A bacterial endosymbiont of the fungus *Rhizopus microsporus* drives phagocyte evasion and opportunistic virulence

**Highlights**
- Bacterial endosymbionts protect fungal spores from phagocytes
- A secreted factor blocks growth and killing by environmental amoebas
- Endosymbionts improve fungal stress resistance
- Endosymbiosis also allows the evasion of vertebrate immune cells and virulence *in vivo*

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**In brief**
How environmental fungi evolved virulence mechanisms to opportunistically infect humans is unclear. Itabangi et al. identify a tri-kingdom interaction, whereby a bacterial endosymbiont living within a fungus causes the generation of a secreted factor that blocks the predatory activity of amoebas and drives virulence in animal models.
A bacterial endosymbiont of the fungus *Rhizopus microsporus* drives phagocyte evasion and opportunistic virulence

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SUMMARY

Opportunistic infections by environmental fungi are a growing clinical problem, driven by an increasing population of people with immunocompromising conditions. Spores of the Mucorales order are ubiquitous in the environment but can also cause acute invasive infections in humans through germination and evasion of the mammalian host immune system. How they achieve this and the evolutionary drivers underlying the acquisition of virulence mechanisms are poorly understood. Here, we show that a clinical isolate of *Rhizopus microsporus* contains a *Ralstonia picketti* bacterial endosymbiont required for virulence in both zebrafish and mice and that this endosymbiosis enables the secretion of factors that potently suppress growth of the soil amoeba *Dictyostelium discoideum*, as well as their ability to engulf and kill other microbes. As amoebas are natural environmental predators of both bacteria and fungi, we propose that this tri-kingdom interaction contributes to establishing endosymbiosis and the acquisition of anti-phagocyte activity. Importantly, we show that this activity also protects fungal spores from phagocytosis and clearance by human macrophages, and endosymbiont removal renders the fungal spores avirulent in *vivo*. Together, these findings describe a new role for a bacterial endosymbiont in *Rhizopus microsporus* pathogenesis in animals and suggest a mechanism of virulence acquisition through environmental interactions with amoebas.

INTRODUCTION

Soil-dwelling fungi must evade predation by phagocytic amoebas and, similarly, pathogenic fungi evade host phagocytic cells that defend against infection.1,2 Interaction with amoebas has been proposed as an evolutionary training ground for pathogenic fungi, enabling them to resist the multifactorial stresses of the mammalian host.3 Mucoralean fungi such as soil-associated *Rhizopus microsporus*, which causes invasive mucormycosis, successfully establish in complex polymicrobial environments that include predatory amoebas. Yet, they lack common strategies fungi employ to evade predation and phagocytosis: *R. microsporus* fails to mask cell wall ligands in swollen spores, has a slow germination rate, and produces relatively low biomass.4,5 Mucorales resting spores also fail to elicit pro-inflammatory cytokine responses and do not produce strong phagocyte chemotaxis. Here, we investigate an as-yet unexplored aspect of *R. microsporus* pathogen biology: the impact of a bacterial endosymbiont on interaction with amoebas and host phagocytes.

Pathogenic Mucorales span multiple genera, with the *Rhizopus* genus causing almost half of all documented cases and high mortality in susceptible patient populations.5–12 Infection occurs through inoculation with dormant, immunologically inert spores. Upon germination, these spores become metabolically active and begin to swell.14,15 Based on analogy to *Aspergillus* spores, frequently used as a model for Mucorales infectious processes, swelling is expected to reveal microbe associated molecular patterns (MAMPs) and induce increasing rates of phagocytosis which must be overcome by rapid hyphal extension.16,17 However, swollen spores in the *Rhizopus* genus are often no more readily phagocytosed than resting spores, and *R. microsporus* spores are phagocytosed at lower rates than other well studied fungal spores.18–21 While in some species, larger spore size (12.3 μm) reduces phagocytosis, the small spore size of *R. microsporus* (5 μm, comparable with other well-phagocytosed particles) makes this unlikely to explain the observed reduced uptake.22,23 A common strategy fungi employ to evade phagocytosis is through masking cell wall ligands.17,24–29 Two classic examples...
of this are the Cryptococcus neoformans capsule and the Aspergillus spore hydrophobin layer, which effectively block phagocytosis by preventing detection of MAMPs.16,17,28–31 However, there is no evidence of hydrophobins in the R. microsporus genome, and evidence of cell wall remodeling that masks MAMPs is limited to the hyphal phase.32 Moreover, the slow germination rate and low biomass of R. microsporus are also predicted to reduce fungal virulence.4,5,22,33–35 Together, these differences suggest an alternate strategy available to Rhizopus for the evasion of phagocytosis during the early stages of germination.

Members of the Mucoromycota, which includes the order Mucorales, are sometimes colonized or influenced by endosymbiotic bacteria.36,37 The soil-dwelling plant bacterial pathogen Ralstonia solanacearum produces a lipopeptide, ralsolamycin, that aids invasion of fungal hypha and can influence chlamydospore development across a wide range of fungi, including members of the Mucoromycota, as well as Ascomycota and Basidiomycota.38,39 Bacteria of the genera Mycetohabitans (formerly Burkholderia rhizoxinica and B. endofungorum) are well-established endosymbionts of R. microsporus and have been identified in patient isolates.40–42 During endosymbiosis, Mycetohabitans spp. influence fungal behavior such as sporulation and mating.43–45 Endosymbiosis is also implicated in plant pathogenesis: the endotoxin rhizoxin is secreted by Mycetohabitans rhizoxina during mutualism with R. microsporus and inhibits plant defenses.42,46,47 While endosymbionts are widespread in patient fungal samples, a role for endosymbionts in preventing fungal phagocytosis has not been established.40,48 Rather, work examining this interaction in the context of phagocyte-deficient mouse models found no correlation between endosymbiont presence and fungal virulence.40 We recently showed that phagocyte activity in the early stages of infection control is critical for predicting Mucorales disease outcome, suggesting that phagocyte deficiency primes the host for infection.49 Together, this raises the hypothesis: do endosymbionts specifically impact fungal interaction with phagocytic cells, including both environmental amoebae and mammalian macrophages and neutrophils, leading to enhanced fungal virulence?

Here, we investigate the interaction of environmental and host phagocytic cells with R. microsporus. We report, for the first time, a role for a bacterial endosymbiont of R. microsporus in modulating the interaction with these phagocytic cells and further expand this to demonstrate a role in the early stages of disease in both zebrafish and murine models of infection. Specifically, we observed a significant reduction in phagocytosis of metabolically activated spores compared with resting spores. We investigate the consequences of endosymbiont status on infection outcome and demonstrate that this bacterial endosymbiont contributes to both fungal stress resistance and immune evasion during the earliest stages of infection, enabling fungal pathogenesis.

**RESULTS**

**A clinical isolate of R. microsporus suppresses phagocytosis by macrophages**

We previously showed that the early stages of host-fungus interaction determine disease outcome in the zebrafish model of mucormycosis.49 Successful control of infection therefore requires both the presence of phagocytes at the site of infection within the first 24 h and their subsequent ability to kill spores. We hypothesized that, in instances where infection control fails, spores might evade phagocytosis. We therefore examined in detail the interactions between sporangiospores from R. microsporus strain FP469-12, a clinical isolate from a patient at the Queen Elizabeth Hospital, Birmingham, and J774A.1 macrophage-like cells.

During these critical early stages of infection, R. microsporus sporangiospores become metabolically active and start to swell, prior to germination.50,51 This swelling is normally associated with exposure of surface MAMPs which should facilitate phagocytosis.1 Contrary to this expectation however, while dormant Rhizopus spores were readily engulfed by J774A.1 cells, swollen spores were taken up significantly less (Figure 1A, p > 0.0001). This was dependent on fungal viability as UV-killing of swollen spores completely restored their uptake (Figure S1A). Larger objects are harder for phagocytes to engulf, and under these conditions swollen spores reached a mean diameter of 7.3 μm after 6 h, compared with 4.6 μm during dormancy. However, as J774A.1 cells engulfed latex beads up to 11.9 μm in diameter at least as well as dormant spores (Figure S1B), size was not a limiting factor.

These findings suggest a novel active mechanism for evading phagocytosis upon spore germination. As fungi are well known...
Figure 2. *R. microsporus* FP469-12 contains a bacterial endosymbiont required for anti-phagocytic activity

(A) PCR screen for the presence of bacterial 16S rDNA. Genomic DNA was isolated from wild-type and ciprofloxacin-treated *R. microsporus* FP469-12 cells, as well as the isolated endosymbiont alone. Presence of 16S rDNA is indicated by the presence of a 1.5-kb PCR product.

(B) Phylogenetic tree showing the relatedness of *R. microsporus* FP469-12 and its endosymbionts to other fungal and bacterial species.

(C) Comparison of untreated, ciprofloxacin-treated, and *R. pickettii* alone conditions under DIC and SYTO9 staining.

(D) Effects of ciprofloxacin on phagocytosis of *R. microsporus* FP469-12 under resting, swollen 2 hrs, and swollen 4 hrs conditions.

(E) Effects of ciprofloxacin on phagocytosis of *R. microsporus* FP469-12 under DMEM and conditioned medium conditions.

(F) Effects of prior co-culture with live fungi, heat-killed fungi, bacteria only, and endo-free swollen spores on phagocytosis.

(G) Effects of prior co-culture with live fungi, heat-killed fungi, bacteria only, and endo-free mycelium on phagocytosis.

