

In vitro inhibitory assessment of *Lactobacillus* spp. supernatant on the growth of *Candida glabrata* and *Cryptococcus neoformans* yeasts.

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Abstract

Antifungal resistance poses a growing challenge to public health, exacerbated by the rise in yeast infections and the limited availability of safe and effective therapeutic options. Given this scenario, strategies based on bioactive compounds, such as cell-free supernatants (CFS) from probiotics, represent promising alternatives to conventional antimicrobials, which are associated with toxicity and the induction of resistance. The objective of this study was to evaluate the effect of *Lactobacillus* spp. supernatants—referred to as cell-free supernatants (CFS) or metabotics—and sodium butyrate (NaBut) on the growth of *Candida glabrata* and *Cryptococcus neoformans*. Minimum inhibitory concentration (MIC) tests were performed according to the BrCAST/EUCAST protocol, using different culture media and dilutions of the tested compounds. The CFS from *L. reuteri* was the most effective, inhibiting the growth of *C. glabrata* and *C. neoformans* in all tested media. All CFS inhibited the growth of *C. glabrata* and *C. neoformans* in RPMI medium. NaBut, in Sabouraud medium, reduced the growth of *C. glabrata* and *C. neoformans* at concentrations ranging from 55 mM to 1.71 mM. The production of organic acids by *Lactobacillus* spp. was inferred, as the pH of the culture media decreased from approximately 6.1 to 4.0. The data indicate that CFS exert an inhibitory effect on pathogenic yeasts. These findings highlight them as potential adjunct strategies for antifungal therapies, enhancing safety and cost-effectiveness, even in the context of microbial resistance.

1. Introduction

1.1. Antimicrobial Resistance—A Global Health Problem

Defined as the ability of microorganisms to resist the effects of antimicrobials, antimicrobial resistance (AMR) has been accelerating increasingly due to the inappropriate use of drugs. The growing threat of AMR represents one of the greatest challenges to global public health in the 21st century, as it can be intrinsic or acquired through genetic mutations or horizontal gene transfer (HGT)(Giono-Cerezo et al. 2020). Various mechanisms, such as alteration of the site of action, efflux pumps, changes in membrane permeability, and enzyme production, contribute to this resistance(Davies and Davies 2010; Munita and Arias 2016; Bottery et al. 2021) .

Although AMR is a natural phenomenon, accelerated by ecological processes (e.g., amensalism) and selective pressure(Schluter 2001; Holmes et al. 2016), the indiscriminate use of antimicrobials in human and animal healthcare settings, along with anthropogenic practices such as livestock farming and agriculture, has driven the selection and spread of resistant microorganisms(Ramos et al. 2022) .

Infections caused by resistant (MR) or multidrug-resistant (MDR) microorganisms are a major concern, as they result in approximately 700,000 deaths annually worldwide. Even more troubling, estimates predict approximately 10 million deaths annually worldwide by 2050(IACG 2019) .

In this context, invasive fungal infections are a major concern, affecting hospitalized, immunosuppressed, and/or immunocompromised individuals(Hellstein and Marek 2019; Bahn et al.

2020; Bermas and Geddes-McAlister 2020). Pathogenic fungi cause severe infections in more than 300 million people worldwide, resulting in 1.6 million deaths annually (Bermas and Geddes-McAlister 2020) .

Growing concern over resistant fungal infections has led the World Health Organization (WHO) to publish guidelines on the major human pathogenic fungi, with a focus on MR and MDR strains. The guide divides pathogenic fungi into three groups: critical priority group (*Cryptococcus neoformans*, with a mortality rate ranging from 41% to 61%, mainly in patients with HIV/AIDS), high mortality group (*Nakaseomyces glabrata*—*Candida glabrata*—with a mortality rate of 20% to 50%), and medium priority (*Pichia kudriavzevii*—*Candida krusei*—with a mortality rate of 44% to 67% in adult patients) (WHO 2022) .

1.2. *Candida glabrata*

1.2.1 Biological Characteristics

C. glabrata is a haploid, non-hyphal-forming yeast. Taxonomically, *C. glabrata* was initially classified in the genus *Torulopsis* due to its lack of hyphae or pseudohyphae; however, in 1978 it was reclassified into the genus *Candida*, since the ability to form filaments was not a reliable distinguishing factor (Rodrigues et al. 2017). In 2022, *C. glabrata* underwent a further reclassification to *N. glabrata* based on phylogenetic studies showing that this yeast is more closely related to yeasts in the *Nakaseomyces* clade.

Nevertheless, the name *C. glabrata* remains in use, as it is more widely accepted in the literature, making it an artificial and poly/paraphyletic genus (Denning 2024) .

Considered the second most commonly isolated species in bloodstream infections among immunocompromised patients, *C. glabrata* is the most common species in terms of antifungal resistance. This is due, in part, to the plasticity that *C. glabrata* exhibits in its ability to resist antifungal treatments, particularly fluconazole, a drug used worldwide to treat infections caused by *Candida* spp. This yeast species exhibits variations in its fungal cell wall, as well as a set of virulence factors that ensure the success of the infection in humans (Timmermans et al. 2018). Studies have shown that *C. glabrata* accounts for 10% to 15% of VVC cases; the characteristic symptoms of this infection include vaginal inflammation (vaginitis), swelling, erythema, vulvar itching, abnormal discharge, vulvar burning, pain, and irritation (Martin Lopez 2015; Paladine and Desai 2018) .

1.2.2 Virulence Factors

Virulence factors represent fundamental components of *C. glabrata*'s pathogenicity. Among the main mechanisms involved are adhesion to host cells and abiotic surfaces, mediated by cell wall proteins of the Epa family (Epithelial adhesin-like proteins), which facilitate colonization of tissues and medical devices, such as catheters (Silva et al. 2011; Frías-De-León et al. 2021)

Biofilm formation is another critical factor, resulting in cellular communities enveloped by an extracellular matrix rich in glycoproteins, polysaccharides, and lipids, which confers increased resistance to antifungals and the host's immune mechanisms (Frías-De-León et al. 2021) .

In addition, *C. glabrata* secretes hydrolytic enzymes, such as proteases (aspartyl proteases or lapinsins) and phospholipases, which degrade cell membrane components and contribute to tissue invasion and evasion of phagocytes(Dabiri et al. 2018). Another key aspect of virulence is its phenotypic and genetic variability, which allows for rapid adaptation to different host microenvironments, such as variations in pH, temperature, and nutrient availability—characteristics associated with metabolic plasticity and multidrug resistance(Hassan et al. 2021) .

1.2.3 Antifungal Resistance and the Mechanism of Action of These Drugs

Fluconazole acts on the biosynthetic pathway of the fungus's lipid component (ergosterol) via the cytochrome P450 pathway. The drug inhibits the catalytic enzyme 14 α -sterol demethylase (which is cytochrome P-450-dependent), resulting in a plasma membrane with structural and functional defects, thereby inhibiting nutrient transport and signaling between yeast cells(Rodrigues et al. 2017; Nocua-Báez et al. 2020) .

Research indicates that *C. glabrata*'s resistance to fluconazole is associated with possible minor mutations in its genes, driven by continuous drug pressure, and is linked to various mechanisms, such as the induction of efflux pumps encoded by ABC or MDR transporter genes. These genes belong to the superfamily of major efflux transporters that lead to a decrease in drug concentration in the yeast cytoplasm. Furthermore, the drug has reduced affinity, making it unable to bind to the yeast membrane ((Rodrigues et al. 2017; Pristov and Ghannoum 2019; Pais et al. 2020)).

A study demonstrated that biofilm-forming *C. glabrata* yeasts still survive under high concentrations of fluconazole and that biofilm formation depends on modulation by the carbon source and pH available in the host organism(Alves et al. 2020) .

1.3 Cryptococcus neoformans

In the context of systemic fungal infections, *Cryptococcus neoformans* emerges as one of the most lethal pathogens. *C. neoformans* is an encapsulated, saprophytic yeast belonging to the family Cryptococcaceae(Kwon-Chung et al. 2014). It is considered an opportunistic fungus, causing infection primarily in immunocompromised patients; it has a mortality rate of 15% among patients with HIV/AIDS, resulting in approximately 223,000 infections and 181,100 deaths annually(Bermas and Geddes-McAlister 2020) .

Other epidemiological data indicate that, even with conventional antifungal treatments, mortality rates from cryptococcal meningitis range from 10% to 70%(Bahn et al. 2020), and approximately 1,000,000 new cases are diagnosed each year—figures considered alarming and concerning by government agencies(Paul et al. 2015). Infections caused by *C. neoformans* are among the most common fungal infections of the central nervous system, causing meningoencephalitis(Samarasinghe and Xu 2018) .

