Microbiome responses in a novel nematode defensive symbiosis

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Abstract

Background Bacteria adapted to live within animals can protect their hosts against harmful infections. Beyond antagonism with parasites, 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 symbiont as a driver of host-associated microbiota composition by using an experimentally-adapted bacterium with protective properties (Enterococcus faecalis) inside 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 E. faecalis presence and adaptation to host environment had minimal impact on microbiota diversity. In addition, the protective effects of E. faecalis against opportunistic Staphylococcus aureus pathogens were still maintained despite multi-species interactions within the microbiota.

Conclusions Our results reveal the degree to which a novel, evolving symbiont can colonise and maintain its conferred phenotype (i.e., parasite-resistance) with minimal change to the host microbiome.

Background

Animals harbor a diversity of microbes in their microbiota. Many of these can be beneficial by protecting hosts against pathogen infection[1, 2]. These defensive microbial symbionts are important in determining infection outcomes in natural host populations[3], but also for hosts in applied contexts[4–6], such as preventing disease transmission in humans[7]. Understanding how a symbiont affects its host organism necessitates understanding the effects of the symbiont on the whole host-microbiome system. Symbionts can limit the success of pathogens, and therefore protect, but can also selectively exclude non-
symbionts [8] and potentially less competitive symbionts from the microbiota. By shifting microbiota composition, symbionts might have adverse effects on hosts by increasing the infection load of pathogens[9]. Whether individual symbionts can shape the composition of other mutualistic constituents of host microbiota, such as core (i.e., essential microbes conserved in the majority of a species microbiomes) members, is unclear. Given the huge impact of microbiota on host biology and health [11], symbiont-mediated shifts in microbial communities could indirectly cause differences 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 defensive bacterium (*Enterococcus faecalis*) that was artificially adapted to an animal host (*Caenorhabditis elegans*) using *in vivo* experimental evolution[12]. This *E. faecalis* population and others herein were taken from the experiment in King et al. (2016) whereby the authors derived a strains capable of directly suppressing infection by a virulent pathogen (*Staphylococcus aureus*) via superoxide antimicrobials. For comparison, we also used a similarly adapted, but non-protective *E. faecalis*, as well as a common ancestor that formed a novel interaction with *C. elegans*. The evolved, protective *E. faecalis* population (*E. faecalis* P), evolved and non-protective *E. faecalis* (*E. faecalis* NP), and the ancestor *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). These populations are genetically divergent, and *E. faecalis* P contains non-synonymous SNPs in genes putatively associated with superoxide production, the main mechanism for pathogen suppression[12]. Our model system provides a powerful empirical approach to disentangling the various aspects of protective symbiont evolutionary biology and action on their role in shaping the host microbiome.
Firstly, the bacterial populations we selected allow us to control for symbiont presence, cost, and the action of the protective mechanism, as well as symbiont evolutionary history on the host-microbiome system. Secondly, our host organism, *C. elegans*, is a well-established model for infection pathogenesis and innate immunity[13–15]. This animal is also now a rapidly-developing model for host-microbiome research[16]. The nematodes have a defined core microbiota[17–19] that they acquire by eating bacteria in their natural habitats[20].

We tested whether this novel defensive symbiosis affected the natural *C. elegans* microbiota. We exposed colonized nematodes to microbial communities in compost, to mimic natural microbiota colonization, and conducted 16S rRNA sequencing to assess how *E. faecalis* shaped the subsequent assembly of host-associated microbiota. Since the evolve *E. faecalils* strain confers greater resistance to *S. aureus*, we reasoned that it might also affect other members of the nematode microbiome. We hypothesized that *E. faecalis* presence, adaptation, and protective phenotype would have distinguishable effects on the *C. elegans* microbiome. Adapted *E. faecalis* had a small impact on driving natural host-microbiota assembly. We also hypothesized that protective effects of *E. faecalis* would remain amongst a natural microbiome, but be less effective at protecting overall. This is motivated by the rationale that fitness constraints imparted by diverse interactions in polymicrobial communities can dilute phenotypes normally observed in reduced systems[22]. Indeed, there were minimal shifts in the abundance of other microbial symbionts, and the symbiont maintained its protective ability amongst a natural microbiota. Interestingly, this result suggests that new, rapidly evolving symbionts can be maintained in the host without disrupting the native microbiome. Overall, we show that beneficial host-microbe relationships conferring protection against pathogens can have minimal implications for the host microbial community.
Results