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for the production of bioactive secondary metabolites, we hypothesized a secreted factor might be responsible. We therefore allowed Rhizopus spores to swell in macrophage medium (Dulbecco’s modified eagle medium [DMEM]) for 1 h before removing the spores and testing the capacity of the conditioned medium supernatant to inhibit phagocytosis of other particles. This conditioned medium was sufficient to inhibit phagocytosis of dormant spores to a similar extent as swollen spores themselves (Figure 1B). Conditioned media also had a cross-protective effect on non-mucormycetes, inhibiting phagocytosis of the ascomycete yeasts Candida albicans and Saccharomyces cerevisiae (Figure 1C). R. microsporus FP469-12 therefore produces a secreted factor (or factors), induced upon spore swelling and metabolic activation, with broad anti-phagocytic activity.

**R. microsporus FP469 harbors a bacterial endosymbiont**

Soil-dwelling fungi are associated with bacterial endosymbionts that modulate their host and environments through the synthesis of secreted metabolites. The plant pathogenic bacterium *Rhizostoma solanacearum* produces a lipopeptide ralsolamycin that induces fungal chlamydospore formation and enables bacterial invasion of fungal cells. In particular, *R. microsporus* isolates can host a endosymbionts that impact plant pathogenesis: *R. microsporus* var. *microsporus* CBS 699.68 pathogenesis in rice seedlings is augmented by the endosymbiont *Mycetohabitans rhizoxinica* producing the secondary metabolite rhizoxin, a toxin that targets a conserved residue in β-tubulin, potently depolymerizing microtubules. However, no involvement for endosymbions in mammalian disease has been proven, either as a requirement for pathogenesis in humans or associated with disease severity in diabetic mice.

To test whether an endosymbiont might influence phagocytosis, we tested for the presence of bacterial endosymbiont 16S rDNA in our *Rhizopus* strain (*R. microsporus* FP469-12), by PCR (Figure 2A). *R. microsporus* FP469-12 was positive for 16S rDNA (lane 1), which was lost upon treatment of the fungus with the antibiotic ciprofloxacin (lane 2). Through enzymatic and physical disruption of the fungal cell wall, we were able to isolate endosymbionts from FP469-12, and 16S rDNA could be amplified from the isolated bacteria, which we term RpFP469 (lane 3). For the remainder of this work, unless otherwise stated, spores treated with ciprofloxacin were germinated, passaged through sporulation twice, and frozen down for stocks before use as endosymbiont-free spores to limit the possibility that ciprofloxacin was affecting outcomes.

To further characterize the endosymbiont isolated from *R. microsporus* FP469-12, it was subjected to whole-genome sequencing. This identified the beta-proteobacterium *Ralstonia pickettii*, a relative of *Ralstonia solanacearum* and *Mycetohabita rhizoxinica* commonly found in soil and water and occasionally associated with contaminated medical equipment as an opportunistic pathogen. This was confirmed by two independent replicates identifying 16S sequences consistent with *R. pickettii* in FP469-12- but not FP469-12-cured genomic extracts (Figure 2A). A phylogeny based on 16S sequences placed *R. pickettii* isolated from FP469-12 as most similar to environmental isolates of *R. pickettii*, and more similar to *R. solanacearum* than to *M. rhizoxinica* or *M. endofungorum*, all known endosymbionts of *R. microsporus* (Figure 2C).

To further validate the presence and clearance of the endosymbiont, we stained *R. microsporus* FP469-12 spores with the nucleic acid-binding dye SYTO9 commonly used to stain endosymbionts within fungi. Endosymbionts were clearly visible in hyphae of the parental FP469-12 strain, but absent after ciprofloxacin treatment (Figure 2D). Magnification confocal imaging enabled endosymbions to be seen more clearly and confirmed they were intracellular in both spores and hyphae (Figure 2E). Importantly, SYTO9 staining was again lost upon ciprofloxacin treatment. Additionally, we were able to independently culture the bacterium using established growth protocols for *R. pickettii* (Figure 2E).

**Endosymbiotic R. pickettii are required for anti-phagocytic activity**

We examined whether the presence of the bacterial endosymbiont was necessary for inhibition of fungal spore uptake by macrophages. Uptake of cured swollen spores of *R. microsporus* FP469-12 was significantly higher than that of the uncured parent (p < 0.0001) (Figure 2F). Media conditioned by endosymbiont-free spores also lost the ability to inhibit phagocytosis (Figure 2G). In addition, while the *R. pickettii* endosymbiont grew well in the absence of the fungus, it only caused a small decrease in

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(B and C) (B) Phylogenetic comparison of *R. microsporus* FP469-12 and (C) its *R. pickettii* endosymbiont based on 28S and 16S sequences respectively. Both were aligned with MUSCLE, bootstrapped and produced with RAxML. Strain *R. microsporus* var. *microsporus* is CBS 699.68, shown to harbor *M. rhizoxinica*. Strains CCF4531 and VPCI are clinical isolates from a nasal and pulmonary mass respectively. (D) SYTO9 staining of *R. microsporus* FP469-12 mycelium for bacterial endosymbions. Spores of parent and ciprofloxacin-treated cells were fermented in VK medium, the mycelial pellet submerged in NaCl and then stained with SYTO9 prior to brightfield and fluorescence imaging. (E) High resolution confocal imaging of endosymbions in both spores and hyphae of *R. microsporus* FP469-12 grown for 16 h, then stained with SYTO9. Top row shows the parental and ciprofloxacin-treated cells, and isolated *R. pickettii*, scale bars represent 10 μm. The boxed regions are enlarged below showing both hyphae (middle row) and spores (bottom row), for each fungal strain (scale bars, 5 μm). (F) Contributions of the fungi and bacteria to the secreted anti-phagocytic activity. *R. microsporus* FP469-12 spores by J774.2 macrophages, upon swelling. (G) Effect of media conditioned by co-cultures of bacterial symbionts and endosymbiont-free fungal spores grown on phagocytosis of *R. microsporus* resting spores. Each graph shows the mean and SEM of 3 independent experiments. *p < 0.05, **p < 0.001, ***p < 0.000, one-way ANOVA with Tukey’s correction for multiple comparisons. See also Figure S2.
phagocytosis when used to condition macrophage medium, although still significantly more than medium conditioned by cured spores (p < 0.001, Figure 2G). Endosymbiotic bacteria are therefore required for the secreted anti-phagocytic activity.

To further test whether the presence of intracellular bacteria was required for the anti-phagocyte activity of *R. microsporus* FP469-12, we reconstituted the endosymbiosis by co-culturing protoplasted, cured *R. microsporus* spores with the isolated *R. pickettii*. Re-establishment of the endosymbiosis was confirmed by both PCR and SYTO9 staining (Figures S2A and S2B). This fully restored the ability of swollen spores to inhibit uptake by macrophages (Figure S2C), as well as the inhibition of phagocytosis by conditioned medium (Figure S2D) and ability of the spores to evade killing by macrophages (Figure S2E).

We next assessed the individual contributions of bacteria and fungus to inhibiting phagocytosis of resting cured fungal spores (Figure 2G). There was a small but not significant decrease in phagocytosis when the conditioned media from the fungus alone was used. Conditioned media from bacteria alone caused a decrease in phagocytosis relative to conditioned media from the fungus alone although not as much as when endosymbiotic (Figure 2G). We therefore investigated whether the activities were synergistic (Figure 2H). Conditioned media generated by co-culturing bacteria with endosymbiont-free swollen spores caused a minor but significant (p = 0.019) augmentation of the inhibitory effect of *R. pickettii*-conditioned medium (Figure 2H). This effect was not present when either fungal mycelium, or heat-killed spores were used for conditioned medium (Figure 2H). While this indicates that both organisms cause a minor additive effect when cultured independently, it never reached the extent seen when growing as a true endosymbiosis. We therefore conclude that the holobiont formed by fungal/bacterial endosymbiosis is required for effective anti-phagocyte activity.

### The Rhizopus/Ralstonia holobiont blocks growth of predatory amoebas

While Mucorales can cause opportunistic infections in susceptible humans, they normally live in environments such as soil.60 The emergence of virulence is consequently driven by environmental interactions, rather than those between fungus and the human immune system. It has thus been proposed that mechanisms allowing evasion of phagocytic immune cells originally evolved to help fungi escape professional phagocytes in the environment, such as predatory amoebas.5 To test whether *R. microsporus* FP469-12 can also inhibit capture by environmental phagocytes, we examined its interactions with the soil amoeba *Dictyostelium discoideum*, a well-established model host for both pathogenic bacteria and fungi.61–65

Consistent with our macrophage data, conditioning *D. discoideum* growth medium (HL5) with swollen *R. microsporus* FP469-12 spores for 4 h reduced the phagocytosis of inert, heat-killed *S. cerevisiae* by 41%, compared with untreated medium (Figure 3A). Time-lapse microscopy after addition of conditioned medium revealed that *D. discoideum* cells were still able to form protrusions and actively migrate over this period, indicating that they remain active and viable. However, the amoebae immediately started accumulating prominent swollen vacuoles (Figure 3B; Video S1). Factors secreted by *R. microsporus* FP469-12 therefore also affect amoeba and inhibit phagocytosis from primitive phagocytes, such as amoeba, to human phagocytes.

