Impacts of a novel defensive symbiosis on the nematode host microbiome

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Abstract

**Background:** Bacteria adapted to live within animals can protect their hosts against harmful infections. Beyond antagonism with pathogens, a ‘defensive’ bacterial symbiont could engage in additional interactions with other colonizing micro-organisms. A single bacterium might thus have cascading ecological impacts on the whole microbiome that are rarely investigated. Here, we assess the role of a defensive symbiont as a driver of host-associated microbiota composition by using a bacterial species (*Enterococcus faecalis*) that was previously experimentally adapted (*Enterococcus faecalis*) to a nematode host model (*Caenorhabditis elegans*).

**Results:** An analysis of 16S rRNA data from *C. elegans* exposed to *E. faecalis* and subsequently reared in soil, reveal that symbiont adaptation to host environment or its protective potential had minimal impact on microbiota diversity. Whilst the abundance of *Pseudomonas* was higher in the microbiota of hosts with protective *E. faecalis* (and another protective species tested), three other genera - *Serratia, Klebsiella* and *Salinispora* - were less abundant in hosts colonized by all *E. faecalis* strains. In addition, the protective effect of *E. faecalis* against opportunistic *Staphylococcus aureus* pathogens was maintained despite multi-species interactions within the microbiota.

**Conclusions:** Our results reveal the degree to which a new, evolving symbiont can colonise and maintain its conferred phenotype (i.e., pathogen-resistance) with minimal disruption to the host microbiota diversity.

**Background**

Animals can harbor a diversity of microbes. Many of these micro-organisms can be beneficial and protect their hosts against pathogen infection [1, 2]. These defensive microbial symbionts are important in determining infection outcomes across natural host populations [3, 4], and for hosts in agricultural or biomedical contexts [5–8]. It has also been suggested that defensive symbionts might prevent the transmission of devastating vector-borne diseases to humans [9].

Hosts are protected when these defensive symbionts block the growth or establishment of pathogens. These symbionts can suppress invading parasites and pathogens directly via toxin production [10] or offer protection through influence on their host immune systems and microbiomes [5, 11]. In addition
to pathogen-suppression, these mechanisms employed by a bacterial symbiont might have cascading ecological impacts on the whole microbiome within the host. This might be the case if the protection mechanism is not highly specific (e.g., bacteriocin secretion), but effective against a broad-spectrum of pathogen isolates or species [12, 13]. With competition thought to dominate species interactions within the microbiome [14], protectors could exclude casual colonizers or less competitive symbionts from the microbiota. By interacting with other species or shifting microbiota composition, symbionts might have negative effects on hosts by inadvertently increasing the infection load of pathogens [15, 16]. The protective phenotype conferred to the host could be diminished or lost. The degree to which individual symbionts can shape the composition of other constituents of host microbiota, such as core (i.e., essential microbes conserved in the majority of a species microbiomes) members, is unclear.

The microbiota can have a huge impact on host biology and health [17]. Thus, symbiont-mediated shifts in microbial communities could ultimately cause differences in in host phenotypes within and across populations.

Here, we test the impact of a novel bacterial symbiont on host microbiome structure, throughout the symbiont's in vivo evolution from initial interactions with the host. We used a bacterium (Enterococcus faecalis) that was experimentally adapted to form a defensive symbiosis with an animal host (Caenorhabditis elegans) using in vivo experimental evolution [10]. The selected E. faecalis populations vary in their ability to directly suppress infection by a virulent pathogen (Staphylococcus aureus) via reactive oxygen species [10]. C. elegans nematodes have a defined core microbiota [18–20] acquired in their natural habitats [20], and the E. faecalis strains used herein allow us to test for impacts of symbiont presence, protective ability, and evolutionary history on the host microbiome. We introduced symbiont-colonized nematodes to microbial communities in compost (to mimic natural microbiota colonization), allowed a microbiome to assemble inside the worm, and then conducted 16S rRNA sequencing. Since E. faecalis can suppress S. aureus colonization using a mechanism that is not species specific (i.e., ROS production), we predicted that other members of the nematode microbiome would also be inhibited. We further hypothesized that the protective effects of E. faecalis would remain in microbiome-colonised hosts, but be less effective overall. It has been
previously shown that fitness constraints from multiple interactions in polymicrobial communities can
dilute phenotypes normally observed in reduced systems [21]. However, the minimal shifts in
microbiome composition we found suggests that novel, rapidly evolving symbionts can be maintained
and remain effective without major disruption to the native microbiome.

