Title: Synergistic cross-kingdom host cell damage between *Candida albicans* and *Enterococcus faecalis*

Short title: Synergistic bacterial-fungal damage

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Abstract

The fungus *Candida albicans* and the Gram-positive bacterium *Enterococcus faecalis* share mucosal niches in the human body. As opportunistic pathogens, both are found to expand population size during dysbiosis, and can cause severe systemic infections in susceptible individuals. Here, we show that the presence of *C. albicans* results in increased host cell damage by *E. faecalis*. Furthermore, *E. faecalis* aggravates oropharyngeal candidiasis in mice. Increased damage is mediated by enterococcal cytolysin, and involves both physical interaction and altered glucose availability. Physical interaction promotes accumulation of bacteria on host cells, facilitating contact of cytolysin with host cells. Glucose depletion by the metabolic activity of the fungus sensitized host cells to cytolysin. This work illustrates how a complex interplay between fungi and bacteria can result in detrimental consequences for the host.

Keywords

Candida / Enterococcus / co-infection / cytolysin

MAIN TEXT

Introduction

Candida albicans is a fungal pathobiont colonizing different mucosal surfaces of the human body. Colonization is asymptomatic in healthy individuals, but translocation across mucosal barriers in hospitalized and/or immunocompromised patients can lead to severe systemic infection (McCarty and Pappas, 2016; Pappas et al., 2003). Besides immunosuppression, disturbance of the mucosal microbiome by antibiotic treatment is a major risk factor for disseminated candidiasis (Pappas et al., 2003), suggesting that fungal-bacterial interactions significantly influence the behaviour of *C. albicans* in the host. While most research has focused on antagonistic effects of bacteria on *C. albicans* virulence or colonization, synergism has been observed for a few bacterial species, including *Enterococcus faecalis* (Bertolini et al., 2021b; Klaerner et al., 1997; Mason et al., 2012a; Mason et al., 2012b; Todd et al., 2019b).

Enterococci are lactic acid producing Gram positive bacteria ubiquitous in nature and colonizers of plants and animals, including humans (Mundt, 1963a; Mundt, 1963b; Noble, 1978). Like *C. albicans*, they are opportunistic pathogens that can cause various infections ranging from urinary tract infections to bacteraemia and endocarditis (Graninger and Ragette, 1992; Hidron et al., 2008). They share various mucosal host niches with *C. albicans*, like the oral cavity (Smyth et al., 1987), urogenital (Kline and Lewis, 2016) and the intestinal tract (Noble, 1978). In the gut and the oral cavity, the presence of *C. albicans* increases the relative abundance of *Enterococcus* spp. after perturbation of the mucosal microbiota by antibiotics (Erb Downward et al., 2013; Mason et al., 2012a; Mason et al., 2012b) or chemotherapy (Bertolini et al., 2019). Furthermore, *Candida* and enterococci are commonly co-isolated from clinical samples, including blood cultures (Hermann et al., 1999; Klotz et al., 2007; Sutter et al., 2008).

While these observations suggest synergistic interactions between *C. albicans* and enterococci, with possible clinical consequences, *E. faecalis* has also been shown to reduce *C. albicans* virulence by secretion of the bacteriocin EntV. Therefore, it was postulated that *E. faecalis* promotes a commensal lifestyle of *C. albicans* by inhibiting fungal filamentation, which in turn would benefit *E. faecalis* by synergistic interactions (Garsin and Lorenz, 2013). Contrary to this hypothesis, however, interaction of *E. faecalis* and *C. albicans* on an oral organoid model led to increased tissue erosion and microbial invasion (Krishnamoorthy et al., 2020). In this model, virulence-associated *E. faecalis* genes were downregulated, whereas fungal virulence-associated genes were upregulated.

One limitation of the previous studies on *C. albicans – E. faecalis* interactions is that only few strains were used. Enterococci are known for high genome plasticity, with a small core genome and a large accessory genome contributing to up to 25% of the total genome size (Cattoir, 2022). Horizontal gene transfer is common in enterococci, involves different types of mobile genetic elements, and has mainly been studied in the context of antimicrobial resistance (Johnson et al., 2021). In addition, the *E. faecalis* pathogenicity island can be horizontally transferred (Johnson et al., 2021) and contains variations between strains that modulate virulence (Shankar et al., 2002). To address the possible impact of strain-to-strain variation of *E. faecalis* on interaction with *C. albicans*, we screened a larger number of strains in an *in vitro* intestinal model. We discovered that the type of bacterial-fungal interaction depends on strain-specific properties of the bacterial partner. Specifically, synergistic host cell damage depended on the *E. faecalis* cytolysin.

We confirmed the *in vivo* relevance of these findings by using a murine model of oral candidiasis. In this model, cytolysin-producing *E. faecalis* aggravated invasion and damage, while a cytolysin-negative EntV-producing strain reduced invasion. The presence of *C. albicans* increased bacterial virulence partly by increasing *E. faecalis* accumulation and aggregation on host cells. In addition, fungal-mediated nutrient depletion sensitized host cells to pore-forming toxins *in vitro*. Thus, multiple mechanisms contribute to synergistic damage caused *C. albicans*-*E. faecalis* coinfection.

Results

Host cell damage after coinfection of an *in vitro* intestinal model with *Candida albicans* and *Enterococcus faecalis* is bacterial strain-dependent

Both antagonistic and synergistic interactions between *C. albicans* and *E. faecalis* have been reported (Bertolini et al., 2019; Cruz et al., 2013; Graham et al., 2017; Hermann et al., 1999; Klotz et al., 2007; Mason et al., 2012b). To determine the type of interaction on enterocytes, we used an *in vitro* cell culture model to quantify host cell damage following either single infection with *C. albicans* or enterococci, and damage after coinfection. The *E. faecalis* type strain, the commonly used lab strains ATCC 29212, OG1RF, and V583, as well as 16 clinical isolates obtained from blood, stool, or urine were analysed to address possible variations between bacterial strains. While for most of the tested *E. faecalis* isolates the coinfection damage was comparable to or slightly lower than the sum of damage caused by fungus and bacteria alone, five *E. faecalis* strains displayed significantly higher coinfection damage under ambient oxygen (21%), reduced oxygen (10%), and low but physiological gut oxygen tension (1% O_2; Fig. 1A-C). The synergistic effect was especially pronounced at medium and low oxygen. By quantification of bacterial and fungal burden, we could exclude that enhanced host cell damage was due to increased proliferation of microbes during coinfection (Fig. S1).

To determine if the overall higher microbial burden during coinfection led to increased host cell damage, we tested if the number of bacterial and fungal cells used for infection affects LDH release from host cells. Increasing the bacterial inoculum over a range of 4 $\log_{10}$ led to only a twofold increase in monoinfection damage, and coinfection damage was similar within a range of 10^4 to 10^6 bacteria per ml (Fig. S2A). Similarly, *C. albicans*-induced damage was relatively stable in monoinfections; however, coinfection damage was concentration-dependent (Fig. S2B). One possible explanation for the lack of a clear dose-dependent increase of enterocyte damage in mono-infections is that bacterial growth in the system is limited, as suggested by the lack of further increase of bacterial CFU from 24 to 30h shown in Fig. S1. For *C. albicans*, high numbers of fungal filaments might multiple layers of fungal cells without increased numbers of fungal filaments accessing and damaging enterocytes. This indicated that synergistic host cell damage could not be explained by the higher microbial burden, but suggested that fungal activities were required. Consistent with this, heat-killed *C. albicans* did not induce synergistic host cell damage during coinfection (Fig. S2C). Likewise, bacterial viability was required for inducing increased damage (Fig. S2D), indicating that both microbes contribute to the synergistic virulence phenotype. Furthermore, synergistic damage developed within the last 6 h of the co-incubation period, in which bacterial CFU exceeded fungal CFU > 100fold (Fig. S1) and damage caused by monoinfections remained stable (Fig. S2E).

154 ***C. albicans* virulence factors contribute to damage during coinfection but are not**
155 **required for synergism**

156 In the experiments presented above, enterocytes were infected with *C. albicans* first, and
157 bacteria were added after 6 h to prevent a possible negative impact of fast bacterial
158 replication on fungal growth or germ tube induction at early stages of infection. We next
159 tested if this preincubation was required for synergistic damage, and observed that
160 synergism became evident and more pronounced with prolonged preincubation of host
161 cells with *C. albicans* (Fig. S2F).

162 *C. albicans* rapidly forms germ tubes and hyphae in cell culture conditions, and thereby
163 could contribute to synergistic damage. To test this, we used *C. albicans* mutants with
164 defects in filamentation (*eed1*Δ/*eed1*Δ and *efg1*Δ*cph1*Δ/*efg1*Δ*cph1*Δ). As expected, these
165 mutants were strongly reduced in their ability to damage host cells during monoinfection
166 (Fig. 1D). These strains were still able to induce synergistic damage during coinfection.
167 Synergistic damage was also observed with a mutant lacking *ECE1* encoding the fungal
168 peptide toxin candidalysin, and the commensal *C. albicans* strain 101 (Fig. 1D). Overall
169 coinfection damage with was lower for the *C. albicans* mutants and 101 compared to the
170 virulent wildtype strains; this is likely due to the lower or absent fungal-mediated damage
171 in coinfection with these strains. To determine if the synergistic effect on coinfection
172 damage is limited to *C. albicans*, we tested additional fungal species: *Saccharomyces*
173 *cerevisiae* and *Candida glabrata*, but not *Candida parapsilosis*, induced increased
174 coinfection damage (Fig. 1E) despite their lack of cytotoxicity in mono-infections. Taken
175 together, these findings suggested that the increased host cell damage during coinfection
176 was mainly caused by bacterial activities.

177 **Enterococcal cytolysin is necessary for enhanced coinfection damage of enterocytes**

178 Several *E. faecalis* factors contributing to virulence and host cell damage have been
179 described (Fisher and Phillips, 2009). We hypothesized that synergism with fungal
180 infection is mediated by a distinct virulence factor, or set of factors, found in all
181 synergistic strains, but absent in non-synergistic isolates. Therefore, we screened the
182 genomes of the *E. faecalis* isolates used for the presence of virulence-associated genes.
183 Synergistic strains belong to different capsule operon types, but share several virulence-
184 associated genes, e.g. encoding aggregation substance, certain pili, adhesins, and
185 gelatinase (Table S1). However, this set of genes was also present in non-synergistic
186 strains, including OG1RF (Table S2). A notable exception was the cytolysin operon
187 (Table S3). Cytolysin is a pore-forming toxin consisting of two peptides and causing
188 haemolysis on horse blood agar plates (HBA). It is an important virulence factor of *E.*
189 *faecalis* causing host cell damage, and is associated with increased virulence in infection
190 models and patient mortality (Garsin et al., 2001; Huycke et al., 1991; Ike et al., 1984; Jett
191 et al., 1995; Jett et al., 1992). All synergistic strains displayed β-haemolysis on HBA, and
192 carried the complete cytolysin operon (*cyl*; Table S3). The two strains that appear to
193 contain the full *cyl* operon but displayed no synergism in co-infections, strain B3 and B8,
194 showed no β-haemolysis on HBA at high oxygen, and only B3 displayed weak haemolysis
195 at low oxygen (Table S3), indicating possible defects in cytolysin regulation or
196 expression.

