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Host-Microbe Interactions and Their Functional Significance in Host Animals and Plants

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Host-Microbe Interactions and Their Functional Significance in Host Animals and Plants

By

Rebecca Choi

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in the

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of the

University of California, Berkeley

Committee in charge:

Professor Michael Shapira, Co-Chair
Professor Todd Dawson, Co-Chair
Professor Peter Sudmant
Professor Ashley Wolf

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Abstract

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Microbiomes are a highly diverse community of microorganisms that have complex interactions with animals and plants. It is well known that these relationships range from being antagonistic, for example, in the case of pathogenic infection, or symbiotic by promoting host fitness. How microbes impact the physiology of their host organisms has been studied across many taxa including vertebrate and invertebrate animals. In animals, the gut microbiome is known to contribute significantly to host health while shifts in composition, or dysbiosis, have been associated with pathology. Understanding specific contributions of gut microbes and their dynamics throughout aging has yet to be explored. In plants, research often focuses on the role of microorganisms in agricultural plants from mitigating pathogenic spread to promoting productivity and growth. Fewer studies explore these interactions in natural systems.

In my dissertation, I examine the gut microbiome in the model organism, *Caenorhabditis elegans,* and then explore the leaf microbiome of the California Coast Redwood (*Sequoia sempervirens*, D. Don). The first two chapters are dedicated to investigating factors that modulate host preference for beneficial microbes and their roles in host fitness, and during aging. The final chapter expands the focus to both the bacterial and fungal microorganisms associated with the leaves of redwoods, an extremely long-lived tree species with a massive aboveground biomass, that represents a natural plant-microbe system shaped over a vast evolutionary timeline.

Chapter 1 examines the preference for specific gut commensals in *C. elegans,* a bacteriovore, that has been established in recent years as a model for gut microbiome research. As a short-lived animal, worms enable experimenting on genetically identical individuals that can reveal trends at the population level. This chapter identifies bacterial strains, specifically in the *Pantoea* family, isolated from the worm gut from populations raised in natural-like compost environments that promoted faster development compared to other environmental or non-colonizing bacteria. Worms show preference for gut isolated *Pantoea* strains over environmental species from the same family and additionally, prevented colonization by invading, pathogenic bacteria.

Chapter 2 continues to examine the functional significance of commensal gut bacteria in *C. elegans* and their dynamics during aging. I track compositional changes in the gut microbiome in worms using a combination of experiments including 16S rRNA gene sequencing, fluorescent microscopy, quantitative PCR, and bacterial colony forming unit (CFU) counts. The combined results reveal an increase in relative abundance of *Enterobacteriaceae*, a trend observed across worm populations raised on natural-like and defined, representative bacterial communities. Additional experiments show that a specific gut isolated *Enterobacter* strain in the *Enterobacteriaceae* family accumulates in the aging worm gut and is associated with a decline in an immune signaling pathway. *Enterobacter* also exacerbates infection susceptibility by an opportunistic pathogen. Finally, I show that this infection susceptibility can be mitigated using commensal gut strain communities that are able to outcompete opportunistic pathobionts.

Chapter 3 characterizes both the bacterial and fungal members found on external and internal surfaces of leaves, or phyllosphere, of the Coast Redwood. The redwood range spans from wetter regions in the north to drier regions in the south that also vary in level of fog exposure and exposure to major fire events. I establish methods for sterile leaf sampling, epiphyte and endophyte microbial harvest, and DNA extraction for 16S and ITS amplicon sequencing to characterize relative microbial composition. Results reveal both geographical and leaf-surface dependent shifts in microbial composition in both bacterial and fungal communities identifying potential ecological factors that may contribute to shaping the phyllosphere microbiome.

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CHAPTER 1: Host preference of beneficial commensals in a microbially-diverse environment

The results presented here were published from the co-authored* paper:

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ABSTRACT

Gut bacteria are often described by the neutral term, commensals. However, the more we learn about their interactions with hosts, the more apparent it becomes that gut commensals often contribute positively to host physiology and fitness. Whether hosts can prefer beneficial bacteria, and how they do so, is not clear. This is of particular interest in the case of the bacterivore *C. elegans*, which depends on bacteria as food source, but also as gut colonizers that contribute to its physiology, from development to immunity. It is further unclear to what extent worms living in their microbially-diverse habitats can sense and distinguish between beneficial bacteria, food, and pathogens. Focusing on *Enterobacteriaceae* and members of closely related families, we isolated gut bacteria from worms raised in compost microcosms, as well as bacteria from the respective environments and evaluated their contributions to host development. Most isolates, from worms or from the surrounding environment, promoted faster development compared to the non-colonizing *E. coli* food strain. *Pantoea* strains further showed differential contributions of gut isolates versus an environmental isolate. Characterizing bacterial ability to hinder pathogenic colonization with *Pseudomonas aeruginosa*, supported the trend of *Pantoea* gut commensals being beneficial, in contrast to the environmental strain. Interestingly, worms were attracted to the beneficial *Pantoea* strains, preferring them over non-beneficial bacteria, including the environmental *Pantoea* strain. While our understanding of the mechanisms underlying these host-microbe interactions are still rudimentary, the results suggest that hosts can sense and prefer beneficial commensals.

INTRODUCTION

Bacterial community composition in plant and animal hosts are typically distinct from those in the surrounding environments (Bäckhed et al., 2005; Hacquard et al., 2015; Wippel et al., 2021). What determines this distinction, and which factors dominate the process of microbiota assembly is an active area of research and a topic of debate (Ley et al., 2008; Gilbert et al., 2018; Sieber et al., 2019). Environmental availability and external environmental factors, such as temperature, toxins and host diet are important (Spor et al., 2011; Smith et al., 2015; Callens et al., 2018; Simon et al., 2019). Equally important is host filtering, which is dependent on host genetics and is dominated by host immunity, internal nutrient availability and behavior (Hacquard et al., 2015; Berg et al.,

2016a; Davenport et al., 2017; Goodrich et al., 2017; Berg et al., 2019), but is also affected by bacterial interactions, which can impact microbiota diversity and are known to affect host physiology and fitness (Lawley and Walker, 2013; Simon et al., 2019; Johnke et al., 2020).

The nematode C. elegans is well-suited for studying host-microbe interactions. Clonal populations and tight genetic control reduce inter-individual variation when examining gut microbiota composition, and a transparent body permits *in vivo* monitoring of bacterial colonization (Shapira, 2017). Over the past several years, work in this system has yielded important insights into the processes that shape the gut microbiota, in particular with respect to the role of host genes (Berg et al., 2019; Ortiz et al., 2021; Zhang et al., 2021). *C. elegans* feeds on bacteria from its environment, but specific bacteria resist digestion and persist in the worm gut giving rise to a characteristic gut microbiota shaped by environmental availability as well as by host genetics (Berg et al., 2016a, 2016b; Zhang et al., 2017, 2021). Work in the past several years has characterized the composition and diversity of the C. elegans gut microbiota, and common taxa were identified across worm populations isolated from different geographical locations (Berg et al., 2016a; Dirksen et al., 2016; Samuel et al., 2016). Bacteria of the *Enterobacteriaceae* family are among the more prevalent, and several species of this family were shown to have positive effects on host development (Berg et al., 2019; Dirksen et al., 2020) [although this may come with a trade-off for adult stress resistance (Slowinski et al., 2020)]. Additional families including Pseudomonadaceae, Xanthomondaceae, Comamonadaceae and Brucellaceae, contain members that have similar contributions to worm development (Watson et al., 2014; Samuel et al., 2016; Dirksen et al., 2020; Zhang et al., 2021).

Gut bacteria are often described as commensals, but the more they are studied, the clearer it becomes that many contribute positively to host functions such as development, as described above, metabolism (Zimmermann et al., 2020), and often, to colonization resistance - preventing invasion by pathogens, but also by non-pathogenic colonizers that may perturb homeostasis. Such colonization resistance depends on competition for nutrients, inter-bacterial warfare mediated by secreted toxins, or activation of host immunity (Lawley and Walker, 2013; Sorbara and Pamer, 2019). In *C. elegans*, colonization resistance has been so far shown to depend on secretion of anti-microbial peptides (Dirksen et al., 2016; Kissoyan et al., 2019) and on immune priming (Montalvo-Katz et al., 2013; Berg et al., 2016b; Willis et al., 2021). While research focuses on beneficial commensals, not all environmentally available bacteria are necessarily beneficial. Whether hosts can distinguish between species or strains that are beneficial and those that are less so is unclear. Previous work with *Enterobacter* commensals isolated from *C. elegans* or from *C. briggsae* demonstrated that commensals of one host species could protect it from a subsequent exposure to a pathogen, but failed to protect the other host species, suggesting a functional adaptation between hosts and their cognate commensals, the basis for which is still unknown (Berg et al., 2016b). If host preference for certain bacteria existed, it could be mediated by differential bacterial abilities to colonize and persist in the worm gut, or

alternatively, by host behavior, as previously described for *C. elegans* preference of food sources that better supported its growth (Shtonda and Avery, 2006; Kim and Flavell, 2020).

In this study, we set out to expand the repertoire of beneficial gut bacteria, focusing on members of the *Enterobacteriaceae* family and of the *Enterobacterales* order, to examine whether some taxa are more likely than others to colonize the worm gut, and to explore the worm's ability to prefer beneficial ones. This analysis identified the genus *Pantoea* [(part of the *Erwiniaceae* family, a recent splinter off the *Enterobacteriaceae* family (Adeolu et al., 2016)], of which gut isolates showed significant contributions to host fitness. Further analysis demonstrated that beneficial *Pantoea* strains were preferred as gut commensals due to a greater ability to colonize than a non-beneficial congeneric, but also through host attraction toward these bacteria.

METHODS

Standard worm and bacterial strains

All experiments were performed using worms of the Bristol N2 strain and raised either on nematode growth medium (NGM) or on Peptone-free medium (PFM). *E. coli* strain OP50 was used as the standard food to raise worms and as a reference point in comparisons with other bacteria. A GFP-expressing version of *P. aeruginosa* strain PA14 was used as a model pathogen. The N2, OP50 and PA14-GFP strains were originally obtained from the *Caenorhabditis* Genome Center (CGC).

Worm growth in compost microcosm and bacterial isolation

Microcosms were prepared with 5 gr oak knoll soil composted for two weeks either with chopped bananas or apples, cured of endogenous non-*C. elegans* nematodes and reconstituted with the original bacterial community, as previously described (Berg et al., 2016a). Initially germ-free L1 larvae were added to microcosms, raised at 20°C for four days, following which adults were harvested, washed intensively and surface sterilized, ascertaining removal of external bacteria by plating final wash solutions on lysogeny broth (LB) plates/no antibiotics (Berg et al., 2016a). Bacteria were released from worms by grinding the latter with a motorized pestle in a volume of 100 µl M9, pelleting debris, and plating bacteria from supernatants on *Enterobacteriaceae*/*Enterobacterales*-selective violet red bile glucose (VRBG) agar (Difco) or on non-selective rich media, tryptic soy (TS) or LB agar. Plates were incubated at 25°C for 48 hours, from which colonies were regrown, and frozen at -80°C till subsequent analyses. Bacteria were similarly cultured from the respective microcosm environments, following resuspension in M9 solution, vortexing to disrupt larger soil aggregates and plating supernatant after spinning down (one minute at 1800 rpm) soil particles.

Twenty bacterial clones were randomly selected from each microcosm environments. Approximately 160 strains were isolated in total, of which 69 (41 from soil, 28 from worms) were taxonomically classified at the genus level following Sanger sequencing of

full-length 16S rRNA gene amplified using primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′).

Development rate measurements

The impact of individual bacterial strains on *C. elegans* growth rate was assessed by comparing the percentage of animals reaching larval L4 stage to that in animals raised on *E. coli* OP50. Each strain was tested in at least two independent experiments. Worm populations were raised from eggs obtained by bleaching gravid worms, or from starvation-synchronized L1 larvae (50-100 worms per plate), on lawns of bacterial isolates (cultured overnight in LB at 28°C, concentrated 10x and plated on NGM plates) for 40 hours at 25ºC, at which point the percentage of L4 larvae was evaluated. Each plate was divided to quarters. Starting to the upper left of the quadrant, the stage of the first 25 worms observed was assessed.