The accumulation of swollen vacuoles indicates that *R. microsporus* FP469-12 may cause additional disruption of intracellular vesicle trafficking. Consistent with this, *R. microsporus* FP469-12 conditioned medium caused a strong, dose-dependent inhibition of *D. discoideum* growth (Figure 3C). In liquid culture, *D. discoideum* grow using macropinocytosis to take up nutrients. We therefore measured macropinocytosis by following uptake of TRITC-dextran by flow cytometry (Figure 3D). This was unaffected by *R. microsporus* FP469-12 conditioned media, ruling out defective nutrient uptake as the cause of inhibited growth. Growth inhibition was completely dependent on the presence of the endosymbiont, as neither ciprofloxacin-treated spores nor ciprofloxacin alone affected *D. discoideum* growth, and this activity could be rescued by reconstitution of the endosymbiosis (Figures 3E and S3A). HL5 medium conditioned with concentrations of the *R. pickettii* endosymbiont well exceeding that present in the endosymbiotic cultures had little effect on *D. discoideum* growth (Figure 3F). Both bacteria and fungi are therefore essential to inhibit amoeba growth.

*R. microsporus/R. pickettii* endosymbiosis inhibits phagosomal maturation and killing

To grow, amoebas need to both capture and digest food captured within phagosomes or macrophagosomes.66 Newly formed phagosomes therefore represent a major interface between amoebae and ingested microbes and are a common site for evolutionary adaptations that help drive virulence. We therefore tested whether *R. microsporus* FP469-12 affected *D. discoideum* phagosomal maturation. Using DQ-BSA coated beads, which increase fluorescence upon proteolysis, we found addition of spore-conditioned medium after phagocytosis severely inhibited degradation (Figure 4A); this was again dependent on the presence of the endosymbiont. Pre-treating cells for 30 min prior to phagocytosis had no additional effect, indicating that inhibition of proteolysis was due to direct interference with the proteolytic machinery, rather than initial lysosomal delivery to the phagosome (Figure S3B).

We also measured whether *R. microsporus* FP469-12-conditioned medium inhibited the ability of amoebas to kill phagocytosed bacteria. Following the phagocytosis of non-pathogenic *Klebsiella pneumoniae* expressing GFP by time-lapse microscopy allows their intracellular survival to be measured, as the GFP-fluorescence becomes quenched upon death and permeabilization in acidic phagosomes.67 In untreated medium, *D. discoideum* killed 50% of engulfed bacteria within 400 s. Bacterial survival was significantly promoted by conditioned medium, and this was dependent on the presence of the endosymbiont (Figure 4B). *Klebsiella* survival was again restored for conditioned medium from spores where the endosymbiosis was reconstituted (Figure S3C). Amoeba normally feed on bacteria in the soil: *D. discoideum* were able to grow almost as fast on isolated *R. picketti* RpFP469 as on *K. aerogenes* bacteria widely used in laboratory culture (Figure S3D). We therefore propose predation constitutes an important evolutionary driver of endosymbiosis, selecting for collaboration between bacteria and fungi to inhibit both uptake and killing by amoeboid predators.
Amoebas are inhibited by a novel, microtubule-independent activity

Endosymbionts of fungi commonly influence their environments via the secretion of bioactive metabolites. For example, \textit{R. solanacearum} produces the lipopeptide ralsolamycin, which enables invasion of target fungi, via the activity of two non-ribosomal peptide synthetase (NRPS) genes.\textsuperscript{38,39} The \textit{M. rhizoxinica} endosymbiont synthesizes the rhizoxin family of secondary metabolites via a trans-acyl transferase non-ribosomal peptide synthase polyketide synthase (trans-AT NRPS PKS) gene cluster.\textsuperscript{32,55} Rhizoxin directly binds the \(\beta\)-tubulin subunit of microtubules, potently causing their depolymerization and mitotic arrest in both plants and humans.\textsuperscript{52} It is thought that this endosymbiosis emerged to help \textit{R. microsporus} damage and saprophytically feed on plant tissue.\textsuperscript{68} Loci for both ralsolamycin and rhizoxin derivatives are widespread: we identified sequences consistent with the \textit{rhi} locus responsible for rhizoxin biosynthesis in \textit{R. solanacearum} genomes, and the analysis of \textit{M. rhizoxinica} genome HKI 454 identified a locus with 40% similarity to ralsolamycin.\textsuperscript{69} We therefore asked whether \textit{R. pickettii} might similarly encode biosynthesis of ralsolamycin or rhizoxin derivatives or other known metabolites. We performed an unbiased genomic analysis of the \textit{R. pickettii} isolated from \textit{R. microsporus} FP469-12, as well as publicly available \textit{R. pickettii} genomes from environmental sources. AntiSMASH-6 software for secondary metabolite clusters revealed similar overall architecture across the three isolates, including biosynthetic pathways for siderophores, bacteriocins, terpenes, and arylopolyenes.\textsuperscript{70} Uniquely, \textit{R. pickettii} RpFP469 encodes a type I polyketide synthase (TIPKS) lacking similarity to known T1PKS, and this locus was specifically expressed under conditions that induced expression of the anti-phagocytic factor (Figure S4A). Blast analysis identified similar loci in \textit{R. pickettii} (FDAARGOS_1535 and K-288, 84%) and \textit{R. insidiosa} (FC1138, 79%) genomes, but not in \textit{R. solanacearum} or \textit{M. rhizoxinica} genomes. Loci consistent with either ralsolamycin or rhizoxin were not identified in any of the tested \textit{R. picketti} genomes.

The microtubule cytoskeleton is important for many cellular functions, including vesicle trafficking, which is important for phagosome maturation. Therefore, we investigated whether...
microtubules might be a target of the *R. microsporus*- *R. pickettii* endosymbiosis. Contrary to this hypothesis, treatment of *D. discoideum* cells expressing GFP-α-tubulin with *R. microsporus* FP469-12 conditioned medium caused no measurable depolymerization of the microtubule cytoskeleton either with or without the endosymbiont (Figure 4C). This was quantified by the extent of the microtubule array as a proportion of the cell area (Figure 4E). As a positive control, we also treated the cells with rhizoxin D. This was highly effective at depolymerizing microtubules, but only at concentrations above 1 μM (Figures 4D and 4E). This is 5 orders of magnitude higher than that required for a similar effect in mammalian cells, even though all residues at the β-tubulin rhizoxin-binding site are conserved. Furthermore, while conditioned medium blocks *D. discoideum* growth without obvious microtubule disruption, rhizoxin D only inhibited growth at concentrations where microtubules were almost completely depolymerized (Figure 4F). We therefore conclude that the *R. microsporus*- *R. pickettii* endosymbiosis inhibits amoeba function via a novel, most likely microtubule-independent, mechanism.

### Endosymbiosis facilitates fungal evasion of macrophages

The data above show that endosymbiosis allows *R. pickettii* and *R. microsporus* FP469-12 to inhibit both phagocytosis and phagosomal killing by amoebae. To test whether this promotes virulence in animals, we first tested how the presence of the endosymbiont affected evasion from J774A.1 macrophages. Coincubation showed that spores containing endosymbiont were highly resistant to clearance by macrophages, with no significant decrease in colony forming units (CFUs) over 24 h. In contrast, spores lacking endosymbionts were cleared much more effectively, with 60% removed within 24 h (Figure 5A, p < 0.0001).
The secreted activity identified above may not be the sole protective mechanism conferred by the endosymbiont. Previous work has shown that endosymbiosis with *M. rhizoxinica* is also beneficial for fungal development, cell wall synthesis, and stress resistance.43,44,71 As the fungal cell wall is the main interface between the engulfed fungi and the antimicrobial activities of the phagosome, we investigated whether *R. pickettii* endosymbiosis also affects *R. microsporus* resistance to phagosome-relevant stresses. In the absence of the endosymbiont, both resting and swollen spores were significantly more sensitive to treatment with 0.01% sodium dodecyl sulphate (SDS), 5-mM NaNO₃, or 1-mM H₂O₂, as well the front-line antifungal amphotericin B (AmB, 0.5 μg/mL) (Figures 5B and 5C). Endosymbiosis with *R. pickettii* therefore increases the resistance of *R. microsporus* to several physiologically important antimicrobial stresses.

As activation of phagocytic receptors by fungal surface ligands is critical for engulfment, we measured how the fungal cell wall changed upon swelling and how this was influenced by the presence of the endosymbionts. Transmission electron microscopy (TEM) of swollen spores with and without the endosymbiont revealed differences in electron density and width of the cell wall interior, suggesting that the endosymbiont influences spore cell wall organization (Figure 5D).

To further characterize the impact of the endosymbiont, fungi were stained and imaged to reveal specific cell wall components. This showed that in the parental FP469-12 strain, total chitin (stained with calcofluor white [CFW]) increased more than 2-fold upon spore swelling (Figure 5E), although surface exposure of chitin (WGA, wheat-germ agglutinin) and total protein (FITC, fluorescein isothiocyanate) were unaltered between resting and swollen spores (Figures 5F and 5G). We were unable to detect any β-glucan exposure, consistent with previous reports showing low glucan in *Rhizopus* species spore cell walls.4,72,73 Instead, mannan (stained with the lectin concavalin A) was detected as a major component of the cell wall at all stages of growth: exposed mannan was detected on the surface of all cells and was particularly high in approximate half of resting spores and swollen (Figures 5H and S4B).

When the endosymbiont was removed, polysaccharide levels still increased, although there was less CFW staining at each time point compared with the parental strain (Figure 5E).
contrast to the parental strain, spores lacking endosymbionts had higher levels of exposed chitin (WGA), total protein (FITC), and mannan levels at all time points (Figures 5G and 5H). Both surface chitin and total protein increased further after 4 h swelling, with a transient increase in mannan (Con A, concanavalin A) at 2 h swelling (Figure 5H, p < 0.0001). Consistent with this broad impact on fungal cell wall organization and stress resistance, we observed a decrease in spore production when the cleared strain was serially passaged (Figure S4C). The presence of the endosymbiont therefore has broad influence on the structure of the cell wall during swelling and protects both the fungus and bacteria from environmental phagocytes and immune cells by multiple mechanisms.