1.3.1 Virulence Factors

One of the main virulence factors of *C. neoformans* is its capsule, which is composed primarily of glucuronoxylomanan (GXM), glucuronoxylomanogalactan (GXMGal), and water. Its function is to provide protection against immune mechanisms by inhibiting phagocytosis by macrophages, dendritic cells, and neutrophils (Casadevall et al. 2019; Zaragoza 2019). The capsule is also important in clinical practice, as it is used as a cryptococcal antigen in diagnosis; it can be visualized using India ink or Chinese ink (Casadevall et al. 2019).

Another notable virulence factor is the formation of melanin, a dark pigment in the cell wall that acts as an antioxidant, protecting the fungus from the immune system by neutralizing oxygen and nitrogen free radicals produced by phagocytic cells and suppressing the inflammatory response (Zaragoza 2019). In addition to capsule and melanin formation, other attributes are crucial to the fungus's pathogenicity, such as heat tolerance at 37°C, biofilm formation, and the secretion of various enzymes—including phospholipases, proteinases, and urease—that characterize the fungus's interaction with the host's immune system and its response pathways (Alspaugh 2015; Kong et al. 2020). In addition, fluconazole-resistant *Cryptococcus* strains have been identified, primarily in less developed socioeconomic regions and due to the indiscriminate use of the drug (Paul et al. 2015; Peng et al. 2018).

1.3.2 Antifungal Resistance and the Mechanism of Action of These Drugs

The treatment of choice for cryptococcosis varies according to the severity of the infection. For individuals with HIV/AIDS, first-line treatment is amphotericin B and flucytosine, and second-line treatment is fluconazole. Amphotericin B binds to the fungal cell membrane, forming porin channels, resulting in cell rupture and cytoplasmic leakage. Amphotericin B requires hospitalization for administration; consequently, in resource-limited countries, other treatment methods are chosen. Furthermore, amphotericin B deoxycholate is considered nephrotoxic. Another formulation of amphotericin B is lipid-based and offers a more effective delivery profile while reducing toxicity; however, the use of this drug is limited due to its high cost (Lemke et al. 2005; Hamill 2013; Bermas and Geddes-McAlister 2020).

Flucytosine is a synthetic antifungal agent that, to prevent frequent fungal resistance, is typically used in combination therapy. Its mechanism of action occurs through two main pathways, interfering with protein synthesis and the fungus's genetic material. In the first pathway, flucytosine (5-fluorocytosine) is incorporated into fungal RNA in place of uridyl acid, disrupting normal protein synthesis. The second pathway involves the inhibition of the enzyme thymidylate synthase. This inhibition blocks thymidine synthesis, thereby halting the production of fungal DNA. This compound has side effects such as hepatotoxicity and gastrointestinal complications, among others (Bennett 1977; Viviani 1995; Vermes 2000).

C. neoformans' resistance to antimicrobials stems from factors related to the natural evolution of strains and the misuse of antifungals in medicine and agriculture. One of the resistance mechanisms is hybridization in *C. neoformans*; that is, hybridization facilitates genetic changes and the fungus's

adaptation to the drug. The mechanism of resistance to amphotericin B is a mutation in sterol $\Delta 8 - 7$ isomerase, an enzyme involved in ergosterol biosynthesis (Bermas and Geddes-McAlister 2020) .

The resistance mechanisms for flucytosine and fluconazole are not well understood, but it is believed that *C. neoformans* resistance to flucytosine is associated with mutations in enzymes and cytosines. Resistance to fluconazole is associated with mutations in ERG11, a gene that encodes the enzyme lanosterol 14 α -demethylase, the target of azole activity. However, the main concern regarding the use of antifungals is the similarity between fungal cells and human cells, which makes these drugs toxic to the human body (Bermas and Geddes-McAlister 2020) .

1.4. Probiotics and Cell-Free Supernatants (CFS)

In the context of the growing challenge of antimicrobial resistance, and considering the limitations of conventional therapeutic approaches—such as the toxicity of antifungals to the human body and the increasing incidence of resistant fungi (Lin 2009) —cell-free supernatants (CFS) are emerging as a promising therapeutic approach. These are preparations obtained from the culture medium of probiotic bacteria (live microorganisms), from which all cellular biomass has been removed, leaving behind a diverse set of secreted soluble metabolites. In the literature, this concept is also referred to by some authors as “metabiotics,” a term that collectively encompasses metabolites and structural components with biological activity capable of modulating the host’s physiological functions and exhibiting antimicrobial activity (Vinderola et al. 2022; Zakrzewska et al. 2022). However, as highlighted by the *International Scientific Association for Probiotics and Prebiotics* (ISAPP), the use of the term “metabiotic” still lacks consensus and a formally established definition that clearly distinguishes it from related concepts (Vinderola et al. 2024) .

This interest in CFS is part of a broader context of research on probiotics and their metabolites for the treatment of fungal infections, which has grown exponentially. When administered in adequate amounts, probiotics provide a y benefit to the host’s health (Buggio et al. 2019; De Gregorio et al. 2020) ; they are notable for their ability to inhibit or reduce the growth of certain pathogenic microorganisms, thereby strengthening the gut microbiota’s defense barrier. Furthermore, there is a direct and indirect relationship between probiotics and CFS, which act as modulators of the immune system (Mundula et al. 2019; Ceccarani et al. 2019; Hu et al. 2019) .

At the same time, the term “postbiotic” is also used in the literature. According to the definition established by ISAPP, postbiotics refer to “a preparation of non-viable microorganisms and/or their components that confers a health benefit to the host” (Favero et al. 2022) .

It is essential to note, however, that CFS do not strictly fit this definition. This is because CFS consist primarily of secreted soluble metabolites, lacking inactivated cellular biomass or intentionally added cellular structural components. ISAPP established this distinction to avoid confusion in scientific reports; that is, if a purified metabolite (such as a vitamin or a fatty acid) were classified as a postbiotic, this would create an inconsistency, in which an identical molecule, chemically synthesized in a

laboratory, could not be designated as such, despite having exactly the same functional effect. Furthermore, the term “biotic” implies something that “came from life” (*after-life*), and pure chemical molecules do not possess these structural characteristics derived from a microorganism(Vinderola et al. 2024) .

CFS offer significant advantages over probiotics, particularly in terms of their safety profile. Because they consist of metabolites, they eliminate the risk of replication and, consequently, of causing bacteremia or opportunistic infections, especially in immunocompromised individuals. Furthermore, there is no possibility of acquiring or transferring antibiotic resistance genes to the gut microbiota(Kumar et al. 2024) .

CFS also possess superior inherent stability, since they do not require colonization because they are not viable cells. Thus, they have a longer shelf life during storage, facilitating distribution logistics and clinical application(Kumar et al. 2024). Furthermore, these metabotics are able to traverse the intestinal mucus layer more quickly than probiotics, whose direct contact with host cells may be limited by mucus(Ali et al. 2023) .

In addition to their practical advantages, the therapeutic potential of CFS is based on a wide range of mechanisms of action, such as enhanced epithelial barrier function(De Marco et al. 2018), modulation of immune responses (Rocchetti et al. 2024), systemic metabolic responses(Liu et al. 2020), and central nervous system signaling(Clapp et al. 2017), as well as regulation of the human microbiota(Nagpal et al. 2018; Favero et al. 2022) .

Metabotics have been identified in various bacterial species of the human microbiota, such as *Bifidobacterium*, *Bacteroides fragilis*, *Escherichia coli*, Nissle 1917, and *Faecalibacterium prausnitzii* (CICENIA et al., 2014)

Among the probiotics that produce metabolites, *Lactobacillus rhamnosus*, *Lactobacillus casei*(Song and Lee 2017), and *Lactobacillus reuteri*(Spinler et al. 2008; Silva 2010) stand out. These *Lactobacillus* species produce high amounts of lactic acid, acetic acid, and hydrogen peroxide(Ceccarani et al. 2019), maintaining an acidic pH of around 4.5 or even lower(Buggio et al. 2019) .

Another important metabolite in the modulation of intestinal inflammation is a short-chain fatty acid called sodium butyrate (NaBut). NaBut is produced by fermentative bacteria in the large intestine using dietary fiber and/or starch as sources (e.g., *Clostridium butyricum* and *Faecalibacterium prausnitzii*)(Qiu et al. 2015; Ji et al. 2016) .

NaBut has been extensively studied for its anti-inflammatory effects and other health benefits, including anticancer activity and promotion of intestinal barrier integrity (both physical and functional)(Kang et al. 2023; Vinderola et al. 2024). It also exhibits promising antifungal effects, with the potential to be used as an adjunct in the treatment of fungal infections, either in combination with antifungal agents or alone(Brandão et al. 2015) .

It is important to emphasize that the vast majority of studies involving *Lactobacillus* spp. use them solely as probiotics. On the other hand, this study evaluated *in vitro* the effect of *Lactobacillus* spp. supernatants, as well as sodium butyrate (NaBut), on the growth of *Candida glabrata* and *Cryptococcus neoformans* yeasts, using protocols for determining the minimum inhibitory concentration.