Preventing infective colonization and fluorescent imaging

L1 worms were grown at 25ºC to early adulthood (36 hours) on PFM plates with lawns of different *Pantoea* strains (prepared as described above). Adult worms were washed off plates, washed twice with M9, transferred to slow killing plates seeded with PA14-GFP (Shapira and Tan, 2008), and further replenished with the respective *Pantoea* strains (50 ml 20-fold concentrated two-day culture). Following additional 40 to 48 hours at 25ºC, 20-30 worms were picked off plates, washed twice with 1 mL M9 in an Eppendorf tube, paralyzed with 10mM levamisole and mounted on 2% to 4% agarose pads to be imaged using a Leica MZ16F stereoscope equipped with a QImaging MicroPublisher 5.0 camera. Images of worms of different experimental groups were all taken using identical exposure settings.

Image analysis

Quantification of fluorescence signal was achieved using a protocol implemented on the Fiji plugin within the ImageJ package v2.10/1.53c (Schindelin et al., 2012). Fluorescent signal per worm was calculated using Integrated Density values, following subtraction of background mean gray values and autofluorescence, and subsequently normalized for worm size.

To bring together results from different experiments for single time point analyses, normalized fluorescent signal values per worm were additionally scaled by the median value in worms raised on OP50 of the respective experiment.

Normalized PA14-GFP signal values correlated well with the number of colonizing bacteria as evaluated by colony-forming unit (CFU) counts (Supplementary Fig. 1).

Paraformaldehyde-killed bacteria

Bacteria were killed by a one-hour incubation at 28ºC with paraformaldehyde (PFA) at a final concentration of 0.5% as previously described (Beydoun et al., 2021). Dead bacteria were then washed five times, concentrated, OD adjusted and plated on the appropriate plates.

CFU counts

Adult worms raised at 20ºC (72 hours) on PFM plates with lawns of the examined bacterial strains were rinsed off and washed as previously described with several extra washes (Dirksen et al., 2020). Three 10-worm samples were taken from each population and washed five times more with 1 mL M9/0.01% Triton. Each 10-worm sample was transferred in a 100 mL volume to tubes containing 10-15 zirconia beads and broken to release bacteria using a PowerLyser (30 sec, 4000 rpm). Lysates were serially diluted, plated on VRBG plates, and colonies appearing after 24 hours of incubation at 28 ºC were counted. Post-wash worm-free supernatant was plated in parallel to ascertain the lack of contaminating external bacteria.

Bacterial choice assays

Worms were raised to young adulthood on OP50 lawns on PFM plates and shifted following three M9 washes to the center of test plates equidistantly spotted (5 uL volume, 1.5 cm from center) with tested strains at similar densities. In pairwise comparisons, plates contained two spots of each strain; worms at each spot were counted following 3 hours at 20ºC, calculating a choice index (CI) as # Worms on strain A - #Worms on strain B / Total # of scored worms (Abada et al., 2009). In multiple strain plates, worms were offered a choice of each of the *Pantoea* strains as well as of OP50, and the percentage in each spot was evaluated after 3 hours.

Statistical analyses

Statistical evaluation was performed in R (v 3.6.3). Plots were generated using the ggplot2 R package (Villanueva and Chen, 2019).

RESULTS

Worm gut commensals and their environmental counterparts frequently promote faster development than the standard E. coli food

To explore *C. elegans* preferences of bacteria from its environment, and bacterial contributions to host fitness, we isolated bacteria from worms raised in compost microcosms, as well as from their immediate environments. Microcosms were made of local soil composted with produce to emulate environments from which *C. elegans* has been previously isolated (Berg et al., 2016a). Bacteria were isolated by culturing on VRBG selective medium (see Methods), to focus on bacteria of the *Enterobacteriaceae* family/*Enterobacterales* order, which were previously shown to be a significant component of the worm gut microbiota and have diverse effects on host fitness (Berg et al., 2016a, 2016b; Samuel et al., 2016; Zhang et al., 2017; Slowinski et al., 2020). Culturing on rich media plates further supplemented isolation efforts with non-*Enterobacterales* isolates. Sixty-nine of the isolates were characterized by full-length 16S (identified at genus-level resolution, although not always unambiguously) and were employed in subsequent analysis (Fig. 1A; Supplementary Table S1, Accession numbers OK487509-OK487574). Among the different genera, some were represented mostly (or exclusively) by environmental isolates (*Erwinia, Pseudomonas, Rahnella*), while others were represented primarily by gut isolates (*Kluyvera* and *Raoultella*). This

may suggest preferred niches for the respective genera. However, it should be noted that bacteria of the *Pseudomonas* genus (which also grew on VRBG with a different colony morphology than *Enterobacterales*), here not found in worm guts, were previously isolated from worms (Montalvo-Katz et al., 2013; Schulenburg and Félix, 2017; Zimmermann et al., 2020).

Initial analysis examined the effects of individual bacterial isolates on host development, quantifying the percentage of worms reaching the last larval stage (L4) after 40 hours. Overall, most isolates promoted faster development than the standard food *E. coli* strain OP50, and similar to the previously described *Enterobacter hormaechei* CEent1 gut commensal (Berg et al., 2016b; Dirksen et al., 2020). In contrast, raising worms on most non-*Enterobacterales* genera, i.e., *Chryseobacterium*, *Leuconostoc* and *Lactococcus*, demonstrated slower or similarly-paced development, compared to OP50, in agreement with previous reports (Dirksen et al., 2016, 2020; Samuel et al., 2016).

Interestingly, among members of the *Erwiniaceae* genus Pantoea (a recent splinter off *Enterobacteriaceae*), gut isolates promoted significantly faster development than an environmental isolate (Fig. 1B and Supplementary Fig. 2). Isolates of the *Rouxiella* genus showed a similar distinction between gut and soil isolates, but not as significant as with *Pantoea* isolates. *Pantoea* bacteria, together with other *Enterobacterales* genera, are known to be part of the *C. elegans* microbiota (Berg et al., 2016a), and a previously identified *Pantoea* gut isolate, BIGb0393, which is part of the CeMBio community has been shown to promote fast development in *C. elegans* despite not being a prolific colonizer (Dirksen et al., 2020). Our screen further suggested that a subset of *Pantoea* bacteria contributed to fast development in *C. elegans*.

Gut Pantoea offer protection from pathogenic colonization by Pseudomonas aeruginosa

To expand examination of additional fitness measures that may be affected by *Pantoea* isolates, we tested their ability to prevent pathogenic colonization by the virulent *Pseudomonas aeruginosa* PA14. We found that worms raised on the three strains recovered from the *C. elegans* gut (the previously described BIGb0393 and the two identified in this study (V8 and T16)) were more resistant to subsequent colonization by PA14-GFP than worms raised on *E. coli*, and additionally, more resistant than worms raised on a *Pantoea* strain isolated from the worm microcosm environment (Fig. 2A, B). Subsequent analyses showed that gut *Pantoea* were better at colonizing the worm gut than the environmental strain (Fig. 2C), similar to previous reports for BIGb0393 (Dirksen et al., 2020). However, better colonization, and presumably competition, did not seem to be at the basis of *Pantoea*'s ability to prevent infection, since CFU analysis of worms following PA14-GFP colonization identified only a few remaining *Pantoea* in the gut, which was not correlated with the extent of PA14-GFP colonization (Fig. 2D), and since dead *Pantoea* were as capable of preventing PA14 colonization as live ones (Fig. 2B). Together, these results suggested that *Pantoea* helped prevent PA14 colonization but not through competition.

Worms prefer beneficial gut Pantoea

Our results suggested that the characterized beneficial *Pantoea* were better able to colonize the worm intestine, which is likely what made them gut commensals. However, behavioral mechanisms were also shown to affect worm interactions with bacteria. Worms are known to avoid pathogenic bacteria (Pradel et al., 2007) or show attraction to food bacteria that better support growth (Shtonda and Avery, 2006). We therefore investigated whether worms preferred specific *Pantoea* strains. Both multiple-choice and pairwise choice assays revealed significant preference of the beneficial *Pantoea* gut isolates over the environmental isolate or over control OP50 (Fig. 3A, B). These results indicate that behavioral mechanisms further promote preference of beneficial commensals over a similar yet non-beneficial congeneric.

DISCUSSION

In exploring the adaptive capacities of environmentally-acquired commensals for *C. elegans* fitness, we identified members of the genus *Pantoea* as multi-purpose beneficial commensals. Gut *Pantoea* promoted fast development and further hindered pathogenic colonization. While the number of strains available for examination was rather low, it appears that benefits were limited to *Pantoea* isolated from the worm gut and were missing from a congeneric soil isolate. Gut *Pantoea* were better at colonizing the worm, and in addition, worms seemed to prefer them over the less beneficial soil *Pantoea* or the *E. coli* food bacteria. This behavior adds to previous reports of worms preferring bacteria that support their growth and development (Shtonda and Avery, 2006), by expanding such preference to bacteria that are beneficial beyond nutrition.

C. elegans behavioral food preferences are linked to its neuronal responses to external bacterial cues (*e.g.*, bacterial odors), helping to pre-emptively avoid pathogenic bacteria and promoting interactions with beneficial ones. In addition, *C. elegans* can exhibit learned behaviors within one to three hours after sampling different food sources, where in some cases, consumption of preferred bacteria has been correlated with increased lifespan and, to a lesser extent, with increased growth rates (Troemel et al., 1997; Law et al., 2004; Shtonda and Avery, 2006; Sowa et al., 2015; Worthy et al., 2018). The capability of *C. elegans* to prefer beneficial microbes may be comparable to that observed eukaryotic models. In *Drosophila melanogaster*, for example, olfactory signals synthesized by gut-associated microbes were shown to modify host foraging behavior, egg laying, and nutritional as well as microbial preferences (Wong et al., 2010; Fischer et al., 2017). Unlike the microbial signals in the drosophila model, the molecular basis for *Pantoea* preference by *C. elegans* is yet unknown. However, an intriguing parallel in the effects that bacteria exerts on their host is presented by a recently described *Providencia* gut commensal, which was shown to promote worm growth and was able to attract worms by modulating host neurotransmission (Samuel et al., 2016; O'Donnell et al., 2020).

Colonization resistance conferred by gut bacteria can be due to direct antagonism of invaders (*i.e.*, resource competition or metabolic warfare) or through induction of host immune responses (Kamada et al., 2013; Montalvo-Katz et al., 2013; Iatsenko et al., 2014; Granato et al., 2019). While the basis of *Pantoea's* ability to promote such resistance is not known, other *Pantoea* species have been reported to produce antimicrobial molecules, effective, amongst others, against *Pseudomonas aeruginosa* (Williams and Stavrinides, 2020). In the example we identified, hindering of pathogen colonization was likely not due to competition, as BIGb0393 conferred protection from PA14 colonization even when dead. Another alternative is immune priming, which was previously shown to provide protection from PA14 infection through activation of the p38 MAPK pathway (Montalvo-Katz et al., 2013). Other studies have shown that immune priming can be conferred by heat-killed bacteria (Lee et al., 2015; Yuen and Ausubel, 2018), suggesting that immune activation may be triggered by recognition of heat-labile microbe-associated molecular patterns (MAMPs), in line with previous studies (Twumasi-Boateng and Shapira, 2012). Lastly, it is also possible that the nutritional value of *Pantoea*, similar in live bacteria as in dead ones, makes for healthier worms, which can then better withstand infection. However, generally better health is more likely to contribute to long-term tolerance of infection and survival than on the initial stages of colonization, which are dependent on specific immune pathways.

The expansion in the reported abilities of *Pantoea* strains to accelerate development and contribute to host fitness present them as important members of the worm gut microbiota. The difference in the effects of, and host affinity to, some members of this taxa (gut isolates) compared to others (the soil isolate), distinguishes this genus from other examined genera. This together with what appears to be mutual adaptations, including better ability of beneficial *Pantoea* to colonize the worm gut (compared to a non-beneficial congeneric) and host preference of its cognate commensals, suggest long-standing interactions between the worm host and its *Pantoea* gut commensals. How these interactions have evolved and how tightly they link the fitness of the two partners, remains to be further studied. Furthermore, research into *C. elegans* interactions with additional *Pantoea* strains is required to ascertain the paradigm proposed here and to provide further support.