197 The *cyl* operon is often located on self-transmissible plasmids (Jett et al., 1992), and
198 sequencing results indicated the presence of a 64 kb plasmid containing the cyl operon in

199 the synergistic strain B1 (Fig. S3). To explore the possible role of cytolysin for synergistic
200 damage, we therefore performed plasmid curing of strain B1 and selected haemolytic and
201 non-haemolytic derivative strains for coinfection experiments. The loss of haemolytic
202 activity was associated with the loss of increased coinfection damage (Fig. 2A).
203 Conversely, transmission of haemolytic activity from B1 to the non-synergistic plasmid-
204 free strain OG1RF by conjugation resulted in increased host cell damage during
205 coinfection (Fig. 2B; Fig. S2G, H). This suggested the presence of a conjugative plasmid
206 in B1, and a link between haemolysis and synergistic damage. However, we cannot
207 exclude that some of the haemolytic colonies obtained in the conjugation experiment were
208 spontaneous rifampicin-resistant mutants of the donor strain. Furthermore, other factors
209 encoded on the putative plasmid could have contributed to the phenotype. We therefore
210 analysed isogenic mutants of *E. faecalis* OG1X (Ehrenfeld and Clewell, 1987; Ike and
211 Clewell, 1984; Ike et al., 1990; Ike et al., 1983) harbouring plasmids encoding the
212 cytolysin operon and/or the gene encoding for the adhesin aggregation substance (AS)
213 (Kreft et al., 1992), which increases adhesion and internalization of *E. faecalis* by colonic
214 enterocytes (Sartingen et al., 2000). Increased damage of host cells during coinfection was
215 consistently observed for cytolysin positive strains (Fig. 2C), while the presence of AS
216 had no effect on damage. Thus, enterococcal cytolysin is required for the enhanced
217 damage during coinfection, and the presence or absence of the *cyl* operon explains the
218 differences in coinfection damage observed for the different *E. faecalis* strains.

219 **The effect of *E. faecalis* on the severity of murine oropharyngeal candidiasis depends**
220 **on the bacterial cytolysin**

221 *C. albicans* and *E. faecalis* do not only share a niche in the gastrointestinal tract but also in
222 the oral cavity. We therefore quantified host cell damage during mono- and coinfection of
223 oral buccal cells (TR146). For these experiments, we used an MOI of 1 to achieve a
224 similar level of damage with *C. albicans* alone as observed with enterocytes. Similar to
225 intestinal cells, coinfection damage was increased with cytolysin positive strains of
226 *E. faecalis* (Fig. 3A). To determine if these strain-dependent effects can be translated to *in*
227 *vivo* infections, we used a murine oropharyngeal candidiasis (OPC) model in which the
228 drinking water was supplemented with different *E. faecalis* strains after *C. albicans*
229 infection. Since we observed apoptotic cell death following infection *in vitro* both for
230 intestinal and epithelial cells (Fig. S4), apoptosis was included as read out for *in vivo*
231 invasion in addition to invasion depth; both parameters were significantly correlated (Fig.
232 3B).

233 Application of haemolytic *E. faecalis* alone, in the absence of *C. albicans* infection, did
234 not cause any histological alterations (Fig. 3C). The consequences of *E. faecalis* on OPC
235 were strain-dependent: The cytolysin-negative strain OG1RF, which was previously
236 shown to reduce *C. albicans* virulence in a *Caenorhabditis elegans* model by production
237 of EntV (Cruz et al., 2013; Graham et al., 2017), led to a significant reduction of the depth
238 of fungal invasion (Fig. 3D) and areas of apoptosis (Fig. 3E). In contrast, the haemolytic
239 strain ATCC 29212 led to more extensive lesions (Fig. 3D, E and Fig. S5A). Similarly,
240 comparison of two *E. faecalis* OG1X derivatives showed enhanced tissue invasion and
241 apoptosis only for the cytolysin-positive strain (Fig. 3F, G and Fig. S5B). By testing six
242 additional *E. faecalis* strains, we confirmed that increased severity of OPC by *E. faecalis*
243 depends on the presence of the *cyl* operon (Fig. 3H, I).

244 Plating of homogenized tongues tissue from uninfected and infected mice confirmed the
245 absence of endogenous *C. albicans* strains (Fig. S6 A, B). Quantification of fungal burden
246 showed a significant but minor increase in *C. albicans* CFU in co-infection with 2/10
247 strains (Figure S6); both *E. faecalis* strains were cytolysin positive. However, no increased
248 fungal burden was observed with the other four haemolytic *E. faecalis* strains tested, three
249 of which led to significantly increased tissue damage. Thus, increased fungal burden
250 might contribute to the augmented severity of OPC for some but not all strains. The effect
251 of *C. albicans* on *E. faecalis* CFU was variable; a significant positive effect was observed
252 for *E. faecalis* ATCC 29212 and OG1RF (Fig. S6A), but not for *E. faecalis* OG1X (Fig.
253 S6B). Importantly, within experiments bacterial burden did not differ between cytolysin-
254 positive and -negative strains in either the presence or absence of *C. albicans* (Fig. S6A-
255 C).

256 **Physical interaction with fungi leads to accumulation of bacteria on host cells**

257 While soluble factors contributed to increased damage of coinfections, physical contact
258 could also be involved. To test this, we separated microbes from host cells and/or each
259 other by using cell culture inserts. Separation of both bacteria and fungi from enterocytes
260 completely abolished host cell damage (Fig. 4A, orange bar), consistent with the
261 requirement of adhesion and invasion for damage caused by *C. albicans* (Sundstrom et al.,
262 2002; Zhu and Filler, 2010), and the lack of solubility of *E. faecalis* cytolysin in most
263 liquid media (Booth et al., 1996; Todd, 1934). The presence of *E. faecalis* in the inserts
264 did not affect host cell damage when *C. albicans* was in contact with enterocytes (Fig. 4A,
265 green/turquoise bars). In contrast, a significant increase of damage by *E. faecalis* was
266 observed if *C. albicans* was applied to the insert (Fig. 4A, purple bars), confirming the
267 role of soluble factors in enterocyte damage. However, the damage in this setting, as well
268 as the sum of the damage caused by either microbe on enterocytes when physically
269 separated from the other, was significantly lower than that observed if both microbes were
270 in physical contact with each other and the host cells (Fig. 4A, pink bar), indicating a
271 significant contribution of contact-mediated mechanisms. Similarly, the presence of *S.*
272 *cerevisiae* in the trans well insert significantly increased enterococcal host cell damage,
273 but damage was lower than that caused by coinfection (Fig. 4A, right). Independent of
274 physical contact, the presence of *C. parapsilosis* did not affect enterocyte damage (Fig.
275 4A, right). One possibility for the higher damage caused by coinfection is that adhesion of
276 bacteria to fungal elements, as observed by electron microscopy (Fig. 4B), increases the
277 contact of bacteria with host cells. To test this, we carefully removed the supernatant after
278 infection and plated the supernatant and lysed host cell layer separately. While the
279 majority of bacteria were found in the supernatant of monoinfections, significantly more
280 bacteria were found on the host cell layer than in the supernatant after coinfection with *C.*
281 *albicans*, reflecting a significant increase of bacteria on enterocytes (Fig. 4C). To test
282 whether this effect is specific for *C. albicans*, we also analysed *S. cerevisiae*, which
283 showed synergistic coinfection damage, and *C. parapsilosis*, which did not.
284 *C. parapsilosis* had no effect on bacterial distribution (Fig. 4C), significantly more
285 bacteria were isolated from supernatant than enterocytes. This is consistent with the failure
286 of this fungus to induce synergistic coinfection damage. *S. cerevisiae* displayed
287 intermediate results, bacterial numbers on enterocytes were increased (Fig. 4C), but the
288 increase was not statistically significant. This could be due to the significantly weaker
289 adhesion of *S. cerevisiae* to enterocytes compared to *C. albicans* (Fig. 4D). This difference
290 was not only observed for enterocytes but also with the plastic surface of cell culture
291 plates (Fig. 4E). Adhesion of *S. cerevisiae* and *C. parapsilosis* was comparable (Fig. 4D,

292 E), suggesting differences in the interaction with *E. faecalis* might mediate the differences
293 in bacterial location during coinfection observed for these species (Fig. 4C).

294 **_Candida_-induced glucose starvation sensitizes enterocytes to bacterial damage and**
295 **pore-forming toxin**

296 To test if soluble factors produced by or changes in media composition due to the
297 metabolic activity of *C. albicans* affect bacterial virulence, we generated *Candida*-
298 conditioned medium (CCM) by growing fungi either alone (CCM-) or on enterocytes
299 (CCM+) in KBM medium for 30 h. These media were then used for infection of
300 enterocytes with *E. faecalis*. Both media led to significantly increased host cell damage
301 during *E. faecalis* monoinfection (Fig. 5A), which was more pronounced than the effect of
302 *C. albicans* in inserts during transwell experiments (Fig. 4A). Therefore, we hypothesized
303 that CCM either contained specific molecules produced by *C. albicans* that cause damage
304 or that nutrient depletion due to the metabolic activity of the fungus affected bacterial
305 virulence. Glucose in naïve KBM (9 mM) was completely depleted after 12 h incubation
306 of *C. albicans*, irrespective of the strain used (Fig. S7A). Of note, in the transwell assay,
307 glucose did not diffuse freely between both compartments, and higher concentrations were
308 observed in the well with *E. faecalis* compared to the insert with *C. albicans* (Fig. S7B).
309 Thus, the stronger effect of CCM compared to transwell experiments could be due to
310 differences in glucose depletion. Furthermore, while naïve KBM contains no lactate, a
311 considerable amount (13 mM) was observed after incubation of enterocytes in medium for
312 the duration of infection experiments. Addition of *C. albicans* resulted in three-fold lower
313 levels of lactate, likely due to its utilization by the fungus. To determine the effect of
314 lactate on host cell damage caused by *E. faecalis*, CCM- was spiked with lactate and used
315 for infection of enterocytes with *E. faecalis*. Lactate addition had no significant effect on
316 *E. faecalis* mediated damage (Fig. S7C). In contrast, glucose supplementation reduced
317 bacterial-induced damage to the level observed with fresh media (Fig. 5A). While glucose
318 deprivation led to higher LDH release during bacterial infection, transcription of $cylL_L$ and
319 $cylL_S$, encoding the two cytolysin subunits, was not altered (Fig. 5B). Similarly, no clear
320 changes in $cylL_L$ and $cylL_S$ were observed for coincubation with *C. albicans* (Fig. S8).
321 This suggested that cytolysin expression is not regulated by glucose. However, while both
322 *C. albicans* and *S. cerevisiae* led to glucose depletion, *C. parapsilosis* did not (Fig. S7D),
323 suggesting a link between glucose and synergistic damage independent of *cyl* expression.
324 Hypothesizing that glucose affects host cell susceptibility to the toxin, we used melittin, as
325 a second small pore forming toxin found in bee venom (Ceremuga et al., 2020). Melittin-
326 induced host cell damage was increased in the absence of glucose (Fig. 5C), suggesting
327 that glucose-depletion by *C. albicans* leads to enhanced effects of bacterial cytolysin by
328 sensitizing enterocytes. Addition of melittin to enterocyte cultures infected with *C.*
329 *albicans*, *S. cerevisiae* or *C. parapsilosis* resulted in synergistic damage with *C. albicans*
330 but not *C. parapsilosis*, similar to the observations with haemolytic *E. faecalis* (Fig. 5D).
331 In contrast to coinfection, no synergism was observed with *S. cerevisiae*. Furthermore,
332 conditioned media of both, *C. parapsilosis* and *S. cerevisiae*, increased damage by *E.*
333 *faecalis* to the extent observed for *C. albicans* (Fig. S7E). Thus, even though glucose
334 depletion affects host cell susceptibility to melittin and bacterial damage, it does not fully
335 explain the synergism during coinfection and the effects of conditioned media.