2. Materials and Methods

2.1. Origin and Isolation of Strains

The *Candida* strain used in this study was *C. glabrata* from the American Type Culture Collection (ATCC 90030), provided by the Clinical Microbiology Laboratory at the University Hospital of Brasília (HuB/UnB). The *Cryptococcus neoformans* strain was H99, mating type α .

The selected *Lactobacillus* spp. strains were *L. casei* (Gamma, Lot AF43180921) *L. reuteri* (Lemma, Lot IE2702), and *L. rhamnosus* (Strain LR-G14, Lemma, Lot HK0202), provided by the Laboratory of Clinical Microbiology and Immunology (LabMiC/UnB).

2.2. Culture Media

Sabouraud Dextrose Agar (SDA) (Neogen do Brasil, São Paulo, Brazil) was used for the initial cultivation of the fungal species.

The culture media used were Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco Thermo, Grand Island, New York, United States), Sabouraud Dextrose Broth, Yeast Malt (YM) medium (Neogen do Brasil, São Paulo, Brazil), and Minimal Medium (MM), a chemically defined medium (15 mM dextrose, 10 mM MgSO_4 , 29.3 mM KH_2PO_4 , 13 mM glycine, and 3 μM thiamine; pH 5.5) were used in the Minimum Inhibitory Concentration (MIC) assays for the fungal species.

Lactobacillus spp. were cultured in Acumedia® 29 LACTOBACILLI MRS BROTH (MRS) culture medium (Neogen do Brasil, São Paulo, Brazil).

2.3. Reagents and Solutions

Regarding reagent preparation, for sodium butyrate (Cayman Chemical® Michigan, United States), a stock solution was prepared and stored at -20°C protected from light at a concentration of 500 mM, diluted in water for injection.

To prepare the cell-free supernatant (CFS) from the *Lactobacillus* spp. culture, fresh, pure cultures were used; these were incubated for 48 hours at 37°C , then centrifuged for 10 minutes at 2000 rpm using an Arcano TDL80-2B centrifuge® centrifuge (Novatécnica, São Paulo, Brazil), and subsequently filtered through sterile membrane filters with a pore size of 0.22 μm . After filtration, the CFAs were immediately used in the assays.

2.4. Measurement of pH and Brix

The initial pH of the MRS medium was determined prior to inoculation with *Lactobacillus* spp. strains. The pH of the CFS samples was measured 48 hours after the cultivation and filtration steps. In both analyses, a digital pH meter (model AK90 hidro, ASKO®) was used.

The Brix degree of the samples was determined using a portable refractometer (Portable Refractometer, code LGAM84381). For each measurement, 1 to 2 drops of the previously homogenized sample were placed on the instrument's prism, ensuring uniform distribution of the solution over the optical surface. Next, the lid was closed to minimize the influence of external light, and the Brix reading was taken directly from the refractometer's digital display.

2.5. *In vitro* Analysis of the Growth Curve – Minimum Inhibitory Concentration (MIC) of CFS

The assays to determine the MIC were performed based on the BrCAST/EUCAST broth microdilution protocols, with modifications. Growth curves were plotted in RPMI 1640, Sabouraud, YM, and MM media, in a total volume of 170 µL per well in 96-well plates, with optical density (OD) readings taken at 600 nm.

In the 96-well plate, each column represented a group (controls or treatments), while the rows corresponded to serial dilutions, starting with the first row A (highest concentration) through the last row H (lowest concentration). To prepare the plates containing CFS from *Lactobacillus* spp., 150 µL of one of the culture media under study was added to all wells, followed by the addition of 150 µL of CFS to the first well, resulting in a final CFS concentration of 50% (v/v). Serial dilutions were performed by transferring 150 µL between consecutive wells until the last one. Subsequently, 20 µL of yeast suspension at a McFarland scale of 0.5 was added, followed by homogenization, and the plate was incubated at $37 \pm 1^\circ\text{C}$ for 24 h.

2.6. *In vitro* Analysis of the Growth Curve – Minimum Inhibitory Concentration (MIC) of NaBut

When preparing the plates for NaBut assays, we started with 2X NaBut at a concentration of 110 mM in the first well, followed by serial dilutions across the wells; finally, culture medium (RPMI or Sabouraud) containing *C. glabrata* or *C. neoformans* adjusted to a McFarland scale of 0.5 was added. The plates were incubated at $37 \pm 1^\circ\text{C}$ for 24 h and subsequently analyzed using an EnSpire® microplate reader (PerkinElmer, Brazil) at 600 nm to determine the optical density (OD).

2.7. Statistical Analyses.

Data analysis was performed using R software, version 4.4.2. Growth curve data were plotted with optical density values (OD 600 nm) on the Y-axis and concentrations (on a numerical scale) on the X-axis for dose-response evaluation. Descriptive statistics (standard error, standard deviation, mean, and median) were calculated to generate box plots, bar charts, and heatmaps. For comparisons between groups, the Student's t-test was used, with a significance level of $p < 0.05$. The following packages were

used to perform these analyses and visualizations: tidyverse, ggpubr, rstatix, ggtext, ggplot2, pheatmap, RColorBrewer, and grid.

The percentage reduction in growth was determined using the formula:

$$\text{Redução de crescimento (\%)} = \frac{\text{tratamento}}{\text{controle}} \times 100$$

Where the treatment represents the optical density (OD) value after the addition of CFS. The control is the OD value observed in the growth control group, without CFS, but in the presence of MRS and RPMI media.

3. Results

3.1. Analysis of the effect of CFS on the growth of *C. glabrata* and *C. neoformans* in different nutritional microenvironments.

Initially, to evaluate the influence of MRS medium on fungal growth, the growth curves of *C. glabrata* and *C. neoformans* were compared in RPMI, Sabouraud, YM, and MM media, either alone or supplemented with 50% MRS (control). This approach aimed to avoid bias resulting from possible effects of MRS on yeast growth, since cell-free supernatants (CFSs) from *Lactobacillus* spp. are derived from cultures in this medium. For *C. glabrata*, it was observed that RPMI, Sabouraud, and YM supplemented with MRS did not alter growth compared to RPMI, Sabouraud, and YM alone ($p = 0.14$, $p = 0.1985$, and $p = 0.0669$, respectively).

As for *C. neoformans*, RPMI ($p = 0.0061$) and MM ($p = 0.0094$) media supplemented with MRS promoted increased growth compared to the controls; whereas YM ($p = 0.5957$) and Sabouraud ($p = 0.9261$) media supplemented with MRS showed no differences in fungal growth (Fig. 1b). These results demonstrate that the effect of MRS varies depending on the fungal species and the culture medium used, making it crucial to consider such interactions when evaluating CFS.

Since there was no inhibition of yeast growth in the presence of 50% v/v MRS (the maximum concentration used in tests with CFS from *Lactobacillus* spp.), it was possible to establish this condition as a positive growth control, allowing the tested samples to be compared at different CFS concentrations and ruling out potential biases from MRS inhibition in the Minimum Inhibitory Concentration (MIC) assays.

3.2. Measurement of pH and Brix

The acid-producing capacity of *Lactobacillus* spp. was evaluated by measuring the pH of MRS before and after the growth process (Fig. 2a). All species studied (*L. rhamnosus*, *L. reuteri*, and *L. casei*) demonstrated the ability to lower the pH after 48 h of growth at 37°C, compared to uninoculated MRS medium. The average initial pH of the MRS was approximately 6.2. In contrast, the CFS derived from

Lactobacillus cultures exhibited lower pH values. The *L. rhamnosus* and *L. reuteri* species exhibited a marked acidification capacity, with mean pH values of 3.95 and 3.88, respectively. *L. casei* also caused a reduction, with a mean of 3.94, although it showed a slightly higher standard deviation (SD = 0.22) compared to the other species. These results demonstrate a clear differentiation between the CFS groups and the control, confirming the production of organic acids by these species.

Regarding the distribution of total soluble solids (Brix degree), it was observed that the control group (MRS) had higher mean values than the CFS groups (Fig. 2b). While the MRS medium had a mean of 5.27 and a standard error (SE) of 0.14, the lactobacilli showed lower values: *L. casei* (mean 4.71 and SE = 0.14), *L. reuteri* (mean 4.6 and SE = 1.5), and *L. rhamnosus* (mean = 4.6 and SE = 1.4) compared to the MRS medium. This reduction in Brix suggests the consumption of nutrients available in the medium during bacterial growth and metabolism for the synthesis of metabolites.

