Ecology and Evolution of Protective Microbes

Anke Kloock

Christ Church

DPhil in Zoology

Department of Zoology, University of Oxford

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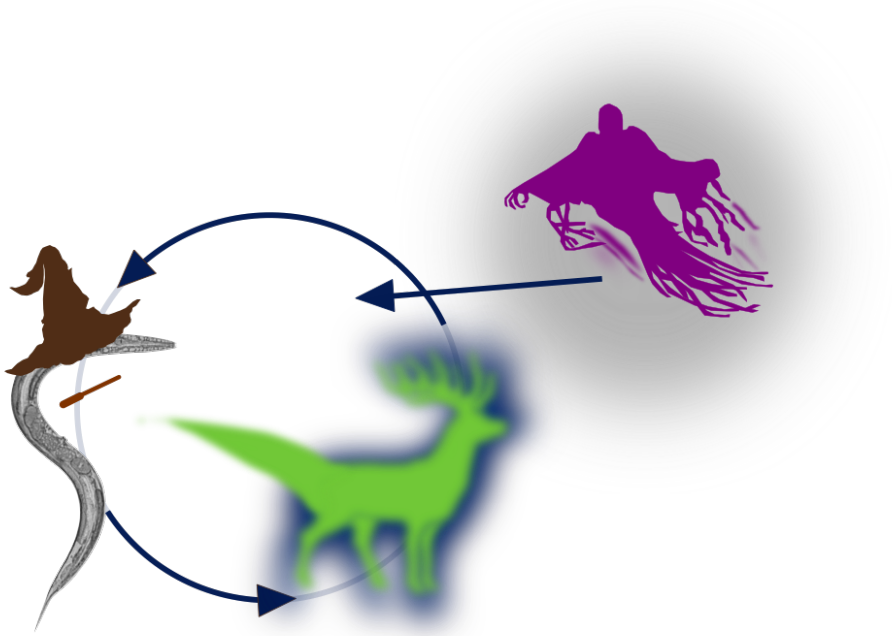

'After all this time?'

'Always!'

Albus Dumbledore and Severus Snape

Joanne K Rowling

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My time as a PhD student at Oxford has taught me one very important lesson in life: “The only stable thing in life is change”. But five offices and three labs, one building closed due to asbestos, two building relocations, a major contamination and a worldwide pandemic later, I have written up my PhD thesis nevertheless. I would not have been able to do so without the support of the people mentioned below:

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- CMD+X, CMD+C, CMD+V, CMD+Z
    - Without these commands I would have been lost! I believe my keyboard might need new keys for these particular letters but finishing with a PhD should be worth it.

Publications

Chapter 2 has been published and the publication is included in the Appendix. Chapter 3 and Chapter 4 are in preparation for submission.

Kloock, A.; Bonsall, M. B.; King, K. C. Evolution and Maintenance of Microbe-Mediated Protection under Occasional Pathogen Infection. *Ecol. Evol.* **2020**. https://doi.org/10.1101/2020.01.24.917138.

During my DPhil I have contributed to two other papers:

Pees, B.; Yang, W.; Peters, L.; Kloock, A.; Fan, L.; Petersen, C.; Zárate-Potes, A.; Schulenburg, H.; Dierking, K. Effector and regulator: Diverse immune functions of *C. elegans* C-type lectin-like domain proteins; *submitted to PLOS pathogens* (July 2020)

Rafaluk-Mohr, C.; Sealey, J.; Ekroth, A.K.E.; Aboobaker, A.; Kloock, A.; Gerth, M.; King, K.C. Host adaptation to microbe-mediated protection limits the Red Queen; *prepared for submission in PNAS*

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Abstract

Every host is colonized by a variety of microbes, some of which can protect their hosts from pathogen infection. Harbouring protective microbes (PMs) has evolutionary implications which often result in these microbes being costly for the host. Here I have investigated the evolutionary and ecological conditions under which the interaction between a host and a protective microbe are established. For this I have used populations of *Caenorhabditis elegans* worm hosts, bacteria possessing protective traits (*Enterococcus faecalis)* and pathogenic bacteria (*Staphylococcus aureus)*. I have experimentally coevolved the host and protective microbe and infected the coevolving system at different intervals and host generations. Furthermore, I have investigated how host sex and mating status affects the interaction of the host with the surrounding bacteria. More generally, to assess how host dynamics are affected by protective microbes, I constructed SIR models in the absence and presence of the protective microbe. My results indicate that even the rare presence of the pathogen is enough to drive the evolution of microbe mediated protection, and that this is independent of the interval or initial pathogen presence. I find that both sexes use the benefits of the PM to increase their reproductive success, even though females invest more in egg production and males more in mate searching behaviour. The SIR models indicate that the protective microbe stabilises host dynamics under a range of different parameters once the cost-benefit ratio is greater than one. Overall these results suggest, that protective microbes have high potential to influence and stabilize host dynamics, even though the two host sexes might benefit differently from the provided protection.

Chapter 1

General Thesis Introduction

You are never alone.

In 1877, Karl Möbius, Professor for Zoology at the University of Kiel described that oyster beds in Cacale, Marannes and Arcachon did not grow as well, as those oyster beds in the British river estuaries and in Schleswig-Holstein, Germany[1,2]. He concluded that these differences between the oyster beds were not due to the oysters themselves, but due to the species surrounding them[1,2]. Today, almost 150 years later, we know that all species are surrounded by other species and in order to understand an organism fully, its whole environment and surrounding species need to be considered[1]. This understanding of the effect of surrounding species has focused on the interaction of species with competitors, predators and mutualists[3,4]. Different forms of mutualism operate within and between species and one form of mutualistic interactions that occurs within an organism is the interaction with surrounding bacteria[5]. As these surrounding bacteria have a short generation time and large population size, they have a great potential for adaptation to the ever-changing environment of the host with several generations of microbes within one host generation[6].

However, the roles of these microbes might not always be so clearly assigned and can change depending on the environment. In 1859 Charles Darwin envisioned a "tangled bank", acknowledging that species co-exist in a complex environment with other species[3,4]. Depending on this complex environment, species interaction can range from parasitic to beneficial. A prominent microbial example for this is *Wolbachia*, which can act as a parasite in one context of species[7] or as nutritional mutualist in another context[8,9]. However, which role each species adopts is not static, but can be dynamic depending on the surrounding environment[3]. Most of the surrounding microbes shape the evolution of their host, by either being harmful[10–12] or harmless[13–15]. Some of these harmless microbes can also transfer

benefits to the host, such as the protection from infections, the latter being referred to as 'protective microbes'[6].

Harmful microbes fall into the category of parasites, which exploit host resources for their own benefit[16]. These dynamics are described under the Red Queen hypothesis, which is derived from the famous story by Lewis Carroll in which the Red Queen says to Alice "It takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"[17]. For host-parasite dynamics, this means that both the host and the parasite are under constant pressure to evolve through the dynamics of the arms race[18,19]. These arms race dynamics of host-parasite coevolution have been described extensively in the literature[20–24]. In contrast to the Red Queen dynamics for hosts and harmful microbes, the dynamics between hosts and harmless microbes are described by the Red King hypothesis. The Red King hypothesis highlights that the largest benefit is gained by the slower evolving species[25]. Examples of the microbes, that provide a benefit to their host through either protection or nutrients, can be found across kingdoms by a variety of interactions between bacteria, fungi and eukaryotes[13–15,26,27]. The presence of microbes that transfer protection to the host can influence the ecological and evolutionary dynamics between host and parasites[28,29].

Interaction between protective microbes and hosts

The interaction between hosts and protective microbes (PMs) involves a host that acquires microbes from the environment; these microbes increases host fitness relative to non-microbe harbouring conspecifics, thus making this trait more prevalent in the population[26]. A fitness increase can be achieved by either providing previously inaccessible nutrients[30], or by providing protection against parasites[6,31]. These PMs can be acquired horizontally, by

uptake from the environment, or vertically, from mother-offspring transfer[32] and may differ in efficacy (vertical transmission may be stronger[6]). Many of these vertically transmitted microbes are referred to as symbionts, as they tend to have a long coevolutionary history with the host[13–15]. Once metabolic pathways evolve to be shared between host and PM, a facultative interaction becomes obligate and can result in a stable interaction between the host and the PM[33]. Examples for obligate symbionts are pea aphids and their endosymbiotic *Buchnera*[31], the arbuscular mycorrhizal between plants and Glomeromycota fungi[34], the stinkbug and their endosymbiotic *Ishikawaella capsulata*[35], the whitefly and their endosymbiotic *Portiera*[36] or the tsetse fly with their endosymbiont *Wigglesworthia*[37]. Some of these histories between host and microbes are such long standing that their phylogenies are highly correlated, which can be found for both obligate (such as for the stinkbug and *Candidatus*[38], or the cockroach and *Blattabacterium*[39]) and facultative (such as for fig wasps and *Wolbachia*[40], *Macaranga* trees and *Crematogaster* ants[41] or for mammals and their associated gut microbiota[42]) symbionts. Studies have highlighted that the performance of the microbes can decline when transferred into a novel host environment[43,44].

Hosts can have a single obligate symbiont (such as the tsetse fly with *Wigglesworthia*[37]), or multiple species of facultative symbionts (such as for the pea aphid with *Spiroplasma*, *Rickettsia*, *Serratia* or *Regiella*[36,45–50]). In contrast, facultative symbionts can be gained and lost without apparent costs or benefit to the host, such as for fig wasps and the removal of their symbiont *Wolbachia*[51]. Any dependency of the host on the PM will favour traits that will conserve the interaction between host and PM and, potentially even favour direct vertical transmission of the PM from parent to offspring[52]. This vertical transmission is however dependent on reproduction of the host[31]. If reproductive age might not be reached due to various reasons (such as infection), horizontal transmission might be more effective[31]. Vertically transmitted symbionts can be passed on from mother to offspring and

thus persist in the population due to two main mechanisms: First, the symbiont can manipulate reproduction so that those mothers that carry a symbiont have more offspring and are overrepresented in the next generation, such as *Wolbachia* in insects, crustaceans, mites and filarial nematodes[53], or second, the symbiont increases host fitness by providing benefits to the host such as protection from infection or nutrients in the example of pea aphids[54].

The establishment and potential coevolution of a PM and a host is hypothesized to occur in two ways: (i) when microbial fitness depends on host fitness (such as in the case of obligate symbionts[31]) either through vertical transmission[55] or through selective culturing of the PM[56,57], or (ii) when it evolves a by-product of intra and interspecific interaction[58,59]. By-product interactions are established when horizontally transferred symbionts provide benefits to a host, that were originally intended for the symbiont itself[60]. As the symbionts still benefit the host, this phenomenon is referred to as by-product mutualism[61]. Overall, these horizontally transferred PMs provide a fitness benefit to the host otherwise the PM would not be expected to invade the host population in the first place[62] or persist in the host population over time[63]. Those PMs that are persisting in the population over a long time establish a close relationship with their host and the coevolved interaction of PM and host will undergo selection as one entity, as those interactions with the most beneficial and least costly properties will proportionally spread more to the next generation. Through this selection process, it is expected that selection on the host will lead to more beneficial and less costly PMs over evolutionary time[64,65].

Host defences against infection

Animal hosts are equipped with several mechanisms to reduce or avoid an infection. One of the first lines of defence is a physical barrier around the body (e.g. the outer skin for humans[66] or the cuticle for nematodes[67]). Another line of defence is pathogen avoidance behaviour, during which a host reduces the risk of infection by minimising physical contact with the source of harm[68], as is proposed for the COVID-19 pandemic by maintaining distance from surrounding people who may be carrying the virus[69]. If these two strategies fail to protect the host, hosts are furthermore equipped with an immune system, which can be activated upon infection[70]. However, this is linked to maintenance and activation costs of the immune system[70]. Costs for maintaining the immune system are constant and independent of an infection, as it involves investment in immune infrastructure[70]. Activation costs are only mounted once an infection occurs[71,72]. The presence of a PM can supplement the host's immune system[5,73,74] and potentially decrease the host's investment costs in an immune system which results in outsourcing the host's intrinsic resistance to infection[26,28,29]. How intensely the host invests in the PM or its own immunity depends on several factors: these include, the prevalence of the parasite (higher parasite prevalence is hypothesized to select for extrinsic and intrinsic resistance[75]), the costs of fighting an infection with the host's own immunity, and the cost over benefit ratio of the PM[75]. The costs of the PM can be of a constitutive set up, as the host provides services to the PM throughout[63] independent of an infection, but can also decrease host survival and reproduction as found for mosquitoes and *Wolbachia*[76–81] or pea aphids and different symbionts, such as *Hamiltonella*[50,82–85], *Regiella*[50,82], *Serratia symbiotica*[84], or X-type symbiont[83]. The costs of resistance to one threat can however also result in the susceptibility to another threat[82,83]. If the costs of the PM are higher than the benefits transferred to the host, the PM can evolve to be a parasite[86]. As such the evolutionary dynamics of PMs can

be highly dependent on these costs and benefits associated with microbe-mediated versus host-encoded resistance to parasites[29]. However, even if costs for harbouring the PM are not too high and the PM obtains basal reproduction without harming the host[87,88], the interaction with the host might be stable across evolutionary time[87,88]. When the costs of the PM are higher, these can also be ameliorated by the presence of a second symbiont[89] or a well-provisioned host[70]. The interaction between the host and the PM is hypothesized to be dependent on parasite prevalence[32,44,55,90,91], which can act as a high selection pressure on the interaction and drive coevolution. The absence of such selection pressure has been hypothesized to result in decline of the spread of PMs, particularly if the PM is costly[632,85,92].

The benefit a PM can provide to the host varies. The benefits can range from providing nutrients[93,94] or provisioning protection against parasites[6]. Nutrient provision by PMs has been shown for plant-sap-sucking aphids[45] or blood-feeding tsetse flies[2395]. In these cases, the PM provides the host with essential amino acids and vitamins, without which the host would not be able to survive[45,95]. During the provision of protection, a third species is involved, which shapes the interaction: a parasite. The interaction between the PM and the parasite can be direct[59,96–98] or indirect, through involvement of the host's immune system[98,99] or via a behavioural change of the host[83,96,97,100]. Direct interaction between the PM and the pathogen and thus protection for the host can be achieved by competing for the same resources[59] or via toxin production[101]. Indirect interaction can be established by a stimulation of the hosts immune system by the PM[96,97,101]. A change in host behaviour can consist of less defensive behaviour in the presence of a PM, such as shown in the aphid system[83]. This behaviour then leads to higher susceptibility to predation[83].

Nature is rarely homogenous

Resistance to one predator as a trade-off to higher susceptibility to another, as observed in the pea aphid[83], shows the complex system in which interactions are imbedded in in nature[3]. A specific set of two species interacts with many other species and depending on the context this interaction can be positive or negative[3]. In nature it can thus be difficult to isolate certain species interactions, as coinfection with several species is likely . To be able to understand such complex interactions, researchers have brought these complex interactions into the lab to simplify them. This introduction of homogeneous conditions in the laboratory reduces any unwanted effects and helps to identify the specific pattern. Nature however is heterogeneous[103] and can vary across space[104] and time (such as during seasonal epidemics[105]). Furthermore, abiotic (such as oxygen concentration[106], resource availability[107–109], environmental productivity[110] or rainfall[111]) and biotic factors (such as parasite presence[112], parasite species[113,114] or contact between hosts[115]) may vary within and between host populations. PMs themselves can also be variable: facultative symbionts have not been observed in every host in a population, even if most of the population carries a PM[36,116]. Furthermore, within one host population different strains of the same PM species have also been observed[116,117]. The effect a PM can have on individual hosts can also vary, depending on the host sex[118]. This heterogeneity is a central theme through my thesis, in which I have investigated temporal pathogen heterogeneity in evolutionary interactions, and the heterogeneity of sex in ecological interactions.

The model system *Caenorhabditis elegans*, *Staphylococcus aureus* and *Enterococcus faecalis*

In this DPhil thesis I advance our understanding of heterogeneity in the context of PMs. For the practical part of this thesis I have used *Caenorhabditis elegans*, which is an established model in various fields of science including developmental biology[119], neurobiology[120], innate immunity[121–124], and experimental evolution[23,125–128]. *Caenorhabditis elegans* provides several advantages that make it a well-suited model organism for this project. *Caenorhabditis elegans* has been isolated from microbe-rich environments[129], where it feeds on microbes, some of which have now also been described as its microbiome[130–132]. This surrounding bacterial community of *C. elegans* can be characterized as beneficial, commensal or parasitic[132]. Due to this close interaction with microbes, *C. elegans* has been used to describe aspects of microbe-mediated protection[98,126,127,133]. In nature it can be found as a self-fertilising hermaphrodite[134], with only few males (<0.5%)[135]. In my DPhil project I have used a strain not found in nature (line EEVD00), which was generated by Henrique Teotonio in Paris[136]. This line consists of females and males, where the females are hermaphrodites and are genetically engineered to not produce sperm (due to the *fog-2(q71)* mutation[136]). This lineage encompasses the genetic diversity of 16 natural worm isolates and has high standing genetic variation[137]. This lack of self-sperm production enforces outcrossing and maintains the high genetic variation over time. Due to the selection pressure applied to these worm populations during experimental evolution, I select certain genotypes over others, rather than waiting for novel mutations to arise. Other studies using *C. elegans* hermaphrodites and males for experimental evolution[23,128,138,139], found that male frequency varied over time[128,138,139]. Whether this frequency of males declines[128] or increases[138,139] depends on the ability of males to cope with an infection of the specific pathogen[128]. As

hermaphrodites and males are differently affected by pathogenic infection, I used a population with a stable sex ratio.

In culture, this nematode is routinely reared on Nematode Growth Medium (NGM) inoculated with *Escherichia coli* OP50[134]. Due to a stock contamination and the realisation of such after the completion of the practical work, NGM plates in my projects were inoculated with *Salmonella*. Here, *Salmonella* occurred as a biofilm (personal observation), which was shown to persist in the worm gut[140], to not affect *C. elegans* lifespan[139] and support worm growth and development[141].

For pathogenic infection, the Gram positive *Staphylococcus aureus* strain MSSA476[142] was used, which kills worms within five days by lysing the intestinal cells lining the gut wall[143]. *Staphylococcus aureus* is known as an opportunistic pathogen across the animal kingdom[144] including humans[142].

The strain OG1RF of *Enterococcus faecalis*[145] was used as a PM against *S. aureus* infection. The effect of this bacteria as a PM has been previously shown in worm hosts[126,127]. It protects the worm by scavenging siderophores from *S. aureus*[133]. If *E. faecalis* and *S. aureus* are coevolving, the scavenging behaviour of *E. faecalis* leads to a reduction in the virulence of *S. aureus*[133]. In my set-up *E. faecalis* has a low mortality[112].

Both bacterial strains are known to co-occur in animal microbiomes[142,146–149]. The exact strains I used here, *S. aureus* MSA4476 and *E. faecalis* OG1RF, were both isolated from humans and are unlikely to have had contact with *C. elegans* prior to these experiments[145]. The evolutionary changes observed in this project are thus novel and cannot stem from any previous contact. It furthermore allows for the exploration of the initial stages of a defensive mutualism.

Scope of this thesis

My thesis focuses on the ecology and evolution of protective microbes, which consists of two experimental chapters and one theoretical chapter. The two experimental chapters focus on heterogeneity in the system, either in the context of pathogen infection or in the context of the sex and mating status of the host. The theoretical chapter focuses on exploring host dynamics in the absence and presence of a PM.

Chapter 1 is this introduction which serves as a general overview of the relevant literature in this field.

In **Chapter 2** I use experimental evolution to test for the effect of pathogen heterogeneity on the coevolution of the host and the PM. After 20 host generations I found, that infrequent pathogen infection throughout the evolution experiment is sufficient to drive the evolution of microbe-mediated protection, and that this is independent of the interval of pathogen infection or the initial presence of the pathogen.

During data collection for Chapter 2, I observed, that female and male worms behaved differently in the presence of the PM. This observation led to **Chapter 3** in which I have assessed the difference between females and males in the presence and absence of the PM. I found that females and males both benefit from the presence of the PM and use this benefit to invest more energy in reproduction. However, females and males use different ways to invest in offspring reproduction in the presence of the PM; females invest more in egg production, while males invest more in mate searching behaviour.

In **Chapter 4** I used a mathematical modelling approach to explore host dynamics in the presence and absence of PMs. I investigate how PMs affect dynamics when hosts pass from being susceptible (S) to infected (I) to recovered (R) states. Under different cost and benefit

ratios, I found that the dynamics are likely to be most stable when the costs are higher than the benefits and the number of infected and susceptible hosts is sufficiently large.

Chapter 5 summarizes the findings of this thesis, discusses the caveats and benefits of this study and highlights areas for future research.

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Chapter 2

Evolution and Maintenance of Microbe-Mediated Protection under Occasional Pathogen Infection

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Abstract

Every host is colonized by a variety of microbes, some of which can protect their hosts from pathogen infection. However, pathogen presence naturally varies over time in nature, such as in the case of seasonal epidemics. I experimentally coevolved populations of *Caenorhabditis elegans* worm hosts with bacteria possessing protective traits (*Enterococcus faecalis*), in treatments varying the infection frequency with pathogenic *Staphylococcus aureus* every host generation, alternating host generations, every fifth host generation or never. I additionally investigated the effect of initial pathogen presence at the formation of the defensive symbiosis. My results show that enhanced microbe-mediated protection evolved during host-protective microbe coevolution when faced with rare infections by a pathogen. Initial pathogen presence had no effect on the evolutionary outcome of microbe-mediated protection. I also found that protection was only effective at preventing mortality during the time of pathogen infection. Overall, my results suggest that resident microbes can be a form of transgenerational immunity against rare pathogen infection.

Introduction

In nature, all plants and animals are colonised by microbes[1–3]. The composition of these microbial communities is highly diverse and includes harmful, neutral, and beneficial microbial species[1], including those that can be important players in host defence against parasites, a phenomenon referred to as 'defensive mutualism'[4,5]. Recognised for over a century, defensive mutualism has been observed in plants[6] and in a range of animals[7–10], including humans[1,11,12] wherein microbes can supplement host immune systems[13–15].

The net benefits of defensive mutualism are dependent upon the presence of pathogens[16–18]. Whilst hosts can benefit from microbe-mediated protection, defensive symbionts can be less beneficial to the host in the absence of enemies, due to metabolic and physiological costs[4]. For example, in the interaction of aphids and the bacterium *Hamiltonella defensa*, the host tissue is harmed by defensive toxins that protect against infection from parasitoids[19]. In some cases, possessing protective microbes might be more beneficial to the host than investing in its own immune system[20]. From the perspective of the symbiont, it is most useful to its host under high pathogen prevalence, and thus can persist in the host population[21]. Nevertheless, a stable symbiotic interaction is hypothesized to be evolved and maintained[22] only when the host benefit of carrying defensive symbionts outweighs any costs. The interactions of obligate and defensive symbionts and hosts can be stable for millions of years[23].

Not all environments are constantly pathogen rich which might shift the balance of costs and benefits during defensive mutualisms, particularly during coevolutionary interactions[17]. Pathogen prevalence can be spatially[24] or temporally variable, the latter in the case of seasonal epidemics (e.g., flu peaks each winter in the northern hemisphere[25] or rabies in North American skunks which peaks in Autumn[26]). Different environmental factors can

influence disease transmission such as an increase in malaria risk in warmer regions after rainfall[27], or an increase in contact rate and thus higher flu infection rate during the winter months[28]. The impact of other temporally heterogeneous factors on the strength and direction of selection on species interactions have been explored (oxygen concentration[29], resource availability[30–32], environmental productivity[33]). Whether the varied presence of pathogens can similarly alter selection for symbiotic interactions has been explored theoretically[34], but remains to be empirically tested.

Here, I examined the impact of temporal variation in pathogen infection on the evolution of microbe-mediated protection. I used *Caenorhabditis elegans* as a worm host and allowed it to be colonised by a bacterium (*Enterococcus faecalis*) that protects against infection by *Staphylococcus aureus*[35]. *Enterococcus faecalis* has been shown to be protective across animal microbiomes[36,37]. It has been previously shown that *E. faecalis* can evolve to provide enhanced protection when residing in *C. elegans* hosts during constant pathogen infection[35,38]. From this, I predict that variation in pathogen infection might limit the evolution of microbe-mediated protection. In the present study, I experimentally co-passaged *C. elegans* with protective *E. faecalis* and infected the host with evolutionary static pathogenic *S. aureus* at different intervals of host evolution. I also examined whether pathogen presence at the initial formation of the coevolving interaction is crucial to the evolution of protection. I show that enhanced microbe-mediated protection emerged out of novel coevolutionary host-microbe interactions and during pathogen infection, regardless of its temporal variability or the time point of first infection. Enhanced protection was only effective during pathogen infection. If hosts survived infection, they could recover and had the same longevity and reproductive output across treatments. These results thus suggest that even occasional pathogen infection can select for defensive mutualism, revealing the potential for this phenomenon to be widespread in nature.

Materials and Methods

Worm host and bacteria system

As a bacteriovore, *Caenorhabditis elegans* interacts constantly with a variety of bacteria either by feeding or hosting them[39–41]. Consequently, *C. elegans* is an established model for studying innate immunity[42], as it can be infected with its natural[41,43] as well as opportunistic pathogens[40,44]. Most pathogens are taken up orally by the worm[45], and some can proliferate and colonize the worm gut[35,38].

