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

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, KCK and MBB wrote the manuscript and agreed on the final version.

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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. 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: (1) Experimental evolution, (2) Defensive Symbiosis, (3) Heterogeneity, (4) Host-pathogen interactions, (5) Protection,

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 beneficial microbial 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 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 infection from parasitoids (Vorburger and 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 and Vorburger, 2012) only when the host benefit of carrying defensive symbionts

44 outweighs any costs. The interactions of obligate and defensive symbionts and hosts can be stable
45 for millions of years (Moran et al., 2005).
46
47 Not all environments are constantly pathogen rich which might shift the balance of costs and
48 benefits during defensive mutualisms, particularly during coevolutionary interactions (King and
49 Bonsall, 2017). Pathogen prevalence can be spatially (King et al., 2009) or temporally variable,
50 the latter in the case of seasonal epidemics (e.g., flu peaks each winter in the northern hemisphere
51 (Finkelman, 2007) or rabies in North American skunks which peaks in Autumn (Gremillion-Smith
52 and Woolf, 1988)). Different environmental factors can influence disease transmission such as an
53 increase in malaria risk in warmer regions after rainfall (Altizer et al., 2006), or an increase in
54 contact rate and thus higher flu infection rate during the winter months (London and Yorke, 1973).
55 The impact of other temporally heterogeneous factors on the strength and direction of selection on
56 species interactions have been explored (oxygen concentration (Dey et al., 2016), resource
57 availability (Friman et al., 2011; Friman and Laakso, 2011; Hiltunen et al., 2012), environmental
58 productivity (Harrison et al., 2013)). Whether the varied presence of pathogens can similarly alter
59 selection for symbiotic interactions has been explored theoretically (Fenton et al., 2011), but
60 remains to be empirically tested.
61
62 Here, we examined the impact of temporal variation in pathogen infection on the evolution of
63 microbe-mediated protection. We used *Caenorhabditis elegans* as a worm host and allowed it to
64 be colonised by a bacterium (*Enterococcus faecalis*) that protects against infection by
65 *Staphylococcus aureus* (King et al., 2016). *Enterococcus faecalis* has been shown to be protective
66 across animal microbiomes (Kommineni et al., 2015; Martín-Vivaldi et al., 2010). It has been

67 previously shown that *E. faecalis* can evolve to provide enhanced protection when residing in
68 *C. elegans* hosts during constant pathogen infection (King et al., 2016; Rafaluk-Mohr et al., 2018).
69 From this, we predict that variation in pathogen infection might limit the evolution of microbe-
70 mediated protection. In the present study, we experimentally co-passaged *C. elegans* with
71 protective *E. faecalis* and infected the host with evolutionary static pathogenic *S. aureus* at
72 different intervals of host evolution. We also examined whether pathogen presence at the initial
73 formation of the coevolving interaction is crucial to the evolution of protection. We show that
74 enhanced microbe-mediated protection emerged out of novel coevolutionary host-microbe
75 interactions and during pathogen infection, regardless of its temporal variability or the time point
76 of first infection. Enhanced protection was only effective during pathogen infection. If hosts
77 survived infection, they could recover and had the same longevity and reproductive output across
78 treatments. These results thus suggest that even occasional pathogen infection can select for
79 defensive mutualism, revealing the potential for this phenomenon to be widespread in nature.
80

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 *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 et al., 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 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 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, 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 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).

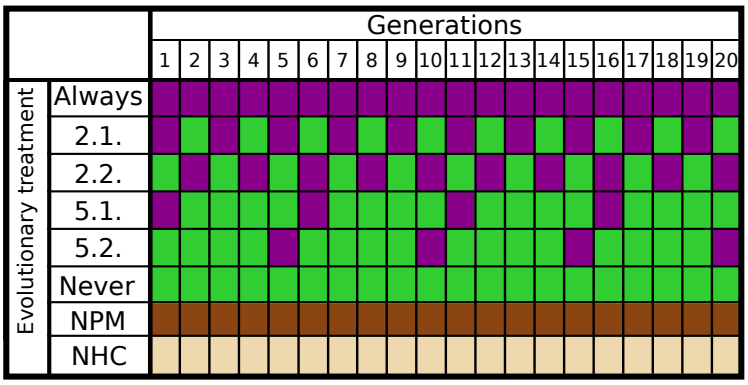


Table 1: Experimental procedure for the evolution experiment. Columns indicate the number of experimental host generations (1- 20), while rows show the eight treatments. Host generations were infected with *S. aureus* (purple) or given food (green), while constantly coevolving with *E. faecalis.* Two 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.

