A novel virulence phenotype rapidly assesses *Candida* fungal pathogenesis in healthy and immunocompromised *Caenorhabditis elegans* hosts

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Running title: Fungal infection reduces multiple measures of host fitness

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Abstract

The yeast *Candida albicans* is an opportunistic pathogen of humans, meaning that despite commensal interactions with its host, it can transition to a harmful pathogen. While *C. albicans* is the predominant species isolated in the human mycobiome and implicated in fungal infection, infections due to non-albicans *Candida* species are rapidly rising. Studying the factors that contribute to virulence is often challenging and frequently depends on many contexts including host immune status and pathogen genetic background. Here, we utilize the nematode *Caenorhabditis elegans* as a perspicuous and efficient model host system to study fungal infections of *Candida* pathogens. We find that in addition to reducing lifetime host survival, exposure to *C. albicans* results in delayed reproduction, which significantly reduced lineage growth over multiple generations. Furthermore, we assessed fungal pathogen virulence in *C. elegans* hosts compromised for innate immune function and detected increased early mortality, reduced brood sizes and delayed reproduction relative to infected healthy hosts. Importantly, by assessing virulence in both healthy and immunocompromised host backgrounds we reveal the pathogen potential in non-*albicans Candida* species. We find that *C. tropicalis* is moderately virulent in both host backgrounds whereas *C. dublineinsis* and *C. parapsilosis* show little to no virulence in healthy hosts but significantly elevated virulence in immunocompromised hosts. Taken together, we present a novel lineage growth assay to measure reduction in host fitness associated with fungal infection and demonstrate significant interactions between pathogen and host immune function that contribute to virulence.

Introduction

Candida species are commensals of the human gastrointestinal microbiota and various other niches in the human body [1]. Despite their commensal existence in many humans, *Candida* species also account for the most fungal infections in the world and are the fourth most prevalent cause of all nosocomial blood stream infections [2,3]. The severity of fungal infection is often dependent on the immune status of the host, with superficial infections occurring in healthy individuals and bloodstream infections in immunocompromised hosts [4-8]. The majority of these infections are associated with

63 *Candida albicans,* however other non-*albicans Candida* species are increasingly
64 becoming implicated in fungal infections [9,10].
65 Several host models have been employed to better understand fungal infection
66 and disease progression. These experimental systems include mouse, zebrafish,
67 nematode, wax moth and human *ex vivo* models have been specifically developed to
68 study *Candida* infections [11]. Most often, virulence phenotypes are limited to survival
69 outcomes or tissue-specific fungal burden [12], the latter of which requires animal
70 sacrifice and cannot address the long-term consequence of fungal disease.
71 Furthermore, animal mortality and sacrifice, particularly in vertebrate models, constrain
72 the number of infected individuals to study and thus reduce the quantitative robustness
73 of these experiments. Nonetheless, these studies provide valuable insight into *Candida*
74 infection and disease and have uncovered important roles for innate immune function of
75 hosts [13-15], specific *C. albicans* genes regulating virulence [16,17] and differences
76 between genetic backgrounds within and between *Candida* species [18].
77 Other major limitations for most studies of *Candida* infection virulence include the
78 time-intensive nature of tracking virulence phenotypes in vertebrate systems such as
79 mice, the genetic intractability of zebrafish and wax moth larvae and the context-
80 independent nature of *ex vivo* models [4]. As such, most studies focus specifically on *C.*
81 *albicans* virulence while many non-albicans *Candida* species are evaluated to a lesser
82 degree. The nematode *Caenorhabditis elegans* has been developed as a model for
83 *Candida* infection to overcome these limitations [19,20] and to screen compounds for
84 antifungal activity [21]. *C. elegans* has proven useful for studying host-microbe
85 interactions [22] because many fungal and bacterial pathogens that cause illness in
86 humans also cause disease in *C. elegans* [23]. Infecting *C. elegans* is relatively simple
87 and often done by replacing or incorporating its standard laboratory food *E. coli* with a
88 desired pathogen which colonizes the gut and causes disease [23]. Furthermore, the
89 innate immune system in *C. elegans* includes the *SEK-1* gene which encodes a MAP
90 Kinase Kinase (MAPKK) that is homologous to the MKK3/6 and MKK4 family of
91 mammalian MAPKKs and activates the *C. elegans* p38 MAP kinase ortholog [24]. This
92 pathway has been suggested to be an ancient and conserved component of *C. elegans'*
93 immune response to pathogens [25]. As such, the *sek-1* mutation increases

susceptibility to microbial colonization [26], including infection with many *Candida* species [27] and is necessary to induce the appropriate antifungal immune defense [28].