336

337

338 **Discussion**

339 *C. albicans* and *E. faecalis* co-exist on mucosal surfaces in the human body as part
340 of the normal microbiota. Furthermore, both thrive under dysbiosis, and the presence of
341 *C. albicans* correlates with higher colonization levels of *E. faecalis* (Bertolini et al.,
342 2021a; Bertolini et al., 2019; Mason et al., 2012a; Mason et al., 2012b), suggesting
343 synergistic interactions. However, antagonistic effects on virulence mediated by the *E.*
344 *faecalis* bacteriocin EntV have also been described (Cruz et al., 2013; Graham et al.,
345 2017). This raises the question if this antagonistic interaction neutralizes the increased risk
346 of infection usually associated with elevated fungal and bacterial colonization. We chose
347 to address this by using an *in vitro* model that allowed us to assess the consequences of
348 coinfection on enterocyte damage using a collection of reference and laboratory strains, as
349 well as clinical isolates.

350 This approach revealed that the consequences of coinfection were highly strain-
351 dependent. The majority of *E. faecalis* strains, including the commonly used laboratory
352 strain OG1RF, led to a neutral outcome, in which the damage after coinfection was similar
353 to the sum of damage inflicted by each microbe alone. A distinct set of strains, however,
354 displayed significantly increased host cell damage after coinfection with *C. albicans*. One
355 feature of these strains was haemolytic growth on horse blood agar plates, which is
356 typically caused by the *E. faecalis* cytolysin (Basinger and Jackson, 1968). We confirmed
357 the presence of the cytolysin operon in these strains, with the exception of strain ATCC
358 19433. This strain furthermore caused haemolysis and detectable enterocyte damage only
359 at high oxygen concentrations, whereas coinfection synergy of the other tested strains was
360 more pronounced at low oxygen. As cytolysin expression has been reported to be higher at
361 low oxygen (Day et al., 2003), this together with the absence of a detectable cytolysin
362 operon strongly suggests that ATCC 19433 carries another factor responsible for the
363 observed haemolysis. Two strains, B3 and B8, showed no or only weak haemolytic
364 activity on HBA and no synergistic damage *in vitro* despite presence of the complete
365 operon in the genome. While not tested in this study, a possible explanation is a lack of
366 cytolysin operon expression in these strains. Importantly, gain or loss of haemolytic
367 activity by plasmid curing, plasmid transfer, and using isogenic mutants correlated with
368 synergistic coinfection damage in different genetic strain backgrounds. This indicates that
369 increased damage caused by coinfections can be attributed to enterococcal cytolysin.

370 Increased toxin production by bacteria in the presence of *C. albicans* was
371 demonstrated for *Staphylococcus aureus* (Todd et al., 2019a; Todd et al., 2019c), but
372 could not be confirmed in this study for *E. faecalis* on the transcriptional level. Attempts
373 to detect and quantify the small cytolysin peptides *in vitro* experiments by unbiased
374 proteomics were unsuccessful. Thus, while we cannot exclude that coinfection with *C.*
375 *albicans* results in increased cytolysin production by *E. faecalis*, it is likely not the main or
376 only mechanism involved.

377 An alternative explanation for the increased enterocyte damage upon coinfection is
378 increased local toxin concentration on enterocytes. The importance of localized toxin
379 secretion has recently been demonstrated for the fungal toxin candidalysin that is released
380 in fungal invasion pockets (Mogavero et al., 2021; Swidergall et al., 2021). Consistent
381 with this hypothesis, physical contact was necessary for full synergistic damage. Of note,
382 cytolysin expression and the cytolytic activity of the produced toxin molecules is affected
383 by the presence of target cells: In the absence of target cells, the toxin subunits $CylL_S$ and
384 $CylL_L$ form a stable complex lacking cytolytic activity. $CylL_L$ has a higher affinity to lipid
385 membranes than $CylL_S$, thereby leading to an excess of free $CylL_S$ in the presence of
386 target cells. Free $CylL_S$ exceeding a threshold can bind to the membrane protein CylR1,

387 which modulates the repressive function of CylR1 and CylR2, resulting in expression of
388 the operon (Coburn et al., 2004; Haas et al., 2002; Roux et al., 2009). As a consequence,
389 cytolysin-positive *E. faecalis* strains can cause haemolysis on blood agar plates, but fail to
390 lyse erythrocytes in liquid media (Todd, 1934). In addition, quorum-sensing autoinduction
391 depending on cell density drives cytolysin expression and adherence at the infection site
392 promotes the toxic effects of cytolysin (Haas et al., 2002). The presence of *C. albicans*
393 resulted in a higher number of bacterial cells in association with enterocytes, and thereby
394 possibly enhanced cytolytic activity.

395 Binding to fungal cells has also been reported for *S. aureus* and oral streptococci
396 (Diaz et al., 2012; Peters et al., 2012). While *S. aureus* binds preferably to Als3 expressed
397 on hyphae (Peters et al., 2012), synergistic damage by *E. faecalis* also occurred with
398 filament-deficient *C. albicans* strains and other yeast species, suggesting a different
399 binding mechanism. The synergistic coinfection damage observed with non-filamentous
400 fungi furthermore implies that pronounced fungal invasion and creation of invasion
401 pockets are not necessary for increased cytolysin-mediated damage, although they did
402 contribute to overall host cell damage. *S. cerevisae*, which led to increased damage in *E.*
403 *faecalis* coinfection, also trended to increase the number of bacteria on host cells. It should
404 be noted that binding of *S. cerevisiae* to host cells and the plastic surface was weaker than
405 that of *C. albicans*. This possibly resulted in the loss of bacteria during washing steps, and
406 consequently underestimation of the number of bacteria in contact with enterocytes during
407 coinfection with *S. cerevisiae*. Furthermore, coinfection with *C. parapsilosis* had no effect
408 on bacterial association with the host cell layer and did not result in synergistic damage.
409 As the adhesion of *C. parapsilosis* and *S. cerevisiae* to host cells and the plastic surface
410 was comparable, different factors might mediate binding to surfaces and interactions with
411 *E. faecalis*.

412 Taken together, our results suggest that physical interactions between fungi and
413 bacteria, resulting in an increased concentration of bacteria on host cells enhances
414 cytolysin-mediated damage. This would be consistent with the enhancing effects that
415 target cells have on cytolysin regulation (Coburn et al., 2004; Haas et al., 2002; Roux et
416 al., 2009), haemolysis on solid but not in liquid media (Todd, 1934), and the lack of
417 damage enhancement if bacteria were located on the insert during transwell experiments.

418 While physical interaction played a prominent role for coinfection damage *in vitro*,
419 culture-conditioned media also had an effect on enterococcal host cell damage. This
420 implied that consumption of nutrients or release of small molecules by *C. albicans* could
421 affect bacterial virulence. This might also explain why synergistic damage *by C. albicans*
422 only occurred if enterocytes were infected with *C. albicans* first, and why the synergistic
423 effect was more pronounced with increasing preincubation time and fungal cell numbers:
424 Ongoing fungal metabolic activity would lead to increased nutrient depletion or
425 conversion over time. Production of small molecules, including secreted metabolites,
426 might be more pronounced in the absence of bacterial nutrient competition. Here, we
427 focused on glucose consumption by *C. albicans* as an activity that would lead to depletion
428 of nutrients available to the bacteria. While glucose depletion increased host cell damage
429 by *E. faecalis*, this was not attributable to increased cytolysin production. Furthermore, the
430 use of KBM medium with a high buffering capacity prevented media acidification during
431 the experiments. Therefore, we considered effects of glucose on host cells. Because
432 recombinant cytolysin is not available, we used melittin as a model pore-forming toxin,
433 and observed that damage by melittin was reduced by glucose supplementation. A
434 protective effect of luminal glucose was also observed for LPS-, and *Giardia*-induced
435 enterocyte apoptosis *in vitro* (Yu et al., 2005; Yu et al., 2008), and ischemia/reperfusion-

436 induced enterocyte damage and barrier dysfunction *in vivo* (Huang et al., 2011). While the
437 mechanisms by which glucose reduces toxin-mediated damage remain unclear, the
438 negative effects of fungal glucose depletion have been demonstrated for *C. albicans* and
439 macrophages. Infected macrophages increase glycolysis and rely on glucose for survival.
440 *C. albicans* rapid glucose uptake depletes glucose available for the macrophage, thereby
441 causing macrophage death (Tucey et al., 2018). This illustrates that nutrient depletion by
442 microbes can affect host cell susceptibility. Interestingly, though, the addition of melittin
443 to enterocytes infected with *C. albicans*, *S. cerevisiae* or *C. parapsilosis* resulted in
444 synergistic damage with *C. albicans* only, whereas coinfection of *E. faecalis* also resulted
445 in synergistic damage with *S. cerevisiae*. This indicates differences between the toxins,
446 and that results obtained with melittin cannot be directly translated to enterococcal
447 cytolysin. Furthermore, infection of enterocytes with *E. faecalis* in conditioned media
448 from all three fungal species resulted in increased host cell damage. This cannot be
449 attributed to glucose depletion, and for *C. parapsilosis* is in stark contrast to the lack of
450 synergistic damage in coinfection experiments. It seems possible that various soluble
451 factors influence host cell susceptibility either positively or negatively, and that the
452 ultimate outcome depends on the quality and quantity of different factors.

453 Cytolysin-dependent synergistic damage *in vitro* was not restricted to enterocytes
454 but also occurred with oral epithelial cells. Since intestinal colonization of mice with
455 neither *C. albicans* nor *E. faecalis* results in detectable epithelial damage (Garsin et al.,
456 2014; Garsin and Lorenz, 2013; Shao et al., 2022), we decided to use a murine
457 oropharyngeal candidiasis (OPC) model in which mucosal damage can be more easily
458 measured to test if cytolysin-dependent synergistic damage occurs *in vivo*. Furthermore,
459 physical interaction between *C. albicans* and *E. faecalis* has been observed during murine
460 OPC (Bertolini et al., 2019). The results confirmed increased host damage by coinfection
461 with *C. albicans* and cytolysin-producing *E. faecalis*. Additionally, the cytolysin-negative
462 strain OG1RF led to reduced fungal invasion depth. This likely reflects EntV-mediated
463 reduction of filamentation and virulence, which was described in other infection models
464 (Cruz et al., 2013; Graham et al., 2017), but was not evident in *in vitro* enterocyte
465 infection. While it appears likely that the physical interactions between *C. albicans* and *E.*
466 *faecalis in vitro* also affect *in vivo* coinfection, the role of metabolism and soluble factors
467 might differ. Regarding glucose availability, for example, it should be noted that normal
468 blood glucose levels (70 – 100 mg/dl) are only slightly lower than the amount of glucose
469 in cell culture media, and remain relatively constant over time, providing continuous
470 supply for tissues. However, changes in local perfusion due to inflammation might create
471 microenvironments in which glucose concentrations are lower independent to microbial
472 activities, or increased blood flow might compensate for increased glucose consumption.
473 It therefore remains unclear if and to which extend *C. albicans*' metabolic activity affects
474 glucose concentrations *in vivo*.

