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RESEARCH ARTICLE
Host-Microbe Biology

Candida glabrata Has No Enhancing Role in the Pathogenesis of *Candida*-Associated Denture Stomatitis in a Rat Model

Junko Yano,[a] Alika Yu,[a] Paul L. Fidel, Jr.,[b] Mairi C. Noverr[a,b]*

[a]Department of Prosthodontics, Louisiana State University Health Sciences Center School of Dentistry, New Orleans, Louisiana, USA
[b]Department of Oral and Craniofacial Biology, Louisiana State University Health Sciences Center School of Dentistry, New Orleans, Louisiana, USA

ABSTRACT Denture stomatitis (DS) is a condition characterized by inflammation of the oral mucosa in direct contact with dentures and affects a significant number of otherwise healthy denture wearers. *Candida*-associated DS is predominantly caused by *Candida albicans*, a dimorphic fungus that readily colonizes and forms biofilms on denture materials. Previous studies showed a requirement for *Candida* biofilm formation on both palate and dentures in infection and identified fungal morphogenic transcription factors, Efg1 and Bcr1, as key players in DS pathogenesis. While both *C. albicans* and *Candida glabrata* are frequently coisolated in mucosal candidiasis, a pathogenic role for *C. glabrata* in DS remains unknown. Using an established rat model of DS, we sought to determine whether *C. glabrata* alone or coinoculation with *C. albicans* establishes colonization and causes palatal tissue damage and inflammation. Rats fitted with custom dentures were inoculated with *C. albicans* and/or *C. glabrata* and monitored over a 4-week period for fungal burden (denture/palate), changes in body weight, and tissue damage via lactate dehydrogenase (LDH) release as well as palatal staining by hematoxylin and eosin (H&E) and immunohistochemistry for myeloperoxidase (MPO) as measures of inflammation. *C. glabrata* colonized the denture/palate similarly to *C. albicans*. In contrast to *C. albicans*, colonization by *C. glabrata* resulted in minimal changes in body weight, palatal LDH release, and MPO expression. Coinoculation with both species had no obvious modulation of *C. albicans*-mediated pathogenic effects. These data suggest that *C. glabrata* readily establishes colonization on denture and palate but has no apparent role for inducing/enhancing *C. albicans* pathogenesis in DS.

IMPORTANCE Many denture wearers suffer from *Candida*-associated denture stomatitis (DS), a fungal infection of the hard palate in contact with dentures. Biofilm formation by *Candida albicans* on denture/palate surfaces is considered a central process in the infection onset. Although *Candida glabrata* is frequently coisolated with *C. albicans*, its role in DS pathogenesis is unknown. We show here, using a contemporary rat model that employed a patented intraoral denture system, that *C. glabrata* established stable colonization on the denture/palate. However, in contrast to *C. albicans* inoculated rats, rats inoculated with *C. glabrata* exhibited minimal changes in weight gain or palatal tissue damage. Likewise, coinoculation with the two *Candida* species resulted in no exacerbation of *C. albicans*-induced DS pathology. Together, our findings indicate that *C. glabrata* has no inducing/enhancing role in DS pathogenesis.

KEYWORDS *Candida albicans*, *Candida glabrata*, biofilms, candidiasis, host-pathogen interactions, mycology

Denture stomatitis (DS) is an inflammatory fungal infection, presenting primarily as inflammation of oral mucosa beneath maxillary dentures (1–7). DS is by far the most common form of oral candidiasis, affecting approximately 70% of otherwise

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Address correspondence to Mairi C. Noverr, mnoverr@tulane.edu.

* Present address: Mairi C. Noverr, Department of Microbiology and Immunology, School of Medicine, Tulane University, New Orleans, Louisiana, USA.

P.L.F. and M.C.N. contributed equally as senior authors.

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healthy denture wearers (8). DS is predominantly caused by *Candida albicans*, a dimorphic fungus that readily colonizes and forms biofilms on denture materials; however, non-*albicans Candida* species can also be associated with infection (9, 10). *Candida glabrata* is the second most common isolate, and up to 50% of patient samples contain more than one species of *Candida*, very often a combination of *C. albicans* and *C. glabrata* (3, 11–13). Manifestations of *Candida*-associated DS can range from being painless and asymptomatic to severe, involving erythematous and edematous palatal mucosa, painful inflammation, papillary hyperplasia (small pebble-like sores), and petechial hemorrhage (pinpoint bleeding) (14, 15). DS can have a negative impact on the quality of life of those affected, with high recurrence rates despite treatment with antifungal therapy (13, 16–21). Chronic DS infection could lead to seeding of the gastrointestinal tract, which serves as a major portal for systemic infection in immunosuppressed or hospitalized patients. Despite its high prevalence, the role of fungal virulence factors in the pathogenesis of DS has not been well defined.