In addition to utilizing innate immune system mutants, most studies measure host mortality in temperature sensitive sterile mutants [17,21,29,30] to more easily track mortality in founder populations since *C. elegans* has a rapid lifecycle and large brood sizes [31,32]. The effects on host reproduction are often overlooked in favor of examination of mortality rates when assessing a pathogen's virulence [33]. Yet, it is important to remember that virulence can be broadly measured as any reduction in host fitness resulting from interactions between a pathogen and its host [34-36]. In this work, we assessed virulence of *Candida* species in *C. elegans* hosts using a novel measure of host fitness that incorporated both host survival and fecundity. We found that fungal pathogens reduced host fitness by delaying reproduction, resulting in long-term consequences for population growth in both healthy and immunocompromised host backgrounds. Using this novel measure, we characterized virulence phenotypes for three non-*albicans Candida* species; *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis* in both healthy and immunocompromised *C. elegans* hosts. Our studies demonstrate that differences in virulence can be identified between pathogen species and that pathogenic potential is often revealed when host immune function is diminished.

Methods

Strains and media

For this study, the fungal pathogens *C. albicans* (SC5314 [37]), *C. dubliniensis* (Wu284 [38]), *C. tropicalis* (ATCC 22109), and *C. parapsilosis* (ATCC 22109) strains were used. *C. elegans* N2 Bristol (WT) and a *sek-1* mutant derivative [24] were used to test host survival, fecundity and population growth. *C. elegans* populations were maintained at 20°C on 100mm petri dishes with 25 mL of lite nematode growth medium (NGM, US Biological) with *E. coli* OP50 as a food source. Nematodes were transferred to a newly seeded *E. coli* plate every 3-4 days. For survival, fecundity and population growth assays, NGM was supplemented with 0.08g/L uridine, 0.08g/L histidine, and 0.04g/L adenine to facilitate growth of auxotrophic *C. albicans* strains and 0.2g/L streptomycin sulfate to inhibit *E. coli* overgrowth so fungal strains could proliferate.

125

126 *Seeding NGM plates for survival, fecundity, and population growth assays*

127 *Candida* strains and *E. coli* OP50 strains were inoculated in 3 mL of YPD or 5 mL
128 of LB, respectively, and cultured at 30°C for 1-2 days. *Candida* culture densities were
129 measured with a spectrophotometer and diluted to a final volume of 3.0 OD_{600} per mL
130 (~6 x 10^7 cells per ml). *E. coli* cultures were pelleted and washed twice with 1 mL of
131 ddH_2O. The supernatant was removed and the pellet was centrifuged for 60 sec at
132 maximum to remove any excess liquid. The pellet was weighed and suspended with
133 sterilized water to a final volume of 200 mg/mL. A mix of 6.25 μL *E. coli*, 1.25 μL
134 *Candida* was brought to a final volume of 50 μL with ddH_2O. The entire 50 μL was
135 spotted onto the center of a 35mm supplemented-NGM Lite agar plate and incubated at
136 room temperature overnight before addition of eggs or transferring nematode. *E. coli*
137 OP50 was used as a control at the same concentration described above.

138

139 *Egg preparation and synchronization for survival, fecundity and population growth*
140 *assays*

141 For survival, fecundity and population growth assays, approximately 100
142 nematodes at the L3/L4 stage were transferred to a 100mm NGM plate seeded with *E.*
143 *coli* OP50 and maintained at 20°C for 2-3 days prior to the start of an experiment. On
144 the first day of an experiment, these NGM plates were washed with M9 buffer and
145 contents (live nematodes and eggs) transferred to 15 mL conical tube and pelleted by
146 centrifugation (2 min at 1200 rpm). The pellet was re-suspended in a 1:4 bleach (5.25%)
147 solution and transferred to a micro-centrifuge tube. The suspension was mixed via
148 inversion for 90-120 sec and subsequently centrifuged (30 sec at 1500 rpm). The pellet
149 was washed with 1 mL M9 buffer three consecutive times to remove excess bleach
150 solution and brought to a final suspension with 500 μL M9 buffer. To determine the
151 concentration of eggs, 10 μL was placed on a concaved slide and eggs counted and the
152 egg suspension was diluted with M9 to a final concentration of 10 eggs/μL. All assays
153 were treated equally on the first day (Day 0) by adding roughly 100 eggs to a treatment
154 or *E. coli* plate (described above).

155

156 *Survival assays*

157 This experimental procedure is a modified version of [39]. Briefly, 72h (Day 3)
158 after adding ~50-75 nematode eggs to a plate, 40 adult nematodes were randomly
159 selected and transferred to newly seeded plates with the same concentration of food as
160 described above and incubated at 20°C. Every other day nematodes were transferred to
161 freshly seeded plates until all nematode populations went extinct. The number of living,
162 dead, and censored worms were scored daily. Each survival assay was replicated at
163 least three independent times.