123 Experimental evolution - Culturing and passaging methods

124 At the start of each generation, worms were bleached as described previously and left in M9 buffer
125 overnight for larvae to hatch (Stiernagle, 2006). Simultaneously, *E. faecalis* clones were cultured
126 overnight in Todd-Hewitt Broth (THB) in 600µl at 30ºC, while food was cultured overnight in LB
127 broth. Subsequently, 9cm NGM plates were inoculated with 300µl of each overnight culture. Plates
128 with freshly inoculated bacteria were dried at room temperature before approximately 1000 L1
129 worms were added to each NGM plate. After these plates dried at room temperature, they were
130 transferred to a 20ºC incubator and left for 48h. Simultaneously, a liquid culture of *S. aureus* was
131 grown in THB from frozen stock, while a liquid culture of food was grown in LB, and both were
132 incubated under shaking conditions at 30ºC. The following day, 100µl of each overnight culture
133 were spread on 9cm plates, *S. aureus* on Tryptone Soy Broth agar (TSB) plates and food on NGM
134 plates and incubated at 30ºC overnight. To transfer worms to the pathogen or food plates,
135 nematodes were washed off the *E. faecalis* plates with M9 buffer and washed three times over
136 small-pore filters to remove all externally attached bacteria, as previously described (Jansen et al.,
137 2015; Papkou et al., 2019; Rafaluk-Mohr et al., 2018). Worms were infected with either *S. aureus*
138 or exposed to food (Table 1) and left at 25ºC for 24h. After this time, worms were then washed off
139 the plates with M9 buffer once more to plate them on NGM plates seeded with food for laying
140 eggs. Roughly 10% of these worms were crushed and plated on *E. faecalis* selective medium (TSB
141 + 100mg/ml Rifampicin). The remaining worms were left on food plates for 48h to allow for egg
142 laying.

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

146 All passaged worms and *E. faecalis* samples were cryopreserved at -80 ºC. A proportion of the
147 offspring of surviving worms were frozen in 40% DMSO, and 100µl of *E. faecalis* liquid culture
148 was mixed with 100µl of glycerol before cryopreservation.
149

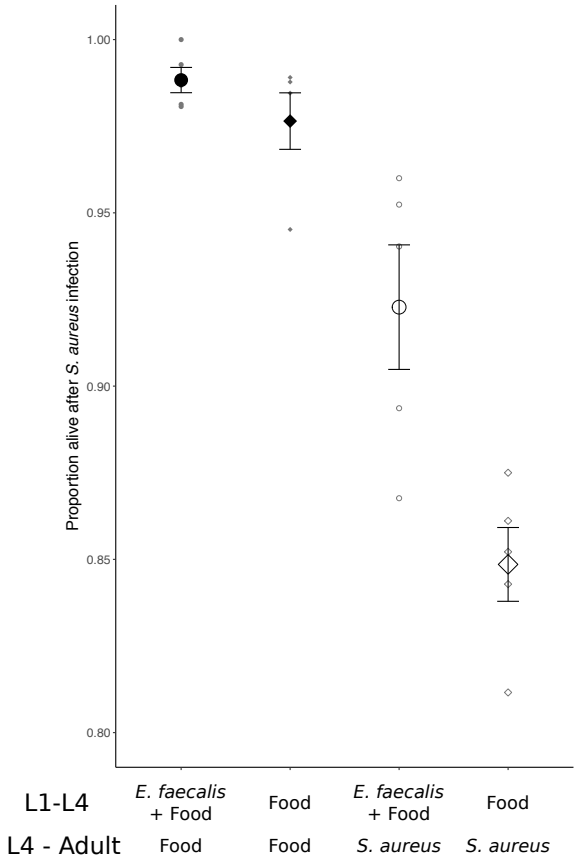
150 ## Host survival and fecundity assays