Colonization by *E. faecalis* did not disrupt microbiome diversity

We exposed *C. elegans* to bacterial treatments and then compost prior to 16S rRNA sequencing. Treatments included *E. faecalis* Anc, *E. faecalis* NP, *E. faecalis* P, *E. coli* OP50, and *P. mendocina*. We used *P. mendocina* since it previously showed to limit colonization by monocultures and therefore also reasoned it would shape a colonizing natural microbiota [15]. 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[23]. 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 ribosomal sequence variants (RSVs), where each RSV represents a unique 16S rRNA read, or a rough proxy for a microbial species. Early colonization by symbionts had a significant effect on observed RSVs and Chao 1 diversity but not Shannon diversity (ANOVA; Tukey-HSD; Figure S1; Tables S1–4), likely indicating major differences were driven by RSV richness and the abundance of rare RSVs. Post-hoc analyses suggested that significant differences were driven by low RSV diversity in samples exposed to *P. mendocina* and not evolved *E. faecalis* strains (ANOVA; Tukey-HSD; adj-Ps < 0.05; exact P-values in Tables S2, S3). Thus, colonization by *E. faecalis* strains had no significant effects on microbiota alpha diversity.

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; $R^2 = 0.201; P = 0.001$). However, this result was driven by the difference between exposures with *P. mendocina* or *E. coli* OP50 to exposures to *E. faecalis* strains. Amongst *E. faecalis* strain exposures, 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 and not by differences between *E. faecalis* strains.

Microbiota differential abundance influenced by symbionts

Early monoculture exposure to commensal microbiota can negatively shape host-microbiota colonization by subsequently increasing parasite colonization[9]. Thus, we sought to understand if early exposure to *E. faecalis* P impacted normal colonization of subsequent microbial colonizers. We measured how the different *E. faecalis* populations influenced significantly changing genera abundance. First, comparing the microbiota in hosts colonized by *E. faecalis* populations and *P. mendocina*, we observed that all three *E. faecalis* populations increased the abundance of an RSV identified as *Enterococcus* (sq10; base mean = 1277), by an average of 12.4 log2fold (s.e. = 0.279) (Figure 3a). We hypothesize that *E. faecalis* populations to which we exposed hosts were at increased abundance, but lack of strain-level resolution sequencing limits our conclusions. Interestingly, *P. mendocina* also increased *Enterococcus* abundance but only by 6.08 log2fold (Figure 3a). In this case, *Enterococcus* was from the environment compost communities only. We also found that *P. mendocina* and *E. faecalis* NP exposures significantly decreased abundance of an RSV previously identified as corresponding to the core *C. elegans* microbiota genus, *Sphingomonas*[17] (sq256; base mean = 0.481), by an average of 26.6 log2fold (s.e. = 0.149).

We also measured differential abundance of microbiota among *E. faecalis* populations (Figure 3b; Table S5). Compared to the ancestor, *E. faecalis* P induced differential
abundance of three RSVs and *E. faecalis* NP of four RSVs, with the only shared one being a decrease in *Tetragenococcus* (sq103; base mean = 2.92). Uniquely, *E. faecalis* P significantly influenced an increase of the core microbe, *Sphingomonas*, by an average of 26.2 log2fold (s.e. 4.21). As expected, no genera found in the compost samples with the potential to be pathogens (i.e., *Bacillus* and *Pseudomonas aeruginosa*)[15,24] increased in abundance with *E. faecalis* colonization.

Protection by *E. faecalis* maintained amidst microbiota

We examined whether within-host evolution of *E. faecalis* P resulted in increased colonization efficacy and protection persistence. 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, adj-P = 0.015, t = 3.39, df = 4.98; CCE to SE = one-tailed t-test, adj-P = 0.015, t = 3.83, df = 4.45).