Results
We allowed *C. elegans* to be colonized early in life with bacterial symbionts, followed with microbiota
assembly in compost, and then we conducted 16S rRNA sequencing of nematodes to examine
microbiota composition. Symbiont treatments included *E. faecalis* Anc, *E. faecalis* NP, *E. faecalis* P,
and *Pseudomonas mendocina*. The evolved protective *E. faecalis* population (*E. faecalis* P), evolved
non-protective *E. faecalis* (*E. faecalis* NP), and the ancestral *E. faecalis* (*E. faecalis* Anc) correspond to
a randomly-selected replicate population from CCE *E. faecalis*, SE *E. faecalis*, and ancestral *E. faecalis*
populations, respectively, in King et al. (2016) [10]. These populations have different genetic
compositions, and *E. faecalis* P contains non-synonymous SNPs in genes putatively associated with
superoxide production, the main mechanism for pathogen suppression [10]. Increased superoxide
production by *E. faecalis* in this system does not differentially affect host survival [22]. We used *P.
mendocina* since it has previously been shown to limit colonization of *Pseudomonas aeruginosa*
pathogens by inducing nematode immune responses [23]. We reasoned this bacterial species might
also shape nematode microbiota if hosts were more likely to resist colonization via immune
mechanisms. We sought to understand how early colonization with symbionts can shape microbiome
diversity, since deviations from normal microbiota diversity due to early colonization can link with
adverse outcomes for health host [24, 25].

**Colonization by *E. faecalis* did not change microbiome diversity**
We retained on average 46,317 16S rRNA reads per microbiome across 75 *C. elegans* microbiome
samples after quality filtering and preprocessing. Reads were processed into unique amplicon
sequence variants (ASVs), where each ASV represents a unique 16S rRNA read, or a rough proxy for a
microbial species. Early colonization by symbionts had a significant effect on observed ASVs ($F_{(4,39)} =$
3.84, P < 0.01) and Chao 1 diversity (\(F_{(4,39)} = 3.67\) P = 0.012) but not Shannon diversity (\(F_{(4,39)} = 0.478, P = 0.75\)) (Figure S1; Tables S1-4), likely indicating major differences were driven by ASV richness and the abundance of rare ASVs. Post-hoc analyses suggested that significant differences were driven by low ASV diversity in samples exposed to *P. mendocina* and not evolved *E. faecalis* strains (Tables S2, S3). Colonization by *E. faecalis* strains had no significant effects on microbiota alpha diversity within nematode hosts.

In our beta diversity analyses, the first two axes explained more than 50% of sample variance (Figure 2; PCo1 = 28.7% and PCo2 = 22.7%) and a marginal batch effect remained after removing it (ANOSIM; \(R^2 = 0.083; P = 0.01\); see “Methods”). Colonization treatment was a small but significant predictor of discernably clustering *C. elegans* microbiota diversity (Figure 2a; ANOSIM; \(R^2 = 0.201; P = 0.001\)). Amongst *E. faecalis* treatments, there were no significant differences in beta diversity (Figure 2b; ANOSIM; \(R^2 = 0.01; P = 0.34\)). This result reveals that the observed differences in beta diversity were driven by differences between symbiont species (*E. faecalis* vs. *P. mendocina*) and not by differences between *E. faecalis* strains.

