

Phocaeicola vulgatus mpk colonization protects epithelial cells from *Candida albicans* infection

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Abstract

Candida albicans is an opportunistic pathogen residing in the gastro-intestinal tract of humans from where it can cause life-threatening systemic infection. Dysbiosis is one predisposing factor for *C. albicans* overgrowth, indicating that commensal bacteria limit fungal growth and convey colonization resistance. *Phocaeicola vulgatus* (formerly classified as *Bacteroides*) is an abundant gut commensal. We show that *P. vulgatus* can protect enterocytes *in vitro* from *C. albicans* damage. The protective effect is most pronounced if the bacteria pre-colonize host cells 6 h prior to addition of *C. albicans*. Colonization of the enterocytes with *P. vulgatus* leads to reduced adhesion of *C. albicans*, shorter hyphae, and increased fungal shedding, while the overall fungal burden is not reduced. The protective effect is contact-dependent but can be elicited to some degree by heat-inactivated bacterial cells. Our findings suggest that multiple mechanisms mediate the protective effect, including activation of self-defenses of the host cells to shed the pathogen, as well as a direct antagonistic interaction between the fungi and the bacteria targeting *C. albicans* filamentation, and thereby hyphae-associated virulence factors.

Keywords *Candida albicans*, *Phocaeicola vulgatus*, *Bacteroides vulgatus*, colonization, antagonism, *in vitro* model

Introduction

The intestinal tract is densely colonized by microorganisms that contribute to host health by providing vitamins, partaking in food degradation, stimulating formation of epithelial barrier function, and training of the immune system (Hou et al. 2022, McCallum and Tropini 2024). Furthermore, the microbiota can control proliferation of facultative pathogenic microbes by competing for nutrients and adhesion sites, producing antimicrobial compounds such as bacteriocins, and indirectly by its effects on barrier function and the immune system (Caballero-Flores et al. 2023, de Nies et al. 2023). Consequently, depletion of the bacterial microbiota by antibiotic treatment is a risk factor for certain opportunistic infections. One example is candidiasis, infections caused by fungi belonging to the genus *Candida* (de Nies et al. 2023, Lass-Flörl et al. 2024), especially *Candida albicans* (Eggimann et al. 2015, Pap-

as et al. 2018) that has been designated as a fungal pathogen of critical priority by the WHO (WHO 2022). Both mucosal and life-threatening disseminated *C. albicans* infections are commonly caused by strains colonizing the individual before dysbiosis facilitates fungal growth and infection (Bougnoux et al. 2006, Papon et al. 2020, Zhai et al. 2020).

Anaerobic bacteria are especially important commensals since their loss is associated with reduced colonization resistance to *C. albicans* (Fan et al. 2015). *Blautia producta* and *Bacteroides thetaiotaomicron* significantly reduce *C. albicans* burden in germ-free and wild-type mice by inducing the production of the intestinal epithelial-derived antimicrobial peptide CRAMP (the equivalent to LL-37 in humans) (Fan et al. 2015). Commensal anaerobes are also producers of short chain fatty acids (SCFA), which correlate negatively with *C. albicans* colonization levels in cecal content of mice and stool samples in humans (Guinan et al. 2019,

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Seelbinder et al. 2020). SCFA act both as positive immunomodulators on epithelial cells and as direct inhibitors of *C. albicans* virulence factors (Mann et al. 2024, McCrory et al. 2024). Additionally, SCFA are consumed by epithelial cells and bacteria under oxygen consumption and thus contribute to a hypoxic epithelial state which limits *Candida* expansion (Kelly et al. 2015, Savage et al. 2024). Apart from SCFA, a variety of bacterial metabolites negatively affect *C. albicans* colonization, such as carboxylic acids and secondary bile acids (Gutierrez et al. 2020, Mirhakkak et al. 2021). Bacteria can also directly target *C. albicans*: The *Enterococcus faecalis* bacteriocin EntV limits yeast-to-hyphae transition and thereby biofilm formation *in vitro* and *in vivo* (Graham et al. 2017). *Serratia marcescens* uses the type VI secretion system to deliver toxic effector proteins into fungal cells (Trunk et al. 2018). *Salmonella enterica* serovar Typhimurium can kill *C. albicans* with *sopB* effectors released by the type III secretion system (Kim and Mylonakis 2011), and *Streptococcus mutans* targets hypha formation using a fatty acid called Streptococcus Diffusible Signal Factor (SDSF) (Vilchez et al. 2010). Effective bacterial antagonization of *C. albicans*, be it direct or indirect, contributes to limitation of *C. albicans* overgrowth and thereby reduces one major risk factor for candidiasis (Mishra and Koh 2021).

Another risk factor for disseminated candidiasis is damage to the mucosal intestinal barrier leading to fungal translocation to the peritoneal cavity or blood stream (Basmaciyan et al. 2019, Lass-Flörl et al. 2024). *In vitro* models mimicking the intestinal epithelial layer have been helpful to understand the site specific bacterial-fungal interaction that limit pathogenicity. In a mixed enterocyte infection model *Lactobacillus rhamnosus* reduced fungal elongation, prevented translocation across the epithelial barrier and promoted fungal shedding from the host cell surface (Graf et al. 2019). *Lactobacillus rhamnosus* reshaped the metabolic environment, depleting *C. albicans* preferred nutrient sources and consequently reduced fungal pathogenicity (Alonso-Roman et al. 2022). In the same model the commensal *Escherichia coli* Nissle 1917 reduced fungal damage by impacting fungal growth even without direct contact, suggesting the involvement of secreted factors (Rebai et al. 2023).

In this study we investigated the effects of the anaerobic commensal *P. vulgatus* mpk (formerly classified as *Bacteroides* (García-López et al. 2019)) on *C. albicans*-mediated damage in a mixed enterocyte infection model. *P. vulgatus* has been studied for its potential use in reducing inflammatory diseases (Waidmann et al. 2003, Steimle et al. 2016, Yoshida et al. 2018, Steimle et al. 2019, Liu et al. 2022, Wang et al. 2022). Furthermore, *P. vulgatus* possesses enzymes facilitating the degradation of mannan on the surfaces of *C. albicans*, providing a nutrient source for the bacteria (Cuskin et al. 2015, Valentine et al. 2019), and potentially affecting fungal virulence.

We found that *P. vulgatus* mpk limits *C. albicans*-mediated host cell damage by reducing early adhesion, and hyphal length while simultaneously enhancing fungal shedding. *P. vulgatus* mpk protects epithelial cells with multiple mechanisms. The major protection is conveyed in a contact-dependent manner with living or heat-inactivated bacteria, while minor protection is observed by bacterial supernatants. *P. vulgatus* mpks colonization most likely primes enterocyte defenses while also directly affecting *C. albicans* hyphae formation.

Results

P. vulgatus mpk and other related strains protects epithelial cells from *C. albicans*-mediated damage in a dose- and time-dependent manner

To determine if colonization with *P. vulgatus* affects host cell damage caused by subsequent *C. albicans* infection, we used a cell culture model based on a 7:3 mix of brush border-expressing C2BBE1 and mucus-producing HT29-MTX cells. *Candida albicans* infection was performed 6 h after addition of bacteria to simulate bacterial colonization. *P. vulgatus* mpk significantly reduced *C. albicans*-mediated damage in a dose-dependent manner (Fig. 1A; Suppl. Fig. 1). The closely related strain *P. vulgatus* ATCC8482, but also strains of related species such as *P. dorei* DSM17855, and *B. thetaiotaomicron* VPI 5482, reduced damage (Fig. 1B; Suppl. Fig. 1). In addition, three out of four clinical isolates (A271, A272, A273, A274) that could not be definitively characterized as either *P. vulgatus* or *P. dorei*, protected enterocytes from *C. albicans* damage (Fig. 1C). Thus, the protective potential seems to be a trait within the genus *Phocaeicola* (and the close relative *B. thetaiotaomicron*) but is not present in all strains. Of note, none of the tested bacterial strains exhibited significant cytotoxic potential in the cell culture model (Fig. 1A–C). Therefore, we will refer to the interaction of these bacteria with enterocytes *in vitro* as “colonization” throughout the manuscript.

We next tested if pre-colonization with bacteria is required for the protective effect by adding bacteria simultaneously with or 6 h after *C. albicans* to the host cells. When added simultaneously, *P. vulgatus* mpk significantly reduced the damage only at the highest ratio of 100 bacteria per fungal cell (Fig. 2A), but damage reduction was less pronounced than for pre-colonized cells (40% damage, Fig. 2A, vs. 15.5% damage with pre-colonization, Fig. 1A). No damage reduction was observed when *P. vulgatus* mpk was added 6 h after *C. albicans* (Fig. 2B). In contrast, *B. thetaiotaomicron* significantly reduced damage if added at a ratio of 100 bacteria per fungal cell after *C. albicans* (76.8%, Fig. 2B), but to a lesser extent than for pre-colonized enterocytes (28.8%, Fig. 1B). Thus, priming of enterocytes by bacterial pre-colonization was most effective against *C. albicans* damage.