164

165 *Fecundity and lineage expansion assays*

166 For both fecundity and lineage expansion assays, 48h (Day 2) after adding
167 nematode eggs to a plate, a single L3/L4 reproductively immature hermaphroditic
168 nematode was randomly selected and transferred (6-10 independent biological
169 replicates per treatment per block) to a newly seeded 35mm petri plate containing either
170 a treatment of *C. albicans* and *E. coli* or *E. coli* alone (described above) containing one-
171 fifth the volume of food and incubated at 20°C. *C. elegans* were transferred to freshly
172 seeded plates in 24 hour time intervals from day two until the end of the experiment.
173 Eggs remained undisturbed on the plate and were incubated at 20°C for an additional
174 24 h to provide enough time to hatch, at which the number of viable progeny per day
175 was scored. Nematodes that died during the assay were scored dead at the time of
176 transfer. Nematodes that crawled off the plate or were otherwise unaccountable for
177 were considered censored and excluded from the analysis.

178

179 *Lineage Expansion Assay*

180 48 hours after adding nematode eggs to a plate (Day 2), a single L3/L4
181 reproductively immature hermaphroditic nematode was randomly selected and
182 transferred (6 biological replicates per treatment) to a freshly seeded 100mm treatment
183 or *E. coli* plate (described above) containing a 6-fold increase in food. On Day 7, each
184 plate was washed with M9 buffer until the majority of nematodes were displaced and
185 subsequently transferred to 15mL conical tubes. Tubes were placed at 4°C for 1h to

186 allow the nematodes to settle at the bottom. All tubes were concentrated to a final
187 volume of 10mL. Six-20uL samples were taken from each population and counted.
188
189 **Results**
190 *Exposure to C. albicans reduces multiple measures of C. elegans fitness*
191 Pathogens affect host fitness by decreasing lifespan and/or reducing fecundity.
192 To determine how *C. albicans* impacts *C. elegans* host fitness, we first measured
193 nematode lifespan in the presence or absence of *C. albicans*. We observed a significant
194 reduction in survival when *C. elegans* was reared on *C. albicans* compared to when it
195 was reared in its absence (Figure 1, $p < 0.0001$ Log-rank test), consistent with
196 previously published results [39]. 50% mortality was reached in eight days in nematode
197 populations exposed to *C. albicans* compared to 16 days in unexposed nematode
198 populations, indicating that *C. elegans* lifespan is substantially decreased by exposure
199 to *C. albicans*.

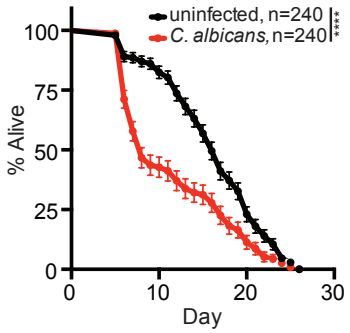

Figure 1: Reduced nematode survival with *C. albicans* infection. Survival curves for *C. elegans* populations that are either uninfected (exposed to just an *E. coli* food source, black) or when infected with *C. albicans* SC5314 (red). Mean data values are plotted with SEM error bars, number of worms analyzed (n) for each treatment is indicated in the legend. **** indicates $p < 0.0001$, Log-rank (Mantel-Cox) test.

200
201 While overall survival was reduced, nematode death was rarely observed earlier
202 than 5 days post-infection. Importantly, adult nematodes produce the majority of their
203 offspring before *C. albicans* infection reduces rates of survival. Given this information
204 and that fecundity is a key component of fitness from an evolutionary perspective, we
205 investigated whether *C. albicans* negatively impacts *C. elegans* reproduction. To test
206 this, we counted the total number of viable progeny (i.e. brood size) produced in the first
207 seven days of reproductive maturity from individual nematodes that were either

208 unexposed or exposed to either heat-killed or live *C. albicans*. As shown in Figure 2A,
209 exposure to *C. albicans* reduced the average number of viable progeny (267±6)
210 compared to the unexposed control (286±4; $p < 0.05$, one-way ANOVA) by ~7%. While
211 statistically significant, the cost of *C. albicans* toward host brood size is modest.
212 Furthermore, the brood size of nematodes exposed to heat-killed *C. albicans* (HK) was
213 not detectably different from uninfected or live *C. albicans* treatments (Figure 2A).