Previous studies using an established rat model of DS showed a requirement for *Candida* biofilm formation on both palatal epithelium and denture surfaces in the initiation of infection and identified regulators of fungal morphogenesis (Efg1) and biofilm formation (Bcr1) as key players in DS pathogenesis (22). While *C. glabrata*, unlike *C. albicans*, does not undergo morphogenesis and thus is considered less virulent, both *Candida* species are frequently coisolated in mucosal candidiasis, including DS (9, 10, 23–25). Although single-species infection by *C. glabrata* alone is relatively rare, oral infections involving *C. glabrata* have shown an increasing trend over the past decade, especially in cancer patients, denture wearers, or those receiving prolonged antibiotic, steroid, or head and neck radiation therapies (10, 26–29). In addition, since *C. glabrata* displays significant resistance to azole antifungal drugs (23, 30–32), successful treatment of DS is likely challenging in cases of coinfection by both *Candida* species.

Despite its presence and ability to establish infection in animal models of oropharyngeal candidiasis (OPC) (33, 34), a pathogenic role for *C. glabrata* in DS remains unknown. In terms of adherence to biotic/abiotic surfaces, biofilm formation, and host tissue invasion, *C. albicans* has a major advantage over *C. glabrata* by its ability to transition from yeast to hyphae. In addition, *C. albicans* hyphal adhesins, such as agglutinin-like sequence (ALS) proteins and hyphal wall protein 1 (HWP1), also play an important role as binding sites for *C. glabrata* and other microorganisms, including *Staphylococcus aureus* (33, 35–39). *C. glabrata* virulence, on the other hand, likely involves cell wall proteins expressed independent of its morphology (35, 40–42). It is possible that cocolonization by *C. glabrata* with *C. albicans* may have additive impacts on virulence and pathogenicity compared to that by either species alone.

Using an established rat model of DS with a contemporary rodent denture system, we sought to determine whether *C. glabrata* alone or in combination with *C. albicans* establishes colonization and/or causes/enhances palatal tissue damage and inflammation.

RESULTS

***C. glabrata* establishes consistent colonization on dentures and palate tissues *in vivo*.** Rats installed with the denture system were inoculated with *C. glabrata* or *C. albicans* individually or the two species together and monitored longitudinally for a 4-week period. Fungal burden measured by swab collection demonstrated a consistent colonization with *C. glabrata* alone on the palate (Fig. 1A) and denture (Fig. 1B), similar to that with *C. albicans*. Coinoculation with the two *Candida* species resulted in a marked, but not statistically significant, increase in *C. glabrata* fungal burden (10- to 100-fold on dentures and palates at 2 to 3 weeks postinoculation). Levels of *C. albicans* were unaffected by coinoculation with *C. glabrata*.

***C. glabrata* has no inducing or enhancing effects on *C. albicans* virulence.** Inoculated rats were evaluated for levels of LDH release by the palate, an indicator of tissue damage. Repeated measures analysis indicated that animals inoculated with *C. albicans*, alone or together with *C. glabrata*, exhibited significant modulation in levels