Colonization-mediated protection was not limited to the mixed culture of C2BBE1 and HT29-MTX cells, but was also observed for each cell line cultured individually, and for the oral epithelial cell line TR146 (Fig. 2C). Increased viability of enterocytes colonized with *P. vulgatus* mpk and infected with *C. albicans* compared to *C. albicans* alone was qualitatively confirmed using a live/dead stain (Suppl. Fig. 2). Both LDH release and live/dead stain measure the loss of cellular integrity and are established markers of cytotoxicity. To address epithelial barrier function, we additionally measured the transepithelial electrical resistance (TEER). The reduction of TEER was significantly mitigated upon *P. vulgatus* mpk colonization (Fig. 3). In contrast to epithelial barrier dysfunction and cellular integrity, induction of apoptosis by *C. albicans* infection was not reduced by *P. vulgatus* mpk colonization (Suppl. Fig. 3). Taken together, these results suggest that *P. vulgatus* mpk colonization reduces necrotic cell death and the

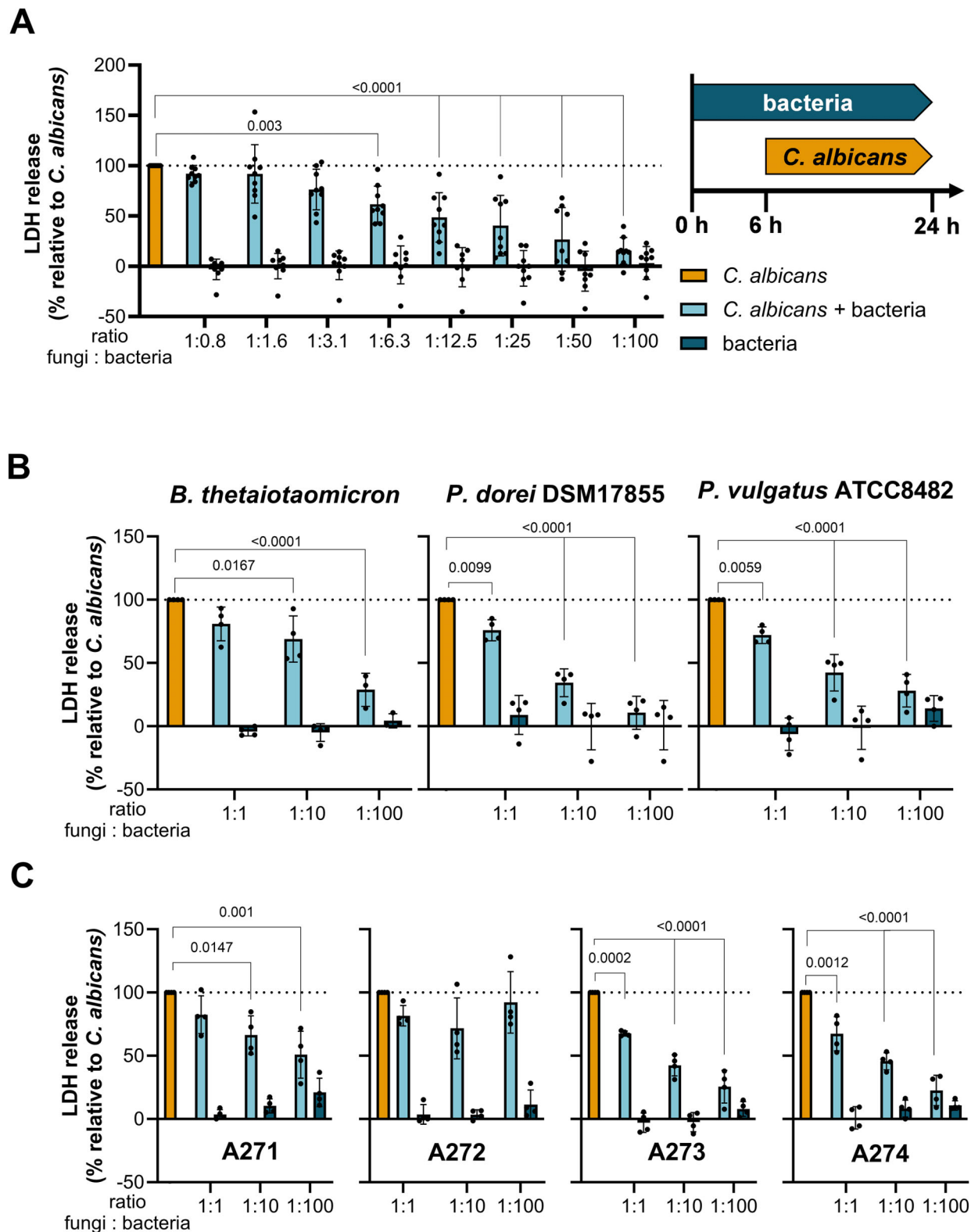


Figure 1 *P. vulgatus* mpk and related strains reduce host cell damage during coinfection with *C. albicans*. Enterocytes were colonized with bacteria for 6 h and subsequently infected with *C. albicans* at MOI 10 for 18 h. The host cell damage was measured by LDH release relative to uninfected host cells treated with triton and then normalized to *C. albicans* damage. Individual data points are plotted, bars represent mean and SD. Statistical analysis was performed by One-Way ANOVA followed by Dunnett's multiple comparisons test comparing the conditions with *C. albicans* and bacteria to *C. albicans* only as control. The *P*-values of statistically significant results are indicated in the graphs. (A) Multiple doses of *P. vulgatus* mpk were tested. *n* = 9 independent experiments. (B) Three strains closely related to *P. vulgatus* mpk were tested at three different doses. *n* = 4 independent experiments. (C) Four clinical isolates not definitively characterized as either *P. vulgatus* or *P. dorei* were tested at three different doses. *n* = 4 independent experiments.

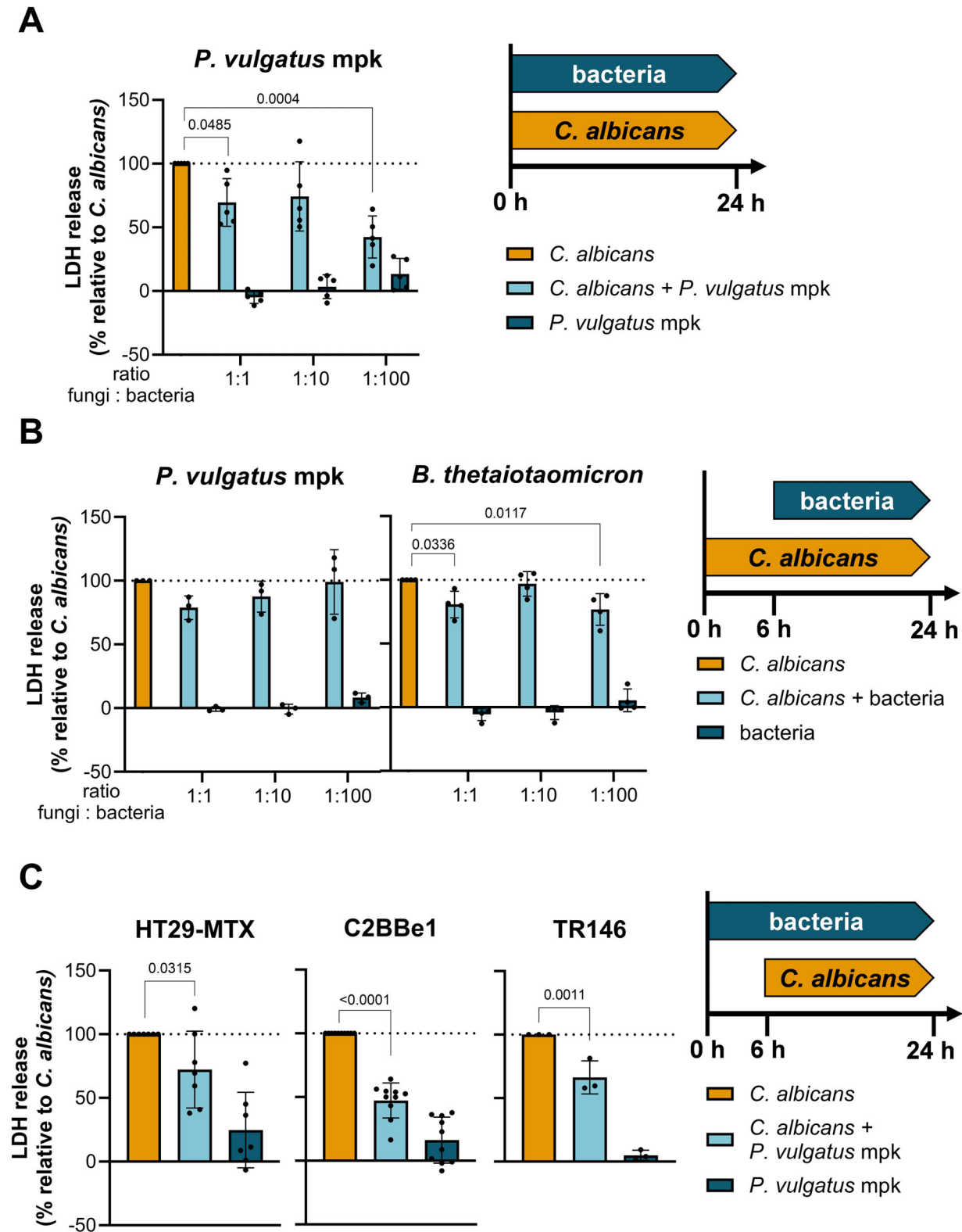


Figure 2 Enterocytes can be protected but not rescued from *C. albicans* damage by bacteria. The host cell damage was measured by LDH release relative to uninfected host cells treated with triton and then normalized to *C. albicans* damage. Individual data points are plotted, bars represent mean and SD. Statistical analysis was performed by (A, B) One-Way ANOVA followed by Dunnett's multiple comparisons test comparing the conditions with *C. albicans* and bacteria to *C. albicans* only as control or (C) unpaired two-sided Student's t-test. The *P*-values of statistically significant results are indicated in the graphs. (A) Enterocytes were simultaneously infected with *C. albicans* at MOI 10 and *P. vulgatus* mpk at three different doses. $n = 5$ independent experiments (B) Enterocytes were first infected with *C. albicans* at MOI 10 and 6 h later colonized with three different doses of *P. vulgatus* mpk or *B. thetaiotaomicron* and further incubated for 18 h. $n = 3$ (*P. vulgatus* mpk) or $n = 4$ (*B. thetaiotaomicron*) independent experiments (C) Three different epithelial cell lines were colonized with *P. vulgatus* mpk at ratio 1:100 (fungi : bacteria) for 6 h and subsequently infected with *C. albicans* at MOI 10 for 18 h. $n = 7$ (HT29-MTX), $n = 10$ (C2BBE1) or $n = 3$ (TR146) independent experiments