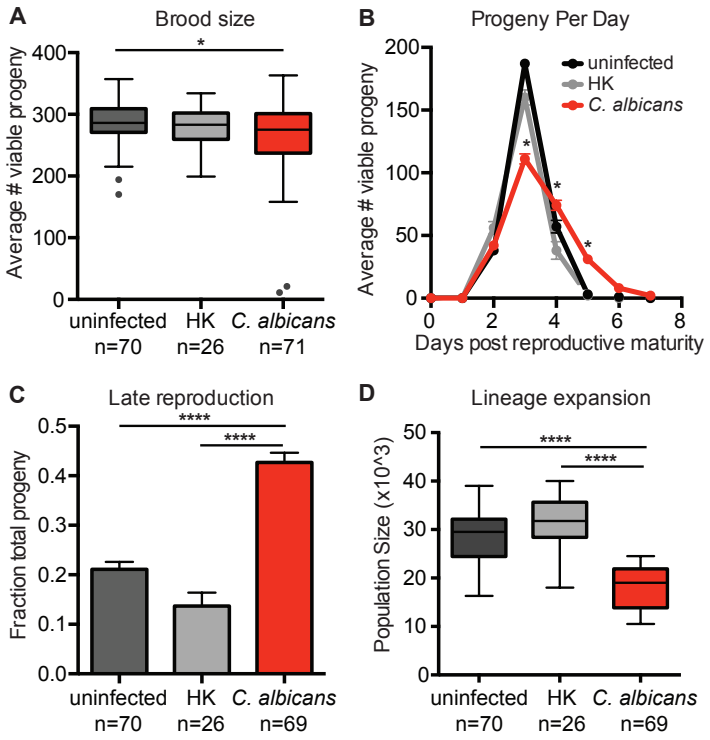

Figure 2. Live *C. albicans* reduces nematode fecundity. A) Box and whiskers plot of average brood sizes for *C. elegans* (N2) exposure to *E. coli* food source alone (uninfected, black), heat-killed *C. albicans* (HK, grey), or live *C. albicans* (red). Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. **B)** Number of viable *C. elegans* progeny produced per day in uninfected, heat-killed and live *C. albicans* treatments. Data represent the mean and error bars indicate SEM. **C)** The fraction of *C. elegans* progeny produced after Day 3 during the 'Late Reproductive' window. Data represent the mean and error bars indicate SEM. **D)** Box and whiskers plot of average population size (representing the number of F1 and F2 progeny) produced from a single founder *C. elegans*. Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. The number (n) of experimental samples analyzed is indicated for each treatment. * $p < 0.05$, **** $p < 0.0001$; one-way ANOVA with Tukey's multiple comparison test.

214

215 Intriguingly, we identified a significant delay in reproductive timing when we
216 measured the number of progeny produced every 24 hrs during the host reproductive
217 window. We observed a large reduction in reproduction on Day 3 in nematodes
218 exposed to *C. albicans* (102±4) relative to unexposed hosts (174±4) (Figure 2B; $p <$
219 0.001 two-tailed t-test) and increased progeny produced on Days 4-6 in nematodes

220 exposed to *C. albicans* (Figure 2B). We calculated the total fraction of reproduction
221 occurring in this 'late' window (Days 4-6) and identified a significant increase in the
222 progeny produced late in *C. elegans* exposed to *C. albicans* compared to unexposed
223 controls (Figure 2C; p=0.0205 two-tailed t-test). Taken together, our data indicate that
224 *C. albicans* severely delays and reduces reproduction, in addition to impacting overall
225 survival in *C. elegans.*
226 To investigate whether the reproductive delay observed in nematodes exposed
227 to *C. albicans* had any long-term consequences, we measured the total progeny
228 produced from a single founder worm over multiple generations through lineage
229 expansion assays. From these experiments, we find that a single nematode can
230 produce ~28,500 F1 and F2 progeny within seven days (Figure 2D). Lineage expansion
231 is significantly impacted in nematodes exposed to live *C. albicans*, with an average
232 population size of ~18,000 F1 and F2 progeny, a reduction of ~35% compared to
233 uninfected and heat-killed control treatments (Figure 2D, $p < 0.0001$ one-way ANOVA).
234 We conclude that delays in reproduction resulting from exposure to *C. albicans* severely
235 impacts *C. elegans* evolutionary fitness, as we detect a dramatic reduction in population
236 size in a timeframe that is unlikely to be severely impacted by mortality (Figure 1) nor
237 the modest reduction in total brood size (Figure 2A).
238
239 *Immunocompromised hosts are susceptible to fungal infection*
240 *C. albicans* is an opportunistic pathogen meaning that it only causes disease
241 under certain conditions and host contexts. A leading host-related risk factor for invasive
242 candidiasis is compromised immune function [40]. Given our novel results regarding
243 fecundity in a healthy *C. elegans* host, we were curious how exposure to *C. albicans*
244 impacts *C. elegans* hosts with compromised immune function. To address this point, we
245 utilized a *C. elegans* strain background carrying a mutation in *SEK*-1, a well-conserved
246 MAP kinase involved in the innate immune signaling cascade [24,26] and antifungal
247 response [28]. We detected substantial mortality in *sek-1* immunocompromised hosts
248 during the course of our seven-day fecundity assays, which we did not observe with
249 healthy wild-type hosts (data not shown). Furthermore, exposure to *C. albicans* resulted
250 in the largest amount of early mortality in immunocompromised hosts compared to heat-