FIGURES

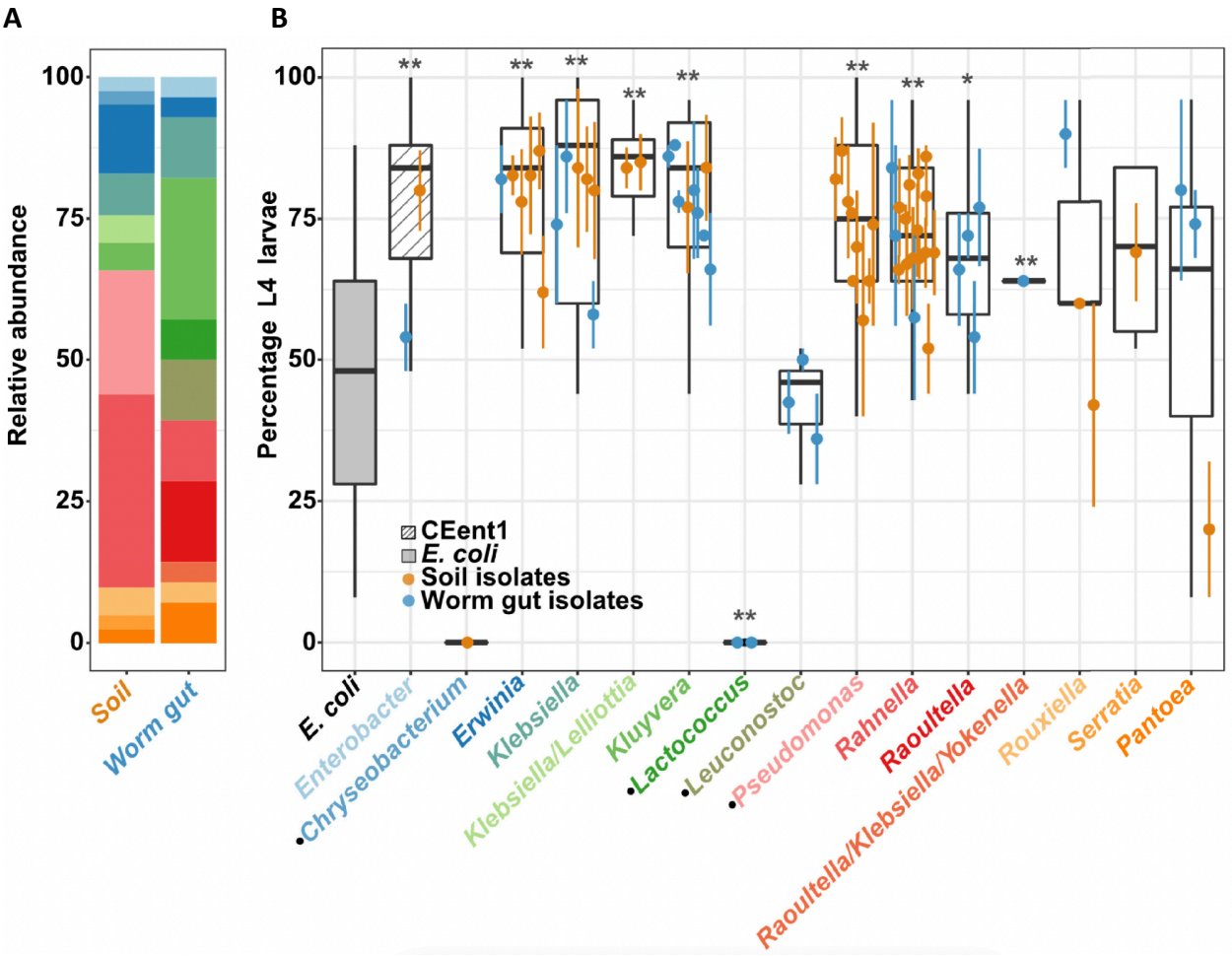


Figure 1. Effects of environmental and worm gut isolates on *C. elegans* development. A. Bacterial isolates distribution. Relative abundance of genera recovered from soil microcosms and from worm guts, identified based on full length 16S Sanger sequencing and colored as in panel B *x*-axis (see also **Supplementary Table 1**). **B.** Percentage of L4 larvae in worm populations raised from eggs for 40 hours at 25°C on environmental or gut bacterial isolates, as designated, grouped at the genus level; N=25/strain/experiment, dots represent averages (± SE) of 2 independent experiments. *E. coli* strain OP50 and *Enterobacter hormaechei* strain CEent1, in the gray and hatched box, respectively, represent values from 41 experiments and provide reference for comparisons. Dotted lines represent the mean value for development on OP50, mean + SD and mean + 2 SD's. Boxes extend from first to third quartiles, with black lines representing the median. * $p<0.05$; **, $p<0.001$, in comparison to *E. coli* (Student's t-test, corrected for multiple testing using Bonferroni). Black dots at *x*-axis labels indicate *non-Enterobacteriales* isolates.

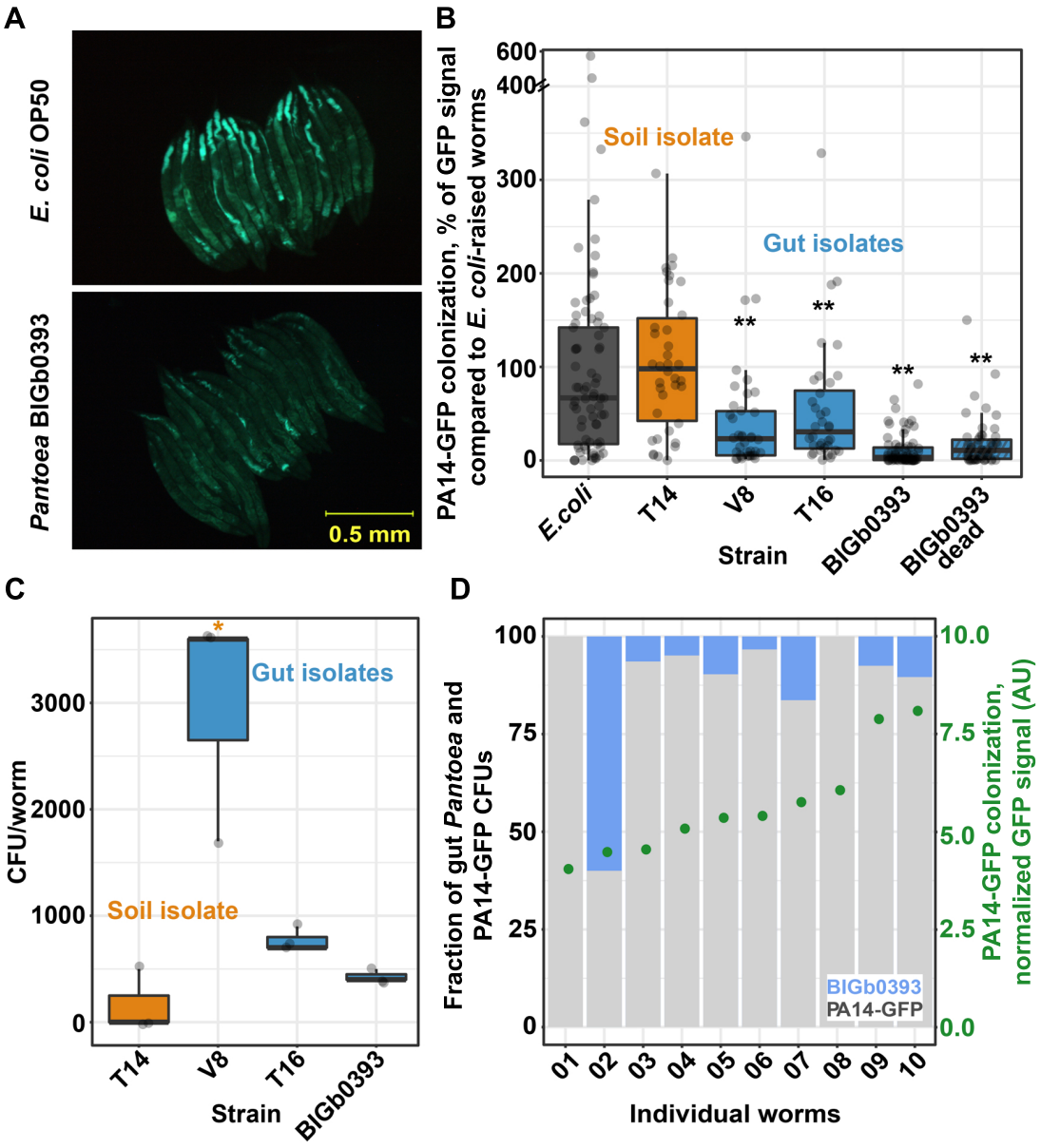


Figure 2. Gut isolates but not an environmental *Pantoea* strain provide protection from pathogenic colonization by *Pseudomonas aeruginosa* PA14. A. Worm colonization by PA14-GFP following 43-45 hours of exposure. **B.** Quantification of PA14-GFP fluorescent signal in images as in A; N=36-75 worms from two or three independent experiments. **C.** Worm colonization by individual *Pantoea* strains. CFU counts were estimated in worms raised for 72 hours starting at the L1 stage. Shown are measurements performed in triplicate (N=10 worms each). B and **C.** * and **, indicate $p < 0.05$ and $p < 0.001$, respectively (Student's t-test). **D.** Gut colonization in individual worms raised on BIGb0393 and shifted for 44 hours to *P. aeruginosa* PA14-GFP. No correlation is observed between *Pantoea* colonization (left *y*-axis, based on CFU counts), and PA14-GFP colonization [right *y*-axis, fluorescent signal, arbitrary units (AU)].

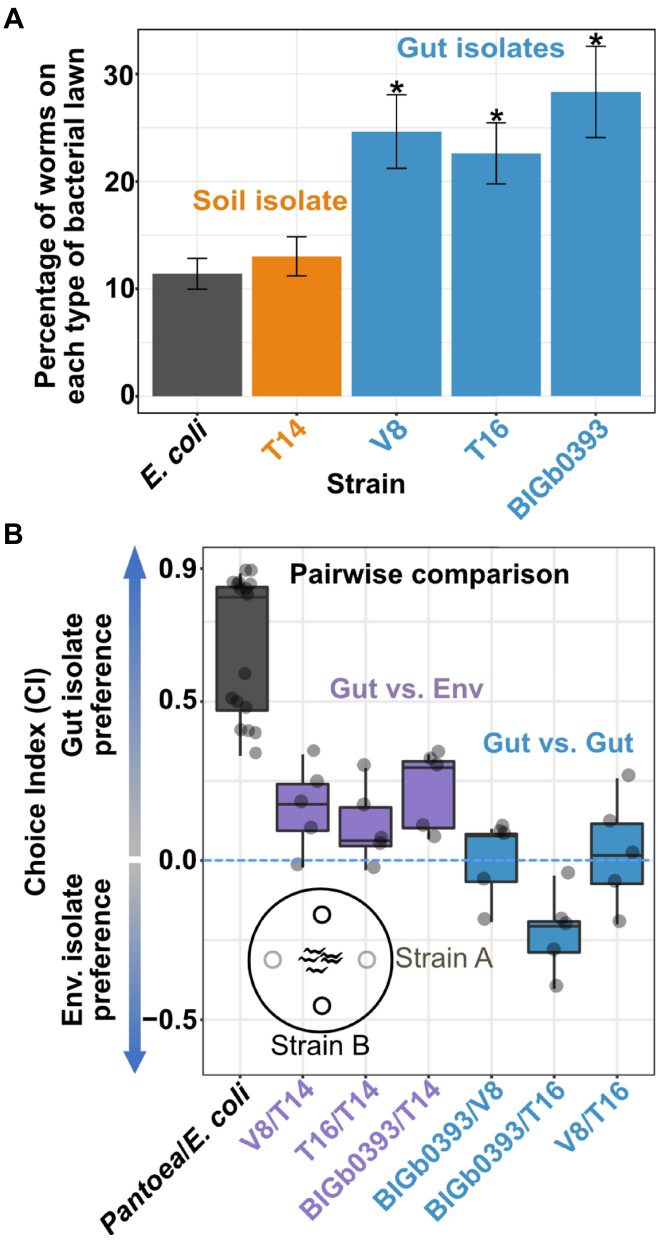
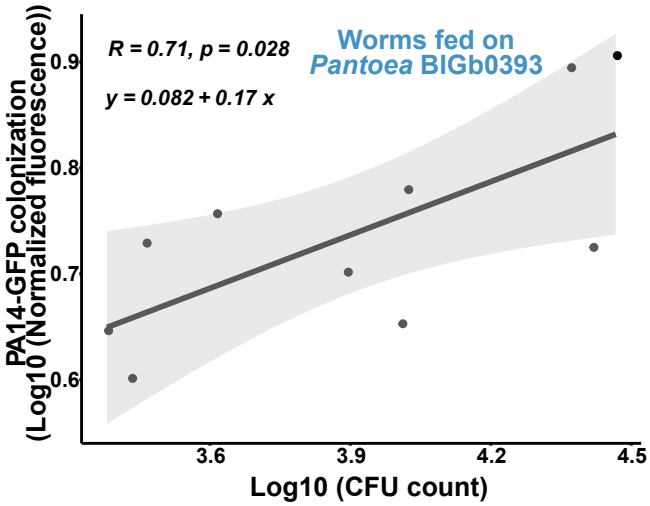
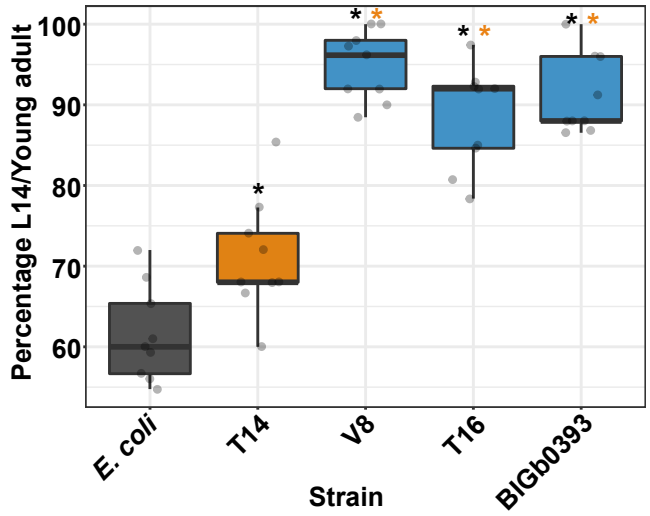


Figure 3. *C. elegans* shows preference towards beneficial *Pantoea*. A. Multi-strain plates. Shown are percentages of young adult worms localized to spots of the designated strains after a three-hour incubation at 25ºC. Shown are averages ± SEM for 10 plates (N=36-164 worms each). *, $p < 0.01$ (Student's t-test). **B**. Pairwise preference comparisons. Shown are choice index values of young adult worms exposed to designated strains for 3 hours. Each dot represents an independent pairwise comparison between the designated *Pantoea* strains (N=21-123 worms per comparison), with boxes defining the 25 to 75 percentile values and lines representing the median value.