3.3. Analysis of the CFS of *L. casei* on the growth of *C. glabrata* and *C. neoformans*

The effect of *L. casei* CFS on the growth of *C. glabrata* and *C. neoformans* was evaluated in different nutritional microenvironments. For *C. glabrata* (Fig. 3a, Fig. 3b), CFS did not exhibit growth-inhibiting activity in complex media such as Sabouraud and YM. In these microenvironments, fungal growth was even stimulated, exceeding the levels observed in the growth controls with or without MRS. In contrast, in RPMI medium (Fig. 3c), CFS promoted a reduction in growth at all tested CFS concentrations, with the reduction being most pronounced at the highest concentration, 50% (v/v), where growth was reduced by 29.98% compared to the control supplemented with MRS.

For *C. neoformans*, the CFS from *L. casei* induced a slight reduction in growth in Sabouraud and YM media (9.81% and 8.96%, respectively) at a concentration of 50% (v/v) (Fig. 3e, Fig. 3f). However, in RPMI medium (Fig. 3g), a more pronounced decrease was observed across all dilutions; at the maximum tested concentration of 50% (v/v), there was a 28.8% reduction compared to the control group supplemented with 50% MRS.

Notable results were also obtained in minimal medium (MM), a chemically defined medium widely used in studies with *Cryptococcus* yeasts. Under these conditions, CFS markedly inhibited growth at all concentrations (Fig. 3h). Although the addition of MRS to MM stimulated fungal growth (Fig. x), the presence of *Lactobacillus* CFS undoubtedly impairs yeast growth.

3.4. Analysis of *L. reuteri* CFS on the Growth of *C. glabrata* and *C. neoformans*

L. reuteri CFS demonstrated inhibitory activity against *C. glabrata* and *C. neoformans*, with variations depending on the nutritional microenvironments and the concentration tested.

For *C. glabrata*, a 33.42% reduction in growth was observed in Sabouraud medium and a 44.49% reduction in YM medium at the first concentration (50% v/v) (Fig. 4a, Fig. 4b). However, in RPMI medium (Fig. 4c), the inhibitory effect was more pronounced and consistent, observed at all tested

concentrations (50% to 0.39% v/v). This pattern corroborates the results previously observed with the CFS of *L. casei*, reinforcing the influence of both the *Lactobacillus* species and the nutritional microenvironments on the modulation of antifungal activity.

Regarding *C. neoformans*, the CFS of *L. reuteri* promoted a 33.49% and 5.75% reduction in growth at concentrations of 50% and 25% (v/v), respectively, in Sabouraud medium. In YM medium, the inhibitory effect was limited to the highest concentration (50% v/v), resulting in a 28.47% reduction in growth (Fig. 4e, Fig. 4f). In RPMI and MM media (Fig. 3g, Fig. 3h), the reduction occurred in a dose-response manner at all concentrations evaluated, following a pattern similar to that observed with other CFS. Notably, in both RPMI and MM, lower concentrations of the CFS resulted in reduced fungal growth, indicating an inverse relationship between metabolite concentration and fungal proliferation.

3.5. Analysis of the CFS from *L. rhamnosus* on the growth of *C. glabrata* and *C. neoformans*

The CFS from *L. rhamnosus* exhibited a pattern of antifungal activity comparable to that of the other CFS tested (*L. casei* and *L. reuteri*), with variations depending on the different nutritional microenvironments and the concentration used.

For *C. glabrata*, a 50% (v/v) concentration of the *L. rhamnosus* CFS resulted in a 19.62% and 13.28% reduction in growth in Sabouraud and YM media, respectively (Fig. 5a, Fig. 5b). In RPMI medium (Fig. 5c), consistent with previous experiments, there was a pronounced reduction in fungal growth at all concentrations evaluated. Specifically, at a concentration of 50% (v/v), the reduction reached 34.28% of fungal growth, which reinforces the consistency of this activity pattern among different *Lactobacillus* species.

With regard to *C. neoformans*, the CFS from *L. rhamnosus* exhibited antifungal activity only at a concentration of 50% (v/v) in Sabouraud medium, at which it reduced fungal growth by 13.95% (Fig. 5e). In YM medium (Fig. 5f), no reduction was observed at any of the tested concentrations; on the contrary, no growth inhibition was observed. On the other hand, in RPMI and MM media (Fig. 5g, Fig. 5h), the CFS from *L. rhamnosus* exhibited growth-inhibiting activity at all concentrations. An interesting phenomenon is that at the lowest concentrations of CFS in RPMI and MM media, a more pronounced reduction in growth is observed than at the median concentrations; this is most likely due to the fact that at the more diluted concentrations of CFS, the medium is closer to RPMI without MRS supplementation.

These data support the hypothesis that the efficacy of CFS varies depending on the *Lactobacillus* species, the target fungus, and the characteristics of the nutritional microenvironment. Such variability underscores the importance of rigorous standardization of experimental conditions for an accurate assessment of the antifungal activity of bacterial metabolites.

3.6. Analysis of Sodium Butyrate (NaBut) on the Growth of *C. glabrata* and *C. neoformans*

The results demonstrated that sodium butyrate (NaBut) exerts distinct effects on fungal growth, depending on both the species analyzed and the nutritional microenvironment.

For *C. glabrata*, a complex response to NaBut was observed. In Sabouraud medium (Fig. 6a), all tested concentrations (ranging from 55 mM to 1.71 mM) exhibited antifungal activity against yeast growth. In contrast, in RPMI medium (Fig. 6b), NaBut stimulated growth. This discrepancy suggests that the nutritional composition of the medium, particularly the sugar content, may modulate the action of NaBut, indicating that its inhibitory effect is more pronounced in nutritionally rich environments.

In the case of *C. neoformans*, NaBut exhibited antifungal activity in both media evaluated, but with distinct sensitivity profiles. In RPMI medium (Fig. 6d), the reduction in growth was limited to the three highest concentrations, with inhibitions of 25.03% (55 mM), 12.44% (27.5 mM), and 7.68% (13.75 mM). On the other hand, in Sabouraud medium (Fig. 6c), the growth-inhibiting effect extended to lower concentrations, being observed as low as 1.78 mM, and the 55 mM concentration resulted in a 33.48% reduction in growth. These results indicate that *C. neoformans* exhibits a greater sensitivity to NaBut compared to *C. glabrata*, especially in nutritionally more complex media.

4. Discussion

Lactobacillus spp. are microorganisms widely recognized as probiotics beneficial to humans, and certain strains exhibit antifungal and immune-modulating activities (Villena et al. 2011; Song and Lee 2017). During fermentation, these bacteria release bioactive components called metabolites, notably lactic acid and antimicrobial peptides. Lactic acid, for example, penetrates the fungal cell membrane, lowering the cytoplasmic pH and compromising fungal viability, while bioactive peptides can disrupt the fungal membrane, causing lysis and inactivation of intracellular molecules (Gerbaldo et al. 2012; Van Der Mei et al. 2014; Catanoze et al. 2022).

Given this antifungal potential, the exploration of metabolites derived from probiotics emerges as a promising strategy to address the challenges posed by traditional treatments, such as increasing fungal resistance and toxicity to available antifungals (Lin 2009). Resistant strains of *C. glabrata* to fluconazole (Castanheira et al. 2022) and *C. neoformans* to amphotericin B, flucytosine, and, particularly, fluconazole have been increasingly reported, indicating a significant evolution in the resistance profile of these pathogens (Cicenia et al. 2014; Agudelo et al. 2015).

It has been observed that certain CFS, produced in specific media by particular *Lactobacillus* species, may exhibit paradoxical effects, promoting fungal growth rather than inhibiting it. These findings suggest that the antifungal effect is species- and context-specific, depending on both the producing bacterium and the conditions of the nutritional microenvironment (Zangl et al. 2020; Katsipoulaki et al. 2024). Thus, not all *Lactobacillus* strains would be suitable for decolonization strategies targeting *C. glabrata* and *C. neoformans*, since, under certain circumstances, they may establish ecological interactions that are beneficial to the pathogen but detrimental to the host (Wu et al. 2022; Lapiere and Richard 2022).

In the experiments conducted, CFS derived from three *Lactobacillus* strains reduced the growth of *C. glabrata* and *C. neoformans*. However, it was found that the antagonistic effects may vary depending on the nutritional microenvironment, possibly due to interactions between metabolites and environmental components, such as pH, fatty acids, vitamins, and carbohydrates. Substrate-rich media, for example, can attenuate the inhibitory effect, promoting compensatory fungal growth (Scorzoni et al. 2007; Scillato et al. 2021).

From this perspective, a relevant finding was observed in the assays conducted with nutritionally restricted media, such as RPMI and MM: at the lowest CFS concentrations, fungal growth was lower than that observed at intermediate concentrations. This behavior can be attributed to the proportional reduction in the nutritional supply derived from the residual MRS medium present in the CFS. While MRS acts as an enriching supplement in minimal basal media, its progressive dilution at lower concentrations restores the conditions of nutritional limitation intrinsic to RPMI and MM (Fig. 3g, Fig. 3h, Fig. 4g, Fig. 4h, Fig. 5g, Fig. 5h).

