TITLE: Probiotic yeast Saccharomyces boulardii (nom. nud.) modulates adhesive properties of Candida glabrata

AUTHORS: Zorica Tomičić, Jure Zupan, Tadeja Matos, Peter Raspor

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Probiotic yeast *Saccharomyces boulardii* (nom. nud.) modulates adhesive properties of *Candida glabrata*

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Complete List of Authors:	Tomičić, Zorica; Institute of Food Technology, University of Novi Sad Zupan, Jure; University of Primorska, Faculty of Health Sciences; University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Chair of Biotechnology, Microbiology and Food Safety (at the time of laboratory work) Matos, Tadeja; University Ljubljana, Faculty of Medicine, Institute of microbiology, Department of immunology Raspor, Peter; University of Primorska, Faculty of Health Sciences; University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Chair of Biotechnology, Microbiology and Food Safety (at the time of laboratory work)
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	while at high pH and at increased concentrations of antimycotics, *S. boulardii* did not manage to repress the adhesion of *C. glabrata* strains. The experiments of *C. glabrata* co-cultures with other species showed that the adhesiveness of two separate cultures could not be used to predict the adhesiveness of their co-culture.

Probiotic yeast *Saccharomyces boulardii* (nom. nud.) modulates adhesive properties of *Candida glabrata*

Zorica Tomičić[a], Jure Zupan[b,c], Tadeja Matos[d], Peter Raspor[b,c]

[a] Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

[b] Faculty of Health Sciences, University of Primorska, Polje 42, 6310 Izola, Slovenia

[c] Chair Of Biotechnology, Microbiology and Food Safety, Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000, Ljubljana, Slovenia (the university in the time of experiments)

[d] Institute of microbiology and immunology, Medical faculty, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia

Zorica Tomičić, zoricateh@yahoo.com; zorica.tomicic@fins.uns.ac.rs

Jure Zupan *, jure.zupan@fvz.upr.si, Tel.: +386156626468

Tadeja Matos, tadeja.matos@mf.uni-lj.si

Peter Raspor, peter.raspor@fvz.upr.si

***Corresponding author: Jure Zupan**

Keywords: adhesion, *Candida glabrata*, *Saccharomyces boulardii*, antimycotics, cell surface hydrophobicity

Abstract

Following the widespread use of immunosuppressive therapy together with broad-spectrum antimycotic therapy, the frequency of mucosal and systemic infections caused by the pathogenic yeast *Candida glabrata* has increased in the past decades. Due to the resistance of *C. glabrata* to existing azole drugs, it is very important to look for new strategies helping the treatment of such fungal diseases. In this study, we investigated the effect of the probiotic yeast *Saccharomyces boulardii* (nom. nud.) on *C. glabrata* adhesion at different temperatures, pH values, and in the presence of fluconazole, itraconazole and amphotericin B. We also studied the adhesion of *C. glabrata* co-culture with *Candida krusei*, *Saccharomyces cerevisiae*, two bacterial probiotics *Lactobacillus rhamnosus* or *Lactobacillus casei*. The method used to assess adhesion was crystal violet staining. Our results showed that despite the non-adhesiveness of *S. boulardii* cells, this probiotic significantly affected the adherence ability of *C. glabrata*. This effect was highly dependent on *C. glabrata* strain and was either antagonistic or synergistic. Regarding the extrinsic factors, temperature did not indicate any significant influence on this *S. boulardii* modulatory effect, while at high pH and at increased concentrations of antimycotics, *S. boulardii* did not manage to repress the adhesion of *C. glabrata* strains. The experiments of *C. glabrata* co-cultures with other species showed that the adhesiveness of two separate cultures could not be used to predict the adhesiveness of their co-culture.

1. Introduction

The incidence of infections caused by *Candida* species (candidosis) has considerably increased over past years. The reason for the increasing prevalence of *Candida* species is mainly due to the introduction and more widespread use of certain medical practices, such as immunosuppressive therapy, the use of broad-spectrum antibiotics, and an increase in the number of invasive surgical procedures, such as organ transplantations (1, 2).

Candida pathogenicity is mediated by a number of virulence factors, including the ability to adhere to medical devices and/or host cells, often leading to the formation of biofilms. Thus, adhesion is an extremely important step in the infection process, and the extent of adhesion is dependent on microbial, host and abiotic surface properties, such as cell-surface hydrophobicity and cell-wall composition (3). The formation of *Candida* biofilms carries important clinical repercussions because of their increased resistance to antifungal therapy and the ability of cells within biofilms to withstand host immune system (4, 5).

Most cases of candidosis have been attributed to *Candida albicans*, but recently non-*albicans Candida* species have been identified as frequent human pathogens. Namely, *Candida glabrata* has emerged as the second most common cause of invasive candidiasis, and an increasing number of reports show its important role in mucosal or bloodstream infections (1, 6). Moreover, the incidence of *C. glabrata* systemic infections deserves a great deal of concern due to the high mortality rate in immunocompromised populations (1, 7). This emergence has been attributed to a low susceptibility to azoles, particularly fluconazole (8), which necessitated the use of highly toxic amphotericin B, and to the high rate at which *C. glabrata* develops resistance to antifungals, requiring the use of alternative antifungal therapy (2, 9, 10). As a different strategy, probiotic organisms have already been tested as potential bio-therapeutic agents against *C. albicans* (11).

An increasing number of potential health benefits are being attributed to probiotic treatments (12). They include various bacterial probiotics, while among yeast only *Saccharomyces boulardii* (nom. nud.) is used extensively as a probiotic and often marketed as a dietary supplement. *S. boulardii* is very efficient as a biotherapeutic agent for the prevention and treatment of intestinal diseases, mainly diarrhea (13, 14), which is the greatest cause of morbidity and mortality among immunocompromised patients (15). On the other hand, there were reports of infections with *S. boulardii* (16), therefore caution and more knowledge is certainly needed.

The main mechanism of action of *S. boulardii* is most probably its ability to interfere with the pathogens' colonization of the mucosa and in this way prevent the infection. Other mechanisms include regulation of intestinal microbial homeostasis, modulation of local and systemic immune responses, stabilization of the gastrointestinal barrier function and induction of enzymatic activity favoring absorption and nutrition (13, 17).

The beneficial effect of *S. boulardii* in the case of *C. glabrata* infections have not been studied yet. Also, the influence of *S. boulardii* presence on the efficiency of *C. glabrata* virulence traits, like adhesion and antimycotic resistance, is not known. Therefore, we tested the adhesion of *C. glabrata* in a co-culture with *S. boulardii* to polystyrene surface at different temperatures, pH values and in the presence of three clinically important antifungal drugs, namely fluconazole, itraconazole and amphotericin B. The relative cell surface hydrophobicity (CSH) of the tested *C. glabrata* strains has been determined as well in order to test for a possible correlation between this physico-chemical property and the ability to adhere to polystyrene surface. In addition, *Candida krusei*, *Saccharomyces cerevisiae*, two bacterial probiotics

(*Lactobacillus rhamnosus* and *Lactobacillus casei*) were tested in a co-culture with *C. glabrata* as well.