Supplementary Figure 1. Significant correlation between *Pseudomonas* colonization levels and its CFU counts. Correlation between *Pseudomonas* PA14 colonization levels and its CFU counts in worms raised on the protective *Pantoea* strain BIGb0393. Fluorescence was estimated in individual worms (N=10) after 43 hours of exposure to PA14-GFP.



Supplementary Figure 2. Development rate of *C. elegans* fed on *Pantoea* strains. The proportions of L4 larvae and young adults were measured after 28 hours incubation post L1 at 25ºC (25 to 57 worms per replica (N=3)). * $p < 0.001$ (Student's t-test).

TABLES

Supplementary Table 1. Gut and worm isolates taxonomical annotation.

Strain name	Accession number	Taxonomy	Isolation source
OB_S_T13	OK487509	Chryseobacterium	Soil
OB_S_V25	OK487510	Enterobacter	Soil
OB_W_T8	OK487511	Enterobacter	Worm gut
OB_S_T3	OK487512	Erwinia	Soil
OB_S_V1	OK487513	Erwinia	Soil
OB_S_V17	OK487514	Erwinia	Soil
OB_S_V27	OK487515	Erwinia	Soil
OB_S_V4	OK487516	Erwinia	Soil
OB_W_V3	OK487517	Erwinia	Worm gut
OB_S_V39	OK487518	Klebsiella/Lelliottia	Soil
OB_S_V40	OK487519	Klebsiella/Lelliottia	Soil
OB_S_V26	OK487520	Klebsiella	Soil
OB_S_V21	OK487521	Klebsiella	Soil
OB_S_V31	OK614026	Klebsiella	Soil
OB_W_T6	OK487522	Klebsiella	Worm gut
OB_W_V38	OK487523	Klebsiella	Worm gut
OB_W_V9	OK487524	Klebsiella	Worm gut
OB_S_V24	OK487525	Kluyvera	Soil
OB_W_T2	OK487526	Kluyvera	Worm gut
OB_W_T22	OK487527	Kluyvera	Worm gut
OB_W_T3C	OK614029	Kluyvera	Worm gut
OB_W_T38	OK487529	Kluyvera	Worm gut
OB_W_V21	OK487530	Kluyvera	Worm gut
OB_W_V39	OK487531	Kluyvera	Worm gut
OB_W_V5	OK487532	Kluyvera	Worm gut
OB_W_T35	OK487533	Lactococcus	Worm gut
OB_W_T40	OK487534	Lactococcus	Worm gut
OB_W_T34	OK487535	Leuconostoc	Worm gut
OB_W_T36	OK487536	Leuconostoc	Worm gut
OB_W_T37	OK487537	Leuconostoc	Worm gut
OB_S_V34C	OM321597	Kluyvera	Soil
OB_S_T14	OK487539	Pantoea	Soil
OB_W_T16	OK487540	Pantoea	Worm gut
OB_W_V8	OK487541	Pantoea	Worm gut

OB_S_T1	OK487542	Pseudomonas	Soil
OB_S_T11	OK487543	Pseudomonas	Soil
OB_S_T18	OK487544	Pseudomonas	Soil
OB_S_T20	OK487545	Pseudomonas	Soil
OB_S_T5	OK614027	Pseudomonas	Soil
OB_S_T8	OK487546	Pseudomonas	Soil
OB_S_T9	OK487547	Pseudomonas	Soil
OB_S_V29	OK487548	Pseudomonas	Soil
OB_S_V32	OK487549	Pseudomonas	Soil
OB_S_T2	OK487550	Rahnella	Soil
OB_S_T6	OK487551	Rahnella	Soil
OB_S_V14	OK487552	Rahnella	Soil
OB_S_V19	OK487553	Rahnella	Soil
OB_S_V2	OK487554	Rahnella	Soil
OB_S_V20	OK487555	Rahnella	Soil
OB_S_V28	OK487556	Rahnella	Soil
OB_S_V35	OK614028	Rahnella	Soil
OB_S_V36	OK487557	Rahnella	Soil
OB_S_V37	OK487558	Rahnella	Soil
OB_S_V5	OK487559	Rahnella	Soil
OB_S_V6	OK487560	Rahnella	Soil
OB_S_V7	OK487561	Rahnella	Soil
OB_S_V8	OK487562	Rahnella	Soil
OB_W_T7	OK487563	Rahnella	Worm gut
OB_W_V2	OK487564	Rahnella	Worm gut
OB_W_V37	OK487565	Rahnella	Worm gut
OB_W_T21	OK487566	Raoultella/Klebsiella/Yokenella	Worm gut
OB_W_T1	OK487567	Raoultella	Worm gut
OB_W_T4	OK487568	Raoultella	Worm gut
OB_W_V1	OK487569	Raoultella	Worm gut
OB_W_V30	OK487570	Raoultella	Worm gut
OB_S_T12	OK487571	Rouxiella	Soil
OB_S_T16	OK487572	Rouxiella	Soil
OB_W_V19	OK487573	Rouxiella	Worm gut
OB_S_V12	OK487574	Serratia	Soil
Code	**Soil + rotten fruit used**	**Type of strain**	**Isolation media**

OB_W_V	Oak+Banana	Worm gut strain	VRBG
OB_W_T	Oak+Banana	Worm gut strain	TSA
OB_S_V	Oak+Banana	Soil strain	VRBG
OB_S_T	Oak+Banana	Soil strain	TSA

CHAPTER 2: An Enterobacteriaceae bloom in aging animals is restrained by the gut microbiome

The results presented here were published in the paper:

Choi, R., Bodkhe, R., Pees, B., Kim, D., Berg, M., Monnin, D., *et al.* (2024). An *Enterobacteriaceae* Bloom in Aging Animals is Restrained by the Gut Microbiome. *AgingBio,* 1, 1–11. doi: 10.59368/agingbio.20240024

ABSTRACT

The gut microbiome plays important roles in host function and health. Core microbiomes have been described for different species, and imbalances in their composition, known as dysbiosis, are associated with pathology. Changes in the gut microbiome and dysbiosis are common in aging, possibly due to multi-tissue deterioration, which includes metabolic shifts, dysregulated immunity, and disrupted epithelial barriers. However, the characteristics of these changes, as reported in different studies, are varied and sometimes conflicting. Using clonal populations of *Caenorhabditis elegans* to highlight trends shared among individuals, we employed 16s rRNA gene sequencing, CFU counts and fluorescent imaging, identifying an *Enterobacteriaceae* bloom as a common denominator in aging animals. Experiments using *Enterobacter hormaechei*, a representative commensal, suggested that the *Enterobacteriaceae* bloom was facilitated by a decline in Sma/BMP immune signaling in aging animals and demonstrated its potential for exacerbating infection susceptibility. However, such detrimental effects were context-dependent, mitigated by competition with commensal communities, highlighting the latter as determinants of healthy versus unhealthy aging, depending on their ability to restrain opportunistic pathobionts.

INTRODUCTION

Aging is a process of multi-tissue deterioration, including muscular atrophy, neurodegeneration, epithelial barrier disruption, immune dysregulation, and metabolic remodeling. Vulnerabilities and pathologies associated with this deterioration directly impact lifespan. In the case of the intestine, age-dependent impairments (immune, barrier, metabolic) further converge to alter the niche that is home to a complex community of microbes, the gut microbiome. However, how such changes affect the gut microbiome is not well understood.

The gut microbiome is increasingly appreciated for its contributions to host function (Nicholson et al., 2012; Dominguez-Bello et al., 2019; Fan and Pedersen, 2021; Morais et al., 2021). Imbalances in composition, or dysbiosis, have been shown to play a causative role in pathology (e.g. obesity) (Turnbaugh et al., 2008; Mariño et al., 2017). Age-dependent dysbiosis was described in flies, mice and humans and was suggested to negatively impact both barrier functions and immune fitness (Clark et al., 2015; O'Toole and Jeffery, 2015; Thevaranjan et al., 2017). Studies in human populations

have shown that microbiomes of healthy octogenarians differed from those of unhealthy individuals of similar age (Wilmanski et al., 2021). Other studies characterized the trajectory of microbiome changes through aging all the way to semi-supercentenarian (105-110 year old) (Biagi et al., 2016), offering further insights into the relationship between age-dependent changes in microbiome composition and host health. Importantly, transplanting microbiomes from young mice to old reduced markers of aging and ameliorated health (Kim et al., 2022; Parker et al., 2022), and a similar transfer in killifish increased lifespan (Smith et al., 2017), demonstrating a causal role for age-dependent changes in microbiome composition in host aging. However, for the most part, such studies could not identify common trends in age-dependent changes that may offer points for intervention to ameliorate pathologies. Perhaps the sole exception is the identification of increased abundance of *Proteobacteria* (now re-named *Pseudomonadota*) in aging animals, both in vertebrates, where they are a minor constituent of the gut microbiome of young individuals, and in invertebrates, where they comprise a larger part in young animals and further increase with age (Clark et al., 2015; Bárcena et al., 2019; Adriansjach et al., 2020; Leite et al., 2021). However, the significance of this bloom and whether it is a universal signature of the aging gut microbiome is yet to be determined.

The nematode *C. elegans*, a useful model for aging research, is now gaining momentum as a model for microbiome research. It offers the advantage of working with synchronized, initially germ-free, clonal populations, which overcome the limitations of inter-individual variation common to vertebrate models to better discern shared patterns in microbiome composition (Shapira, 2017). As a bacterivore, *C. elegans* ingests bacteria from its environment. While some bacteria are digested as food, others persist, giving rise to a characteristic gut microbiome, similar across populations raised in the lab in different environments or in worms isolated from different geographical locations, yet distinct from the respective microbial environments (Berg et al., 2016a; Dirksen et al., 2016; Zhang et al., 2017). As in other organisms, gut commensals were shown to provide diverse benefits to their host, including faster development and resistance to pathogens (Berg et al., 2016b; Dirksen et al., 2016; Zhang et al., 2017; Slowinski et al., 2020; Pérez-Carrascal et al., 2022). The power of *C. elegans* as a genetic model further enabled identification of some of the genes, many of which are immune regulators (Berg et al., 2019; Zhang et al., 2021; Taylor and Vega, n.d.), that help control commensals and their function and shape microbiome composition.

We used *C. elegans* to characterize changes in gut microbiome composition during aging, identifying a bloom in bacteria of the *Enterobacteriaceae* family that was associated with an age-dependent decline in immune DBL-1/BMP signaling. The *Enterobacteriaceae* bloom was found to be potentially harmful, increasing vulnerability to infection. However, competing commensals, or a diverse microbiome, were able to mitigate these detrimental effects. The results presented highlight an *Enterobacteriaceae* bloom as a hallmark of normal aging and suggest that the outcomes of this bloom are context-dependent, determined by the ability of the rest of the gut microbiome to restrain it, distinguishing between healthy and non-healthy aging.

