 23.57

FLU: fluconazole; CAS: caspofungin; AMB: amphotericin B. K: non-infected untreated control; F: infected untreated control; KP: probiotic-only control (non-infected); FP: infected + probiotic (post-infection); FPP: probiotic administered 14 days before infection and continued 5 days post-infection; FP-CAS/AMB/FLU: infected + probiotic (post-infection) + antifungal; FPP-CAS/AMB/FLU: infected + probiotic (pre- and post-infection) + antifungal; KPP: probiotic-only (19 days, non-infected). Statistical comparisons between experimental groups were performed using Student's t-test. The following p -values were obtained: F vs FP ($p = 0.159$), F vs FP-CAS ($p = 0.001$), F vs FP-AMB ($p = 0.022$), F vs FP-FLU ($p = 0.045$), F vs FPP ($p = 0.204$), F vs FPP-CAS ($p = 0.011$), F vs FPP-AMB ($p = 0.013$), F vs FPP-FLU ($p = 0.025$), FP vs FPP ($p = 0.299$), CAS-FP vs CAS-FPP ($p = 0.217$), AMB-FP vs AMB-FPP ($p = 0.181$), and FLU-FP vs FLU-FPP ($p = 0.494$). Differences with $p < 0.05$ were considered statistically significant.

respectively. When compared to the F group, no statistically significant difference was observed in the FPP group. However, the differences in the CAS-FPP, AMB-FPP, and FLU-FPP groups were significant. These findings show that CAS was the most effective antifungal agent against infection (Table 5, Figure 2). In the analysis, significant differences in mean *C. parapsilosis* colony counts were observed between several groups. Specifically, when compared to the F group (mean = 133.3 ± 24.94), the in CAS-FP group showed a statistically significant reduction (mean = 32 ± 15.58) ($p = 0.001$). Similarly, F (mean = 133.3 ± 24.94) compared to AMB-FP (mean = 66 ± 7.30) showed a significant difference ($p = 0.022$), as did the comparison with FLU-FP (mean = 80 ± 7.12) ($p = 0.045$). In addition, when compared to the F group (mean = 133.3 ± 24.94), significant differences were found for the CAS-FPP (mean = 48.5 ± 19.11) ($p = 0.011$), AMB-FPP (mean = 46.5 ± 20.22) ($p = 0.013$), and FLU-FPP (mean = 91.5 ± 23.57) ($p = 0.025$) groups.

Discussion

The limited availability of clinically effective antifungal drugs, coupled with increasing resistance and high mortality rates, poses significant challenges in the treatment of invasive fungal infections. Recent studies have highlighted a growing trend of antifungal resistance in *Candida parapsilosis* isolates, including the emergence of resistant strains capable of displacing susceptible populations, potentially triggering nosocomial outbreaks [4,32-34].

Globally, the rise in antifungal resistance has undermined the efficacy of conventional therapies, resulting in recurrent infections and posing a serious public health threat. This highlights an urgent need for novel therapeutic strategies, including the development of new antifungal agents. However, the research and clinical validation of such agents are time-intensive processes. Therefore, alternative approaches, such as adjunct therapies, are gaining traction. Notably, resistance rates to frontline antifungal agents such as amphotericin B (1.3%), voriconazole (4.7%), and fluconazole (15.2%) in *C. parapsilosis* further emphasize this need, as suggested by Yamin et al. [35].

In response to these challenges, several innovative strategies have been proposed, including phage therapy, antimicrobial peptides, antisense antimicrobials, immunotherapy (monoclonal antibodies), gene editing, phytotherapy, photodynamic therapy, nanotechnology, and synthetic biology [36]. Alongside these innovative approaches, microbiome-based treatment strategies

have also emerged as a significant research area. Among these microbiome-based treatment strategies, the investigation of the effects of probiotic and antifungal treatment combinations is gaining increasing importance. Probiotics, when used in conjunction with antifungal drugs, may offer a potential solution for treating infections and preventing resistance.

Food-derived probiotic yeast applications have been shown to reduce the virulence of *C. albicans* and NAC species and yield positive outcomes against infection formation [26]. The potential synergistic effect of probiotics and antifungal agents may prevent the emergence of drug resistance and can be used in combination therapy with antifungal drugs. Previous studies have suggested that the use of probiotics in conjunction with antimicrobial agents can exhibit synergistic effects and that some probiotic species can even act as natural antibiotics [37,38].