Symbiont-conferred phenotypes can be diluted in natural contexts[25]. Thus, we investigated protection persistence of *E. faecalis* P after hosts were colonized by their natural microbiota. We found that protection by *E. faecalis* P was maintained amongst a natural microbiota (Figure 4b). Early exposure to *E. faecalis* P, resulted in ~16.5% lower mortality upon *Staphylococcus aureus* pathogen exposure than with early exposure to *E. faecalis* Anc or *E. faecalis* NP (Figure 4b; Wilcoxon test; one-tailed; adj-Ps = 0.016). Our results also indicate that initial colonization was a significant predictor of 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; P = 0.556; t = -0.839; df = 1; Figure S2). Moreover, the relative abundance of *Enterococcus* post compost exposure did not predict decreased *S. aureus*-induced
mortality (Pearson’s; $P = 0.422$; $t = 1.28$; df = 1; Figure S3). These results suggest that *E. faecalis* population 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, benefitting their hosts in both natural and applied settings [26]. These symbionts can suppress invading parasites and pathogens directly via toxin production[12] or offer protection through influence on their host immune systems and microbiomes[4,27]. We hypothesized that beneficial interactions between protective bacterial species with the host and competition with other colonizing micro-organisms would impact the host-microbiome system, and tracked changes over evolutionary time. Here, we found that during the process of adaptation of a protective bacterial symbiont, minimal effects on the *C. elegans* microbiome were triggered. Symbionts can have synergistic or antagonistic effects on others, effectively shifting symbiont services and costs[9,27]. We found that microbiota diversity and structure were not greatly affected by the early colonization of *E. faecalis*, regardless of evolutionary history or protective phenotype. Out of all early exposure treatments, only *P. mendocina* significantly decreased alpha diversity. In human systems, low microbiome diversity has been associated with adverse health outcomes[23], but this link remains untested in nematodes. Across all populations, the *E. faecalis* species was found to minimally drive beta-diversity and therefore microbiome assembly. These results are not surprising since convergence towards a “normal” microbiome regardless of early colonization is common in other hosts[28].

The minimal effects we did notice included increased abundance of the core microbe
Sphingomonas in the E. faecalis P treatment and decreased abundance of Tetragenococcus from the E. faecalis P and NP treatments. 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 [29] [30].

The pattern of E. faecalis protective ability amongst strains was maintained in nematodes with a natural microbiome, albeit the strength of defence was slightly reduced relative to [12], suggesting a dilution effect. A dilution effect due to multi-species interactions is consistent with other systems showing that fitness constraints imparted by diverse interactions in polymicrobial communities can change[31] and dilute[22,27] phenotypes normally observed in reduced systems. Indeed, symbiont strains with distinct function can persist in hosts[32], allowing for additive symbiont genetic and phenotypic diversity. E. faecalis P’s sustained protection amongst a natural setting is promising since in some natural systems microbes protective effects can be completely ameliorated [25].

Conclusions

In conclusion, an E. faecalis strain evolved to protect C. elegans against infection, had minimal impact on the host microbiome. This work also supports the idea that we can expand methods for yielding beneficial components of the microbiome beyond current methods to include engineering by experimental evolution with vast implications in applied fields. For example, as E. faecalis Pdid not alter the microbiota diversity or core microbiome members to a significant degree, yet still provided strong protection, this suggests similarly-derived bacteria could be robust therapeutics, offering beneficial effects without disrupting microbiome health. However, it must be re-emphasized that the protective strains herein were evolved in the absence of a complex microbial community with the animal host. Given that microbes can evolve more rapidly, resulting in more
extreme phenotypes amidst complex communities (refs), future research should test whether protective effects can be enhanced via evolution in hosts with microbiota present.

Methods
Host-symbiont-pathogen system
*C. elegans* used were Bristol N2, from *Caenorhabditis* Genetic Center. Bacterial *E. faecalis* strains were *E. faecalis* OG1RF (aka Anc) [34], a strain from the human gastrointestinal tract, and randomly selected *E. faecalis* SE and *E. faecalis* CCE from previously evolved lineages[12]. *Pseudomonas mendocina* used was previously isolated from the natural *C. elegans* microbiome[15]. The *S. aureus* strain used was MSSA476[35], a disease-causing pathogen.

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[12,36].