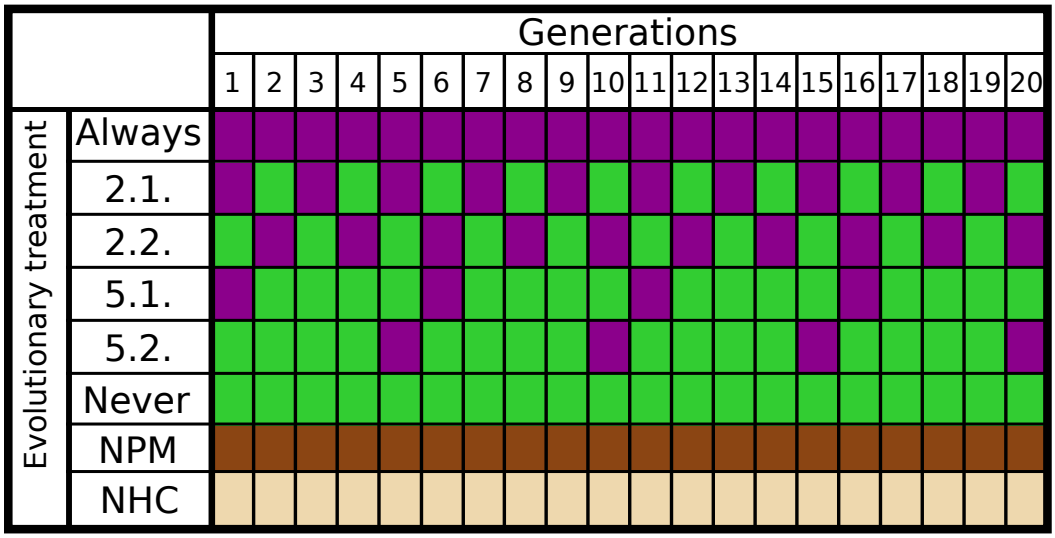
Naturally, *C. elegans* is a self-fertilising hermaphrodite[46], but in this experiment obligate outcrossing worm populations (line EEVD00) with males and females (hermaphrodites that carry the *fog-2(q71)* mutation) were used[47]. This lineage was generated by Henrique Teotonio (ENS Paris) and encompasses the genetic diversity of 16 natural worm isolates[47]. Worms were kept on Nematode Growth Medium (NGM), inoculated with *Salmonella*, hereafter referred to as food. Worms were infected with the pathogenic *S. aureus* (MSSA476)[48], which is virulent and kills worm hosts by lysing the intestinal cells lining the gut wall[49]. Worms were exposed to *E. faecalis* (OG1RF)[40], which was isolated from the human digestive system, but has been previously shown to colonize and proliferate in the worm gut[35,38,50], where it provides protection.

Experimental evolution - Design

Six single clones of *E. faecalis* (one for each of the six replicate populations) and a single population of *C. elegans* were the ancestors (hereafter referred to as the Ancestor) for all evolving populations. To account for potential differences in virulence, a stock of four

clones of *S. aureus* was used for pathogen infections. Both *C. elegans* and colonising *E. faecalis* were allowed to evolve in presence of each other, while *S. aureus* was kept evolutionarily static. Infection with *S. aureus* was varied over host evolutionary time (indicated by purple in Table 1) to represent temporal heterogeneity in pathogen infection, including a range from always to every 2nd generation, every 5th generation, and never (Table 1). Moreover, I included differences in whether pathogens were present at the initial formation of the symbiotic interaction or later (2.1. vs. 2.2., and 5.1. vs. 5.2. in Table 1). Controls for lab adaptation were maintained for the host (No Protective-Microbe control, NPM in Table 1) and *E. faecalis* (No Host Control, NHC in Table 1).

Table 1: Experimental procedure for the evolution experiment. Columns indicate the number of experimental host generations (1- 20), while rows show the eight treatments. Host generations were infected with S. aureus (purple) or given food (green), while constantly coevolving with E. faecalis. Two controls for lab effects on host evolution (dark brown, No Protective Microbe, NPM) and E. faecalis evolution (light brown, No Host Control, NHC) were also included, where the NPM treatment was only ever exposed to food alone. Each evolutionary treatment consisted of six independent evolutionary replicates.



Experimental evolution - Culturing and passaging methods

At the start of each generation, worms were bleached as described previously and left in M9 buffer overnight for larvae to hatch[51]. Simultaneously, *E. faecalis* clones were cultured overnight in Todd-Hewitt Broth (THB) in 600µl at 30ºC, while food was cultured overnight in LB broth. Subsequently, 9cm NGM plates were inoculated with 300µl of each overnight culture. Plates with freshly inoculated bacteria were dried at room temperature before approximately 1000 L1 worms were added to each NGM plate. After these plates dried at room temperature, they were transferred to a 20ºC incubator and left for 48h. Simultaneously, a liquid culture of *S. aureus* was grown in THB from frozen stock, while a liquid culture of food was grown in LB, and both were incubated under shaking conditions at 30ºC. The following day, 100µl of each overnight culture were spread on 9cm plates, *S. aureus* on Tryptone Soy Broth agar (TSB) plates and food on NGM plates and incubated at 30ºC overnight. To transfer worms to the pathogen or food plates, nematodes were washed off the *E. faecalis* plates with M9 buffer and washed three times over small-pore filters to remove all externally attached bacteria, as previously described[38,52,53]. Worms were infected with either *S. aureus* or exposed to food (Table 1) and left at 25ºC for 24h. After this time, worms were then washed off the plates with M9 buffer once more to plate them on NGM plates seeded with food for laying eggs. Roughly 10% of these worms were crushed and plated on *E. faecalis* selective medium (TSB + 100mg/ml Rifampicin). The remaining worms were left on food plates for 48h to allow for egg laying.

To passage *E. faecalis*, roughly 100 *E. faecalis* colonies were picked and grown up shaking overnight in 600µl THB at 30ºC, while worms were bleached and left to hatch overnight. This cycle was repeated for 20 experimental host generations.

All passaged worms and *E. faecalis* samples were cryopreserved at -80 ºC. A proportion of the offspring of surviving worms were frozen in 40% DMSO, and 100µl of *E. faecalis* liquid culture was mixed with 100µl of glycerol before cryopreservation.

Host survival and fecundity assays

All assays were conducted at the end of the evolution experiment on archived samples. Plates were randomized and fully encoded during each experiment to ensure the experimenter was blind to different treatments whilst collecting data.

Basic procedures were adopted from the experimental evolution, but with the following alterations to keep the assays feasible with higher accuracy when scoring dead and alive worms: 400 L1 worms were exposed to 200µl of food and *E. faecalis* on 6cm NGM plates, while 60µl *S. aureus* overnight culture was used to inoculate 6cm TSB plates.

To assess microbe-mediated protection of different combinations of worms and *E. faecalis*, 400 L1s were exposed to 50:50 mixtures of *E. faecalis* and food for 48h. Worms were then washed off these plates as described above and infected with *S. aureus* for 24h at 25°C. Survival in form of counting dead and alive worms was then scored.

To assess any long-term fitness consequences after protective microbe exposure and pathogen infection, long-term survival and fecundity were measured. Worms were exposed as described for the survival assays. Subsequently, five females and five males were picked onto 3cm food seeded NGM plates at 25°C and then transferred to new plates every 36h to avoid any confusion between offspring produced and original adults. At each time point, survival was scored. To measure fecundity, the number of worm eggs on the plates at 120h since bleaching were counted.

Statistical Analysis

Statistical analyses were carried out with RStudio (Version 1.1.463 for Mac)[54], graphs created with the ggplot2 package (Version 2.1.0)[55] and edited with Inkscape (Version 0.91)[56]. All host survival and fecundity data were analysed with nested binomial mixed effects models (R package lme4)[57], followed by a Tukey multiple-comparison tests (R package multcomp)[58]. Life-span data were analysed with Kaplan Meier Log Rank[59,60] test with FDR correction for multiple testing.

Results

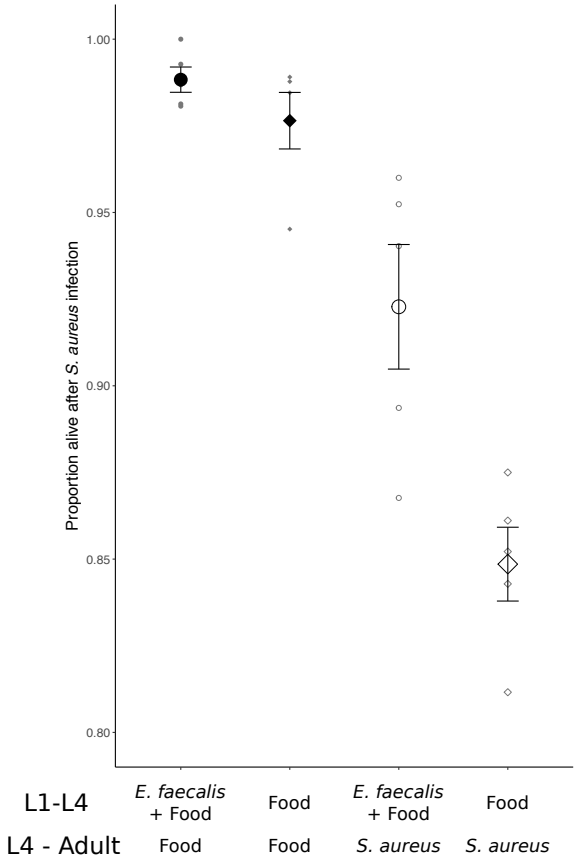


Figure 1: **Host survival showing protective effects of *E. faecalis***. Early exposure of worms to *E. faecalis* (both ancestors) provides some degree of protection from the infection of *S. aureus*. 24h host survival levels reveal a benefit to *E. faecalis* colonisation independent of pathogen presence or absence. Circles indicate those treatment being exposed to *E. faecalis* and food in the earlier stage (L1-L4), while squares indicate food alone treatment in the earlier stage (L1-L4). Filled symbols indicate those treatments being exposed to food in the later stage, while open symbols indicate those treatments being exposed to the pathogen *S. aureus* in the later stage. Each symbol indicates the mean ± S.E of five replicates. Axis scales were chosen to be the same across all plots.

Before the start of the evolution experiment, the starting conditions were tested. Confirming previous results, *E. faecalis* showed some spontaneous host-protective potential against *S. aureus*. Worms raised on *E. faecalis* and food survived better than those raised on food alone, independent of food or pathogen present at the later stage (General Linear Model, X^2=10.205, df=1, p=0.001; Figure 1). Worms infected with *S. aureus* in later life survived worse than those being exposed to food (General Linear Model, X^2=119.643, df=1,

p<0.001; Figure 1). These results demonstrate the beneficial and protective effects for the host after exposure to the protective microbe *E. faecalis*.

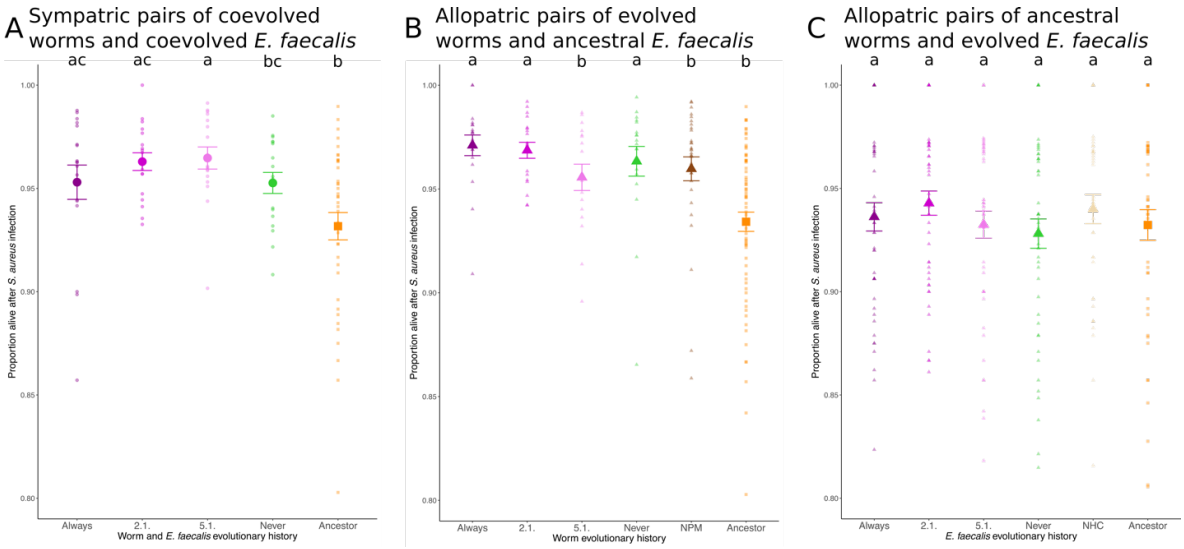


Figure 2: **Host survival for coevolving sympatric and allopatric pairs of worms and *E. faecalis***. Microbe-mediated protection was assessed for (A) sympatric pairs of coevolved worms and *E. faecalis,* (B) allopatric pairs of evolved worms and ancestral *E. faecalis*, and (C) allopatric pairs of ancestral worms and evolved *E. faecalis*. Bigger symbols represent mean ± S.E. and consists of six biological replicates and four technical replicates. Smaller symbols indicate the data distribution. Circles indicate sympatric pairs of coevolved *E. faecalis* and worms, squares indicate ancestral pairs of *E. faecalis* and worms and triangles indicate allopatric pairs of *E. faecalis* and worms. Letters indicate results of a GLMM, followed by a Tukey Post-hoc Test. The same letter indicates no significant difference. Axis scales were chosen to be the same across all plots.

Infection with *S. aureus* over evolutionary time in the evolution experiment led to the substantial enhancement of microbe-mediated protection, with the evolutionary background of the sympatric pair of host and *E. faecalis* having a significant impact on host survival (Mixed Effects Model, X^2=42.479, df=4, p<0.001; Figure 2A). Higher microbe-mediated protection in comparison to the Ancestor occurred in all evolutionary histories involving pathogen presence across the temporal heterogeneity treatments in my evolution experiment (Always, 2.1. and 5.1.). However, this did not occur in the pathogen absence (Never) treatment. Host evolutionary history alone had a significant effect on host survival (Mixed Effects Model, X^2=35.779, df=5, p<0.001; Figure 2B), but did not reveal the same pattern as for sympatric pairs. No effect of bacteria evolutionary history alone on infected host survival was observed (Mixed Effects Model, X^2=3.2511, df=5, p=0.6613; Figure 2C).

Taken together, enhanced microbe-mediated protection evolved only as a product of coevolution and pathogen presence for sympatric pairs; this occurred regardless of the temporal heterogeneity.

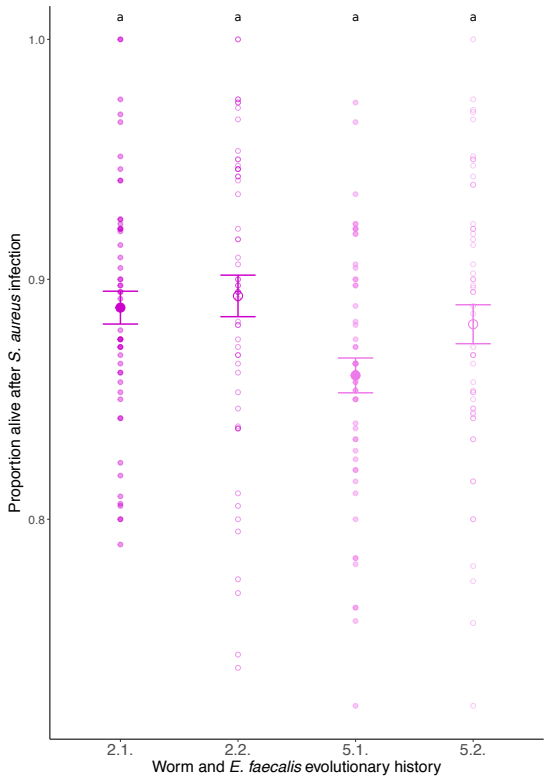


Figure 3: **Host survival in evolutionary treatments differing in initial pathogen exposure time points**. The time point of initial infection varied for infection to the pathogen every two generations (2.1. and 2.2) or every five generations (5.1. or 5.2.) but does not influence the outcome. Closed symbols indicate initial pathogen presence (Host generation 1), open symbols indicate later pathogen presence (Generation 2 for 2.1. and 2.2. and Generation 5 for 5.1. and 5.2.). Bigger symbols represent mean ± S.E and consists of six biological replicates and four technical replicates of the sympatric pairs. Smaller symbols indicate the data distribution. Letters indicate results of a GLMM, followed by a Tukey Post-hoc Test. The same letter indicates no significant difference. Axis scales were chosen to be the same across all plots.

As an additional form of pathogen heterogeneity, the impact of the timing of initial pathogen infection on the evolution of microbe-mediated protection was investigated. An effect of different initial pathogen infection time points on host survival following pathogen infection was observed (Mixed Effects Model: X^2=7.945, df=3, p=0.04716 Figure 3), although a Tukey Post-Hoc test revealed no significant differences (Table A1).

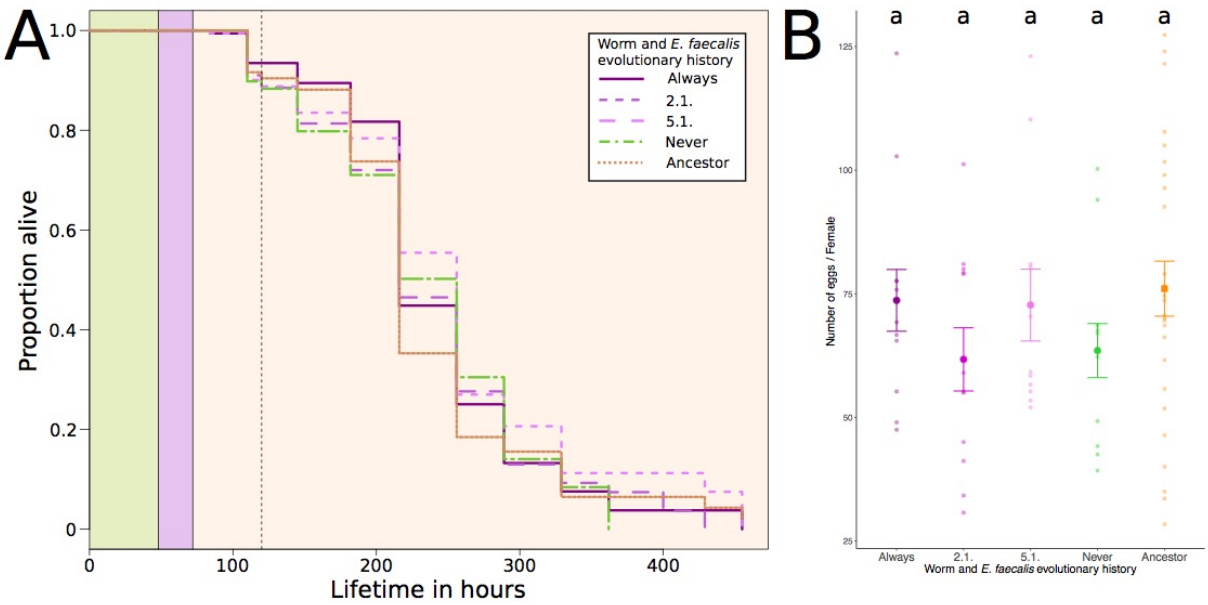


Figure 4: **Long-term survival and fecundity of *E. faecalis*-colonised hosts that survived pathogen infection**. (A) Long-term host survival was measured. Survival curves for sympatric pairs of worms and *E. faecalis* are shown as Kaplan-Meier estimates. Worms were exposed to *E. faecalis* and food (green), then to *S. aureus* (purple), and long-term survival was monitored on food (orange). The dotted line indicates the time point at which fecundity was measured. (B) Number of eggs/Female across sympatric pairs of coevolved worms and *E. faecalis*. Bigger symbols represent mean ± S.E. and consists of six biological replicates and four technical replicates. Smaller symbols indicate the data distribution. Circles indicate sympatric pairs of coevolved *E. faecalis* and worms, squares indicate ancestral pairs of *E. faecalis* and worms. Letters indicate results of a GLMM, followed by a Tukey Post-hoc Test. The same letter indicates no significant difference.

Furthermore, I investigated the long-term consequences to hosts colonised by *E. faecalis* after 24h of pathogen infection. No significant differences were observed in the long-term survival post-infection of worm hosts colonised by their sympatric *E. faecalis* across treatments (Kaplan Meier Log Rank Test, FDR corrected, all comparisons $p>0.05$, Figure 4A). In addition, I did not find significant differences in fecundity among sympatric host-*E. faecalis* pairs (Mixed Effects Model, $X^2=3.9418$, df=4, $p=0.4278$, Figure 4B).

Discussion

It has been shown that hosts receive the greatest benefits from protective microbes under constant pathogen infection. I hypothesized that variation in pathogen presence over time would limit the evolution of microbe-mediated protection due to the reduced benefits to the host and bacterial symbiont. In my study, enhanced pathogen defence emerged out of host-symbiont coevolutionary interactions only when pathogens were present, independent of the interval or initial presence of the pathogen. Notably, the ultimate strength of microbe-mediated protection that evolved was not impacted by the number of host generations between pathogen infections, the proportion of generations infected, or the presence of the pathogen at the first host-microbe interaction. These results suggest that resident microbes can be a form of transgenerational immunity against rare pathogen infections.

I found that microbe-mediated protection is maintained even in the prolonged absence of pathogen, but that pathogen presence is necessary for microbe-mediated protection to evolve, as previously hypothesized[16–18]. This result is unlike previous work showing that the scale of heterogeneity in abiotic conditions can affect the strength of selection for traits in some symbiotic interactions[33]. This discrepancy is potentially due to costs in my symbiotic system being ameliorated (at least in terms of host survival) in well-provisioned hosts, as hosts are provided with food alongside *E. faecalis* and are thus rescued from starvation (also see[61]). Although protective symbionts can incur costs (e.g.,[19]) for their hosts, with potential for impacts on coevolutionary interactions[17], it is possible that potential costs of bacterial colonisation might be only detectable when hosts are stressed[62] or that the costs were not strong enough for me to detect[63]. Different measures of cost remain to be explored (e.g. lifespan in the complete absence of a protective microbe and a pathogen).

Higher protection also does not always come with higher costs, as found in the black bean aphid-*Hamiltonella defensa* interaction[64]. Thus, protective traits in an organism's commensal microbiota could be selected for under pathogen infection and easily maintained in subsequent uninfected generations.

Microbe-mediated protection was strongest between sympatric pairs when pathogens were present over evolutionary time, consistent with previous findings[38]. In my study, protection emerged during coevolution after only 20 host generations, and not due to the independent evolution of either interacting species, but due to the coevolution of both species[17]. The time-scale of these interactions is short compared to the longer shared evolutionary histories shared by other defensive mutualisms[65–67]. Nevertheless, my findings reveal the potential for microbe-mediated protection to become enhanced during the formation of a coevolving host-microbiota relationship.

In conclusion, my results show that enhanced protection in host-microbe interactions can rapidly evolve and be maintained even under infrequent pathogen infection, suggesting that resident microbes can be a form of stable, transgenerational immunity. The protective benefit of an organism's microbiota might remain undetected for several host generations until pathogens re-emerge. Future research on the failure of pathogens to transmit within host populations should consider the contribution of the protective microbiota to prevent disease spread.

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Appendix

Experimental Procedure

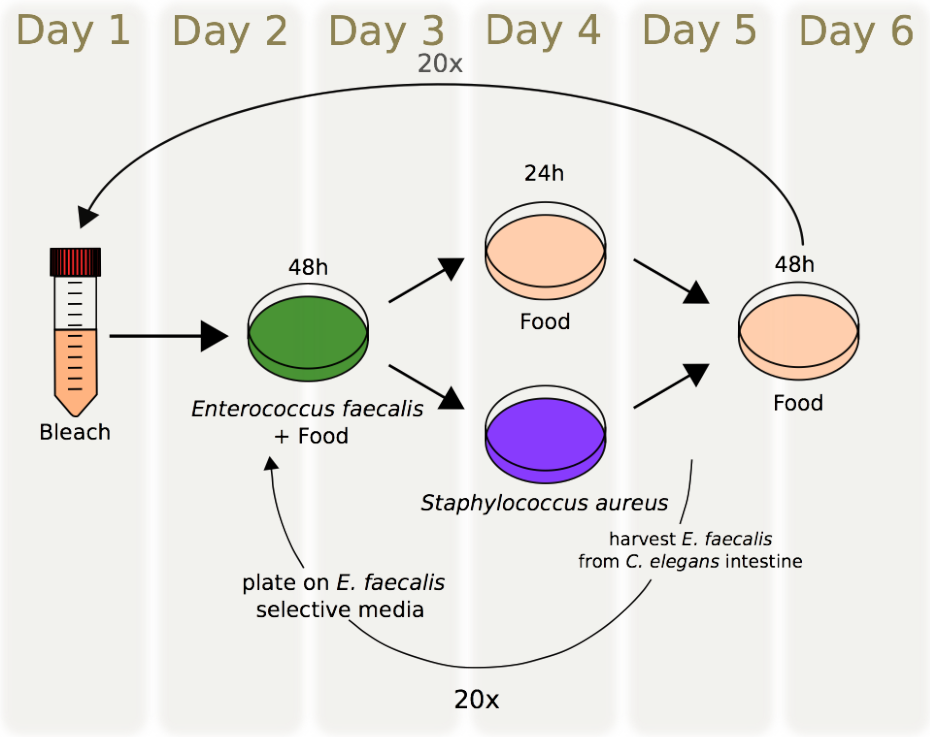

Figure A1: The experimental procedure of the evolution experiment in detail.

The procedure of the evolution experiment can be seen in Figure S1 and will be the following:

1. On the first day of each generation, worms were bleached[51]. During this step worms were removed from the plates by washing with M9 buffer. Afterwards a 50:50 mixture of 10% NaClO and 5M NaOH was used to bleach all the remaining bacteria in the solution and to release eggs from bleached adult worms. Only eggs survive this step and were then left in the M9 buffer over-night. All bleached eggs hatched overnight, but do not develop any further and thus lead to increased synchronisation for each worm population. Every bleach solution was plated out on a TSA plate to control for carried over contaminations. 100 colonies of *E. faecalis* were picked from the plates that were grown on *E. faecalis* selective medium for 48 hours at 30°C. These colonies were picked into 600µl of THB and then grown up over-night. Simultaneously, a single colony of *Salmonella* food was picked and added to 25ml of LB broth to be grown under shaking conditions over night.
2. On the second day of each generation, the overnight shaken and hatched worms were exposed to 600µl of a 50:50 mixture of *E. faecalis* and food. At this step the population size was adjusted to only contain 1000 individuals. Worms remained on these plates with *E. faecalis* for 48 hours.
3. On the third day of each generation, TSA plates to which worms were going to be exposed on day 4 were inoculated with 100µl of either *S. aureus* or food and were incubated at 30°C overnight.