475 In summary, this study demonstrates that the outcome of *C. albicans – E. faecalis*
476 coinfection depends on strain-specific bacterial properties, and we identified cytolysin as
477 the factor aggravating host damage *in vitro* and *in vivo*. Therefore, species identification
478 alone might not be sufficient to predict the risk of infections in patients with concurrent
479 bacterial and fungal overgrowth. Furthermore, both physical interactions between the
480 microbes and metabolic activity altering the environment and affecting host cell resilience
481 contribute to increased damage during coinfection.
482
483
484 **Materials and Methods**
485

486 **Strains and culture conditions**
487 All strains used in this study are listed in Table S4 and S5. Strains were stored as glycerol
488 stocks at -80 °C and streaked on solid media prior to experiments. Fungal overnight
489 cultures were prepared by inoculating 10 ml yeast extract peptone dextrose (YPD; 2%
490 glucose, 2% peptone and 1% yeast extract) with a single colony and incubation at 30 °C
491 and 180 rpm. Bacteria were streaked on Columbia CNA Agar (HiMedia) or Brain Heart
492 Infusion (BHI) Agar (Carl Roth) containing 5% horse blood (Thermo Scientific). For
493 overnight cultures, colonies were transferred into 5 ml liquid Todd-Hewitt-Broth medium
494 (THB) (Carl Roth) and incubated at room temperature (RT) at 180 rpm. For experiments,
495 overnight cultures were inoculated to OD_{600} 0.1 in fresh THB and grown for
496 approximately 2 h at 37 °C and 180 rpm till they reached an OD_{600} of approximately 1.
497 The bacterial OD_{600}-CFU-correlation was determined by plating serial dilutions of such
498 cultures on lysogeny broth agar (LB; 1% tryptone, 0.5% yeast extract, 1% sodium
499 chloride) and quantification of colony forming units (CFUs) after 24 h incubation at 37
500 °C. Heat inactivation was achieved by incubating cultures diluted to the desired
501 concentrations for 1 h at 65 °C in a water bath. Killing was confirmed by plating on THB
502 (bacteria) or YPD (fungi) agar plates.
503
504 ***In vitro* infection models**
505 For enterocyte infection a 7:3 mixture of the two human cell lines C2BBe1 (brush-border
506 expressing enterocytes, ATCC CRL2102) and mucus producing HT29-MTX-E12 (mucus
507 producing, European Collection of Authenticated Cell Cultures Cat. No.: 12040401) cells
508 were grown at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM,
509 Gibco) supplemented with 10% v/v fetal calf serum (FCS) (Bio&Sell), 0.01 mg/ml Holo-
510 Transferrin (Sigma), and 1% non-essential amino acids (Glibco) (Hilgendorf et al., 2000).
511 Cells were differentiated for 14 days with medium exchanges twice a week. TR146 cells
512 (human oral buccal cells, European Collection of Authenticated Cell Cultures Cat. No.:
513 10032305) were grown in DMEM/F12 (Gibco) with 10% FCS for 48 h. Fungal overnight
514 cultures were washed twice with phosphate buffered saline (PBS), counted in a Neubauer
515 Chamber (Hecht Assistent), and set to 2×10^7 cells per ml in PBS. Bacteria were washed
516 twice with PBS and diluted to a cell density of $2x10^6$ per ml based on the CFU-OD_{600}-
517 correlation. Host cells (mixed enterocytes or oral buccal cells) were washed once with
518 PBS and KBM (Gold Keratinocyte Medium; Lonza Walkersville) or conditioned KBM
519 was added. KBM was used instead of the respective cell culture maintenance medium due
520 to its higher buffering capacity to prevent media acidification by bacteria during infection.
521 Then, host cells were infected with $1x10^6$ fungal cells per ml (MOI = 1; e. g. 10 µl of
522 $2x10^7$ cells stock solution in 200 µl for the 96-well plate format; 50 µl for 24-well plates;
523 250 µl for 6-well plates) unless stated otherwise, and incubated for 6 h at 37 °C, with 5%
524 CO_2 and either 21%, 10%, or 1% O_2. After 6 h (or earlier, if indicated) $1x10^5$/ml
525 enterococci were added. After additional 24 h of incubation supernatants were removed,
526 and samples were processed as described below. Due to the fast growth of the bacteria,
527 bacterial CFU exceeded fungal CFU at the end of the experiment (Fig. 1S). Trans-well
528 experiments were performed as described above in 24 well plates using inserts with a 0.4
529 µm pore size (Merck). In order to assess host cell susceptibility to small pore forming
530 toxins, 1 µM of melittin (Sigma) was added to the host cells as described in (Ceremuga et
531 al., 2020).
532
533 **Quantification of host cell damage**
534 Host cell damage was quantified by measuring lactate dehydrogenase (LDH) release into
535 the supernatant using the Cytotoxicity Detection Kit (Roche) according to the

536 manufacturer's instructions. For each experiment, supernatants from uninfected cells and
537 uninfected cells lysed with 0.25% Triton X-100 (Sigma) served as negative and positive
538 controls, respectively. Infection-mediated cell damage was calculated by subtracting the
539 non-infected control values from all other samples relative to the high control.

540 $$\%rel.LDH = \frac{absorbance(sample) - absorbance(uninfected)}{absorbance(highcontrol) - absorbance(uninfected)} x100$$

541

542 **Quantification of host cell apoptosis**

543 To measure apoptosis, 5% DMSO (Carl Roth) was added as positive control to non-
544 infected host cells for 4h. Apoptotic cells were measured by using FAM-DEVD-OPH *in*
545 *vitro* Apoptosis Detection Reagent (ICT-6356) according to the manufacturer's protocol.
546 Briefly, 700 µl of supernatant was removed and 10 µl staining solution was added to each
547 well. After 1-hour incubation at room temperature, cells were scrapped off followed by
548 washing once with PBS. Cells were resuspended in 300 µl of PBS and mixed gently to
549 disrupt clumping. Apoptotic cells were quantified on a fluorescence plate reader (Tecan
550 infinite M200 pro) at Ex/Em = 490/525 nm.

551

552 **Quantification of total microbial burden during host cell infection**

553 To quantify microbial burden, 10 µl supernatants were removed at the indicated time
554 points for host cell damage quantification the wells were then treated with 1 mg/ml
555 Zymolyase 20T (amsbio) for 30 min at 37 °C. The well content was scraped off, removed
556 from the well, and further incubated for 30 min to separate fungal hyphae. The samples
557 were serially diluted and plated on YPD agar containing 50 µg/ml gentamicin and 100
558 µg/ml doxycycline (for quantification of fungi) and LB agar containing 50 µg/ml nystatin
559 (for quantification of bacteria). Colonies were counted after 24 h incubation at 37 °C.

560

561 **Quantification of bacterial cell numbers in infection supernatants and on host cells**

562 Single and coinfections were carried out as described above. At the end of infection, the
563 supernatant was used to carefully wash off loose bacteria by pipetting up and down once
564 before it was removed from the cell layer. The remaining cell layer was then washed once
565 and resuspended in PBS by rigorous pipetting. Both supernatant and cell fraction were
566 serially diluted and plated on LB agar to quantify bacterial CFUs.

567

568 **Generation of *C. albicans* conditioned media**

569 Enterocytes or empty wells containing KBM only were inoculated as described above
570 with $1x10^6$ cells/ml *C. albicans* in 30 ml and incubated for 30 h at 37 °C with 5% CO2
571 and 1% O_2. The supernatant was removed, filtered through a 2 µm Filtropur sterile filter
572 (Sarstedt), and stored at -80 °C. Conditioned medium was used either directly or spiked
573 with glucose (Carl Roth) or L-(+)-lactate (Sigma) to a final concentration of 9 mM or 13
574 mM, respectively.

575

576 **Quantification of glucose and lactate in cell culture supernatants**

577 Host cells were either uninfected or infected with ether $1x10^6$ cells/ml fungi, $1x10^5$
578 cells/ml *E. faecalis*, or both. Over a course of 10 h each hour one droplet of supernatant
579 was taken and the glucose (mmol/l) measured using a Contour blood sugar meter
580 (Ascensia Diabetes Care, Germany). Lactate in cell culture media or culture supernatants
581 was quantified by the Institute of Clinical Chemistry and Laboratory Diagnostics, Jena
582 University Hospital, Jena, Germany, using routine methods.

583

584 ***E. faecalis* plasmid curing and conjugation**

585 Plasmid curing was performed as described by McHugh and Swartz (McHugh and Swartz,
586 1977). Briefly enterococcal overnight cultures were diluted to $1x10^5$ cells/ml in 10 ml
587 fresh THB medium containing 8 µg/ml novobiocin (Sigma) and incubated at 37 °C
588 without shaking for 18 h. Afterwards the culture was serially diluted and plated on CNA
589 Colombia Agar (HiMedia) supplemented with 5% horse blood (Thermo Scientific)
590 overnight to determine haemolytic activity of colonies. Non-haemolytic colonies were
591 subcultured twice on CNA Colombia Agar (HiMedia) supplemented with 5% horse blood
592 to confirm absence of haemolysis before long-term storage in glycerol at -80°C. Strains
593 that underwent the curing procedure but were still haemolytic were used as controls in
594 infection experiments. To facilitate plasmid transfer by conjugation, filter mating was
595 performed as described by Haug *et al.* (Haug et al., 2010). In short, donor (B1) and
596 recipient (OG1RF) overnight cultures were mixed 1:3 and 100 µl of cells were dropped
597 onto a sterile 0.45 µm nitrocellulose filter (GVS North America) placed on a nonselective
598 THB agar plate. The plates were incubated overnight, the filter removed, and washed with
599 5 ml sterile PBS in a 50 ml centrifuge tube by vigorous vortexing. The suspension was
600 then serially diluted and plated on CNA Colombia Agar supplemented with 5% horse
601 blood and 50 µg/ml rifampicin (Sigma) overnight to select for the recipient strain OG1RF
602 (rifampicin-resistant). Haemolytic colonies were subcultured twice on CNA Colombia
603 Agar supplemented with 5% horse blood to confirm acquisition of haemolytic activity
604 before long-term storage in glycerol at -80°C. Non-haemolytic colonies from the same
605 experiments were used as negative control strains.
606
607 **Bacterial DNA Isolation**
608 Genomic DNA from 10 ml Enterococci overnight cultures was isolated using the Qiagen
609 Genomic-tip 100/G (Qiagen) Kit according to the manual for gram positive bacteria.
610 Genomic DNA for smaller volumes was isolated with the kit Qiagen DNeasy Blood &
611 Tissue according to the manual section for gram positive bacteria.
612
613 **Whole Genome Sequencing of enterococcal DNA**
614 DNA was quantified using the Qubit High Sensitivity DNA kit (ThermoFisher). DNA was
615 fragmented using Covaris microTUBE AFA Fiber tubes (Covaris). Fragmented DNA was
616 used to prepare sequencing libraries using the TruSeq Nano kit (Illumina) to obtain 550 bp
617 insert size libraries. Quality of sequencing libraries was assessed using the QIAxcel
618 Advanced System (Qiagen). Quantity was determined using Qubit High Sensitivity DNA
619 kit (Thermo Fisher). Sequencing libraries were sequenced on Illumina Nextseq creating
620 300bp paired-end reads. Sequencing coverage was 100-fold.
621 The raw binary base call files produced from the Illumina Nextseq were converted to fastq
622 using Illumina's bcl2fastq conversion software (v2.20.0.422). Before analysis, general
623 quality readings were collected using FastQC (0.11.4 34) (Andrews, 2010), for quality
624 control of data generated on Illumina platforms. Reference sequence data for cytolysin
625 operon was retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG -
626 www.genome.jp) entry for the Enterococcus faecalis ATCC 29212 (T03320) p1plasmid
627 (genes DR75_2951 - DR75_2958). These sequences were then compared to sequence data
628 using the BBduk (Decontamination Using Kmers) tool from the BBMap suite (38.90)
629 (Bushnell, 2014.), while simultaneously trimmed of lower quality regions, using the
630 flags/options: quality trim reverse/forward ends (qtrim) = rl, trim read length equal to zero
631 modulo 5 to correct for erroneous Illumina extra sequencer bases (ftm) = 5, trim regions
632 with average quality below (trimq) = 10, kmer length (k) = 31, allowing one mismatch
633 (hdist) = 1. Each cytolysin gene sequence was probed separately and the resulting kmer
634 contamination statistics files were then used to determine its presence/absence in every