METHODS

Worm strains

C. elegans strains included wildtype N2, *dbl-1(nk3)*, and the *dbl-1* overexpressing strain BW1940, obtained from the *Caenorhabditis* Genome Center (CGC); transgenic strains expressing GFP from the *spp-9* promoter: TLG690, texIs127[spp-9p::GFP], TLG707, texIs127;*dbl-1*(*nk3*) and TLG708, texIs127;texIs100[*dbl-1*p::GFP::*dbl-1*], were gratefully received from Tina Gumienny (Lakdawala et al., 2019; Madhu et al., 2020) and *eat-2(ad1116)* mutants were gratefully received from Andrew Dillin. Gain-of-function (gof) *sma-4(syb2546)* mutants were generated using CRISPR/Cas9 editing to insert a GFP tag at the N-terminus of the endogenous *sma-4* locus (C-terminal tags disrupt function by blocking Smad complex formation (Wu et al., 2001; Wang et al., 2002). GFP::SMA-4 animals display gain-of-function phenotypes such as long body size (measured in images using ImageJ), presumably due to loss of autoinhibition mediated by interaction between N-terminal MH1 and C-terminal MH2 domains (Hata et al., 1997).

Bacterial strains and communities

Escherichia coli strain OP50 and the Gram-positive pathogen, *Enterococcus faecalis* strain V583 were obtained from the CGC. Two defined communities of worm gut commensals were used: CeMbio, with twelve strains, represents the worm core gut microbiome (Dirksen et al., 2020) and SC20, a subset of twenty strains of the previously described SC1 (Berg et al., 2019), with eight species out of the 20 of the *Enterobacteriaceae* family (Table 1). In addition, a subset of CeMbio strains was used in experiments testing effects on susceptibility to *Enterococcus faecalis* infection, including only strains that are sensitive to gentamycin, which is used in *E. faecalis* plates, to prevent enrichment of gut commensals through the environment. Additional commensals, of the genus *Pantoea,* family *Erwiniaceae* (a recent splinter off *Enterobacteriaceae),* included BIGb0393 (also in CeMbio) and the recently characterized *Pantoea cypripedii* strains V8 and T16 (Pérez-Carrascal et al., 2022). Bacterial communities were prepared for experiments by growing individual strains in LB at 28°C for two days, adjusting cultures to 1 OD, concentrating 10-fold and mixing equal volumes from each culture. 100-200 μL aliquots of the mix were plated on either minimal nematode growth medium (NGM) or on peptone-free medium (PFM), which further limits bacterial growth (Dirksen et al., 2020), as described, and air-dried for 2 to 12 hours prior to the addition of worms.

Construction of fluorescently-tagged Enterobacter hormaechei CEent1-dsRed

E. hormaechei CEent1, previously misidentified as *E. cloacae* (Berg et al., 2016b), is a member in both the CeMbio and SC20 communities. The construction of its dsRed-expressing derivative was achieved by integrating the dsRed gene into the functionally neutral attTn7 site in the CEent1 genome using the site-specific Tn7-mini transposon system (McKown et al., 1987) (Supplementary Fig. 1). Transposon insertion was achieved through triparental mating with a donor strain and a transposase-expressing helper strain, both based on the DAP-auxotrophic and *pir1*-positive *E. coli* strain BW29427, containing the conjugative RP4 mating system as a chromosomal insert.

This strain, as well as other *E. coli* strains and plasmids used in constructing the fluorescently-tagged strains, were gratefully received from the Goodrich-Blair lab, University of Tennessee Knoxville. Briefly, the original GFP in the mini-transposon, carried on plasmid pURR25 (Supplementary Fig. 1), was replaced with dsRed by digesting the plasmid with BseRI and NheI to cleave out the GFP coding sequence (Teal et al., 2006), amplifying the dsRed gene from pBK-miniTn7-ΩGm-DsRed (Murfin et al., 2012) using primers 5'-TAC GTG CAA GCA GAT TAC GG-3' and 5'-ATC CAG TGA TTT TTT TCT CCAT-3,' and ligating the amplified dsRed to the linearized pURR25 vector. The modified pURR25 plasmid, carrying the *pir1*-dependent oriR6K, as well as dsRed and the antibiotic resistance genes KanR and StrR, was re-introduced by electroporation into its original host strain, constituting the donor strain. The transposase plasmid, pUX-BF13, in the helper strain, also included the *pir1*-dependent oriR6K and AmpR antibiotic resistance, along with the Tn7-transposase (Bao et al., 1991).

Recipient strain CEent1 (*pir1*-negative), donor, and helper strains were each cultured until they reached an OD600 of 0.4 and then mixed in a 1:1:1 ratio in SOC DAP media for one hour at 37 °C. The mixture was spread on LB DAP plates for an additional 24-hour incubation to promote conjugation. Bacteria subsequently underwent several rounds of re-streaking on Kan+/DAP– plates to select for integrant CEent1 cells and to dilute-out the *pir1*-negative plasmids which cannot be replicated in CEent1 (Supplementary Fig. 1). Integrant clones were verified as CEent1 by sequencing a 200bp fragment of the CEent1 gene for *gyrB* using the primers 5'-GCA AGC AGG AAC AGT ACA TT-3' and 5'-TCG GCT GAT AAA TCA GCT CTT TC-3'.

Microcosm experiments

Compost microcosms harboring diverse microbial communities were prepared from local soil composted with produce for up to two weeks essentially as previously described (Berg et al., 2016a; Trang et al., 2022). Briefly, local soils were supplemented with banana peels or chopped apple, composted soils were split into two parts: one part (6g in a glass vial) autoclaved to eliminate native nematodes and the other (10g soil) suspended in M9 buffer to obtain a microbial extract which was concentrated and added to the autoclaved samples to reconstitute the original microbial community.

Synchronized worm populations were prepared by bleaching gravid worms, releasing eggs that hatched in M9 salt solution to provide germ-free L1 larvae. In continuous aging experiments, synchronized populations of germ-free L1 worms were raised at 20°C in separate vials containing the same compost and harvested at advancing ages up to day five of adulthood (D5) (Fig. 1A). The final time point was determined by the need to distinguish between the original cohort (post-reproductive, or post-gravid, at D5), and progeny (mid-stage gravids), which could not be achieved in subsequent time points. In experiments with fixed-time colonization, worms were raised on live E. coli until the L4 stage to ensure proper development, then transferred to kanamycin-killed *E. coli* (Shapira and Tan, 2008), from which worms were further transferred at advancing ages to microcosm environments for two days before harvesting for analysis. For the

earliest time point (gravids, day zero of adulthood) worms spent only four hours on dead *E. coli* before transferring to microcosms. This was deemed necessary to minimize carry over of live *E. coli* which can colonize older worms. A two-day exposure time was selected for all worm populations as it is the time required to obtain well-colonized worms in microcosms initiated with L1 larvae, used for the earliest time point. Worm harvesting was carried out using a Baermann funnel as previously described (Trang et al., 2022).

Soil samples (1g) were taken from microcosms of the same compost batch used to grow worms ("soil"), or from the same microcosm from which worms were harvested ("grazed soil").

Experiments with defined bacterial communities
Aging experiments on bacterial communities/strains were carried out similarly to the description for microcosm experiments, with bacteria seeded on NGM or PFM plates as described. In continuous aging experiments, worms were transferred to fresh plates every day during the reproductive phase to separate the original cohort from their progeny, enabling carrying on experiments into later stages of adulthood.

DNA extraction
Worms harvested in microcosm experiments (100-500 per group, except for the last time point of the continuous aging experiment in which only 8 post-gravid worms could be retrieved) were extensively washed, surface-sterilized by letting them crawl for an hour on plates with 100 μg/mL gentamycin and used for DNA extraction with the QIAGEN PowerSoil DNA isolation kit (Cat. #12888) as previously described (Berg et al., 2019).

Worms harvested from plates with defined communities (100-150 per group) were washed three times with M9, paralyzed with 25mM levamisole (Acros Organics) to close their intestine, and surface-sterilized with 2% bleach in M9, as previously described (Trang et al., 2022). DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen Cat. # 47016) according to manufacturer instructions, with the following modification: to break open worms prior to the first step of the protocol, worms were incubated in the kit's buffer for 10 minutes at 60°C, then crushed with added zirconium beads using a PowerLyzer (2000 RPM 2 x 30 seconds).

16S rRNA gene sequencing and analysis
DNA samples from worms and their respective environments were used to generate sequencing libraries of the 16S V4 region. Libraries from microcosms experiments were prepared using tailed primers, as previously described (Berg et al., 2016a), and sent for 150 paired-end sequencing to the UC Davis sequencing facility. Analysis of bacterial 16S amplicon data was carried out using the QIIME2 software pipeline (Bolyen et al., 2019). Sequence reads were demultiplexed and filtered for quality control, with 85.6% of all reads passing quality filtering, providing an average read of 121,053 reads per sample. Sequences were aligned and clustered into operational taxonomic units (OTU)

based on the closed reference OTU picking algorithm using the QIIME2 implementation of UCLUST (Edgar, 2010), and the taxonomy of each OTU was assigned based on 99% similarity to reference sequences based on Greengenes release 13_8. Prior to diversity analysis, all communities were rarefied to 116,543 sequences per sample. Shannon's diversity and Faith's phylogenetic diversity were calculated to assess alpha diversity of soil and worms gut microbiotas (Faith, 1994; Shannon, 1997). Shannon diversity is a composite measure of both richness and evenness, while Faith method takes the phylogenetic distance of species into account. Weighted UniFrac distances were calculated to assess beta diversity and used in principal coordinates analysis (PCoA). Raw data and metadata can be accessed at https://www.ncbi.nlm.nih.gov/sra with accession number PRJNA982115.

Fluorescence imaging

For each time point examined, 30-40 worms were picked off CEent1-dsRed, or CEent3-GFP containing plates (with the single strain, or with the fluorescent strains as part of a community), washed with M9, paralyzed with 10 mM Levamisole, and mounted on a slide with a 2-4% agarose pad. Imaging was performed with a Leica MZ16F equipped with a QImaging MicroPublisher 5.0 camera. Quantification of fluorescent signal was conducted using the Fiji plugin within the ImageJ package v2.10/1.53c or 1.53f51 (Schindelin et al., 2012). Integrated Density values were measured per worm after subtracting background mean gray values and autofluorescence and normalized for worm area.

Quantifying bacterial load by colony-forming unit (CFU) counting

For each time point examined 10-15 worms were washed three times in M9-T (M9/0.0125% TritonX-100), paralyzed with 25 mM Levamisole, and surface sterilized by a three-minute incubation in 2% bleach (Dirksen et al., 2020). After two washes of 1mL of PBS-T (PBS/0.0125% Triton-X 100), worms were collected in a final volume of 100μL. Samples of final wash were plated and incubated at 28°C for 2 days to verify removal of external bacteria. To release bacteria from the worm gut, worms were crushed with 10-15 zirconium beads in 100μL of PBS in a PowerLyzer at 4000 RPM for 30 to 45 seconds. Released bacteria were diluted and plated either on non-selective LB agar plates (for all bacteria) or *Enterobacteriaceae*-selective VRBG plates. CFUs were counted after 1-2 days of incubation at 28°C. CFUs for *E. hormaechei* and *Lelliottia amnigena* could be distinguished on VRBG plates based on morphology.

Quantifying bacterial load via quantitative PCR (qPCR)

When CFU counts on selective VRBG medium could not offer the required resolution between Enterobacteriaceae and other bacteria, relative bacterial load was measured with real-time quantitative PCR using the eubacterial 16S rRNA gene primers 806f (5'-AGATACCCCGGTAGTCC-3') and 895r (5'-CYGYACTCCCCAGGYG-3'), and the Enterobacteriaceae-specific 16S specific primers Ent_MB_F (5'-ACCTGAGCGTCAGTCTTTGTC-3') and R (5'-GTAGCGGTGAAATGCGTAGAGA-3') (Berg et al., 2019). qPCR was performed using Bio-Rad SsoAdvanced Universal SYBR Green qPCR Supermix and an Applied Biosystems StepOne Plus real-time PCR

system. Cycling (eubacterial primers): 95°C for 5 min, 45 x [95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec], 72°C for 5 min; and for the Enterobacteriaceae specific primers: 95°C for 5 minutes, 40 x [95°C for 15 sec, 60°C for 30 sec], 72°C for 5 minutes.
Ct values for bacterial 16S were normalized to worm material by subtracting Ct values obtained for worm actin using the pan-actin primers (Livak and Schmittgen, 2001; Shapira et al., 2006).