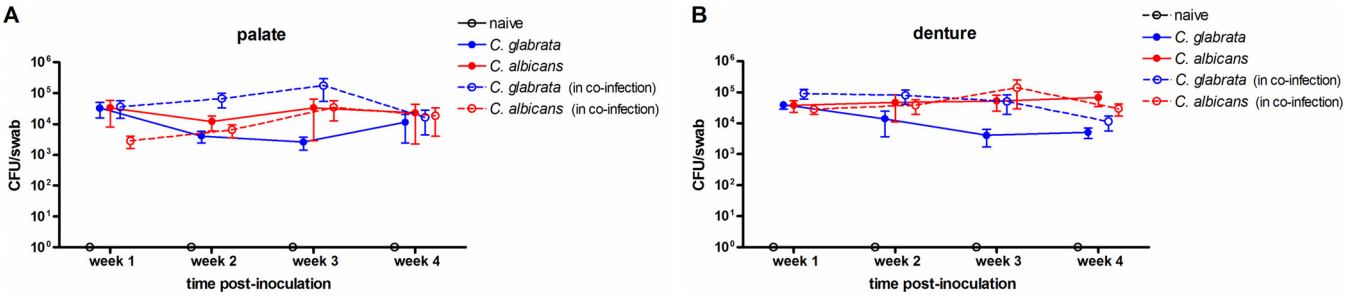

FIG 1 Fungal burden on dentures and palate tissues in rats inoculated with *C. albicans* and/or *C. glabrata*. Rats fitted with dentures were inoculated 3 times at 3-day intervals with 1×10^9 CFU *C. albicans*, *C. glabrata*, or both species together (5×10^8 CFU each). Swab samples of the palate (A) and denture (B) were collected weekly for a period of 4 weeks postinoculation. Fungal burden was assessed from overnight cultures of swab suspension fluid from the removable denture and associated palate tissue. Figures represent cumulative results from 2 independent experiments with 2 to 5 animals per group. Data were analyzed using repeated measures ANOVA (longitudinal data for each group) and one-way ANOVA (individual time points between groups) followed by the unpaired Student's *t* test (experimental versus control groups at individual time points).

of lactate dehydrogenase (LDH) over the course of infection ($P = 0.003$ and $P = 0.002$, respectively) (Fig. 2). In contrast, inoculation with *C. glabrata* alone induced minimal palatal LDH release with no apparent change under a consistent state of colonization. An indirect measure of virulence during infection is stunted weight gain over time, indicating a sign of DS-related discomfort in eating due to tissue damage in the oral cavity. Consistent with the lack of palatal tissue damage, colonization by *C. glabrata* alone resulted in normal weight gain comparable to that by naive animals over the 4 week period (Fig. 3). Conversely, animals inoculated with *C. albicans* alone or together with *C. glabrata* exhibited stunted weight gain (Fig. 3).

***C. glabrata* does not promote inflammation.** Palate tissues from inoculated rats at 4 weeks postinoculation were examined for evidence of inflammation. Histological analysis of palatal mucosa of rats inoculated with *C. glabrata* alone revealed few or no cellular infiltrates in lamina propria, with intact epithelial layers similar to naive tissues (Fig. 4, hematoxylin and eosin [H&E]). In contrast, palates from rats inoculated with *C. albicans* alone or together with *C. glabrata* demonstrated copious amounts of cellular infiltration as well as epithelial thinning and sloughing. Finally, the expression of the inflammatory marker myeloperoxidase (MPO) was markedly elevated by *C. albicans* colonization alone compared to that by *C. glabrata* colonization alone, with the combination of the two species showing moderate expression (Fig. 4, anti-MPO).

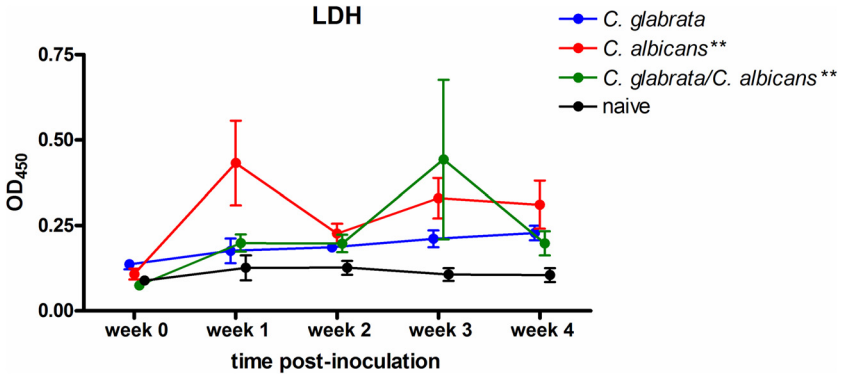

FIG 2 Palatal tissue damage over time in rats inoculated with *C. albicans* and/or *C. glabrata*. Rats fitted with dentures were inoculated 3 times at 3-day intervals with 1×10^9 CFU *C. albicans*, *C. glabrata*, or both species together (5×10^8 CFU each). Swab samples of the palate over the removable denture portion were collected weekly for a period of 4 weeks postinoculation. Swab suspension fluid was tested for LDH levels. Figure represents cumulative data from 2 independent experiments with 2 to 5 rats per group. Data were longitudinally analyzed by repeated measures ANOVA (significance indicated on graph legend) and comparatively analyzed by one-way ANOVA (individual time points between groups) followed by the unpaired Student's *t* test at specific time points. **, $P < 0.01$.