2. Materials and methods

2.1. Strains and growth conditions

A total of 48 *C. glabrata* strains, isolated from clinical samples, were examined to determine a correlation between adhesion and CSH. For the co-culture adhesion assays the following strains were used: four *C. glabrata* strains strains (ZIM 2344, ZIM 2367, ZIM 2369, and ZIM 2382), a probiotic *S. boulardii* strain isolated from commercially available probiotic food supplement, *C. krusei* strain CBS573, clinical *S. cerevisiae* strain YJM311, two bacterial probiotics *L. rhamnosus* strain ZIM B542 and *L. casei* strain ZIM B538. All strains were obtained from the Collection of Industrial Microorganisms (ZIM) at the Biotechnical Faculty, University of Ljubljana, Slovenia. These strains were preserved in glycerol at −80 °C, and they were revitalized from frozen stocks by cultivation on the *M*alt *E*xtract *A*gar (MEA) plates (Merck KGaA, Darmstadt, Germany) and incubated 2 days at 37 °C before performing the adhesion assays. Bacterial probiotics *L. rhamnosus* and *L. casei* were grown in an anaerobic container at 37 °C for 4 days before use in the test.

The influence of the temperature was tested in the *M*alt *E*xtract *B*roth (MEB) medium (Merck, KgaA, Darmstadt, Germany) at 28 °C, 37 °C, 39 °C and 42 °C. In order to analyze the effect of pH on adhesion, yeasts were grown in MEB medium adjusted with HCl (Merck KGaA, Darmstadt, Germany) to reach pH 4.0, and with NaOH (Merck KGaA, Darmstadt, Germany) to reach pH 5.5, 7.0, and 8.5.

2.2. Relative cell surface hydrophobicity

The CSH of *C. glabrata* strains was determined using the *M*icrobial *A*dhesion *T*o *H*ydrocarbon (MATH) test of Rosenberg (1984) (18) with modifications. Yeasts were cultivated in 6 ml of *Y*east *P*eptone *D*extrose (YPD) medium (Sigma-Aldrich, St. Louis, USA) at 30 °C for 24h. After the cultivation, cells were centrifuged at 1500 × g for 3 min and washed twice with the *P*hosphate *B*uffered *S*aline (PBS) (Oxoid, Hampshire, England). Subsequently, yeasts were re-suspended in 6 ml of 4 M a ammonium sulphate (Merck KGaA, Darmstadt, Germany) in PBS, which increase hydrophilicity of the aqueous phase (18) and adjusted to an optical density of 0.7-0.8 at 650 nm (A_0). Cell suspension aliquots of 1.4 ml were transferred to 2 ml centrifuge tubes and 0.2 ml of xylene (Merck KGaA, Darmstadt, Germany) was added to start the assay. A tube without the addition of xylene was used as a control. The tubes were vortexed for 1 min and allowed to stand for 15 min to ensure the complete separation of the two phases. After the separation of the phases, a volume of 300 μl of the lower aqueous phase was gently removed and the optical density of samples (A) and control (A_0) was measured at 650 nm. The CSH was assessed using the formula: CSH (%) = $(1-A/A_0) \times 100$ %. The assays were performed in triplicates.

2.3. Adhesion assay

Adhesion assays were performed as previously described (19) with a few modifications. Prior to testing, strains were grown on MEA plates at 37 °C for 48h. After the incubation, a loopful of actively growing cells was suspended in the appropriate MEB medium. The concentration of cells were determined and adjusted to 2×10^7 cells/ml by using the Bürker-Türk counting chamber (Brand, Wertheim, Germany), a microscope with camera (Leica DFC290) and an image processing software ImageJ as described before (20). The assay was initiated by the addition of 200 µl cell suspensions into 96-well polystyrene microtiter plate (Nunc, Roskilde, Germany), which were than incubated at 37 °C for 24 hours, except if stated differently. For co-culture tests, the cell suspensions of each organism were mixed immediately before use.

The antimycotics tested in this study, fluconazole, itraconazole and amphotericin B, were purchased from Sigma Chemical Co. (St. Louis, USA). The selection of antimycotics concentrations used in the adhesion assay was based on the MICs obtained by the preliminarily performed microdilution modification of the Reference method for broth dilution antifungal susceptibility testing of yeast (CLSI standard M27-A2) (21). Amphotericin B and itraconazole were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA) before dilution in the MEB medium. The final concentration of dimethyl sulfoxide in microtiter wells did not exceed 1 %. Fluconazole was dissolved in sterile distilled water. In all experiments a positive (assay medium without antimycotics and with yeast strains) and a negative control (growth medium without yeast strains) were included.

After incubation period, non-adherent cells were removed by washing three times with 150 µl sterile distilled water. After 10 min drying with hair drier, the yeast cells in the wells were stained with 100 µl 0.5% crystal violet (Merck KGaA, Darmstadt, Germany) and left on bench for 20 min. The redundant crystal violet was removed by inverting the plates and the wells were washed three times with sterile distilled water and dried for another 10 min with hair drier. After adding 100 µl of 33% acetic acid into each well, the plates were shaken for 3 min to release the dye from the cells. The amount of adhered cells, i.e. the concentration of the released crystal violet was determined by measuring the optical density at 584 nm (OD_{584}) using a microplate reader (Tecan, Mannedorf/Zurich, Switzerland).

3. Results

In the present study, we examined whether the presence of the probiotic yeast *S. boulardii* affects the adhesion of the pathogenic yeast *C. glabrata* to polystyrene in dependence to growth temperatures, pH, inoculum size, and antimycotics fluconazole, itraconazole and amphotericin B.

3.1. Relative cell surface hydrophobicity and adhesion to polystyrene

Since the CSH is often connected to adhesion and flocculation (22, 23), 48 *C. glabrata* strains were examined by the water-hydrocarbon (xylene) biphasic assay (18) as described in the Methods. The degree of hydrophobicity is expressed as the percentage of cells transferred from the aqueous phase to the non-polar phase. The correlation between adhesion to polystyrene and CSH was tested in Fig. 1.

The results showed a wide distribution of the *C. glabrata* strains over the range of hydrophobicity from 0 to 90%, regarding the use of xylene as organic solvent (Fig. 1). No correlation with the adhesion to polystyrene was observed (R^2=0.02). Moreover, the majority of strains were weakly adhesive to polystyrene, while only three strains showed moderate (ZIM 2367, ZIM 2369) to strong (ZIM 2344) adhesiveness to polystyrene. These 3 strains, together with the strain ZIM 2382, which showed highest CSH (90%), were selected for further experiments with the probiotic *S. boulardii*.