151 All assays were conducted at the end of the evolution experiment on archived samples. Plates were
152 randomized and fully encoded during each experiment to ensure the experimenter was blind to
153 different treatments whilst collecting data.
154 Basic procedures were adopted from the experimental evolution, but with the following alterations
155 to keep the assays feasible with higher accuracy when scoring dead and alive worms: 400 L1
156 worms were exposed to 200µl of food and *E. faecalis* on 6cm NGM plates, while 60µl *S. aureus*
157 overnight culture was used to inoculate 6cm TSB plates.
158 To assess microbe-mediated protection of different combinations of worms and *E. faecalis*, 400
159 L1s were exposed to 50:50 mixtures of *E. faecalis* and food for 48h. Worms were then washed off
160 these plates as described above and infected with *S. aureus* for 24h at 25°C. Survival in form of
161 counting dead and alive worms was then scored.
162 To assess any long-term fitness consequences after protective microbe exposure and pathogen
163 infection, long-term survival and fecundity were measured. Worms were exposed as described for
164 the survival assays. Subsequently, five females and five males were picked onto 3cm food seeded
165 NGM plates at 25°C and then transferred to new plates every 36h to avoid any confusion between
166 offspring produced and original adults. At each time point, survival was scored. To measure
167 fecundity, the number of worm eggs on the plates at 120h since bleaching were counted.
168

169 ## Statistical Analysis

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

175 ## Results



176

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

184

185 Before the start of the evolution experiment, the starting conditions were tested. Confirming
186 previous results, *E. faecalis* showed some spontaneous host-protective potential against *S. aureus*.
187 Worms raised on *E. faecalis* and food survived better than those raised on food alone, independent
188 of food or pathogen present at the later stage (General Linear Model, X^2=10.205, df=1, p=0.001;
189 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*.

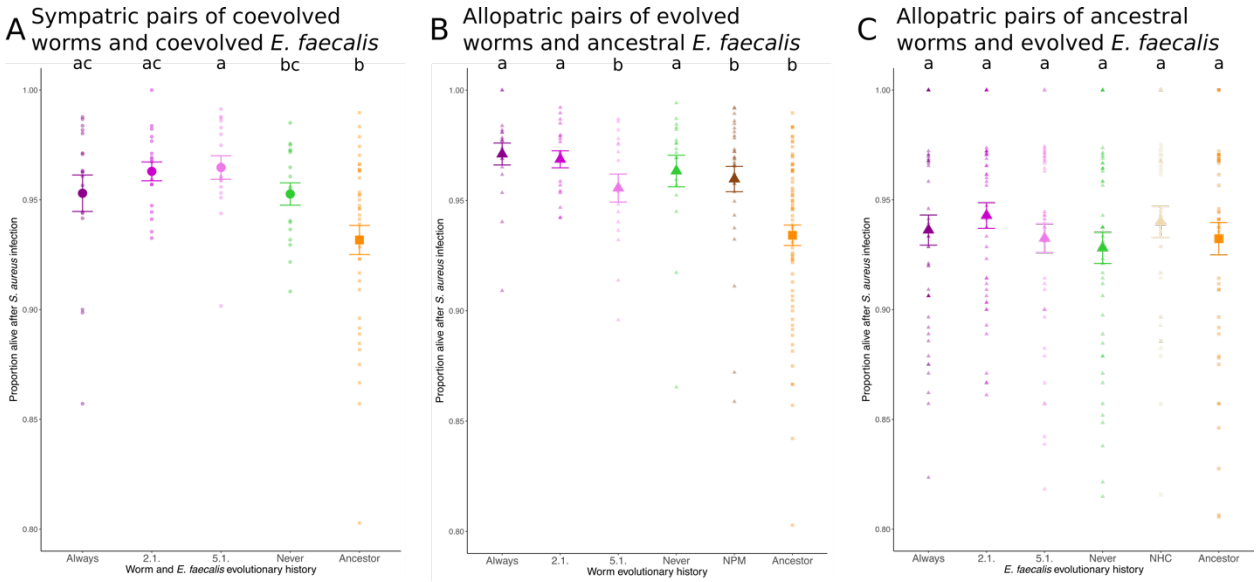
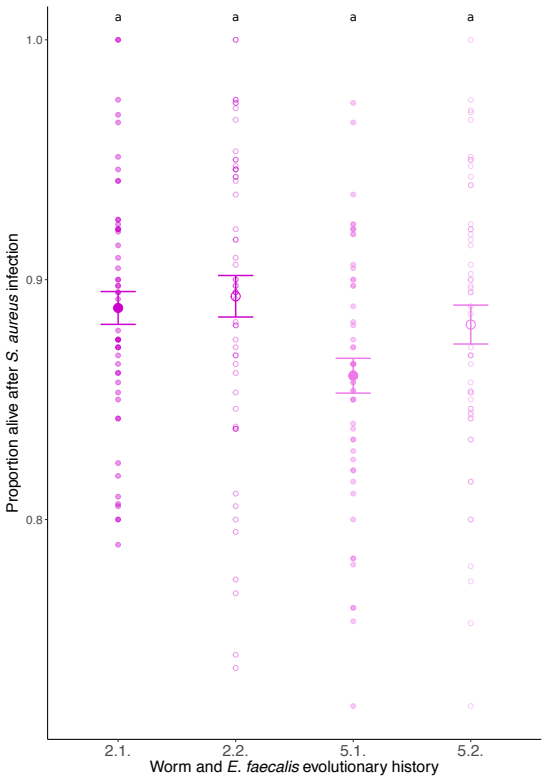