It is essential to emphasize that, although MRS medium alone has a growth-stimulating effect, the presence of metabolites in the CFS competes with this nutritional benefit. This is clearly observed in the reduced growth of pathogens when compared to controls containing RPMI supplemented with MRS. The consistency of this pattern across all tested *Lactobacillus* strains reinforces the premise that antifungal activity is mediated by bioactive compounds generated during fermentation, whose inhibitory potency overrides the stimulatory effect of the remaining components of the original culture medium. This dynamic is even more evident when analyzing the role of the MRS medium, which, due to its high concentration of sugars, amino acids, and other essential nutrients, proved particularly favorable for fungal growth, possibly by providing substrates (Zhang et al. 2020). Nevertheless, the metabolites produced by *Lactobacillus* spp. during fermentation—especially organic acids and antimicrobial peptides—have a proven effect in reducing fungal growth (Mishra et al. 2024). A dose-response relationship was therefore observed: the higher the CFS concentration, the lower the fungal growth, indicating that the medium's composition and the metabolic profile of the fermented compounds modulate the fungal response.

The experiments conducted with both *C. glabrata* and *C. neoformans* showed similar response patterns consistent with the medium's nutritional composition, highlighting the differential efficacy among *Lactobacillus* strains. Notably, the CFS from *L. reuteri* exhibited the broadest antifungal spectrum, standing out as the only strain capable of reducing fungal growth in Sabouraud medium down to a concentration of 25% (v/v). Furthermore, it was found that both the CFS of *L. reuteri* and that of *L. rhamnosus* promoted a reduction in growth at a concentration of 50% (v/v) in this same medium.

With regard to the YM medium, inhibitory activity was also observed for the *L. reuteri* CFS at 50% (v/v), confirming its superior potency compared to the other CFS tested in nutritionally rich microenvironments. However, in nutritionally restricted media (RPMI and MM), all CFS tested exhibited a persistent antifungal effect at various dilutions. This discrepancy reinforces the evidence that *C. glabrata*

possesses a superior proliferative capacity in carbohydrate-rich substrates(Jiang et al. 2015), which tends to attenuate the efficacy of the metabolites in media such as YM and Sabouraud. At the same time, the results obtained for *C. neoformans* corroborate the literature, which describes greater vulnerability of this pathogen in nutritionally limited microenvironments(Zhang et al. 2020; Spaggiari et al. 2022), where the inhibitory action of CFS becomes more pronounced.

In this context, the reduction in growth observed for the pathogens in the presence of the CFS from *L. reuteri* is consistent with previous evidence regarding the antimicrobial potential of this strain. Spaggiari et al. (2022), for example, demonstrated that the supernatant of *L. reuteri* not only inhibited the growth of *C. parapsilosis* but also reduced its ability to adhere to cells, although without interfering with pseudohyphal formation(Spaggiari et al. 2022). The clinical relevance of this activity was reinforced by Oncel et al. (2015), who reported that supplementation with *L. reuteri* was as effective as nystatin in preventing mucocutaneous candidiasis in preterm infants(Oncel et al. 2015) .

Similarly, the metabolic profile of *L. rhamnosus* and *L. casei* also plays a crucial role in modulating fungal pathogenicity. These species are known for producing lactic acid and antimicrobial peptides capable of reducing the virulence of *C. albicans*, including biofilm formation on dental prostheses(Song and Lee 2017). These findings are corroborated by MacAlpine et al. (2021), who demonstrated that the filtered supernatant of *L. rhamnosus* blocks the morphological transition of *C. albicans* to hyphae, one of the main determinants of its virulence. In this study, the compound identified as 1-ABC was responsible for this effect by interacting with the Yak1 kinase, thereby inhibiting filamentation and biofilm formation(MacAlpine et al. 2021) .

As for NaBut, a histone deacetylase inhibitor, it exhibited different effects depending on the medium and the species. In *C. glabrata*, NaBut inhibited growth in Sabouraud medium, but the opposite was observed in RPMI medium. In *C. neoformans*, however, it reduced growth in both media, with a greater effect in Sabouraud medium. These results are consistent with studies demonstrating NaBut's modulatory role on epigenetic factors linked to virulence, such as capsule and melanin formation(Nguyen et al. 2011; Brandão et al. 2015). This finding suggests that NaBut may exert broader regulatory functions, affecting epigenetic pathways associated with fungal pathogenicity, without directly acting on cell wall integrity(Braun et al. 1987) .

One study observed the inhibition of fungal growth and virulence factors in *C. neoformans*, *C. albicans*, and *C. parapsilosis* by NaBut. The researchers found that NaBut interfered with the expansion of the *C. neoformans* capsule—the primary virulence factor used by this fungus to evade the immune system—and, furthermore, NaBut increased the phagocytic capacity of macrophages(Nguyen et al. 2011). Another study demonstrated that NaBut reduced biofilm formation by *C. albicans*, *C. krusei*, and *C. parapsilosis* resistant to amphotericin B; furthermore, NaBut enhanced the efficacy of amphotericin B(Al-Dhaheri and Douglas 2010) .

5. Conclusions

The present study robustly demonstrates that cell-free supernatants (CFS) from *Lactobacillus* spp., particularly *L. reuteri*, exert potent antifungal activity against the pathogenic yeasts *Candida glabrata* and *Cryptococcus neoformans*. The efficacy of these metabolites was found to be superior to the stimulatory nutritional effect of MRS medium, indicating that inhibition is mediated by bioactive compounds, such as organic acids and antimicrobial peptides, generated during bacterial fermentation.

A particularly relevant finding is the influence of the nutritional microenvironment on the modulation of the fungal response. While complex media may mitigate the inhibitory action, the dose-response effect observed in chemically defined media reinforces the potency of CFS and Nabut as agents for reducing microbial growth. This evidence aligns with previous studies highlighting the prominent antifungal activity of CFS derived from *Lactobacillus* species, particularly in the control of *Candida* spp. infections.

However, it is essential to identify which metabolites are responsible for the fungistatic or fungicidal actions in CFS for the future development of biotechnological products against resistant fungal infections. Finally, our findings position *Lactobacillus* metabolites and Nabut as promising adjunctive strategies for the development of new antifungal therapies. Such approaches not only offer a viable alternative in the face of growing resistance to conventional drugs but also present significant advantages in terms of safety, cost-effectiveness, and biotechnological efficacy, paving the way for strengthening the therapeutic arsenal against invasive fungal infections.

Declarations

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7. Authors' Contributions

CA planning, execution, editing, and review, TNGS execution and FB planning, guidance, and review.

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9. Data Availability

No datasets were generated or analysed during the current study.

10. Statements and Declarations

Ethics Approval and Consent to Participate

Not applicable.

Competing Interests

The authors declare no competing interests.

Disclosure of the Use of AI and AI-Assisted Technologies in Writing

During the preparation of this manuscript, the authors used DeepL to translate the text from Portuguese into English, as well as Manus AI and NotebookLM to assist with language refinement, grammatical editing, and the organization of bibliographic references. Following the use of these tools, the authors carefully reviewed and revised the manuscript as necessary and accept full responsibility for the accuracy, integrity, and content of the published work.