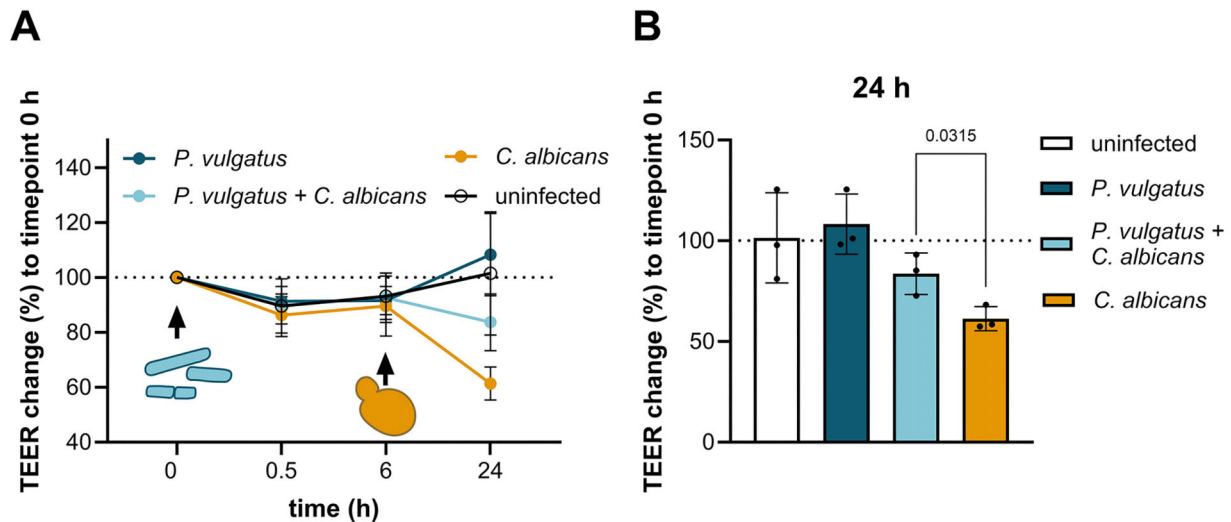


Figure 3 *P. vulgatus* mpk minimizes transepithelial electrical resistance (TEER) reduction during coinfection with *C. albicans*. (A) Enterocytes were seeded on a cell culture insert. After maturation they were colonized with bacteria for 6 h and subsequently infected with *C. albicans* at MOI 10 for 18 h. The TEER was measured during the course of infection and normalized to the TEER at timepoint 0 h. Mean and SD are plotted. (B) Relative TEER values at timepoint 24 h. Individual data points are plotted, bars represent mean and SD. For statistical analysis an unpaired two-sided Student's t-test was performed. $n = 3$ independent experiments.

associated reduction of barrier function caused by *C. albicans* infection.

P. vulgatus mpk and *B. thetaiotaomicron* do not impair fungal replication

To test if reduced damage was the consequence of reduced fungal growth in the presence of bacteria, host cells and supernatants were removed and plated. Neither presence of *P. vulgatus* mpk (Fig. 4A) nor *B. thetaiotaomicron* (Fig. 4B) resulted in reduced *C. albicans* CFU. Furthermore, *P. vulgatus* mpk did not affect *C. albicans* CFU in the absence of enterocytes, but *C. albicans* prolonged viability of *P. vulgatus* mpk in long-term oxygen-rich cultures (Suppl. Fig. 4). This is likely mediated by oxygen-depleted microniches generated by the fungus, as previously reported by others for co-culture of *C. albicans* with obligate anaerobic *Bacteroides* species and *Clostridium difficile* in the absence of host cells (van Leeuwen et al. 2016, Valentine et al. 2019).

Heat-killed *P. vulgatus* mpk provides protection against *C. albicans*-mediated damage

To investigate if protection by *P. vulgatus* mpk is contact-dependent, we performed transwell experiments, where the microbes were separated by a hanging insert with a 0.4 μm pore size. *P. vulgatus* mpk only conferred protection if in direct contact with both the host cells and *C. albicans* (Fig. 5A). To further characterize the relative contribution of metabolic activity and soluble factors, heat-killed bacteria and supernatants of enterocytes colonized with *P. vulgatus* mpk were tested. Heat-killed *P. vulgatus* mpk cells provided dose-dependent protection to the mixed enterocyte model and C2BBE1 and HT29-MTX cells, respec-

tively (Fig. 5B). In contrast, supernatants collected after 6 h had only a moderate protective effect, which was significant only if derived from host cells incubated with a high number of bacteria (Fig. 4C). Together with the observation that colonization prior to infection mediated the strongest protective effect, this suggested that the response of enterocytes to *P. vulgatus* colonization contributed to reduced *C. albicans*-mediated damage.

P. vulgatus mpk and *B. thetaiotaomicron* do not induce LL-37 production but induce fungal shedding in cell culture

B. thetaiotaomicron has been shown to mediate colonization resistance against *C. albicans* in mice by inducing increased expression of the antimicrobial peptide CRAMP, the murine homologue to human LL-37 (Fan et al. 2015). To investigate whether LL-37 production played a role in our enterocyte model, possibly explaining the protective effect mediated by heat-killed *P. vulgatus* mpk (Fig. 5B), the concentration of the antimicrobial peptide was measured in supernatants of colonized enterocytes. Incubation of enterocytes with *P. vulgatus* mpk or *B. thetaiotaomicron* did not lead to increased LL-37 production (Suppl. Fig. 5A). In mice, the increased production of CRAMP was linked to activation of hypoxia-inducible factor (HIF) (Fan et al. 2015). We therefore measured accumulation of the HIF-1 α subunit as an indicator for HIF activation. In line with the results for LL-37, HIF was not significantly activated in cells incubated with either *P. vulgatus* mpk or *B. thetaiotaomicron* (Suppl. Fig. 5B; Suppl. Fig. 6). To test if host cells responded differently to *C. albicans* in the presence of either *P. vulgatus* mpk or *B. thetaiotaomicron*, we quantified the amounts of IL-10, TGF- β , IL-6, TNF- α , and IL-8 in cell culture supernatants. With the exception of IL-8, cytokines levels were beyond the detection limit. No significant differences in IL-8 were observed between the treatment groups (Suppl. Fig. 5C).

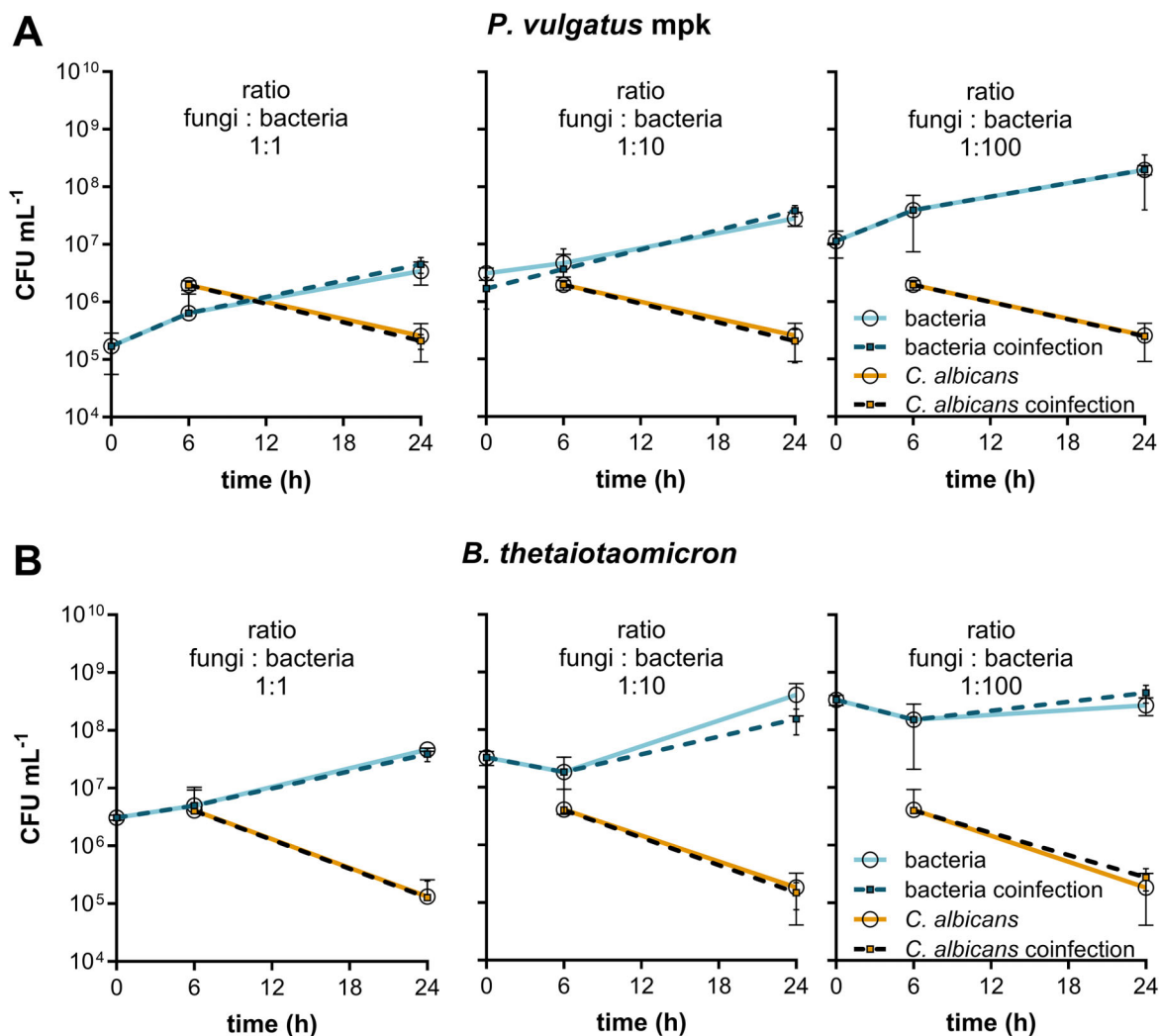


Figure 4 Bacteria and *C. albicans* do not affect each other's growth. Enterocytes were colonized with either (A) *P. vulgatus* mpk or (B) *B. thetaiotaomicron* at three different ratios as indicated for 6 h and subsequently infected with *C. albicans* MOI 10 for 18 h. At time point 0 h, 6 h and 24 h bacterial and/or fungal burden was determined by CFU plating. Mean and SD are plotted. n = 3 independent experiments.

