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

Running title: Evolution of microbe-mediated protection

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Authorship Contributions

KCK and AK conceived the project. KCK, MBB, and AK designed the project. AK performed the evolution experiment and all subsequent assays. AK and MBB performed the statistical analysis. AK and KCK wrote the manuscript, and all authors agreed on the final version.

Acknowledgments

We would like to thank the King group for help and support during the performance of the evolution experiment, especially Charlotte Rafaluk-Mohr and Maria Ordovas-Montanes. AK was supported by a fellowship from the Studienstiftung des Deutschen Volkes. KCK is grateful for a Leverhulme Trust project grant (RPG-2015-165) and ERC Starting Grant (COEVOPRO 802242).

Conflict of Interest Statement

The authors declare no conflict of interest.

Abstract

Every host is colonized by a variety of microbes, some of which can protect their hosts from pathogen infection. As microbe-mediated protection can be costly, it is hypothesized, that these costs only pay off in the presence of a pathogen. However, pathogen presence naturally varies over time and can expand once pathogen infection spreads to a new host, as during occasional spill-over from reservoir hosts. 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 spill-over infections by a human pathogen occurring rarely over evolutionary history. 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 occasional pathogen attack or spill-over events.

Keywords: (1) Experimental evolution, (2) Defensive Symbiosis, (3) Heterogeneity, (4) Host-pathogen interactions, (5) Protection, (6) Spill-over

Introduction

In nature, all plants and animals are colonised by microbes (Barriere, 2006; Ley et al., 2006; Vántus et al., 2014). The composition of these microbial communities is highly diverse and includes harmful, neutral, and helpful microbe species (Ley et al., 2006) including those that can be important players in host defence against parasites, a phenomenon referred to as 'defensive mutualism' (King, 2019; May and Nelson, 2014). Recognised for over a century, defensive mutualism has been observed in plants (Mendes et al., 2011) and in a range of animals (Dillon et al., 2000; Dong et al., 2009; Jaenike et al., 2010; Koch and Schmid-Hempel, 2011), including humans (Kamada et al., 2013; Ley et al., 2006; Maynard et al., 2012) wherein microbes can supplement host immune systems (Abt and Artis, 2013; Hooper et al., 2012; McFall-Ngai et al., 2013).

The net benefits of defensive mutualism are dependent upon the presence of pathogens (Clay et al., 2005; King and Bonsall, 2017; Lively et al., 2005). Whilst hosts can benefit from microbe-mediated protection, defensive symbionts are often metabolically and physiologically costly in the absence of enemies (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 infection from parasitoids (Vorburger and Gouskov, 2011). In some cases, the costs of possessing the protective microbe might be less energetically costly than investing in the host's own immune system (Cayetano et al., 2014). 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 and Vorburger, 2012) only when the host benefit of carrying defensive symbionts

outweighs any costs. The interactions of obligate symbionts and hosts can be stable for millions of years (Moran et al., 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 and Bonsall, 2017). Pathogen prevalence can be spatially (King et al., 2009) or temporally variable, the latter in the case of seasonal epidemics (e.g., flu peaks each winter in the northern hemisphere (Finkelman, 2007). Infection might occur even less often and be asynchronous with host generations due to host range expansion (Phillips et al., 2010) or pathogen spill-over from reservoir hosts (Lo Iacono et al., 2016). Pathogen spill-over can occur between unrelated species and be very harmful, such as zoonotic tuberculosis in humans caused by *Mycobacterium bovis* (Olea-Popelka et al., 2017). Ejeh *et al* observed that there were seasonal differences in zoonotic tuberculosis transmission (Ejeh et al., 2013). The impact of other temporally heterogeneous factors on the strength and direction of selection on species interactions have been explored (oxygen concentration (Dey et al., 2016), resource availability (Friman et al., 2011; Friman and Laakso, 2011; Hiltunen et al., 2012), environmental productivity (Harrison et al., 2013)). Whether the varied presence of pathogens can similarly alter selection for symbiotic interactions remains to be directly tested, but has been explored theoretically (Fenton et al., 2011), and in terms of symbiont spread through a host population (Oliver et al., 2014).