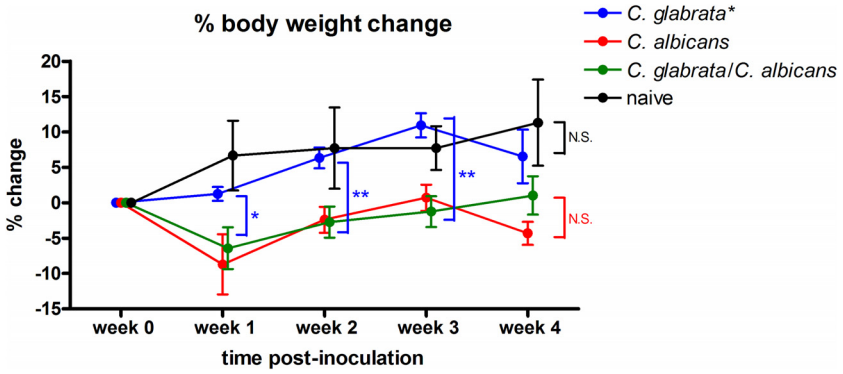

FIG 3 Body weight change over time in rats inoculated with *C. albicans* and/or *C. glabrata*. Rats fitted with dentures were inoculated 3 times at 3-day intervals with 1×10^9 CFU *C. albicans*, *C. glabrata*, or both species together (5×10^8 CFU each). Rats were weighed weekly for a period of 4 weeks postinoculation to assess the percent weight change (% weight change = [weight at time point/weight at week 0 prior to inoculation] × 100). Figure represents cumulative data from 2 independent experiments with 2 to 5 rats per group. Data were longitudinally analyzed by repeated measures ANOVA (significance indicated on graph legend) and comparatively analyzed by one-way ANOVA (individual time points between groups) followed by the unpaired Student's *t* test at specific time points (significance indicated on data points). *, $P < 0.05$; **, $P < 0.01$; N.S., not significant.

DISCUSSION

In the present study using the contemporary rodent denture system, we demonstrated that *C. glabrata* has the ability to establish consistent colonization on both denture surfaces and palate tissues. *C. glabrata* is typically difficult to establish consistent colonization in experimental model systems involving biotic surfaces, presumably due to the lack of morphologic transition to hyphae as a virulence factor. For example, *C. glabrata* alone showed poor colonization on oral or vaginal reconstituted human epithelium (RHE) *in vitro* (35, 43, 44). *In vivo* models of murine oropharyngeal candidiasis (OPC) and vulvovaginal candidiasis (VVC) require corticosteroid-induced immunosuppression and a streptozotocin-induced diabetic state, respectively, to achieve consistent colonization (33, 34, 45). In the present DS model using immunocompetent rats, however, dentures appeared to serve as a stable reservoir for *C. glabrata* to sustain colonization. Indeed, *C. glabrata* is capable of growing on a variety of abiotic surfaces (34, 46, 47). The trend toward increased *C. glabrata* burden during cocolonization with *C. albicans* is consistent with recent evidence showing enhanced colonization by *C. glabrata* in a mouse OPC model following coinoculation with *C. albicans* (33). However,