Another possible mechanism of protecting enterocytes from *C. albicans*-mediated damage is the physical removal of the fungal cells from the host cell surface—termed fungal shedding. This has been shown for *C. albicans* infection of enterocytes in the presence of lactobacilli: *C. albicans* adheres to the enterocytes at early time points but is shed into the supernatant after 24 h due to detachment of host cells (Graf et al. 2019). We tested whether *P. vulgatus* mpk induced fungal shedding by determining the fungal burden in the cell layer and the supernatant after infection. In the mixed enterocyte model, the number of fungal cells in the supernatant was significantly increased and fungal cells attached to enterocytes was significantly reduced if enterocytes were colonized with *P. vulgatus* mpk prior to infection (Fig. 6A). This effect was not observed in C2BBE1 cells (Fig. 6B), a brush border expressing cell line, suggesting that mucus production might facilitate fungal shedding. However, cell layer-associated fungal burden was not significantly reduced by *P. vulgatus* mpk for infected HT29-MTX cells, which are known to produce mucus (Lesuffleur et al. 1991) (Fig. 6C), although detachment of structures that likely represent a *C. albicans*-mucosal biofilm was observed when

handling *P. vulgatus* mpk-colonized HT29-MTX cells infected with *C. albicans* (Fig. 6D). Detachment was also occasionally observed for HT29-MTX cells infected with *C. albicans* only (Fig. 6D), and this might have affected quantification of cell layer-associated fungal burden (Fig. 6C). To test if differences in mucus production correlate with fungal shedding, we quantified mucin-2 (MUC2), the major constituent of the colon mucus (Fig. 6E, Suppl. Fig. 7). Although a tendency of increased MUC2 was observed in supernatants of *C. albicans*-infected HT29-MTX cells in the presence of *P. vulgatus* mpk, this difference was not statistically significant. No increased MUC2 production was observed in the mixed enterocyte model.

P. vulgatus mpk reduces early adhesion and hyphal length of *C. albicans*

We hypothesized that *P. vulgatus*, in addition to supporting fungal shedding, interferes with crucial steps of infection leading to damage, and therefore quantified adhesion and filamentation. Adhesion of *C. albicans* to enterocytes was determined by two meth-

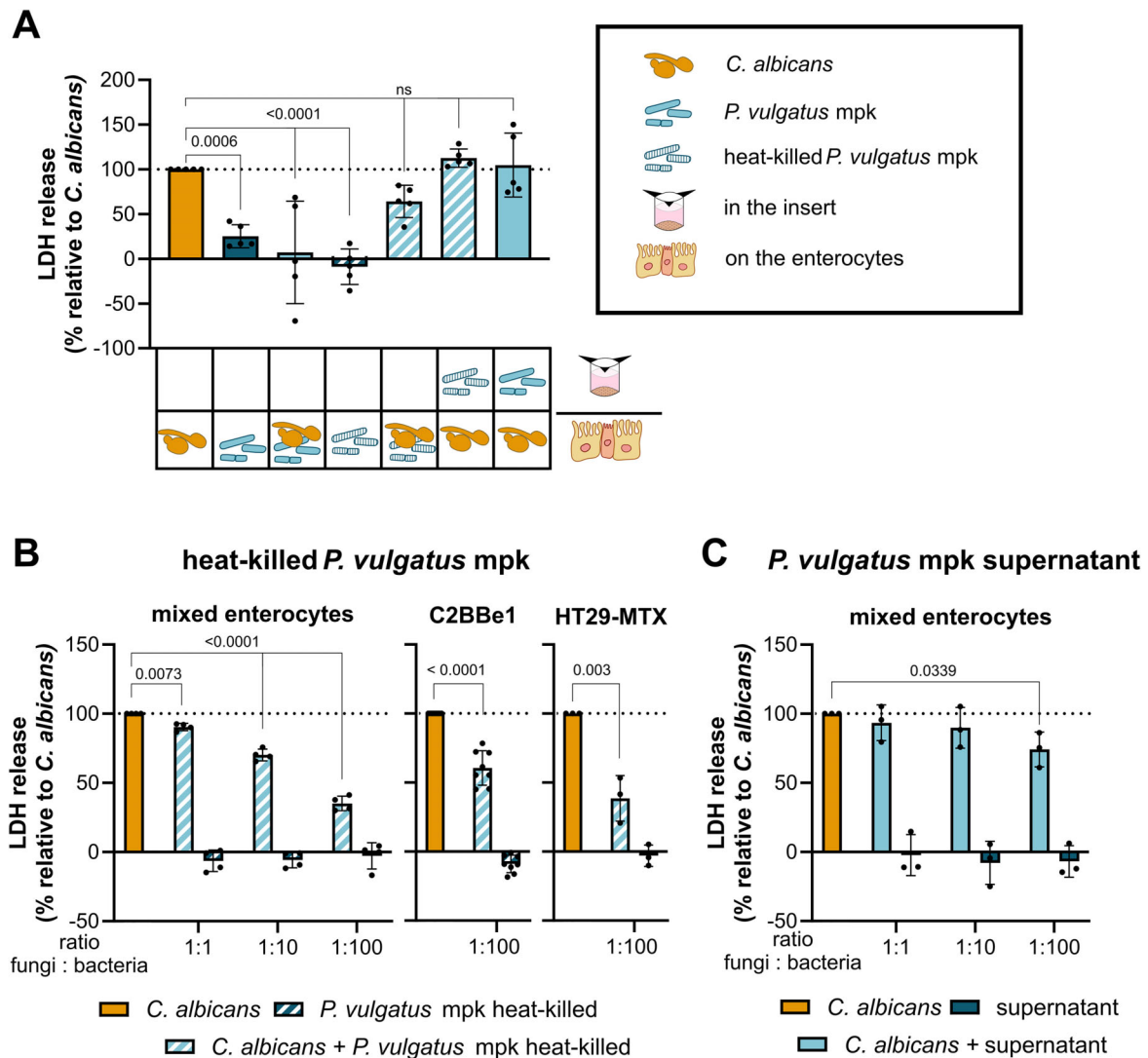


Figure 5 *P. vulgatus* mpk protection is contact-dependent and partially independent of metabolic activity. Enterocytes were colonized with *P. vulgatus* mpk(A) at ratio 1:100 (fungi : bacteria) (B, C) or at three different ratios as indicated for 6 h and subsequently infected with *C. albicans* at MOI 10 for 18 h. The host cell damage was measured by LDH release relative to uninfected host cells treated with triton and then normalized to *C. albicans* damage. Individual data points are plotted, bars represent mean and SD. Statistical analysis was performed by One-Way ANOVA followed by Dunnett's multiple comparisons test comparing the conditions with *C. albicans* and bacteria to *C. albicans* only as control [A, B (mixed enterocytes), C] or unpaired two-sided Student's t-test (B, C2BBE1, and HT29-MTX). The *P*-values of statistically significant results are indicated in the graphs. (A) Microbes were separated from the enterocytes via a hanging cell culture insert (0.4 μ m pore size). *n* = 5 independent experiments. (B) Impact of heat-killed *P. vulgatus* mpk on *C. albicans* damage. *n* = 4 (mixed enterocytes), *n* = 8 (C2BBE1) or *n* = 3 (HT29-MTX) independent experiments. (C) Impact of sterile-filtered culture supernatants of enterocytes colonized for 6 h with *P. vulgatus* mpk. *n* = 3 independent experiments.

ods: (i) Plating of CFU after washing to quantify fungal cells associated with the cell layer (adhesion and invasion) for time points up to 3 h after infection, when invasion begins. Adhesion was not affected by *P. vulgatus* mpk 1 h after infection, but significantly reduced at 2 h and 3 h. (Fig. 7A). (ii) Staining of extracellular fungi and microscopic analysis was performed at 3 h and 4 h after infection to quantify adherent fungal cells. This confirmed reduced adhesion in the presence of *P. vulgatus* mpk after 3 h and revealed reduced adhesion at 4 h (Fig. 7B, E). Furthermore, *C. albicans* formed shorter hyphae on enterocytes pretreated with *P. vulgatus* mpk, but only if the bacteria were alive (Fig. 7C–E). This effect on filamentation appears to be independent of host cells, as the pres-

ence of living *P. vulgatus* mpk also negatively affected the growth of *C. albicans* microcolonies without enterocytes (Fig. 8). Thus, *P. vulgatus* protection of host cells from *C. albicans*-mediated damage is not only the result of increased shedding but also reduced fungal adhesion and filamentation.