Here, we examined the impact of temporal variation in spill-over pathogen infection on the evolution of microbe-mediated protection. We 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* (King et al., 2016). *Enterococcus faecalis* has been shown to be protective across animal microbiomes (Kommineni et al., 2015; Martín-Vivaldi et al., 2010). Pathogenic *S. aureus* is novel to *C. elegans* and is known to expand its range of host species, including different domestic animals, wild hosts, such as rodents, non-human primates and bats (Mrochen et al., 2018; Peton and Le Loir, 2014; Schaumburg et al., 2012). It was 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 et al., 2018). From this, we predict that variation in pathogen infection might limit the evolution of microbe-mediated protection. In the present study, we 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. 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 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 attack or spill-over in the community 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 (Cabreiro and Gems, 2013; Garsin et al., 2001; Schulenburg and Ewbank, 2004). Consequently, *C. elegans* is an established model for studying innate immunity (Gravato-Nobre and Hodgkin, 2005), as it can be infected with its natural (Jansson, 1994; Schulenburg and Ewbank, 2004) as well as opportunistic pathogens (Garsin et al., 2001; Tan et al., 1999). Most pathogens are taken up orally by the worm (Marsh and 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-fertilising 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 et al., 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 *Escherichia coli* OP50 (Brenner, 1974), 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 et al., 2003). By using this human isolate of *S. aureus* (Holden et al., 2004), we can mimic pathogenic spill-over from a human background to a nematode infection. Worms were exposed to *E. faecalis* (OG1RF) (Garsin et al., 2001), which was isolated from the human digestive system, but was previously shown to colonize and proliferate in the worm´s gut (Ford et al., 2017; King et al., 2016; Rafaluk-Mohr et al., 2018), 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 Figure 1) to represent temporal heterogeneity in pathogen spill-over, including a range from always to every 2nd generation, every 5th generation, and never (Figure 1). Moreover, we included differences in whether pathogens were present at the formation of the symbiotic interaction or later (2.1. vs. 2.2., and 5.1. vs. 5.2. in Figure 1). Controls for lab adaptation were maintained for the host (No Protective-Microbe control, NPM in Figure 1) and *E. faecalis* (No Host Control, NHC in Figure 1).