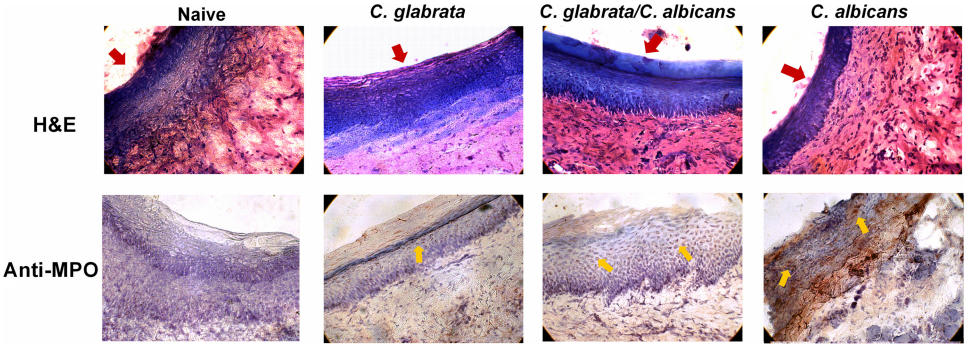

FIG 4 Histological analysis of palatal inflammation in rats inoculated with *C. albicans* and/or *C. glabrata*. Rats fitted with dentures were inoculated 3 times at 3-day intervals with 1×10^9 CFU *C. albicans*, *C. glabrata*, or both species together (5×10^8 CFU each). Palate tissue was harvested at 4 weeks postinoculation. Frozen tissue sections were stained with hematoxylin and eosin (H&E) for histopathological analysis or with anti-myeloperoxidase (MPO, brown-red) or isotype control (mouse IgG1) antibodies. Red arrows indicate the apical surface of the palate epithelium. Yellow arrows represent cells positively stained for MPO. Figure shows a representative result of 2 independent experiments. Magnification, ×400.

the lack of any statistically significant increase is more in line with studies reporting no changes in *C. glabrata* burden between mono- and cocolonization (34, 45). Hence, the observation is likely a minor attribute overall and does not appear to be suggestive of a synergistic outcome.

Biofilm formation by *C. albicans* has been exhaustively studied *in vitro* and *in vivo*, where hyphae provide scaffold structures that are essential for developing robust biofilms (22, 48–52). Furthermore, there is increasing evidence demonstrating that microorganisms preferentially bind to *C. albicans* hyphae in a polymicrobial environment (37, 39). This is presumably due to the fact that fungal adhesins are abundantly expressed on hyphal cell walls (33, 35–37, 53–55). Adherence to the hyphal surface and growth within biofilms are advantageous to many planktonic microbes in which the fungal polysaccharide extracellular matrix can provide protection from host defense and resistance to environmental stress and antimicrobials (56, 57). Interestingly, recent studies showed that despite its ability to colonize murine mucosal surfaces, colonization with *C. glabrata* alone did not result in appreciable biofilm formation on oral and vaginal epithelia (33, 45). This suggests that robust biofilm formation is not required for the survival of *C. glabrata* at mucosal sites. Although biofilms were not evaluated in our present study, we expect biofilm growth to be minimal on both palate mucosa and dentures in the absence of *C. albicans*. Support for this comes from our previous finding that hypha-deficient mutant strains of *C. albicans* failed to form mature biofilms despite sustained colonization (22). We hypothesize that the stable colonization of the palatal mucosa by *C. glabrata* or hypha-deficient *C. albicans* mutants is facilitated by the denture that serves as an adherence catalyst and feeder system for the mucosal tissue.

Contrary to its vigorous adhesion and colonization capacity, our results indicated that *C. glabrata* alone was not competent to cause a similar pathology observed in *C. albicans*-associated DS (tissue damage, weight loss, or palate inflammation) nor could it enhance *C. albicans* pathogenicity under coinoculated conditions. The lack of a pathogenic role for *C. glabrata* in monospecies colonization appears to be a common feature in several *in vitro* and *in vivo* models. Studies using oral epithelial cell culture showed no notable increase in proinflammatory cytokine production in response to *C. glabrata* alone (58, 59). Similarly, recent reports from both mouse OPC and VVC studies indicated that *C. glabrata* monoinfection resulted in only mild weight loss (OPC) and vaginal inflammation (VVC) (33, 45). Hence, our model, as well as others, has not been able to provide any clear evidence for a pathogenic role for *C. glabrata* monospecies infection at mucosal sites. It is possible, however, that *C. glabrata* monoinfections result in a more appreciable pathology in denture wearers under immunocompromising conditions (e.g., use of chemotherapies, prolonged antibiotics, advanced age).