### Microbiota differential abundance influenced by symbionts

Early exposure to a symbiont can negatively shape host-microbiota by subsequently increasing pathogen colonization [26]. We sought to understand if early exposure to *E. faecalis* P impacted the normal colonization of subsequent microbiota members. We measured how the different *E. faecalis* strains influenced significantly changing genera abundance. Comparing the microbiota in hosts colonized by *E. faecalis* strains and *P. mendocina* relative to the non-symbiont control (i.e., OP50), we observed that all three *E. faecalis* strains increased the abundance of an ASV identified as *Enterococcus* by an average of 7.13 log2fold (Figure 3; ANCOM; adj-P < 0.01). We hypothesize that the *E. faecalis* strains to which we exposed hosts were at increased abundance, but lack of strain-level resolution sequencing limits our conclusions. In this case, *Enterococcus* was from the environment compost communities only. We found that *P. mendocina* and *E. faecalis* P exposures
significantly increased abundance of *Pseudomonas* by an average of 5.82 log2fold (Figure 3; ANCOM; adj-P < 0.01). We also observed a decrease in abundance of *Serratia, Klebsiella* and *Salinispora* in the *E. faecalis* P, NP, and Anc treatments (Figure 3; ANCOM; adj-P < 0.01). Interestingly, the *E. faecalis* Anc exposures uniquely decreased the abundance of *Corynebacterium* (Figure 3; ANCOM; adj-P < 0.01). We also measured the differential abundance of microbiota among *E. faecalis* strain exposures but did not find any significant differences.

**Protection by *E. faecalis* maintained amidst microbiota**

We examined whether within-host adaptation by *E. faecalis* P [27] resulted in increased colonization efficacy and protection persistence in the nematode-microbiome system. Nematodes were colonized by, on average, 3.43x more *E. faecalis* P colony forming units (cfus) (mean = 8201 cfus; s.e. = 1540), than *E. faecalis* Anc (mean = 2664; s.e. = 543) and *E. faecalis* NP (mean = 2125; s.e. = 365), a finding that was significant (Figure 4a; CCE to Anc = one-tailed t-test t = 3.39, df = 4.98, adj-P = 0.015; CCE to SE = one-tailed t-test, t = 3.83, df = 4.45, adj-P = 0.015).

Symbiont-conferring phenotypes can be diluted in natural contexts. We therefore investigated protection persistence of *E. faecalis* P in hosts with a microbiota, and found that protection was maintained (Figure 4b). Early exposure to *E. faecalis* P, resulted in ~16.5% lower mortality during infection with *Staphylococcus aureus* compared to that with *E. faecalis* Anc or *E. faecalis* NP (Figure 4b; Wilcoxon test; one-tailed; adj-Ps = 0.016). Our results also indicate that initial colonization by the symbiont was a significant predictor of reduced mortality (Figure 4c; Pearson’s; R = -0.775; t = -4.42; df = 13; p < 0.01). We also found that *E. faecalis* colonization did not correlate with the relative abundance of *Enterococcus* taxa (Pearson’s, R = -0.642; t = -0.839; df = 1; p = 0.556; Figure S2).

Moreover, the relative abundance of *Enterococcus* after microbiome assembly did not predict decreased *S. aureus*-induced mortality (Pearson’s; R = 0.788; t = 1.28; df = 1; p = 0.422; Figure S3). These results suggest that *E. faecalis* strain type and early colonization abundance, but not *Enterococcus* relative abundance in the greater microbiota, was important for protection against *S. aureus* pathogens.
Discussion
Defensive bacteria have been found in the microbiota of a diversity of animal species [28]. We hypothesized that competition between defensive symbionts and other colonizing micro-organisms would impact microbiome diversity and structure, and we examined changes caused by ancestral and evolved strains. Here, we found that early colonization by an experimentally-adapted protective symbiont did not have significant effects on *C. elegans* microbiome diversity. The abundances of three microbiome components were reduced, a pattern consistent across nematodes colonized by all strains of the symbiont species tested.

Symbionts can have synergistic or antagonistic effects on others, effectively shifting symbiont services and costs [11, 26]. We found that nematode host microbiota diversity was not greatly affected by the early colonization of *E. faecalis*, regardless of evolutionary history or protective ability. Out of all early exposure treatments, only *P. mendocina* significantly decreased microbiome alpha diversity. This result could suggest that symbionts that protect by launching the host immune response might cause more substantive shifts in microbiome structure, particularly if host control plays a key role in maintaining the microbiome [29]. In human systems, low microbiome diversity has been associated with adverse health outcomes [24], but this link remains largely untested in wild animal systems (although see [30]). Across all strains, *E. faecalis* was found to minimally drive beta-diversity and therefore microbiome assembly. Convergence towards a “normal” microbiome regardless of early colonization is common in other hosts [31].