635 strain. The sequence data was then also probed using the sequences of other known
636 *Enterococcus* virulence factors retrieved from the Virulence Factors of Pathogenic
637 Bacteria database (http://www.mgc.ac.cn/VFs/main.htm), to determine their presence in
638 the strains.
639 Additional sequencing was conducted using the Nanopore sequencing technology (ONT).
640 The ZymoBIOMICS DNA Miniprep Kit (Zymobiomics) was used to isolate bacterial
641 DNA for long read sequencing. DNA quantification steps were performed using the
642 dsDNA HS assay for Qubit (Invitrogen, US). DNA of the samples was size selected by
643 clean up with 0.45 × volume of Ampure XP buffer (Beckman Coulter, Brea, CA, USA)
644 and afterwards eluted in 50 µl EB buffer (Qiagen, Hilden, Germany). The library was
645 prepared from 1 µg DNA using the SQK-LSK114 kit (Oxford Nanopore Technologies,
646 Oxford, UK) according to the manufacturer's protocol. Reads of the samples were
647 basecalled using *Dorado* (v7.3.11) (Oxford Nanopore Technologies) using the high super
648 accuracy basecalling model. The basecalled reads were assembled and polished using *Flye*
649 (v2.9.3), *Medaka* (v1.11.3), and *Racon* (v1.4.20).
650
651 **PCR of enterococcal genomic DNA**
652 Polymerase chain reaction with Phusion High-Fidelity DNA-Polymerase (ThermoFisher)
653 was performed as stated in the manual with the following settings. 35 cycles, denaturation
654 10 s, annealing at 55 °C 30 s, elongation 45 s, final extension 10 min. Genomic DNA was
655 diluted 1:100 and used as 1 µl. Final reaction volume 20 µl. All primers used to amplify
656 cytolysin genes are shown in Table S4.
657
658 **Quantitative real-time PCR**
659 To measure cytolysin transcription single- and coinfections were carried out as described
660 above for 18 h post enterococcal infection. To assess if contact to *C. albicans* alone is
661 sufficient to trigger cytolysin transcription single- and cocultivations were carried out
662 using KBM medium mixed with lysed frozen enterocytes (10^6 cells/ml) to allow optimal
663 enterococcal growth. Microbes were scraped off, washed once with ice cold distilled water
664 to remove enterocytes, collected into ceramic bead tubes (Genaxxon bioscience), and
665 stored at -80 °C till further use. Frozen cell pellets were resuspended in 1 ml TRI Reagent
666 (RiboPure™ RNA Purification Kit, #AM1924, Invitrogen). Cells were mechanically
667 disrupted on a FastPrep-24 cell-homogenizer (MP Biomedicals) with two 30 s runs at 6.0
668 m/s and with 5 min on ice incubation between each run. Samples were centrifuged at
669 13,000 rpm for 10 min at 4°C and further processed using the RiboPure RNA Purification
670 Kit (#AM1924, Invitrogen) according to the manufacturer's protocol. The purified RNA
671 was treated with TURBO DNase (#AM2238, Ambion) by adding 0.1 volume 10×
672 TURBO DNase buffer and 1 µl TURBO DNase to each sample. DNase activity was
673 inactivated by adding 0.1 volume DNase Inactivation Reagent. The quality and quantity of
674 the RNA was determined by utilizing the Bioanalyzer device (Agilent) and Nanodrop
675 (ThermoFisher), respectively. Subsequently, 1500 µg RNA was transcribed into cDNA by
676 using the SuperScript IV Reverse Transcriptase (#18090010, Invitrogen) according to the
677 manufacturer's protocol. The following qRT-PCR was conducted in biological and
678 technical triplicates with the Brillant II SYBR Green QPCR Master Mix (#600828,
679 Agilent) in the Stratagene Mx3005 device (Agilent). In brief, 1:10 dilutions of each cDNA
680 sample were used. The primer efficiency was determined with genomic DNA of the *E.*
681 *faecalis* strain ATCC 29212 in 1:10 dilutions from 100 µg/ml to 0.1 µg/ml. The respective
682 gene expression was calculated with the Δct method relative to the housekeeping gene
683 *gyrA*. All primers are listed in Table S4
684

685 **Mouse model of oropharyngeal candidiasis**
686 Six to seven weeks old male C57BL/6J mice were purchased from Jackson laboratories
687 (USA) and housed for 1 week in a pathogen free facility prior to infection. The mice were
688 randomly assigned to the infection groups. No antibiotics were used for infections with the
689 other strains. Oral *C. albicans* infection was induced as described previously (Millet et al.,
690 2020; Solis and Filler, 2012; Swidergall et al., 2018). Briefly, on day -1 and +1 post oral
691 fungal infection mice were given a subcutaneous injection of 150 mg/kg cortisone acetate
692 (Sigma-Aldrich). For inoculation, the animals were sedated, and a swab saturated with $1 \times$
693 10^6 *C. albicans* cells SC5314 (Fonzi and Irwin, 1993) was placed sublingually for 75 min.
694 Six hours post *C. albicans* infection *E. faecalis* was added to the drinking water for 24 h (2
695 $\times 10^7$ CFUs in 50 ml). This approach was chosen to resemble the *in vitro* experiments, in
696 which synergistic damage was most pronounced if fungi were allowed to adhere to host
697 cells before enterococci were added. Water consumption was monitored in some
698 experiments and found to be 5 ml ± 0.4 ml/ mouse and 24 h. Thus, the effective infectious
699 dose for *E. faecalis* is estimated to be 2×10^7 CFU/mouse.
700 Preliminary experiments showed that *E. faecalis* OG1X + pAM714 and OG1X +
701 pAM9058, respectively, could not be retrieved from the tongues of mice infected by the
702 protocol described above. We considered two possible reasons: Loss of the plasmids due
703 to the lack of selective pressure, and reduced fitness in competition with other bacteria,
704 especially endogenous enterococci. The later hypothesis was based on the observation that
705 these strains grew more slowly *in vitro* than other *E. faecalis* strains used. Therefore, mice
706 to be infected with these strains were started on antibiotics (gentamicin 15 µg/mL;
707 erythromycin 50 µg/mL) in the drinking water at day -2, and antibiotic supplementation
708 was continued throughout the experiment. Erythromycin was used to ensure plasmid
709 maintenance, gentamicin to suppress endogenous bacteria (OG1X is gentamicin resistant).
710 The mice were euthanized by pentobarbital overdose followed by cervical dislocation two
711 days after *C. albicans* infection. For CFU enumeration the tongues were harvested, cut in
712 half, weighed, homogenized and quantitatively cultured. Sabouraud dextrose agar (SDA)
713 containing chloramphenicol (50 µg/ml) was used for quantification of *C. albicans*. BHI
714 Agar with 5% horse blood, gentamicin (15 µg/ml)/erythromycin (50 µg/ml), and
715 fluconazole (250 µg/mL; Sigma-Aldrich) was used for selective culture of *E. faecalis*
716 OG1X pAM714 and OG1X pAM9058 based on the resistance marker located on the
717 respective plasmid. The presence or absence of haemolysis confirmed that the isolated
718 colonies showed the anticipated phenotype. Selection by antibiotics was not possible for
719 the other *E. faecalis* strains. Therefore, *Enterococcus* ChromoSelect Agar (Sigma-Aldrich)
720 supplemented with fluconazole (250µg/mL) was used as selective medium for CFU
721 quantification for all other strains. The presence/absence of haemolytic activity of colonies
722 growing on *Enterococcus* ChromoSelect Agar was confirmed by plating randomly
723 selected colonies on BHI Agar with 5% horse blood.
724 For histology and immunhistochemistry, tongues were cut in half, fixed in zinc buffered
725 formalin, and embedded in paraffin. Three non-consecutive sections per tongue (sections
726 from different parts of the tongue) were randomly selected and stained with Periodic Acid
727 Schiff (PAS) were imaged by light microscopy, and the depth of individual fungal lesions
728 relative to surface of the tongue (Solis et al., 2017) were determined using PROGRES
729 GRYPHAX® software version 1.1.8.153 (Jenoptik). Apoptotic cells were stained by
730 immunohistochemistry using the ApopTag *in situ* apoptosis detection kit (EMD Millipore)
731 as described previously (Soliman et al., 2021). Briefly, three non-consecutive paraffin-
732 embedded sections per tongue were randomly selected, rehydrated in Histo-Clear II
733 (National Diagnostics) and alcohols followed by washing with PBS. The sections were
734 pre-treated with 20 µg/mL Proteinase K (Ambion) in PBS 15 min at RT. Endogenous

735 peroxidases were blocked by incubation of the slides for 15 min in 3% hydrogen peroxide.
736 Sections were incubated with equilibration buffer (EMD Millipore) for 30 sec at RT,
737 followed by terminal deoxynucleotidyl transferase (TdT; EMD Millipore) at 37°C for 1 h.
738 Sections were further exposed to anti-Digoxignenin for 30 min at RT, and the positive
739 reaction was visualized with 3-diaminobenzidine substrate (Thermo Scientific). After
740 counterstaining the specimens with 0.5% methyl green (Sigma), they were imaged by
741 bright field microscopy. For quantification, apoptotic areas were quantified using
742 PROGRES GRYPHAX® software (Jenoptik). Samples were blinded before evaluation.
743
744 **Ethics statement**
745 All animal work was approved by the Institutional Animal Care and Use Committee
746 (IACUC) of the Lundquist Institute at Harbor-UCLA Medical Center (protocol number
747 30927-01R).
748
749 **Statistical analysis**
750 Statistical analyses were performed using Graphpad Prism version 9.2.0. The type of
751 statistical analysis used is indicated for each data set in the figure legend.
752
753
754
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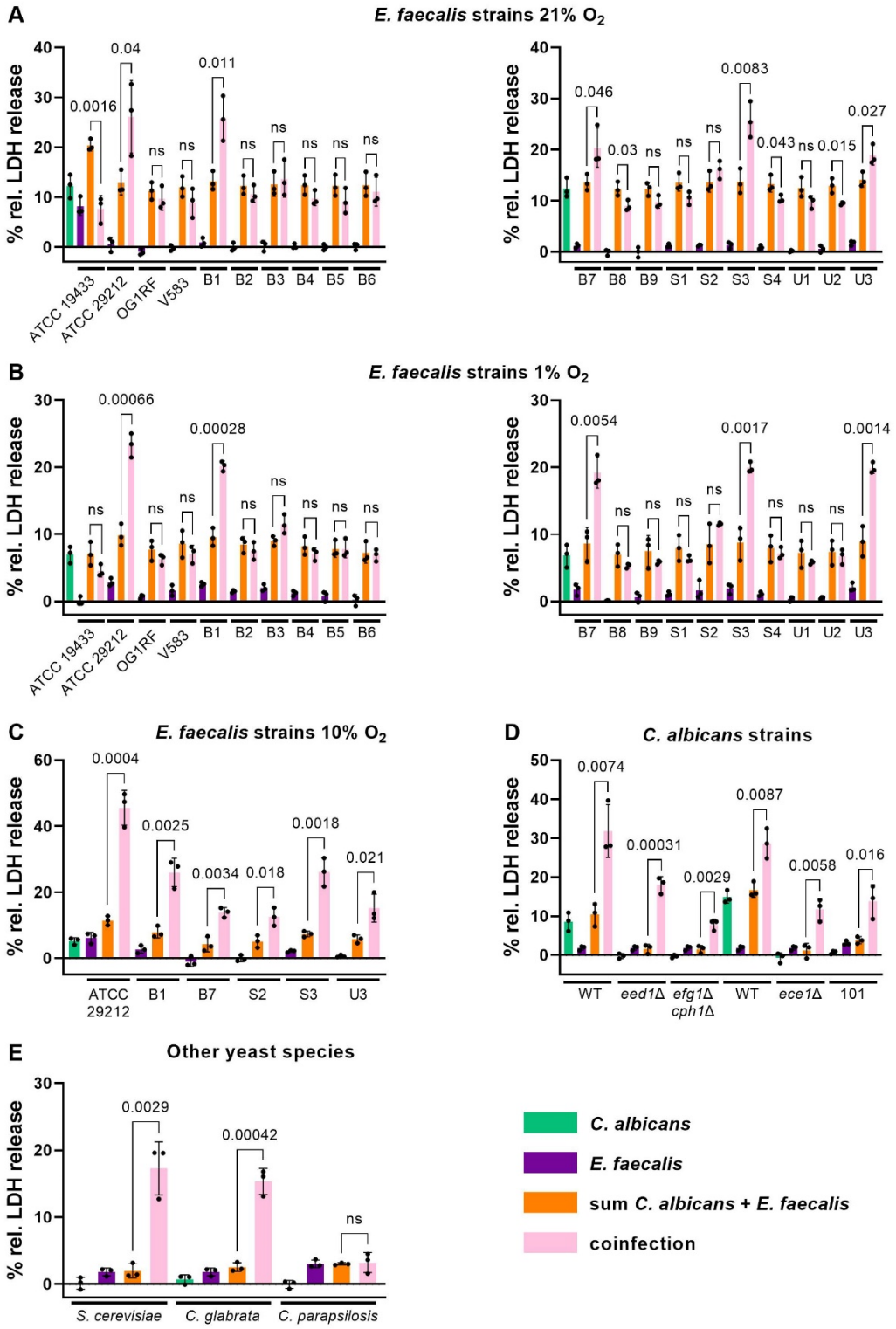
All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