251 killed *C. albicans* treatments and unexposed controls (Figure 3A). In addition to
252 increased early mortality, *C. albicans* exposure reduced average brood size by nearly
253 50% (84±6) in immunocompromised hosts compared to uninfected (157±5) and heat-
254 killed (127±13) controls (Figure 3B, $p < 0.0001$, one-way ANOVA). It is important to note
255 that in all treatments (unexposed, heat-killed, and live *C. albicans*),
256 immunocompromised hosts produced significantly less progeny than healthy hosts
257 (Table S1), but even more so when exposed to live *C. albicans*.

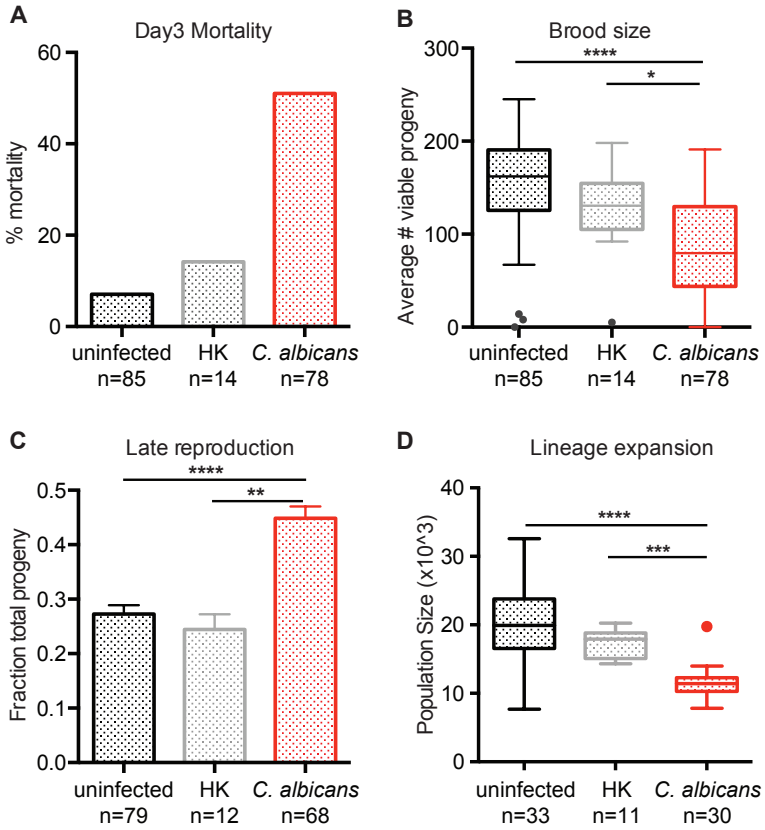

Figure 3. Live *C. albicans* severely impacts host fitness in immunocompromised nematodes. A) Percent mortality observed on or by Day3 in immunocompromised (*sek-1*) hosts exposed to *E. coli* food source alone (uninfected, black), heat-killed *C. albicans* (HK, grey), or live *C. albicans* (red). **B)** Box and whiskers plot of average brood sizes of *sek-1 C. elegans* treated with the same conditions as (A). Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. **C)** The fraction of *sek-1 C. elegans* progeny produced after Day3 in the 'Late Reproductive' window. Data represent the mean and error bars indicate SEM. D) Box and whiskers plot of average population size (representing the number of F1 and F2 progeny) produced from a single founder *sek-1 C. elegans*. Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. The number (n) of experimental samples analyzed is indicated for each treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Tukey's multiple comparison test.

258

259 One explanation for the overall reduction in average brood size is the increased
260 early mortality we observe in immunocompromised hosts. To address this possibility,