The minimal effects we did notice included an increased abundance of the core microbe *Pseudomonas* in the *E. faecalis* P treatment and decreased abundance of *Serratia, Klebsiella* and *Salinispora* in all treatments with *E. faecalis* strains. The results could be linked to microbe-microbe interactions or a host-mediated response, such as increased ROS inducing higher expression of genes associated with antagonism to other bacteria or with host colonization [32, 33]. The loss of *Serratia* was the most significant. Reductions in the abundance of this genus from the microbiome might thus have consequences for nematode longevity and fitness not investigated in this study. Some strains and species of *Serratia* can be beneficial or pathogenic to *Caenorhabditis* [34, 35], and pathogenic *S.
*marcescens* can drive nematode evolution and reproductive strategy [36].

The pattern of *E. faecalis* protective ability amongst strains was maintained in nematodes with a natural microbiome. However, the strength of defence was slightly reduced relative to single colonization of this symbiont [10], suggesting a dilution effect caused by the microbiome. This finding is consistent with other studies showing that fitness constraints imparted by diverse interactions in polymicrobial communities can change [37] and dilute [11, 38] phenotypes normally observed in reduced systems. Indeed, symbiont strains with distinct functions can persist in hosts [39], allowing for additive symbiont genetic and phenotypic diversity. *E. faecalis* P’s sustained protection amongst a more natural-like setting is promising for the application of probiotic microbes to hosts with a diverse microbiota. In some wild species-rich communities, microbe-mediated host protection can be diminished [21].

**Conclusions**

An *E. faecalis* strain experimentally evolved to protect *C. elegans* against infection had minimal impact on the host microbiome. This finding supports the idea that we can expand methods for yielding beneficial components of the microbiome beyond current methods to include genetic engineering of probiotics [8] or using experimental evolution [27]. For example, defensive *E. faecalis* did not alter the microbiota diversity or core microbiome members to a significant degree, yet still provided strong protection against a deadly pathogen. These results highlight that similarly-derived bacteria could be robust therapeutics, offering beneficial effects without disrupting microbiome health. However, it must be re-emphasized that the protective strains studied herein were evolved in the absence of a complex microbial community with the animal host. The community context can alter the evolutionary outcomes of individual microbe species [40, 41]. Future research could test whether microbe-mediated protection can be enhanced via evolution in hosts with a resident microbiota.

**Methods**

**Host-symbiont-pathogen system**

We used *C. elegans* Bristol N2 strain obtained from *Caenorhabditis* Genetic Center. They were
maintained using standard procedures (www.wormbook.org), and fed *Escherichia coli* OP50 on Nematode Growth Medium (NGM). Bacterial *E. faecalis* strains were *E. faecalis* OG1RF (aka Anc) [42], a strain from the human gastrointestinal tract, and randomly selected *E. faecalis* SE (NP herein) and *E. faecalis* CCE (P herein) from previously evolved lineages [10]. The *Pseudomonas mendocina* strain used was previously isolated from the soil, and was found to be capable of colonizing nematodes as part of their microbiome [23]. The *S. aureus* strain used was MSSA476 [43], a disease-causing pathogen that has previously been demonstrated to cause harm to worm hosts during infection [27].

**Experimental approach**

We provide a graphical abstract of assays related to our results in Figure 1.

**C. elegans exposures to food and bacteria**

These nematodes are easily reared in a gnotobiotic setting, sans intensive gnotobiotic procedures, allowing for controlled assembly of microbes in their gastrointestinal tract [10, 44]. Culturing of and *C. elegans* exposure to *E. faecalis* Anc, *E. faecalis* NP, or *E. faecalis* P were the same as in King et al. (2016) [10], with slight adjustments, including a different washing procedure that was described by Ford et al. (2016) [45]. This procedure was confirmed to remove the majority of externally adhering bacteria by Berg et al. (2016) [19]. In short, this included removing cutaneous microbes by washing nematodes three times with M9 buffer over a filter tip and spinning at 800 g. For all experiments, eggs were obtained from gravid nematodes by bleaching. Approximately 1000 nematodes were exposed as L1s to OP50 on NGM at 20°C and allowed to develop for 24 h. After being washed, they were transferred to the food control OP50 (since this bacterium is ground by the pharyngeal grinder and typically does not colonize *C. elegans*) or one of four symbiont treatment exposures – *E. faecalis* strains (Anc, NP, or P), or *P. mendocina* - at 25°C for 24 h. All bacteria were cultured overnight in lysogeny broth (LB) (OP50 and *P. mendocina*) or Todd-Hewitt Broth (*E. faecalis* strains and *S. aureus*) before being plated on NGM (OP50, 100μL) or Tryptone Soy Broth Agar (TSA; *E. faecalis* strains, *P. mendocina*, *S. aureus*; all at 60μL) and cultured for 24 h at 30°C. Culture and exposure procedures were consistent across all assays (RNA extraction, soil exposure, gut accumulation, and protection persistence), with differences only in replicates, batch numbers and treatment exposures, referred to
as the standard experimental exposure.