**Endosymbiosis with *R. pickettii* is required for immune evasion and virulence in vivo**

Finally, we investigated the influence of bacterial-fungal symbiosis in Mucorales pathogenesis in vivo. Using our recently established zebrafish (*Danio rerio*) model of infection,49 wild-type larvae were infected with either resting or swollen wild-type or cured spores (Figure S5A). Over 96-h post-infection (hpi), *R. microsporus* FP469-12 spores containing the endosymbiont killed a significant proportion of the larvae, with swollen spores being more virulent than resting (65% mortality at 96 hpi, compared with 40%, Figure 6A). In contrast, spores lacking the endosymbiont were completely avirulent, with neither swollen nor resting spores statistically different from mock injection in this model (p > 0.05). This correlated with CFUs over time: while there were no differences in initial inocula, fungal CFUs from fish infected with endosymbiont-free spores were significantly reduced within just 2 h and continued to be more rapidly cleared than wild-type spores over 96 h (Figure 6B). This was independent of whether fish were infected with resting or pre-swollen spores, although in both cases, resting spores were more rapidly cleared (Figure 6C). Together, these data suggest that the endosymbiont aids immune evasion during the initial phase of infection and that this is exacerbated by metabolic pre-activation of spores.

Disease outcome correlates with the peak number of phagocytes at the site of infection (carrying capacity) in the first 24 h, and successful infection control requires spore killing.18,49 To investigate the role of the endosymbiont in modulating host defense, we quantified immune cell recruitment using transgenic zebrafish where either macrophages or neutrophils were fluorescently labeled (Tg(mpeg1:G/Ub:NfsB-mCherry or Tg(mpz:GFP)i114, respectively) (Figure S5). Zebrafish were therefore infected with wild-type or endosymbiont-free resting spores and the number of phagocytes recruited to the site of infection counted over the following 96 h (for survival curves, see Figures S6A and S6B). 24 hpi, both macrophage and neutrophil recruitment was significantly lower in larvae injected with spores containing the endosymbiont than those without (Figures 6D and 6E, p < 0.0001). This difference was sustained over 48 h but became less significant at 72 hpi, most likely due to different disease progression as endosymbiont-free spores are cleared (Figure 6A). Similar results were observed with spores pre-swollen before injection (Figures S6C and S6D). While fish that failed to recruit macrophages after 24 h did not survive until the end of the assay regardless of endosymbiont status, only macrophages recruited to

![Figure 6](https://example.com/figure6.png)

**Figure 6. Effect of endosymbiont status on fungal infection of zebrafish**

(A) Survival of AB wild-type zebrafish injected via the hindbrain with resting or swollen spores of *R. microsporus* FP469-12 in the presence or absence of endosymbiotic bacteria. PVP indicates mock-injected fish. Three biological replicates of populations of 10 fish each were examined (n = 30). Statistical differences were determined using Mantel-Cox with Bonferroni’s correction for multiple comparisons (5% family-wise significance threshold = 0.025).

(B and C) Effect of endosymbiont status on fungal survival (CFUs) following hindbrain injections of AB wild-type zebrafish with (B) resting or (C) swollen *R. microsporus* FP469-12 spores. Three biological replicates of 5 fish per condition were examined (n = 15).

(D and E) Effect of the endosymbiont on in vivo recruitment of macrophages and neutrophils to the site of infection. Figure S5 shows representative images and Figure S6 shows equivalent data with swollen spores. Statistical significance was assessed by two-way ANOVA with Tukey’s correction for multiple comparisons or pairwise t tests where sample number was unequal due to fish death, *p < 0.05; **p < 0.001; ***p < 0.0001 unless otherwise indicated.
endosymbiont-free infections efficiently contained the infection. While it remains an open question whether recruitment is inhibited by secreted factors, or other changes in fungal biology mediated by the endosymbiont, the presence of *R. pickettii* enhances *R. microsporus* virulence *in vivo* by multiple mechanisms, leading to suppression of the immune response and fungal clearance.

As endosymbiont removal promoted spore clearance in zebrafish, we attempted to model the impact of antibiotic treatment immediately prior to or during infection on disease outcome. Concomitant treatment of the fish water with 60 µg/mL ciprofloxacin had no effect on fish survival or spore CFU upon infection with either resting or swollen spores (Figures S6E and S6F). Unfortunately, it is not possible to differentiate whether this is due to lack of effect or insufficient drug permeability. Therefore, we instead performed a small proof of concept pilot study in immune-competent mice (*n* = 5 per group). Mice were infected intra-tracheally with resting spores that had been untreated or treated with ciprofloxacin for 3 h immediately prior to inoculation and CFUs measured after 4 and 48 h. Similar to the rapid spore killing observed in fish (Figure 6B), within 4 h, there was a reduction in CFUs recovered from mouse lungs infected with ciprofloxacin-treated resting spores (*p* = 0.019) (Figure 7A). We ruled out a direct inhibitory effect of ciprofloxacin on fungal survival as swollen spores treated with ciprofloxacin were as resistant to host killing as untreated swollen spores (*p* = 0.095) (Figure 7C). After 48 h, while 3/5 mice infected with untreated resting spores remained positive by CFU, all 5 mice infected with ciprofloxacin-treated resting spores cleared the infection (Figure 7B). While underpowered, this was statistically significant (*p* = 0.0384, chi-square test, Newcombe/Wilson with continuity correction, with 60% attributable risk). This effect was lost when mice were infected with pre-swollen spores, where any secreted factors are washed away prior to infection (Figure 7C). Therefore, while endosymbiosis most likely evolved to promote survival in the environment, it also confers protection from phagocytic immune cells in animals, demonstrating an important role in facilitating opportunistic infection.

**DISCUSSION**

In this work, we describe an endosymbiosis between the opportunistic fungal pathogen *Rhizopus microsporus* and the gram-negative bacterium *Ralstonia pickettii*. Both are widely distributed in the environment, normally living in the soil where they will be preyed upon by microbial phagocytes, such as amoebas. A wide diversity of fungi harbor bacterial endosymbionts that influence fungal phenotypes related to metabolism, cell wall organization, development, and plant host colonization. Endobacteria are also a source of potent mycotoxins that can influence fungal pathogenesis in plants and insects. Here, we demonstrate a novel interaction, whereby endosymbiosis between *R. microsporus* and *R. pickettii* enables the biosynthesis and secretion of factors that inhibit both the growth of amoebas, and their ability to capture and kill other microbes. We propose that the tri-kingdom interaction with amoebas provides an additional evolutionary driver for endosymbiosis, enabling both bacteria and fungus to evade predation in the environment.

While not all isolates of *R. microsporus* (*Rm*) harbor endosymbionts, there are examples of *Rm*-Mycetohabitans endosymbionts that are essential for asexual sporangiospore and sexual zygospore production. In these partnerships, endobacteria reside within the fungal cytoplasm and are vertically transmitted through sporulation and mating. Here, we similarly observe vertical transmission of *R. pickettii* in sporangiospores, with multiple bacteria apparent per spore, and observed a reduction in sporangiospore production after repeated passage of cured fungi, suggesting large scale regulatory changes as a result of endosymbiont loss. Consistent with mutualism, the presence of the *R. pickettii* endosymbiont caused widespread changes to its host; endosymbiont removal altered *R. microsporus* cell wall structure and sporangiospore viability and reduced resistance to reactive nitrogen and oxygen species as well as engulfment and phagocyte-mediated killing. Loss of the endosymbiont also increased sensitivity to the front-line antifungal treatment amphotericin B. This indicates altered ergosterol content and suggests an additional way in which the endosymbiont may influence the outcome of human infections.

Endosymbionts of fungi can employ multiple strategies to modulate their hosts and environment. Our analysis of the *R. pickettii* genome did not identify loci consistent with known metabolites ralosolamycin or rhizoxin but did identify a unique T1PKS that was specifically expressed during interaction with the fungus. Endosymbiosis-dependent expression of biosynthetic gene...
clusters (BGCs) has been observed in R. microsporus-M. rhizoxinica interactions, including for conserved factors that support invasion and endosymbiosis. However, the R. pickettii locus lacked similarity to known metabolites and not present in the closely related environmental R. pickettii isolates 12D and 12J. The locus does share 79% similarity with the emerging pathogen R. insidiosa. There was no evidence that this locus shared similarity with Rhizopus species genomes, excluding horizontal gene transfer of the locus from the host fungus. Future work will further investigate the nature of this product.

Most studies of R. microsporus secreted mycotoxins have focused on molecules generated by M. rhizoxinica and related endosymbionts of the Burkholderia genus. Although Ralstonia, Burkholderia, and Mycetohabitans spp. are related and share the same niche, the anti-amoebic activity of the R. microsporus FP469-12 endosymbiosis differs in several important ways. First, while M. rhizoxinica alone is sufficient for rhizoxin-derivative biosynthesis, media conditioned with the isolated R. pickettii endosymbiont from R. microsporus FP469-12 was ineffective against either D. discoideum or macrophages, indicating in this case both fungi and bacteria are necessary for the secreted bioactivity. The mechanism of action also differs compared with rhizoxin; while rhizoxin potently depolymerizes the microtubule cytoskeleton, this appeared unaffected in amoebas treated with rhizoxin; while rhizoxin potently depolymerizes the secreted bioactivity. The mechanism of action also differs, indicating in this case both fungi and bacteria are necessary for the secreted bioactivity. The presence of the endosymbiont confers resistance to macrophages in a similar manner to amoebae, inhibiting both phagocytosis and killing. The presence of the endosymbiont is also essential for virulence in a zebrafish model of infection. While the widespread changes in fungal physiology that occur upon endosymbiont removal indicate that evading amoebas is not the sole driver of endosymbiosis, we suggest that it is an important contributory factor, and significant for the evolution of virulence.