Discussion

Candidiasis is commonly caused by colonizing strains, with increased fungal colonization facilitated by dysbiosis as a major clinical risk factor (Bognoux et al. 2006, Papon et al. 2020, Zhai et al. 2020). Thus, identification of bacterial species and strains

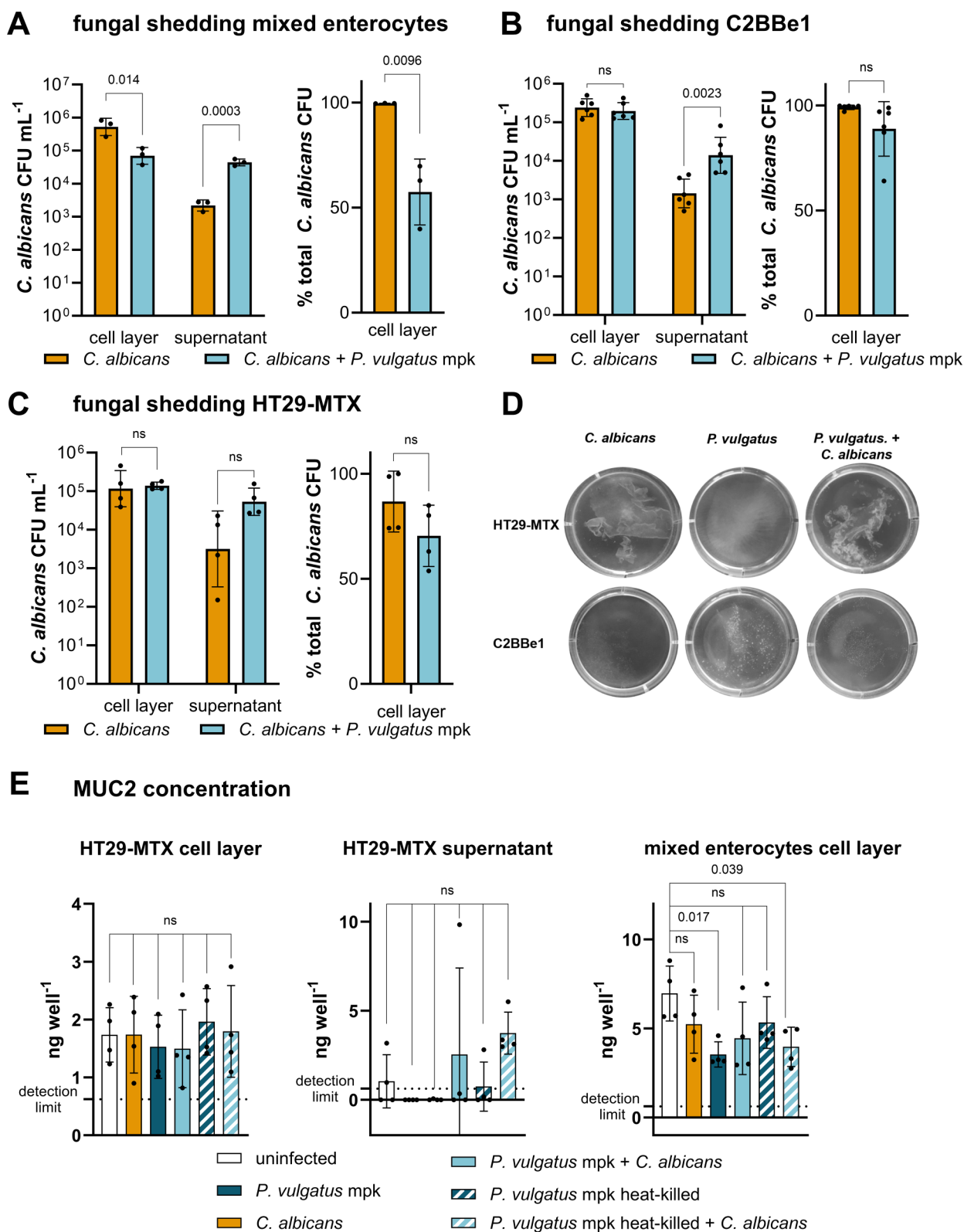


Figure 6 *P. vulgatus* mpk induces fungal shedding. (A–D) Enterocytes were colonized first with *P. vulgatus* at ratio 1:1000 (fungi : bacteria) and 6 h later infected with *C. albicans* MOI 1. (A–C) After 24 h overall incubation, the fungal burden in the supernatant and the triton-disrupted host cell layer was determined by CFU plating. Left: Absolute CFU. Right: CFU retrieved from the host cell layer as percentage of total CFU. Individual data points are plotted, bars represent geometric mean and geometric SD. Statistical analysis was performed on log-transformed data by unpaired two-sided Student's t-test. (A) Mixed enterocytes were used. $n = 3$ independent experiments. (B) C2BBe1 were used. $n = 6$ independent experiments. (C) HT29-MTX were used. $n = 4$ independent experiments. (D) Representative images of cell cultures wells colonized with *P. vulgatus* mpk and/or infected with *C. albicans* are shown. (E) Mixed enterocytes and HT29-MTX cells were colonized with bacteria at ratio 1:100 (fungi : bacteria) for 6 h and subsequently infected with *C. albicans* at MOI 10 for 18 h. MUC2 in the cell layer and the supernatant was quantified by ELISA. Bars represent mean and SD. Statistical analysis by one-way ANOVA followed by Dunnett's multiple comparisons test comparing all treatments to the uninfected control.

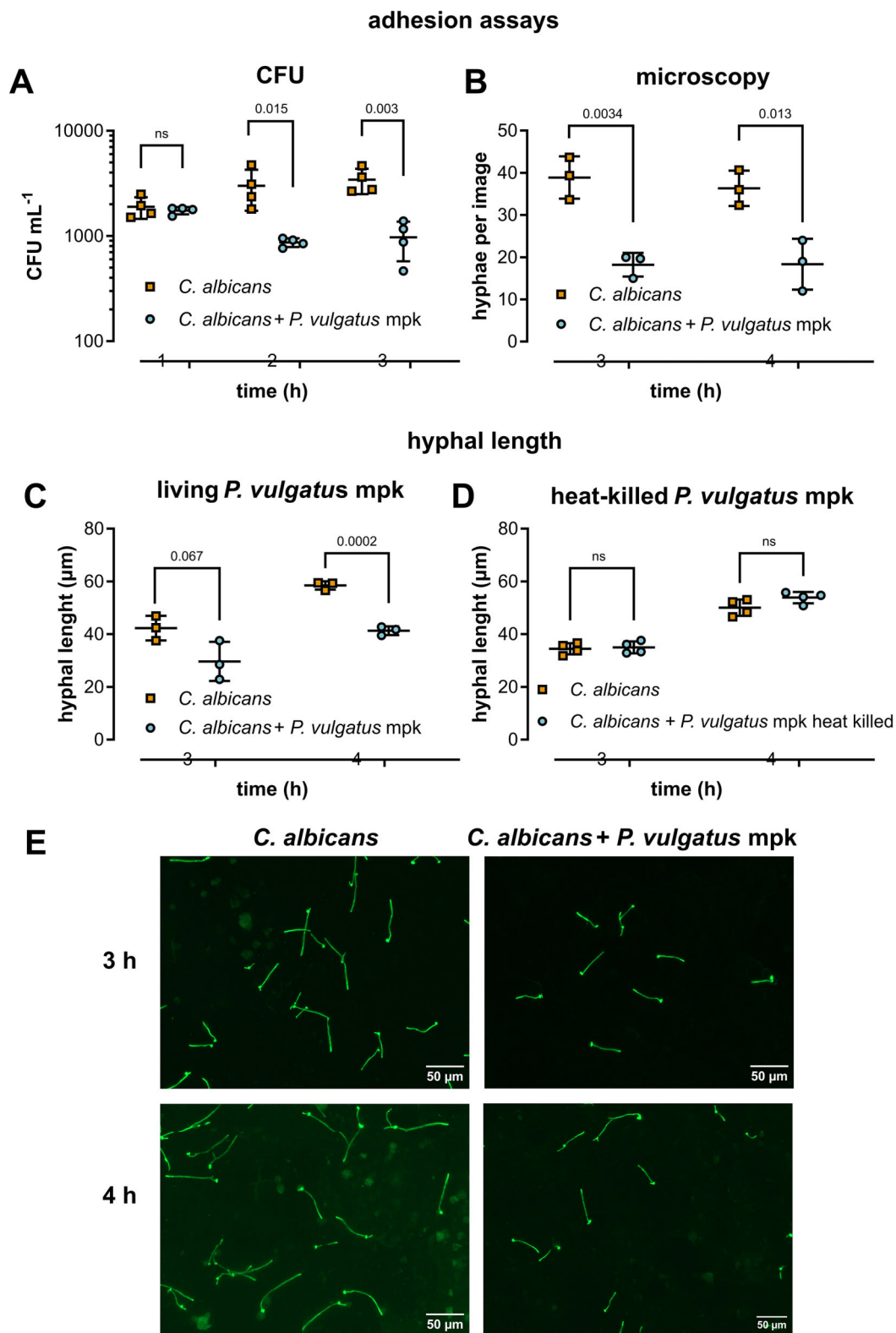


Figure 7 Living *P. vulgatus* mpk reduces early adhesion and hyphal length of *C. albicans*. Enterocytes were colonized first with *P. vulgatus* at ratio 1:1000 (fungi : bacteria) and 6 h later infected with *C. albicans* MOI 1. Individual data points are plotted, lines and bars represent mean and SD. For statistical analysis an unpaired two-sided Student's t-test was performed (ns = not significant). (A) The enterocytes were washed to remove unattached hyphae, treated with zymolyase to release attached hyphae and then plated to determine CFU. n = 4 independent experiments. (B–D) For microscopy-based analysis the enterocytes were cultivated on a glass slide. After infection they were washed, fixed and *C. albicans* was stained with fluorescent antibodies. (B) The number of hyphae in 16 images (20x objective) per condition was counted. n = 3 independent experiments. (C and D) The longest hyphae originating from the mother yeast was measured. (C) n = 3 independent experiments with 200 hyphae analyzed in each experiment. (D) *P. vulgatus* mpk was heat-inactivated before colonization. n = 4 independent experiments with 200 hyphae analyzed in each experiment. (E) Representative images of the experiments B and C.