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Figures

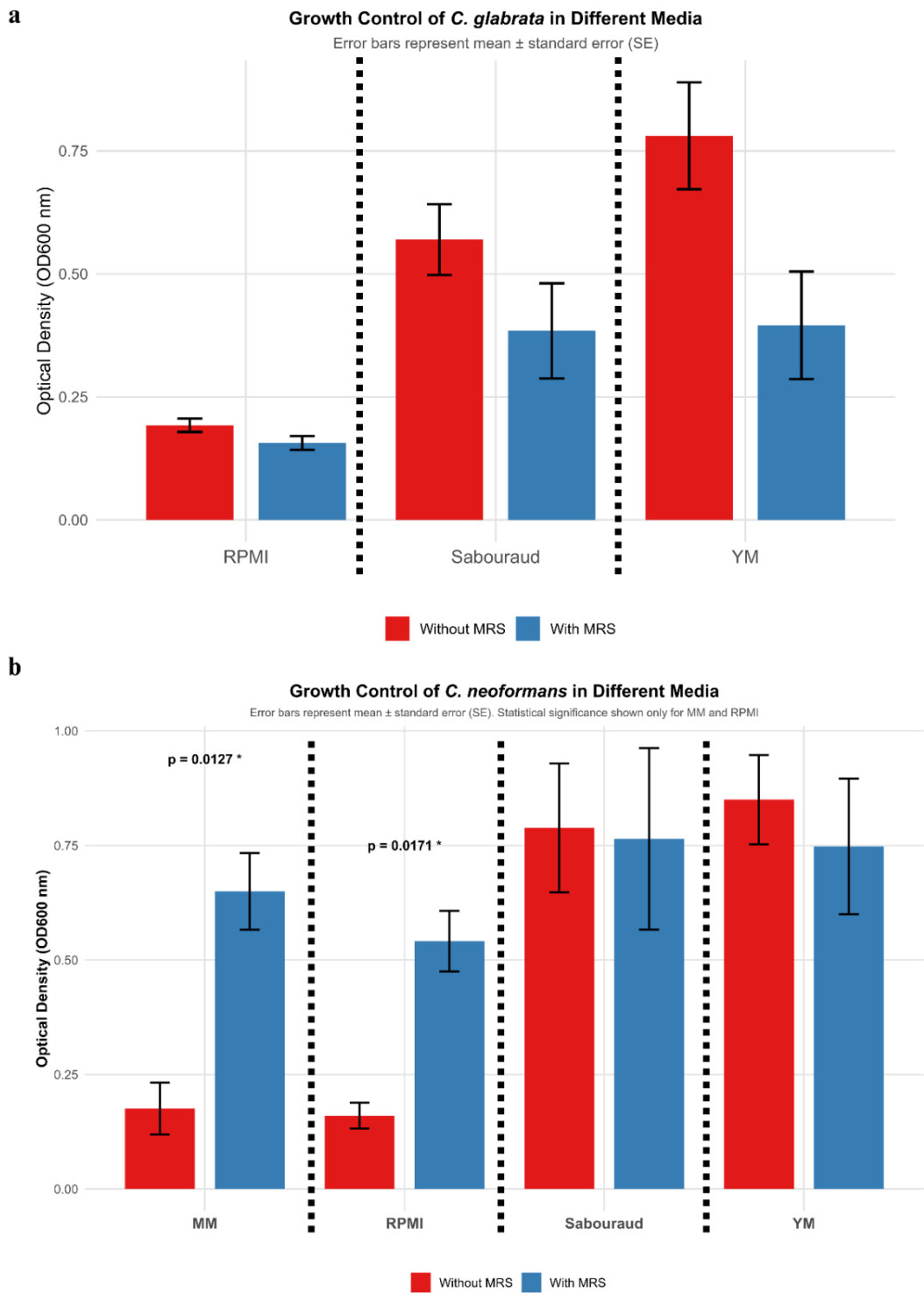


Figure 1

Effect of MRS on different nutritional microenvironments. (a) Effect of MRS on the growth of *C. glabrata*. (b) Effect of MRS on the growth of *C. neoformans*. The yeasts were cultured in media with different nutritional compositions, supplemented or not with 50% MRS. The analysis was performed after 24 hours of growth at 37 °C. The Y-axis indicates optical density (600 nm), while the X-axis represents the

groups tested. The error bars represent the standard error of the mean. Statistical test: Student's t-test, comparing each culture medium supplemented with MRS to the respective medium without MRS

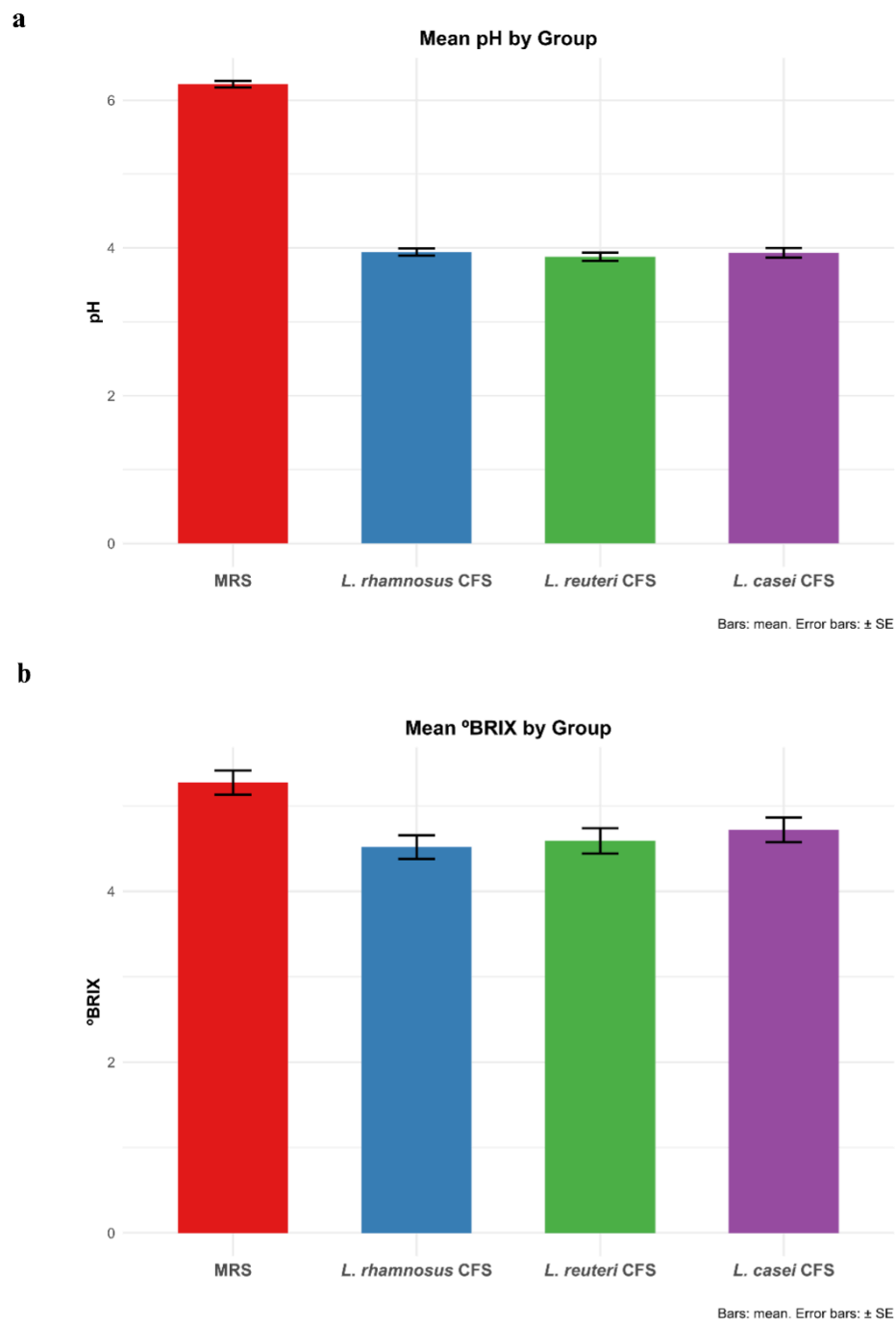


Figure 2

Distribution of pH and °Brix values (a) pH measurement in *Lactobacillus* CFS. (b) °Brix measurement in *Lactobacillus* CFS. pH and °Brix analyses were performed after 48 hours of incubation at 37 °C and