While a broadening diversity of basal saprophytic fungi are a major emerging cause of clinically important superficial and disseminated fungal infections, the role of endosymbionts in pathogenesis has yet to be clinically defined. Two groups recently reported that although bacterial endosymbionts, including rhizoxin and rhizoxin producers, are widely found among mucormycete clinical isolates, these endosymbionts did not impact pathogenesis during mucormycosis in both mouse and fly models. In a diabetic mouse model infected with Rhi zobus isolates harboring rhizoxin-producing Burkholderia endosymbionts, no impact of treatment with ciprofloxacin 24 hpi was observed. In contrast, we found that pretreatment of resting spores with ciprofloxacin before infecting immune-competent mice significantly reduced CFUs retrieved after 4 h, and spores could no longer be recovered after 48 h. This benefit was however lost when spores were pre-swollen prior to infection, indicating that to be therapeutically effective, endosymbiont removal would need to occur early in the infection. While our study only used a small number of mice (5 per group) and is therefore prone to the challenges associated with underpowered models of infection, similar patterns were observed in zebrafish, with much larger sample sizes. While most studies to date have focused on Burkholderia spp endosymbionts and the rhizoxin and rhizoxin mycotoxins, our work indicates a broader diversity of both endosymbionts and virulence mechanisms. The relevance of fungal-bacterial endosymbiosis to human infections therefore needs readdressing, using larger-scale studies of the prevalence and diversity of endosymbionts in both clinical and environmental isolates.

Our findings highlight the potential importance of bacterial symbiosis in R. microsporus pathogenesis. However, we urge caution in extending our findings to all Mucorales or fungal endosymbionts. While a number of studies have demonstrated general features common to host-Mucorales interactions, there are also likely to be species-specific aspects. In addition, not all clinical isolates profiled harbor endosymbionts, suggesting that they may augment, but are not constitutively required for, pathogenesis. Overall however, this work points to a new role for bacterial endosymbionts in fungal immune evasion, driven by environmental interactions with amoebae. The observation that endosymbiont elimination impacts phagocytosis, cell wall organization, stress resistance, and antifungal drug susceptibility as well as resistance to macrophage-mediated killing raises the prospect that endosymbiont-targeted treatments may be useful in the treatment and management of a subset of opportunistic fungal infections.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:
The authors declare no competing interests.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Dectin-1 IgG antibody | Gordon Brown lab<sup>105</sup> | Fc Dectin-1; RRID:AB_2904615 |
| goat anti-mouse IgG-48 | Sigma Aldrich | Cat# SAB4600387-50UL; RRID:AB_2904614 |
| **Bacterial and Virus Strains** |        |            |
| GFP-expressing Klebsiella pneumoniae fluorescence | Pierre Cosson, University of Geneva. | N/A |
| Ralstonia pickettii RpFP469 | Isolated from *R. microsporus* FP469-12, originating from a human infection at the Queen Elizabeth Hospital Birmingham Trauma Centre, UK, by Dr. Deborah Mortiboy | Strain ID RpFP469 |
| **Biological Samples** |        |            |
| Rhizopus microsporus strain FP469-12.6652333 | Isolated from a human infection at the Queen Elizabeth Hospital Birmingham Trauma Centre, UK, by Dr. Deborah Mortiboy | Strain ID FP469-12.6652333 |
| Candida albicans | Neil Gow, University of Exeter | Strain ID SC5314 |
| Saccharomyces cerevisiae | Neil Gow, University of Exeter | Strain ID AM13/001 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| HL5 medium | Formedium | Cat# HLG0102 |
| DQ-green BSA | Invitrogen | Cat# D12050 |
| Alexa594-NHS ester | Invitrogen | Cat# A37572 |
| LoFlo medium | Formedium | Cat# LF0501 |
| **Critical Commercial Assays** |        |            |
| Live/Dead BacLight bacterial viability kit | ThermoFisher Scientific | Cat# L7007 |
| DNeasy® powerlyzer® microbial kit | Qiagen | Cat# 12255-50 |
| Phusion High Fidelity PCR kit | New England Biolabs | Cat# E0553S |
| **Deposited Data** |        |            |
| Ralstonia pickettii RpFP469 whole genome and RNAseq | Sequence Read Archive (SRA) | Accession # SRR16523121 |
| *Rhizopus microsporus* FP469-12 whole genome | Sequence Read Archive (SRA) | Accession # SRR16610381 |
| *Ralstonia solanacearum* 16S | GenBank | Accession # ABO24604.1 |
| *Candidatus Glomeribacter gigasporarum* 16S | GenBank | Accession # AJ251633.1 |
| *Mycetohabitans endofungorum* 16S | GenBank | Accession # AM420302.1 |
| *Ralstonia pickettii* 12J 16S | GenBank | Accession # CP001644.1 |
| *Mycetohabitans paraburkholderia rhizoxinica* HKI 454 16S | GenBank | Accession # FR687359.1 |
| *Rhizopus microsporus* CCF4531 23S | GenBank | Accession # HG324080.1 |
| *Rhizopus microsporus* VPCI 12B/P/10 23S | GenBank | Accession # KJ417570.1 |
| *Rhizopus microsporus* var. *oligosporus* 23S | GenBank | Accession # MH869766.1 |
| *Rhizopus microsporus* var. *microsporus* 23S | GenBank | Accession # MH870925.1 |
| *Rhizopus microsporus* var. *chinensis* 23S | GenBank | Accession # MH871901.1 |
| *Rhizopus microsporus* var. *rhizopodiformis* 23S | GenBank | Accession # MH873059.1 |
| **Experimental Models: Cell Lines** |       |            |
| J774A.1 | ATCC | Strain ID TIB-67 |
| Dictyostelium discoideum Ax2 axenic strain | www.dictybase.org | Strain ID DBS0235521 |

(Continued on next page)
## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact Elizabeth Ballou (E.Ballou@Exeter.ac.uk).

### Materials availability

Strains unique to this study will be made available to other users upon request.

### Data and code availability

Genomic sequencing data is available through the Sequence Read Archive (SRA) on NCBI and accession codes for sequences referenced in this work are: *R. picketii*: SRA: SRR16523121 and *R. microsporus*: SRA: SRR16610381. This work did not lead to the generation of any new code.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### *R. microsporus* strain and culture

*Rhizopus microsporus* strain FP469-12.6652333 was isolated from a human infection at the Queen Elizabeth Hospital Birmingham Trauma Centre by Dr. Deborah Mortiboy. This was routinely cultured on Sabouraud dextrose agar (SDA) (EMD Millipore co-operation) or potato dextrose agar (PDA) to induce sporulation at room temperature. Sporangiospores were collected by gentle disruption of the mycelium in the presence of PBS. Harvested spores were filtered through a 70 μm filter to exclude hyphal fragments, then washed and counted by haemocytometer. Routine liquid cultivation was performed in 500 mL conical flasks containing 250 mL of serum free

## Table

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental Models: Organisms/Strains** |
| wild type AB zebrafish | Steve Renshaw, University of Sheffield | N/A |
| GFP-neutrophil zebrafish line | Steve Renshaw, University of Sheffield | Genotype Tg(mpx:GFP) | 114 |
| GFP-macrophage zebrafish line | Steve Renshaw, University of Sheffield | Genotype Tg(mpeg1:G/U:NlsB-mCherry) |
| CD-1 20-23g male mice | Envigo, Indianapolis, IN, USA | Strain ID Hsd:ICR (CD-16) |
| **Oligonucleotides** |
| 5’CCGAATTCCGTCGACAAACAGATTTGAT CCTGGCTCAAG3’ | Primer 16S rDNA Universal F |
| 5’CCGGGGATCCAAAGCTTACTTACAGCCTACCTT GTTACGACTT 3’ | Primer 16S rDNA Universal R |
| 5’GTCTTCTCTCTTAAATTGGTTC3’ | Primer RmACT1-F |
| 5’-CCATCGAGAAGCTTACGCTTTAAAGAC-3’ | Primer RmACT1-R |
| 5’ATGATCTGATGCTAGATTGATC3’ | Primer Rp-F |
| 5’ACTGATCGTCGCCTTGTTG3’ | Primer Rp-R |
| **Dicystostelium GFP-tubulin expression vector** | Plasmid pJSK336 |
| **Software and Algorithms** |
| ImageJ | https://imagej.nih.gov/ij/ | N/A |
| kraken (v1) | https://ccb.jhu.edu/software/kraken/ | N/A |
| Graphpad Prism 7 | Graphpad | N/A |
| Hisat2 (Version 2.0.5) | http://www.ccb.jhu.edu/software/ hisat/index.shtml | N/A |
| HTSeq (Version 0.10.0) | https://htseq.readthedocs.io/en/ release_0.10.0/ | N/A |
| edgeR (Version 3.16.5) | https://bioconductor.org/packages/ release/bioc/html/edgeR.html | N/A |
| RAxML | https://github.com/stamatak/standard- RAxML | N/A |
| MUSCLE | https://www.ebi.ac.uk/Tools/msa/muscle/ | N/A |
Dulbecco’s modified eagle’s media (with 1% penicillin/streptomycin and L-glutamine) (DMEM). Fungi grown in this way was used for metabolic activation of spores, to prepare swollen spore supernatants, and for DNA isolation.