4. On the fourth day of each generation, worms were washed off the plates seeded with *E. faecalis* by filter tip washing. For this purpose, worms were washed off the plates with twice 1.5ml of M9 buffer +1% Triton X100, as previously described[38,52,53].This worm and bacteria suspension was spun down for 1 minute, 1 ml of the supernatant was discarded and the rest of the pellet was pipetted on the top of a filter of a filter tip and spun down for 3 min. Worms were left on the top of the filter were washed with 400µl of M9 three times, before being re-suspended in 100µl of M9 to bring onto plate. During this method, most of the externally attached bacteria are washed off the worms to ensure that worm survival can be attributed to gut colonization of *E. faecalis* and not external attachment of the protective microbe. *Enterococcus faecalis* remains in the worm's intestine and will establish a protective effect. After worms were transferred to the plates containing either *S. aureus* or food, all plates were moved to 25°C.
5. On the fifth day of each generation, worms were washed off the plates again by filter tip washing (as described for day 4 and previously [38,52,53]). Worms were left on plates seeded with food at 20°C for 48 hours to lay eggs. The amount of transferred bacteria (either *S. aureus* or food can be neglected to have any influence on the further development of the worms. These plates were then used for bleaching on day 1 of the following generation. 10% of the worm mixture was separated and used to isolate *E. faecalis*. For this purpose, the suspension of worms was crushed and then plated on TSA plates with Rifampicin, as the
E. faecalis strain carries a Rifampicin resistance. The plated gut content was allowed to grow at 30°C for 48 hours.

Statistical results

Table A1: All statistical results summarized, including the statistical test, the specifics associated with each test, the relevant degrees of freedom and p-values.

<table>
<tr><th>Figure</th><th>Results</th></tr>
<tr><td>1</td><td>Binomial GLM between the bacterial diets
<table>
<tr><th>Comparison</th><th>X^2</th><th>Degrees of Freedom</th><th>p-value</th></tr>
<tr><td>L1-L4 stage</td><td>10.205</td><td>1</td><td>0.001401</td></tr>
<tr><td>L4 – Adult</td><td>119.643</td><td>1</td><td>$<2.2 \times 10^{-16}$</td></tr>
<tr><td>Interaction</td><td>0.053</td><td>1</td><td>0.871</td></tr>
</table></td></tr>
<tr><td>2A</td><td>Binomial GLM
Evolution effect: X^2: 42.479, Df=4, $p=1.327 \times 10^{-8}$
Tukey Post-hoc comparisons:
<table>
<tr><th>Comparison</th><th>Estimate Std.</th><th>Error</th><th>z-value</th><th>p-value</th></tr>
<tr><td>Always – Ancestor</td><td>-0.5490</td><td>0.1354</td><td>-4.055</td><td><0.001</td></tr>
<tr><td>2.1. - Ancestor</td><td>-0.5902</td><td>0.1348</td><td>-4.378</td><td><0.001</td></tr>
<tr><td>5.1. – Ancestor</td><td>-0.8224</td><td>0.1448</td><td>-5.680</td><td><0.001</td></tr>
<tr><td>Never – Ancestor</td><td>-0.3174</td><td>0.1290</td><td>-2.460</td><td>0.0985</td></tr>
</table></td></tr>
<tr><td>2B</td><td>Binomial GLM
Evolution effect: X^2: 35.779, Df=5, $p=1.051 \times 10^{-6}$
Tukey Post-hoc comparisons:
<table>
<tr><th>Comparison</th><th>Estimate Std.</th><th>Error</th><th>z-value</th><th>p-value</th></tr>
<tr><td>Always – Ancestor</td><td>-0.92335</td><td>0.19399</td><td>-4.760</td><td><0.001</td></tr>
<tr><td>2.1. - Ancestor</td><td>-0.84208</td><td>0.18860</td><td>-4.465</td><td><0.001</td></tr>
<tr><td>5.1. – Ancestor</td><td>-0.49494</td><td>0.18527</td><td>-2.671</td><td>0.0791</td></tr>
<tr><td>Never – Ancestor</td><td>-0.78893</td><td>0.19395</td><td>-4.068</td><td><0.001</td></tr>
<tr><td>NPM – Ancestor</td><td>-0.52794</td><td>0.18847</td><td>-2.801</td><td>0.0560</td></tr>
</table></td></tr>
<tr><td>2C</td><td>Binomial GLM</td></tr>
</table>

<table>
<tr><td></td><td>Evolution effect: X^2: 3.2511, Df=5, p= 0.6613</td></tr>
<tr><td>3</td><td>Binomial GLM
Evolution effect: X^2: 7.945, Df=3, p= 0.04716

<table>
<tr><th>Comparison</th><th>Estimate Std.</th><th>Error</th><th>z value</th><th>p-value</th></tr>
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<tr><td>5.1. - 2.1.</td><td>-0.26298</td><td>0.12387</td><td>-2.123</td><td>0.1414</td></tr>
<tr><td>5.2. - 2.1.</td><td>-0.07281</td><td>0.12634</td><td>-0.576</td><td>0.9373</td></tr>
<tr><td>5.1. - 2.2.</td><td>-0.31337</td><td>0.12399</td><td>-2.527</td><td>0.0538</td></tr>
<tr><td>5.2. - 2.2.</td><td>-0.12320</td><td>0.12646</td><td>-0.974</td><td>0.7585</td></tr>
<tr><td>5.2. - 5.1.</td><td>0.19017</td><td>0.09467</td><td>2.009</td><td>0.1795</td></tr>
</table></td></tr>
<tr><td>4A</td><td>Kaplan Meier Estimates
<table>
<tr><th>Comparison</th><th>p-value</th><th>FDR corrected p-value</th></tr>
<tr><td>Always – Ancestor</td><td>0.16086355</td><td>0.3217271</td></tr>
<tr><td>2.1. - Ancestor</td><td>0.71526638</td><td>0.7152664</td></tr>
<tr><td>5.1. – Ancestor</td><td>0.04131051</td><td>0.1652421</td></tr>
<tr><td>Never – Ancestor</td><td>0.04131051</td><td>0.7152664</td></tr>
</table></td></tr>
<tr><td>4B</td><td>Binomial GLM
Evolution effect: X^2: 3.8418, Df=4, p= 0.4278</td></tr>
</table>

Chapter 3

Sex Matters: Effects of Sex and Mating in the Presence and Absence of a Protective Microbe

In preparation as: Kloock, A.; Peters, L.; Rafaluk-Mohr, C.; Bonsall, M. B.; King, K. C. Sex matters: effects of sex and mating in the presence and absence of a protective microbe

Abstract

Males and females invest differently in reproduction and possibly in the immune system. In order for the two sexes to increase their reproductive output, I expect females to invest more in immunity, while I expect males to invest more in mating behaviour due to male-male competition. Microbe-mediated protection (MMP) is known to influence strategies of immune investment and reproduction. How this interplays with sex specific differences in different life history traits following mating is yet to be explored. Here, I investigated the sex-specific differences in host life history traits between female and male *Caenorhabditis elegans* in the context of mating under the influence of pathogenic infection by *Staphylococcus aureus* in the presence or absence of MMP by *Enterococcus faecalis*. My results show that during pathogen infection, males survive better than females, independent of MMP. Once provided with MMP, females are able to invest more in offspring production, while males show higher behavioural activity. This pattern can be explained by Bateman's principle, which states that females optimise their reproductive success by offspring quality, while males increase their reproductive success by offspring quantity. These results highlight the different strategies potentially employed by the two sexes in dealing with a pathogen in the absence or presence of MMP.

Introduction

Mating is costly. Females need to invest more directly into reproduction (such as the production of eggs)[1], while males have to invest more in obtaining mates with females, e.g. through male-male competition[2], ornate phenotypes[3] or searching for mates[4–6]. These traits are energy costly, and as such it is hypothesized that there are trade-offs between survival and reproduction in both females and males[7], albeit being different in the two sexes. Survival depends on the availability of energy, mainly food[8], but also on patterns of longevity and infection responses[9]. As the available energy resources are often limited, a trade-off between reproduction and infection is almost inevitable[7,10,11]. However, females and males might allocate their resources differently, given different investments in reproduction. While females might invest the majority of their energy into staying healthy enough to reproduce and by such produce a high quality of offspring, males might invest the majority of their energy into finding potential mating partners and by such produce a high quantity of offspring[12,13]. This pattern follows Bateman's principle[14–16], in which females maximise their reproductive success by mating with high quality partners, while males maximise their reproductive success by mating with as many females as possible[2,12].

This difference in energy allocation into reproduction can also be observed in the face of infections. Males are observed to have reduced survival[17,18], higher infection load[17,18], reduced lysozyme-like activity and hemocytes[19], and reduced body size[18], suggesting that males invest less in immunity than females. Males were however observed to have a higher activity (observed in the form of higher escape behaviour)[17], which is also known as a responding to an infection[20]. Females on the other hand were observed to have increased immune activity[19] and to survive pandemics[12], slavery or famines better than males do[21]. This increase in immune activity could however be the reason for higher auto immune diseases in females[22].

Given these differences in energy investment into immunity between the sexes and the differences in how to maximise reproductive success I hypothesized that the two sexes would benefit differently from the presence of protective microbes[23]. Protective microbes can be important in host defence in the face of infection, a phenomenon referred to as "defence mutualism"[24,25], where microbes can supplement the host's immune system[26–28]. Defensive mutualisms has been observed across kingdoms, including plants[29,30], vertebrates[31,32], insects[33,34] and *C. elegans*[35,36] (reviewed in[37]). The potential of defensive mutualism to enhance survival[38,39] as well as offspring production[40] has been observed repeatedly[38–40]. However, in most of these examples, only the population-level effects have been considered, while few studies have focussed on individual behaviours and/or sex differences between the hosts[23].

To test for differences in life history traits between the two sexes of hosts in the presence or absence of microbe-mediated protection (MMP) during infection, I used an established experimental system using *Caenorhabditis elegans* as a host, *Enterococcus faecalis* as a protective microbe and *Staphylococcus aureus* as a pathogen[35,41–43]. Here, I used a population of *C. elegans* made up of males and genetically engineered hermaphrodites, that only carry eggs and cannot produce sperm, and thus are referred to as females[44]. Due to this lack of self-sperm the females are required to mate with males to produce viable offspring. *Caenorhabditis. elegans* males are known to actively search for females to mate with[6] and in the absence of females this behaviour is increased[6]. For my experiment, I either separated females and males as single sex plates or let them mate, before introducing mating treatments. These treatments ranged from unmated, short-term mated to lifetime mated individuals for both sexes to test for differences between offspring production and effects of mating. Both mated treatments (short-term and lifetime mated) females produce eggs throughout their lifetime and I assessed the differences between these different mating

regimes under three different bacterial diets: food only, pathogen infection and pathogen infection with MMP. I measured a range of life history traits including survival during pathogen infection, longevity, behavioural activity and offspring production. I investigate the differences in life history traits for the two sexes, for the effect of mating on each sex, and in the presence or absence of MMP.

Materials and Methods

Worm and bacteria system

In nature, *Caenorhabditis elegans* can be found in microbe rich environments[45], where bacteria serve as the nematodes main diet and also form their microbiota[46,47]. These nematodes are an established model to study innate immunity[48] as they can be infected with a variety of bacteria which are naturally co-occurring[46,49] or with opportunistic animal and human pathogens[50,51].

Caenorhabditis elegans is naturally a self-fertilising hermaphrodite[52] with a low proportion of males (less than 0.5%)[53]. However, for my experiments I used an obligate outcrossing worm population (line EEVD00) where worms carry the *fog-2(q71)* mutation, preventing hermaphrodites from producing sperm[44]. In culture, the nematode is routinely kept on Nematode Growth Medium (NGM)[52], inoculated with *Salmonella*, hereafter referred to as food, which was previously shown to support worm growth and development[54]. For pathogenic infection, the Gram positive *Staphylococcus aureus* strain MSSA476[55] was used, which kills worms by lysing the intestinal cells lining the gut wall[56]. The strain OG1RF of *Enterococcus faecalis*[50] was used as a protective microbe against *S. aureus* infection, as previously shown[35,43].

Pathogenic infection and long-term survival analysis

Plates were randomized and fully coded during each experiment to ensure the experimenter was blind to different treatments during data collection. All results shown for food,

pathogenic infection with or without MMP have been generated from different set ups for each bacterial diet but results within each bacterial diet can directly be correlated.

Starved worms were transferred to new plates inoculated with food 2.5 days before bleaching to allow for egg laying. Worms were then bleached as described previously[57] and left in M9 buffer overnight to allow larvae to hatch and arrest in the first larval stage. Simultaneously, the bacteria (on which worms were raised to L4 stage) were grown in overnight cultures: Either *E. faecalis* overnight in 25ml of Todd-Hewitt Broth (THB), or food in 25ml of Lysogeny broth (LB), both at 30ºC in a shaking incubator. Subsequently, 6cm NGM plates were inoculated with either 400µl of food or 200µl of food mixed with 200µl of *E. faecalis*. Plates with freshly inoculated bacteria were dried at room temperature before approximately 600 L1 worms were added to each NGM plate and transferred to 20ºC for 42h. At the same time, a liquid culture of *S. aureus* was grown in THB from a frozen stock, while food was grown in LB. Both cultures were incubated under shaking conditions at 30ºC overnight. The following day, 20µl of the *S. aureus* overnight culture was pipetted onto 3cm on Tryptone Soy Broth agar (TSB) plates. Simultaneously, 6cm NGM plates were inoculated with 150µl food. These plates were used to split worms into groups of only females, only males or 50:50 mixed for 6-8 h (time point when the first eggs appeared on the plate) as outlined in Table 1. After worms had mated, 50 worms were placed onto the *S. aureus* lawn with a platinum wire pick and left at 25ºC for 24h in the set up described in Table 1.

Table 1: Mating Treatments: Three different mating treatments (Unmated, Short-term mated and Lifetime mated) were set up for both sexes (Female in Red and Male in Blue), which were either single sex plates for each sex (Females only or Males only) or a 50:50 mixed population. Worms were left on these mating plates for 6-8h, before three different mating treatments were set up (unmated, short-term mated, and lifetime mated, the darker the colour the longer the mating period) for each sex.

Females				Males			
Treatment	L1-L4	6-8h	Adult	Adult	6-8h	L1-L4	Treatment
unmated Female	Mixed Population	Females only	not premated Female				
short term mated Female	Mixed Population	50:50	premated Female				
lifetime mated Female	Mixed Population	Females only	not premated Female	not premated Male	Males only	Mixed Population	lifetime mated Male
lifetime mated Female	Mixed Population	Females only	not premated Female	premated Male	50:50	Mixed Population	lifetime mated Male
lifetime mated Female	Mixed Population	50:50	premated Female	not premated Male	Males only	Mixed Population	lifetime mated Male
lifetime mated Female	Mixed Population	50:50	premated Female	premated Male	50:50	Mixed Population	lifetime mated Male
				premated Male	50:50	Mixed Population	short term mated Male
				not premated Male	Males only	Mixed Population	unmated Male

Survival upon pathogenic infection was scored after 24h, counting all live and dead worms present on the plates. Worms were considered dead if they did not respond to touch with a platinum wire pick. After survival was scored, 10 worms were transferred to 3cm NGM plates seeded with 150µl food and placed at 25ºC. Worms are then transferred to new plates every 24h with a platinum wire until no further offspring production occurred, to allow for more accurate tracking of adult individuals. Survival was scored every day until all worms were dead.

For the food alone treatment, the long-term survival assay followed a similar protocol except that the experimental procedure was carried out at 20 ºC, as usually done with *C. elegans*[58], with 20 worms on each plate, and worms were split into only females, only males or 50:50 mixed when worms were 46h old. The lifetime mated treatment consisted of two different groups of mixed worms; those that were pre-mated and those that were not.

Activity analysis

Males and females were previously observed to show different activities, may it be due to infection[17] or due to higher mate searching behaviour[6]. After 24h on the pathogen infection plates, the behavioural activity level of worms was determined. To measure this activity, the number of worms at the edge of the plate was counted and divided by all alive worms present on the plate. Worms were considered at the edge of the plate, if they could not be seen from atop.

Avoidance analysis

Avoidance behaviour is known as a response to pathogen defence[59]. The proportion of missing worms was calculated 24h after pathogen infection and whenever worms were transferred to new plates during the long-term survival analysis. To define the proportion of missing worms, the number of initially exposed worms minus the counted alive and counted dead worms was divided by the number of initially exposed worms. For the long-term avoidance analysis, the cumulative number of dead worms was used, while only the last time point of each experiment was plotted.

Offspring production

To measure the female investment into reproduction, the presence or absence of offspring on a plate was noted during pathogen infection and during long term survival. Unmated females might sometimes produce and lay unfertilised eggs, which do not develop into viable larvae (personal observation). As no exact numbers of produced eggs could be

counted due to feasibility, offspring production is defined as a proportion of how many plates had offspring over the total amount of plates per treatment.

Statistical Analysis

Statistical analyses were carried out with RStudio (Version 1.1.463 for Mac)[60]. Figures were created with the ggplot2 package (Version 2.1.0)[61]and edited with Inkscape (Version 0.91)[62]. All data, except for the long-term survival and offspring production data, was analysed with nested binomial mixed effects models (GLMM - R package lme4)[63] to test for an effect of sex, the mating status or an interaction between the two. If the interaction was shown to have a significant effect, a Tukey multiple-comparison tests (R package multcomp)[64] was performed. The long-term survival data was analysed with Kaplan Meier Log Rank test with FDR correction for multiple testing[65,66]. The offspring data was analysed using a Wilcoxon Rank Test[67].

Results & Discussion

Females suffer more during pathogen infection

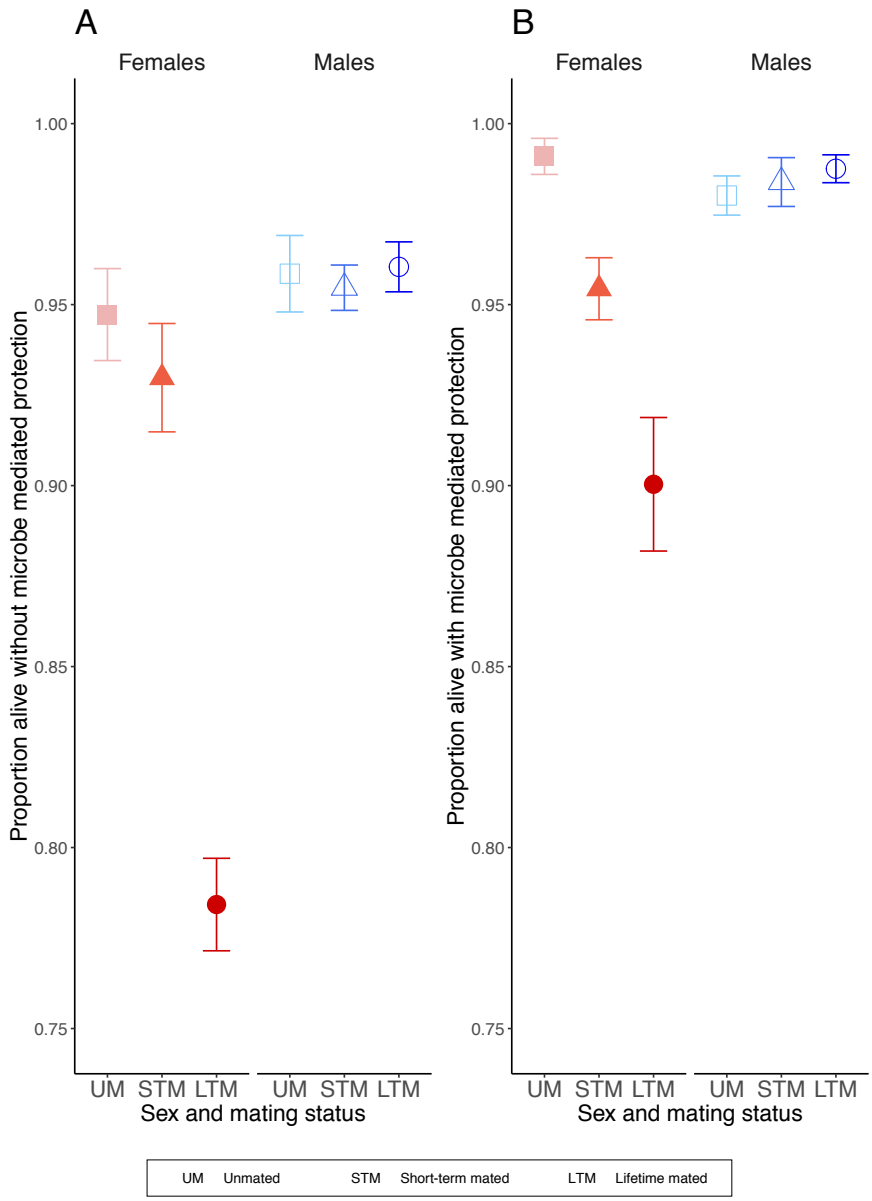

Figure 1: Survival of different sexes and mating treatments after 24h pathogen infection (A) Without MMP females suffer more from mating than males do, while males survive better overall. (B) With MMP, females are suffering more from short term and lifetime mating, while males survive better overall. (A, B) Each point represents the mean ± the standard error of the mean of four biological replicates and three or four technical replicates.

To assess differences in pathogen infection between the two sexes and different mating treatments, survival after 24h pathogen infection was measured in the absence (Figure 1A) and presence of MMP (Figure 1B). In the absence and presence of MMP, males survived significantly better than females ($p<0.001$, GLMM, $X^2=121.171$, $df=1$ and $p<0.001$,

GLMM, X^2=20.864, df=1, respectively). Females were significantly affected by the mating status ($p<0.001$, GLMM, X^2=84.252, df=2 and $p<0.01$, GLMM, X^2=10.759, df=2, respectively), as in the absence of MMP only lifetime mated females survived significantly worse than short-term mated and unmated females during pathogen infection ($p<0.001$, GLMM, X^2=71.09, df=3) with no difference between short-term mated and unmated females ($p>0.05$, GLMM, X^2=71.09, df=3) , while in the presence of MMP all comparisons between females were significant ($p<0.05$, GLMM, X^2=71.09, df=3). Males did not show any influence of the mating status ($p>0.05$, GLMM, X^2=312.54, df=3 and $p>0.05$; GLMM, X^2=71.09, df=3 respectively).

Our analysis revealed that males survive pathogen infection better than females independent of MMP (Figure 1A & 1B), while females benefit more from MMP during pathogen infection. This could be explained by a lower bacterial count in males than in females (personal observation). The potential of MMP to enhance survival[35,38,39] as well as offspring production[40] has been shown repeatedly. So far, these effects have mainly been considered at the population level. However, the role of MMP might have different effects on individual behaviours, differences between the sexes and/or differences between adults and juveniles[23]. In the presence of MMP, pathogenic infection is not entirely rescued, but sufficiently weakened, as shown in this system[35,38]. A potential explanation for the observed phenotype could be mechanical gut integrity, which can be different between males and females as observed in *Drosophila*[68]. The pathogen used here, *S. aureus*, is known to accumulate in the worms gut and to kill worms by distention of the intestinal lumen[56]. If gut integrity would thus be more easily damaged in *C. elegans* females, but not in males (personal observation, Figure S2), this could serve as a potential explanation as to why females are more harshly affected by pathogenic infection with *S. aureus*.

Lifetime mated females suffer most

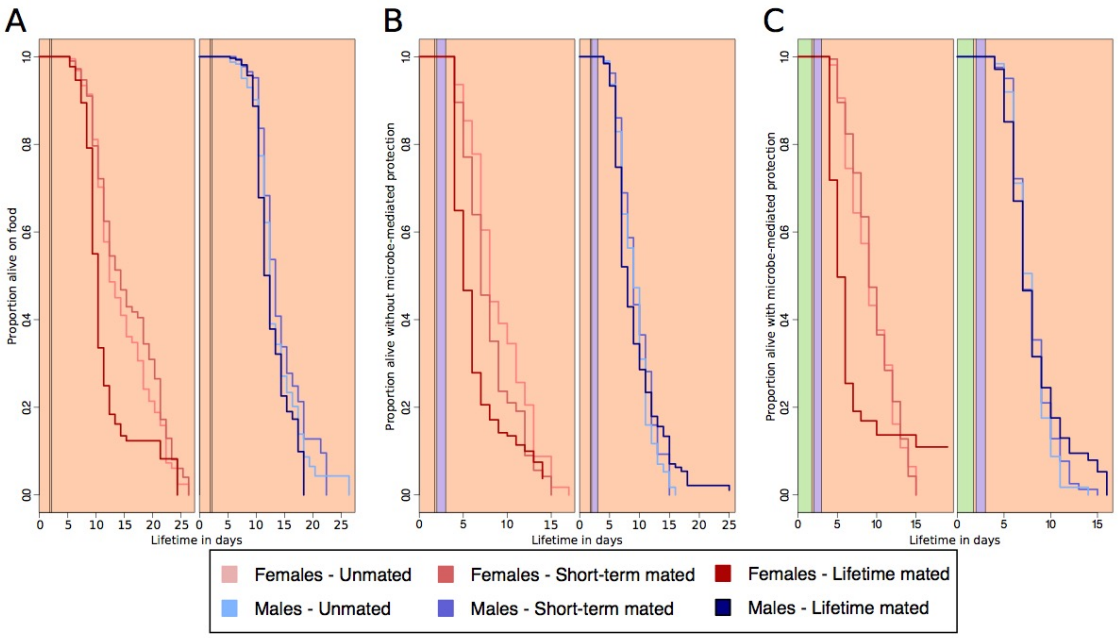

Figure 2: Survival of different sexes and mating treatments on food alone (A) or after pathogen infection without MMP (B) or after pathogen infection with MMP (C). (A) When only ever being exposed to food (indicated in orange), lifetime mated females survive worse than any other females, while only lifetime mated males survive worse than short-term mated males. (B) After pathogen infection (indicated by purple) without MMP, lifetime mated females survive worse than short-term mated females, which survive worse than unmated females. Males do not show any effect of mating on survival, while males live overall longer than females do. (C) After pathogen infection with MMP (indicated by green), lifetime mated females survive worse than short-term mated females, while males do not show any effect of mating. (A-C) Each curve represents the Kaplan Meier Survival estimate for three or four technical replicates and four or five biological replicates.