filtration. The bars represent the standard error of the mean

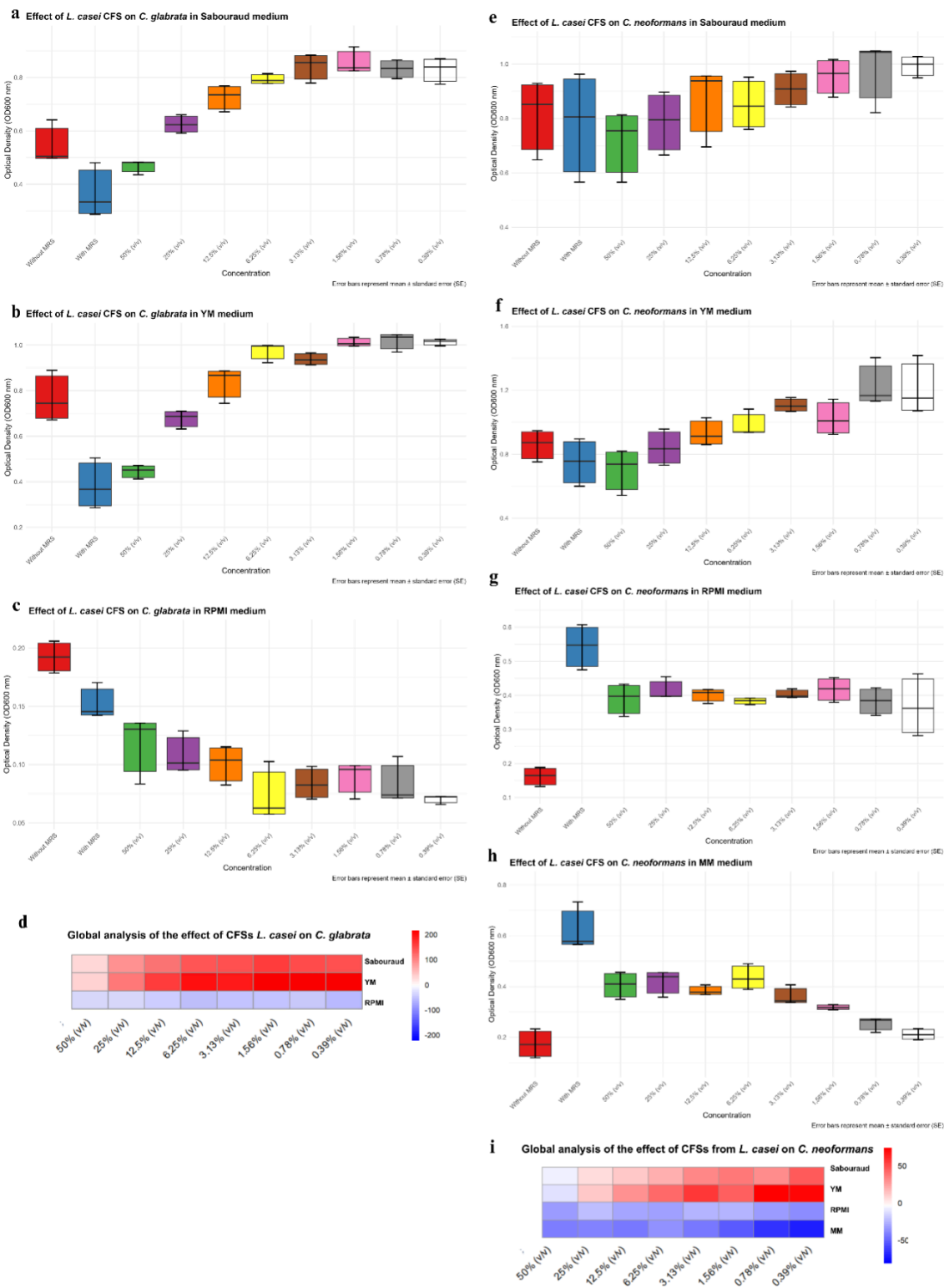


Figure 3

The effect of *L. casei* CFS on the growth of *C. glabrata* and *C. neoformans* depends on different nutritional microenvironments. (a, b, c) Dose-response effect of *L. casei* CFS on the growth of *C. glabrata*. (d) Overall analysis of the effect of CFS from *L. casei* on the growth of *C. glabrata*. (e,f,h) Dose-response

effect of CFS from *L. casei* on the growth of *C. neoformans*. (i) Overall analysis of the effect of CFS from *L. casei* on the growth of *C. neoformans*. The analyses were performed after 24 hours of growth. The Y-axis indicates optical density (600 nm). The X-axis indicates the serial concentrations of the analyzed compounds v/v (1: 50%; 2: 25%; 3: 12.5%; 4: 6.25%; 5: 3.12%; 6: 1.56%; 7: 0.78%; 8: 0.39%). The control group without MRS (culture media only: Sabouraud, YM, RPMI, or MM) is represented by the red-, and the control group supplemented with 50% MRS is represented by the blue. The analyses represent biological triplicates with similar data among them. The bars represent the standard error of the mean

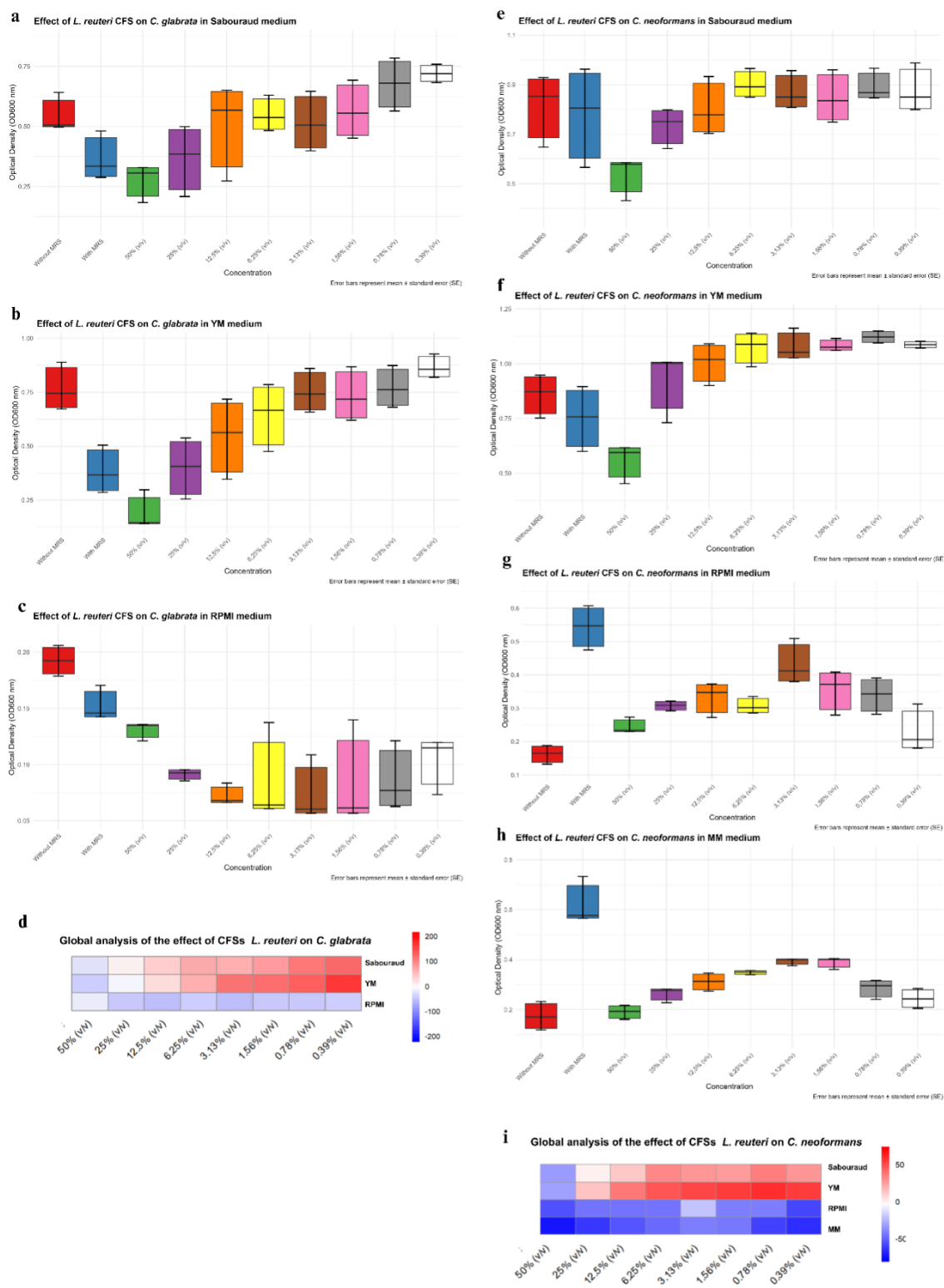


Figure 4

The effect of *L. reuteri* CFS on the growth of *C. glabrata* and *C. neoformans* depends on different nutritional microenvironments. (a,b,c) Dose-response effect of *L. reuteri* CFS on the growth of *C. glabrata*. (d) Overall analysis of the effect of *L. reuteri* CFS on the growth of *C. glabrata*. (e,f,h) Dose-response effect of *L. reuteri* CFS on the growth of *C. neoformans*. (i) Overall analysis of the effect of *L. reuteri* CFS on the growth of *C. neoformans*. The analyses were performed after 24 hours of growth. The Y-axis indicates optical density (600 nm). The X-axis indicates the serial concentrations of the analyzed compounds v/v (1: 50%; 2: 25%; 3: 12.5%; 4: 6.25%; 5: 3.12%; 6: 1.56%; 7: 0.78%; 8: 0.39%). The control group without MRS (culture media only: Sabouraud, YM, RPMI , or MM) is represented in red, and the control group supplemented with 50% MRS is represented in blue. The analyses represent biological triplicates with similar data among them. The bars represent the standard error of the mean