**R. picketti strain and culture**

Raistonia picketti strain RpfFP469 was isolated from *R. microsporus* FP469-12.665233 spores incubated in SabDEX overnight and then macerated with sterilized glass beads. The resulting supernatant was filtered through decreasing filter sizes (70 μm to 3 μm) to remove fungal particles and plated onto LB agar to allow bacterial growth. Isolated colonies were incubated in LB liquid medium at 30 degrees, 200rpm overnight, then washed and stained with the Live/Dead BacLight bacterial viability kit (Thermofisher Scientific) according to the manufacturer’s instructions.

**Macrophage cell line culture**

J774A.1 murine macrophage-like cells were maintained in DMEM supplemented with 10% foetal bovine serum, 1% Streptomycin (100 μg/mL), penicillin (100 U/mL), and 1% L-glutamine (2 mM). The cells were cultivated in a humidified environment at 37°C enriched with 5% CO₂ and used between 3 and 15 passages after thawing.

**D. discoideum culture**

All experiments were performed using the Ax2 axenic strain, routinely subcultured in HL5 medium (Formedium) at 22°C. For growth curves, cells were seeding cells at 1 x 10⁷/ml in the appropriate medium and counted at regular intervals until out of exponential growth. GFP-α tubulin was expressed using extrachromosomal vector pJSK336109, cells were transformed by electroporation and transformants selected in 20 μg/mL G418. Using ImageJ110 maximum intensity projections of images from a Perkin-Elmer Ultra-view VoX (60X 1.4NA objective) were then used to manually draw around the tips of the microtubule array, and calculate the areas covered as a proportion of the whole cytosol.

**Zebrafish maintenance and infection**

Infections were performed in wild type AB zebrafish, as well as GFP-neutrophil (Tg(mpx:GFP)i114) lines.74 Macrophage-specific mCherry expression was achieved by crossing Tg(mpeg1:Gal4-FF)g125 with Tg(UAS-E1b:NfsB.mCherry)c264, referred to as Tg(mpeg1:G/U:NfsB-mCherry).75 Zebrafish were cultivated under a 14 h-10 h light-dark cycle at 28°C at the University of Birmingham (BMS) Zebrafish Facility. All zebrafish care protocols and experiments were performed in accordance with the UK animals (scientific procedures) act, 1986. Following collection of the eggs, at 40–60 eggs per 25 mL E3 media (plus 0.1% methylene blue and 1 mg/mL 1-phenyl-2-thiourea (PTU)) for 24 h and larvae were incubated at 28°C. PTU at this concentration prevented melanization of the embryos. All media and reagents used was purchased from Sigma Aldrich unless otherwise mentioned.

**Mouse husbandry**

All experiments were performed using immunocompetent CD-1 20-23g male mice. Group size was determined from previous experiments as the minimum number of mice needed to detect statistical significance (P < 0.05) with 90% power. Mice were randomly assigned to groups by an investigator not involved in the analysis, and the fungal inocula were randomly allocated to groups. Mice were house in individually ventilated cages (IVCs), 5 per cage, and were provided with food and water ad libitum. Inocula were delivered in an unblinded fashion.

**Ethical statement**

All maintenance protocols and experiments were performed in accordance to Animals (scientific procedures) Act, 1986 as required by United Kingdom (UK) and European Union (EU). All work was performed under appropriate Biosafety Level 2 conditions (BSL2). All zebrafish care and experimental procedures were conducted according to Home Office legislation and the Animals (Scientific Procedures) Act 1986 (ASPA) under the Home Office project license 40/3681 and personal licenses I13220C2C to Kerstin Voelz and ICDB92D64 to Herbert Itabangi. Mouse studies were approved by the Institutional Animal Care and Use Committee of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, according to the NIH guidelines for animal housing and care under protocol 11671.

**METHOD DETAILS**

**Generation of Conditioned medium**

This was obtained by incubating at 4x10⁵ spores/mL in 250 mL of either serum free DMEM (for macrophage experiments) or HL5 medium (Formedium, for *D. discoideum* experiments) at 37°C with shaking at 200 rpm for 4 h. The medium was then centrifuged at 3990 rpm for 5 mins and the supernatant filter sterilised through a 0.45 μm filter.

Endosymbiont-free (cured) fungal strains were obtained by cultivating fungi in the presence of 60 μg/mL ciprofloxacin for a month. Fungi were then maintained on ciprofloxacin plates for 3 months before the endosymbiont was entirely cleared. Absence of bacteria was routinely confirmed through SYTO9 staining and PCR screening for bacterial 16S rDNA, as described below.

Spore stress resistance was measured by incubating at 10⁶ spores/mL in serum-free DMEM containing the indicated concentrations of hydrogen peroxide (H₂O₂), sodium nitrite (NaNO₂, Fisherscientific), sodium dodecyl sulphate (SDS) (Fisher scientific), sodium
chloride (NaCl, Sigma-Aldrich) or Amphotericin B (AmB, Sigma Aldrich). The spores were incubated at 37°C and 5% CO₂ for 24 or 48h before CFUs were determined by plating serial dilutions on SDA agar.

**Reintroduction of *R. pickettii* in *R. microsporus***

Reintroduction of the endosymbiont was achieved as described previously, with some modifications.43 Cured *R. microsporus* spores were harvested and washed 3x with PBS before incubation at 37°C for 40 min with 30 mg/ml of lysing enzymes from *Trichoderma harzianum* (Sigma L1412) to generate protoplasts. Spores were then transferred in SCS buffer pH 5.8 (20mM sodium citrate, 20mM citric acid, 1M D-sorbitol) and co-cultured with *R. pickettii* on plates containing PDA. A plug of PDA was removed and replaced with a plug of LB agar covered with an inoculum of the bacteria. An inoculum of the fungus was placed directly on the bacteria and plates incubated at 30°C for 2 weeks.

To test for the presence of endobacteria, 12h-germinated spores were sheared by pipetting (first round 70x and a second round 20x) before centrifugation at first 32 g for 5 min, then 2,060 g for 20 min. After each centrifugation step, supernatant aliquots were plated onto Pseudomonas Agar® supplemented with 0.5% yeast extract and 1% glycerol (ThermoFisher Scientific CM0559) and incubated at 30°C.

At the same time, supernatants and cell pellet aliquots were retained for DNA isolation. Briefly, cell pellets and supernatants were disrupted using glass-beads and vortex (4 cycles, 1 min vortex: 1 min ice) before snap freezing in liquid nitrogen before immediate transferrance to a water bath at 85°C for 20 min, before snap freezing again. DNA was then purified by phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. Primers amplifying the 16S rDNA of *Ralstonia* spp. were used. RalGS-F (5’CTGGGGTCGATGACGGTA3’) and RalGS-R (5’ATCTCTGGCTTCGTTAGTGGC 3’) were used to identify the endosymbiont at the genus level (*Ralstonia* (5’CTGGGGTCGATGACGGTA3’) and RalGS-R (5’ATCTCTGCTTCGTTAGTGGC 3’) to identify it at species level (*R. pickettii* (amplicon 546 bp) and *R. microsporus* (amplicon 600 bp)).40

**Phylogenies**

Trees were built based on 28S and 16S sequences respectively. Both were aligned with MUSCLE, bootstrapped and produced with RAxML.117,118 GenBank accession numbers for each sequence used were: *Ralstonia solanacearum* AB024604.1; *Candidatus Glom- eribacter gigasporarum* AJ251633.1; *Mycetohabitans endofungorum* AM420302.1; *Ralstonia pickettii* 12J CP001068.1; *Ralstonia pickettii* 12D CP001644.1; *Paraburkholderia rhizoxinica* HKI 454 FR687359.1; *Rhizopus microsporus* CCF4531 HG324080.1; *Rhizopus microsporus* VPCI 128/P/10 KJ417570.1; *Rhizopus microsporus* var. *oligosporus* MH869766.1; *Rhizopus microsporus* var. *microtus* MH870925.1; *Rhizopus microsporus* var. *chinensis* MH871901.1; *Rhizopus microsporus* var. *rhizopodiformis* MH873059.1

**Cell wall analysis**

For microscopy, 2x10⁸ spores/ml were incubated for 30 minutes at room temperature in PBS containing either 100 μg/ml fluorescein isothiocyanate isomer 1 (FITC) (Sigma-Aldrich) in 0.1 M sodium bicarbonate buffer (pH 7.45) (Sigma Aldrich), 25–50 μg/ml RhTRITC-concanavalin A (ThermoFisher scientific) or 250 μg/ml calcofluor white (Sigma Aldrich). Spores were then imaged at 63x magnification on a Zeiss Axio Observer Z1 equipped with structured illumination (Apotome) using a Flash 4 sCMOS camera (Hamamatsu). The same exposure was used across all samples. Staining intensity was quantified for each cell using ImageJ to measure relative fluorescence within computationally identified regions of interest.