Figure 2: **Host survival for coevolving sympatric and allopatric pairs of worms and *E. faecalis***. Microbe-mediated protection was assessed for (A) sympatric pairs of coevolved worms and *E. faecalis,* (B) allopatric pairs of evolved worms and ancestral *E. faecalis*, and (C) allopatric pairs of ancestral 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 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 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<0.001; Figure 2B), but did not reveal the same pattern as for sympatric pairs. No effect of bacteria evolutionary history

211 alone on infected host survival was observed (Mixed Effects Model, X^2=3.2511, df=5, p=0.6613;
212 Figure 2C). Taken together, enhanced microbe-mediated protection evolved only as a product of
213 coevolution and pathogen presence for sympatric pairs; this occurred regardless of the temporal
214 heterogeneity.



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

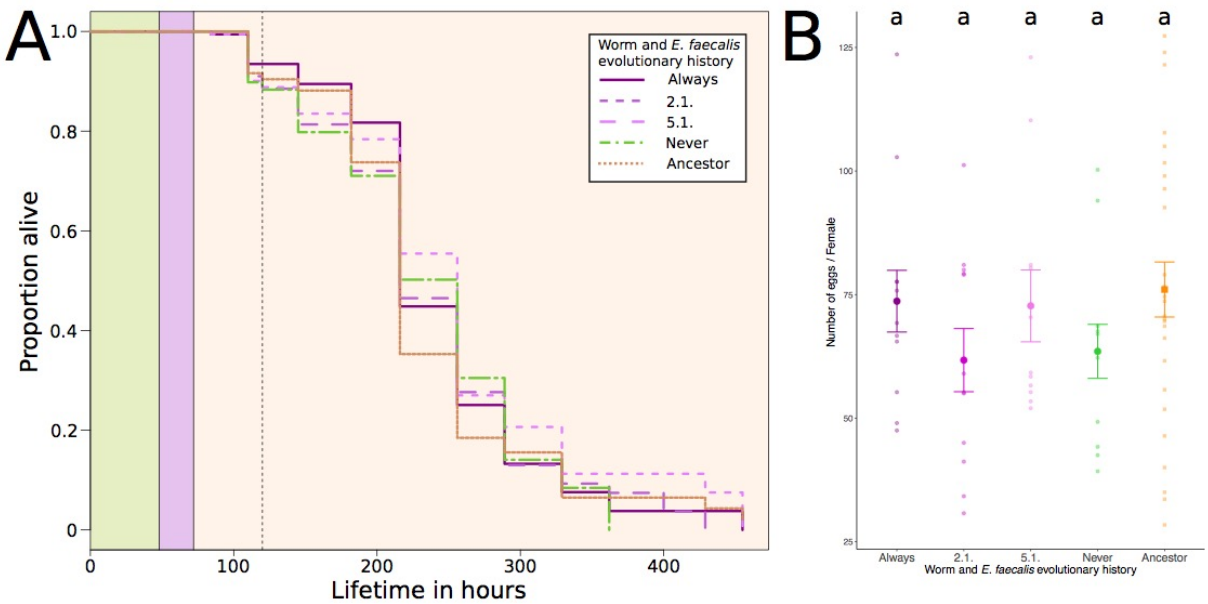
224

225 As an additional form of pathogen heterogeneity, the impact of the timing of initial pathogen
226 infection on the evolution of microbe-mediated protection was investigated. An effect of different
227 initial pathogen infection time points on host survival following pathogen infection was observed

228 (Mixed Effects Model: X^2=7.945, df=3, p=0.04716 Figure 3), although a Tukey Post-Hoc test

229 revealed no significant differences (Table A1).