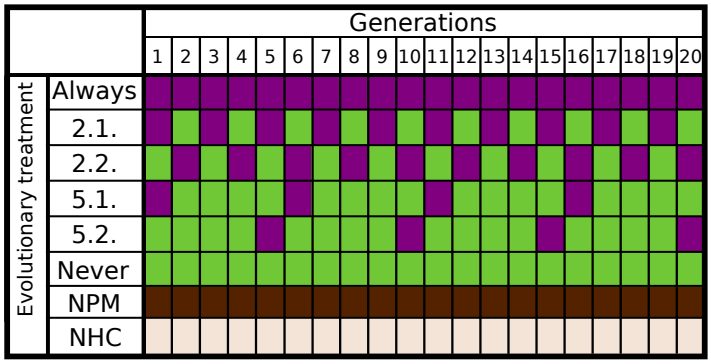

Figure 1: Experimental procedure for the evolution experiment. Columns indicate the number of experimental host generations (1- 20), while rows show the eight treatments. Host generations were infected with *S. aureus* (purple) or given *E. coli* food (green). Two controls for lab effects on host evolution (dark brown, No Protective Microbe, NPM) and *E. faecalis* evolution (light brown, No Host Control, NHC) were also included. Each evolutionary treatment consisted of six independent 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 (Stiernagle, 2006). Simultaneously, *E. faecalis* clones were cultured overnight in Todd-Hewitt Broth (THB) in 600µl at 30ºC, while *E. coli* OP50 food was cultured overnight in LB broth. Subsequently, 9cm NGM plates 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 *E. coli* OP50 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 *E. coli* OP50 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 (Jansen et al., 2015; Papkou et al., 2019; Rafaluk-Mohr et al., 2018). Worms were infected with either *S. aureus* or exposed to food (Figure 1) and left at 25ºC for 24h. Worms were then washed off the plates with M9 buffer once more to plate them on NGM plates seeded with *E. coli* OP50 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 *E. coli* OP50 food and *E. faecalis* on 6cm NGM plates, while only 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 *E. coli* OP50 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 *E. coli* OP50 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 hosts 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), graphs created with the ggplot2 package (Version 2.1.0) and edited with Inkscape (Version 0.91). Host survival to *E. coli* OP50 food and ancestral *E. faecalis* and *S. aureus* were analysed with Wilcoxon Rank Tests with False Discovery Rate (FDR) correction for multiple testing. Host survival data across evolutionary treatments were analysed with nested binomial mixed effects models (R package lme4), followed by a Tukey multiple-comparison tests (R package multcomp). Life-span data were analysed with Kaplan Meier Log Rank test with FDR correction for multiple testing, and host fecundity was analysed with a nested linear mixed model (R package lme4).

Results

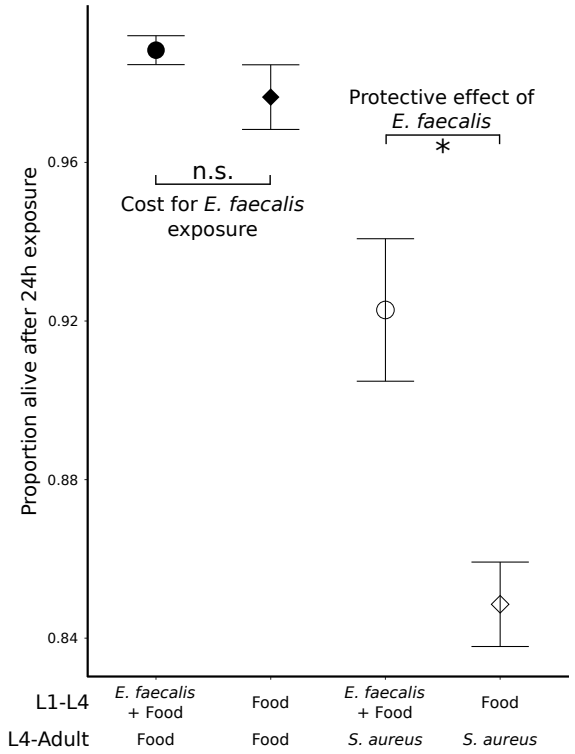

Figure 2: **Host survival showing the costs and protective effects of *E. faecalis***. Early exposure of worms to *E. faecalis* (both ancestors) provides some degree of protection from the infection of *S. aureus*. 24h host survival levels do not reveal a cost to *E. faecalis* colonisation, however worms were also given food during the L1-L4 stage. 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.

Confirming previous results, *E. faecalis* showed some host-protective potential against *S. aureus*. Survival of worms raised on *E. faecalis*/food and then infected with *S. aureus* was greater than worms raised on food alone and then infected with *S. aureus* (Wilcoxon Rank Test, p=0.03; Figure 2, open symbols). These results demonstrate the protective effect of *E. faecalis*. Moreover, those worms raised on *E. faecalis*/food survived similarly to those worms raised on food alone

(Wilcoxon Rank Test, p=0.42; Figure 2, filled symbols) indicating no cost of *E. faecalis* colonisation as measured by 24h host survival.