1006 **Figures and Tables**



1007
1008 **Figure 1: Strain-dependent synergy during *Candida-Enterococcus* coinfection of**
1009 **enterocytes results in increased host cell damage**

1010 Enterocytes were infected with 10^6 cells/ml *C. albicans* for 6 h followed by addition of 10^5
1011 cells/ml *E. faecalis* and further incubation for 24 h. Damage was measured by release of
1012 host cell lactate dehydrogenase (LDH) into the supernatant, normalised to non-infected
1013 controls, and is displayed as percent damage relative to Triton X-100-lysis. Green:

1014 monoinfection with *C. albicans*; dark purple: monoinfection with *E. faecalis*; orange: sum
1015 of single infections; light pink: coinfection. Bars represent mean and SD, n = 3. The sum
1016 of LDH release caused by bacterial and fungal monoinfection was compared to the LDH
1017 release following coinfection using two-tailed unpaired Student's t-tests. Significant
1018 values are indicated by p-values. ns: not significant ($p > 0.05$). (**A**) Damage after infection
1019 with *C. albicans* SC5314 and different *E. faecalis* strains at 21 % O_2 ,5 % CO_2. (**B**)
1020 Damage at 1 % O_2, 5 % CO_2. **C:** Damage at 10 % O_2, 5 % CO_2. (**D, E**) Damage after
1021 infection with *E. faecalis* B1 and different *C. albicans* strains (**D**) or other yeast species
1022 (**E**). WT: Parental strain of the respective deletion mutants (SC5314 for *eed1*Δ and
1023 *efg1*Δ*cph1*Δ; BWP17 for *ece1*Δ).
1024

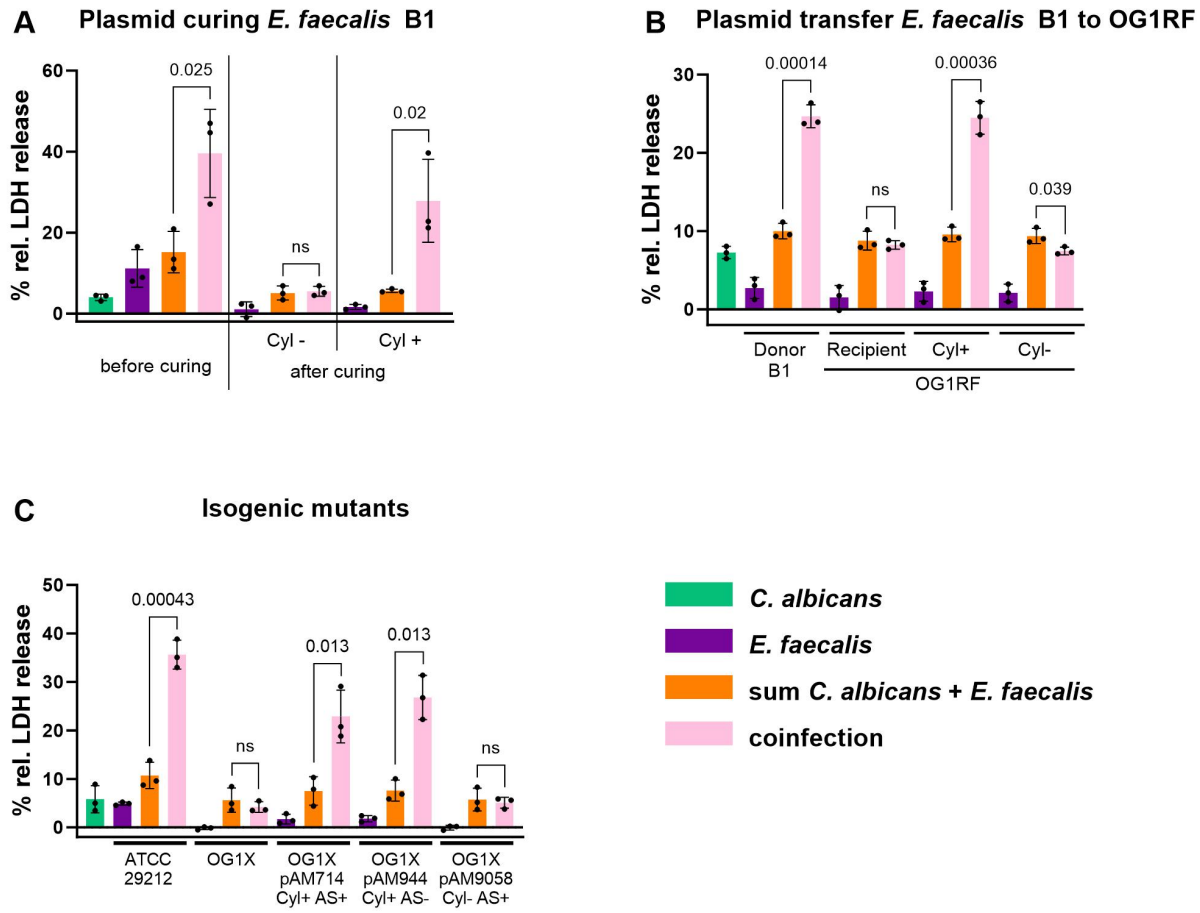
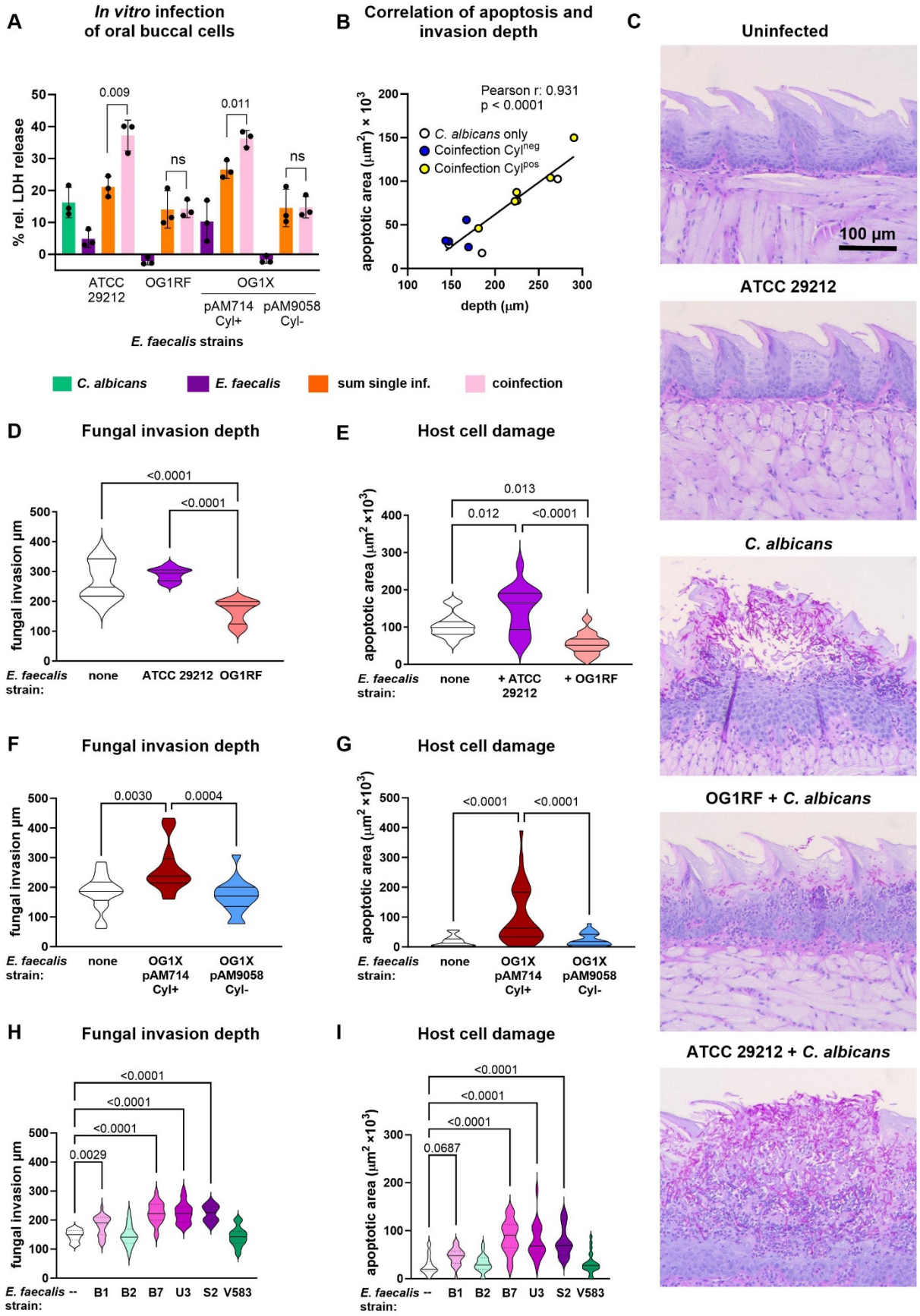


Figure 2: Synergistic coinfection damage is mediated by the *E. faecalis* cytolysin.