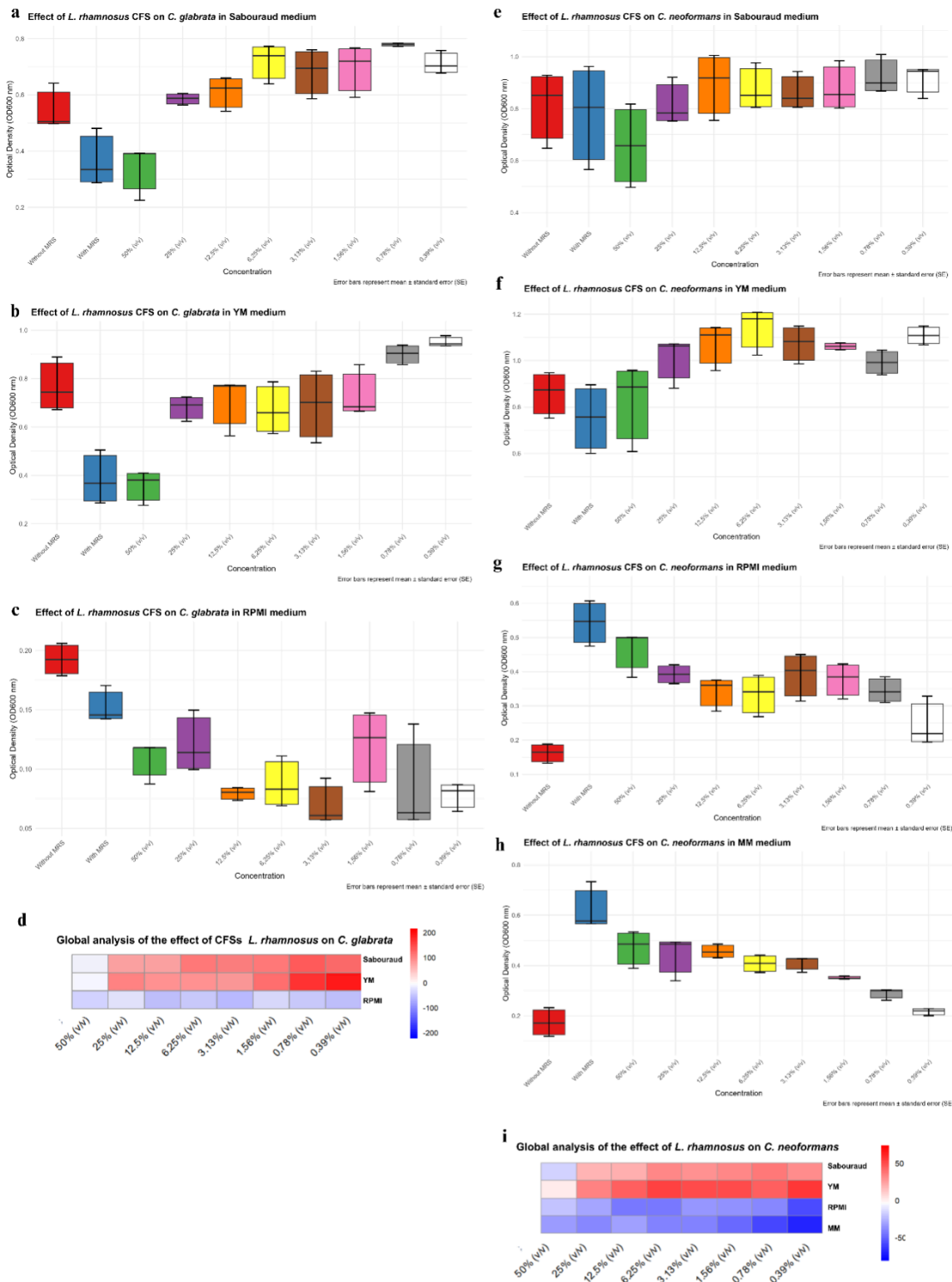


Figure 5

The effect of *L. rhamnosus* CFS on the growth of *C. glabrata* and *C. neoformans* depends on different nutritional microenvironments. (a,b,c) Dose-response effect of *L. rhamnosus* CFS on the growth of *C. glabrata*. (d) Overall analysis of the effect of *L. rhamnosus* CFS on the growth of *C. glabrata*. (e,f,h) Dose-response effect of *L. rhamnosus* CFS on the growth of *C. neoformans*. (i) Overall analysis of the effect of *L. rhamnosus* CFS on the growth of *C. neoformans*. The analyses were performed after 24 hours of

growth. The Y-axis indicates optical density (600 nm). The X-axis indicates the serial concentrations of the analyzed compounds v/v (1: 50%; 2: 25%; 3: 12.5%; 4: 6.25%; 5: 3.12%; 6: 1.56%; 7: 0.78%; 8: 0.39%). The control group without MRS (culture media only: Sabouraud, YM, RPMI, or MM) is represented by the color red, and the control group supplemented with 50% MRS is represented by the color blue. The analyses represent biological triplicates with similar data among them. The bars represent the standard error of the mean

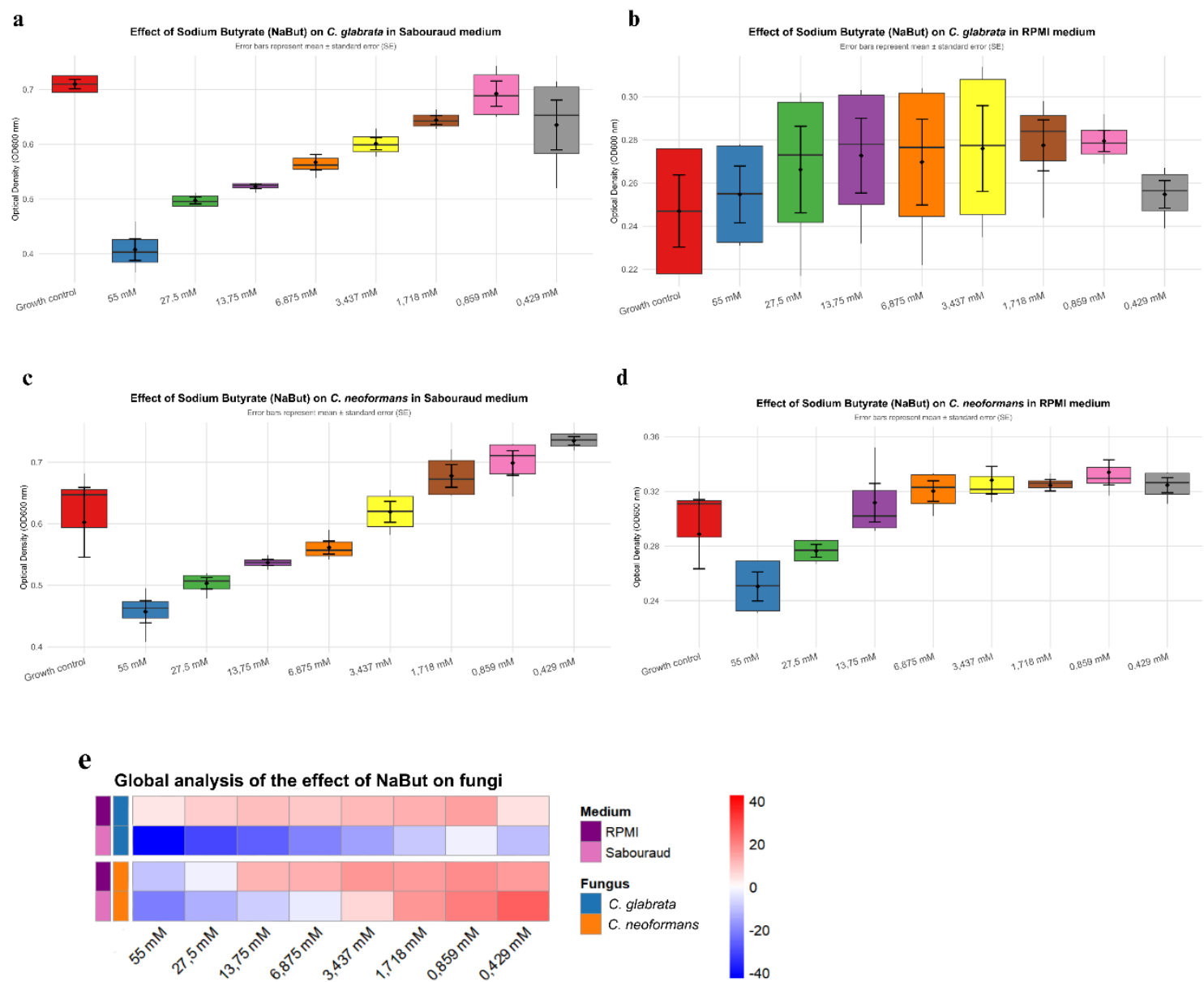


Figure 6

The effect of sodium butyrate (NaBut) on the growth of *C. glabrata* and *C. neoformans* depends on different nutritional microenvironments. (a,b) Dose-response effect of NaBut on the growth of *C. glabrata*. (c,d) Dose-response effect of NaBut on the growth of *C. neoformans*. (e) Overall analysis of the effect of NaBut on the growth of *C. glabrata* and *C. neoformans*. The analyses were performed after 24

hours of growth. The Y-axis indicates optical density (600 nm). The X-axis indicates the serial concentrations of NaBut (1 = [55 mM], 2 = [27.5 mM], 3 = [13.75 mM], 4 = [6.875 mM], 5 = [3.437 mM], 6 = [1.718 mM], 7 = [0.859 mM], and 8 = [0.429 mM]). The analyses represent biological replicates with similar data among them. The bars represent the standard error of the mean