Survival assays

For infection resistance experiments, synchronized worm populations were raised from L1 on PFM plates with *E. coli* OP50, CEent1, or designated communities and shifted at L4, or at day four of adulthood, to *E. faecalis* plates prepared with Brain Heart Infusion Agar containing 25 μg/mL gentamicin and seeded with bacteria a day before the transfer of worms. *E. faecalis* strain V583 was previously shown to proliferate in the worm gut, killing adult worms within four days (Garsin et al., 2001). Raising worms on CEent1 until L4 before shifting to pathogen allows for worm colonization, while maintaining previously described infection parameters to facilitate comparisons (Garsin et al., 2001; Berg et al., 2016b). Assays were carried out at 25°C and dead or live worms were counted every day (Sifri et al., 2002). For lifespan assays, worms were raised, on designated strains or communities in PFM plates at 20°C and scored daily for survival beginning at L4(t_0).

Statistical analyses

Statistical tests were conducted in R (v 3.6.3). Survival curves were statistically compared using Kaplan-Meier analysis and log-rank tests using the survdiff R package (Therneau et al., 2024) and all graphs were created with the ggplot R package (Villanueva and Chen, 2019).

RESULTS

C. elegans aging involves an expansion of bacteria in the Enterobacteriaceae family

To characterize changes in gut microbiome composition during *C. elegans* aging, worms were raised in fifteen identical natural-like microcosm environments, harvested at designated time points, three populations at a time, and analyzed using 16S rRNA gene sequencing (Fig. 1A, continuous aging). For each time point, the environmental compost microbiome of one of the three samples was similarly analyzed. As previously observed, worm gut microbiomes were distinct from their environment (Fig. 1B) (Berg et al., 2016a). Furthermore, worm microbiomes changed with age, but these changes appeared to be independent of availability of bacteria in the environment, where the microbial community remained largely unaltered during the experiment (Fig. 1B, grazed soil). Most prominently, we observed an expansion in gut *Enterobacteriaceae*, particularly in post-reproductive worms (post-gravid, day five of adulthood). This increase could be due to ecological succession inside the gut, shaped by interbacterial interactions; alternatively, it could be determined by age-dependent changes in the host and in the gut niche, directly affecting gut bacteria. To distinguish between the two

possibilities, we carried out microcosm experiments where worms were raised on dead *E. coli* (*i.e.*, fed, but not colonized) and shifted to the complex microcosm community at advancing ages for a fixed amount of time, preventing age-associated differences attributed to ecological succession over accumulating time (Fig. 1A right). While the initial soil microbiome in this experiment showed relatively higher microbial diversity compared to the experiment described in Fig. 1B, the *Enterobacteriaceae* expansion re-emerged, suggesting that this expansion was associated with age-dependent changes in the intestinal niche rather than with the time of exposure, and highlighting it as a potential hallmark of worm aging (Fig. 1C).

Gut bacterial diversity (alpha diversity) decreases during aging, which was more pronounced in the fixed-colonization-time aging experiment (Fig. 1E) but was also seen in post-gravid worms in the continuous aging experiment (Fig. 1D). Declines were observed in worm microbiome diversity both with respect to species richness and evenness, represented by the Shannon Index, as well as with respect to phylogenetic diversity, represented by the Faith Index (Fig. 1D, E). In addition, worm gut microbiomes of different ages differed from one another. Principal coordinate analysis (PCoA) based on weighted UNIFRAC distances showed that in both experiments worms of a specific age harbored similar gut microbiomes, which were distinct from worm microbiomes in other ages, similar to trends observed in aging mice (Fig. 1F, G) (Langille et al., 2014). Together, these results support a role for the age-modified intestinal niche in driving age-dependent changes in microbiome composition, including a prominent expansion of *Enterobacteriaceae* as well as a general decline in bacterial diversity.

An Enterobacteriaceae expansion is a recurring theme in worms aging in different microbial environments

Microcosm experiments provide natural-like microbial diversity. However, to investigate the *Enterobacteriaceae* bloom in greater detail we turned to defined communities of characterized gut commensals. Two communities were used, each representing a slightly different paradigm: the publicly available CeMbio community, which includes two *Enterobacteriaceae* strains, *Enterobacter hormaechei* (CEent1) and *Lelliottia amnigena* (JUB66), but in the worm gut is dominated by *Stenotrophomonas* and *Ochrobactrum* (Dirksen et al., 2020), and SC20, which has a higher proportion of *Enterobacteriaceae* (eight out of 20 species), including CEent1 (Table 1). We used a CEent1-dsRed derivative included in both communities to enable imaging of gut colonization. In both, colonization with CEent1-dsRed increased with age, in line with the *Enterobacteriaceae* bloom observed in natural-like microcosm experiments (Fig. 2A, C). To examine whether the age-dependent bloom was specific to gut *Enterobacteriaceae* or also involved increases in the abundance of other members of the gut microbiome, we used CFU counts and qPCR to evaluate gut bacterial load. In worms aging on CeMbio, an increase in total bacterial load (CFUs on non-selective LB) was observed, from 10^3 bacterial cells/worm at D0 (L4 larvae) to around 10^5 cells/worm at D12 of adulthood, a roughly 100-fold increase (Fig. 2B). However, a steeper increase was observed in the *Enterobacteriaceae* load (CFUs on selective VRBG plates), from being barely detectable at day 0 to about 5% of the total gut microbiome by old age (~5000

cells/worm), close to a 10^4-fold increase. A similar trend was observed in worms raised on the *Enterobacteriaceae*-rich SC20 community, as demonstrated with qPCR using universal *Eubacteria* primers or *Enterobacteriaceae*-specific primers and normalized to worm DNA (represented by actin genes). This evaluation showed a much steeper increase in *Enterobacteriaceae* strains compared to the increase in the total bacterial load (Fig. 2D). These results support the notion that an *Enterobacteriaceae* bloom is a hallmark of aging, regardless of the initial conditions such as high or low environmental diversity, or high or low initial proportion of *Enterobacteriaceae*. This bloom involves a large increase in total bacterial load, but a proportionally larger increase in the *Enterobacteriaceae* load per worm.

An Enterobacteriaceae bloom in aging animals can have detrimental consequences

Previous work showed that the gut commensal CEent1, a representative Enterobacteriaceae, protected young animals from infection with the intestine-proliferating pathogen *Enterococcus faecalis* (Sifri et al., 2002; Berg et al., 2016b). However, in worms disrupted for DBL-1/BMP immune signaling, gut abundance of CEent1 increased and the otherwise beneficial commensal became an exacerbating factor during infection (Berg et al., 2019). We used CEent1 to examine the functional significance of the age-dependent *Enterobacteriaceae* bloom. Worms raised on CEent1 and shifted to *E. faecalis* at the end of larval development showed higher pathogen resistance compared to worms raised on the *E. coli* control, as previously shown. In contrast, worms raised on CEent1 to middle age before shifting to *E. faecalis* (day four of adulthood, midway through the *Enterobacteriaceae* bloom (Figs 1 and 2)), showed significantly lower pathogen resistance compared to worms raised on *E. coli* controls (Fig. 3A, B). Thus, the *Enterobacteriaceae* bloom has the potential to be detrimental in aging worms.

Changes in the intestinal niche associated with an age-dependent decline in DBL-1/BMP signaling may underlie the Enterobacteriaceae bloom

What may be the cause for the Enterobacteriaceae bloom? Experiments in microcosm environments and with defined communities showed that environmental availability was not a likely cause (Fig. 1B, 2A-D). Ecological succession, driven by accumulating effects of interactions over time, also did not appear to contribute to the expansion (Fig. 1C). This was further supported by a comparison of CEent1-dsRed colonization in worms raised continuously, through larval development and into aging, on CEent1-dsRed monocultures versus worms shifted to CEent1-dsRed in different ages for a fixed duration of two days (see Methods), which showed comparable age-dependent increases in *Enterobacteriaceae* abundance (Fig. 4A). We next examined whether age-dependent decline in bacterial uptake may play a role in causing the bloom. To this end, we compared CEent1-dsRed colonization, as part of the SC20 community, in wildtype worms, which show an age-dependent decline in pharyngeal pumping (and thus bacterial uptake), and eat-2 mutants, which lack a pharyngeal receptor for the neurotransmitter acetylcholine resulting in slow pumping rate, which does not change considerably during aging (Fig. 4B inset). In both strains we observed a similar course

of age-dependent increases in CEent1-dsRed colonization (Fig. 4B), indicating that age-dependent changes in bacterial uptake likely did not contribute to the *Enterobacteriaceae* bloom.

DBL-1/BMP signaling is a conserved regulator of development, body size and immunity (Savage-Dunn and Padgett, 2017). Previous work identified a role for DBL-1-dependent immune regulation in shaping the worm gut microbiome, particularly affecting *Enterobacter* strains, which bloomed when genes encoding different components of this pathway were disrupted (Berg et al., 2019). Gene expression data in Wormbase (https://wormbase.org) suggested that expression of the pathway's components may decline at the end of larval development. To examine whether this indicated an age-dependent decline in DBL-1 signaling and downstream gene expression, which might affect the gut microbiome, we used a transgenic worm strain expressing GFP from the *spp-9* promoter, previously shown to be negatively regulated by DBL-1 signaling (Lakdawala et al., 2019). Fluorescent imaging demonstrated age-dependent increases in the expression of the GFP reporter, indicating a decline in DBL-1 signaling in aging worms (Fig. 5A). In agreement, the effects of either disruption or over-expression of the *dbl-1* ligand gene on GFP expression from the *spp-9* promoter diminished with age. The significance of *dbl-1* disruption was similarly lessened in aging worms for colonization with CEent1, which in middle-aged *dbl-1* mutants was comparable to that seen in wildtype animals, indicating a decline in DBL-1 signaling and in its involvement in controlling gut *Enterobacteriaceae* abundance during early aging (Fig. 5B). Additional experiments replicated the observation of lessened importance of dbl-1 disruption for CEent1 colonization as worms age (Fig. 5C, red box). These experiments also included *sma-4*(*syb2546*) mutants, which carry a gain-of-function (gof) mutation in DBL-1's transcriptional mediator and show a 20% increase in body length compared to wildtype animals (Fig. 5C inset). *sma-4*(gof) mutants provided further support for a decline in DBL-1 control of gut bacteria, showing lower CEent1-dsRed colonization compared to wildtype animals in early days of adulthood, increasing to wildtype levels by day five of adulthood (blue box compared to dotted line in Fig. 5C). While the effects of the *sma-4*(gof) mutation waned with age, not capable of restoring long-term control over CEent1 colonization, it was able to delay the CEent1 bloom. This had beneficial consequences, as *sma-4*(gof) mutants were partially protected from CEent1's detrimental effects on infection resistance in day four adults (Fig. 5D). Together, these results suggest that an age-dependent decline in DBL-1/BMP signaling alters the intestinal niche, permitting preferential accumulation of *Enterobacteriaceae*, which can be detrimental. Boosting DBL-1 signaling may mitigate the bloom and its consequences, but only partially.

Commensal communities can effectively mitigate the detrimental consequences of the Enterobacteriaceae bloom

The ability of the *sma-4*(gof) mutation to partially mitigate the detrimental effects of CEent1 expansion suggested that protection from an *Enterobacteriaceae* bloom is possible. Considering that the decline in DBL-1 signaling was also associated with increased abundance of non-*Enterobacteriaceae* bacteria (Fig. 2), we examined whether other bacteria could compete with CEent1 and help prevent its detrimental

effects. Recent work identified three members of the genus *Pantoea* as common *C. elegans* gut commensals that effectively colonize the worm gut and are capable of competing with an invading pathogen (Pérez-Carrascal et al., 2022). Wildtype worms raised on a community of the three commensals in addition to CEent1-dsRed (in equal parts) and shifted to *E. faecalis* in middle-age (which with CEent1 alone would lead to compromised infection resistance), were as resistant as worms raised on *E. coli* alone, and significantly more resistant than worms raised on a similar inoculum of CEent1-dsRed mixed with *E. coli* (Fig. 6A). While mortality on *E. faecalis* plates was attributed to the pathogen, 96.7% of the worms raised on the CEent1-dsRed/*E. coli* mix, which died in any of the days of the infection assay, were heavily colonized with CEent1-dsRed (Fig. 6A inset), indicating proliferation alongside *E. faecalis*. In contrast, only 62.5% of the worms who were initially raised on the CEent1-dsRed/*Pantoea* mix were colonized, indicating that the Pantoea community was able to mitigate CEent1 proliferation in some of the worms and to reduce mortality in the population.