3.2. Validation of the method

Three preliminary adhesion assays were performed to examine how the inoculum concentration and the ratio between *C. glabrata* and *S. boulardii* affect the adhesion of both strains to polystyrene. As shown in the Fig. 2A, the inoculum concentration of *C. glabrata* ZIM 2369 in the range between 4×10^6 and 4×10^7 cells/ml have no statistically significant effect on the amount of adhered cells after 24h; even the adhesion with the inoculums of 4×10^6 and 4×10^7 cells/ml were not statistically different (P = 0.178).

In the second experiment, we inoculated fixed concentration of *C. glabrata* ZIM 2369 cells (2×10^7 cells/ml) with a range of *S. boulardii* cell concentrations (from 5×10^6 to 1.6×10^8 cells/ml). The results clearly show that the adhesion of *C. glabrata* was highly dependent on the inoculum size of *S. boulardii* over the selected concentration range (Fig. 2B). The adhesion of cells was exponentially reduced with the increased *S. boulardii* inoculum. At the lowest *S. boulardii* concentration used (5×10^6 cells/ml, representing 20% of cells in the co-culture) the adhesion of *C. glabrata* was reduced by 31% as compared to control cells not treated with the probiotic strain. When the concentration of *S. boulardii* cells represent $^1/_3$ of cells in the co-culture (at 1×10^7 cells/ml), the adhesion of *C. glabrata* was reduced by 50%. It has to be emphasized that this reduction in adhesion is not due to the lower amount of *C. glabrata* in the co-culture, because the results in 2A showed that inoculum size did not have significant influence on final adhesion result.

In the third experiment we wanted to have cumulative inoculum size of 2×10^7 cells/ml in all co-culture combinations, therefore we mixed *C. glabrata* cells and *S. boulardii* cells in different ratios. We tested three *C. glabrata* strains. The results (Fig. 2C) show that i) *S. boulardii* cells are completely non-adhesive to polystyrene (at the ratio of 100 % *S. boulardii*, no adhesion was detected), ii) the presence of *S. boulardii* significantly affected the adhesion of *C. glabrata*, and iii) this effect was dependent on *C. glabrata* strain. In the case of the strains ZIM 2344 and ZIM 2369, the effect was antagonistic – the adhesion of both strains was significantly inhibited by *S. boulardii* in a dose-dependent manner (Fig. 2C). At the ratio 50/50 % of the co-cultures, a significant 50% and 65% reduction in the adhesion of *C. glabrata* ZIM 2344 and ZIM 2369, respectively, was observed. However, with the strain ZIM 2382 on the other hand, the effect was even weakly synergistic. The experiment was repeated 3 times with this strain, but the result was always the same; at the ratio around 30% *S. boulardii* / 70% *C. glabrata* ZIM 2382, the adhesion of this co-culture was significantly induced when compared with the single *C. glabrata* culture (P = 0.0025).

3.3. The influence of temperature on S. boulardii and C. glabrata co-culture adhesion to polystyrene is strain-dependent

The influence of *S. boulardii* on the adhesion of *C. glabrata* strains to polystyrene at 28, 37, 39 and 42 °C is presented in Fig. 3. Again, the effect of *S. boulardii* was highly dependent on *C. glabrata* strains. We observed two different patterns, similar to the observations above: the adhesion in the case of ZIM 2344 and ZIM 2369 was relatively equally decreased over all tested temperature range, while the adhesion in the case of ZIM 2367 and ZIM 2382 was significantly stimulated at 28 °C ($P < 0.05$) and repressed at 42 °C ($P < 0.05$).

3.4. Low pH stimulates adhesion of C. glabrata to polystyrene

Adhesion assays were performed over a pH range of 4.0-8.5. The results indicate that pH has a weak influence on the adhesion of a co-culture *C. glabrata / S. boulardii*. Namely, as seen from Fig. 4, the adhesion of *C. glabrata* was slightly better in acidic medium. As in all other experiments, the strain ZIM 2382 acted differently; in this experiment the level of adhesion was tripled by the presence of *S. boulardii*, which was evident over the whole pH range. Also, this strain was highly dependent on pH, with highest adherence at pH 4 and lowest at pH 8.5. It is also worth mentioning that at pH 8.5 we did not observed the repression of *C. glabrata* adhesion by *S. boulardii* in any co-culture combination.

3.5. The effect of antimycotics on the adhesion of a co-culture C. glabrata/S. boulardii

In this part of the study we investigated the effect of antimycotics on the relationship between pathogenic yeast *C. glabrata* and probiotic yeast *S. boulardii* during biofilm formation to polystyrene as presented in Fig. 5. Growing single cultures and co-cultures were challenged with increasing concentrations of two azoles, fluconazole and itraconazole, and a polyene antimycotic, amphotericin B.

The lowest concentrations of antimycotics which significantly decreased the adhesion of *C. glabrata* in the co-cultures were generally higher than the MICs determined according to the CLSI method; the MIC values of the strains ZIM 2369 and ZIM 2367 were for fluconazole 8 and 4 µg/ml, for itraconazole 1 and 2 µg/ml and for amphotericin B 0.0625 and 0.125 µg/ml, and respectively. Interestingly, despite the inhibitory effect of *S. boulardii* on the adhesion of *C. glabrata* ZIM 2369, high concentrations of antimycotics had relatively smaller effect on the co-culture adhesion than on the adhesion of the single culture. It was expected that in the case of *S. boulardii* inhibition, antimycotics would additionally decrease the level of adhesion if compared with single cultures, but this did not happen. Namely, even at high concentration of fluconazole (125 µg/ml) a complete suppression of adhesion of the co-culture with *C. glabrata* ZIM 2369 was not achieved; in this case the adhesion of the co-culture at 125 µg/ml fluconazole was two-times higher than in a single culture, despite the facts that i) the adhesion at no antimycotic added was two-times lower than in a single culture, and ii) the MIC for this strain was as low as 8 µg/ml fluconazole. Similarly, the level of adhesion of *C. glabrata* ZIM 2369 in the co-culture with *S. boulardii* was constant over the whole tested range of itraconazole (0 – 2 µg/ml), whereas the MIC is at 1 µg/ml.

In the case of ZIM 2367, again, induced adhesion was observed when *S. boulardii* was added. Another interesting observation was that at higher concentrations of both azoles, the induction effect by *S. boulardii* disappeared, most probably due to the suppression of *S. boulardii* growth.

As expected, the results indicated that amphotericin B was the most effective against both *C. glabrata* isolates. Exposure to various concentrations of amphotericin B significantly reduced the adherence ability of *Candida* strains or rather its growth in a single culture and in a co-culture with *S. boulardii*. The adhesion was completely suppressed at 1 µg/ml, which was up to 16-fold higher than the corresponding MICs.