The lack of any enhanced pathology under coinoculated conditions was surprising considering that coinoculation resulted in fungal burden (i.e., biomass) that was virtually doubled on both palate and dentures despite the reduced inoculum for each species (5×10^8 for a total of 1×10^9). In fact, one inflammatory marker, MPO, was actually decreased under coinoculated conditions. This result is likely due to the fact that DS occurs in immunocompetent subjects, both clinically and in our experimental model using immunocompetent rats. In agreement with this, studies in an immunocompetent mouse model of VVC (45), which resulted in a similar additive effect in fungal burden under coinoculated conditions, showed no changes in inflammatory response/tissue damage. On the other hand, studies using an immunosuppressed mouse OPC model (33) showed increased tissue damage and invasion during coinfection. Similarly, *in vitro* studies using a 3-dimensional (3-D) human oral mucosa model (60) or oral RHE model (43), which do not include immune cells, demonstrated *C. glabrata* strain-dependent effects on promoting tissue damage and invasion, even in the context of coinfection with *C. albicans* (43). While it is possible that the results in the DS model were strain dependent, the *C. glabrata* isolate chosen was based on its strong mucosal colonization capacity (45) and use in other model systems (VVC and intra-abdominal infection) (45, 61). Hence, while the isolate was not an oral isolate, it appeared representative for experimental models. Moreover, more recent studies in the

intra-abdominal model using an oral *C. glabrata* isolate in parallel with the vaginal isolate yielded similar results (M. C. Noverr, unpublished observations), further supporting that strain-dependent attributes of *C. glabrata* pathogenicity in the DS model were unlikely. Additionally, in the OPC model, intimate binding of *C. glabrata* with *C. albicans* hyphae was observed, indicating that *C. glabrata* possibly exploits *C. albicans* to establish colonization and gain invasion into the oral epithelium under immunocompromised conditions (33, 43). In the VVC model, coinoculation with *C. glabrata* and *C. albicans* displayed a more interspersed presence throughout the tissue, with little interaction or colocalization, suggesting that the two species exist independent of each other (45). Therefore, interspecies interactions may also play pathogenic roles in OPC versus VVC. Because *C. albicans* rarely invades the hard palate, the likelihood that the two species would interact such to exploit each other in DS is low. Taken together, these arguments support the interpretation that there is no apparent contribution of *C. glabrata* in *C. albicans*-mediated DS pathogenesis.

Despite these results, a pathogenic potential of *C. glabrata* should not be underestimated due to its inherent resistance to azole compounds. Inadequate diagnosis and treatment of seemingly noninvasive *C. glabrata* infections could lead to more severe yet underreported cases of *C. glabrata*-associated candidiasis (e.g., fungal otitis, candidemia, candiduria) (62–67), which could potentially be a life-threatening condition if not treated in a timely manner. There is also the issue of microbial access to the gastrointestinal tract, where a continuous gastrointestinal exposure to *Candida* originating from denture biofilms could have a detrimental effect in denture wearers under immunocompromising conditions or those with advanced age who are at risk for immunosuppression. Indeed, patients with chronic DS have increased *Candida* carriage in the gastrointestinal tract, with similar species isolated from the oral cavity and feces (68). We also observed both *C. albicans* and *C. glabrata* in feces of inoculated mice, albeit in lower numbers than in the oral cavity (data not shown). As such, the rodent denture system represents an excellent model to further investigate these important pathogenesis questions along the entire oro-gastrointestinal tract.

MATERIALS AND METHODS

Animals. Male CD hairless rats (7 weeks old) were purchased from Charles River Laboratories (Willington, MA). All rats were maintained in an AAALAC-accredited animal facility at Louisiana State University Health Sciences Center (LSUHSC) under a protocol approved by LSUHSC Institutional Animal Care and Use Committee. The animals were weaned onto gel diet A76 (ClearH2O, Westbrook, ME) and acclimated for at least 1 week prior to denture installation. The animals were maintained on the gel diet for the remainder of the study to minimize the accumulation of food debris on the denture.

***Candida* species strains.** *C. albicans* strain DAY185, a prototrophic derivative of SC5314, was a gift from Aaron Mitchell (Carnegie Melon University, Pittsburgh, PA). *C. glabrata* strain LF 574.92 was provided by Jack Sobel (Wayne State University, Detroit, MI). Both *Candida* strains were grown in yeast extract-peptone-dextrose (YPD) broth for 18 h at 30°C with shaking at 200 rpm to reach a stationary-phase culture. Following incubation, the culture was washed 3 times in sterile phosphate-buffered saline (PBS) and enumerated on a hemocytometer using trypan blue dye.