230



231

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

241

242 Furthermore, we investigated the long-term consequences to hosts colonised by *E. faecalis* after

243 24h of pathogen infection. No significant differences were observed in the long-term survival post-

244 infection of worm hosts colonised by their sympatric *E. faecalis* across treatments (Kaplan Meier

245 Log Rank Test, FDR corrected, all comparisons p>0.05, Figure 4A). In addition, we did not find

246 significant differences in fecundity among sympatric host-*E. faecalis* pairs (Mixed Effects Model,

247 X^2=3.9418, df=4, p=0.4278, Figure 4B).

248 **Discussion**

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

259

260 We found that microbe-mediated protection is maintained even in the prolonged absence of
261 pathogen, but that pathogen presence is necessary for microbe-mediated protection to evolve, as
262 previously hypothesized (Clay et al., 2005; King and Bonsall, 2017; Lively et al., 2005). This result
263 is unlike previous work showing that the scale of heterogeneity in abiotic conditions can affect the
264 strength of selection for traits in some symbiotic interactions (Harrison et al., 2013). This
265 discrepancy is potentially due to costs in our symbiotic system being ameliorated (at least in terms
266 of host survival) in well-provisioned hosts, as hosts are provided with food alongside *E. faecalis*
267 and are thus rescued from starvation (also see (Dasgupta et al., 2019)). Although protective
268 symbionts can incur costs (e.g., Vorburger & Gouskov, 2011) for their hosts, with potential for
269 impacts on coevolutionary interactions (King and Bonsall, 2017), it is possible that potential costs
270 of bacterial colonisation might be only detectable when hosts are stressed (Lively, 2006) or that

271 the costs were not strong enough for us to detect(Little et al., 2002). Different measures of cost
272 remain to be explored (e.g. lifespan in the complete absence of a protective microbe and a
273 pathogen). Higher protection also does not always come with higher costs, as found in the black
274 bean aphid-*Hamiltonella defensa* interaction (Cayetano et al., 2015). Thus, protective traits in an
275 organism's commensal microbiota could be selected for under pathogen infection and easily
276 maintained in subsequent uninfected generations.
277
278 Microbe-mediated protection was strongest between sympatric pairs when pathogens were present
279 over evolutionary time, consistent with previous findings (Rafaluk-Mohr et al., 2018). In our study,
280 protection emerged during coevolution after only 20 host generations, and not due to the
281 independent evolution of either interacting species, but due to the coevolution of both species
282 (King and Bonsall, 2017). The time-scale of these interactions is short compared to the longer
283 shared evolutionary histories shared by other defensive mutualisms (Jousselin et al., 2003; Quek
284 et al., 2004; Shoemaker et al., 2002). Nevertheless, our findings reveal the potential for microbe-
285 mediated protection to become enhanced during the formation of a coevolving host-microbiota
286 relationship.
287
288 In conclusion, our results show that enhanced protection in host-microbe interactions can rapidly
289 evolve and be maintained even under infrequent pathogen infection, suggesting that resident
290 microbes can be a form of stable, transgenerational immunity. The protective benefit of an
291 organism's microbiota might remain undetected for several host generations until pathogens re-
292 emerge. Future research on the failure of pathogens transmit within host populations should
293 consider the contribution of the protective microbiota to prevent disease spread.