3.6. Interactions of C. glabrata with other microorganisms

Besides the *S. boulardii* strain, we studied the adhesion of the *C. glabrata* co-culture with other species as well. We tested three strains of *C. glabrata*, *C. krusei, S. cerevisiae* and two bacterial probiotics *L. rhamnosus* and *L. casei* (Table 1).

In single cultures, *C. krusei and S. cerevisiae* showed lower adhesion ability as compared with *C. glabrata*, while the probiotic strains were non-adhesive to polystyrene.

The results presented in the Table 1 indicate that the co-culture of two *C. glabrata* strains (ZIM 2344 and ZIM 2369) was less adherent (OD = 0.64) than each of those two cultures separately (OD = 0.95 and 0.74, respectively). But on the other hand, the assay showed that *C.*

glabrata strains in the co-cultures with non-adhesive pathogenic strains *C. krusei* and *S. cerevisiae*, showed weaker adhesion than in co-cultures with non-adhesive probiotic strains (*S. boulardii*, *L. rhamnosus* and *L. casei*). Interestingly, when *C. glabrata* ZIM 2344 was co-cultured with *C. glabrata* ZIM 2382 or ZIM 2369 the adhesion was equally strong (P = 0.94), regardless to the fact that ZIM 2382 is much less adherent as a single culture (OD = 0.09) than ZIM 2369 (OD = 0.74). The inhibitory effect on *C. glabrata* adhesion was observed by *C. krusei* CBS573 and *S. cerevisiae* YJM311 (Table 1), indicating that these two strains are mutually antagonistic in community growth. Furthermore, both *Lactobacillus* strains used in the present study were not able to adhere to polystyrene and showed similar antagonistic effect on the adhesion of *C. glabrata* strains.

4. Discussion

To our knowledge we have shown for the first time the effect of *S. boulardii* cells on the adhesive properties of *C. glabrata*. We have shown that the presence of *S. boulardii* cells significantly suppressed the adhesion of the two most adhesive *C. glabrata* strains used in the study (ZIM 2344 and ZIM 2369) to polystyrene (Fig. 2). Despite to the fact that *S. boulardii* was not adhesive in any of the tests in this study, it seems that *S. boulardii* still manages to occupy a portion of well surface during incubation and disrupt *C. glabrata* growth and/or adhesion We could also speculate that *S. boulardii* rather attaches to *C. glabrata* cells and in this manner interrupts flocculation and adhesion of *C. glabrata*. This hypothesis can be further supported by the reports about *S. boulardii* cell wall galactomannans as a prebiotic factor (24), since several studies showed that the presence of *S. boulardii* cells or the extract from its spent medium reduced *C. albicans* filamentation and adhesion to plastic surfaces *in vitro* (11).

On the other hand, the interaction between the *C. glabrata* ZIM 2382 and *S. boulardii* happened differently. The apparent increase in the number of adherent *C. glabrata* at certain concentration ratios could be explained by the flocculation between organisms (23). Despite the contradiction with the discussion above, in this case, *S. boulardii* cells may present a link between *C. glabrata* cells. Same effect was observed also with *S. cerevisiae* YJM311, despite being a clinical strain, but not with bacterial species or *C. krusei* (Table 1). It would be interesting in further studies to examine the cell wall properties of *C. glabrata* ZIM 2382 and 2344, like adhesines, and search for differences. Another explanation could include one of the first definitions of probiotics as being capable to support growth of other organisms. Nevertheless, considering both observations, the fact is that the effect of *S. boulardii* is highly dependent on *C. glabrata* strains. Further, we can also conclude that the adhesiveness of two separate cultures cannot be used to predict the adhesiveness of their co-culture.

The CSH is considered an important pathogenic attribute of *Candida* spp. pertaining to its adhesion and retention on host surfaces. It is also known that hydrophobic yeasts are more virulent than their hydrophilic counterparts (22). Regarding the correlation between CSH and adhesion to polystyrene, the findings of other authors are inconsistent. Using different species of *Candida*, Klotz and co-authors observed that fungal adherence to plastic surfaces were correlated with CSH (25). In the present study, CSH of *C. glabrata* strains did not correlated with amount of cells adhered to polystyrene (Fig. 1). Other researchers have also failed to find a correlation between the hydrophobicity of microbial strains and attachment to a surface. In addition, Camacho *et al.* (26) did not find a correlation between the CSH and adherence for *Candida* cells on siliconized latex catheters, demonstrating that CSH alone was not a predictor for adhesion levels. Also, Hazen (27) states that although CSH appears to be involved in the adhesion of *C. albicans* to human epithelial cells, this is not the predominant adhesion mechanism. Further, they claim that the contribution of hydrophobicity to adhesion is strain dependent, which also connects to our findings described above.

Beside strain specificity, environmental factors have major influence on cell growth, spread and adhesion. Adaptation to changing environments is a requirement for the survival of many microorganisms. Pathogens, such as *C. glabrata*, cover a wide range of host niches, with changing niche-specific conditions that are encountered during the process of infection (28). The ability to grow and attach at the temperature characteristic of the human fever is a highly important virulence trait of a pathogen. Our results demonstrated two types of cell response to different temperatures: i) the strains ZIM 2344 and ZIM 2369 showed only weak decrease in adhesion at elevated temperatures (42 °C), as in single and in co-culture with *S. boulardii*, while ii) the strains ZIM 2367 and ZIM 2382 had maximal adhesion in mid-range, at 37 and 39 °C, with minimums at 28 and 42 °C (Fig. 3). What is even more interesting is the shift in the "*S. boulardii* effect" from highly stimulative at 28 °C to repressive at 42 °C. Namely, adhesion of a co-culture at 28 °C was increased for 100% when compared to a single culture, while at 42 °C the adhesion was decreased for around 30% when compared to a single culture. It could be speculated that the concentration of *S. boulardii* cells was smaller at 42 °C because of slower propagation and therefore had smaller influence on *C. glabrata* adhesion. However, this hypothesis fails with the cases of ZIM 2344 and ZIM 2369, where co-cultures showed similar decrease in adhesion at 28 and 42 °C.

The diverse niches occupied by *C. glabrata* within the host environment vary greatly in terms of their ambient pH. Within the human host such pH levels can range from the relatively acidic regions of the stomach and vaginal tract through to the more neutral and basic regions found in the bloodstream and many organs (29). In many pathogenic fungi, ambient pH has been considered as an important factor in adherence to host tissue. If the single cultures from our experiments are considered, uniform conclusions could be reached that *C. glabrata* strains were capable of adhering at all pH values, but with significant preference to more acidic environment (Fig. 4). This finding is in accordance to the niches, such as vaginal tract, where *C. glabrata* is typically found (1). But again, in the co-cultures we observed different responses from the same above-mentioned groups of the *C. glabrata* strains. Both strains, ZIM 2344 and ZIM 2369, showed weakest adhesion at neutral pH, but what is interesting is that at pH 8.5 we observed for the first time with these two strains the indication of the "*S. boulardii* stimulative effect" (Fig. 4). In the case of ZIM 2382, the adherence of the co-culture was several times higher over entire pH range if compared to single cultures. The explanation behind this phenomenon remains opened.