Compost preparation

Overripe bananas were supplemented to Westland Multi-Purpose Compost with added John Innes (Westland Horticulture; Dungannon, UK) to enrich microbiota via carbohydrates. They were left to compost at 20°C for 5 days before the mixture was disrupted and washed to create a microbial extract. To create the microbial extract, we added 2mL M9 to 5 g compost in a 50mL conical tube, vortexed vigorously for 60 seconds, transferred a 10mL aliquot to a 15mL conical tube and centrifuged the mixture for one minute at 300 g, and created a glycerol stock (25%) of the wash that was immediately stored at -80°C. To reconstitute compost with microbes prior to worm addition, 5g of autoclaved compost was supplemented with 1mL microbial wash and incubated for 48h at 25°C.

Worm compost exposure and harvesting

Five replicates of each treatment repeated over three replicate batches were used for compost exposures. Following the standard treatment exposure, nematodes were repeatedly washed and transferred to microbial enriched soil for 24h, after which ~700 nematodes were harvested over 2h using a Baermann funnel lined with tissue paper [19], then washed again and immediately stored at -80°C until DNA extractions.

DNA extractions

Genomic DNA was isolated from compost exposed nematodes (~700) or soil (0.25g) using the MO BIO PowerSoil DNA Isolation Kit (12888; MO BIO Laboratories; Carlsbad, CA, USA), with slight adjustments. For homogenization and cell lysis, we attached the MO BIO kit’s PowerBead Tubes to the Benchmark Scientific BeadBlaster Homogenizer (D1030-E; Benchmark Scientific; South Plainfield, NJ, USA) and homogenized and lysed cells for 60 seconds at 2800 rpm. Final gDNA was released from the silica membrane using 40μL sterile, nuclease-free water (Promega; Madison, WI, USA).

16S rRNA library preparation

The 16S rRNA V4 region was amplified from the worm microbiome gDNA using the 515F Golay-barcoded primers and 806R, primers revised by Apprill et al. and developed by Caporaso et al. and listed on the Earth Microbiome Project (EMP) 16S protocol site (http://www.earthmicrobiome.org/emp-
standard-protocols/16s/). Samples were prepared in accordance with the standard EMP 16S rRNA protocol. 25µL polymerase-chain reactions (PCR) contained 10µL Platinum Hot Start MM (2X) (company), 11µL nuclease-free water, 1µL of each forward and reverse primer (0.20 uM final concentrations), and 2µL Genomic DNA (gDNA) template. No-template controls (NTCs) contained nuclease free water in lieu of gDNA. Reactions were held at 94°C for 3min to denature the DNA, and amplification took place for 35 cycles at 94°C for 45 sec, 50°C for 60 sec and, 72°C for 90 sec. The cycles were followed by a hold at 72°C for 10 min. Amplicons were visualized on a 1.5% agarose gel.
gDNA was quantified using the Qubit 2.0 (Thermofisher, Bartlesville, OK) and amplicons were pooled at equimolar ratios (~ 240ng per sample). The combined amplicon pool was then cleaned using the Qiagen PCR Purification Kit (Qiagen, Germantown, MD). The multiplexed library was quality checked and sequenced with the MiSeq 2x250nt PE v2 protocol at the W.M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign; Urbana, IL, USA).