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Appendix

Experimental Procedure

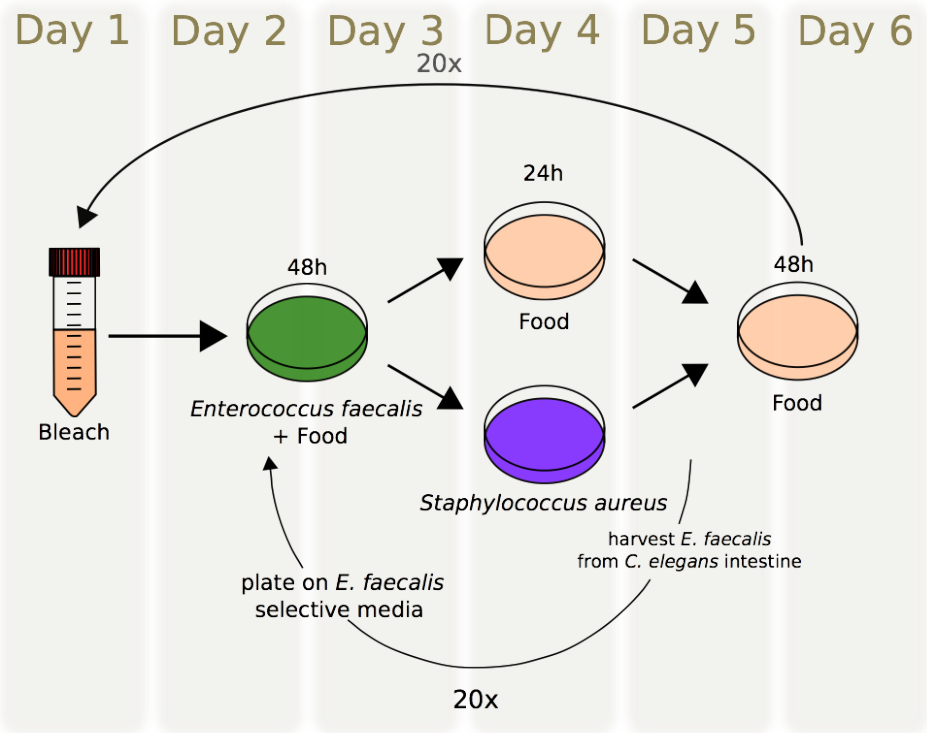

Figure A1: The experimental procedure of the evolution experiment in detail.

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(Stiernagle, 2006). 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(Jansen et al., 2015; Papkou et al., 2019; Rafaluk-Mohr et al., 2018).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 (Jansen et al., 2015; Papkou et al., 2019; Rafaluk-Mohr et al., 2018)). 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.

Figure	Results
1	Binomial GLM

Comparison	X^2	Degrees of Freedom	p-value
L1-L4 stage	10.205	1	0.001401
L4 – Adult	119.643	1	$<2.2 \times 10^{-16}$
Interaction	0.053	1	0.871

2A Binomial GLM

Evolution effect: X^2: 42.479, Df=4, $p=1.327 \times 10^{-8}$

Tukey Post-hoc comparisons:

Comparison	Estimate Std.	Error	z-value	p-value

	Always – Ancestor	-0.5490	0.1354	-4.055	<0.001
	2.1. - Ancestor	-0.5902	0.1348	-4.378	<0.001
	5.1. – Ancestor	-0.8224	0.1448	-5.680	<0.001
	Never – Ancestor	-0.3174	0.1290	-2.460	0.0985

2B

Binomial GLM

Evolution effect: X^2: 35.779, Df=5, p=1.051×10^{-6}

Tukey Post-hoc comparisons:

Comparison	Estimate Std.	Error	z-value	p-value
Always – Ancestor	-0.92335	0.19399	-4.760	<0.001
2.1. - Ancestor	-0.84208	0.18860	-4.465	<0.001
5.1. – Ancestor	-0.49494	0.18527	-2.671	0.0791
Never – Ancestor	-0.78893	0.19395	-4.068	<0.001
NPM – Ancestor	-0.52794	0.18847	-2.801	0.0560

2C

Binomial GLM

Evolution effect: X^2: 3.2511, Df=5, p= 0.6613

3

Binomial GLM

Evolution effect: X^2: 7.945, Df=3, p= 0.04716

Comparison	Estimate Std.	Error	z value	p-value
2.2. - 2.1.	0.05039	0.10064	0.501	0.9576
5.1. - 2.1.	-0.26298	0.12387	-2.123	0.1414
5.2. - 2.1.	-0.07281	0.12634	-0.576	0.9373
5.1. - 2.2.	-0.31337	0.12399	-2.527	0.0538
5.2. - 2.2.	-0.12320	0.12646	-0.974	0.7585
5.2. - 5.1.	0.19017	0.09467	2.009	0.1795

4A

Kaplan Meier Estimates

Comparison	p-value	FDR corrected p-value
Always – Ancestor	0.16086355	0.3217271
2.1. - Ancestor	0.71526638	0.7152664
5.1. – Ancestor	0.04131051	0.1652421
Never – Ancestor	0.04131051	0.7152664

4B	Binomial GLM Evolution effect: X^2: 3.8418, Df=4, p= 0.4278

525