In medical treatment, we hardly control the temperature or the pH of the infection site, but we can fight against pathogens with antimicrobials. There are not many effective antifungal agents due to the scarcity of fungus-specific targets discovered and the rapid development of drug resistance among pathogenic fungi. Also microorganisms that form biofilms are very often resistant to antifungal agents (4, 30). One of the solutions to this problem might be the inhibition of biofilm formation. In this study, we have shown the effect of three clinically important antifungal drugs on the relationship between *C. glabrata* and *S. boulardii* during biofilm formation to polystyrene. We observed poor activity of azoles against *C. glabrata* (Fig. 5), which is consistent with many observations (7, 9, 31). The results show interesting dynamics; as observed in our other experiments, the presence of *S. boulardii* suppressed the adhesion of the strain ZIM 2369 and stimulated the adhesion of the strain ZIM 2367. However, it seems that the increased concentrations of azoles deactivated the "*S. boulardii* effect" in both cases, since the adhesion of co-cultures at high azole concentrations is comparable with the adhesion of single cultures, but not in all cases. Azoles seem to affect *S. boulardii* more than *C. glabrata*, which possibly resulted in the overgrowth of *C. glabrata*.

The presence of other microorganisms, which colonize most of human surfaces, increases the complexity of such adhesion studies. In general, many of today's infectious diseases are directly linked to biofilms in which multiple species coexist in biofilm consortia. Colonization due to the non-*albicans Candida* species is rising, and in recent years a significant increase in bloodstream invasion due to *C. glabrata* and *C. krusei*, especially in debilitated patients with malignancies and bone marrow transplant recipients, is of serious concern (32). Among the species analyzed in the co-cultures, the strongest inhibitory effect on *C. glabrata* adhesion was observed by the strains *C. krusei* CBS 573 and *S. cerevisiae* YJM311, which are both nearly non-adhesive in single cultures. Both *Lactobacillus* strains used in this study showed a similar inhibitory effect on the adhesion of *C. glabrata* strains. Many lactobacilli are known to inhibit the growth of *Candida* spp. in different ways, such as competition for adhesion sites or production of different antagonistic metabolites which inhibit its growth (33). The use of probiotic bacteria to reduce yeasts prevalence in biofilms remains a worthwhile approach. Development of new technologies based on the control of the *Candida* spp. biofilm growth is, thus, foreseen as a major breakthrough in medicine and will have a strong impact in the clinical practice and preventive medicine. On the other hand, the pair *C. glabrata* ZIM 2382 and *S. cerevisiae* YJM 311 was more adhesive than each of both strains in single cultures. Therefore we should explore the mechanisms behind such interactions, which decide between repression and stimulation of biofilm formation.

In conclusion, the results of our studies indicate that *S. boulardii* can have a significant inhibitory effect on the adhesion of *C. glabrata*. Besides, at specific strain ratios we also observed a slight stimulative effect with some *C. glabrata* strains, which highlights the importance of strain specificity and opens further research interests to examine cell wall surfaces of tested strains, which may explain these differences. When environmental conditions are considered, pH and temperature seem not to be decisive factors for the interaction between *C. glabrata* and *S. boulardii*. Antimycotics on the other hand showed more impact, since *S. boulardii* did not manage to have such influence on the co-culture adhesion at higher antimycotics concentrations. However, it can be speculated that *S. boulardii* could substitute the effect of antimycotics in some concentration range and with specific strain types. This would certainly change the view on treating yeast infections.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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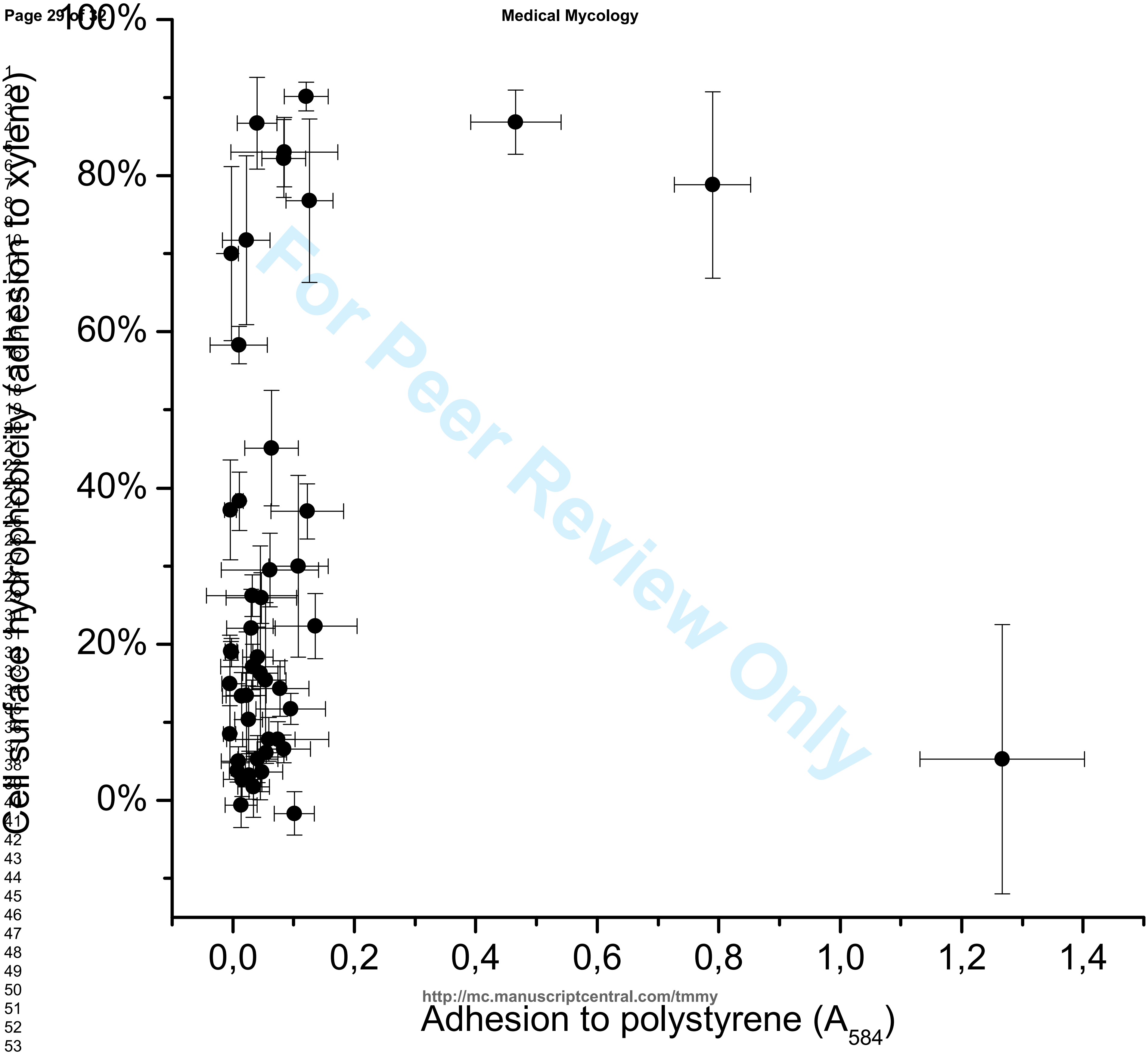
Table 1. The adhesion of the pathogenic yeast *Candida glabrata* in a co-culture with other yeast and bacterial species. The values represent optical densities at 584 nm after the adhesion assay using crystal violet (see Materials and Methods). Inoculum concentration of each strain in a co-culture was 1×10^7 cells/ml, resulting in sum concentration 2×10^7 cells/ml. The experiments were performed with eight replicates and the arithmetic mean of the absorbance values was used.