Gut accumulation enumeration and protection persistence

Five replicates of each treatment from the same batch were used for gut accumulation enumeration and protection persistence assays. Following the standard treatment exposure, nematodes were repeatedly washed and then either transferred to microcentrifuge tubes containing ten 1 mm zirconia/silica beads in 50µL M9, for the gut accumulation enumeration, or advanced to soil exposures for the protection persistence assay. The nematodes were homogenized and bacteria were released using the Benchmark Scientific BeadBlaster Homogenizer (D1030-E; Benchmark Scientific; South Plainfield, NJ, USA) for 45s at 2800 rpm. Dilution series of the mixture were plated on TSA and cfus were enumerated after incubating at 30°C for 24 h. For the protection persistence assay nematodes were transferred to plates with S. aureus and exposed for 24 h at 25°C. After exposure, we calculated mortality by counting alive and dead nematodes.

16S rRNA bioinformatic processing and analyses

PhiX sequences were first removed from my library using Bowtie2 by mapping my reads against an index built from a phiX genome (found at
support.illumina.com/sequencing/sequencing_software/igenome.html). Demultiplexed, paired-end fastq files were then processed in R (3.4.0) using DADA2 as previously described [46]. In short, this included filtering and trimming, error rate estimation, dereplication of reads into unique sequences, and ribosomal variant inference. We then merged paired-end reads, constructed a ribosomal sequence variant (ASV) table (sample x sequence abundance matrix), and removed chimeras. We also used DADA2’s native implementation of the Ribosomal Database Project (RDP) naïve Bayesian classifier trained against the GreenGenes 13.8 release reference fasta (https://zenodo.org/record/158955#.WQsM81Pyu2w) to classify ASVs taxonomically. For DADA2 and phyloseq processing we provide a reproducible R Markdown file (Supplementary File 1).

We described early exposure to symbionts effects on subsequent microbiota assembly and diversity using both within (alpha) and between (beta) sample diversity measurements. Observed ASVs indicates the number of ASVs per sample, the Shannon metric is an equal weighted metric for species richness and evenness, and the Chao 1 index is a metric weighted towards rare ASVs that also incorporates richness and evenness.

We created visualizations and conducted statistical analyses on the ASV table in R (3.4.0). To calculate alpha diversity measurements of observed ASVs, Shannon’s index and Chao 1, we used phyloseq’s (1.16.2) [47] estimate_richness function. Phyloseq was also used to perform ordinations, using PCoA on UniFrac distance scores [48]. To perform differential abundances analyses, we used the ANCOM package [49]. Other R packages used include: ggplot2, for visualizing data and making figures (2.0.0); Rcpp for C++ parallelization in R; optparse (1.3.2.) to parse command line options; stats (3.2.3) to conduct statistics; and data.table (1.9.6) to handle data frames. For our 16S rRNA analyses we have also provided an R markdown file outlining a fully reproducible workflow (Supplementary File 1).

**Statistical analysis**

For all analyses we used R (v3.5.3). For all tests, we report exact n-values in figure legends. We also provide complete ANOVA tables, including F-values and degrees of freedom, as supplementary tables. For t-tests and Wilcoxon tests, we provide P-values, t-values and degrees of freedom in results.
Our microbiome samples were prepared in three independent batches, with 5 biological replicate per treatment in each batch, yielding a total of 75 *C. elegans* microbiome samples. After pre-processing, we retained 65 *C. elegans* microbiome samples. For taxa, pre-processing included removing taxa from samples found in non-template controls, and removing taxa not observed at least once in 20% of samples. We corrected for a batch effect in beta diversity and differential taxa abundance analyses using a variance stabilizing transformation, which normalizes taxa count data based on depth factor and produces a matrix with values that are homoscedastic. We corrected for a batch effect in alpha diversity measurements by testing for batch as a significant predictor, then pruning batch three, which accounted for the majority of outliers.

To examine whether the different symbiont exposures affected microbiota alpha diversity, we used Analysis of variance (ANOVA) tests with Tukey’s honest significant difference (HSD) for post-hoc comparisons. After rarefying, we retained 45 *C. elegans* microbiome samples for alpha diversity tests. We report exact n-values in figure legends and complete ANOVA tables, with F values and degrees of freedom.