To assess differences in longevity between the two sexes and different mating treatments on different bacterial diets, survival was measured on food alone (Figure 2A), after pathogen infection without MMP (Figure 2B) and after pathogen infection with MMP (Figure 2C). The two sexes only showed significant differences after pathogen infection without MMP ($p<0.001$, Kaplan Meier Survival Estimate (KMSE)), while no such difference could be observed on food alone ($p=0.62$, KMSE) or after pathogen infection with MMP ($p=0.32$, KMSE). Lifetime mated worms survived worse than unmated and short-term mated worms on all three diets (all $p<0.05$, KMSE), while short term and unmated worms showed no significant difference on all three diets (all $p>0.05$, KMSE).

Life-time mated females survive significantly worse than short-term and unmated females on each diet (Figure 2A-C). As this is present on each diet, it suggests that the act of mating is costly and life shortening[69], as short-term mated females, that also produce costly offspring, do not show reduced survival over a lifetime (Figure 2 A-C). The lifespan of the generically used *C. elegans* wild strain N2 ranges from 11.8 to 20 days at 20°C[70,71], with the upper limit of around 20 days corresponding to the long-term survival observed in my study, which used the strain EEVD00[44] (Figure 2). In the hermaphroditic N2 strain of *C. elegans*, the mating span was found to not have an effect on the lifespan[72]. In my set up, lifetime mating worms were mating until they are parted by death (Table 1), while in other studies worms mate for no longer than 12h[72–74]. Mating activates immune genes expression in some species (e.g.[75]), which results in earlier deaths linked with increased rates of mating (such as in *Drosophila*[76], or in birds[77]). During mating males transfer genetic material in forms of sperm, along with seminal fluid, which damages *C. elegans* mothers[74] by male induced demise[69,72,78], which could explain the highly reduced lifespan phenotype that I can observe here for lifetime mated females. Hermaphrodites are protected from this phenomenon by the presence of self-sperm[72,73]. However, the females that I used here lack self-sperm (hermaphrodites with the *fog-2* mutation[44]) and might thus also lack the linked protection, which would explain the detrimental effect of male mating on self-sperm depleted hermaphrodites. Increased survival might however also be connected with behaviour: In *C. elegans* it was shown that higher survival can be affected by pathogenic avoidance behaviour[59].

Male and female behaviour is linked to the presence of the other sex

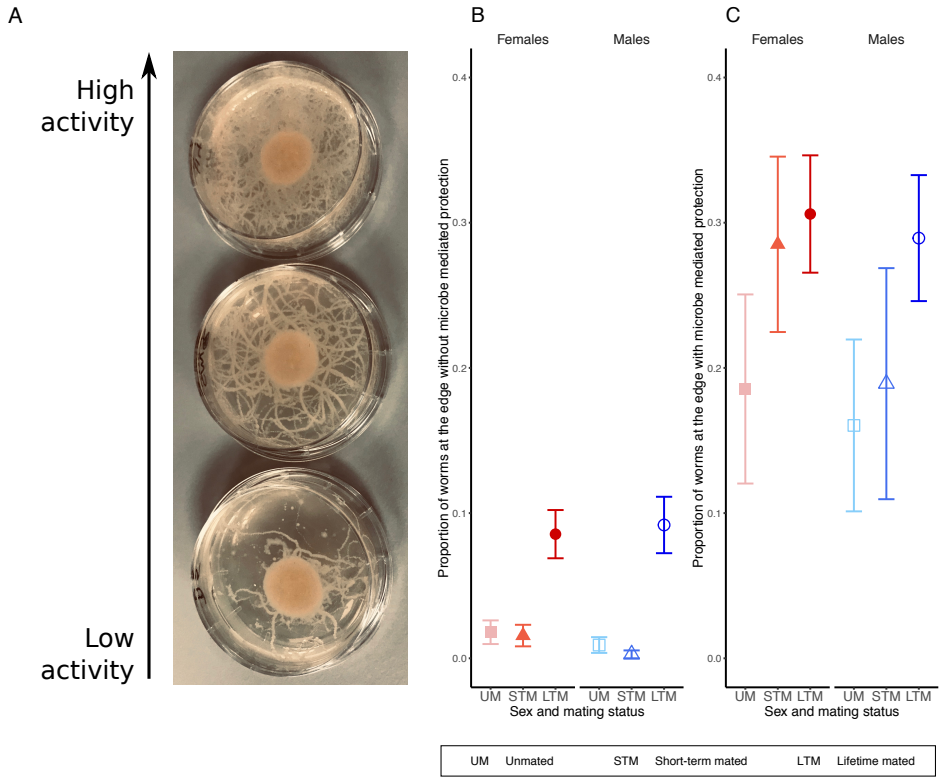

Figure 3: Activity of different sexes and mating treatments either after 24h pathogen infection. (A) Picture of three pathogenic plates with different activity levels from low to high. (B) Without MMP after pathogen infection plates with lifetime mated worms of both sexes show higher proportion of worms at the edge of the plate. (C) With MMP after pathogen infection plates with lifetime mated worms of both sexes show higher proportion of worms at the edge of the plate. (B, C) Each point represents the mean ± the standard error of the mean of four biological replicates and three- four technical replicates, with each 50 worms.

When plates were assessed for worm survival, different levels of activity were observed (Figure 3A). To assess differences in these behavioural observations between the two sexes and different mating treatments, the proportion of worms at the edge of the pathogenic plates was counted. Independent of the presence or absence of MMP, lifetime mated worms showed a higher proportion of worms at the edge ($p<0.001$, GLMM, $X^2=122.56$, df=5 and $p<0.001$, GLMM, $X^2=51.8544$, df=2). This increased activity for lifetime mating worms can be a hint to increased avoidance behaviour, which is itself a mechanism to respond to pathogenic infection[59].

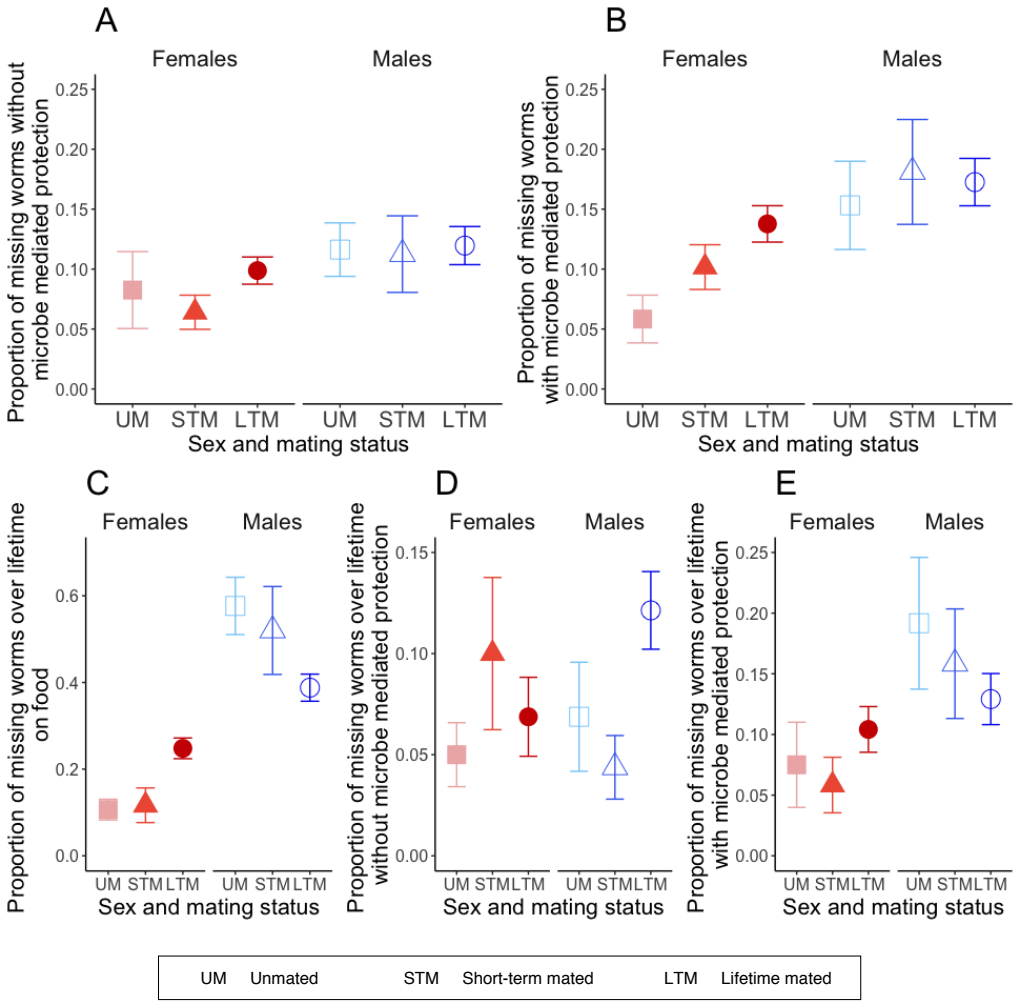

Figure 4: Proportion of missing worms of different sexes and mating treatments either during pathogen infection (A&B) and over lifetime (C-E, only the last time point is plotted). (A) During pathogen infection without MMP, a higher proportion of worms is missing for males than for females. (B) During pathogen infection with MMP, males showed a higher proportion of missing worms than females did. (C) Proportion of missing worms on food over lifetime. Females showed lower proportion of missing worms in comparison to males. (D) Proportion of missing worms without MMP over lifetime with no differences detected. (E) Proportion of missing worms with MMP over lifetime, where males show higher proportion of missing worms than females do. (A-E) Each point represents the mean ± the standard error of the mean of four or five biological replicates and three or four technical replicates.

To assess the differences in these behavioural observations between the two sexes and different mating treatments, the proportion of missing worms was calculated during pathogen infection (Figure 4A & 4B) and over lifetime (Figure 4C-E). During pathogen infection in the presence (Figure 4A) and absence of MMP (Figure 4B), males were found to have a higher proportion of missing worms than females ($p<0.001$, GLMM, $X^2=17.5919$, df=1 and $p<0.001$, GLMM, $X^2=35.586$, df=1, respectively). In the presence of MMP, more lifetime mated females than unmated females went missing ($p<0.001$, GLMM, $X^2=57.68$,

df=5), while all other comparisons between the different combinations of sexes and mating treatments were not significant ($p>0.05$, GLMM, $X^2=57.68$, df=5). Over a lifetime, males show a higher proportion of missing worms than females when grown on food alone ($p<0.001$, GLMM, $X^2=154.21$, df=1) and after pathogen infection with MMP ($p=0.002$, GLMM, $X^2=9.198$, df=1), while the difference between the sexes was not significant after pathogen infection without MMP ($p=0.23$, GLMM, $X^2=1.42$, df=1). Interestingly on food alone, lifetime mated worms showed opposite patterns in comparison to unmated worms: for females there were more lifetime mated females than unmated females missing ($p<0.01$, GLMM, $X^2=201.31$, df=5), while for males there were less lifetime mated males than unmated males missing ($p<0.01$, GLMM, $X^2=201.31$, df=5). No more significant differences were observed. These missing worms are either actively crawling up the plastic edge of the petri dish, where they then die due to desiccation[59], or dig into the agar, where they are unable to return from due to the experimental procedure.

The increased activity observed in Figure 3 was thought to be a first indication of the proportion of missing worms that is shown in Figure 4. This was however not the case, as the activity level was mainly determined by the mating status, while the proportion of missing worms was more determined by the worm's sex. The mating status also affected the proportion of missing worms in opposite directions in lifetime mated worms on food: females would be missing with a higher proportion in the presence of males while males would stay with a higher proportion in the presence of females. This indicates higher mate searching behaviour for females by males, which is increased when females are not presented on the plate (as on the unmated and short-term mated male plates). This also indicates, that the proportion of missing worms observed here is not pathogenic avoidance behaviour[59], for which one would expect, that worms during pathogenic infection without

MMP should show the highest proportion of missing worms. This is however not the case as this behaviour is not observed for pathogen infection in the absence of MMP, as the worms are not provided with enough energy to display such behaviour. Once they are provided with sufficient energy (with food alone and in the presence of MMP), a tendency towards this pattern can be seen, but is not significant. MMP can cushion the effect of pathogenic infection, but do not entirely rescue a survival phenotype[35,38]. It is important to note that this energy constraint only occurs in early stages of a worm's life, as the proportion of missing worms plateaus after the first few days (Figure S1).

MMP enables females to invest in offspring production during pathogenic infection

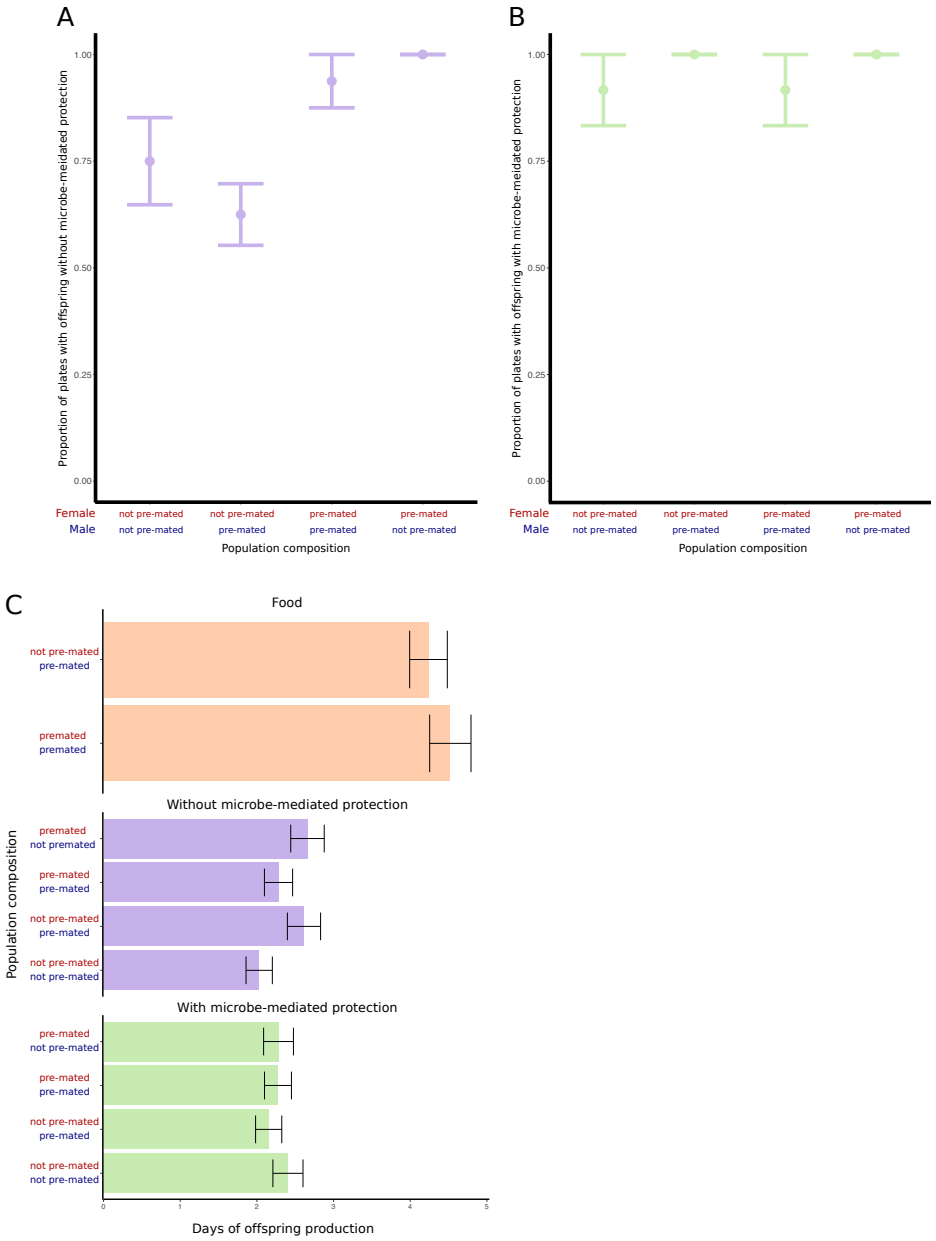

Figure 5: Differences in offspring production during pathogen infection without (A) and with (B) MMP and during lifetime (C). (A) During pathogen infection but without MMP, there are less plates with offspring, despite the presence of males. (B) During pathogen infection, but with MMP, there are no differences visible between pre-mated and not pre-mated females. (C) Over a lifetime no difference between pre-mated and not pre-mated females for the days of offspring production can be observed independent on whether worms were raised on food, infected with the pathogen with or without MMP. (A, B) Each point represents the mean ± the standard error of the mean of three or four technical replicates.

During the survival assays, it was observed, that some plates did not have offspring at all, despite having both sexes on the plate. When comparing pre-mated females with not-pre-

mated females, a Wilcoxon Rank test confirmed that there is a difference between these female treatments in the absence of MMP (Wilcoxon Rank Test (WRT), pre-mated females vs. not pre-mated females p=0.004, Figure 5A). By contrast there is no difference between these treatments in the presence of MMP (WRT, pre-mated females vs. not pre-mated females, p=1, Figure 5B). Analysis of the days during which offspring is produced over a lifetime revealed no significant difference for any of the diets (WRT, pre-mated females vs. not pre-mated females, p=0.522 on food alone; p=0.5557 after pathogenic infection without MMP and p=0.9901 after pathogenic infection with MMP; Figure 5).

Here I observe, that during pathogen infection without MMP those females that have not encountered males before do not produce offspring in the same way that those females provided with MMP do (Figure 5A and 5B). Once worms are provided with enough energy though, this phenotype can be rescued (Figure 5C). This suggests, that during pathogen infection females invest great parts of their energy into fighting the infection, and do not invest anything into reproduction or even mating. Once the female is provided with MMP, the amount of available energy is increased, and with a fixed amount of energy being allocated to immunity by the presence of MMP, the female can invest more energy into reproduction, which was observed in an aphid system[23]. Due to the fact that I only scored for the presence/absence of offspring, I would not be able to detect any change of timing in offspring production, as was described for fecundity compensation in this system of *C. elegans* and *S. aureus*[79].

Conclusion

In conclusion I investigated sex differences of *C. elegans* hosts in the presence or absence of MMP during pathogen infection. My results reveal that during pathogen infection, males survive better than females, independent of MMP. MMP enables females to invest more in offspring production, while males show higher levels of behavioural activity. The observed patterns can be explained by Bateman's principle[14–16]. Females will maximise their reproductive success by making high quality mate choices. Their reproductive success is also determined by the available energy, as egg production is generally more costly[12]. Furthermore, the benefits for survival beyond the end of reproduction and the patterns of senescence may be limited; for instance hermaphrodites that show dramatic shrinking and death shortly after mating. This study highlights, that even though a population response is observed to defensive mutualists, the response of the individual might differ from the population response, depending on the diet, sex, the mating status or an interaction of these factors.

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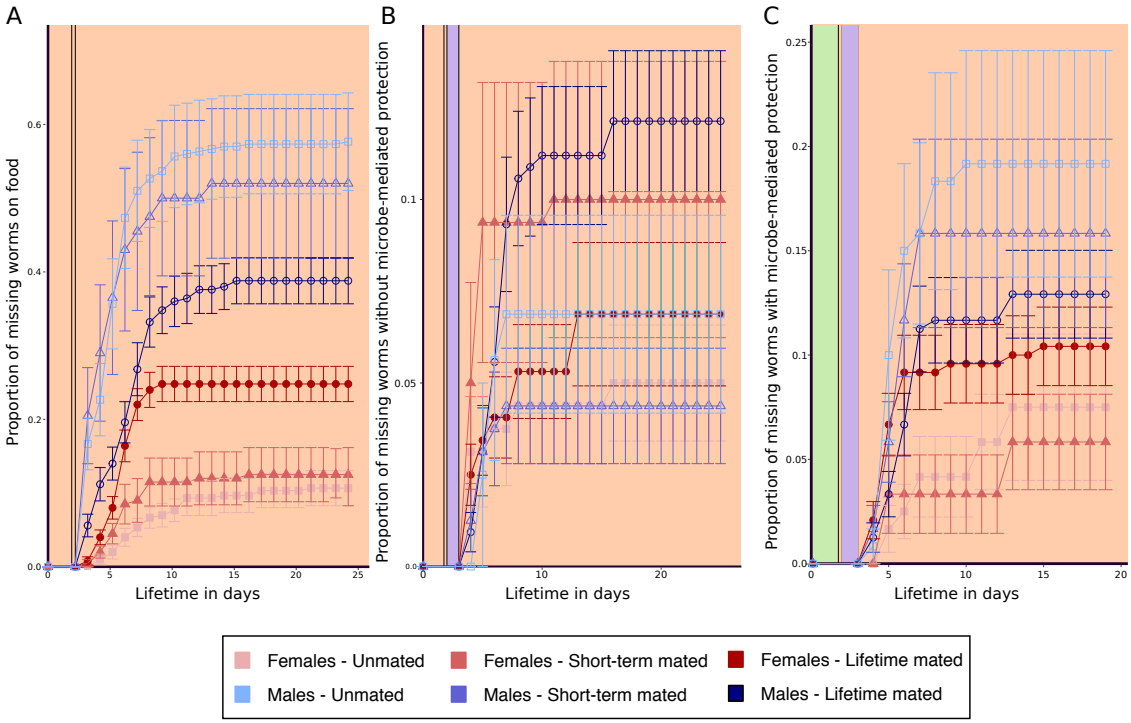
Supplemental Material

Supplemental Figures



Supplemental Figure S1: Lifelong proportion of missing worms on food (A), in the absence of microbe-mediated protection(B) and in the presence of microbe-mediated protection (C). All graphs show the mean +- the standard error of the mean across time.

Supplemental Figure S2: Females show hints of lost gut integrity, while male gut integrity is still intact. Worms that were fed food, coloured with a blue food dye. If gut integrity is still fully intact, blue dye can only be seen in the intestine (as in the lower worm – a male), while if the gut integrity is out of balance, the blue dye can be found in the whole worm body cavity (as in the upper worm – a female).