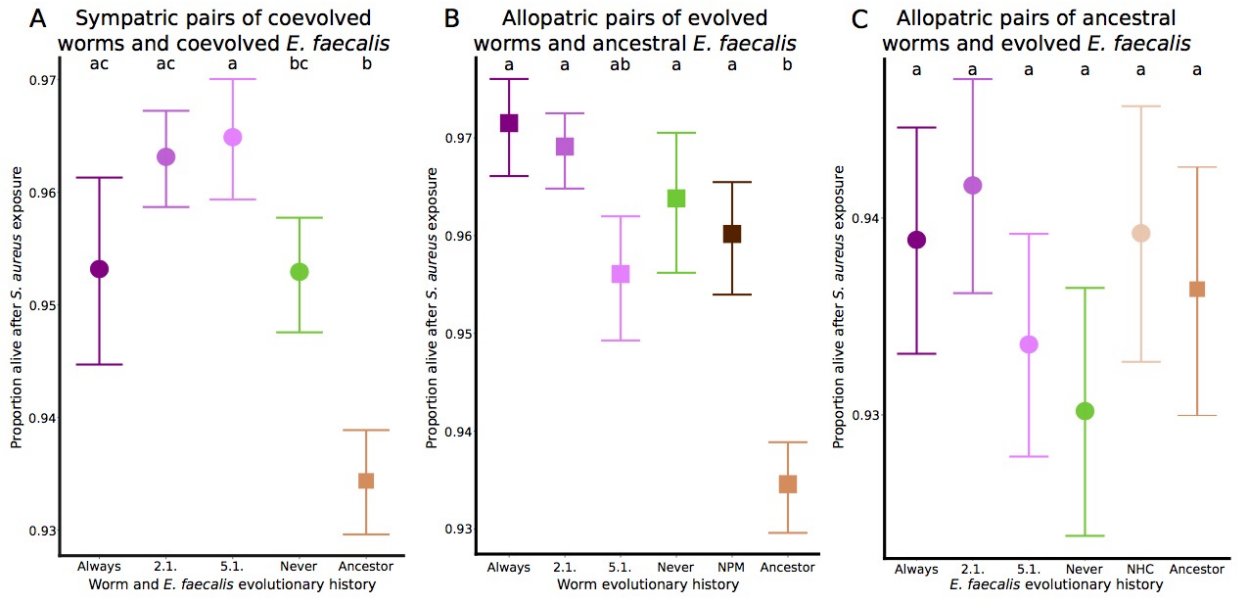

Figure 3: **Host survival for coevolving sympatric and allopatric pairs of worms and *E. faecalis***. Microbe-mediated protection was assessed for (A) sympatric pairs of coevolved worms and *E. faecalis,* (B) allopatric pairs of evolved worms and ancestral *E. faecalis*, and (C) allopatric pairs of ancestral worms and evolved *E. faecalis*. Each symbol represents mean ± S.E. and consists of six biological replicates and four technical replicates. Circles indicate (co)evolved *E. faecalis* treatment, while squares indicate ancestral *E. faecalis* treatment.

Infection with *S. aureus* over evolutionary time in the experiment led to the significant enhancement of microbe-mediated protection, with the evolutionary background of the host and *E. faecalis* had a significant effect on host survival (Mixed Effects Model, X^2=25.849, df=4, p<0.001; Figure 3A). Higher microbe-mediated protection occurred in all evolutionary histories involving infection at some point in the evolution experiment (Always, 2.1. and 5.1.). Host evolutionary history had a significant effect on host survival (Mixed Effects Model, X^2=40.458, df=5, p<0.001; Figure 3B), but this difference was independent of the pathogen or protective

microbe, as it is also demonstrated in the absence of pathogen infection or the protective microbe (NPM control). These results suggest that worms have adapted under the procedure of the evolution experiment. No effect of bacteria evolutionary history alone on infected host survival was observed (Mixed Effects Model, X^2=2.3891, df=5, p=0.7931; Figure 3C). Taken together, enhanced microbe-mediated protection evolved only as a product of coevolution and pathogen presence, the latter regardless of its 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 explored. No significant effect of different initial pathogen infection time points on host survival upon *S. aureus* infection was demonstrated (Mixed Effects Model: A: X^2=0.1407, df=1, p=0.7076 and B: X^2=1.6179, df=1, p=0.2034; Figure S1).