To examine whether mitigating the detrimental effects of CEent1 proliferation was unique to *Pantoea*, worms were raised on a subset of seven members of CeMbio (see Methods), with or without BIGb0393 (one of the protective *Pantoea* strains, which is also a member of CeMbio) and with a large excess of CEent1-dsRed (50% of total, to ensure effective colonization), and shifted at middle age to *E. faecalis*. Raising worms on the CeMbio subsets, with or without BIGb0393, conferred significantly higher resistance to infection than in worms raised on CEent1-dsRed alone (Fig. 6B). Again, fewer of the dead worms were colonized with CEent1-dsRed among those raised on CeMbio/CEent1-dsRed (51.4%), compared to those raised on CEent1-dsRed alone (84.6%). These experiments demonstrate that over proliferation of *Enterobacteriaceae* and its detrimental consequences in aging worms can be mitigated with more than one combination of gut commensals. Lastly, in the context of such a community, CEent1, or other *Enterobacteriaceae*, did not compromise the lifespan of their host, as worms grown on CeMbio with or without its *Enterobacteriaceae* members had a comparable lifespan (Fig. 6C).

DISCUSSION

Our experiments identify an *Enterobacteriaceae* bloom as a hallmark of the gut microbiome in aging *C. elegans*. This bloom was observed in worms raised in natural-like microcosm environments with varying initial microbial diversity, as well as in worms raised on defined bacterial communities differing in the environmental availability of *Enterobacteriaceae*, indicating that it is independent of initial conditions. The *Enterobacteriaceae* bloom is not due to bacteria-driven ecological succession or to age-dependent changes in bacterial uptake. Rather, it is due to intrinsic age-dependent changes in the intestinal niche, suggesting that the bloom is a signature of chronological age. Our results demonstrate that increased gut abundance of *Enterobacteriaceae* strains may have detrimental consequences for aging animals, at least for infection resistance. However, in the context of a community, even such with restricted diversity, the detrimental consequences of this bloom can be mitigated. Our results highlight the

Enterobacteriaceae bloom as a hallmark of chronological aging but suggest that the consequences of this bloom are context-dependent, with microbiome composition representing the context that can differentiate between healthy or unhealthy aging.

It is accepted that aging is accompanied by gut dysbiosis (Claesson et al., 2011; Clark et al., 2015; O'Toole and Jeffery, 2015; Smith et al., 2017; Thevaranjan et al., 2017). Human studies have documented diverse changes in gut microbiome composition. Among those, increased abundance of *Proteobacteria/Pseudomonadota*, and specifically of *Enterobacteriaceae*, is a recurring theme (Odamaki et al., 2016; Leite et al., 2021). In line with this, our results show a replicable age-associated expansion of *Enterobacteriaceae*, suggesting that it may be an evolutionarily conserved signature of aging. What causes this bloom is not clear. A study in fruit flies described gut dysbiosis characterized by a biphasic change in the microbiome of aging flies, in which a midlife bloom of *g-proteobacteria* led to intestinal barrier dysfunction and a subsequent increase in *a-proteobacteria* (Clark et al., 2015). While barrier dysfunction was suggested as the cause for gut dysbiosis (Salazar et al., 2018), what initiated the *g-proteobacteria* bloom was not clear. The *Enterobacteriaceae* bloom we observed in worms may be analogous to the initial phase of dysbiosis in flies. In worms, initiation of the *Enterobacteriaceae* bloom was associated with a decline in DBL-1/BMP signaling. DBL-1 signaling was previously shown to play a central role in controlling gut *Enterobacteriaceae* (Berg et al., 2019). Thus, its age-dependent decline may change the host intestinal niche making it more permissible for *Enterobacteriaceae* expansion. Supporting a causal role for DBL-1 signaling in the *Enterobacteriaceae* bloom, gof mutants for the SMA-4 BMP mediator showed a delay in *Enterobacter* colonization as well as attenuated infection susceptibility. These results suggest that decline of immune signaling during aging is an important factor in initiating dysbiosis. However, at least in the case of SMA-4, revamping the immune pathway to mitigate the detrimental effects of the bloom was only partially successful, suggesting that additional changes in the gut niche may take place to promote the *Enterobacteriaceae* bloom, and further limiting the potential of DBL-1/BMP reactivation in aging worms as an intervention to alleviate gut dysbiosis.

Enterobacteriaceae blooms are associated with increased susceptibility to infection (Lupp et al., 2007; Bailey et al., 2010; Shaler et al., 2021; Schlechte et al., 2023). In agreement with this, expansion of *E. hormaechei* CEent1 in aging worms compromised infection resistance and survival. Continued gut accumulation of CEent1 following a shift to pathogen plates further suggests that in old worms, CEent1 (and perhaps additional *Enterobacteriaceae* members) was an opportunistic pathogen, or a pathobiont. Previous results support the notion that CEent1 was a pathobiont, as worms growing on CEent1 alone have a shorter lifespan compared to a those raised on an *E. coli* diet (Berg et al., 2016b). However, in the context of a community (CeMbio), inclusion or removal of CEent1 did not have any effect on lifespan, supporting the importance of a diverse microbiome in keeping pathobionts in check as the host ages.

The genetic tractability and short lifespan of *C. elegans* has made it a useful model for aging research. Its more recent establishment as a model for microbiome research adds to that the advantages of longitudinal microbiome analysis in clonal host populations, while having greater control over bacterial availability, to facilitate studies of host-microbiome interactions during aging. Using this model, we identified what seems to be an evolutionary conserved signature of dysbiosis in aging animals and have begun to dissect its causes as well as its consequences. As often seen in different scenarios of gut dysbiosis, the *Enterobacteriaceae* bloom that we identified is associated with pathology. However, this pathology can be circumvented by manipulating the gut microbiome using various commensal communities. Thus, while an *Enterobacteriaceae* bloom seems to be an inevitable consequence of aging, its extent and outcomes can be restrained by other members of the gut microbiome. What differentiates between communities that can or cannot achieve this remains to be seen.

FIGURES

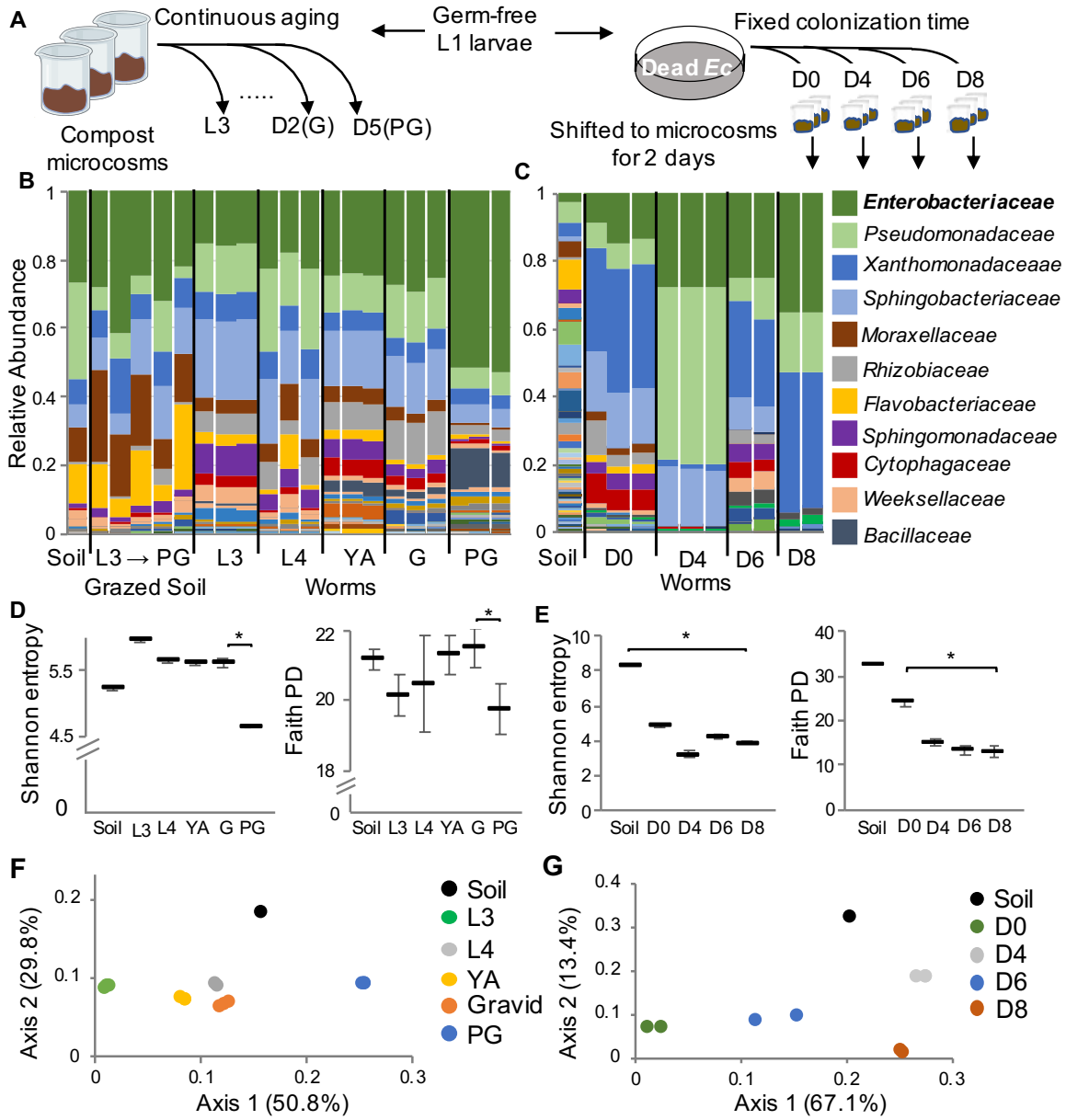


Figure 1. A gut *Enterobacteriaceae* bloom in worms aging in natural-like environments. A. Two sampling schemes for worm microbiome analysis during late development and early aging. Dead Ec stands for dead *E. coli*. **B,C.** 16S rRNA gene sequencing of microbiome composition in worms raised continuously in microcosm environments (B) or in worms of advancing ages, shifted for two days to microcosm environments (C). Bars represent microbiomes in microcosm environments or in the gut of worms raised in these microcosms (each bar represents a population of 100 worms). Taxa are shown at family level resolution. B, L3, L4, larval stages; YA, young adults; G, gravids (D2, second day of adulthood); PG, post-gravid (D5). C, D0, early gravids. **D,E.** Alpha diversity represented by Shannon and Faith phylogeny indices; *, p<0.05, t-test. **F,G.** Principal Coordinate Analysis based on weighted UNIFRAC distances between microbiomes.