Statistical results

Table S1: Summary of all statistical results. The following abbreviations were used: U=unmated, STM= short-term mated, LTM= Lifetime mated

<table>
<tr><th>Figure</th><th>Result</th></tr>
<tr><td>1A</td><td>
<u>Pathogen Infection without MMP</u>
Generalized mixed effects model
<table>
<tr><th>Effect</th><th>X^2</th><th>Df</th><th>p-value</th></tr>
<tr><td>Mating</td><td>84.252</td><td>2</td><td>$<2.2x10^{-16}$</td></tr>
<tr><td>Sex</td><td>121.171</td><td>1</td><td>$<2.2x10^{-16}$</td></tr>
<tr><td>Interaction</td><td>43.693</td><td>2</td><td>$3.252x10^{-10}$</td></tr>
</table>
Analysis of the interaction:
Generalized mixed effects model
$X^2=312.54$, Df=3, $p<2.2x10^{-16}$
Results of the Tukey Post-Hoc Test:
<table>
<tr><th colspan="2">Comparisons</th><th>Estimate Std.</th><th>Error</th><th>z-value</th><th>p-value</th></tr>
<tr><td>F- STM</td><td>F-U</td><td>-0.2810</td><td>0.2191</td><td>-1.282</td><td>0.56150</td></tr>
<tr><td>F-LTM</td><td>F-U</td><td>-1.5767</td><td>0.1750</td><td>-9.012</td><td><0.001</td></tr>
<tr><td>M</td><td>F-U</td><td>0.2637</td><td>0.1873</td><td>1.408</td><td>0.48141</td></tr>
<tr><td>F-LTM</td><td>F-STM</td><td>-1.2957</td><td>0.1603</td><td>-8.081</td><td><0.001</td></tr>
<tr><td>M</td><td>F-STM</td><td>0.5446</td><td>0.1738</td><td>3.134</td><td>0.48141</td></tr>
<tr><td>M</td><td>F-LTM</td><td>1.8404</td><td>0.1131</td><td>16.268</td><td><0.001</td></tr>
</table>
</td></tr>
<tr><td>1B</td><td>
<u>Pathogen Infection with MMP</u>
Generalized mixed effects model
<table>
<tr><th>Effect</th><th>X^2</th><th>Df</th><th>p-value</th></tr>
<tr><td>Mating</td><td>10.759</td><td>2</td><td>0.0046095</td></tr>
<tr><td>Sex</td><td>20.864</td><td>1</td><td>$4.932x10^{-6}$</td></tr>
<tr><td>Interaction</td><td>16.078</td><td>2</td><td>0.0003226</td></tr>
</table>
Analysis of the interaction:
Generalized mixed effects model
$X^2=71.09$, Df=3, $p=2.5x10^{-15}$
Results of the Tukey Post-Hoc Test:
</td></tr>
</table>

Comparisons		Estimate Std.	Error	z-value	p-value
F- STM	F-U	-1.7075	0.5631	-3.032	0.01166
F-LTM	F-U	-2.4503	0.5197	-4.715	< 0.001
M	F-U	-0.5632	0.5439	-1.036	0.71377
F-LTM	F-STM	-0.7428	0.2910	-2.552	0.04760
M	F-STM	1.1442	0.3338	3.428	0.00316
M	F-LTM	1.8870	0.2492	7.572	< 0.001

2A

Lifetime Analysis on Food

Kaplan Meier Estimates

Comparisons		p-value	FDR corrected	
Female	Male	0.6199	0.6199	
U	STM	0.05122	0.0683	
U	LTM	3.33×10^{-16}	6.66×10^{-16}	***
STM	LTM	0	0	***

Comparisons		p-value	FDR-corrected	
F- U	F-STM	0.0531	0.0638	
F-U	F-LTM	0	0	***
F-STM	F-LTM	0	0	***
M-STM	M-LTM	0.0046	0.0092	***
M-LTM	M-U	0.0236	0.0354	***
M-STM	M-U	0.5932	0.5932	

2B

Lifetime Analysis after pathogen infection without MMP

Kaplan Meier Estimates

Comparisons		p-value	FDR corrected	
Female	Male	0	0	***
U	STM	0.2263	0.2633	
U	LTM	1.55×10^{-10}	3.11×10^{-10}	***
STM	LTM	4.87×10^{-7}	6.5×10^{-7}	***

Comparisons		p-value	FDR-corrected	
F- U	F-STM	0.0016	0.0032	***
F-U	F-LTM	0	0	***
F-STM	F-LTM	1.03×10^{-9}	3.09×10^{-9}	***
M-U	M-STM	0.26	0.3119	
M-U	M-LTM	0.4268	0.4268	
M-STM	M-LTM	0.0750	0.1125	

2C

Lifetime Analysis after pathogen infection with MMP

Kaplan Meier Estimates

Comparisons		p-value	FDR corrected	
Female	Male	0.3177	0.3177	
U	STM	0.2688	0.3177	
U	LTM	2.58×10^{-8}	5.17×10^{-8}	***
STM	LTM	5.40×10^{-12}	2.16×10^{-11}	***

Comparisons		p-value	FDR-corrected	
F- U	F-STM	0.4029	0.5996	
F-U	F-LTM	1.57×10^{-13}	4.71×10^{-13}	***
F-STM	F-LTM	0	0	***
M-U	M-STM	0.4734	0.5996	
M-U	M-LTM	0.4997	0.5996	
M-STM	M-LTM	0.7244	0.7224	

3B

Activity analysis without MMP

Generalized mixed effects model

Effect	X^2	Df	p-value
Mating	109.8893	2	$<2 \times 10^{-16}$
Sex	0.0968	1	0.7557
Interaction	8.2846	2	0.0159

Analysis of the interaction:

Generalized mixed effects model

X^2=122.56, Df=5, p<2.2x10^{-16}

Results of the Tukey Post-Hoc Test:

Comparison		Estimate Std.	Error	z-value	p-value
F- STM	F-U	-0.19589	0.39950	-0.490	0.9956
F-LTM	F-U	1.62482	0.29287	5.548	<0.001
M-LTM	F-U	1.71553	0.28918	5.932	<0.001
M-STM	F-U	-1.90172	0.75765	-2.510	0.0998
M-U	F-U	-0.85757	0.49241	-1.742	0.4565
F-LTM	F-STM	1.82071	0.31236	5.829	<0.001
M-LTM	F-STM	1.91142	0.30895	6.187	<0.001
M-STM	F-STM	-1.70583	0.76539	-2.229	0.1908
M-U	F-STM	-0.66168	0.50426	-1.312	0.7434
M-LTM	F-LTM	0.09071	0.14694	0.617	0.9872
M-STM	F-LTM	-3.52654	0.71554	-4.928	<0.001
M-U	F-LTM	-2.48239	0.42480	-5.844	<0.001
M-STM	M-LTM	-3.61725	0.71405	-5.066	<0.001
M-U	M-LTM	-2.57310	0.42227	-6.094	<0.001
M-U	M-STM	1.04415	0.81775	1.277	0.7647

3C

Activity analysis with MMP

Generalized mixed effects model

Effect	X^2	Df	p-value
Mating	51.8544	2	5.95x10^{-12}
Sex	0.0059	1	0.9389
Interaction	0.208	2	0.9012

Results of the Tukey Post-Hoc Test for the effect of Mating:

Comparisons		Estimate Std.	Error	z-value	p-value
STM	U	0.3380	0.2008	1.683	0.2109
LTM	U	0.8564	0.1748	4.898	<0.001
LTM	STM	0.5184	0.1847	2.807	0.0139

4A

Proportion of missing worms during pathogen infection without MMP

Generalized mixed effects model

Effect	X^2	Df	p-value
Mating	5.6135	2	0.0604
Sex	17.5919	1	2.738×10^{-5}
Interaction	2.5445	2	0.2802

4B

Proportion of missing worms during pathogen infection with MMP

Generalized mixed effects model

Effect	X^2	Df	p-value
Mating	14.213	2	0.0008198
Sex	35.586	1	2.44×10^{-9}
Interaction	12.672	2	0.0017717

Analysis of the interaction:

Generalized mixed effects model

$X^2=57.68$, Df=5, $p=3.66 \times 10^{-11}$

Results of the Tukey Post-Hoc Test:

Comparisons		Estimate	Std. Error	z value	p-value
F-STM	F-U	0.61565256	0.2219	2.77507149	0.0582
F-LTM	F-U	0.95634029	0.1949	4.90788243	0.0000
M-LTM	F-U	1.23348919	0.1918	6.43190203	0.0000
M-STM	F-U	1.2943663	0.2051	6.30974485	0.0000
M-U	F-U	1.09022263	0.2090	5.21692009	0.0000
F-LTM	F-STM	0.34068773	0.1609	2.11689384	0.2683
M-LTM	F-STM	0.61783662	0.1572	3.93083671	0.0012
M-STM	F-STM	0.67871374	0.1732	3.91820727	0.0012
M-U	F-STM	0.47457007	0.1778	2.66968986	0.0773

M-LTM	**F-LTM**	0.2771489	0.1160	2.3899288	0.1528
M-STM	**F-LTM**	0.33802601	0.1369	2.46868646	0.1273
M-U	**F-LTM**	0.13388234	0.1426	0.93863936	0.9334
M-STM	**M-LTM**	0.06087711	0.1325	0.45962075	0.9973
M-U	**M-LTM**	-0.1432666	0.1384	-1.0354932	0.9017
M-U	**M-STM**	-0.2041437	0.1563	-1.3058182	0.7737

4C

Lifetime analysis of proportion of missing worms on food

Generalized mixed effects model

Effect	X^2	Df	p-value
Mating	0.2805	2	0.8691
Sex	154.2064	1	$<2.2x10^{-16}$
Interaction	41.8466	2	$8.187\ x10^{-10}$

Analysis of the interaction:

Generalized mixed effects model

$X^2=201.31$, Df=5, $p=<2.2x10^{-16}$

Results of the Tukey Post-Hoc Test:

Comparisons		Estimate	Std. Error	z value	p-value
F-STM	**F-U**	0.3543	0.3105	1.1413	0.859974
F-LTM	**F-U**	1.1295	0.2436	4.6363	< 0.001
M-LTM	**F-U**	1.8341	0.2352	7.7982	< 0.001
M-STM	**F-U**	2.5055	0.2545	9.8447	< 0.001
M-U	**F-U**	2.5860	0.2273	11.3780	< 0.001
F-LTM	**F-STM**	0.7751	0.2838	2.7317	0.0667
M-LTM	**F-STM**	1.4797	0.2756	5.3682	< 0.001
M-STM	**F-STM**	2.1512	0.2838	7.5788	< 0.001
M-U	**F-STM**	2.2316	0.2726	8.1850	< 0.001
M-LTM	**F-LTM**	0.7046	0.2032	3.4678	0.0067
M-STM	**F-LTM**	1.3760	0.2204	6.2426	< 0.001
M-U	**F-LTM**	1.4565	0.1958	7.4374	< 0.001

	M-STM	**M-LTM**	0.6714	0.2085	3.2211	0.0156
	M-U	**M-LTM**	0.7519	0.1837	4.0935	< 0.001
	M-U	**M-STM**	0.0805	0.2027	0.3969	0.9987

4D

Lifetime analysis of proportion of missing worms on food

Generalized mixed effects model

Effect	X^2	Df	p-value
Mating	3.8327	2	0.14715
Sex	1.4218	1	0.23311
Interaction	7.9230	2	0.01903

Analysis of the interaction:

Generalized mixed effects model

X^2=13.63, Df=5, p=0.01814

Results of the Tukey Post-Hoc Test:

Comparisons		Estimate	Std. Error	z value	p-value
F_STM	**F-U**	0.7520	0.4489	1.6750	0.5355
F-LTM	**F-U**	0.3420	0.4249	0.8051	0.9647
M-LTM	**F-U**	0.9810	0.4014	2.4438	0.1346
M-STM	**F-U**	-0.1413	0.5298	-0.2667	0.9998
M-U	**F-U**	0.3420	0.4790	0.7140	0.9791
F-LTM	**F_STM**	-0.4099	0.3449	-1.1885	0.8353
M-LTM	**F_STM**	0.2290	0.3155	0.7258	0.9775
M-STM	**F_STM**	-0.8933	0.4682	-1.9080	0.3847
M-U	**F_STM**	-0.4099	0.4098	-1.0003	0.9137
M-LTM	**F-LTM**	0.6389	0.2802	2.2799	0.1938
M-STM	**F-LTM**	-0.4834	0.4451	-1.0859	0.8816
M-U	**F-LTM**	0.0000	0.3833	0.0000	1.0000
M-STM	**M-LTM**	-1.1223	0.4228	-2.6543	0.0802
M-U	**M-LTM**	-0.6389	0.3571	-1.7893	0.4597
M-U	**M-STM**	0.4834	0.4971	0.9724	0.9228

<table>
<tr><td>4E</td><td>Generalized mixed effects model

<table>
<tr><th>Effect</th><th>X^2</th><th>Df</th><th>p-value</th></tr>
<tr><td>Mating</td><td>0.7457</td><td>2</td><td>0.688754</td></tr>
<tr><td>Sex</td><td>9.1983</td><td>1</td><td>0.002422</td></tr>
<tr><td>Interaction</td><td>4.0926</td><td>2</td><td>0.129212</td></tr>
</table></td></tr>
<tr><td>5A</td><td>Wilcox-Rank-Test
Not pre-mated females vs. pre-mated females: p=0.004201</td></tr>
<tr><td>5B</td><td>Wilcox-Rank-Test
Not pre-mated females vs. pre-mated females: p=1</td></tr>
<tr><td>5C</td><td><u>Food:</u>
Wilcox-Rank-Test
Not pre-mated females vs. pre-mated females: p=0.5222
<u>Without microbe-mediated protection</u>
Wilcox-Rank-Test
Not pre-mated females vs. pre-mated females: p=0.5227
<u>With microbe-mediated protection</u>
Wilcox-Rank-Test
Not pre-mated females vs. pre-mated females: p=0.9901</td></tr>
</table>

Chapter 4

Host Dynamics are Stabilised in the Presence of a Protective Microbe

In preparation as: Kloock, A.and Bonsall, M.B. Host dynamics are stabilised in the presence of a protective microbe

Abstract

Species interact with a diverse community of microbes, and some of these microbes are able to protect a host from an infection. These protective microbes can transfer traits to the host, that dampen infection transmission or help the host recover from an infection. Harbouring protective microbes has evolutionary implications which often result in these microbes being costly for the host. However, how the ecological dynamics are affected in the presence of the protective microbe is poorly understood. Here I have developed an SIR framework to explore host dynamics in the absence and presence of protective microbes. My analysis and simulations indicate, that protective microbes are able to dampen infection, independent of the cost-benefit ratio under different ecological conditions. Overall, protective microbes are able to stabilise the host dynamics under a range of different conditions and parameter settings. These results indicate that not only do protective microbes operate to minimize how infection spreads in host dynamics, but also affect the broader scale population dynamics.

Introduction

Microbes are ubiquitous. Animal and plant hosts are never just an isolated organism. Each organism serves as host for a variety of microbes[1], which can be a combination of different microorganisms such as archaea, lower and higher eukaryotes, viruses and bacteria[2]. The bacteria among this collection of microorganisms can show a great variety and range from harmful to harmless, which can depend on the context of the interaction with their host[3,4]. A prominent example here is *Wolbachia*, which can act as a parasite in one context[5] or as a nutritional mutualist in another context[6]. Most of these microbes shape the evolution of their host, by either being harmful[7–9], or by transferring benefits to the host in form of protection[10–14]. The availability of these protective traits to the host[15,16] is referred to as 'defensive mutualism'[17,18]. Defensive mutualism can be observed across kingdoms, and include plants, animals and humans[3,10,12].

The interaction between hosts and protective microbes (PMs) involves a host that acquires microbes from the environment; these microbes increase host fitness relative to a host that does not harbour microbes, thus making the trait selectively favourable and, over time, more prevalent in the population[19]. This fitness increase can be achieved by the microbe providing access to previously inaccessible nutrients[20], or by providing protection against parasites[13,21].

The microbial species colonising a host can be transmitted either vertically, from parent to offspring, or horizontally, by environmental exposure[22]. Horizontal transmission can result in the host acquiring facultative PMs, which may not necessarily show such strong coevolution with their respective host. Nevertheless, the protection provided by this class of microbes can be strong[19,23]. Vertically transmitted PMs often show close coevolution with their respective host over a long time scale which can result in dependence on each other[10–

[12] and sometimes eventual obligate symbiosis[24]. Both horizontal and vertical transmission of PMs are commonly observed in nature[25,26], even though neither of the two transmission modes of facultative PMs was observed to span the whole host population. This may be due to imperfect maternal transmission[27,28], spontaneous loss of symbionts or costs associated with harbouring these facultative PMs[29].

The presence of PMs can complement or supplement the host immune system[30–32] and, through this, reduce investment costs and activation of a host's immune system[19,33,34]. However, harbouring facultative PMs is expected to be costly to the host[35,36]; this cost can be displayed in different ways such as reduction in longevity or lifetime reproductive success[36,37]. In the presence of infection, however, these costs are normally outweighed by the protective benefits that the microbe provides to the host[24]. These costs and benefits may also depend on population-level aspects such as the abundance or the density of the PM[34].

Infection dynamics can be described by an 'SIR' model[38,39]. Here hosts are considered susceptible (S), can become infected (I) and eventually recover (R) from an infection[39]. For infectious diseases it is a popular model framework and can be extended in different ways such as to include an exposed group of hosts (SEIR)[40], or to take quarantined individuals into account[41]. While SIR models have been extended to more complex ecological interactions (e.g., the effect of a predator on disease dynamics[42]), the presence of PMs on infection dynamics has not been investigated. In this study, I developed an SIR framework to investigate the role of vertically transmitted PMs on the ecological dynamics of infection dynamics. For this I explore three dynamical scenarios: (I) equilibrium conditions after an initial peak of infection[43], (II), equilibrium conditions after transient dynamics[29,44] and (III) unbounded, exponential growth conditions[45,46]. To explore the effects of PMs I investigate these dynamics in the presence and absence of the PM. In the presence of the PM, I investigate the cost versus benefit effects of harbouring PMs. The results highlight that a

PM can play a crucial role in host-infection dynamics, independent of different cost-benefit ratios, and, that PMs could dampen dynamics in major infection outbreaks.

Model construction

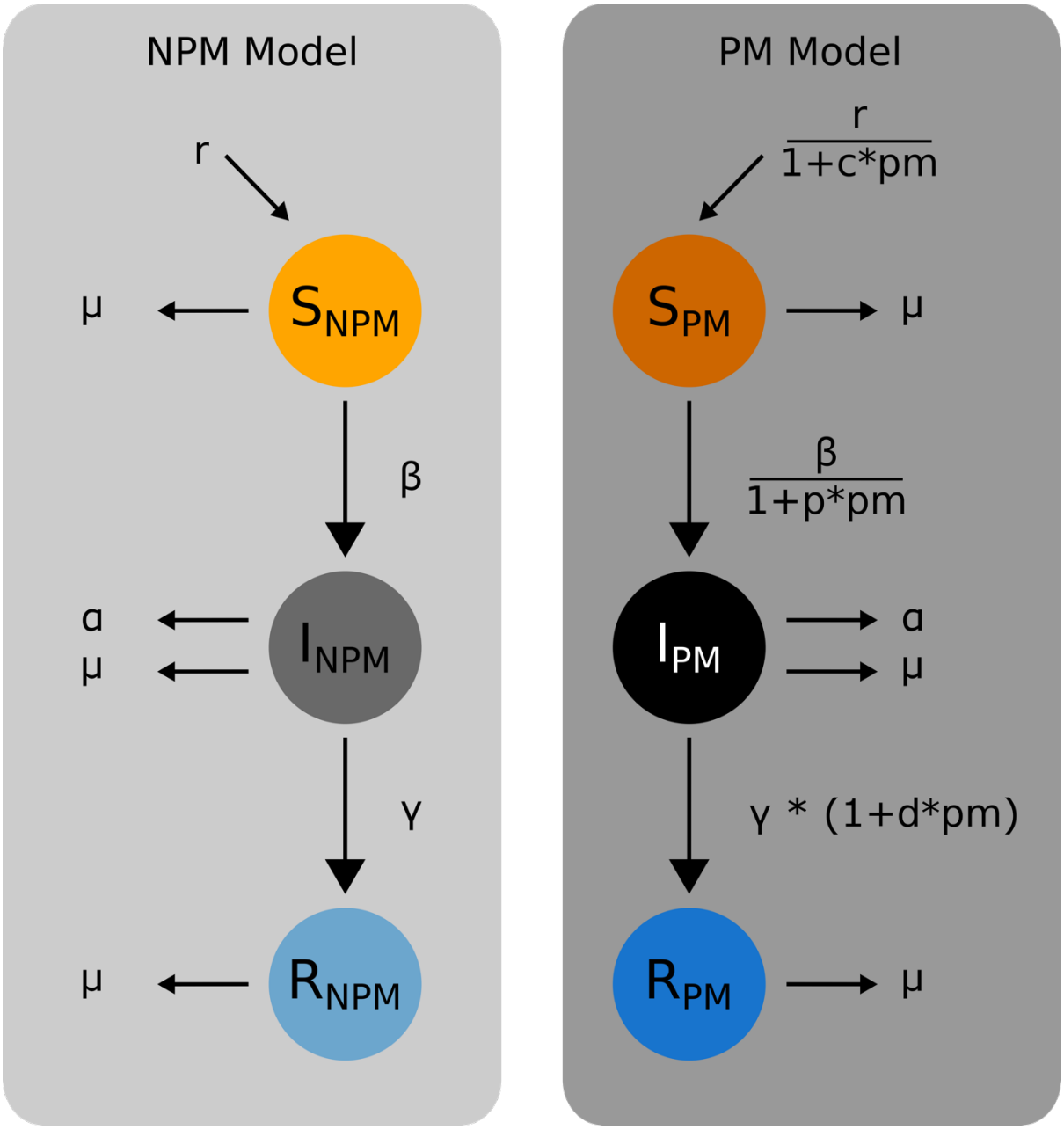


Figure 1: Schematic diagram illustrating the structure of the SIR model in the absence (NPM) or presence (PM) of the protective microbe. The SIR framework is defined by susceptible (S_{NPM} or S_{PM}), infected (I_{NPM} or I_{PM}) and recovered (R_{NPM} or R_{PM}) hosts. The differences between the NPM and PM model capture the cost and benefits of the PM. Parameters definitions are given in the text and values are defined in Tables 1 & 2

I used an SIR model to describe the dynamics of susceptible (S), infected (I) and recovered (R) hosts in the absence (NPM Model) and the presence of the protective microbe (PM Model). Schematics for the model frameworks can be seen in Figure 1 and are described in the differential equations (1) – (6) with an explanation of each parameter given in Table 1.

NPM Model:

$$\frac{\partial S}{\partial t} = r(S + I + R) - \beta SI - \mu S \quad (1)$$

$$\frac{\partial I}{\partial t} = \beta SI - I(\alpha + \mu) - \gamma I \quad (2)$$

$$\frac{\partial R}{\partial t} = \gamma I - \mu R \quad (3)$$

PM Model:

$$\frac{\partial S}{\partial t} = \frac{r(S+I+R)}{1+c*pm} - \frac{\beta SI}{1+p*pm} - \mu S \quad (4)$$

$$\frac{\partial I}{\partial t} = \frac{\beta SI}{1+p*pm} - I(\alpha + \mu) - \gamma I(1 + d * pm) \quad (5)$$

$$\frac{\partial R}{\partial t} = \gamma I(1 + d * pm) - \mu R \quad (6)$$

In equation (1) r is the birth rate, β is the transmission rate and μ is the background death rate. In equation (2), α is the disease induced death rate and γ is the recovery rate. In addition to these parameters in the PM model (equations 4-6), I define cost ($1 + c\ pm$), and benefit (($1 + p\ pm$) and ($1 + d\ pm$)) functions, where c, d and p are variables describing the strength of the cost and benefits. The parameter pm describes the density of the PM. These definitions are also given in Table 1.

Table 1: Explanation of Parameter values for the two models

Parameter	Explanation
S	Number of susceptible hosts
I	Number of infected hosts
R	Number of recovered hosts
r	Birth rate
β	Transmission rate
μ	Background death rate
α	Fatal infection rate
γ	Recovery rate
pm	Density of the PM
c	Costs of the PM

p	Protective Effect of the PM
d	Increased recovery in the presence of the PM

I investigated three scenarios with different expected ecological dynamical outcomes: equilibrium conditions after an initial peak (Scenario 1), equilibrium conditions after transient dynamics (Scenario 2) and exponential growth conditions (Scenario 3). The parameter values that generate these scenarios are given in Table 2.

Table 2 Parameter values for the three investigated scenarios

Parameter	Scenario 1	Scenario 2	Scenario 3
r	0.0025	0.003	0.0004
β	0.0002	0.0006	0.0005
μ	0.002	0.002	0.002
α	0.002	0.2	0.002
γ	0.005	0.08	0.005
pm	0.2	0.6	0.9
c	0.1	0.6	0.7
p	0.2	0.8	0.9
d	0.2	0.8	0.9

Together with these three different scenarios, I also explored, how the dynamics are changing under different cost-benefit ratios. The parameter values used for different cost-benefits ratios are given in Table 3. All other parameters are as mentioned in 2.

Table 3: Parameter values for different costs – benefits ratios

Parameter	Costs < Benefit	Costs = Benefit	Costs > Benefits
c	0.3	0.6	0.9
d	0.3	0.3	0.3
p	0.3	0.3	0.3

As PMs provide protection to their host, the number of infected hosts can be taken as a measure of efficiency of the PM[13]. The host is hypothesized to receive the highest benefit from the PM, whenever the PM provides sufficient protection from an infection. To investigate this at the population-level, I have determined the number of infected hosts at the peak of the initial epidemic.

Model assumptions

It is assumed that the PM is transmitted vertically from mother to offspring, and that the PM cannot be lost over a host's lifetime. Infection can only occur once in a host's lifetime and hosts are immune against reinfection once they have recovered from infection. Infected individuals are considered infectious as long as they are infected with the disease. The colonisation of the host by the PM is assumed to be described by the parameter *pm* and does not vary across different individuals of the population or the stage of infection. The density and the costs of the PM are not correlated, despite other examples from the literature[34]. I assume, that fatal infection is not affected by the presence of the PM.

Stability analysis

To analyse the dynamical stability, I used the Routh Hurwitz conditions[47] and determined the parameter combination that gives rise to stable dynamics. Mathematica (version 12.0.0.0)[48] was used to determine the Ruth Hurwitz conditions; R studio (version 1.1.463)[49] was used to generate plots of these results with the ggplot package[50]. To complement this stability analysis, I performed a sensitivity analysis. For this, the values mentioned in Table 2 were varied by ±50% for all three scenarios. A mean and standard error of the proportion changes in host numbers was determined.

PM dynamics

To assess the dynamics of the PM in the Scenario 2, I varied the parameter values for the PM (pm, c, d, p) ranging from 0 to 1, while the rest of the parameters was kept as described in Table 2. To investigate the effects of each parameter, the maximum number of infected hosts (in the first major epidemic peak) as well as the average across the whole simulation was determined.

Results

Dynamics of the NPM and PM Models

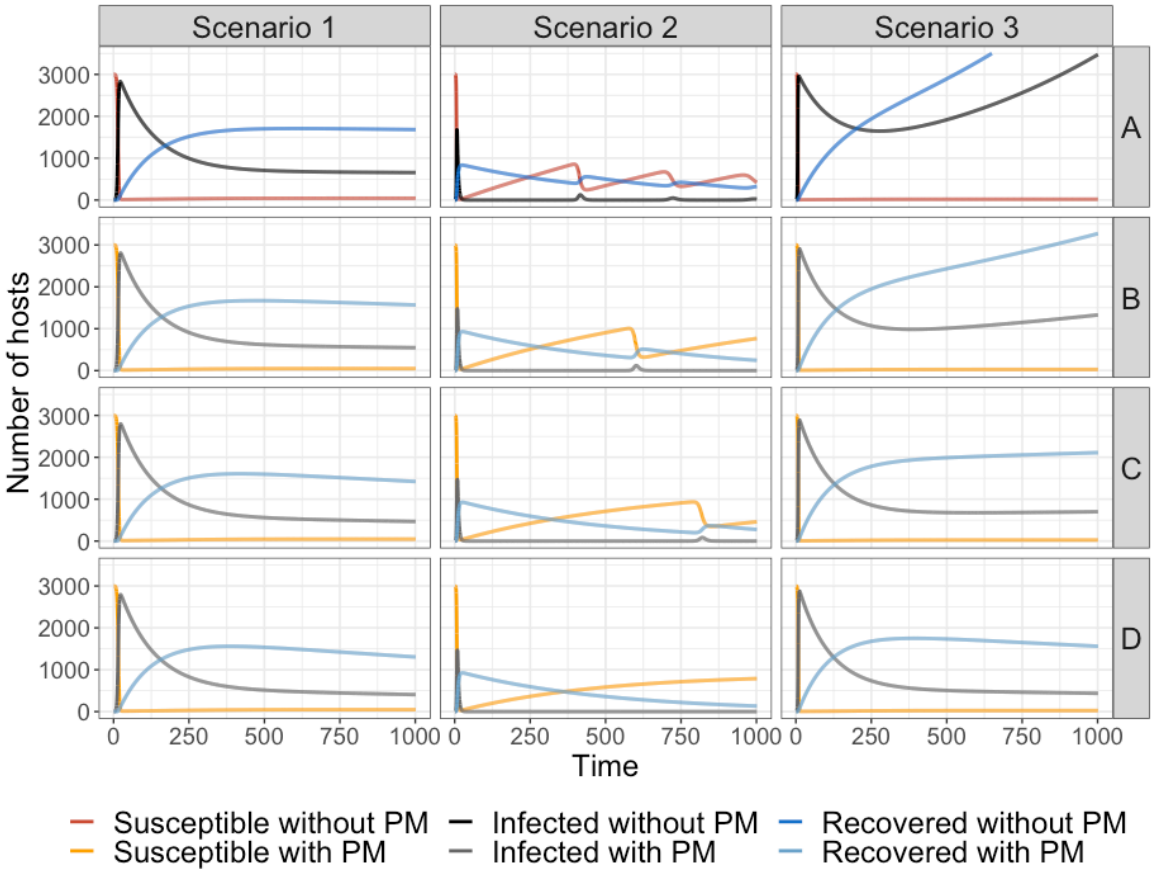


Figure 2: Different parameters of the equations result in three different scenarios: Equilibrium conditions after an initial peak (Scenario 1- left column) equilibrium conditions after transient dynamics (Scenario 2 - middle column), and exponential growth conditions (Scenario 3 - right column). The first row (A) shows the dynamics in the absence of the PM, while the rest of the columns show the presence of the PM under ratios of costs and benefits: The second row (B) shows conditions in which the costs are half as big as the benefit (c=0.3, p=0.3, d=0.3), the third row (C) shows conditions in which the costs and the benefits are of the same impact (c=0.6, p=0.3, d=0.3)and the last row (D) shows conditions in which the costs are 1.5 times greater than the benefits (c=0.9, p=0.3, d=0.3). Colours represent the different states a host can be in, orange for susceptible hosts, black and grey for infected hosts and blue for recovered hosts. The darker colour represents the NPM state, while lighter colours represent the PM state.