261 we re- analyzed brood size only from hosts that survived past Day3 and found that while
262 this did slightly increase average brood size across all treatments, it was not significant
263 between analyses (p=0.2356 two-tailed t-test) and exposure to *C. albicans* still
264 significantly reduced average brood size (94±6) relative to uninfected (163±5) and heat-
265 killed controls (141±9) (Figure S1, $p < 0.0001$ one-way ANOVA). For hosts that survived
266 past three days, we also detected a significant delay in reproductive timing upon
267 exposure to *C. albicans*, with nearly 45% of all reproduction occurring in this late
268 window (Figure 3C), similar to what we observe in healthy hosts (Table S1).
269 We predicted that lineage expansion in immunocompromised hosts exposed to
270 *C. albicans* would be more dramatically impacted relative to wild-type nematodes
271 exposed to *C. albicans*, given the reproductive delays but also early mortality, and
272 overall reduced brood sizes observed. Indeed, we determined that the average
273 population size was reduced by ~45% in immunocompromised hosts exposed to *C.*
274 *albicans* compared to uninfected or heat-killed controls (Figure 3D). Furthermore, this
275 reduction in population size is significantly greater in immunocompromised hosts
276 compared to healthy hosts (Figure S2, p = 0.035 two-tailed t-test). Interestingly, we also
277 detected a significant reduction in population size in immunocompromised hosts
278 exposed to heat-killed *C. albicans* relative to uninfected immunocompromised hosts.
279 This reduction in population size in heat-killed treatments may result from the increased
280 early mortality in immunocompromised nematodes. Taken together, our results indicate
281 that immunocompromised hosts are more susceptible to fungal infection than healthy
282 wild-type hosts.
283
284 *Host immune status reveals pathogenic potential in other Candida species*
285 While *C. albicans* is the predominant fungal agent in invasive candidiasis, non-
286 *albicans Candida* species (NACs) are estimated to account for 35-65% of
287 candidaemia [41]. Here, we measured the impact on host fitness of three additional
288 *Candida* species: *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* analyzing both
289 healthy and immunocompromised *C. elegans* hosts. By evaluating NACs in both healthy
290 and immunocompromised hosts, we can begin to assess their pathogenic potential, and
291 thus extend the utility of *C. elegans* as a model host system for detecting virulence in

292 *Candida* species. For healthy hosts infected with *C. dubliniensis* and *C. tropicalis,* we
293 detected modest levels of early mortality, and we detected no early mortality in healthy
294 hosts infected with *C. parapsolsis*. However, there was increased early mortality in
295 immunocompromised hosts exposed to all three *Candida* species, with the highest
296 mortality observed for *C. parapsilosis* (Figure 4A). In regards to early mortality, we
297 determined that *C. parapsilosis* has high virulence in immunocompromised hosts, but
298 none in healthy hosts.

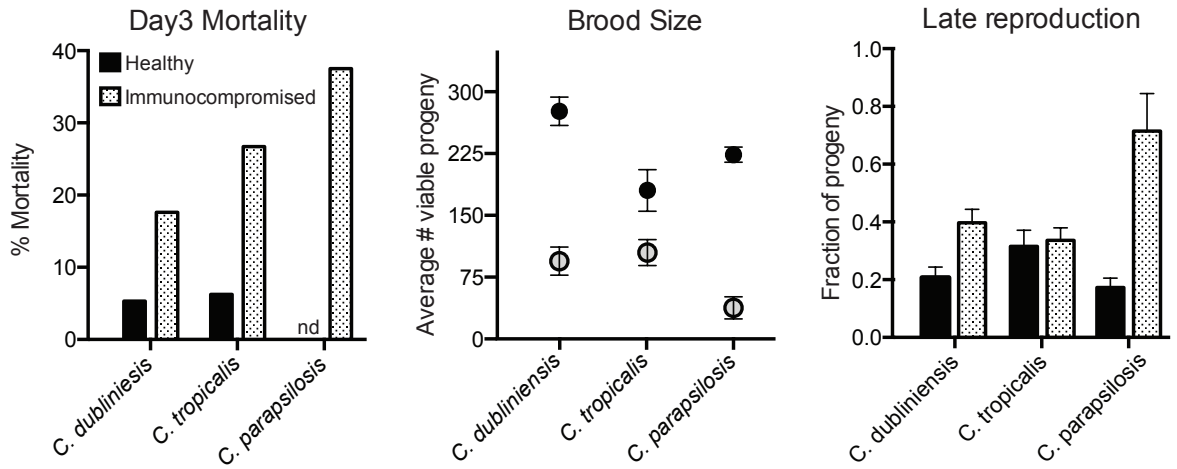

Figure 4. Non-*albicans Candida* species differentially impact host fitness depending on host immune status. A) Percent mortality observed on or by Day3 in N2 (healthy, black) or *sek-1* ('immunocompromised,' grey) *C. elegans* hosts exposed to *C. dubliniensis, C. tropicalis* and *C. parapsilosis*. **B)** Average brood sizes of N2 and *sek-1 C. elegans* hosts exposed to *C. dubliniensis, C. tropicalis* and *C. parapsilosis*. Data represent the mean and error bars indicate SEM. **C)** The fraction of N2 and *sek-1 C. elegans* progeny produced after Day 3 in the 'Late Reproductive' window when exposed to *C. dubliniensis, C. tropicalis* and *C. parapsilosis.* Data represent the mean and error bars indicate SEM. Refer to Table S1 for experimental sample sizes (n). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Tukey's multiple comparison test.