Culturing and *C. elegans* exposure of and to *E. faecalis* Anc, *E. faecalis* NP, or *E. faecalis* P were the same as in King et al. (2016), with slight adjustments including a different washing procedure that was described by Ford et al. (2016). This procedure was confirmed to remove the majority of externally adhering bacteria by Berg et al. (2016). In short, this included removing cutaneous microbes by washing worms three times with M9 over a filter tip and spinning at 800 g. In brief, for all experiments eggs were obtained from gravid worms by bleaching, approximately 1000 worms were exposed as L1s to *E. coli* OP50 at 20°C and allowed to develop for 24 h, then filter tip washed and transferred to treatment exposures—the non-colonized exposure control *E. coli* OP50 (since *E. coli* OP50 are ground
by the pharyngeal grinder and typically do not colonize *C. elegans* [36]), *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) (*E. coli* OP50 and *P. mendocina*) or THB (*E. faecalis* strains and *S. aureus*), before being plated on NGM (*E. coli* OP50, 100ul) or TSA (*E. faecalis* strains, *P. mendocina*, *S. aureus*; all at 60ul) and cultured for 24 h at 30°C. Culture and exposure procedures were consistent in all assays (RNA extraction, soil exposure, gut accumulation, and protection persistence), with differences only in replicates, batch numbers and treatment exposures, and is now referred to as the standard experimental exposure. For the soil exposure experiment, wormswere also early exposed to *P. mendocina*, which was cultured overnight in LB then, the same as *E. faecalis*, plated (60ul) on TSA and grown overnight at 30°C. The *E. faecalis* NP and *E. faecalis* P strains used were randomly selected lineages from the previous evolution experiment [12]. The same evolved lineages were used for all batches. For *E. faecalis* P this was lineage CCE-A and for *E. faecalis* NP it was SE-A. Throughout the experiments, for cultures and plating of all treatments, each colony was twice streaked to ensure that they were isogenic.

**Compost preparation**

Overripe bananas were supplemented to Westland Multi-Purpose Compost with added John Innes (Westland Horticulture; Dungannon, UK) to enrich microbiota via carbohydrates and left to compost at 20°C for 5 days before 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 prior to addition of worms [18].

**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 - where treatments were *E. coli* OP50 (control), *E. faecalis* strains (Anc, NP, or P), or *P. mendocina* - worms were extensively filter tip washed and transferred to microbial enriched soil for 24h, after which ~700 worms were harvested over 2h using a Baermann funnel lined with tissue paper, then filter tip washed again and immediately stored at −80°C until DNA extractions.

**DNA extractions**

gDNA was isolated from compost exposed worms (~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 40ul 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 by Apprill et al. and developed by Caporaso et al. [37,38] 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. 25ul polymerase-chain reactions (PCR) contained 10ul Platinum Hot Start MM (2X) (company), 11ul nuclease-free water, 1 ul of each forward and reverse primer (0.20 uM final concentrations), and 2ul 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 - where treatments were *E. coli* OP50 (control), *E. faecalis* strains (Anc, NP, or P), or *P. mendocina* - worms were extensively filter tip washed and then either transferred to microcentrifuge tubes containing ten 1 mm zirconia/silica beads in 50ul M9, for the gut accumulation enumeration, or advanced to soil exposures for the protection persistence assay. For gut accumulation enumerations, the worms were homogenized and gut bacteria released using the Benchmark Scientific BeadBlaster Homogenizer (D1030-E; Benchmark Scientific; South Plainfield, NJ, USA) for 45 seconds 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 worms 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 worms.

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 [39] as previously described
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 (RSV) 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 [40] trained against the GreenGenes 13.8 release reference fasta (https://zenodo.org/record/158955#.WQsM81Pyu2w) to classify RSVs taxonomically. For DADA2 and phyloseq processing we provide a reproducible R Markdown file (Supplementary File 2).

We described early exposure to symbionts effects on subsequent microbiota assembly and diversity using both within (alpha) and between (beta) sample diversity measurements. Observed RSVs indicates the number of RSVs 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 RSVs that also incorporates richness and evenness.

We created visualizations and conducted statistical analyses on the RSV table in R (3.4.0). To calculate alpha diversity measurements of observed RSVs, Shannon’s index and Chao 1, we used phyloseq’s (1.16.2) [41] estimate_richness function. Phyloseq was also used to perform ordinations, using PCoA on UniFrac distance scores[42]. To perform differential abundances analyses, we used the DESeq2 package [43]. Other R packages used include: ggplot2, for visualizing data and making figures (2.0.0) [44]; Rcpp for C++ parallelization in R[45]; 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 2).