In a study on the use of probiotic yeasts in treatment, it was reported that metabolites produced by *S. cerevisiae* could prevent methicillin-resistant *S. aureus* (MRSA) infections [39]. Oral administration of *S. boulardii* to infants has been reported to reduce fungal colonization and invasive fungal infections [40]. Another study focusing on the effects of probiotics against *Candida* infections in adolescents showed that a probiotic cocktail containing *Lactobacillus* spp. and *Saccharomyces* spp. led to a reduction in *C. albicans* colonization [41]. Studies on interactions between probiotic yeast and *Candida* infections have shown that *S. cerevisiae* inhibits *Candida* adhesion to epithelial cells and forms a barrier on biotic surfaces [42]. Another study reported that in patients exposed to prolonged, continuous antibiotic use, yeast probiotics were more suitable and effective therapeutic agents than bacterial probiotics for preventing and treating all diseases associated with diarrhea resulting from fluctuations in the normal gastrointestinal microbiota [43].

Nevertheless, in our study, a 28% reduction in biofilm quantity was observed when *C. parapsilosis* was inoculated after in vitro probiotic inoculation. Consistent with our results, another study reported a 34% reduction in *C. parapsilosis* adhesion when probiotic yeasts were incubated with *Candida* strains [44]. Similarly, it was reported that the probiotic *S. cerevisiae* (KTP) strain inhibited *C. parapsilosis* biofilm formation by more than 50% during the early biofilm formation stage [26]. Additionally, another study demonstrated that *Lactobacillus* spp. probiotics reduced the pathogenicity of *Candida* species and inhibited biofilm formation. This inhibitory effect

varied depending on the type of probiotic [45]. Given this data, it is evident that *S. cerevisiae* exhibits competition and interference in *C. parapsilosis* biofilm formation. This further suggests that *S. cerevisiae* may have an inhibitory effect on *C. parapsilosis* biofilm-associated infections.

The demonstrated antifungal efficacy of probiotics alone suggests the potential for a synergistic effect when used in combination with antifungals. However, a review of the literature revealed no studies on the combined use of probiotics with antifungal drugs. In our study, we examined the change in susceptibility to antifungal agents in biofilms formed by *C. parapsilosis* and *S. cerevisiae*. In vitro experiments showed that the application of probiotics before and after biofilm formation resulted in a 4-fold decrease in the SMIC50 values of *C. parapsilosis* biofilm for AMB, a 16-fold decrease for FLU, and a 2-fold decrease for CAS. Antifungals tested in combination with probiotics showed a higher biofilm inhibition rate at concentrations where *C. parapsilosis* exhibited SMIC. This finding suggests that administering *S. cerevisiae* alongside antifungals enhances the inhibition of *C. parapsilosis* biofilm formation and integrity.

In the in-vivo experiments, assessed by colony counts of *C. parapsilosis* from the kidneys, the observed antifungal efficacy in the groups where probiotics were administered 14 days before infection (FPP) and in the groups where probiotics were administered in conjunction with antifungal treatments post-infection (FP) was ranked from highest to lowest as CAS, AMB, and FLU.

When probiotics were administered with CAS post-infection (FP-CAS), a significant reduction was observed in the colony counts of *C. parapsilosis* isolated from the kidneys. Similarly, with FLU, lower *C. parapsilosis* colony counts were detected in the group receiving probiotics in conjunction with antifungal treatment post-infection compared to the group receiving probiotics before infection. When treated with AMB, lower *C. parapsilosis* colony counts were found when probiotics were administered before infection and in conjunction with antifungal treatment post-infection (FPP-AMB). These findings suggest that the timing of probiotic administration may vary depending on the class of antifungal agent used. These differences may be related to the diversity of the mechanisms of action of antifungal drugs.

When comparing in vitro and in vivo results, a synergistic effect with probiotics was observed in all experimental groups with AMB and CAS, but not for FLU, based on FIC_i result classification. The timing

factor was influenced the extent of the observed synergistic effect. In the in vitro experiments, probiotics were applied either 60 minutes before or 60 minutes after *C. parapsilosis* biofilm formation, while in the in vivo experiments, these times were adjusted to 14 days before infection and 24 hours after infection. In both experimental conditions, and consistent with the in vitro synergistic effect results, the in vivo experiments showed a greater reduction in *C. parapsilosis* colony counts in the CAS-treated groups that received probiotics post-infection. The application of probiotics with FLU did not show a difference among the groups treated simultaneously in the in vitro experiments. In the in vivo experiments, treated with FLU, higher *C. parapsilosis* counts were detected in the FLU-treated groups that received probiotics post-infection (FP-FLU) compared to other antifungals. These results support the potential use of probiotics as adjunctive agents in antifungal therapy, depending on the class of antifungal.