299

300 We also detected different pathogen virulence levels depending on host immune
301 function for average brood size (Figure 4B) and reproductive delays (Figure 4C). In
302 healthy hosts, *C. dubliniensis* had no significant impact on average brood size (276±17)
303 or reproductive timing (21% late) compared to uninfected controls (286±5 brood size
304 and 21% late). However, *C. dubliniensis* was much more virulent in
305 immunocompromised hosts, where we observed a 40% reduction in total brood size
306 (94±17, Figure 4B) and increased reproductive delays compared to uninfected hosts
307 (Figure 4C). A similar pattern is observed for *C. parapsilosis*, in which healthy hosts
308 were not significantly impacted in regards to reproductive timing (17% late, Figure 4B)
309 and only modest reductions in average brood size (224±9) were observed compared to

310 uninfected healthy hosts. However, *C. parapsilosis* infection greatly reduced average
311 brood size (38±13) and delayed reproductive timing (72% late) in immunocompromised
312 hosts.
313 Not all *Candida* species exhibited elevated virulence in immunocompromised
314 hosts. For example, *C. tropicalis* reduced average brood size by about 37% in healthy
315 hosts (180±25) and 34% in immunocompromised hosts (105±16) (Figure 4B) compared
316 to uninfected treatments. Furthermore, we saw similar significant increases in
317 reproductive delays in healthy (32% late) and immunocompromised (34%) hosts (Figure
318 4C). If we compare these three *Candida* species to each other, we see significant
319 differences for both average brood size (Figure 4B, p=0.0012 and p=0.015 comparing
320 healthy and immunocompromised hosts, respectively, one-way ANOVA) and
321 reproductive timing in immunocompromised hosts (Figure 4C, p = 0.0029 one-way
322 ANOVA), but not in healthy hosts (p = 0.0573 one-way ANOVA). In healthy hosts, *C.*
323 *tropicalis* significantly reduced average brood size more than *C. dubliniensis,* but in
324 immunocompromised hosts, no significant difference is detected. In
325 immunocompromised hosts, *C. parapsilosis* displayed significantly more virulence in
326 regards to both average brood size and reproductive timing compared to *C. tropicalis*
327 and *C. dubliniensis*. Given the virulence phenotypes of these yeasts, our results
328 suggest that fungal pathogenicity depends not only on the species of pathogen but also
329 on the immune status of the host.
330
331 **Discussion**
332 Here we utilized a *C. elegans* experimental host system to identify novel
333 measures of host fitness associated with fungal infection. By tracking the number of
334 progeny produced per day, we can quickly and quantitatively assess three aspects of
335 host fitness: early mortality, total viable offspring produced and reproductive timing. We
336 show that exposure to *Candia albicans* in healthy *C. elegans* hosts not only reduces
337 survival (Figure 1), it modestly reduces total progeny produced, and dramatically delays
338 reproductive timing (Figure 2). This reproductive delay has long-term consequences for
339 lineage expansion, as single founder nematodes exposed to *C. albicans* reduces its
340 population growth by ~30% (Figure 2D). We found similar delays in reproductive timing

341 in immunocompromised hosts exposed to *C. albicans*, although immunocompromised
342 hosts are more susceptible to infection and have higher incidence of early mortality and
343 smaller brood sizes compared to healthy hosts (Figure 3). Importantly, the delayed
344 reproduction phenotype in infected *C. elegans* is easily ascertained, highly quantitative
345 and reproducible and can be used as a screening method to detect relatively small
346 differences in virulence across *Candida* strains or other fungal species. Furthermore, by
347 utilizing different host contexts, this assay revealed the pathogenic potential of other
348 important, yet understudied, *Candida* species (Figure 4), including *C. parapsilosis*,
349 which has dramatically higher levels of virulence in immunocompromised hosts
350 compared to healthy hosts.

351 The reductions in host fitness associated with *C. albicans* and other *Candida*
352 species indicate that we have established a bona fide model of fungal infection.
353 Importantly, these reductions in host fitness cannot be attributed to *Candida* species
354 being a sub-optimal food source or that the hosts are starving because we deliver the
355 pathogen with *E. coli*, the standard food source for *C. elegans*. Further, we do not
356 detect any host larval development into dauer, an alternative lifestage in response to
357 starvation and overpopulation [32]. While pathogen avoidance is a common defense
358 strategy for *C. elegans* [42], we censored any worms that have crawled off the plate or
359 have disappeared during the course of our experiment and removed them from our data
360 analysis. Furthermore, the reductions in host fitness depend on ingesting live *C.*
361 *albicans*, as heat-killed treatments do not cause significant reductions in total brood
362 size, reproductive timing, or lineage expansion (Figure 2, 'HK'). Microscopic analysis
363 reveals that fungal cells inhabit the gut of *C. elegans* ([39] and data not shown) and we
364 can extract viable host-associated fungal cells (data not shown). Taken together, these
365 data support a model of fungal infection in the host *C. elegans*.