Statistical analysis
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. We provide both significant and non-significant P-values in results. 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 test how treatment exposures affect 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 [42], and performed PCoA on the distance matrix. To test how treatment exposures affect 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.
ANOSIMs were conducted with 999 permutations, and ANOSIM R statistics ($R^2$) and exact P-values are reported in the results and figure legends.

For differential abundance of taxa analyses, we corrected for batch effects by incorporating batch as a term in the design formula of our DESeq2 analysis. Again, this test used the 65 pre-processed C. elegans microbiome samples, with exact n-values reported in the figure legend. We employed the main DESeq2 algorithm, DESeq, to conduct a differential count analysis based on the negative binomial distribution. In short, this includes estimating size factors, estimating dispersion, negative binomial general linear model fitting, and calculating Wald statistics.

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. With each test, we reported degrees of freedom, P-values, and T-statistics in results and figure legends. 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. Testing for colonization via CFU accumulation, we used one-tailed t-tests with Holm corrected P-values, with the hypothesis that colonization was highest amongst C. elegans exposed to E. faecalis P. Testing for differences in C. elegans protection against S. aureus by treatment, we used one-tailed Wilcoxon ranked sum tests with Holm corrected P-values, with the hypothesis that protection was highest by E. faecalis P. Exact n-values are reported in figure legends and P-values, test statistics and degrees of freedom are
reported in results. To test the correlation between colonization and protection persistence, we again calculated Pearson’s correlation coefficient and provided the test statistic, degrees of freedom and P-value in the results.

Availability of data and materials
The packages and pipelines used are available, with documentation, on their respective sites and repositories. Concerning 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 Files 2–4). The 16S rRNA dataset supporting the conclusion of this article is available in the EMBL-EBI repository under the primary accession number PRJEB26987.

Declarations

Competing interests
The authors declare that they have no competing interests.

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Author Contributions
D. D., G. M. P. and K. C. K. designed the study. D. D. conducted the pre-exposure experiments and DNA extractions. D. D. conducted the bioinformatic and statistical analyses. D. D. and K. C. K. drafted the manuscript. All authors reviewed and approved the final manuscript.

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**Ethics approval and consent to participate**

Not applicable.

**Consent to publish**

Not applicable.

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Figures
Graphical assay abstract. C. elegans exposed to different bacterial strains underwent 16S rRNA analysis and phenotypic assays. Colonization enumeration was conducted on C. elegans colonized with strains E. faecalis Anc, E. faecalis P, or E. faecalis NP. Microbiota profiling was conducted for C. elegans colonized with E. faecalis Anc, E. faecalis P, E. faecalis NP, P. mendocina, or E. coli OP50. Protection persistence was conducted for C. elegans colonized with E. faecalis Anc, E. faecalis P, or E. faecalis NP.
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 (ANOSIM; R2 = 0.201; adj-P = 0.001; perm = 999). 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 (ANOSIM; R2 = 0.01; adj-P = 0.340; perm = 999). Ellipses are drawn at 95% confidence intervals.
Figure 3

Microbes that significantly differed in abundance under exposure treatments. a. Log2fold change of significantly differentially abundant RSVs identified comparing microbiota of C. elegans colonized by different symbionts or food (DESeq2; adj-P < 0.05). c. Log2fold change of significantly differentially abundant RSVs identified comparing microbiota of C. elegans.
E. faecalis P colonizes hosts at higher density and protects against parasite-induced mortality in a natural microbiome context. a. C. elegans gut bacterial
CFUs after colonization by evolved or ancestral E. faecalis (one-tailed t-test; adj-Ps < 0.05). b. C. elegans mortality after symbiont, compost, and parasite exposures (one-tailed Wilcoxon test; adj-Ps = 0.016). c. Correlation between E. faecalis colonization abundance and mortality under S. aureus infection after compost exposure (Pearson’s; R = -0.775; t = -4.42; df = 13; P << 0.01). Data for CFUs and transcript levels collected at the same time points and from same batches, hence direct comparisons. n = 5 populations per treatment. Error bars = ± s.e.

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