For flow cytometry, resting *R. microsporus* FP 469-12 spores were pre-germinated for the indicated times in serum-free DMEM at 37°C, 200 rpm, before fixation in 4% methanol-free formaldehyde, and incubation with either 0.01 μg/ml Dectin-1 IgG antibody, washed, and 1:200 goat anti-mouse IgG-488 secondary antibody (b-glucan); Calcofluor white (CFW, chitin; 250 μg/ml); or Conca- navalinA (ConA-488; 50 μg/ml). At least 10,000 cells were measured using an Attune NxT flow cytometer and compared to a pooled secondary-only control. Data from three independent experiments.

**TEM**

*R. microsporus* spores were collected and allowed to swell as described above. Samples were processed via high-pressure freezing using a Bal-Tec HPM 010 high-pressure freezer (Boeckler Instruments, Tucson, AZ) and transferred to an RMC FS-7500 freeze substitution unit (Boeckler Instruments, Tucson, AZ) before substitution in 2% osmium tetroxide, 1% uranyl acetate, 1% methanol, and 5% water in acetone. Samples were then transitioned from –90°C to room temperature over 2 to 3 days, rinsed in acetone, and embedded in LX112 epoxy resin (Ladd Inc., Burlington, VT). 70 to 80 nm sections were then cut on a Leica Ultracut UC7 microtome, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

**SYTO9 staining of bacterial endosymbionts**

A small sample of mycelia pellet was aseptically submerged into 200 mL of 0.85% sodium chloride (NaCl) for 1 h, washed 2x in PBS and stained with the Live/Dead BacLight bacterial viability kit (Thermofisher Scientific). Mycelia were incubated in stain solution for...
15 minutes, fixed with 4% PFA, mounted on glass slides and imaged using a 63x oil objective under phase contrast on a Zeiss Axio Observer Z1 microscope. For confocal imaging, 1x10^5/ml spores in 10mls SabDex were incubated for 4 hrs, 150 rpm, then collected, washed 3x with sterile PBS, and stained with the Live/Dead BacLight bacterial viability kit (Thermofisher Scientific) according to the manufacturer’s instructions. Images were acquired on a Zeiss LSM 900 with Airyscan 2.

**Screening and identification of bacterial endosymbiont**

PCR screening for the presence of endosymbionts was performed using Universal primers (5'CCGAA TTGCTGACACACAG AGTTTGA TCCGGGCTCAG3' / 5'CCCGGATCCAGCTTGACCTTTGTTACGACTT 3') which amplify a 1.5 kb rDNA product. DNA was obtained from 1x10^7 spores in a screw cup with beads and homogenised using a bead beater (Bertin technologies) at 6500x g for 1 min. DNA was then extracted with a DNeasy PowerLyzer microbial kit (Qiagen) following the manufacturer’s instructions. PCR was performed using Phusion kit (New England Biolabs) and 35 cycles, of 95°C for 2 min, 60°C for 30s, and 72°C for 1 min. The resulting PCR product was then sequenced, identifying *R. picketti*. For full- genome sequencing, bacterial genomic DNA was extracted using the DNeasy PowerLyzer Microbial kit (Qiagen) and sequenced by MicrobesNG (University of Birmingham, UK). The sample was identified by kraken (v1) as being highly similar to *R. picketti* J12. Transcriptional analysis of *R. picketti* was assessed following growth for 4 h at 30°C, 80-150 rpm in VK, DMEM, HL5, or DMEM+cured *R. microsporus* hyphae. Triplicate biological replicates were prepared for each condition. RNA was extracted using the modified Qiagen RNA extraction method as described previously. Briefly, TRIzol was used to lyse the samples, which were then either immediately frozen at -20°C and stored for RNA extraction or placed on ice for RNA extraction. After lysis, 0.2 ml of chloroform was added for every 1 ml of TRIzol. Samples were incubated for 3 min, then spun at 12,000 g at 4°C for 15 min. To the aqueous phase, an equal volume of 100% ethanol (EtOH) was added, before the samples were loaded onto RNaseo RNA extraction columns (Qiagen). The manufacturer’s instructions were followed from this point onwards. RNA quality was checked by Agilent, with all RNA integrity number (RIN) scores above 7. One microgram of total RNA was used for cDNA library preparation. Library preparation was done in accordance with the NEBNext pipeline, with libraries quality checked by Agilent. Samples were sequenced using the Illumina NextSeq platform; 150-bp paired-end sequencing was employed (2 x 150 bp) (>10 million reads per sample). Data was analyzed using Hisat2 (Version 2.0.5), HTSeq (Version 0.10.0) and edgeR (Version 3.16.5).

**Phagocytosis assays**

For phagocytosis by macrophages, 1x10^5 J774A.1 cells were seeded per well in a 24 well plate in DMEM and incubated overnight. Next day, cells were washed 2x in pre-warmed PBS, before incubation with 1 mL pre-warmed serum-free DMEM at 37°C +5% CO2 for 1 h. Cells were then washed 2x with PBS, before addition of 5x10^6 pre- FITC stained spores in serum-free DMEM (MOI = 1:5). The co-culture was incubated 37°C +5% CO2 for 1 h before fixation in 4% PFA for 15 min, and washing with PBS prior to counter staining with Concanavalin A (Con-A) or calcofluor white (CFW) for 30 min. Cells were then washed in PBS and imaged on a Nikon T1 microscope. Uptake rate was quantified as the number of phagocytes containing at least one spore, >1000 phagocytes assessed per sample.

Phagocytosis of UV killed spores was performed as above, except prior to addition, spores were irradiating twice for 15 min in a UV PCL-crosslinker at 1200 μJ/cm² in PBS, cooling on ice between treatments as previously described. Successful killing was confirmed by plating for CFUs. Latex beads, C. albicans SC5314 and S. cerevisiae AM13/001 were all processed similarly to spores including washing with PBS prior to addition to the macrophages.

Phagocytosis by *D. discoideum* was measured by incubating 2 x 10^6 amoebae in 2ml untreated or conditioned HL5 in 3cm glass-bottomed microscopy dishes for 1 hour, prior to addition of 1 x 10^5 TRITC-labelled heat killed *S. cerevisiae* (prepared as in). After 30 minutes, fluorescence of unengulfed yeast was quenched by addition of 100 μl 0.4% trypan blue solution (Sigma Aldrich), before fluorescence microscopy and quantification of number of yeast engulfed per amoebae, counting >1000 cells quantified per sample.

**Phagosomal proteolysis** was measured as previously described, using 3 μm silica beads co- labelled with DQ-green BSA and Alexa594 (performed as in). Amoebae were washed twice in LoFlo medium (Formedium), before resuspension at 3 x 10^7/ml in HL5 medium and 100 μl well seeded in clear-bottomed black-walled 96-well plates (Greiner). After 2 hours, 10 μl reporter beads were added to triplicate wells at a bead:cell ratio of 1:2, and the plate spun down at 1,200 rpm for 10 seconds to synchronise uptake. Free beads were then removed by tapping the inverted plate on a paper towel and washing twice in HL5. 100 μl conditioned, or control media was then added to wells before the plate was placed in a plate reader, and fluorescence measured at 500/520nm and 594/630nm (excitation/emission) every minute. For pre-treatment with conditioned medium, it was added 30 minutes prior to bead addition, and used in all subsequent washes/incubations. Proteolysis was calculated by the increase in DQ-BSA fluorescence over time, normalised to bead uptake, determined by Alexa594 fluorescence.

**Phagosomal killing** was performed as following the quenching of GFP-expressing Klebsiella pneumoniae (gift from Pierre Cosson, University of Geneva). 10 μl of a saturated overnight bacterial culture in LB was diluted in 280 μl HL5 (or conditioned medium) and allowed to settle as a drop in a glass-bottomed microscopy dish (Mat-Tek). After 15 minutes, 1.5 ml of *D. discoideum* culture at 1 x 10^6 cells/ml was carefully added and GFP and bright-field imaged recorded every 20 seconds for 40 minutes at 20x magnification. Movies were then manually analysed to determine the time of GFP- quenching post-engulfment.
Spore viability
Resistance to killing by macrophages was assessed as following phagocytosis at the indicated time points. Macrophages were lysed with 1 mL sterile water and aggressively washed to collect adherent spore cells. The lysate was serially diluted and 5 μL plated out on SAB agar for CFUs.

Zebrafish infections
Prior to injection, fungal spores collected from 10 day old cultures on SabDex plates were washed 3x in PBS and stained with Calcofluor White in 0.1 M of NaHCO₃ for 30 min. Swollen spores were pre-incubated in DMEM at 37 °C, 200 rpm for 4 hr prior to staining. Spores were the washed 3 times in PBS, and resuspended at 10⁵ spores/mL in 10% (w/v) polyvinylpyrrolidone-40 (PVP) in PBS with 0.05% phenol red in ddH₂O. PVP was used as a carrier medium to increase density and prevent clogging of microinjection needles. Zebrafish were injected into adults infected by a protocol by Brothers et al., and zebra fish development assessment in accordance to Kimmel et al. The fish were injected at prim-25 stage following manual dechorionation and anesthesia with 160 mg/mL of Tricaine in ddH₂O. Micro-injection was performed with 2 nL of 10% (PVP) in PBS or R. microsporus spore suspension through the otic vesicle into the hind brain to achieve an inoculum dose of approximately 50-100 spores per fish. Following injection, larvae were anesthetized with 160 μg/mL Tricaine in E3 media in a 96 well plate and screened by fluorescence microscopy, (Zeiss Axioserver Zi microscope equipped with Apotome system) for the presence of spores. Only larvae with approximate correct inoculum were selected, and transferred to individual wells of a 24 well plate containing E3 media (plus 0.1% methylene blue ±60 μg/mL Ciprofloxacin). The fish were monitored over a period of 96 hours post infection for survival whereupon they were killed by 1600 mg/mL Tricaine overdose and treated with bleach overnight before disposal.