Rat denture stomatitis model. Each rat was housed separately in an individual cage throughout the study period and handled according to institutionally recommended guidelines. A custom-fitted rodent denture system, consisting of fixed and removable portions, was employed (patent 8753113) (69, 70). For custom fitting, impressions of the palate were taken from individual rats using light-body VPS impression material (Aquasil Ultra LC; Dentsply Caulk). Impressions were used to produce stone mold templates for the fabrication of the fixed and removable denture components. For installation, rats were anesthetized by intraperitoneal injection with 90 mg/kg ketamine plus 10 mg/kg xylazine and remained sedated for at least 1 h to complete the installation process. The fixed portion of the denture containing nickel magnets was anchored to the rear molars by orthodontic ligature wires. The removable portion embedded with an aluminum rod was attached to the fixed portion via the nickel magnets and fitted over the anterior palate. The removable portion can easily be detached for sampling and replaced, which allows for longitudinal analyses. The rats installed with the dentures were given an additional acclimation period to ensure normal food and water intake. For inoculation, rats were anesthetized by isoflurane inhalation and inoculated by applying an oral gel (PBS semisolidified with 5% carboxymethylcellulose; Sigma) containing *C. albicans* (1×10^9), *C. glabrata* (1×10^9), or the two species together (5×10^8 each) on the palate beneath the removable denture. The rats remained anesthetized until the removable denture was securely reinstalled with the gel inoculum in place. Inoculation was performed a total of 3 times separated by 3-day intervals, and rats were monitored weekly over a 4-week period for oral outcome parameters, signs of distress, and weight changes. Control animals (naive) were rats with dentures installed and given gel alone.

Quantification of microbial burden. To assess fungal burden on the denture and palate tissue, rats were anesthetized by isoflurane inhalation, and the removable portion of the denture was detached using sterile forceps. The intaglio surface of the denture and the palate were swabbed with individual sterile cotton tipped applicators. Swabbing was performed by gently sliding the cotton applicator on the denture surface or the hard palate along the ridges of the rugae. Swab tips were immersed in 200 μl PBS and vigorously mixed. To assess fungal burden, serial dilutions of the swab supernatants were cultured on Sabouraud dextrose agar (BD Diagnostics) for 24 h at 37°C. CFUs were enumerated and expressed as CFU/swab.

Assessment of palatal tissue damage. To determine tissue damage, the levels of lactose dehydrogenase (LDH) release in palates were measured by an LDH assay kit as per the manufacturer's instructions (Abcam). The activity of LDH in the supernatants of palate swab suspensions was measured with a colorimetric probe. The absorbance was read at a wavelength of 450 nm using a Multiskan Ascent microplate photometer (Labsystems). The results were expressed as the optical density at 450 nm (OD_{450}).

Microscopic evaluation of palatal tissues. Palate tissue was excised from euthanized rats at 4 weeks postinoculation. Tissue specimens were placed in Tissue-Tek cryomolds (Miles Corp.) containing optimum cutting temperature (OCT) medium (Sakura Finetek) and stored at −80°C. Frozen tissue was sectioned (6 μm) and collected on glass slides. The slides were either processed for a hematoxylin and eosin (H&E) staining for histology or fixed in ice-cold acetone for 5 min and stored at −20°C until use. For immunohistochemical analysis, tissue sections were hydrated in PBS and processed using a cell and tissue staining kit (horseradish peroxidase [HRP]-3-amino-9-ethylcarbazole; R&D Systems). Briefly, tissue slides were blocked with peroxidase, goat serum, avidin, and biotin blocking buffers and then incubated with monoclonal mouse anti-rat myeloperoxidase (MPO) antibody (10 μg/ml; R&D Systems) or isotype control antibody (mouse IgG1) overnight at 4°C. The slides were washed and incubated with biotinylated anti-mouse IgG antibodies for 1 h at room temperature followed by streptavidin-HRP for 30 min. The slides were then reacted with AEC chromogen substrate, counterstained with CAT hematoxylin (Biocare Medical), and preserved in aqueous mounting medium (R&D Systems). Images were captured at ×400 magnification.

Statistics. All experiments included groups of 2 to 5 rats and were repeated twice. Longitudinal data of fungal burden, LDH levels, and percent weight change were analyzed by repeated measures analysis of variance (ANOVA) to identify changes over time within each group. Data were further analyzed using a one-way ANOVA followed by the Tukey's *post hoc* multiple-comparison test to identify differences between groups at specific time points. The Student's *t* test was used to compare the experimental groups to relevant control groups. Statistical significance was defined at a confidence level where P was <0.05. All statistical analyses were performed using Prism software (Graph Pad).

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