366 The host reproductive delay we observed in *C. elegans* upon fungal infection is a
367 robust, highly quantitative measure of virulence that makes it amenable to screen a
368 variety of host and pathogen genetic backgrounds that has been challenging in
369 mammalian and insect models. *C. elegans* is easily maintained in the lab, has a short
370 lifecycle that generates a large number of progeny, and has been a fundamental model
371 genetic organism [32]. Previous work has shown that upon exposure to *C. albicans, C.*

372 *elegans* have a transcriptional response consistent with that of infection [28]. In this
373 work, we utilized a mutant *C. elegans* strain in which *SEK-1*, an important MAPK in
374 innate immunity and whose homologs include the MKK3/6 and MKK4 family of
375 mammalian MAPKKs, to determine the severity of fungal infection in
376 immunocompromised hosts. We demonstrate that deficiencies in innate immune
377 function result in hosts with high susceptibility to fungal infection and in the future can
378 extend this analysis for many other host backgrounds that are mutant for immune
379 function.
380 Previous studies using *C. elegans* as a host for fungal infection have described
381 additional phenotypes beyond reduced survival, including a deformed anal region (DAR)
382 [39,43]. It is possible that DAR may be contributing to the delayed reproductive
383 phenotype we observe in our experiments, however, the frequency of DAR phenotypes
384 was low and we did not observe any detectable differences between hosts exposed to
385 *C. albicans* and hosts unexposed (data not shown), nor did we observe dramatic
386 reductions in total number of viable offspring produced between these two groups of
387 hosts (Figure 2A), indicating that exhibiting a DAR phenotype does not necessarily
388 impede egg laying. While this deformity is a result of a local defense reaction of the
389 worm due to extracellular signal-regulated kinase activation of the innate immunity
390 MAKK cascade, it is a distinct marker from delayed reproduction for infection [44].
391 Wildtype reproductive timing utilizes the highly conserved DAF-2 DAF-16 insulin-like
392 signaling pathway [23], but has also been implicated in innate immunity and bacterial
393 infection [45] and is potentially disrupted in *C. elegans* hosts infected with *Candida*.
394 Impacts on host reproduction have often been overlooked in studies regarding
395 *Candida* virulence, yet host reproduction is an important evolutionary measure of an
396 organism's fitness. Not only can we utilize these fecundity assays to assess the roles of
397 host immune function on fungal pathogenesis, we also readily screen diverse pathogen
398 strain backgrounds and species. A large-scale, international 10-year study identified 31
399 species of *Candida* associated with clinical samples. While *C. albicans* is still the most
400 prevalent, its isolation from clinical isolates is decreasing with corresponding increases
401 isolation of *C. glabrata, C. tropicalis* and *C. parapsilosis* [46]. Despite its close
402 evolutionary relationship with *C. albicans*, *C. dubliniensis* does not seem to be highly

403 pathogenic [47]. However, other non-*albicans Candida* species have been increasingly
404 implicated in fungal infections of humans [48]. For example, *C. tropicalis* and *C.*
405 *parapsilosis* are common fungal species isolated in patients with candidemia (7 - 48%
406 and 11 - 27% respectively and depending on geographic region), candiduria (8 – 44%
407 and 0.5 – 11% respectively, and oral candidosis (5 – 13% and 7 – 15%, respectively)
408 ([48] and references therein). Despite the increasing incidence of non-*albicans Candida*
409 infections [46], experimental studies using these pathogens remains limited. Here, we
410 analyzed the virulence phenotypes of three non-albicans *Candida* species, *C.*
411 *dubliniensis*, *C. tropicalis* and *C. parapsilosis* in both healthy and immunocompromised
412 host backgrounds. In healthy *C. elegans* hosts, *C. dubliniensis* is the least virulent; with
413 no significant differences in brood size or reproductive timing compared to uninfected
414 hosts. *C. parapsilosis* is moderately virulent in healthy hosts; there is no reproductive
415 delay, but reduced brood sizes are detected. For both *C. dubliniensis* and *C. tropicalis*,
416 virulence is more severe in immunocompromised hosts. In contrast, *C. tropicalis* has
417 similar degrees of virulence between healthy and immunocompromised host
418 backgrounds. Together, our results regarding virulence in NACs are consistent with
419 published case reports where there is higher incidence of isolating *C. tropicalis* in fungal
420 infections affecting patients with healthy immune function (oral candidosis and
421 candiduria) and infections affecting patients with compromised immune function
422 (candidemia), whereas we see significant *C. parapsilosis* virulence only in
423 immunocompromised hosts. Therefore, the *C. elegans* fecundity assays we developed
424 in this study can reveal the pathogenic potential by assessing virulence in multiple host
425 genetic backgrounds and across pathogens.
426
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