Strain	*Cg* ZIM 2344	*Cg* ZIM 2369	*Cg* ZIM 2382	*Ck* CBS573	*Sc* YJM311	*Sb* -	*Lr* ZIM B542	*Lc* ZIM B538	average
Cg ZIM 2344	0.95 ± 0.09	0.64 ± 0.10	0.63 ± 0.10	0.41 ± 0.09	0.34 ± 0.09	0.63 ± 0.09	0.57 ± 0.11	0.53 ± 0.11	
Cg ZIM 2369	0.64 ± 0.10	0.74 ± 0.11	0.35 ± 0.08	0.27 ± 0.07	0.20 ± 0.03	0.47 ± 0.08	0.55 ± 0.07	0.49 ± 0.08	
Cg ZIM 2382	0.63 ± 0.10	0.35 ± 0.08	0.09 ± 0.04	0.04 ± 0.02	0.20 ± 0.06	0.24 ± 0.05	0.11 ± 0.08	0.10 ± 0.04	
Ck CBS573	0.41 ± 0.09	0.27 ± 0.07	0.04 ± 0.02	0.04 ± 0.02	-	-	-	-	0.24
Sc YJM311	0.34 ± 0.09	0.20 ± 0.03	0.20 ± 0.06	-	0.03 ± 0.02	-	-	-	0.25
Sb -	0.63 ± 0.09	0.47 ± 0.08	0.24 ± 0.05	-	-	0.00	-	-	0.45
Lr ZIM B542	0.57 ± 0.11	0.55 ± 0.07	0.11 ± 0.08	-	-	-	0.00	-	0.41
Lc ZIM B538	0.53 ± 0.11	0.49 ± 0.08	0.10 ± 0.04	-	-	-	-	0.00	0.37
average	0.50	0.40	0.14						