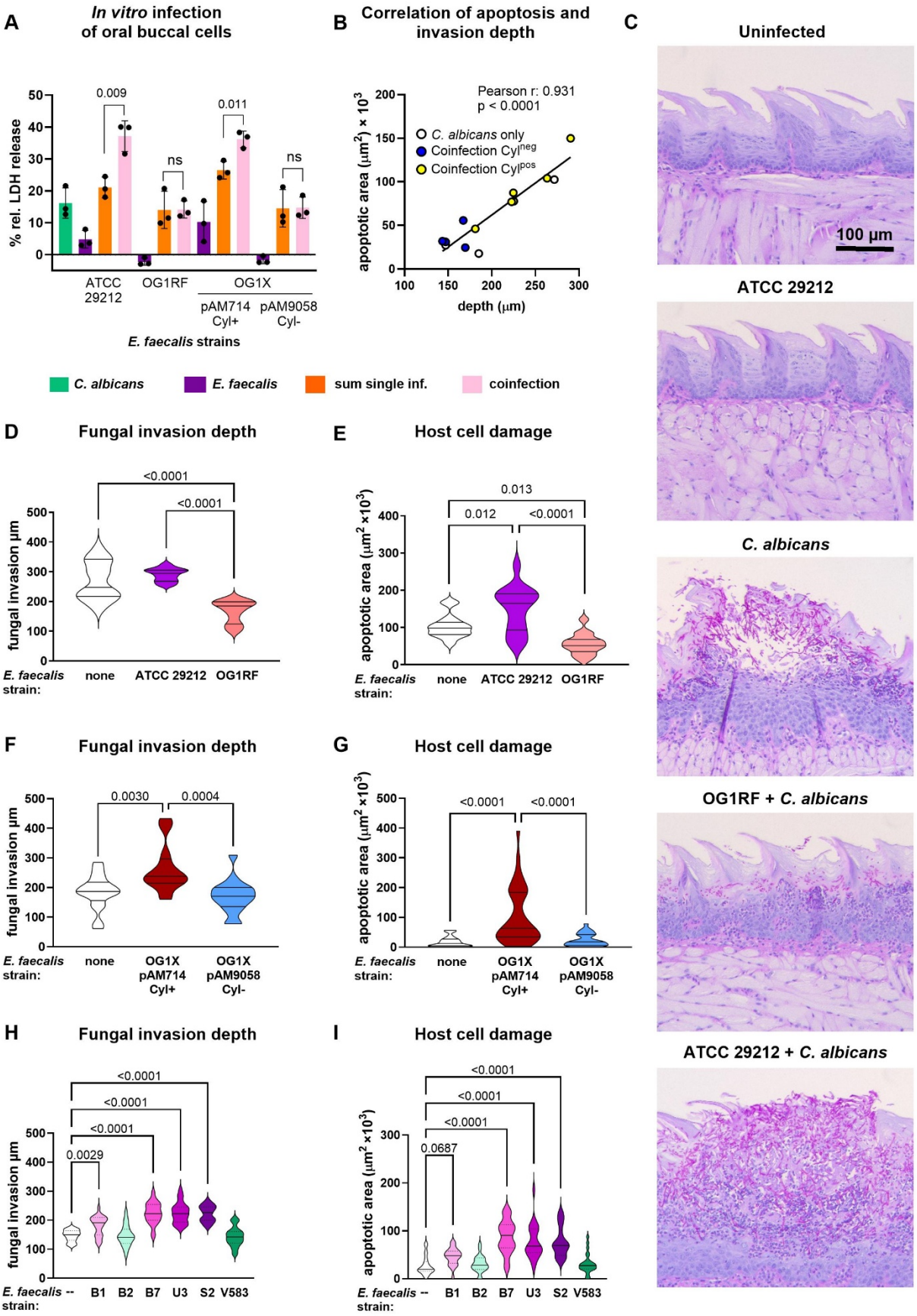
Enterocytes were infected with 10^6 cells/ml *C. albicans* SC5314 for 6 h at 1 % O_2 followed by addition of 10^5 cells/ml *E. faecalis* and further incubation for 24 h. Damage was measured by release of host cell lactate dehydrogenase (LDH) into the supernatant, normalised to non-infected controls, and is displayed as percent damage relative to Triton X-100-lysis Green: monoinfection with *C. albicans*; dark purple: monoinfection with *E. faecalis*; orange: sum of single infections; light pink: coinfection. Bars represent mean and SD, n = 3. The sum of LDH release caused by bacterial and fungal monoinfection was compared to the LDH release following coinfection using two-tailed unpaired Student's t-tests. Significant values are indicated by p-values. ns: not significant ($p > 0.05$). (**A**) Loss of haemolytic activity after plasmid curing of *E. faecalis* B1 coincides with loss of synergistic coinfection damage. Cyl-: non-haemolytic *E. faecalis* B1 obtained by plasmid curing; Cyl+: *E. faecalis* B1 isolated obtained from the same plasmid curing approach which retained haemolytic activity. (**B**) Transfer of haemolytic activity from *E. faecalis* B1 (donor) to *E. faecalis* OG1RF (recipient) by conjugation results in synergistic coinfection damage. Cyl+: haemolytic OG1RF derivative; Cyl-: non-haemolytic OG1RF derivative obtained from the same conjugation experiment. (**C**) Coinfection with OG1X (plasmid free) and OG1X derivatives harbouring the following plasmids: pAM714: cytolysin-positive/agglutination substance-positive; pAM944: cytolysin-positive/agglutination substance-negative; pAM9058: cytolysin-negative/agglutination substance-positive) and ATCC 29212 (haemolytic) as control. Synergistic coinfection coincides with a functional cytolysin operon.


A
In vitro infection of oral buccal cells
% rel. LDH release
0.009
ns
0.011
ns
ATCC 29212
OG1RF
OG1X
pAM714 Cyl+
pAM9058 Cyl-
E. faecalis strains
C. albicans
E. faecalis
sum single inf.
coinfection
B
Correlation of apoptosis and invasion depth
Pearson r: 0.931
p < 0.0001
C. albicans only
Coinfection Cyl^neg
Coinfection Cyl^pos
apoptotic area (µm²) × 10³
depth (µm)
C
Uninfected
100 µm
ATCC 29212
C. albicans
OG1RF + C. albicans
ATCC 29212 + C. albicans
D
Fungal invasion depth
<0.0001
<0.0001
fungal invasion µm
E. faecalis strain:
none
ATCC 29212
OG1RF
E
Host cell damage
0.013
0.012
<0.0001
apoptotic area (µm² ×10³)
E. faecalis strain:
none
+ ATCC 29212
+ OG1RF
F
Fungal invasion depth
0.0030
0.0004
fungal invasion µm
E. faecalis strain:
none
OG1X pAM714 Cyl+
OG1X pAM9058 Cyl-
G
Host cell damage
<0.0001
<0.0001
apoptotic area (µm² ×10³)
E. faecalis strain:
none
OG1X pAM714 Cyl+
OG1X pAM9058 Cyl-
H
Fungal invasion depth
<0.0001
<0.0001
<0.0001
0.0029
fungal invasion µm
E. faecalis -- strain:
B1
B2
B7
U3
S2
V583
I
Host cell damage
<0.0001
<0.0001
<0.0001
0.0687
apoptotic area (µm² ×10³)
E. faecalis -- strain:
B1
B2
B7
U3
S2
V583

1049

1050

1051


A
In vitro infection of oral buccal cells
% rel. LDH release
0.009
0.011
ns
ns
ATCC 29212
OG1RF
OG1X
pAM714 Cyl+
pAM9058 Cyl-
E. faecalis strains
C. albicans
E. faecalis
sum single inf.
coinfection
B
Correlation of apoptosis and invasion depth
Pearson r: 0.931
p < 0.0001
C. albicans only
Coinfection Cyl^neg
Coinfection Cyl^pos
apoptotic area (µm²) × 10³
depth (µm)
C
Uninfected
100 µm
ATCC 29212
C. albicans
OG1RF + C. albicans
ATCC 29212 + C. albicans
D
Fungal invasion depth
fungal invasion µm
<0.0001
<0.0001
E. faecalis strain:
none
ATCC 29212
OG1RF
E
Host cell damage
apoptotic area (µm² ×10³)
0.013
0.012
<0.0001
F
Fungal invasion depth
0.0030
0.0004
OG1X pAM714 Cyl+
OG1X pAM9058 Cyl-
G
Host cell damage
<0.0001
<0.0001
H
Fungal invasion depth
<0.0001
<0.0001
<0.0001
0.0029
E. faecalis --
B1
B2
B7
U3
S2
V583
I
Host cell damage
<0.0001
<0.0001
<0.0001
0.0687

1052

1053 **Figure 3: Damage of oral cells *in vitro* and murine oropharyngeal candidiasis *in vivo* are**
1054 **aggravated by coinfection with cytolysin-producing *E. faecalis* strains**

1055 (**A**) Human oral buccal cells (TR146) were infected with 10^5 cells/ml *C. albicans* SC5314
1056 for 6 h at 1 % O_2 followed by addition of 10^5 cells/ml *E. faecalis* and further incubation
1057 for 24 h. Damage was measured by release of host cell lactate dehydrogenase (LDH) into
1058 the supernatant, normalised to non-infected controls, and is displayed as percent damage
1059 relative to Triton X-100-lysis. Green: monoinfection with *C. albicans*; dark purple:
1060 monoinfection with *E. faecalis*; orange: sum of monoinfections; light pink: coinfection.
1061 Bars represent mean and SD, n = 3. The sum of LDH release/apoptosis caused by bacterial
1062 and fungal monoinfection was compared to coinfection using two-tailed unpaired
1063 Student's t-tests. Significant values are indicated by p-values. ns: not significant ($p >$
1064 0.05). (**B-I**) Six to seven weeks old male corticosteroid-treated C57BL/6J mice were
1065 sublingually inoculated with *C. albicans* SC5314 for 75 min. Six hours later *E. faecalis*
1066 was added to the drinking water for 24 h (2×10^7 CFUs in 50 ml). (**B**) Correlation of
1067 apoptotic area and invasion depth. Mean values of all experimental groups (C-H) were
1068 analysed by Pearson correlation. (**C-E**) Coinfection with *E. faecalis* ATCC 29212 and
1069 OG1RF, respectively, n=5 mice per group. (**C**) Representative histographs of PAS-stained
1070 tongue sections, scale bar 100 µm. For each animal (n=5 mice per group), three non-
1071 consecutive sections per tongue (sections from different parts of the tongue) were
1072 randomly selected and stained. (**D, F, H**) Invasion depth was measured for each lesion
1073 visible on histological sections. Three non-consecutive section per mouse were analysed.
1074 (**E, G, I**) Size of apoptotic area was measured for each lesion visible on histological
1075 sections. Three non-consecutive section per mouse were analysed. (**D, E**) 5 mice/group.
1076 (**F, G**) Coinfection with *E. faecalis* OG1X pAM714 (cytolysin positive: Cyl+) and
1077 pAM9058 (cytolysin negative: Cyl-), respectively. Data from two independent
1078 experiments, n=6 mice/group in total. (**H, I**) Coinfection with different *E. faecalis* strains.
1079 Data from two independent experiments, n=5 mice/group in total. Pink/purple shades:
1080 cytolysin positive; green shades: cytolysin negative. (**D-I**) Data was analysed by 1-way
1081 ANOVA with Tukey's multiple comparisons test to compare all groups (**D-G**) or
1082 Dunnett's multiple comparisons test to compare coinfection with *C. albicans* only (**H, I**).
1083 P-values < 0.05 are indicated in the graphs.
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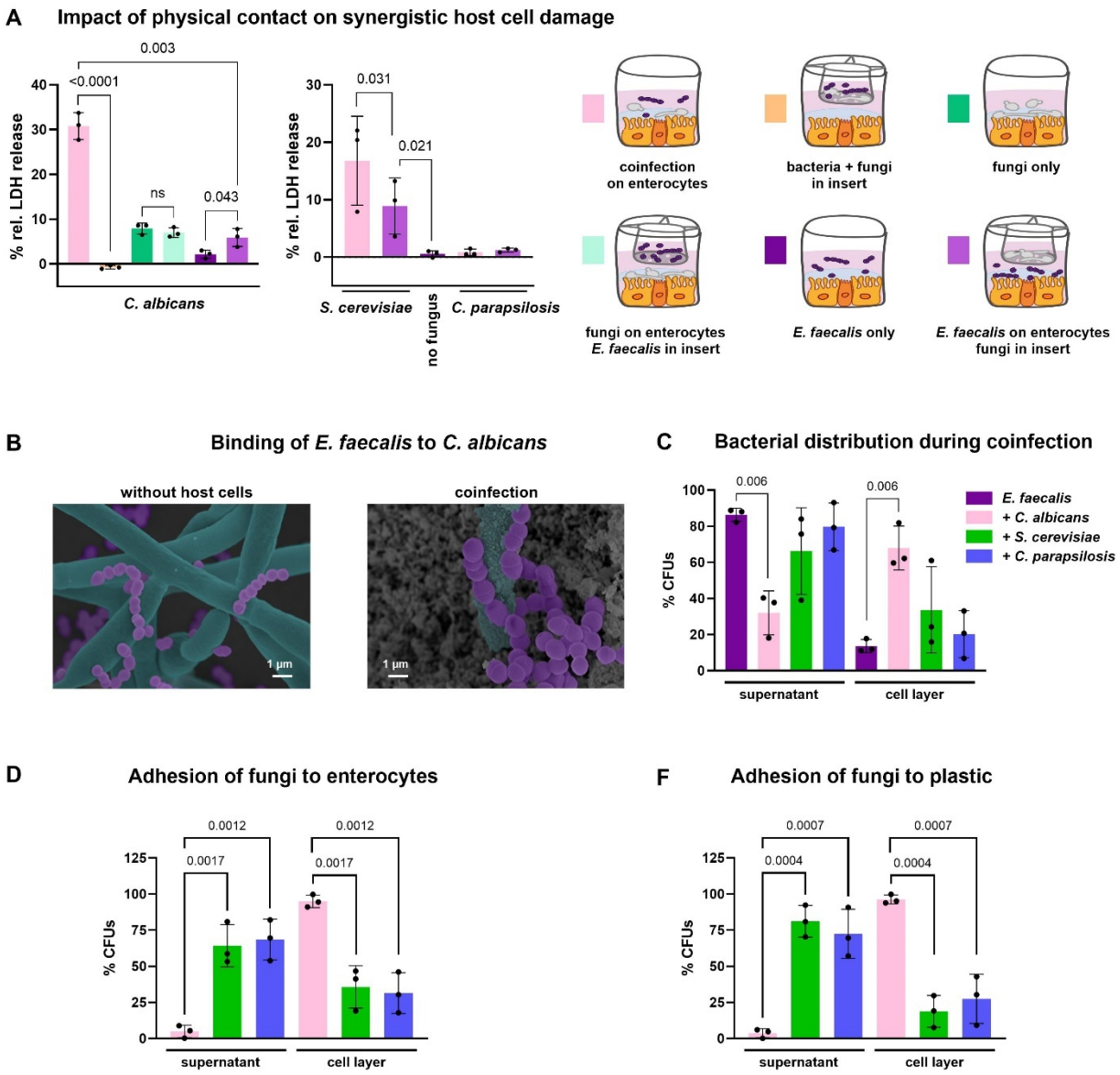