To calculate beta diversity we first built a distance matrix based on samples’ weighted UniFrac scores [48], and performed PCoA on the distance matrix. To test whether the symbiont exposures affected microbiota beta diversity, we used Analysis of Similarity (ANOSIM) tests. For these comparisons, we analyzed high-level beta diversity differences by using the 65 *C. elegans* microbiome samples that were pre-processed and variance stabilized. The exact amounts of replicates remaining per treatment are reported in figure legends. All ANOSIMs were conducted with 999 permutations, and ANOSIM R statistics ($R^2$).

For differential abundance of taxa analyses, we corrected for batch effects by incorporating batch as a term in the design formula of an ANCOM analysis [49]. Again, this test used the 65 pre-processed *C. elegans* microbiome samples, with exact n-values reported in the figure legend.

To analyze how transcript abundances related to *Enterococcus* abundance amongst the microbiome and initial *Enterococcus* accumulation in *C. elegans*, we calculated Pearson’s correlation coefficients.

We also calculated Pearson’s correlation coefficient to analyze how *Enterococcus* abundance in the
microbiome related to initial colonization and protection persistence. Since these comparisons were not within the same batch, but rather between batches from different experiments, we had to aggregate treatment samples and were limited to one data point per treatment.

To test for treatment differences in colonization and protection, we first analyzed data distributions and then used parametric or nonparametric tests where appropriate. We used one-tailed t-tests (with Holm corrected P-values) to test whether *E. faecalis* P accumulated more than other *E. faecalis* strains in nematode guts. To test for differences in symbiont-mediated protection against *S. aureus* across treatments, we used one-tailed Wilcoxon ranked sum tests (with Holm corrected P-values). To test whether there was a correlation between colonization and protection persistence, we calculated Pearson’s correlation coefficient.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

All authors have consented to publication of this manuscript

**Availability of data and materials**

The packages and pipelines used are available, with documentation, on their respective sites and repositories. The main pipelines used, DADA2 (https://github.com/benjjneb/dada2), and phyloseq (https://joey711.github.io/phyloseq/) are all open-source and publicly available. R markdown files for implementing these packages on our data are available in supplementary files (Supplementary File 1). The 16S rRNA dataset supporting the conclusion of this article is available in the EMBL-EBI repository under the primary accession number PRJEB26987.

**Competing interests**

The authors declare that they have no competing interests.

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Author Contributions

D.D., K.C.K., and G.M.P. designed the study. D.D. and J.S. carried out the experiments. D.D. conducted the DNA extractions, as well as the bioinformatic and statistical analyses. D.D. and K.C.K. wrote the manuscript. All authors have read and approved the manuscript.

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Figures
Figure 1

Graphical assay abstract. C. elegans exposed to different defensive symbiont strains underwent 16S rRNA analysis and phenotypic assays. Symbiont colonization enumeration, microbiota profiling, and protection persistence was assessed.
Figure 2

Principal coordinate analyses (PCoA) on weighted UniFrac scores of C. elegans microbiota. a. PCoA on weighted UniFrac scores by early exposure treatment. Symbiont treatment was as
a significant predictor of ecosystem distance. b. PCoA on weighted UniFrac scores comparing microbiota from E. faecalis strain exposures. Early colonization by E. faecalis strains was not a significant predictor of ecosystem distance. Ellipses are drawn at 95% confidence intervals. Ancestor: Anc; Protective E. faecalis: P; Non-protective E. faecalis: NP; E. coli OP50 food: OP50; P. mendocina: Pm.

Figure 3

Microbes that significantly differed in abundance under exposure treatments. Log2fold change of significantly differentially abundant ASVs identified comparing microbiota of C. elegans with colonization by different symbionts to food. There were no significant differences between C. elegans colonized with different E. faecalis. Ancestor: Anc; Protective E. faecalis: P; Non-protective E. faecalis: NP; E. coli OP50 food: OP50; P. mendocina: Pm.
E. faecalis P colonizes hosts at higher density and protects against pathogen-induced mortality in a natural microbiome context. a. Within-host colony-forming units of evolved and ancestral E. faecalis symbiont. b. C. elegans mortality after symbiont, compost, and pathogen exposures. c. Correlation between E. faecalis colonization abundance and pathogen-induced mortality in hosts with a microbiota. n = 5 populations per treatment.

Error bars = ± s.e. Ancestor: Anc; Protective E. faecalis: P; Non-protective E. faecalis: NP; E. coli OP50 food: OP50; P. mendocina: Pm.

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