Absorbance scale	1.0	0.8	0.6	0.4	0.2	0.0

Abbreviations: *Cg*, *Candida glabrata*; *Ck*, *Candida krusei*; *Sc*,


100%
80%
60%
40%
20%
0%
Cell surface hydrophobicity (adhesion to xylene)
0,0
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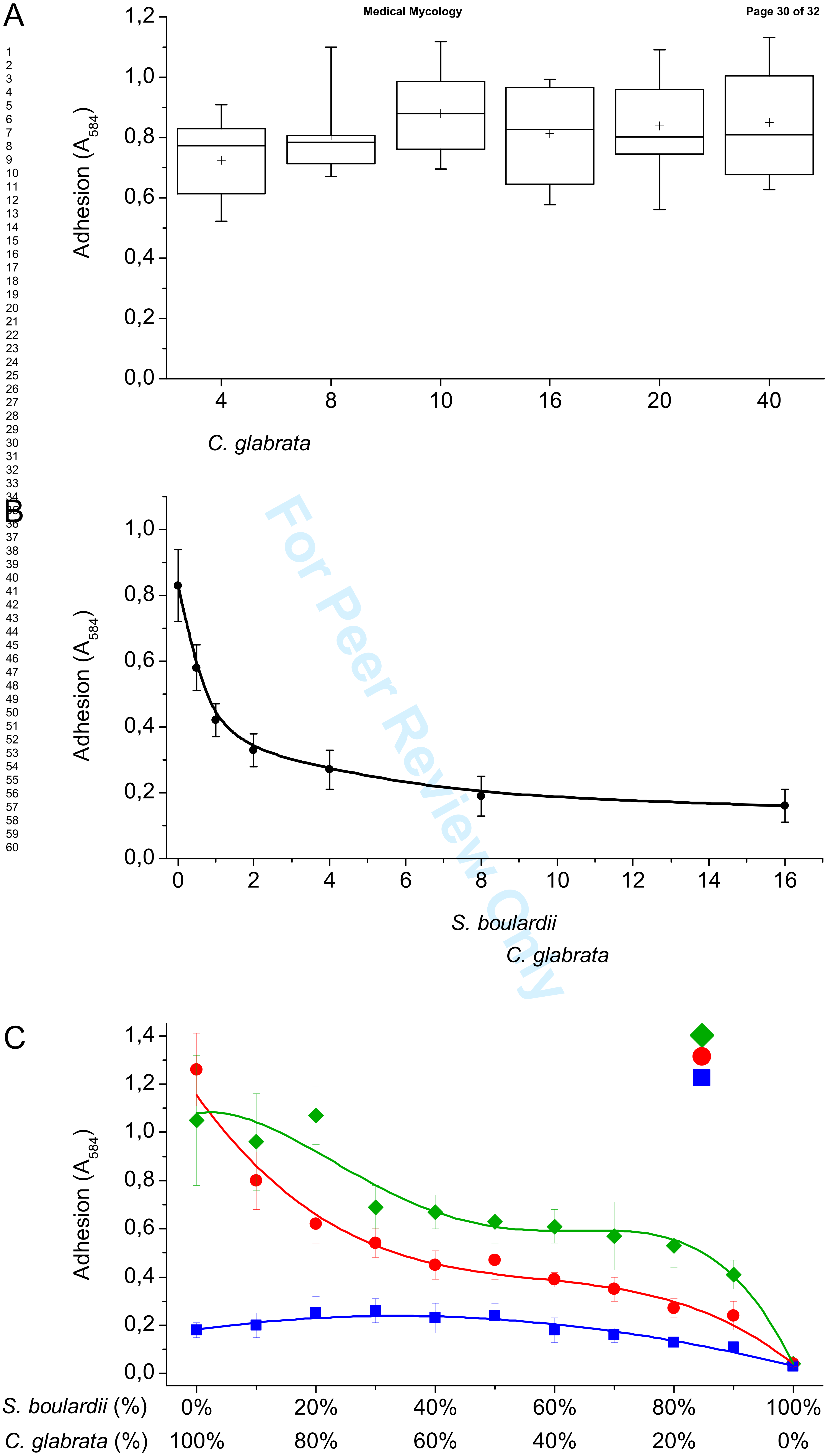
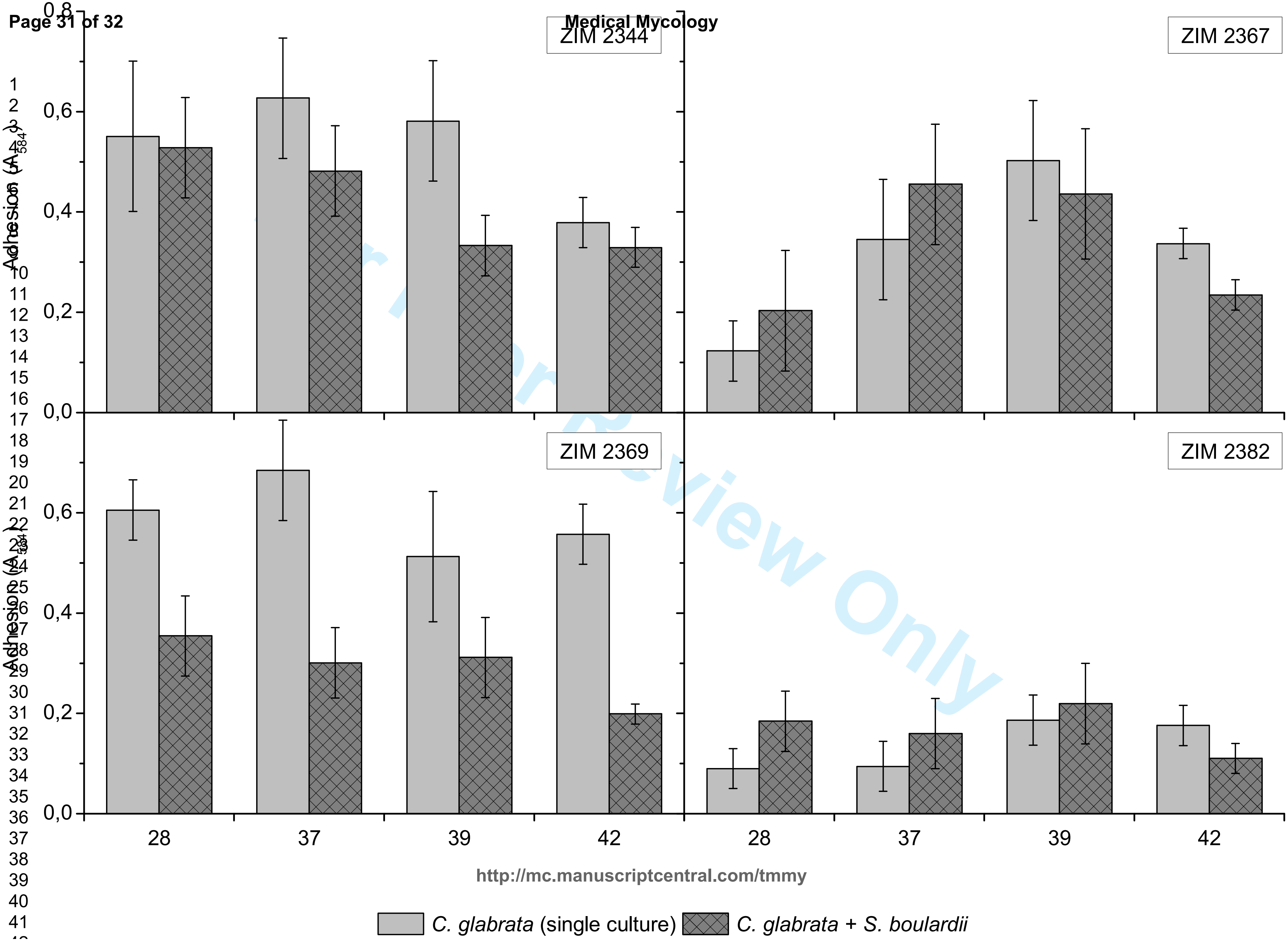
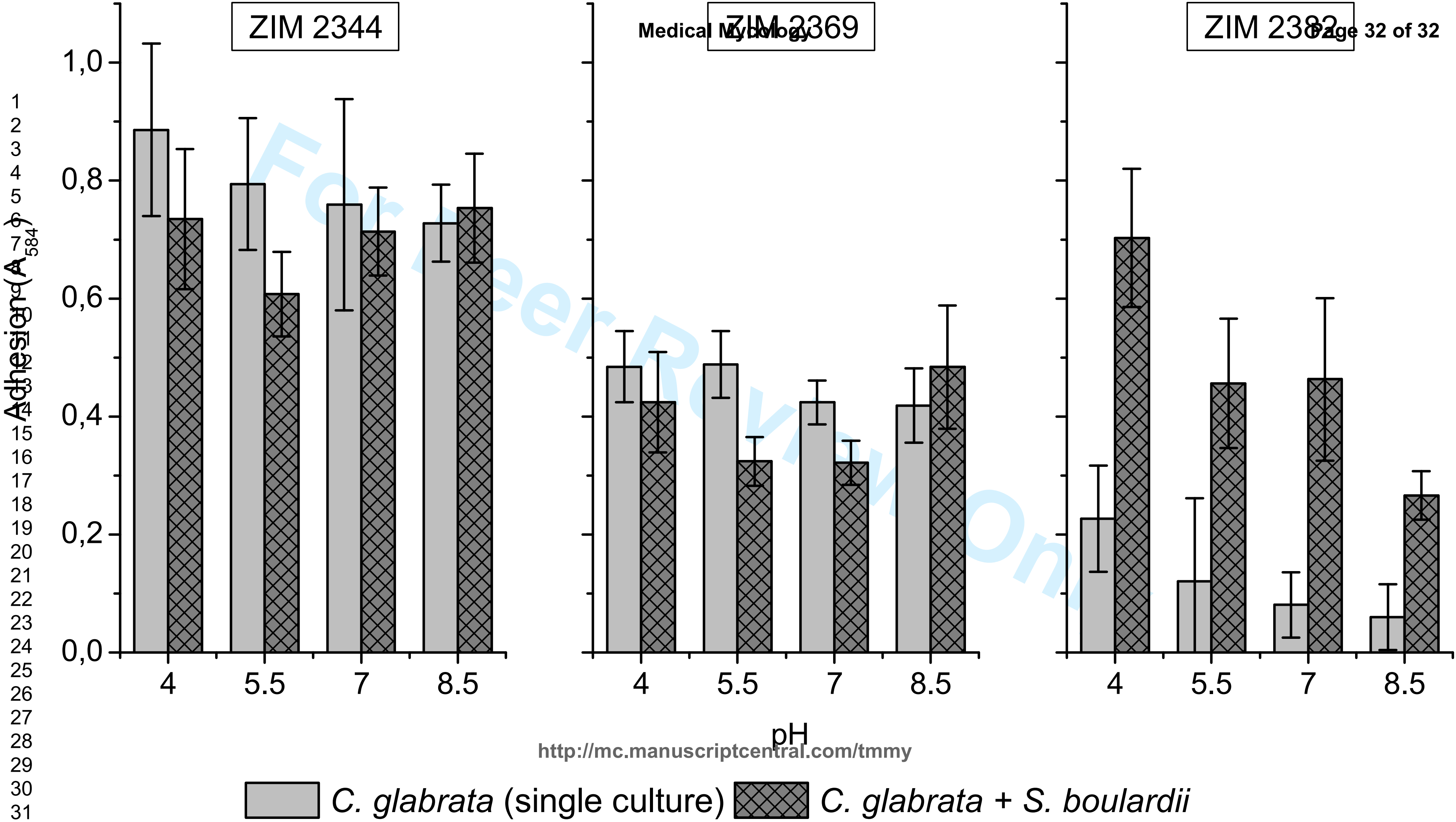
0,4
0,6
0,8
1,0
1,2
1,4
Adhesion to polystyrene (A_{584})