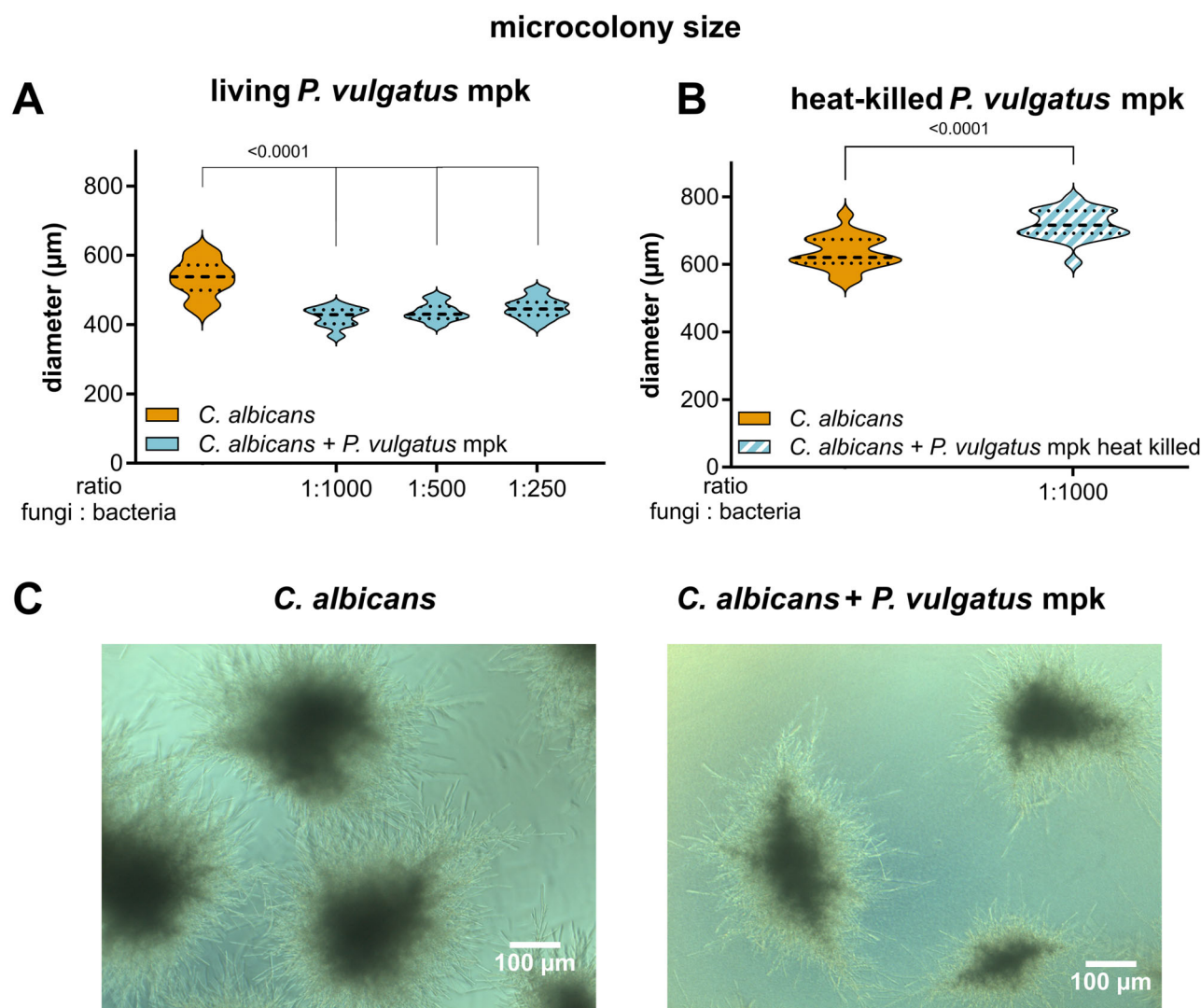


Figure 8 Living *P. vulgatus* mpk reduces the size of *C. albicans* microcolonies. *Candida albicans* (1×10^2 cells ml^{-1}) and *P. vulgatus* mpk (ratio fungi : bacteria as indicated) were co-incubated in KBM medium for 24 h at 37°C. Afterwards the fungal microcolonies were imaged with a microscope and the size determined by calculating the average of the longest and shortest diameter for each microcolony. (A and B) $n = 3$ independent experiment with 20 colonies measured per condition in each experiment. Horizontal lines represent the median and the quartiles. For statistical analysis an unpaired two-sided Student's *t*-test was performed. The *P*-values are depicted in the graphs. (B) *P. vulgatus* was heat-inactivated before incubation. (C) Exemplary pictures for co-incubation with living *P. vulgatus*.

that limit colonization and/or fungus-induced epithelial damage, and thereby subsequent translocation and dissemination, could lead to the development of microbiome-based prophylactic approaches for patients at risk for developing disseminated candidiasis (Matsubara et al. 2016).

Our data indicate that *P. vulgatus* mpk acts as a potent antagonist against *C. albicans* infection by reducing fungal pathogenicity in an enterocyte model. While the bacteria did not reduce fungal burden, they effectively reduced fungal adhesion and filamentation, and enhanced fungal shedding from the mucosal surface. Thus, rather than mediating colonization resistance by limiting proliferation or killing of the fungus, the bacteria reduced virulence. Understanding the molecular mechanisms behind this phenomenon could contribute to the development of novel therapies based on an anti-virulence strategy, an approach that might help

to treat infections without evolutionary pressure for resistance development (Muhlen and Dersch 2016).

Fungal shedding has been described by Graf and Last et al. for lactobacilli (Graf et al. 2019) using the same enterocyte model as in this study. In the case of lactobacilli, fungal shedding was independent of HT29-MTX cells and *L. rhamnosus* colonization did not significantly influence mucin production. Instead, shedding was found to be mediated bacteria adhering to fungal cells, forming large aggregates, and associated with host cell apoptosis. However, inhibition of apoptosis did not reduce lactobacilli-associated shedding (Graf et al. 2019). We observed increased apoptosis only in *C. albicans*-infected cells, independent of the presence of *P. vulgatus* mpk, and inhibition of apoptosis did not reduce lactobacilli-associated shedding (Graf et al. 2019). This suggests other mechanisms, and we tested if mucus is involved. We found

no evidence for *P. vulgatus*-induced upregulation of mucus production, but have not investigated mucin modification in response to sensing of bacteria by enterocytes (de Ram et al. 2024). Mucin modification has been described for both viable and non-viable bacteria, and thus might explain some of the protection mediated by heat-killed *P. vulgatus*. However, C2BBel and TR146 cells, which do not produce mucin at measurable quantities, were also protected by *P. vulgatus* mpk colonization, indicating mucin-independent protective mechanisms.

Activation of HIF (Rius et al. 2008, Nizet and Johnson 2009) resulting in increased production of the antimicrobial peptide LL-37 (CRAMP in mice) has been suggested by Fan et al. as a major mechanism by which *B. thetaiotaomicron* colonization in germ-free mice limits *C. albicans* colonization (Fan et al. 2015). Surprisingly, we did not observe increased LL-37 production or HIF activation by *B. thetaiotaomicron* and *P. vulgatus* in our model. Since we used the same *B. thetaiotaomicron* strain as Fan et al., it is likely that differences between our *in vitro* model and the *in vivo* situation explain the differing results. However, the lack of LL-37 induction is consistent with our finding that *C. albicans* CFU were not affected by the presence of *B. thetaiotaomicron* or *P. vulgatus*. We observed that *C. albicans* infection led to induction of HIF (Suppl. Fig. 5), supporting the notion of the generation of oxygen-depleted microniches by the fungus as described in cultures without host cells (van Leeuwen et al. 2016, Valentine et al. 2019).

The lack of increased LL-37 production does not exclude the possibility that induction of other antimicrobial peptides, by other pathways, are responsible for the protective effect. Sensing of *P. vulgatus* LPS via TLR4 and TLR2 can activate NF- κ B sensing (Cuiv et al. 2011, Di Lorenzo et al. 2020), and NF- κ B activation of epithelial cells limits fungal-mediated damage and promotes barrier integrity (Bohringer et al. 2016, Sprague et al. 2024). NF- κ B activation promotes maintenance of cell-cell junction, which limit intercellular translocation; however, transcellular invasion is the main route of infection of enterocytes by *C. albicans* (Allert et al. 2018). Yet, NF- κ B activation might limit fungal mediated damage by other means, as it has been suggested that membrane repair mechanisms of epithelial cells might be involved (Lapaquette et al. 2022, Westman et al. 2022, Sprague et al. 2024). NF- κ B activation usually results in increased production of cytokines, such as IL-8 (Savkovic et al. 1997, Neuschäfer-Rube et al. 2013), and increased secretion of IL-8 by HT29 cells after stimulation with *P. vulgatus* has been reported (Ohkusa et al. 2009). We could not detect a significant induction of IL-8 (or other cytokines) by *P. vulgatus*; however, as we did not specifically analyze signaling pathways, cell-cell junctions or repair pathways, additional studies are necessary to determine how epithelial cells respond to the bacteria, and if such a response increases resilience to *C. albicans* infection. In addition, while our transwell experiments clearly indicate that contact of *P. vulgatus* mpk to *C. albicans* is necessary for full protection, the possible role of direct contact between *P. vulgatus* mpk and enterocytes yet needs to be determined.