To understand the dynamical effect of PMs, I have used different sets of parameters to investigate three main dynamics: equilibrium conditions after an initial peak (Scenario 1), equilibrium conditions after transient dynamics (Scenario 2) and exponential growth (Scenario 3) (Figure 2). The exact parameter values for each set of dynamics are given in Table 2 and 3. Each dynamical scenario was modelled in the absence and presence

of the PM. In the presence of the PM the cost-benefit ratio of the PM was also investigated. Under equilibrium conditions after an initial peak in infection, the absence or presence of the PM did not influence the overall dynamics (Figure 2). Under equilibrium conditions after transient dynamics and exponential dynamics, the presence of the PM acts to stabilizes the dynamics (Figure 2). This stability is more pronounced when the costs are higher than the benefits of the PM.

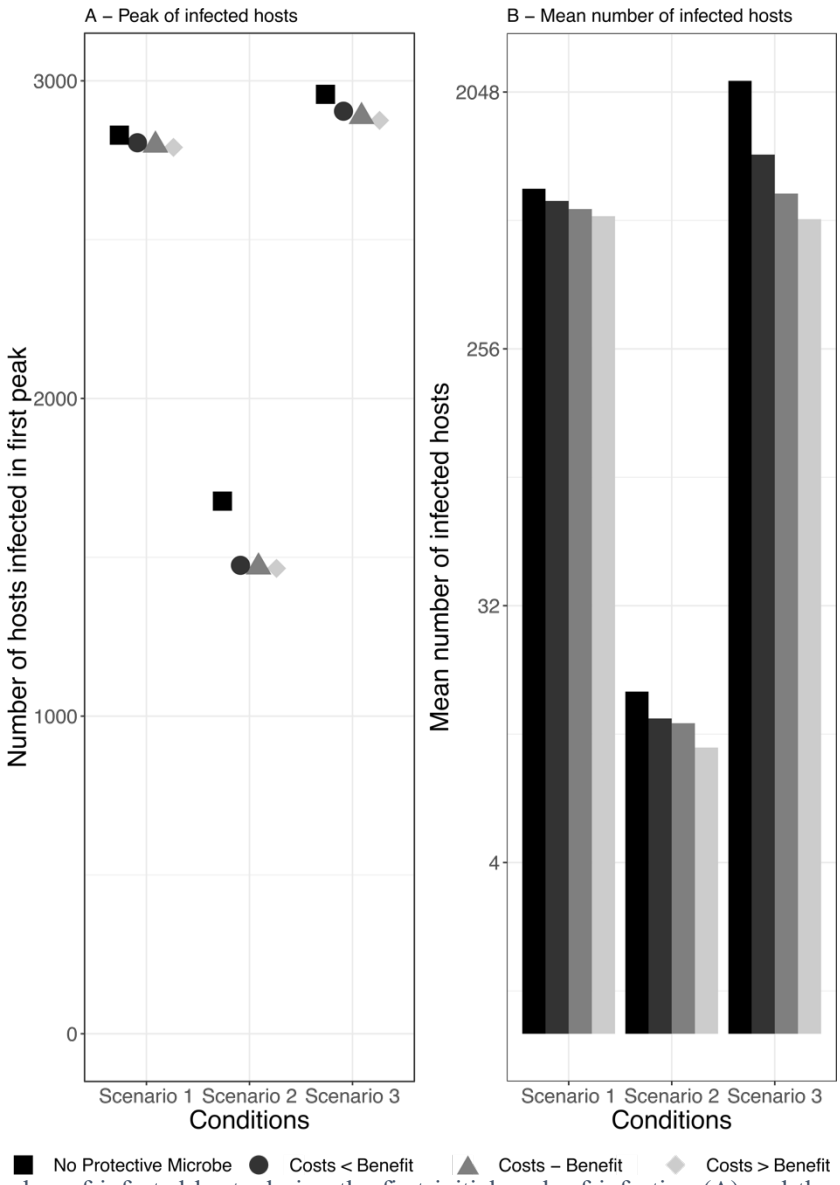


Figure 3: Number of infected hosts during the first initial peak of infection (A) and the mean number of infected hosts across the simulation (B) for all dynamical scenarios from 2. In all scenarios hosts without the PM experience the highest number of infections. In the presence of PMs, infections are lowest when the costs of harbouring the PM outweigh the benefits. Black shading indicates the NPM state, while different shades of grey indicate different PM states.

PMs provide protection from infection. Figure 3 shows, as expected, that the highest number of infected hosts occurs in the absence of the PM. In the presence of PMs, the cost-benefit ratio only leads to small differences on the peak number of infections in the epidemic (Figure 3A). However, these differences are more pronounced across the whole disease dynamics (the average of infected hosts over the simulation) (Figure 3B). Interestingly, the lowest number of infected hosts can be found when costs are higher than the benefits.

Stability analysis

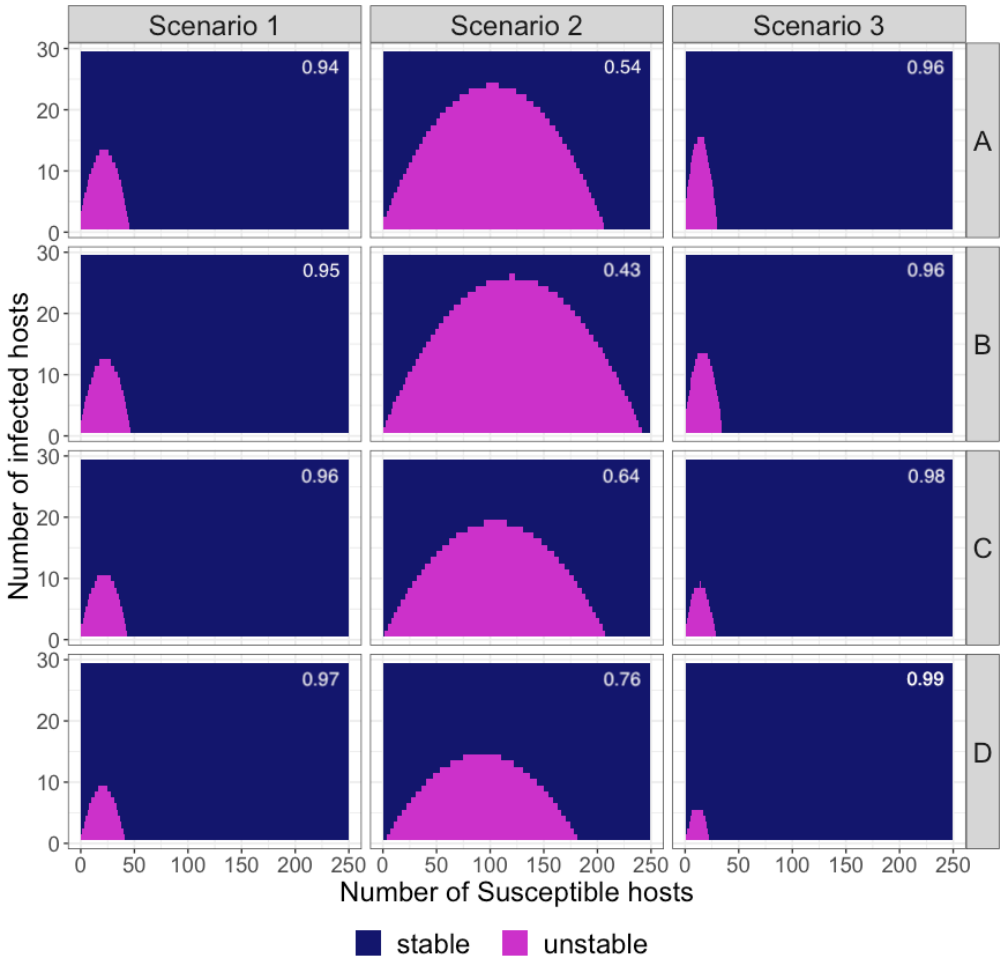


Figure 4: Stability of the different scenarios under Routh Hurwitz conditions. Three different scenarios (as in Figure 2) were investigated under NPM conditions (A - first row) and different ratios of costs and benefits of the PM (second to fourth row; B with costs half as big as the benefit (c=0.3, p=0.3, d=0.3); C with costs and benefits of the same impact (c=0.6, p=0.3, d=0.3); D with costs 1.5 times greater than the benefits (c=0.9, p=0.3, d=0.3)). Magenta indicates unstable conditions, while blue indicates stable conditions. The number in the top right corner refers to the stable area in the shown plot.

The scenarios from Figure 2 are stable under a certain combination of susceptible and infected host numbers. All scenarios are unstable with low numbers of infected and susceptible hosts (Figure 4). If the costs of the PM are equal or higher than the benefits of the PM, the different dynamics show a greater stability than under NPM conditions, while if the costs of the PM are lower than the benefits, the NPM scenario is more stable.

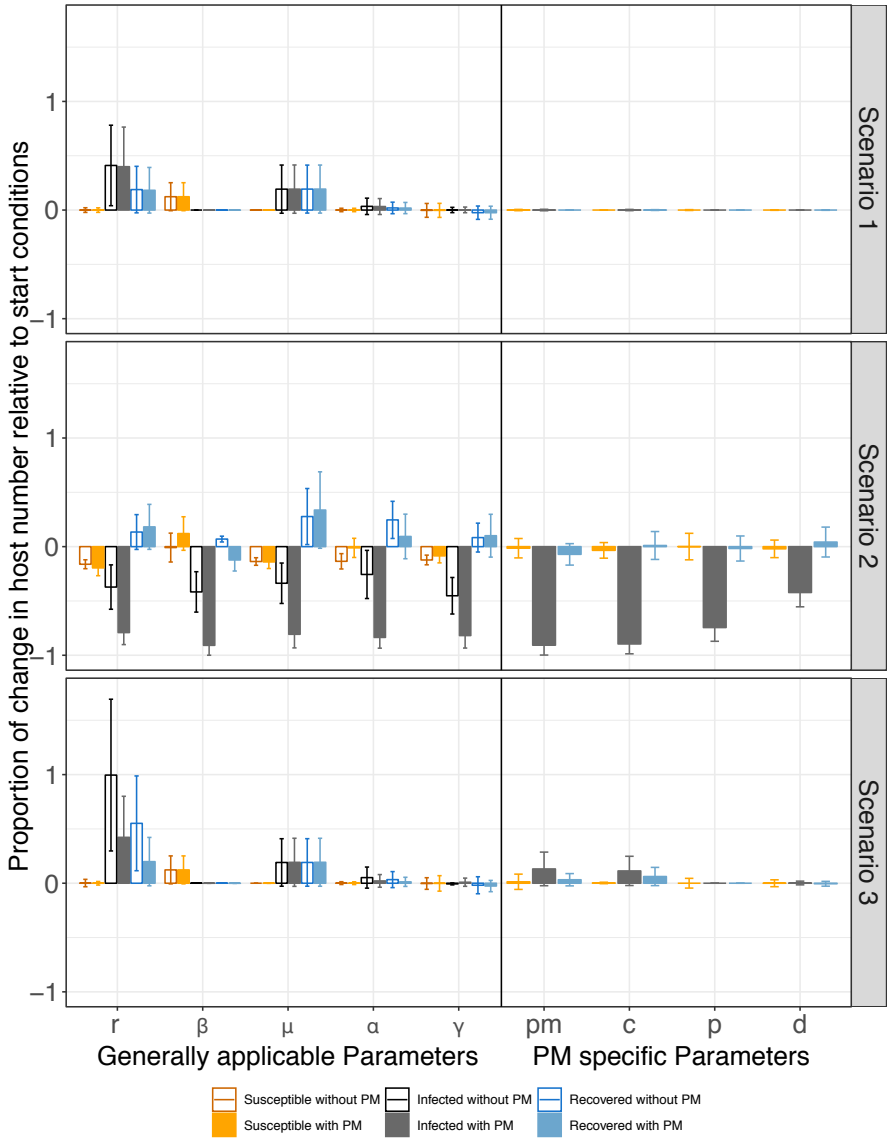


Figure 5: Sensitivity analysis for the three scenarios from Figure 2 in the absence and present of the protective microbe: Equilibrium conditions after an initial peak (top row) equilibrium conditions after transient dynamics (middle row), and exponential growth (bottom row). The y-axis shows the proportion of change in host number relative to the start conditions. Empty bars depict the absence of the PM, while filled bars depict the presence of the PM. Each bar represents the mean ± standard error for the change in host number ranging from -50% to 50% of start conditions. The infected stage is most affected across the different scenarios. *r* and *μ* have the most impact in the first and third scenario independent of the presence or absence of the PM, while the infected stage for the second scenario is equally influenced by almost all parameters. The bars for the parameters *pm*, *c*, *d* and *p* are thicker, as these parameters are not present in the NPM state.

To understand the influence of different parameters on the dynamics, I have performed a sensitivity analysis[51]. It revealed that under equilibrium conditions after an initial peak (Scenario 1) and under exponential growth conditions (Scenario 3), r (birth rate) and μ (background death rate) have the most influence on the dynamics by affecting the infected and recovered stage (Figure 5). Under the equilibrium conditions after an initial peak (Scenario 1), these birth and death rates have similar effects in the absence or presence of the PM. In contrast, under exponential growth conditions (Scenario 3), r has a larger influence in the absence of the PM. Under the same conditions the density of the PM (pm), as well as the costs (c) also have a larger effect compared to other parameters. Under equilibrium conditions after transient dynamics (Scenario 2), all parameters have the same effect in the presence or the absence of the PM, with the infected stage in the presence of the PM being the stage most affected by alterations in parameters. This can be explained by low numbers of infected hosts in this scenario where a small absolute change can lead to a large proportional change. Under these conditions, r and μ positively influence the number of recovered hosts, while most other parameters influence host numbers negatively (Figure 5). Under exponential growth conditions (Scenario 3), r (birth rate) and μ (background death rate) have the most influence on the dynamics by affecting the infected and recovered stage (Figure 5), just as in Scenario 1. In Scenario 3, the effect of the birth rate is however greater in the absence of the PM than in the presence of the PM. Additionally, the density (pm) and the costs (c) of the PM affect the dynamics.

PM dynamics

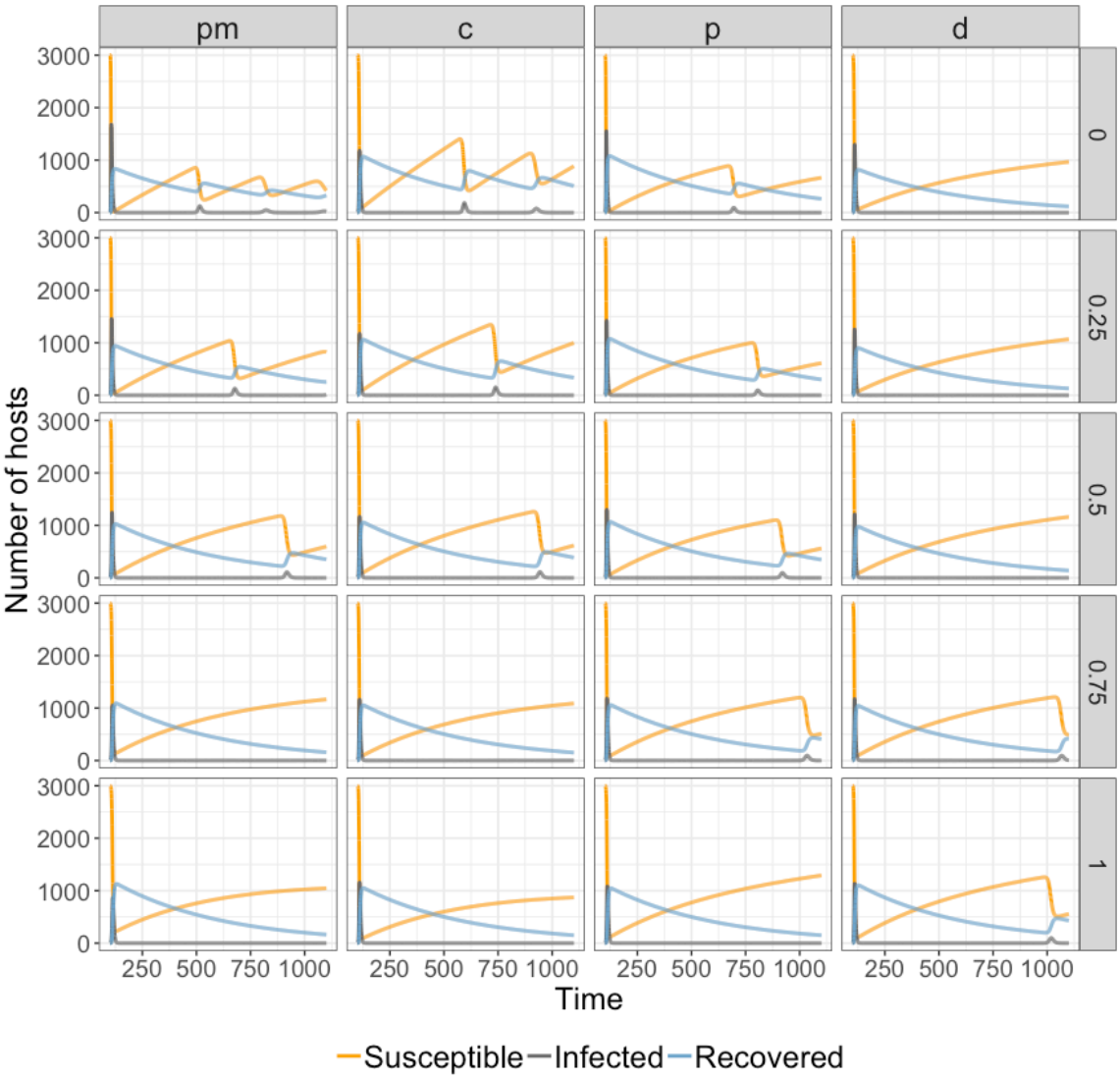


Figure 6: Scenario 2 with different parameter values for the parameters important for the PM. Parameters include *pm* (left column), *c* (second to left column), *p* (second to right column) and *d* (right column). The values for each of these parameters range from 0 (second row), 0.25 (third row), 0.5 (fourth row), 0.75 (fifth row) to 1 (sixth row). The first row of each column shows the dynamics in the total absence of the PM. All other parameters are kept as listed in Table 2.

To assess the influence of these parameters in Scenario 2, I have investigated the conditions under parameter conditions ranging from 0 to 1 (Figure 6). The parameters *pm*, *c* and *p* act to stabilise host dynamics and decrease the infection peaks as the value for each of these parameters increases. For parameter *d* it is the opposite. For smaller *d* values, more stable dynamics are likely.

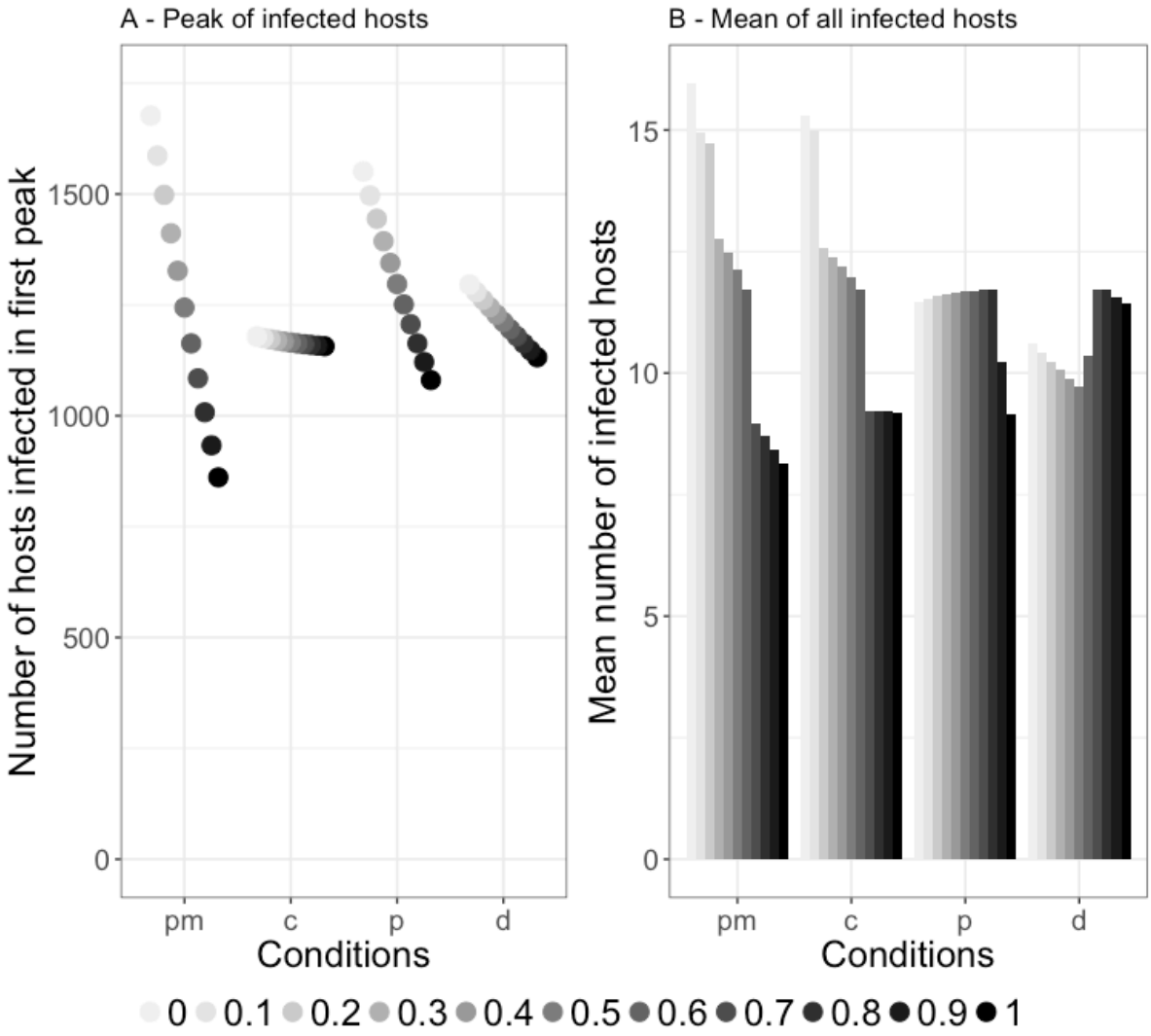


Figure 7: Number of infected hosts during the first initial peak of infection (A) and as a mean across the simulation (B) from Figure 6. The peak of infected hosts decreases the higher each parameter is, except for *c*, where a change is only subtle. For the mean of infected hosts, all numbers decrease with an increase in parameter, except for the parameter *d*.

Figure 7A shows that the increases in *pm*, *p* and *d* reduce the size of the epidemic (reducing the number infections in the first initial peak). The cost parameter *c* has little influence on the size of the epidemic. In comparison to this, the average of infected hosts over the whole of the epidemic dynamic decreases with an increase in the parameters *pm*, *c* and *p*, but increases with an increase in the parameter *d* (Figure 7B).

Discussion

Here I have explored the effects of the presence and absence of a vertically transmitted protective microbe (PM) on host dynamics using a disease dynamic (an SIR) framework. My results indicate, that the largest differences in number of infected hosts can be found between the presence and absence of the PM as shown before[34], while the exact cost-benefit ratio of the PM is less important. Interestingly, whenever the costs are higher or equal to the benefits, the PM is able to stabilise the dynamics the most. This is surprising, as high costs are hypothesized to be the reason for a heterogenous spread of facultative PMs within a host population[27,28,36]. The costs of the PM are known to affect host lifespan[36] and lifetime reproductive effect[37]. These can be caused by direct mechanisms (such as damage of soma due to toxin production by the PM in the absence of a parasite[36]) or indirect mechanisms (such as activation of host encoded resistance to an infection in the absence of the PM[34,52]). Here I consider the cost of a PM as an ecological cost on the amount of offspring that is produced in the population, as it has been described before[37]. This can thus lead to a trade-off between offspring quality and quantity on the host population level. The trade-off between quality and quantity of offspring has been described at an individual level as 'Bateman's principle'[53–55]. Bateman's principle describes females' investment in offspring quality and males' investment in offspring quantity.