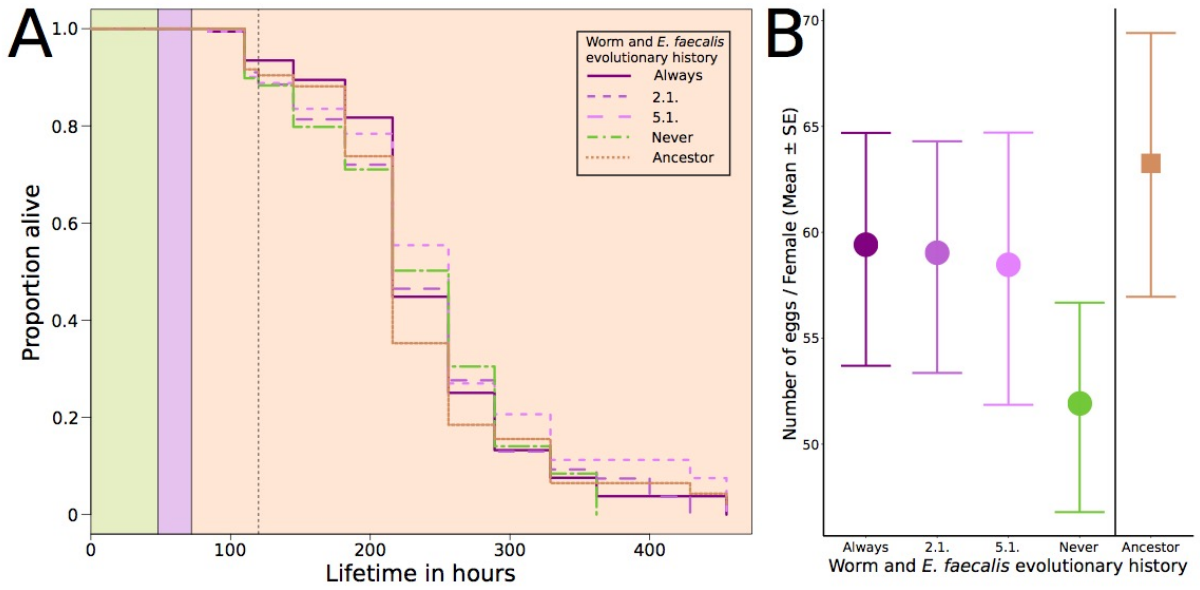

Figure 4: **Long-term survival and fecundity of *E. faecalis*-colonised hosts that survived pathogen exposure**. (A) Long-term host survival was measured. Survival curves for sympatric pairs of worms and *E. faecalis* are shown as

Kaplan-Meier estimates. Worms were exposed to *E. faecalis*/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*. Each symbol represents mean ± S.E. and consists of six biological replicates and four technical replicates. Circles indicate coevolved *E. faecalis* treatment, while squares indicate ancestral *E. faecalis* treatment.

Furthermore, we investigated the long-term consequences to hosts colonised by *E. faecalis* after 24h of pathogen exposure. 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, we did not find significant differences in fecundity among worm-*E. faecalis* pairs (Mixed Effects Model, $X^2=3.9163$, df=4, $p=0.4175$, Figure 4B).

Discussion

It has been shown that hosts receive the greatest benefits from protective microbes under constant pathogen infection. We hypothesized that variation in novel 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 defence emerged out of host-symbiont coevolutionary interactions only when pathogens were present, independent of the interval of pathogen presence. 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 occasional pathogen attack or spill-over events, even by novel pathogens.