Besides increased shedding, we observed reduced adhesion and filamentation of *C. albicans* in the presence of viable *P. vulgatus*. Adhesion is essential for *C. albicans*-induced damage (Martin et al. 2021, Mogavero et al. 2021), as reduced adhesion will result in less invasion. The observed reduction in adhesion can be the consequence of different mechanisms, including changes in mucus composition, competition for adhesion sites with bacte-

ria, or effects on fungal adhesins. Competition for adhesion sites between bacteria and *C. albicans* has been described for *Lactobacillus rhamnosus* GG and other probiotic bacteria (Mailänder-Sánchez et al. 2017, Chantanawilas et al. 2024). Additionally, *B. thetaiotaomicron* and *P. vulgatus* possess mannose degrading enzymes (Cuskin et al. 2015, Charlet et al. 2018, Valentine et al. 2019, Charlet et al. 2020), that might modify the mannosylation pattern of *C. albicans* surface proteins and thereby affect adherence negatively (Sandin et al. 1982). Reduced adhesion could also be the consequence of shorter hyphae as hyphae express specific adhesins (de Groot et al. 2013). Furthermore, the main damage factor produced by *C. albicans*, the peptide toxin candidalysin, is only expressed by hyphae (Moyes et al. 2016, Mogavero et al. 2021), and several other hypha-associated genes have been linked to *C. albicans* virulence. Additionally, invasion by both induced endocytosis and active penetration is linked to the hyphal morphology of *C. albicans*, and a prerequisite for host cell damage (Jacobsen et al. 2012). Also, the cytotoxic effect of candidalysin requires adhesion and formation of an invasion pocket to facilitate locally concentrated toxin secretion (Moyes et al. 2016, Mogavero et al. 2021). Therefore, reduced filamentation is expected to result in reduced host cell damage by decreasing adhesion, invasion, and production of damaging virulence factors.

Candida albicans filamentation is affected by mucus (41, 67, 68), however, as we observed no increase in mucus production and reduced microcolony formation in the presence of *P. vulgatus* without enterocytes, *P. vulgatus* likely affects *C. albicans* filamentation directly or by modifying the media composition. Antagonistic bacteria are well documented to affect hyphal growth by producing molecules that target hyphae or by forcing metabolic shifts by using up nutrients in the environment (Vilchez et al. 2010, Graham et al. 2017, Graf et al. 2019, Salvatori et al. 2020, Alonso-Roman et al. 2022, Rebai et al. 2023). While we observed some reduction of *C. albicans*-induced damage by supernatants of *P. vulgatus*-colonized enterocytes, the effect was less pronounced than in coinfections. This suggests that bacterial depletion of nutrients plays only a moderate role in modifying *C. albicans* cytotoxicity, but further experiments are necessary to clarify this.

Bacterial Type III, Type IV, and Type VI secretion systems (T3SSs, T4SSs, T6SSs) can inject effector molecules into microbes or host cells in a contact-dependent manner, and have been described or proposed to possess antifungal activity (Kim and Mylonakis 2011, Trunk et al. 2018, Fu et al. 2026). Within the Bacteroidales only one strain—*Bacteroides fragilis*—has so far been identified to possess a T3SS (Hu et al. 2017), and while conjugation-associated T4SS-like machinery is common in *Bacteroides* and *P. vulgatus*, T4SSs that inject effector molecules have not been described (Gordils-Valentin et al. 2024, Ortanez and Degnan 2024). In contrast, T6SSs are commonly found in *Bacteroides* species, including *P. dorei* DSM17855 used in this study, but not in *B. thetaiotaomicron* VPI 5482 and *P. vulgatus* ATCC 8482 (Coyne et al. 2016), strains that showed protective potential in our infection model. Thus, T6SSs might be involved in modulating *C. albicans* virulence, but do not explain the protection conveyed by all strains.

In summary, *P. vulgatus* mpk protects epithelial cells from *C. albicans*-induced damage in a multifactorial way. Living or dead bacteria stimulate the host cell defenses and increase HT29-MTX dependent shedding of fungal cells. Additionally, living bacteria reduce adhesion and hyphal length of *C. albicans* by a yet unknown mechanism. The major protective effect is contact-

dependent with only a minor contribution of soluble factors or nutrient depletion by bacteria. While the exact mechanisms and their relative contribution needs further elucidation, our findings indicate that the commensal *P. vulgatus* mpk can attenuate *C. albicans* virulence. Importantly, the observation that heat-inactivated *P. vulgatus* mpk cells provided some protection opens the possibility to use non-viable bacteria as a safe option in immuno-compromised individuals as an anti-virulence strategy. However, further studies are necessary to fully understand how and to which extent *P. vulgatus* mpk affects *C. albicans*, to test whether these interactions impact *C. albicans* colonization and translocation efficacy *in vivo*, and to determine the safety profile of viable *P. vulgatus* mpk.

Material and methods

Cultivation of microbes

The strains used in this study are listed in [Supplementary Table 1](#). Bacteria were grown anaerobically at 37°C in either supplemented Brain Heart Infusion medium (BHIS) (37 g l⁻¹ BHI, Roth; 1 g l⁻¹ cysteine, Roth; hemin 5 mg l⁻¹, Sigma-Aldrich; resazurin 1 mg l⁻¹, Sigma-Aldrich; NaHCO₃ 2 g l⁻¹, Roth) (Bacic and Smith 2008) or on Tryptic Soy agar (TSA) blood plates (15 g l⁻¹ tryptone, Difco; 5 g l⁻¹ soytone, Roth; 5 g l⁻¹ NaCl, Roth; 15 g l⁻¹ agar-agar, Roth; 50 ml l⁻¹ defibrinated sheep blood, Thermo Fisher). Bacterial cultures in BHIS were harvested in the exponential growth phase after 24 h. After washing twice in phosphate-buffered saline (PBS) the cell number was adjusted using a predetermined OD₆₀₀-CFU correlation factor ([Supplementary Table 1](#)). For heat-inactivation the bacterial solutions were heated to 80°C for 15 min. Structural integrity of heat-killed bacterial cells was confirmed microscopically. *Candida albicans* was grown aerobically in YPD (20 g l⁻¹ peptone, BD; 10 g l⁻¹ yeast extract, Roth; 20 g l⁻¹ glucose, Roth) or on YPD agar plates. Liquid cultures inoculated from a single colony were grown at 30°C for 16 h at 180 rpm. After centrifugation the cells were washed twice with PBS and the cell number determined by microscopy with a Neubauer chamber. The desired cell number was adjusted in PBS.

For fungal-bacterial co-cultures, the individual microbial suspensions were adjusted to OD₆₀₀ 0.1 in 5 ml BHIS and mixed in a 1:1 ratio or kept as monocultures. Incubation was performed under normoxic (21% oxygen) and hypoxic (0.2% oxygen, Whitley H35 Hypoxia station) conditions at 37°C and 180 rpm shaking. Samples were taken every 24 h, treated with 20 U ml⁻¹ zymolyase (Amsbio # 120491-1) for 1 h at 37°C with 500 rpm shaking to detach hyphal clumps before serial dilutions were plated on YPD agar (fungal CFU) or TSA blood agar with 75 µg ml⁻¹ Nystatin (bacterial CFU). YPD agar plates were incubated aerobically at 30°C for 24 h, TSA blood agar plates anaerobically at 37°C for 48 h.

Cultivation of human epithelial cells

The enterocyte cell lines C2BBel (CRL-2102™, ATCC; [RRID: CVCL_1096](#)) and HT29-MTX (HT29-MTX-E12, Sigma; [RRID: CVCL_G356](#)) were cultivated in DMEM (high glucose DMEM, #41965, Gibco) supplemented with 10% (V/V) FCS, 0.01 mg ml⁻¹ holo-transferrin (#616397, Merck) and 1% V/V non-essential amino acids (#11140, Gibco), and mixed in a 7:3 ratio amounting

to 10⁵ cells ml⁻¹. After 14 days the medium was changed to KBM™ Gold (#00192151, Lonza) before the infection. The oral cell line TR146 (#10032305, Merck) was cultivated in a 1:1 mixture of DMEM and Ham's Nutrient Mixture F12 Gibco (#11520396, Gibco) supplemented with 10% (V/V) FCS. The cells were used for infection after 48 h.

Infection of epithelial cells

For all epithelial cell experiments the highly buffered KBM™ Gold medium was used to prevent excessive acidification by bacteria. After 24 h incubation the pH of the media (7.4) was reduced to 7.0 in uninfected cells and 6.7 with both *C. albicans* (MOI 10), *P. vulgatus* mpk at ratio 1:100 (fungi : bacteria), and the corresponding coinfection (triple testing with a pH strip). Thus, the pH was comparable under all infection conditions and unlikely to affect filamentation. The fungal multiplicity of infection (MOI) relates to the concentration of epithelial cells originally seeded (10⁵ cells ml⁻¹). MOI 1 (10⁵ cells ml⁻¹) or MOI 10 (10⁶ cells ml⁻¹) was used as indicated. The bacterial concentration varied as indicated; where coinfection was compared to bacteria only, the same number of bacteria was used. The microbial suspensions were adjusted to 5% of the total volume in the well (10 µl for 200 µl total volume in 96-well plates) and added to the wells. PBS was added to control wells. If not indicated otherwise, the bacterial suspension was added first, and the cells were incubated for 6 h before fungi were added, and incubation proceeded for 18 h. To obtain supernatants, media was collected after 6 h incubation with bacteria, centrifuged at 3200 × g for 5 min, sterile-filtered and stored at -80°C.

Quantification of epithelial cell damage, viability, and apoptosis

Damage was assessed by quantification of the release of lactate dehydrogenase in the supernatants of infected enterocytes using a cytotoxicity detection kit (#11644793001, Sigma-Aldrich) according to the manufacturer's instructions. Enterocytes were lysed with 0.25% (V/V) Triton-X 100 (Sigma-Aldrich) as a high damage control. PBS treated enterocytes served as a low damage control. LDH assays were performed in 96-well plate format, with technical triplicates (three wells per condition); the mean of the technical triplicates was used for data visualization and statistical analysis. The only exception were transwell assays that were performed in 24-well plates without technical replicates.