A
Adhesion (A_{584})
1,2
1,0
0,8
0,6
0,4
0,2
0,0
4
8
10
16
20
40
C. glabrata
B
Adhesion (A_{584})
1,0
0,8
0,6
0,4
0,2
0,0
0
2
4
6
8
10
12
14
16
S. boulardii
C. glabrata
C
Adhesion (A_{584})
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
S. boulardii (%)
0%
20%
40%
60%
80%
100%
C. glabrata (%)
100%
80%
60%
40%
20%
0%


Adhesion (A_{584})
0,0
0,2
0,4
0,6
0,8
ZIM 2344
ZIM 2367
ZIM 2369
ZIM 2382
28
37
39
42
C. glabrata (single culture)
C. glabrata + *S. boulardii*


ZIM 2344
ZIM 2369
ZIM 2382
Adhesion (A584)
1,0
0,8
0,6
0,4
0,2
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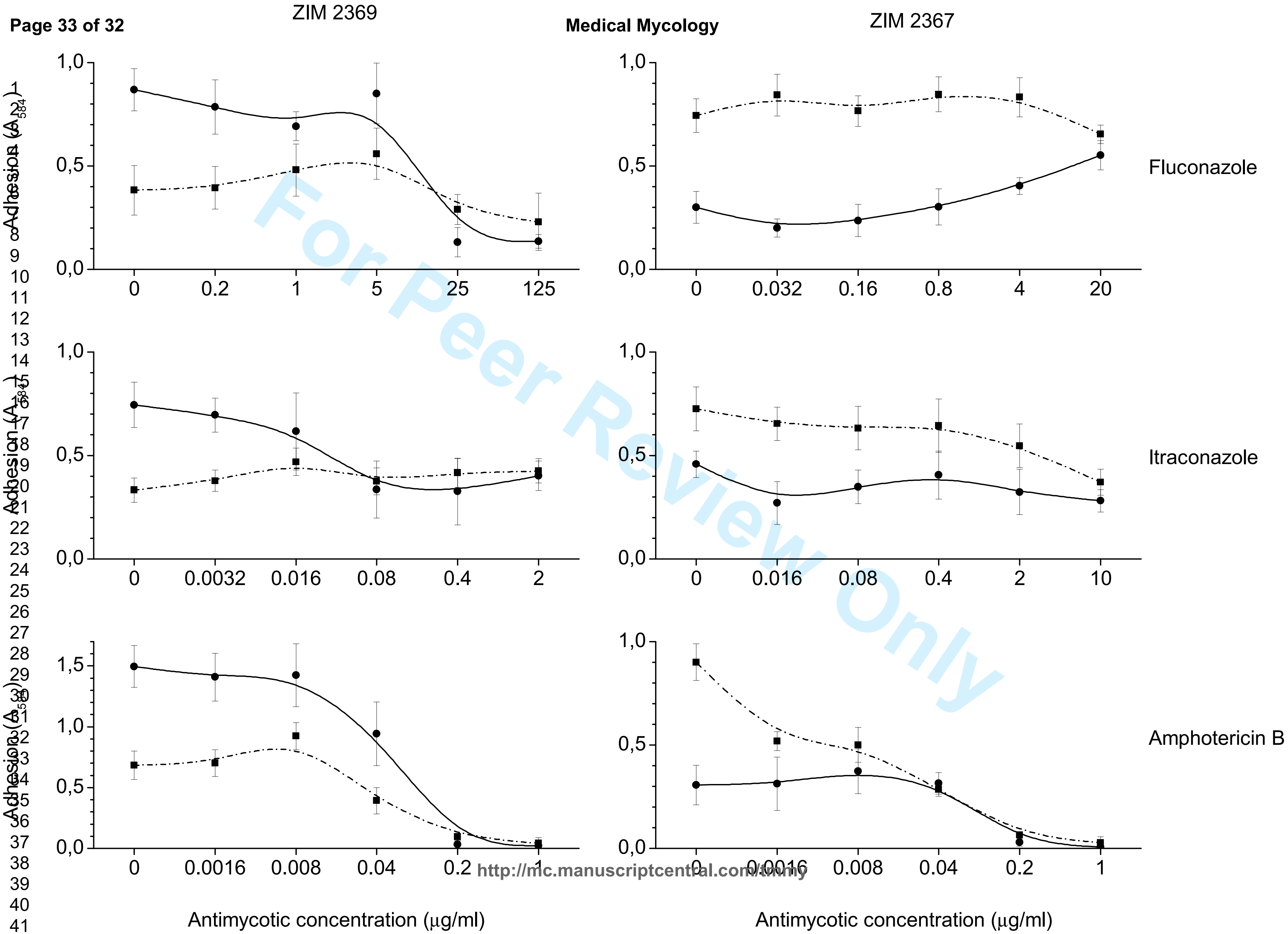
4
5.5
7
8.5
pH
C. glabrata (single culture)
C. glabrata + S. boulardii


ZIM 2369
ZIM 2367
Adhesion (A_{584})
1,0
0,5
0,0
0
0.2
1
5
25
125
0
0.032
0.16
0.8
4
20
Fluconazole
Adhesion (A_{584})
0
0.0032
0.016
0.08
0.4
2
0
0.016
0.08
0.4
2
10
Itraconazole
Adhesion (A_{584})
1,5
1,0
0,5
0,0
0
0.0016
0.008
0.04
0.2
1
Amphotericin B
Antimycotic concentration (μg/ml)
Antimycotic concentration (μg/ml)