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 by King & Bonsall (King and Bonsall, 2017). 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 (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 and Bonsall, 2017), it is possible that potential costs of bacterial colonisation might be only detectable when hosts are stressed (Lively, 2006). Higher protection

also does not always come with higher costs, as found in the black bean aphid-*Hamiltonella defensa* interaction (Cayetano et al., 2014). 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, which was already hypothesized by mathematical models in 2017 (King and Bonsall, 2017). The time-scale of these interactions is short compared to the longer shared evolutionary histories shared by other defensive mutualisms (Jousselin et al., 2003; Quek et al., 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 attack, 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 spill-over or to transmit within host populations should consider the contribution of the protective microbiota to prevent disease spread.

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Supplemental Material

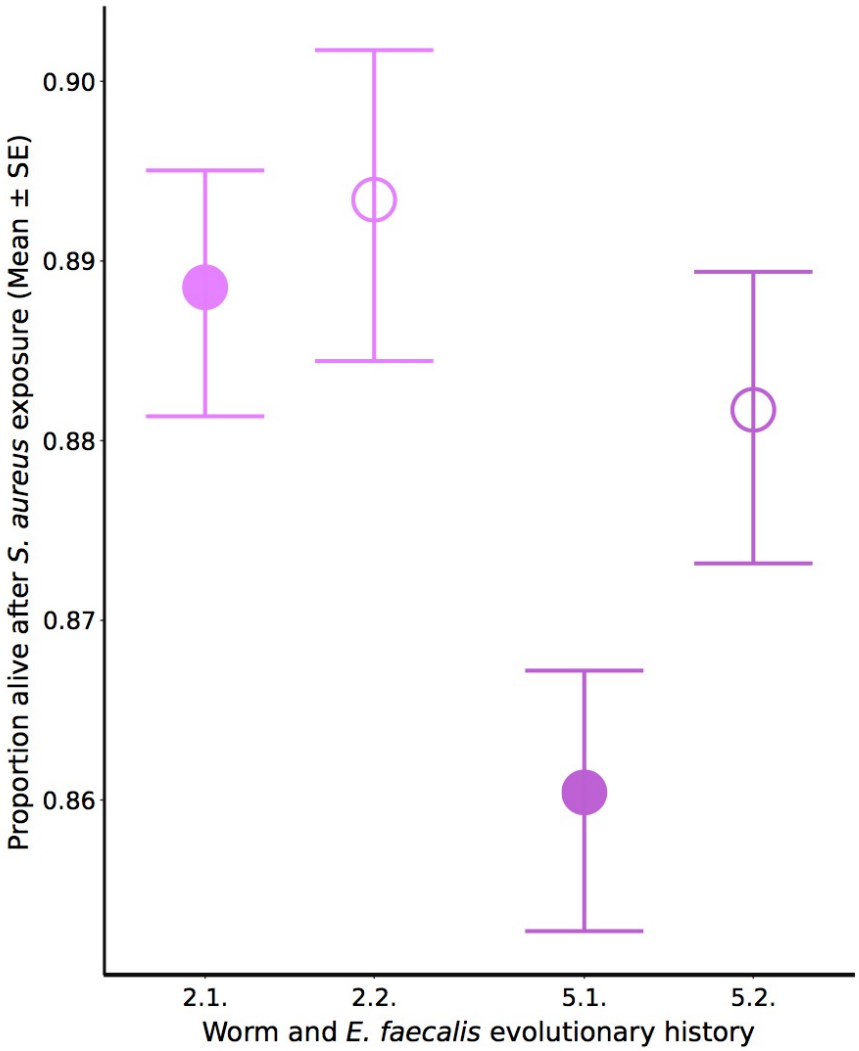

Figure S1: Host survival in evolutionary treatments differing in initial pathogen exposure time points. The time point of initial infection varied for exposure to the pathogen every two generations (2.1. and 2.2) or every five generations (5.1. or 5.2.) but does not influence the outcome. Each symbol represents mean ± S.E and consists of six biological replicates and four technical replicates.