Recovery of fungal spores
Following injection, 5 fish per biological repeat were euthanized with 1600 μg/mL Tricaine at each timepoint, before homogenisation in 100 μL of E3 media containing penicillin-streptomycin (5000 U/mL-5 mg/mL) and gentamicin (10 mg/mL) using pellet pestles. Extracts were then plated out on SDA containing 100 U/mL-100 μg/mL penicillin-streptomycin and 30 μg/mL gentamicin, incubated at room temperature for between 24 and 48 h and colony forming units (CFUs) examined.

Phagocyte recruitment
To quantify phagocyte recruitment into the hindbrain, Tg(mpx: GFP) or Tg(mpeg1:G/U:NfsB-mCherry) transgenic zebrafish were injected as above. At each timepoint, fish were imaged using an Axio Observer Z1 microscope equipped with Apotome (Carl Zeiss), to reconstruct the 3D volumes. Positive phagocyte recruitment was defined by accumulation of >10 neutrophils or macrophages at the site of infection. At least 3 biological repeats were performed with 5 fish per condition to give a total of 15 fish per group.

Mouse infections
Immunocompetent CD-1 20-23g male mice (n=12 per group) were randomly assigned to one of two treatment groups and the groups housed in IVCs. Rhizopus microsporus spores (FP469-12.6652333) were collected from Sabouraud agar plates in 10 mL of PBS + 0.01% Tween 20, washed once with endotoxin-free PBS and resuspended in serum-free DMEM at 4x10⁷/ml. To mimic the early stages of infection, swollen spores were pre-germinated in serum-free DMEM with or without Ciprofloxacin (60 μg/mL) for up to 3 hrs at 37 °C, 200 rpm, sufficient to swell but not form germ tubes. Mice were infected intratracheally with 25μl serum-free DMEM containing 10⁶ resting or swollen spores. Mice (n=5 per group) were sacrificed by pentobarbital overdose and lungs were collected at two different time points: 4 h or 48 h post infection. Lungs were homogenization in 2 ml of PBS, and 200 μl were plated directly from the concentrated samples and also from serial dilutions onto potato dextrose agar + 0.1% Triton plates and incubated at 37 °C. Innoculum was verified via lung CFU from two mice directly after infection. Data were tested for normality using Shapiro-Wilk and analysed using the Mann-Whitney U test. Data are reported as Median with 95% CI.

QUANTIFICATION AND STATISTICAL ANALYSIS
All data was analysed in Graphpad Prism 7 using the nonparametric, Mann-Whitney U tests, and log rank tests, with Dunn’s correction for multiple comparisons where appropriate and as indicated in the figure legends and main text. Differences with p value ≤0.05 were considered significant. All experiments were performed in triplicate, with the number of events measured indicated in figure legends. Data are plotted as Box-and-Whiskers with maximum and minimum values, except where data shown are percentages or relative values.
A bacterial endosymbiont of the fungus

*Rhizopus microsporus* drives phagocyte evasion

and opportunistic virulence

Herbert Itabangi, Poppy C.S. Sephton-Clark, Diana P. Tamayo, Xin Zhou, Georgina P. Starling, Zamzam Mahamoud, Ignacio Insua, Mark Probert, Joao Correia, Patrick J. Moynihan, Teclegiorgis Gebremariam, Yiyou Gu, Ashraf S. Ibrahim, Gordon D. Brown, Jason S. King, Elizabeth R. Ballou, and Kerstin Voelz
Figure S1: Inhibition of phagocytosis requires live spores and is not due to spore size, related to Figure 1. (A) Phagocytosis of R. microsporus FP 469-12 UV-inactivated resting and swollen (2 and 4hr) spores by J774A.1 macrophages. (B) Phagocytosis of swollen (4h, 6.5 μm) R. microsporus FP 469-12 spores and latex beads (11.9 μm) by J774A.1 macrophages. For both experiments the number of phagocytes containing at least one spore or bead were scored as positive. Graphs show mean ± SEM of 3 independent repeats. ns = p>0.05, ***=p<0.0001, One-Way ANOVA with Tukey’s correction for multiple comparisons.
Figure S2: Reconstitution of endosymbiosis restores anti-phagocyte activity, related to Figure 2 (A) PCR confirmation of endosymbiont clearance and reintroduction. PCR was performed on cell pellets of the cells indicated using 3 different primer pairs. The first column shows PCR with Ralstonia genus primers (546bp), the second with R. pickettii-specific primers (210bp), and the third with Rhizopus microsporus-specific primers (600bp). (B) Confocal images of SYTO9 stained reconstituted spores. Left hand panel shows a maximum intensity projection (scale bar = 10 μm), boxed regions showing a spore and hyphae are enlarged in the right hand panels (scale bar = 5 μm). (C) Phagocytosis of spores of endosymbiont-containing parental strain (R.m./R.p), endosymbiont-free strain (R.p.) and reconstituted endosymbiosis (Recon.) but J774.2 macrophages. (D) Inhibition of heat-killed S. cerevisiae phagocytosis by J774.2 macrophages in media conditioned by the same strains. (E) Clearance of fungal spores by macrophages, measured by reduction in CFUs after 24 hours co-incubation. All graphs show the mean ± S.D. of 3 independent experiments. ** P<0.001, One way ANOVA with Tukey's correction for multiple comparisons.
Figure S3: Reconstitution of endosymbiosis also restores activity against amoeba, related to Figure 3 (A)
Effect of reintroduced endosymbionts on the ability of conditioned media to inhibit D. discoideum growth. Media was conditioned by the fungal spore densities for 4 hours prior to spore removal and addition to cultures of amoeba. (B) Pretreatment of D. discoideum with conditioned medium has no additional effect on phagosome proteolysis. Cells were pretreated with medium conditioned by R. microsporus FP 469-12 spores with and without endosymbionts for 30 minutes prior to addition of DQ-BSA proteolysis reporter beads. (C) Effect of media conditioned by 4 x 10^8 reconstituted endosymbiotic spores/ml on phagosomal killing, measured by survival of bacteria (GFP-K. aerogenes) after engulfment by D. discoideum.*** p<, Log-rank(Mantel-Cox) test.(D) Shows colonies of D. discoideum forming on lawns of either K. aerogenes or the isolated endosymbiotic R. pickettii strain.
Figure S4: Endosymbionts affect host gene expression and cell wall composition, related to Figure 5. (A) Volcano plot comparing RNAseq data from R. pickettii across inducing (DMEM+fungus and HL-5) vs. non-inducing (VK and DMEM) conditions. Differentially expressed genes identified in red are those with an FDR of <0.001 and Log Fold Change ±2. RPIC_RS00140 encodes the unique T1PKS. (B) Total Chitin (CFW) and Mannan (ConA) exposure in resting and swollen spores analysed by flow cytometry (>10,000 cells), including Median Fluorescence Intensity (left) and Percent High (right) for each population. Data representative of three independent repeats is shown. (C) Sporangiospore counts for FP469-12 with and without the endosymbiont. FP469-12 was treated with 60μg/ml ciprofloxacin and serially passaged two times in the presence of ciprofloxacin. Sporangiospores were collected and frozen down into stocks. Holobiont and cured spores were cultured on SDA in the dark at 25°C for 7 days and sporangiospores collected and counted by haemocytometer. Serial passage was performed 5 times, with triplicate plates.
Figure S5: Zebrafish larval infection model, related to Figure 6. (A) Representative micrographs of zebrafish larvae infected with CFW-stained swollen parent spores as analysed in Figure 6. (B) Representative images of macrophage-reporter zebrafish (mpeg1:G/U:NfsB-mCherry) showing recruitment to sites of infection with CFW-stained fungal spores. (C) Equivalent images of infected GFP-neutrophil (mpx:GFP i114)-reporter fish.
Figure S6: Effect of endosymbiont status on infection of zebrafish with swollen spores, related to Figure 6. (A) Kaplan-Meier survival curves of transgenic macrophage-reporter (Tg(mpeg1:G/U:NfsB-mCherry)) zebrafish following hindbrain injection with R. microsporus FP 469-12 spores with and without endosymbionts. (B) Equivalent survival data of neutrophil-reporter (Tg(mpx:GFP)i114) zebrafish. n=12 fish per condition, statistical differences were determined using Mantel-Cox with Bonferroni’s correction for multiple comparisons (5% family-wise significance threshold = 0.025). (C) and (D) Effect of infection with pre-swollen spores (4hr) on macrophage and neutrophil recruitment respectively. Three biological replicates of 4 fish per condition were examined (n=12). Statistical significance was assessed by Two-way ANOVA with Tukey’s correction for multiple comparisons or pairwise t-tests where sample number was unequal due to fish death. (E) Survival curves for impact of 60 μg/ml ciprofloxacin on survival of wildtype AB fish upon hindbrain infection with resting or swollen parental R. microsporus FP 469-12 spores (n=30). Injected fish were then cultivated in E3 medium with or without 60 μg/ml Ciprofloxacin and monitored for mortality (n=30). Statistical differences were determined using Mantel-Cox with Bonferroni’s correction for multiple comparisons (5% family-wise significance threshold = 0.025). (F) Shows CFU’s recovered from fish treated as in (E). No statistical significance was observed at any time point. In all panels *=p<0.05; **=p<0.001; ***=p<0.0001 unless otherwise indicated.