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1087 **Figure 4: Fungal-bacterial contact leads to accumulation of bacteria at the host cell layer**
1088 **and downregulation of gelatinase expression**

1089 (**A**) Mono- and coinfection of enterocytes with *C. albicans* SC5314 and *E. faecalis* ATCC
1090 29212 (left) or *E. faecalis* ATCC 29212 and *S. cerevisiae* and *C. parapsilosis*, respectively
1091 (right) using transwell inserts with 0.4 µm pores size for spatial separation. Enterocytes
1092 were infected with 10^6 fungal cells/ml for 6 h of followed by addition of 10^5 cells/ml *E.*
1093 *faecalis* and further incubation for 24 h. Damage was measured by release of host cell
1094 lactate dehydrogenase (LDH) into the supernatant, normalised to non-infected controls,
1095 and is displayed as percent damage relative to Triton X-100-lysis. The scheme below
1096 depicts the location of fungal/bacterial cells in the well or insert, respectively. Data is
1097 shown as mean with SD, n = 3. Statistical significance was analysed for *C. albicans* by

1098 two-tailored unpaired Student's t-test with p-values indicated in the graph (ns = not
1099 significant; $p > 0.05$). For *S. cerevisiae* and *C. parapsilosis*, damage caused by coinfection
1100 was compared to *E. faecalis* on enterocytes with fungi in the insert by 1-way ANOVA
1101 followed by Dunnett's multiple comparisons test; significant differences are indicated by
1102 p-value in the graph. For *S. cerevisiae*, coinfection damage and damage by *E. faecalis* on
1103 enterocytes with fungi in the insert was analysed by ratio paired t-test due to variations of
1104 coinfection damage between experiments. (**B**) Scanning electron micrographs,
1105 representative images from two independent experiments. The images were coloured
1106 manually; turquoise: fungi, fuchsia: bacteria. Without host cells: *E. faecalis* and *C.*
1107 *albicans* coculture in KBM for 24 h. Coinfection**:** Enterocyte coinfection with *E. faecalis*
1108 ATCC 29212 and *C. albicans* SC5314 (24 h). (**C**) Ratio of bacterial cells recovered from
1109 the supernatant or the host cell layer after monoinfection with *E. faecalis* ATCC 29212 or
1110 coinfection with *C. albicans* SC5314, *S. cerevisiae* or *C. parapsilosis*. Data is shown as
1111 mean with SD, n = 3, and was analysed by comparing coinfection data for supernatant and
1112 cell layer, respectively, to monoinfection by 1-way ANOVA followed with Dunnett's
1113 multiple comparisons test. Statistical significance ($p > 0.05$) is indicated by p-values in the
1114 graph. (**D, E**) Ratio of fungal cells recovered from the supernatant and the host cell layer
1115 (**D**) or plastic surface (**E**) after 10^6 fungal cells/ml were incubated for 30 h. Data is shown
1116 as mean with SD, n = 3, and was analysed by 1-way ANOVA followed with Dunnett's
1117 multiple comparisons test comparing *C. albicans* to *S. cerevisiae* and *C. parapsilosis*,
1118 respectively. P-values are indicated in the graph.
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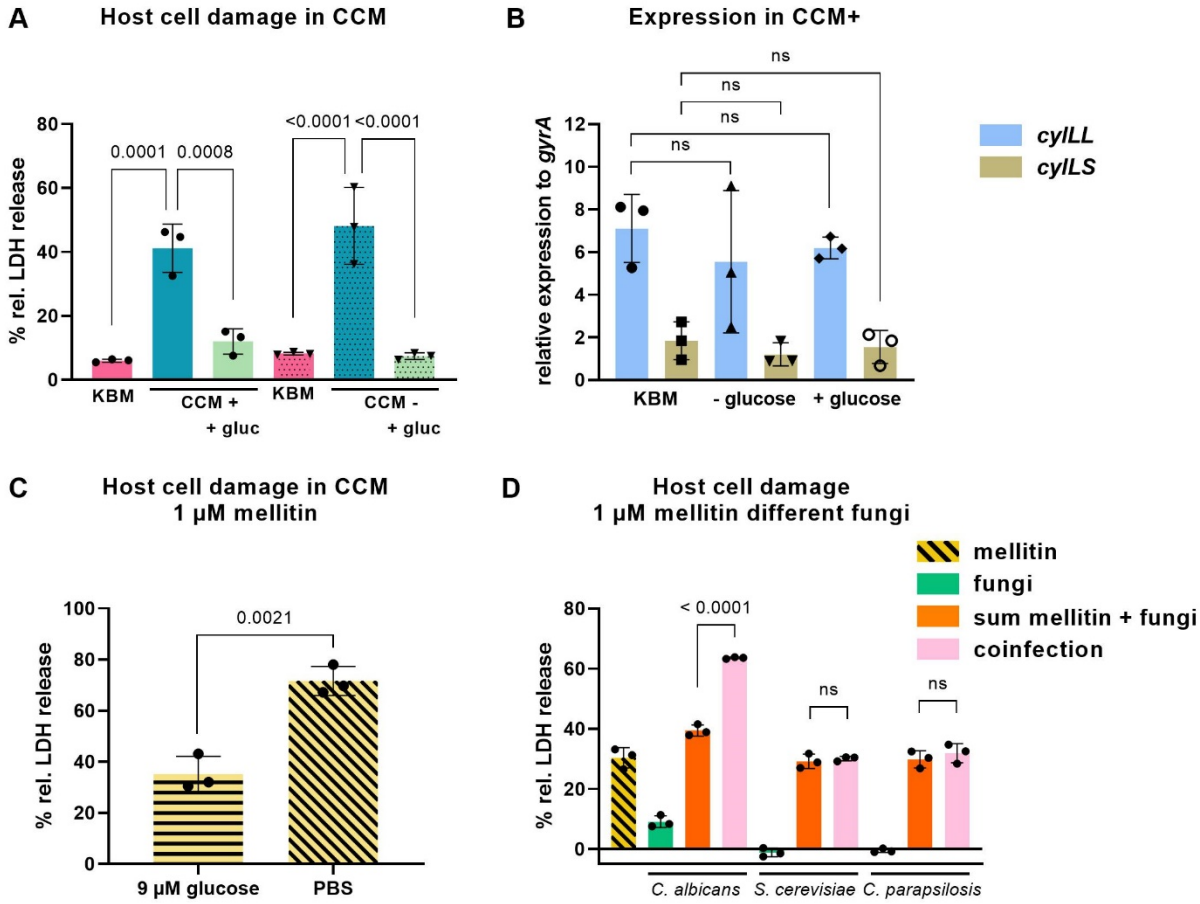

Figure 5: Glucose depletion does not lead to upregulation of cytolysin secretion but sensitizes host cells to damage

(**A**) *C. albicans* SC5314 conditioned media (CCM) were prepared by incubating *C. albicans* SC5314 for 30 h (1% O_2, 5% CO_2) in KBM with (CCM+) or without enterocytes (CCM-). The medium was sterile filtered and used for infection of enterocytes with *E. faecalis* ATCC 29212. Conditioned media were used directly or supplemented with 9 mM glucose (+gluc). Data (n=3) was analysed by 1-way ANOVA with Tukey's multiple comparisons test to compare all groups; p-values < 0.05 of comparisons within the same type of CCM (+/-) are indicated in the graphs. (**A, C, D**) Damage was measured by release of host cell lactate dehydrogenase (LDH) into the supernatant, normalised to non-infected controls, and is displayed as percent damage relative to Triton X-100-lysis. (**B**) Expression of the large (*cylLL*) and small (*cylLS*) cytolysin subunits normalized to the housekeeping gene *gyrA* in *E. faecalis* ATCC 29212 cultured in CCM and controls for 18 h. Data (n=3) was analysed for each gene by 1-way ANOVA with Tukey's multiple comparisons test to compare all groups. (**C**) Melittin-induced enterocytes damage in CCM+ supplemented with or without glucose (PBS as control). n = 3, data is shown as mean with SD, groups were compared with two-tailored unpaired Student's t-test. (**D**) Effect of addition of melittin on enterocyte damage during infection with *C. albicans* SC5314, *S. cerevisiae*, or *C. parapsilosis* (n = 3 independent experiments). The sum of damage caused by the fungus and melittin separately (orange) was compared to the damage during coincubation (pink) for each fungal species with two-tailored unpaired Student's t-test.