The relative damage of the different treatments was calculated using the following formula:

$$\frac{\text{treatment} - \text{low damage control}}{\text{high damage control} - \text{low damage control}} \times 100.$$

For comparisons, relative damage was normalized to the corresponding *C. albicans* damage for each individual experiment. Data without normalization is provided in the supplement ([Suppl. Fig. 8-11](#)).

Live/dead staining was performed with Fixable Green Dead Cell Stain Kit (#L34969, Thermo Fisher Scientific) and counterstaining with DAPI after formaldehyde fixation. 3.3% digitonin (#HN76.3, Roth) was used as high damage control.

The FAM-DEVD-OPH *in vitro* Apoptosis Detection Reagent (Immunochemistry Technologies, SKU: 6356) was used to quantify apoptosis. The reagent binds to activated caspase enzymes present inside apoptotic cells. The experiment was conducted in

the 24-well format and the infection was performed as stated above. As positive control, 5% DMSO (final concentration) was added 4 h before the experiment's endpoint. For staining, 700 μ l cell culture medium were removed and 10 μ l staining solution were added to each well. After 1 h incubation at 37°C cells were scraped from the wells, transferred to a 1.5 ml Eppendorf tube and washed twice in 300 μ l PBS. Total 95 μ l of the suspension were added to a black ELISA plate in triplets and measured at 490 and 520 nm using a plate reader. The mean of the technical triplicates was used for data visualization and statistical analysis.

Transepithelial electrical resistance (TEER) measurement

Enterocytes were seeded on cell culture insert with a 5 μ m pore size membrane (#10107341, Fisher Scientific). After maturation and infection as described above the TEER was measured with a disinfected chopstick electrode (STX2, World precision instruments) at multiple timepoints.

Quantification of microbial burden

To determine the fungal and bacterial burden the host cell layer was detached by scraping with a pipette tip and removed with the media. The samples were treated with 20 U ml⁻¹ zymolyase (Amsbio # 120491-1) for 1 h at 37°C with 500 rpm shaking to detach hyphal clumps before serial dilutions were plated on YPD agar (fungal CFU) or TSA blood agar with 75 μ g ml⁻¹ Nystatin (bacterial CFU) as described above.

Quantification of LL-37

LL-37 in supernatants was quantified using the Human LL-37 ELISA Kit (#HK321, Hycult Biotech) according to the manufacturer's instructions.

Transwell assay

Epithelial cells were seeded in a 24-well plate format in 1 ml medium per well as described above. A hanging cell culture insert with 0.4 μ m pore size (#PTHT24H48, Merck & #83.3932.041, Sarstedt) was placed in the wells and filled with 200 μ l media. The microbes were either added to the well or the insert. Epithelial damage was quantified by measuring LDH release as described above.

Shedding of hyphae from the epithelial surface

To quantify shedding, the fungal burden in the supernatant and in the cell layer was determined separately. The protocol was adapted from Graf et al. (2019). Epithelial cells were cultured in a 24- or 6-well plate. Following infection as described above, the supernatants were removed carefully with a cut 1 ml pipette tip in order to collect floating hyphal clumps. The volume was replaced with PBS containing 0.25% Triton X-100 to disrupt the host cell layer. After thorough scratching and resuspending the

well content was removed. CFUs were determined as described above.

Quantification of HIF-1 α

Epithelial cells in a 6-well plate (5 ml volume per well) were infected as described above. Total 200 μ M CoCl₂ served as a positive control and was added at the time of *C. albicans* infection. After incubation, the epithelial cells were put on ice and washed with 5 ml ice-cold PBS per well. Total 150 μ l ice-cold sample buffer [10% (V/V) glycerol, 2% SDS (W/V), 5% (V/V) β -mercaptoethanol, 12.4% (V/V) 0.5 M TRIS (pH 6.8), 52.6% (V/V) ddH₂O; all chemicals were purchased from Roth] were added to each well. The cells were scraped off with a cell scraper and transferred to a cooled 1.5 ml tube. For denaturation the cell lysate was boiled at 98°C for 10 min at 300 rpm. After 15 min centrifugation at 13 000 $\times$ g, 125 μ l of the supernatant were transferred to a new tube and stored at -20°C until further use. The denaturation step was performed before the centrifugation to guarantee the lysis of the nucleus where HIF-1 α resides after stabilization.

To separate the proteins, standard SDS-PAGE with a 7.5% separation gel and a 4% collection gel was performed. Afterwards proteins were transferred to a polyvinylidene difluoride membrane. For chemiluminescence detection the membranes were cut to separate the actin (45 kDa) and the HIF-1 α bands (120 kDa). The membranes were incubated in blocking buffer pH 7.6 [0.1 M TRIS, Roth; 1 M NaCl, Roth; 9 mM Tween 20, Serva; 1% BSA (for actin), Serva or 5% milk powder (for HIF-1 α), Roth] at 40 rpm for 1 h. Afterwards they were incubated with the primary antibody solution [blocking buffer, 1:200 purified mouse anti-HIF-1 α (#610959, BD Transduction Laboratories) or 1:1000 rabbit anti-pan-actin (#D18C11, Cell Signaling Technology)] over night at 4°C. Afterwards the membranes were washed 3 $\times$ for 10 min in washing buffer (0.1 M TRIS, Roth; 1 M NaCl, Roth; 9 mM Tween 20, Serva; pH 7.6) and then incubated in the secondary antibody solution [blocking buffer, 1:500 Anti-mouse IgG HRP-linked (#7074, Cell Signaling Technology) or 1:500 Anti-mouse IgG HRP-linked (#7076, Cell Signaling Technology)] for 1 h at room temperature and 60 rpm. After three more washing steps the membranes were treated with the Luminescence kit Western Lightning Plus-ECL (#NEL104001EA, PerkinElmer) according to the manufacturer's instruction. Using an ultra-sensitive camera, pictures of the membranes were taken at 1 min exposure time for actin and 10 min for HIF-1 α . The colors of the pictures were inverted and then the intensity of the bands was converted into relative numerical values using *ImageJ* software. The relative intensity of the HIF-1 α band was normalized to the relative intensity of the corresponding actin band. Finally, the fold change of the normalized band intensity compared to the uninfected sample was calculated and plotted.

Quantification of adhesion, hyphal branching, and hyphal length

Glass slides were placed in a 24-well plate before seeding the enterocytes. After 14 days maturation infections were performed as described above with *C. albicans* (MOI 1) and *P. vulgatus* [ratio 1:1000 (fungi: bacteria)]. Three hour and four hour after the addition of *C. albicans* the wells were washed three times with 1 ml PBS and

then fixed overnight with 4% buffered formalin at 4°C. The glass slides were stained with a primary antibody solution [PBS; 1:2000 *C. albicans* polyclonal antibody (#PA1-27158, Fisher Scientific)] followed by a secondary antibody solution [PBS; 1:5000 Alexa Fluor 488 goat anti-rabbit antibody (#A27034, Fisher Scientific)] and placed inverted on a microscope slide using Prolong Mounting Medium (#P36982, Fisher Scientific). Cells were not permeabilized, therefore only extracellular (adherent) but not invasive (intracellular) fungal cells were stained. Microscopic images were obtained with a Zeiss Axio Observer 7 microscope and Zeiss AxioCam Cc1 camera. For adhesion the number of fungal cells per 4×4 tile images was counted in each sample. Total 200 hyphae were measured manually in each sample: The longest hyphae originating from the mother yeast was measured for hyphal length. Any filaments that grew from the mother yeast or the longest hyphae were considered branches.

Additionally, adhesion was quantified by CFU. Therefore, supernatants were removed after 1 h, 2 h, or 3 h, and the enterocytes washed three times with 1 ml PBS. To detach hyphae, the cells they were treated for 30 min at 37°C with 20 U ml⁻¹ zymolyase (Amsbio # 120491-1). Subsequently the enterocytes were scratched out of the wells and resuspended in PBS. CFU were determined by plating serial dilutions on YPD.

Microcolony assay

In a 24 well-plate format *P. vulgatus* mpk (1×10^8 cells ml⁻¹) and *C. albicans* (1×10^2 cells ml⁻¹) were co-incubated in KBM-Gold™ for 24 h at 37°C under cell culture conditions. Microscopic images were taken with Zeiss Axio Vert.A1 microscope and the Zeiss AxioCam Cc1 camera. Colonies grown on the edge of a well or growing into each other were excluded from analysis. All other colonies within a well (minimum of 20 per experiment and condition) were analyzed with ImageJ/Fiji. The colony diameter was calculated as the mean of the longest and shortest diameter measured.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism 10 software package (GraphPad Software, Inc, San Diego, CA, USA). The datasets were tested for normal distribution by the Shapiro–Wilk test. A One-Way ANOVA was performed to compare the means of three or more groups. Dunnett's test was used to compare the means of each treatment group to a control group (uninfected or *C. albicans* infection, as indicated in the figure legends). An unpaired two-sided Student's t-test was performed to compare the means of two groups. Differences between the groups were considered significant at $P < 0.05$.

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Author contributions

Conceptualization: I.D.J., M.J.N., M.L.H., J.B., I.A., A.S.; Data curation: M.L.H.; Formal analysis: I.D.J., I.A., J.B., M.L.H., A.W.; Funding acquisition: I.D.J.; Investigation: F.G., I.A., J.B., M.L.H., A.W.; Methodology: I.D.J., F.G., I.A., M.J.N., A.S., J.S.F., M.L.H., J.B.; Project administration: I.D.J., M.J.N., M.L.H.; Resources: I.D.J., A.S., J.S.F.; Supervision: M.J.N., I.D.J., M.L.H.; Visualization: I.A., J.B., M.L.H.; Writing original draft: I.D.J., M.L.H.; Writing—review & editing: M.L.H., M.J.N., I.A., J.B., F.G., A.S., J.S.F., A.W., I.D.J.

Supplementary material

Supplementary material is available at *FEMSML Journal* online.

Conflicts of interest

None declared.

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