On an evolutionary timescale, the costs of the PM have been hypothesized to play a crucial role in the dynamics between the host and the PMs[29]. Despite incurring these costs for acquiring the PM, the host might still invest in the maintenance of a PM, if the overall net benefit of the PM's maintenance is less costly than activation of the host's own immune system[56]. Some hosts have outsourced their resistance to infection to a PM[57], while in other systems hosts do better with both intrinsic resistance and the presence of the PM[56]. This investment in the maintenance of the PM is mainly dependent on the likelihood of becoming

infected[22,58–60]. While, here I only focus on the ecological implications of the costs on the population, and do not consider the evolutionary dynamics, if the higher costs to benefits were maintained over evolutionary time, the overall dynamics might look different, as the PM would evolve to become a parasite with higher costs than benefit[29].

In the ecological timescale that I assessed here, all dynamics are unstable at low numbers of infected and susceptible hosts, while the number of susceptible hosts has a larger influence on the stability. The presence of the PM stabilizes these dynamics even more by increasing conditions that lead to higher stability. The rate of birth and background death have the largest influence on the dynamics, mostly via influencing the infected and recovered state. Interestingly, the population size is increasing whenever the background death rate is increasing particularly in Scenario 1 and 3. This phenomenon has been described as a 'hydra effect'[61], which describes density dependent effects that lead to increased population size despite increased mortality rate[61].

Under transient dynamics, most PM parameters can drastically reduce the initial epidemic peak, while the costs of the PM (parameter *c*) are only able to do so slightly. In this model, I have defined costs to affect the birth rate, as previously described[37]. The effect of this will only play out over longer ecological time and does not affect the initial endemic peak that much. Over longer ecological time, most parameter combination lead to a decrease in the mean number of infected hosts, while only the benefits associated with host recovery (*d*) leads to an increase in mean number of infected hosts. The parameters for the PM (*pm*, *c*, *d*, and *p*) of the constructed model under conditions for Scenario 2 (equilibrium after transient dynamics) are all affecting the route in which the equilibrium is approached, while no parameter setting explored here resulted in unstable conditions. While the host dynamics are all mathematically correct, the biological application might only be relevant with increased host population size. In Figure 6 the number of infected hosts drops below one

after the first epidemic peak and would thus go extinct without immigration of infected hosts again[62]. This suggests elimination of the infection after the first endemic peak under the influence of the PM.

Even when I varied the density of the PM, I did so for the whole set of dynamics and assumed that this density of the PM is stable across the simulation. The density of the PM was however found to have an effect on the level of protection[34]. Alongside this PM density effect there are other factors that are known to have an effect on the protection provided by the PM, such as host age structure, genotype by genotype interaction or superinfection[28,36,63]. Here I also assume, that the PM is perfectly vertically transmitted from mother to offspring, when even small deviations from perfect maternal transmission can influence the result[29]. Further directions for future work could focus on integrating these different assumptions into further understanding of the ecological effects of protective microbes, such as allowing PM density to change over the time course of infection or allowing imperfect maternal transmission.

In conclusion, my results highlight, that despite high costs of the PM, a vertically transmitted PM can act to stabilise host dynamics during an infection. If the number of susceptible and infected hosts is sufficiently large, the dynamics are stable and are mainly influenced by the rate of reproduction and background death. These results indicate, that the interaction between a PM and a host are more complex than have currently been considered; more attention needs to be given to the interaction between the ecological and evolutionary dynamics of defensive mutualisms.

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Chapter 5

General Thesis Discussion

This thesis deals with the ecology and evolution of protective microbes. Much research has focused on the various aspects of microbial communities, such as identifying community members[1,2], the interactions between microbes[3–5], and understanding potential effects on the host[6]. Despite these findings, many genes of the model organism I used here, *Caenorhabditis elegans*, are still lacking functional annotation and a defined phenotype[7]. *Caenorhabditis elegans*, was the first multicellular organism to be completely sequenced[8] and most of the work on this model system has been performed on the generic laboratory strain N2 under laboratory conditions[7], while nature is much more heterogenous than the laboratory conditions. It is thus possible, that certain phenotypes can only be described under more natural conditions[7]. Here I have used a different worm strain to N2 (the line EEVD00[9]) and have reared worms on different bacteria than the generic *Escherichia coli* OP50[10]. With these settings I have investigated evolutionary and ecological differences of the host and an associated protective microbe.

After providing an overview of the current literature in the introductory **Chapter 1**, in **Chapter 2** I described a large-scale evolution experiment in which I varied pathogen infection temporally. My results indicate that the presence of the pathogen is required for an enhancement of microbe-mediated protection. The interval of pathogen infection or the initial pathogen presence is, however, not a significant driving factor in the persistence of microbe-mediated protection. I could not detect any long-term fitness costs.

In **Chapter 3**, I focussed on the effect of host sex and mating with a protective microbe (PM). For this I set up different mating treatments (unmated, short-term mated and lifetime mated) and then exposed these groups to different treatments: food alone, pathogen infection alone or pathogen infection in the presence of a PM. My results indicate that females and males both benefit from the presence of the PM but use the acquired benefits differently. In the presence of the PM, females invest more energy in egg production, while

males display higher mate searching behaviour. Overall, females are more susceptible to the effects of mating and pathogen infection.

In **Chapter 4**, I used a mathematical modelling approach to explore the effect of a PM under different conditions. I have modelled host dynamics in the absence and presence of the PM and found that the PM is able to stabilise different forms of ecological dynamics (equilibrium conditions after an initial peak, equilibrium conditions after transient dynamics or exponential growth). I further investigated whether the cost-benefit ratio affects host dynamics and found the largest stabilising effect to occur when the costs are equal to or higher than the benefits. The dynamics are unstable if the number of susceptible and infected hosts is small, exact numbers depend on the scenario tested. The higher the cost-benefit ratio, the lower this number of susceptible and infected hosts can be to still assure stabile conditions. The rate of reproduction and the background death rate had the strongest effect on the dynamics, mostly affecting the infected state. Different parameter settings for the PM indicate, that the PM is able to stabilise the host dynamics under a range of different parameter settings.

Synthesizing the chapters

While I was collecting data for Chapter 2, I observed a difference between female and male worms while counting the long-term survival data. Males were living twice as long as females. I also observed that females and males were found in different areas of the plate. Given these observations, I conceived the experiments for Chapter 3. Worm populations from Chapter 2 are thus relatable to lifetime mated worms from Chapter 3. The results from Chapter 3 indicate that females suffer more from pathogen infection, despite the presence of a PM. The prevalence of a survival phenotype observed in Chapter 2 might thus be driven

by differences in mortality rates between females and males. During data collection for Chapter 2, I suspected that avoidance behaviour might play a role in survival, however, I confirmed by an experiment (data not shown) and by data in Chapter 3 that this is not the case. Lifetime mated males in Chapter 3 do not show higher avoidance behaviour compared to lifetime mated females but seem to be able to cope better with pathogenic infection. One possible explanation is mechanical gut integrity. *Drosophila* males have stable gut integrity even during aging, while *Drosophila* females show high age-related intestinal gut integrity degradation[11]. I have observed a similar pattern for lifetime mated worms, namely that females showed reduced gut integrity, while males did not (data not shown). As this was not properly tested, but only made through observation, it remains an open conjecture but is a useful direction for future experiments. These future experiments could consist of either performing microscopy to identify the structure of the gut, or performing a so called "*smurf* assay", during which the worms are fed food with blue dye, and afterwards one can observe where the blue dye can be found[6]. If the gut integrity is maintained, the blue dye should only be found in the gut, while it leaks into the body cavity if gut integrity is not maintained.

This thesis focuses on the ecology and evolution of protective microbes, which I investigated in two experimental chapters and one theoretical chapter. Nevertheless, the results from the mathematical modelling and from the two experimental chapters while complimentary, may not be directly compared. During the experiments, hosts were infected with *S. aureus* by environmental exposure (Chapter 2 & 3), but not due to contact between different hosts (as in Chapter 4). All hosts were susceptible and infected at the same time, while there might only have been differences in how quickly a specific host recovered. To make the model and the experiments comparable, the term for contact between the hosts would need to be removed, and a measure for infection via environmental exposure would need to be introduced. The type of model is however well suited for an infection that is

being transferred directly from host to host, with negligible environmental effects such the existence of a long-lived, free-living pathogen stage, such as Measles or Ebola[12–14]. Another fundamental difference between the experiments and the developed model is host age and generation time. In the experiments in Chapter 2 and 3 there are no overlapping generations. All hosts in the experiment always have the same age and developmental stage, while in the model developed in Chapter 4 hosts are allowed to reproduce continuously.

One advantage of developing a mathematical model of PMs over the experiments is the easy investigation of costs and benefits of a PM. Each panel of Figure 2 and Figure 6 in Chapter 4 would require a separate experiment over many generations and might even require changing host and infection system, as costs and benefits might not be readily amenable to alteration. One option to get around conducting these experiments would be to use long term studies from the wild[15]. However, these long-term studies might be influenced by heterogeneity that is more difficult to control, such as the pronounced drought on the Galapagos islands in 1977 that had consequences on the beak and body size of Darwin's finches[15].

Influence of heterogeneity

To my knowledge, the influence of pathogen heterogeneity on (co)evolution between a host and a protective microbe has not been investigated. There have been studies that tested for constant pathogen presence[16–19], or heterogenous effects[20–24] on the coevolution of two species, but none combines both. This is thus the first study, that reported that it is the presence of the pathogen rather the heterogeneity of pathogen infectivity that has a major effect on the coevolution of the host and a protective microbe.

The diversity in a particular system will always be affected by disturbances, may it be seasonal changes[25] or differences in weather[26]. If the disturbance in a system is at an intermediate level, Joseph Connell hypothesized in 1978, that the diversity in such system should be the highest, when compared to systems with low or high rate or disturbances (Intermediate disturbance hypothesis - IDH)[27]. This IDH has been tested in the field for effects of dilution disturbances on natural plankton communities[28] or for effects of disturbance in marine intertidal communities[29] and in laboratory set ups for effects of oxygen concentration on *C. elegans*[30], for effects of dilution disturbance on *Daphnia*[31] and phytoplankton[32], for effects of resource pulses on *Serratia* and *Tetrahymena*[23,24], for effects of temperature fluctuations on *Serratia*[33] or for effects of productivity on *Pseudomonas fluorescens* and a phage[22]. The interval of the disturbance affects the outcome in some studies[22–24,31,32], but not in all[28]. Shorter intervals affected the evolution the strongest, either by slowing down evolution[22,23,32], by increasing clonal diversity[31], or by increasing fluctuations, which suggests an evolution of generalists[24]. Sometimes only the absence vs. the presence of disturbances was tested, while no intervals of disturbance were taken into account[29]. While all of these above described examples are abiotic factors, the effect of biotic factors such as pathogens[16] and herbivores[34] has also been described[16,34], despite not testing for heterogenous effects but only comparing absence and presence of a particular factor. Heterogeneity can also be observed for the PM. Within one host population different strains of PMs are present[35–40] that do not fully spread across the whole population[41–43]. This observation suggests costs for the maintenance of PM for those hosts that carry a PM, while those hosts that do not carry a PM should not experience any costs.

Costs of the protective microbe

Here I could not detect any long-term fitness costs to the maintenance and enhancement of microbe-mediated protection (Chapter 2). I measured these fitness costs in terms of longevity after exposure to the PM and pathogen infection, and in terms of offspring production at the stage that is under selection during experimental evolution. No observable differences were detected in these traits between those hosts that have evolved higher microbe-mediated protection and the ancestor. PMs are however suggested to be associated with costs[43–48]: in the absence of infection, it is expected that PMs should have a fitness cost. In the presence of infection, every host with a PM has an advantage over those hosts without a PM[48]. If there are no costs to the system, every host that faces a potential infection would carry a PM and would thus be superior to those hosts not carrying a PM[48]. It is however not observed, that a facultative PM had spread through the whole population, which suggests the presence of costs[41–43].

It is thus more likely that the costs in my project are too low to be detectable[49], I used a suboptimal measurement to detect the costs or the costs have been ameliorated by the provision of food, as it was previously shown[50]. As PMs are suggested to come with costs to reproduction and/or longevity[43,51], I could have just used a suboptimal measurement of costs. I would thus propose to measure reproduction and longevity in the sole presence of food alone, in the sole presence of the PM or the sole presence of the pathogen. Furthermore, reproduction could also be measured as lifetime reproductive success, rather than as a single point in time. Methodological improvements might also be required. For instance, the method I used to determine differences in egg production could also be improved: eggs present on the plate were counted, which make up most of the individuals that make it into the next generation. To capture the picture more fully, one could also count the eggs inside a mother; these eggs will also be released upon bleaching[52].

The absence of detection of costs in this system could also be related to the density of the PM[51]. Lower PM densities could lead to lower absolute costs, despite the transfer of a fully protective effect. I have however not controlled for any density effects of the PM, as I assumed all grown bacterial cultures would reach log phase in an over-night culture. It has been hypothesized previously that higher PM density leads to a lower pathogen fitness[53]. In the mathematical model developed in Chapter 4, the costs of the PM are not linked with PM density and I assumed that the density of the PM is static over the course of an infection. If this would be adjusted to represent a more dynamic situation where a lower density of the PM at the beginning of the colonization, or with a higher PM density upon pathogen infection, I would hypothesize, that the host dynamics would be stabilized the most when the costs of the PM are lower or equal to the benefits of the PM. Furthermore, one could also allow the pathogenic infection to be density dependent. Higher pathogen burden was shown to be correlated with higher mortality rate[54]. It was furthermore shown, that the presence of a PM was able to decrease the pathogen burden within a host[54]. I would thus hypothesize, that if one allows the PM and the pathogenic infection to be density dependent, that a high PM density and thus high PM costs would result in the highest infection reduction and thus in the highest host benefit[54], just as I have shown it in Chapter 4.

If hosts have outsourced resistance to infection to a PM[55], one would expect that in the absence of the PM those hosts with the highest pathogen contact would perform worse. However, it has been suggested that in high pathogen prevalence environments, hosts would show higher fitness with a combination of own activated immunity and the presence of a PM[48]. As I observe differences between females and males in how they respond to an infection and given that females seem to be more susceptible to an infection, there is also the possibility that costs were not detected as I always took population-level measurements,

rather than separated males and females, as in Chapter 2. Measuring sex-specific proportions of survival might help to further understand the present phenotype.

In the mathematical model, a higher cost-benefit ratio stabilises the dynamics. In this model, costs were only modelled as a reduction in offspring production. This translates into hosts producing less offspring, but due to the benefits of the PM having a higher quality of offspring. Smith and Fretwell hypothesized in 1974, that an optimum fraction of the energy available to the host should be used to maximise total reproduction over a host's lifetime and that fewer offspring would be produced if there is a higher effort per parent invested into each produced offspring[56]. This trade-off between quality and quantity underpins the principle that has been proposed for the difference between the sexes (Bateman's principle[57–59]). While I have only considered costs on reproduction in the model in Chapter 4, the costs can also affect lifespan[51]. If these costs were to be integrated into the model, I would expect that those host populations with higher benefits over costs would do better. Moreover, if the costs are too high, then the PM is likely to act as a pathogenic infection and this is mostly likely to result in antagonistic coevolution[60].

The influence of bacterial diet and different host genotypes

As a pathogen I have used *S. aureus* MSSA476. In Chapter 2 I showed that *E. faecalis* can transfer protection against this particular strain of *S. aureus*. The ancestral survival of the ancestral worm strain to MSSA476 in the presence of *E. faecalis* is already substantial (around 93%)[61]. If I would have chosen a more virulent *S. aureus* strain, against which *E. faecalis* also transfers protection (such as was shown previously[62]), my results might have been more clear, and the detection of costs might have been easier. In comparison to previous work, I also observe reduced levels of pathogenicity of the same *S. aureus* strain[62]. This could be due to the worm strain used, as other studies have used a generic

hermaphroditic strain N2[62], while I used the strain EEVD00[9]. This latter strain possess the genetic diversity of 16 natural isolates[63] that might have a higher natural resistance to *S. aureus* infection. This pattern in resistance and a difference between lab-adapted and more natural hosts has already been described before[49] just as differences in survival for different natural isolates of *C. elegans* after infection with *Bacillus thuringiensis*[64] or *Serratia marcescens*[65]. Future work could compare different natural isolates and test for differences in resistance to *S. aureus.*

Different bacterial diets were tested in Chapter 3. Normally, *C. elegans* is kept on *Escherichia coli* OP50[10]. To my knowledge, sex differences have only been explored on the normal laboratory food, *Escherichia coli* OP50[66–69]. Here, I have used a non-pathogenic strain of *Salmonella* as food, that formed biofilms on the plates (personal observation). *Salmonella* in biofilms is known to persist in *C. elegans* and to not shorten lifespan[70]. Despite not knowing exactly the details of this *Salmonella* strain, I report that worm development and growth was not impacted, just as described for other *Salmonella* species and strains[71,72]. Worm growth is known to be supported by other bacterial strains such as the worm's natural microbiota[1,6]. The availability of macronutrients can, however, be different depending on the bacterial strain[73–75]. It would be important to identify if the specificity of the *Salmonella* strain that might have a different nutrient provision than the generic *E. coli* OP50 strain.

While for the *E. coli* OP50 strain, it is known that gut colonisation can be negligible when worms are young[72], strains of *Salmonella* have been shown to colonize the gut[72,76–78]. If the used *Salmonella* strain is forming biofilms in the worm's gut[70], this could be another explanation for the reduced pathogenicity of *S. aureus*, that I described earlier. Further research is needed to identify how this strain of *Salmonella* is behaving in the gut. This research could be driven by questions such as "What is the interaction between *E. faecalis*

and *Salmonella* or *S. aureus* and *Salmonella*?" or "Does *Salmonella* itself possess protective properties?". So far, most *Salmonella* research has focussed on pathogenic strains of *Salmonella*[76,78–82], while few studies have reported strains that promote worm growth and development[71,72]. The difference between the pathogenicity of MSSA476 in Chapter 3 and previous results[16,62] could also be attributed to the yet to be identified differences between *E. coli* OP50 and *Salmonella*. A study with the same worm, *E. faecalis,* and *S. aureus* strain, observed a lower level of survival when worms were raised on *E. coli* OP50 and then infected with *S. aureus* (around 80%)[16]. These differences furthermore point to differences between *Salmonella* and *E. coli* OP50 as the reason for the high baseline survival (around 93% in my project) upon *S. aureus* infection.

The set-up of the experiments in the Chapter 3 did not allow for a direct comparison of bacterial diets, as it was not logistically feasible to do all comparisons at the same time. Alternative experimental designs for larger experiments to test differences between bacterial diet and mating would open new and interesting questions such as "Do those males with the PM perform better during pathogenic infection than those males without the PM during pathogenic infection?" Furthermore, it would also be interesting to test what the effect of diet and the PM is in the absence of a pathogen.

Caenorhabditis elegans hermaphrodites (or mothers in my project) were observed to show matricide (intrauterine egg hatching with the results of parental death[83]) under starvation conditions, stress, high population densities or old age[84]. Matricide can be rescued, once worms are provided with enough nutrients[84] and can ensure that the produced offspring are provided with sufficient energy to develop, even if this comes at the cost of parental survival[84]. Both the bacteria I used here (*E. faecalis* OG1RF and *S. aureus*) are known to introduce a starvation response in *C. elegans*[85]. *Enterococcus faecalis* OG1RF is also known to introduce high rate of matricide[86], which is also referred to as "bagging". One

could also argue the two strains introduce bagging for different reasons. *Enterococcus faecalis* might introduce bagging to increase transmission from mother to offspring, while *S. aureus* infected worms show bagging to provide their offspring with sufficient nutrients to enable them to survive the surrounding pathogenic conditions. This matricide can only be observed for the birth giving sex, which are hermaphrodites (or females in my project), which could also contribute to higher levels of female death rates.

The influence of sex

The results from Chapter 3 highlight that the two sexes are affected differently by pathogenic infection and also benefit differently from the presence of the PM. From other systems it is known that females and males fight infection differently[87]. In comparison to males, females have to invest more energy into the offspring, as the egg production is more costly than sperm production[87]. This translates into females having to acquire more resource and thus having a higher risk of pathogenic infection. I observe that females show higher bacterial colonisation than males do, which is even further increased after mating (personal observation), despite other studies having found the opposite[88,89]. While the proportion of missing worms would normally be interpreted as avoidance behaviour, I argue here, that (in addition to the differences due to mating) this phenotype is due to the availability of bacterial diets and thus different energy availability.

Differences exist in other female and male life history traits, such as longevity. Most studies that report sex differences in longevity come from wild populations[90]. In most studies, females live longer: such as in lions, prairie dogs, and primates[91], even though there are examples where males live longer, such as in the pilot whales, where males live twice as long as females do[92]. Male lifespan is expected to be reduced whenever male-male

competition is high, while female lifespan is expected to be reduced whenever females are providing costly and intensive parental care[93]. Chapman and colleagues hypothesized, that the effects of decreased lifespan are more apparent in experiments than they would be in nature, due to high nutrition provided throughout the experiment[94]. High nutrition environments were provided throughout my study.

The sex of a host is, however, not the only thing that contributes to the difference between the sexes. As I show in Chapter 3, the effect of mating is also responsible, as females are more affected by mating than males. In some species mating has been shown to activate expression in immune genes[95,96], but not in other species[97]. For some animals this results in increased mortality rates linked with increased rates of mating (e.g., *Drosophila*[98] or birds[93,99]). This idea of the effects of increased mating translates to my treatment of lifelong mating, where males are mating with females repeatedly. Females suffer the most from mating, as they can potentially be physically damaged by mating (in *C. elegans* a copulatory plug can be put on the female's vulva[100]). In addition to these external costs, females might also carry internal costs. If a female's immune system and reproduction is regulated by the same gene that acts antagonistically, then females may suffer more from an infection during reproduction[101]. In *C. elegans*, one gene that has these pleiotropic characteristics is *ins-11*[67,102,103]. This gene has been hypothesized to be involved in epidermal wounding due to mating[102]. The involvement of *ins-11* could be investigated with two main methods: the removal or the overexpression of the gene *ins-11*. Removal of the gene could be facilitated by the generation of knock-out mutants[104], the knock-down via RNA interference (RNAi)[105] or the use of CRISPR/Cas9[106]. Knock out mutants might however be difficult to integrate into this particular worm strain, as one has to introduce a mutation in *ins-11*, which can only happen by chance, followed by many PCRs to confirm the homozygote mutation in *ins-11*[104]. The RNAi machinery does not knock-out genes entirely, but only supresses a gene

function[105]. The typical RNAi library is however designed to work best on the generic N2 strain[107]. To avoid incompatibility between the RNAi library and the used worm strain, one could design their own RNAi strains[108] to knock down specific genes that are not covered by the Ahringer library or not designed for other worm strains. Whether *ins-11* can be knocked down in this worm strain needs to be tested. CRISPR/Cas9 is the most promising candidate for a removal of *ins-11*. Once *ins-11* is identified, one could furthermore follow up with an overexpression of either the full gene or only the promotor region to identify the expression pattern of this gene[109].

Conclusion

I have found that the host characteristics, such as sex and mating status, as much as the environment, such as pathogen presence, are important for the interaction between a host and a PM. Even the rare presence of a pathogen in the coevolutionary dynamics between host and PM is sufficient to select for enhanced microbe-mediated protection. Host sex is also important to consider, as the females and males respond differently to infection as well as to acquiring protective traits from the PM. The mathematical modelling revealed that the PM is able to stabilise the infection dynamics and reduce infection, independently of the exact cost-benefit ratio. These results advance our understand of microbe-mediated protection and make further steps into understanding the ecology and evolution of microbial communities.

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Appendix

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ORIGINAL RESEARCH

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Evolution and maintenance of microbe-mediated protection under occasional pathogen infection

Anke Kloock | Michael B. Bonsall | Kayla C. King

Department of Zoology, University of Oxford, Oxford, UK

Correspondence
Anke Kloock, Department of Zoology, University of Oxford, 11a Mansfield Road, Oxford OX1 3SZ, UK.
Email: anke.kloock@zoo.ox.ac.uk

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Abstract

Every host is colonized by a variety of microbes, some of which can protect their hosts from pathogen infection. However, pathogen presence naturally varies over time in nature, such as in the case of seasonal epidemics. We experimentally coevolved populations of *Caenorhabditis elegans* worm hosts with bacteria possessing protective traits (*Enterococcus faecalis*), in treatments varying the infection frequency with pathogenic *Staphylococcus aureus* every host generation, alternating host generations, every fifth host generation, or never. We additionally investigated the effect of initial pathogen presence at the formation of the defensive symbiosis. Our results show that enhanced microbe-mediated protection evolved during host-protective microbe coevolution when faced with rare infections by a pathogen. Initial pathogen presence had no effect on the evolutionary outcome of microbe-mediated protection. We also found that protection was only effective at preventing mortality during the time of pathogen infection. Overall, our results suggest that resident microbes can be a form of transgenerational immunity against rare pathogen infection.

KEYWORDS
defensive symbiosis, experimental evolution, heterogeneity, host–pathogen interactions, protection

1 | INTRODUCTION

In nature, all plants and animals are colonized by microbes (Barrière, & Felix, 2005; Ley, Peterson, & Gordon, 2006; Vántus, Kovács, & Zsolnai, 2014). The composition of these microbial communities is highly diverse and includes harmful, neutral, and beneficial microbial species (Ley et al., 2006), including those that can be important players in host defense against parasites, a phenomenon referred to as "defensive mutualism" (King, 2019; May & Nelson, 2014). Recognized for over a century, defensive mutualism has been observed in plants (Mendes et al., 2011) and in a range of animals (Dillon, Vennard, & Charnley, 2000; Dong, Manfredini, & Dimopoulos, 2009; Jaenike, Unckless, Cockburn, Boelio, & Perlman, 2010; Koch & Schmid-Hempel, 2011), including humans (Kamada, Seo, Chen, & Núñez, 2013; Ley et al., 2006; Maynard, Elson, Hatton, & Weaver, 2012) wherein microbes can supplement host immune systems (Abt & Artis, 2013; Hooper, Littman, & Macpherson, 2012; McFall-Ngai et al., 2013).