In patients who have been in ICU for an extended period or those with hematologic malignancies who develop neutropenia, the clinical presentation of candidiasis often resembles bacterial sepsis due to fever and other sepsis-associated clinical findings. When antibacterial treatment is prolonged and unresponsive, opportunistic fungal pathogens are considered. In such cases, antifungal agents are added to the treatment protocol. Particularly in patients with suspected invasive candidiasis, initiating probiotic therapy beforehand may mitigate the adverse effects of prolonged antibiotic use. Moreover, the prophylactic use of probiotics, especially in immunocompromised patients, may positively impact treatment by balancing the gut microbiota and helping to prevent opportunistic infections like *Candida*. Probiotics, with a wide range of effects, including immunomodulation, maintenance of epithelial stability, biofilm inhibition, facilitation of ion transport, and reduction of *intracellular* toxicity, may be a promising treatment option in the future. Although *S. cerevisiae* is widely used as a probiotic and is generally regarded as safe, rare cases of *Saccharomyces*-associated fungemia have been reported, particularly in severely immunocompromised patients. Therefore, while the present findings highlight the potential benefits of *S. cerevisiae* in antifungal strategies, its safety profile should be carefully considered in clinical applications, especially in high-risk patient populations.

In patients planned for antifungal treatment, the use of *S. cerevisiae* as a probiotic may reduce the severity of infection due to its inhibitory effect on *C.*

parapsilosis biofilm and enhance the efficacy of antifungal therapy through its synergistic effect. *S. cerevisiae* can be considered an option in microbiome-based treatment strategies, especially for *Candida* infections.

Conclusions

The increasing antifungal resistance of *C. parapsilosis* and the detection of high MIC values against first-line antifungals such as FLU, AMB, and CAS pose challenges in the treatment of *C. parapsilosis*. Therefore, alternative treatment methods need to be investigated. Given that the development of new antifungal agents often takes a long time, research and development of different treatment strategies are ongoing. In this context, microbiome-based treatment strategies are emerging. Microbiome-based therapies can enhance the efficacy of existing antimicrobials, prevent the development of resistance, and reduce the toxic effects of drugs, thereby extending the lifespan of the host organism. Probiotics, with their wide-ranging beneficial effects may be a promising treatment option in the future.

This study aimed to investigate the potential effects of the probiotic *S. cerevisiae* on *C. parapsilosis* biofilm formation and its efficacy with antifungal treatment. Our findings showed that the probiotic *S. cerevisiae* reduced *C. parapsilosis* biofilm formation and increased the antifungal susceptibility of *C. parapsilosis* when applied in conjunction with antifungal agents. Additionally, the efficacy of *S. cerevisiae* in antifungal treatment was evaluated in an experimental mouse model. The data obtained demonstrated that the use of the probiotic *S. cerevisiae* in the treatment of *C. parapsilosis* infections showed a synergistic effect with AMB and CAS, but not with FLU, compared to the use of antifungals alone. The potential use of *S. cerevisiae* as an adjunctive agent in the treatment of *C. parapsilosis* infections is also supported by in vivo experiments. Specifically, in mice infected with *C. parapsilosis*, the application of *S. cerevisiae* showed that CAS and AMB antifungals were more effective compared to FLU, suggesting that CAS and AMB may represent more effective treatment options for invasive candidiasis or candidemia caused by *C. parapsilosis*. In this context, the identification of *Candida* species in the clinical presentation of invasive candidiasis, especially in immunosuppressed patients, is important for guiding clinicians. The findings suggest that the application of the probiotic *S. cerevisiae* in conjunction with antifungals in the treatment of *C. parapsilosis* infections could be an alternative

therapeutic option. The results of this study provide a promising foundation for the development of new therapeutic strategies in clinical practice. Nevertheless, further experimental and well-designed clinical studies are necessary to clarify the role of probiotic–antifungal combinations before clinical implementation.

Acknowledgements

We would like to thank Prof. Dr. Mustafa Altındış from Department of Microbiology, Sakarya University, Sakarya, Türkiye for providing the probiotic strain. Many thanks to the Scientific Research Project Council of Kocaeli University (KOU-BAP).

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Conflict of interest

No conflict of interest is declared.

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