The net benefits of defensive mutualism are dependent upon the presence of pathogens (Clay, Holah, & Rudgers, 2005; King & Bonsall, 2017; Lively, Clay, Wade, & Fuqua, 2005). While hosts can benefit from microbe-mediated protection, defensive symbionts can be less beneficial to the host in the absence of enemies, due to metabolic and physiological costs (King, 2019). For example, in the interaction of aphids and the bacterium *Hamiltonella defensa*, the host tissue is harmed by defensive toxins that protect against

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infection from parasitoids (Vorburger & Gouskov, 2011). In some cases, possessing protective microbes might be more beneficial to the host than investing in its own immune system (Martinez et al., 2016). From the perspective of the symbiont, it is most useful to its host under high pathogen prevalence and thus can persist in the host population (Palmer et al., 2008). Nevertheless, a stable symbiotic interaction is hypothesized to be evolved and maintained (Kwiatkowski & Vorburger, 2012) only when the host benefit of carrying defensive symbionts outweighs any costs. The interactions of obligate and defensive symbionts and hosts can be stable for millions of years (Moran, Tran, & Gerardo, 2005).

Not all environments are constantly pathogen-rich, which might shift the balance of costs and benefits during defensive mutualisms, particularly during coevolutionary interactions (King & Bonsall, 2017). Pathogen prevalence can be spatially (King, Delph, Jokela, & Lively, 2009) or temporally variable, the latter in the case of seasonal epidemics (e.g., flu peaks each winter in the Northern Hemisphere (Finkelman, 2007) or rabies in North American skunks, which peaks in Autumn (Gremillion-Smith & Woolf, 1988). Different environmental factors can influence disease transmission such as an increase in malaria risk in warmer regions after rainfall (Altizer, Dobson, Hosseini, Hudson, & Pascual, 2006) or an increase in contact rate and thus higher flu infection rate during the winter months (London & Yorke, 1973). The impact of other temporally heterogeneous factors on the strength and direction of selection on species interactions have been explored (oxygen concentration [Dey, Proulx, & Teotónio, 2016], resource availability [Friman & Laakso, 2011; Friman, Laakso, Koivu-Orava, & Hiltunen, 2011; Hiltunen, Friman, Kaitala, Mappes, & Laakso, 2012], environmental productivity [Harrison, Laine, Hietala, & Brockhurst, 2013]). Whether the varied presence of pathogens can similarly alter selection for symbiotic interactions has been explored theoretically (Fenton, Johnson, Brownlie, & Hurst, 2011), but remains to be empirically tested.

Here, we examined the impact of temporal variation in pathogen infection on the evolution of microbe-mediated protection. We used *Caenorhabditis elegans* as a worm host and allowed it to be colonized by a bacterium (*Enterococcus faecalis*) that protects against infection by *Staphylococcus aureus* (King et al., 2016). *Enterococcus faecalis* has been shown to be protective across animal microbiomes (Kommineni et al., 2015; Martín-Vivaldi et al., 2010). It has been previously shown that *E. faecalis* can evolve to provide enhanced protection when residing in *C. elegans* hosts during constant pathogen infection (King et al., 2016; Rafaluk-Mohr, Ashby, Dahan, & King, 2018). From this, we predict that variation in pathogen infection might limit the evolution of microbe-mediated protection. In the present study, we experimentally copassaged *C. elegans* with protective *E. faecalis* and infected the host with evolutionary static pathogenic *S. aureus* at different intervals of host evolution. We also examined whether pathogen presence at the initial formation of the coevolving interaction is crucial to the evolution of protection. We show that enhanced microbe-mediated protection emerged out of novel coevolutionary host–microbe interactions and during pathogen infection, regardless of its temporal variability or the time point of first infection. Enhanced protection was only effective during pathogen infection. If hosts survived infection, they could recover and had the same longevity and reproductive output across treatments. These results thus suggest that even occasional pathogen infection can select for defensive mutualism, revealing the potential for this phenomenon to be widespread in nature.

2 | MATERIALS AND METHODS

2.1 | Worm host and bacteria system

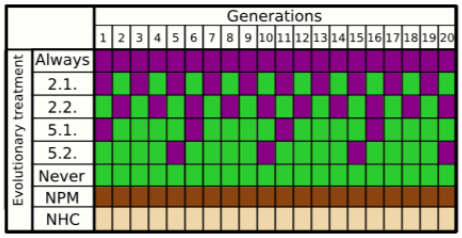
As a bacteriovore, *Caenorhabditis elegans* interacts constantly with a variety of bacteria either by feeding or by hosting them (Cabreiro & Gems, 2013; Garsin et al., 2001; Schulenburg & Ewbank, 2004). Consequently, *C. elegans* is an established model for studying innate immunity (Gravato-Nobre & Hodgkin, 2005), as it can be infected with its natural (Jansson, 1994; Schulenburg & Ewbank, 2004) as well as opportunistic pathogens (Garsin et al., 2001; Tan, Mahajan-Miklos, & Ausubel, 1999). Most pathogens are taken up orally by the worm (Marsh & May, 2012), and some can proliferate and colonize the worm gut (King et al., 2016; Rafaluk-Mohr et al., 2018).

Naturally, *C. elegans* is a self-fertilizing hermaphrodite (Brenner, 1974), but in this experiment obligate outcrossing worm populations (line EEVD00) with males and females (hermaphrodites that carry the *fog-2(q71)* mutation) were used (Theologidis, Chelo, Goy, & Teotónio, 2014). This lineage was generated by Henrique Teotonio (ENS Paris) and encompasses the genetic diversity of 16 natural worm isolates (Theologidis et al., 2014). Worms were kept on Nematode Growth Medium (NGM), inoculated with *Salmonella*, hereafter referred to as food. Worms were infected with the pathogenic *S. aureus* (MSSA476; Holden et al., 2004), which is virulent and kills worm hosts by lysing the intestinal cells lining the gut wall (Sifri, Begun, Ausubel, & Calderwood, 2003). Worms were exposed to *E. faecalis* (OG1RF; Garsin et al., 2001), which was isolated from the human digestive system, but has been previously shown to colonize and proliferate in the host gut (Ford, Williams, Paterson, & King, 2017; King et al., 2016; Rafaluk-Mohr et al., 2018), where it provides protection.

2.2 | Experimental evolution—Design

Six single clones of *E. faecalis* (one for each of the six replicate populations) and a single population of *C. elegans* were the ancestors (hereafter referred to as the Ancestor) for all evolving populations. To account for potential differences in virulence, a stock of four clones of *S. aureus* was used for pathogen infections. Both *C. elegans* and colonizing *E. faecalis* were allowed to evolve in the presence of each other, while *S. aureus* was kept evolutionarily static. Infection with *S. aureus* was varied over host evolutionary time (indicated by purple in Table 1) to represent temporal heterogeneity in pathogen infection, including a range from always to every 2nd generation,

TABLE 1 Experimental procedure for the evolution experiment



every 5th generation, and never (Table 1). Moreover, we included differences in whether pathogens were present at the initial formation of the symbiotic interaction or later (2.1. vs. 2.2., and 5.1. vs. 5.2. in Table 1). Controls for laboratory adaptation were maintained for the host (No Protective Microbe control, NPM in Table 1) and *E. faecalis* (No Host Control, NHC in Table 1).

Columns indicate the number of experimental host generations (1–20), while rows show the eight treatments. Host generations were infected with *Staphylococcus aureus* (purple) or given food (green), while constantly coevolving with *Enterococcus faecalis*. Two controls for laboratory effects on host evolution (dark brown, No Protective Microbe, NPM) and *E. faecalis* evolution (light brown, No Host Control, NHC) were also included, where the NPM treatment was only ever exposed to food alone. Each evolutionary treatment consisted of six independent evolutionary replicates.

2.3 | Experimental evolution—Culturing and passaging methods

At the start of each generation, worms were bleached as described previously and left in M9 buffer overnight for larvae to hatch (Stiernagle, 2006). Simultaneously, *E. faecalis* clones were cultured overnight in Todd-Hewitt Broth (THB) in 600 μl at 30°C, while food was cultured overnight in LB broth. Subsequently, 9-cm NGM plates were inoculated with 300 μl of each overnight culture. Plates with freshly inoculated bacteria were dried at room temperature before approximately 1,000 L1 worms were added to each NGM plate. After these plates dried at room temperature, they were transferred to a 20°C incubator and left for 48 hr. Simultaneously, a liquid culture of *S. aureus* was grown in THB from frozen stock, while a liquid culture of food was grown in LB, and both were incubated under shaking conditions at 30°C. The following day, 100 μl of each overnight culture was spread on 9-cm plates, *S. aureus* on Tryptone Soy Broth agar (TSB) plates and food on NGM plates, and incubated at 30°C overnight. To transfer worms to the pathogen or food plates, nematodes were washed off the *E. faecalis* plates with M9 buffer and washed three times over small-pore filters to remove all externally attached bacteria, as previously described (Jansen et al., 2015; Papkou et al., 2019; Rafaluk-Mohr et al., 2018). Worms were infected with either *S. aureus* or exposed to food (Table 1) and left at 25°C for 24 hr. After this time, worms were then washed off the plates with M9 buffer once more to plate them on NGM plates seeded with food for laying eggs. Roughly, 10% of these worms was crushed and plated on *E. faecalis* selective medium (TSB + 100 mg/ml rifampicin). The remaining worms were left on food plates for 48 hr to allow for egg laying.

To passage *E. faecalis*, roughly 100 *E. faecalis* colonies were picked and grown up shaking overnight in 600 μl THB at 30°C, while worms were bleached and left to hatch overnight. This cycle was repeated for 20 experimental host generations.

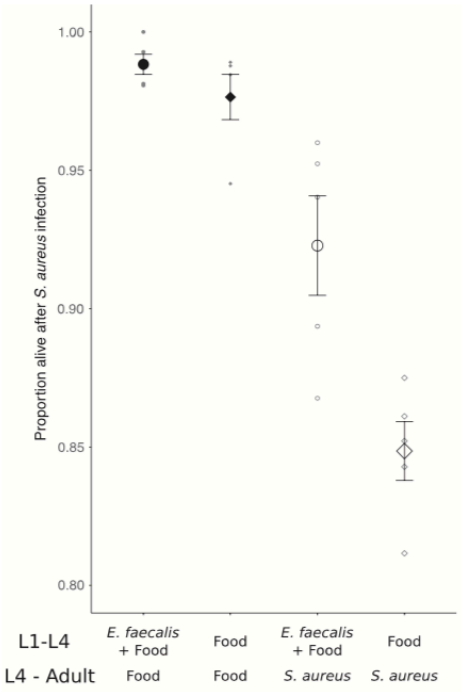


FIGURE 1 Host survival showing protective effects of *Enterococcus faecalis*. Early exposure of worms to *E. faecalis* (both ancestors) provides some degree of protection from the infection of *Staphylococcus aureus*. 24-hr host survival levels reveal a benefit to *E. faecalis* colonization independent of pathogen presence or absence. Circles indicate those treatment being exposed to *E. faecalis* and food in the earlier stage (L1–L4), while squares indicate food alone treatment in the earlier stage (L1–L4). Filled symbols indicate those treatments being exposed to food in the later stage, while open symbols indicate those treatments being exposed to the pathogen *S. aureus* in the later stage. Each symbol indicates the mean ± *SE* of five replicates. Axis scales were chosen to be the same across all plots

All passaged worms and *E. faecalis* samples were cryopreserved at −80°C. A proportion of the offspring of surviving worms were frozen in 40% DMSO, and 100 µl of *E. faecalis* liquid culture was mixed with 100 µl of glycerol before cryopreservation.

2.4 | Host survival and fecundity assays

All assays were conducted at the end of the evolution experiment on archived samples. Plates were randomized and fully encoded during each experiment to ensure the experimenter was blind to different treatments while collecting data.

Basic procedures were adopted from the experimental evolution, but with the following alterations to keep the assays feasible with higher accuracy when scoring dead and alive worms: 400 L1 worms were exposed to 200 µl of food and *E. faecalis* on 6-cm NGM plates, while 60µl of *S. aureus* overnight culture was used to inoculate 6-cm TSB plates.

To assess microbe-mediated protection of different combinations of worms and *E. faecalis*, 400 L1s were exposed to 50:50 mixtures of *E. faecalis* and food for 48 hr. Worms were then washed off these plates as described above and infected with *S. aureus* for 24 hr at 25°C. Survival in form of counting dead and alive worms was then scored.

To assess any long-term fitness consequences after protective microbe exposure and pathogen infection, long-term survival and fecundity were measured. Worms were exposed as described for the survival assays. Subsequently, five females and five males were picked onto 3-cm food seeded NGM plates at 25°C and then transferred to new plates every 36 hr to avoid any confusion between offspring produced and original adults. At each time point, survival was scored. To measure fecundity, the number of worm eggs on the plates at 120 hr since bleaching was counted.

2.5 | Statistical analysis

Statistical analyses were carried out with RStudio (version 1.1.463 for Mac), and graphs were created with the ggplot2 package (version 2.1.0) and edited with Inkscape (version 0.91). All host survival and fecundity data were analyzed with nested binomial mixed-effects models (R package lme4), followed by a Tukey multiple comparison tests (R package multcomp). Life span data were analyzed with Kaplan–Meier log-rank test with FDR correction for multiple testing.

3 | RESULTS

Before the start of the evolution experiment, the starting conditions were tested. Confirming previous results, *E. faecalis* showed some spontaneous host-protective potential against *S. aureus*. Worms

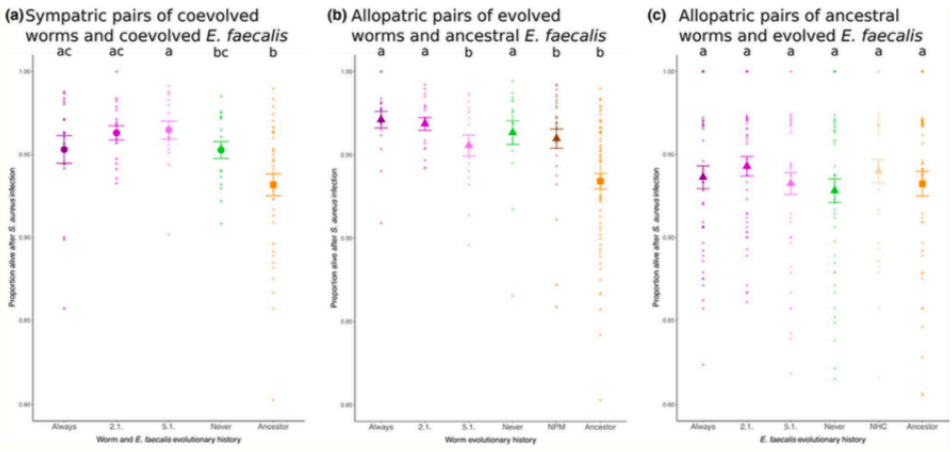


FIGURE 2 Host survival for coevolving sympatric and allopatric pairs of worms and *Enterococcus faecalis*. Microbe-mediated protection was assessed for (a) sympatric pairs of coevolved worms and *E. faecalis*, (b) allopatric pairs of evolved worms and ancestral *E. faecalis*, and (c) allopatric pairs of ancestral worms and evolved *E. faecalis*. Bigger symbols represent mean ± *SE* and consist of six biological replicates and four technical replicates. Smaller symbols indicate the data distribution. Circles indicate sympatric pairs of coevolved *E. faecalis* and worms, squares indicate ancestral pairs of *E. faecalis* and worms, and triangles indicate allopatric pairs of *E. faecalis* and worms. Letters indicate results of a GLMM, followed by a Tukey post hoc test. The same letter indicates no significant difference. Axis scales were chosen to be the same across all plots

raised on *E. faecalis* and food survived better than those raised on food alone, independent of food or pathogen present at the later stage (general linear model, $X^2 = 10.205$, $df = 1$, $p = .001$; Figure 1). Worms infected with *S. aureus* in later life survived worse than those being exposed to food (general linear model, $X^2 = 119.643$, $df = 1$, $p < .001$; Figure 1). These results demonstrate the beneficial and protective effects for the host after exposure to the protective microbe *E. faecalis*.

Infection with *S. aureus* over evolutionary time in the experiment led to the substantial enhancement of microbe-mediated protection, with the evolutionary background of the sympatric pair of host and *E. faecalis* having a significant impact on host survival (mixed-effects model, $X^2 = 42.479$, $df = 4$, $p < .001$; Figure 2a). Higher microbe-mediated protection in comparison with the Ancestor occurred in all evolutionary histories involving pathogen presence across the temporal heterogeneity treatments in our evolution experiment (always, 2.1. and 5.1.). However, this did not occur in the pathogen absence (never) treatment. Host evolutionary history alone had a significant effect on host survival (mixed-effects model, $X^2 = 35.779$, $df = 5$, $p < .001$; Figure 2b), but did not reveal the same pattern as for sympatric pairs. No effect of bacteria evolutionary history alone on infected host survival was observed (mixed-effects model, $X^2 = 3.2511$, $df = 5$, $p = .6613$; Figure 2c). Taken together, enhanced microbe-mediated protection evolved only as a product of coevolution and pathogen presence for sympatric pairs; this occurred regardless of the temporal heterogeneity.

As an additional form of pathogen heterogeneity, the impact of the timing of initial pathogen infection on the evolution of microbe-mediated protection was investigated. An effect of different initial pathogen infection time points on host survival following pathogen infection was observed (mixed-effects model: $X^2 = 7.945$, $df = 3$, $p = .04716$ Figure 3), although a Tukey post hoc test revealed no significant differences (Table S1).

Furthermore, we investigated the long-term consequences to hosts colonized by *E. faecalis* after 24 hr of pathogen infection. No significant differences were observed in the long-term survival postinfection of worm hosts colonized by their sympatric *E. faecalis* across treatments (Kaplan–Meier log-rank test, FDR-corrected, all comparisons $p > .05$, Figure 4a). In addition, we did not find significant differences in fecundity among sympatric host– *E. faecalis* pairs (mixed-effects model, $X^2 = 3.9418$, $df = 4$, $p = .4278$, Figure 4b).

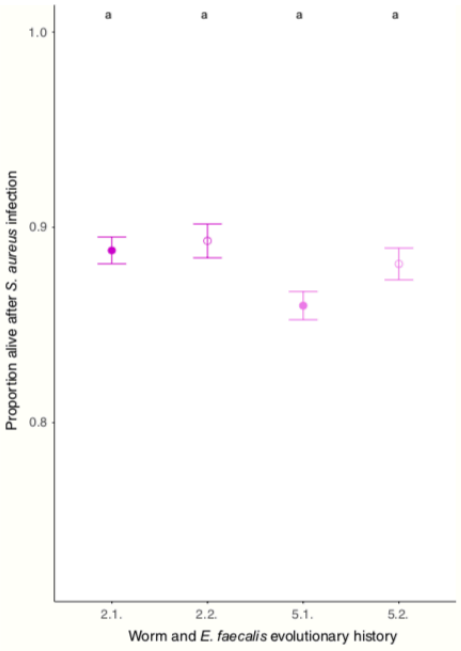


FIGURE 3 Host survival in evolutionary treatments differing in initial pathogen exposure time points. The time point of initial infection varied for infection to the pathogen every two generations (2.1. and 2.2) or every five generations (5.1. or 5.2.) but does not influence the outcome. Closed symbols indicate initial pathogen presence (host generation 1); open symbols indicate later pathogen presence (generation 2 for 2.1. and 2.2. and generation 5 for 5.1. and 5.2.). Bigger symbols represent mean ± *SE* and consist of six biological replicates and four technical replicates of the sympatric pairs. Smaller symbols indicate the data distribution. Letters indicate results of a GLMM, followed by a Tukey post hoc test. The same letter indicates no significant difference. Axis scales were chosen to be the same across all plots

4 | DISCUSSION

It has been shown that hosts receive the greatest benefits from protective microbes under constant pathogen infection. We hypothesized that variation in pathogen presence over time would limit the evolution of microbe-mediated protection due to the reduced benefits to the host and bacterial symbiont. In our study, enhanced pathogen defense emerged out of host–symbiont coevolutionary interactions only when pathogens were present, independent of the interval or initial presence of the pathogen. Notably, the ultimate strength of microbe-mediated protection that evolved was not impacted by the number of host generations between pathogen infections, the proportion of generations infected, or the presence of the pathogen at the first host–microbe interaction. These results suggest that resident microbes can be a form of transgenerational immunity against rare pathogen infections.

We found that microbe-mediated protection is maintained even in the prolonged absence of pathogen, but that pathogen presence is necessary for microbe-mediated protection to evolve, as previously hypothesized (Clay et al., 2005; King & Bonsall, 2017; Lively et al., 2005). This result is unlike previous work showing that the

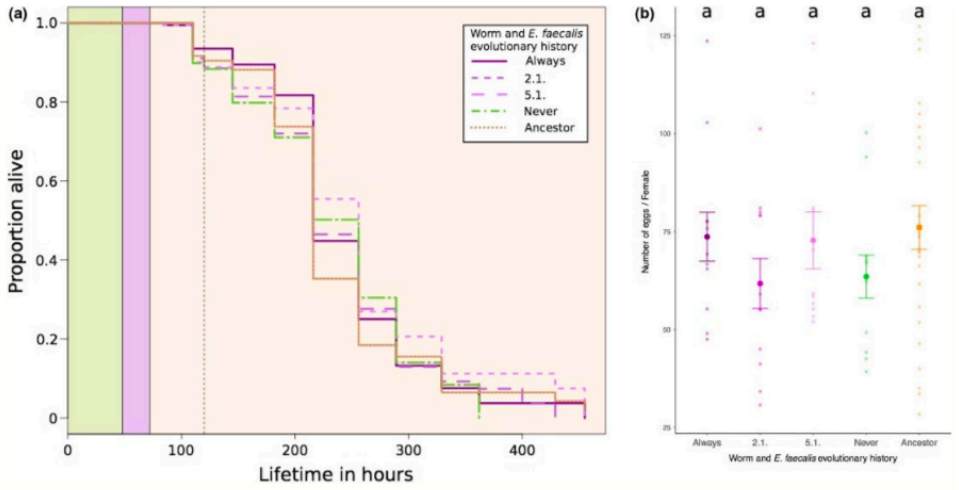


FIGURE 4 Long-term survival and fecundity of *Enterococcus faecalis*-colonized hosts that survived pathogen infection. (a) Long-term host survival was measured. Survival curves for sympatric pairs of worms and *E. faecalis* are shown as Kaplan–Meier estimates. Worms were exposed to *E. faecalis* and food (green), and then to *Staphylococcus aureus* (purple), and long-term survival was monitored on food (orange). The dotted line indicates the time point at which fecundity was measured. (b) Number of eggs/female across sympatric pairs of coevolved worms and *E. faecalis*. Bigger symbols represent mean ± *SE* and consist of six biological replicates and four technical replicates. Smaller symbols indicate the data distribution. Circles indicate sympatric pairs of coevolved *E. faecalis* and worms; squares indicate ancestral pairs of *E. faecalis* and worms. Letters indicate results of a GLMM, followed by a Tukey post hoc test. The same letter indicates no significant difference

scale of heterogeneity in abiotic conditions can affect the strength of selection for traits in some symbiotic interactions (Harrison et al., 2013). This discrepancy is potentially due to costs in our symbiotic system being ameliorated (at least in terms of host survival) in well-provisioned hosts, as hosts are provided with food alongside *E. faecalis* and are thus rescued from starvation (also see Dasgupta et al., 2019). Although protective symbionts can incur costs (e.g., Vorburger & Gouskov, 2011) for their hosts, with potential for impacts on coevolutionary interactions (King & Bonsall, 2017), it is possible that potential costs of bacterial colonization might be only detectable when hosts are stressed (Lively, 2006) or that the costs were not strong enough for us to detect (Little, Carius, Sakwinska, & Ebert, 2002). Different measures of cost remain to be explored (e.g., life span in the complete absence of a protective microbe and a pathogen). Higher protection also does not always come with higher costs, as found in the black bean aphid–*Hamiltonella defensa* interaction (Cayetano, Rothacher, Simon, & Vorburger, 2015). Thus, protective traits in an organism's commensal microbiota could be selected for under pathogen infection and easily maintained in subsequent uninfected generations.

Microbe-mediated protection was strongest between sympatric pairs when pathogens were present over evolutionary time, consistent with previous findings (Rafaluk-Mohr et al., 2018). In our study, protection emerged during coevolution after only 20 host generations, and not due to the independent evolution of either interacting species, but due to the coevolution of both species (King & Bonsall, 2017). The time scale of these interactions is short compared to the longer shared evolutionary histories shared by other defensive mutualisms (Jousselin, Rasplus, & Kjellberg, 2003; Quek, Davies, Itino, & Pierce, 2004; Shoemaker et al., 2002). Nevertheless, our findings reveal the potential for microbe-mediated protection to become enhanced during the formation of a coevolving host–microbiota relationship.

In conclusion, our results show that enhanced protection in host–microbe interactions can rapidly evolve and be maintained even under infrequent pathogen infection, suggesting that resident microbes can be a form of stable, transgenerational immunity. The protective benefit of an organism's microbiota might remain undetected for several host generations until pathogens re-emerge. Future research on the failure of pathogens transmit within host populations should consider the contribution of the protective microbiota to prevent disease spread.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Anke Kloock: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Investigation (equal); Visualization (lead); Writing-original draft (lead). **Michael B. Bonsall:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Supervision (equal); Writing-review & editing (equal). **Kayla C. King:** Conceptualization (equal); Data curation (supporting); Formal analysis (supporting); Funding acquisition (lead); Supervision (equal); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT

All evolved worm and bacteria strains are cryopreserved and can be provided upon request. Raw data and all scripts that were used for statistical analysis are available via the following link: https://osf.io/vpm9b/.

ORCID

Anke Kloock https://orcid.org/0000-0002-4618-148X
Michael B. Bonsall https://orcid.org/0000-0003-0250-0423
Kayla C. King https://orcid.org/0000-0003-1393-9220

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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