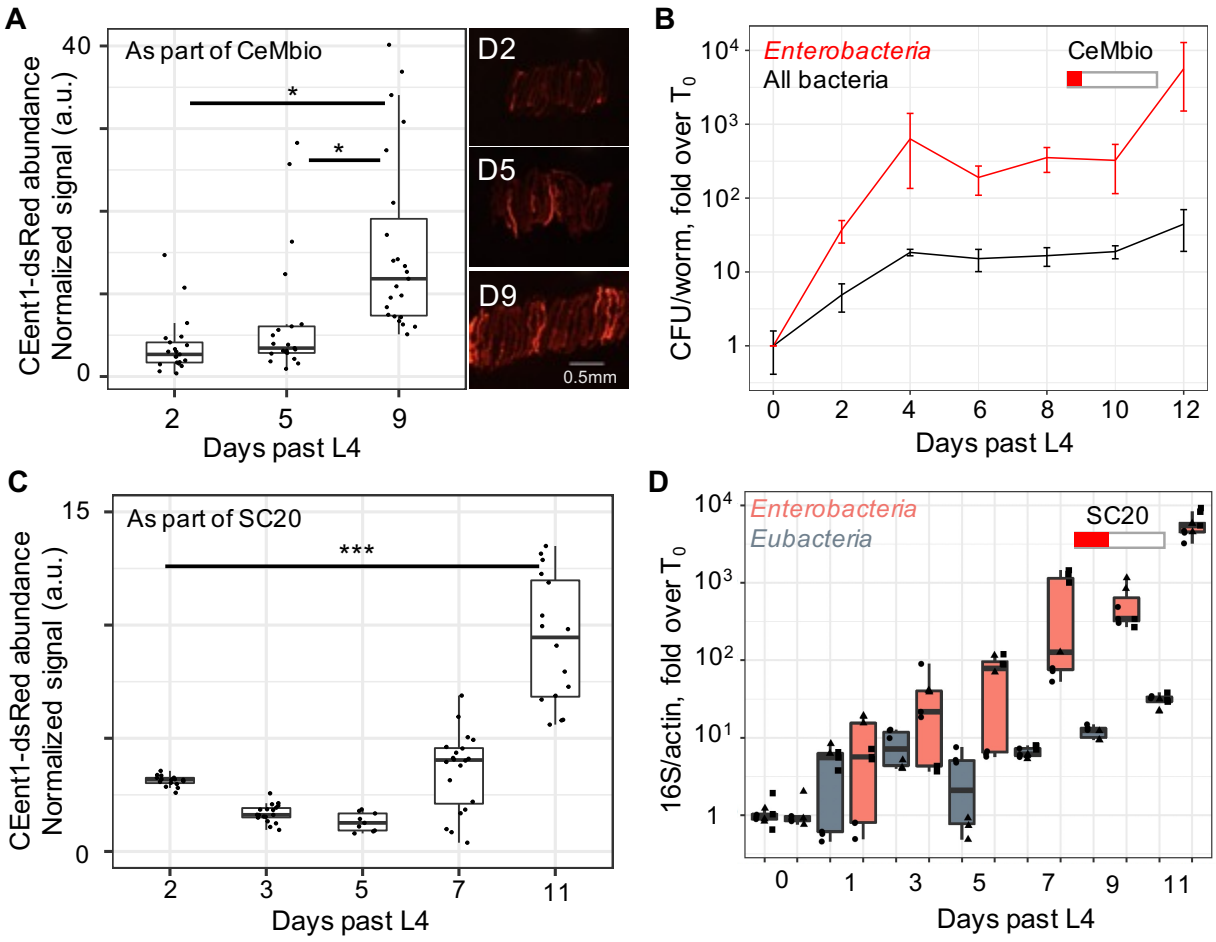


Figure 2: An *Enterobacteriaceae* bloom is a common denominator of aging worms raised in different microbial environments. A. Colonization of individual aging worms raised on CeMbio with CEent1-dsRed, n=21-23/group; $p< 0.01$, pairwise t-tests; **B.** Bacterial load in aging worms raised on CeMbio, based on CFU counts of *Enterobacteriaceae* on VRBG plates and of total bacteria on LB plates. Shown are averages ± SDs for 3 plates per time point (n=4-12 worms/time point). Bar with red coloration (B,D) indicates the fraction of *Enterobacteriaceae* in the designated communities. **C.** CEent1 colonization in aging worms raised on SC20 with CEent1-dsRed (n=9-18 worms/time point); $p<0.001$, pairwise t-tests. **D.** Fold change in bacterial load in worms aging on SC20, assessing bacterial load with qPCR using primers specific for *Enterobacteriaceae* 16S or Eubacterial 16S, normalized to worm DNA assessed by qPCR with primers specific for C. elegans actin (shapes represent replicate plates, each evaluated by qPCR in duplicate or triplicate).

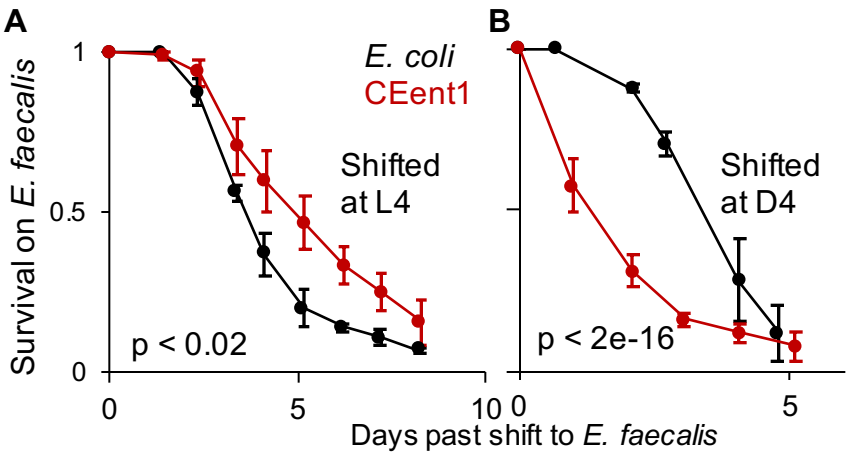


Figure 3: *Enterobacter hormaechei* CEent1 bloom in aging worms is associated with increased susceptibility to infection. Survival curves for wildtype worms raised on designated monocultures and shifted to plates with the pathogen, *E. faecalis* at L4 (A, n=85-99/group), or at day four of adulthood (B, n=96-99). *p*-values calculated with the log rank test.

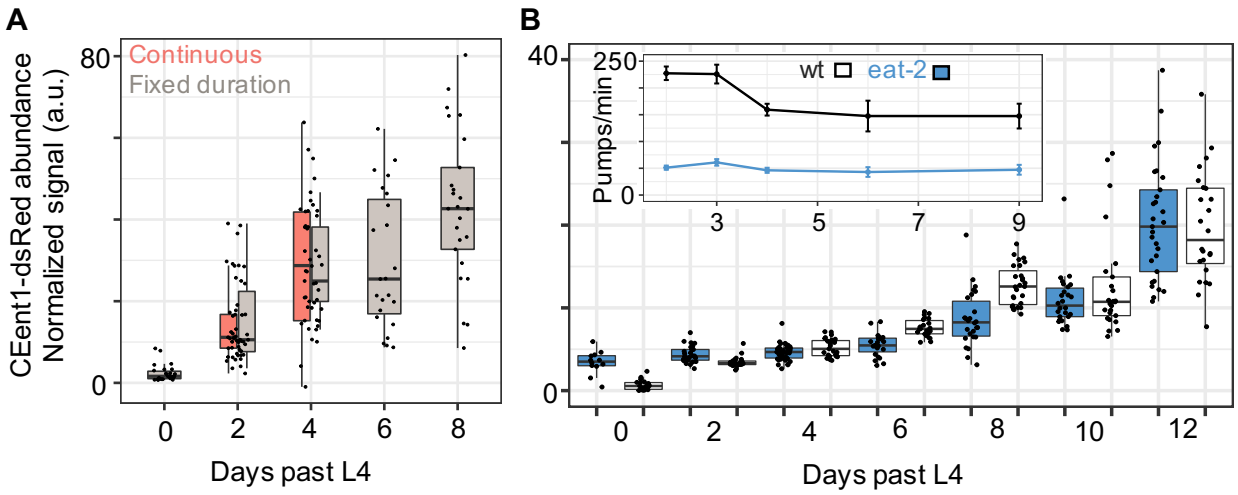


Figure 4: Neither duration of exposure to bacteria nor rate of uptake contributes to the *Enterobacteriaceae* bloom. A. Colonization of aging worms by CEent1-dsRed following continuous exposure from larval stages or shifting worms for two days ending at the designated time points (fixed duration) (n=22-27 worms/group/time point). Box and whisker plots show median values, marked with a line, 25th and 75th percentile values delineating the box. **B.** CEent1-dsRed colonization in wildtype and *eat-2* worms raised on the SC20 community (n=10-36 worms/group/time point); **inset** demonstrates age-dependent declines in pumping rates; n=4-10 worms/time point).

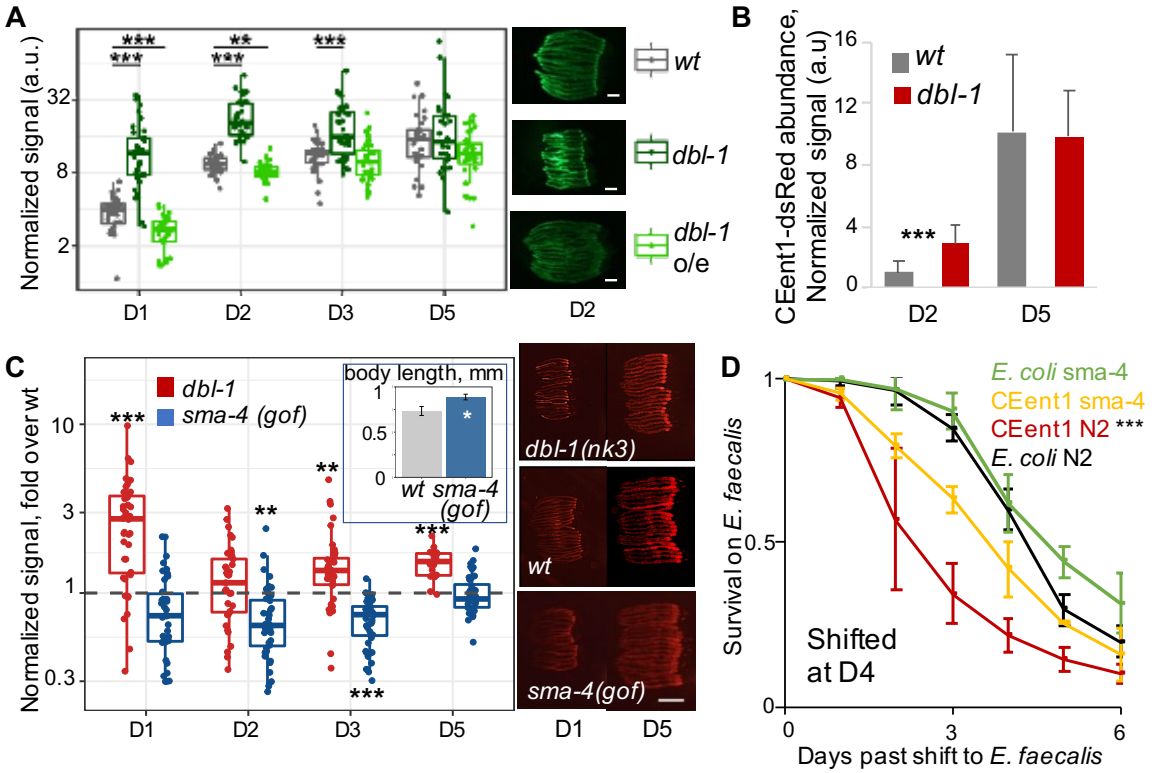


Figure 5: The *Enterobacteriaceae* bloom is associated with an age-dependent decline in DBL-1/BMP signaling. A. GFP expression from the *spp-9* promoter in designated strains; representative images (scale bar=200 μM) and quantification (n=27-37/group/time point); ***p*<0.01, and ****p*<0.001, Kruskal-Wallis rank sum test and *post hoc* Wilcoxon test (mutant vs wt), Bonferroni corrected. **B.** CEent1-dsRed colonization in worms of designated strains at designated days of adulthood; ***, *p*<0.001, t-test. **C.** Colonization of designated strains with CEent1-dsRed as part of CeMbio (n=39-40/group/time point), quantified in images as those shown on the right (scale bar=500 μM) and presented as fold over wt median (dotted line). *P*-values as in A. **Inset.** Body length of young adult worms of designated strains measured using ImageJ (n=9-12); *, *p*<0.0001, t-test. **D.** Survival of worms of designated strains raised on *E. coli* or CEent1 and shifted to *E. faecalis* at D4 of adulthood (n=106-112/group); ****p*<0.001, log rank test, compared to worms raised on *E. coli*.

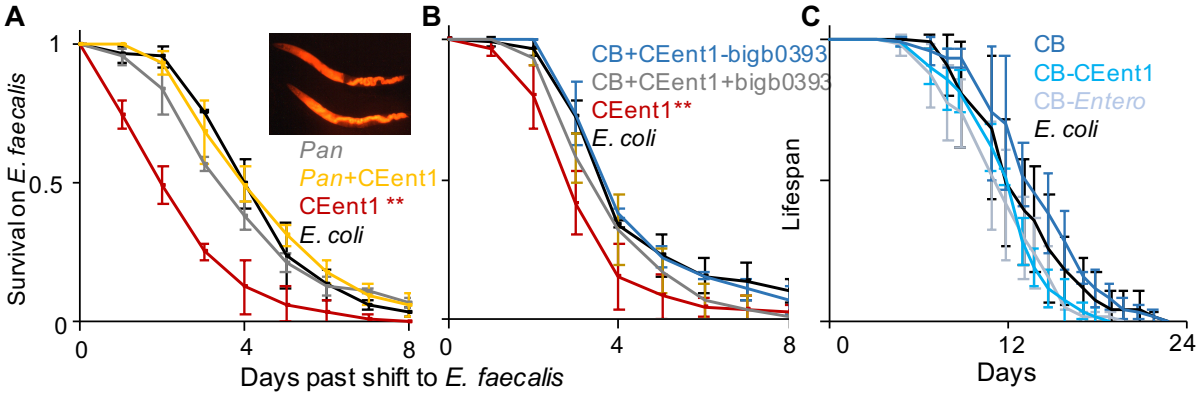
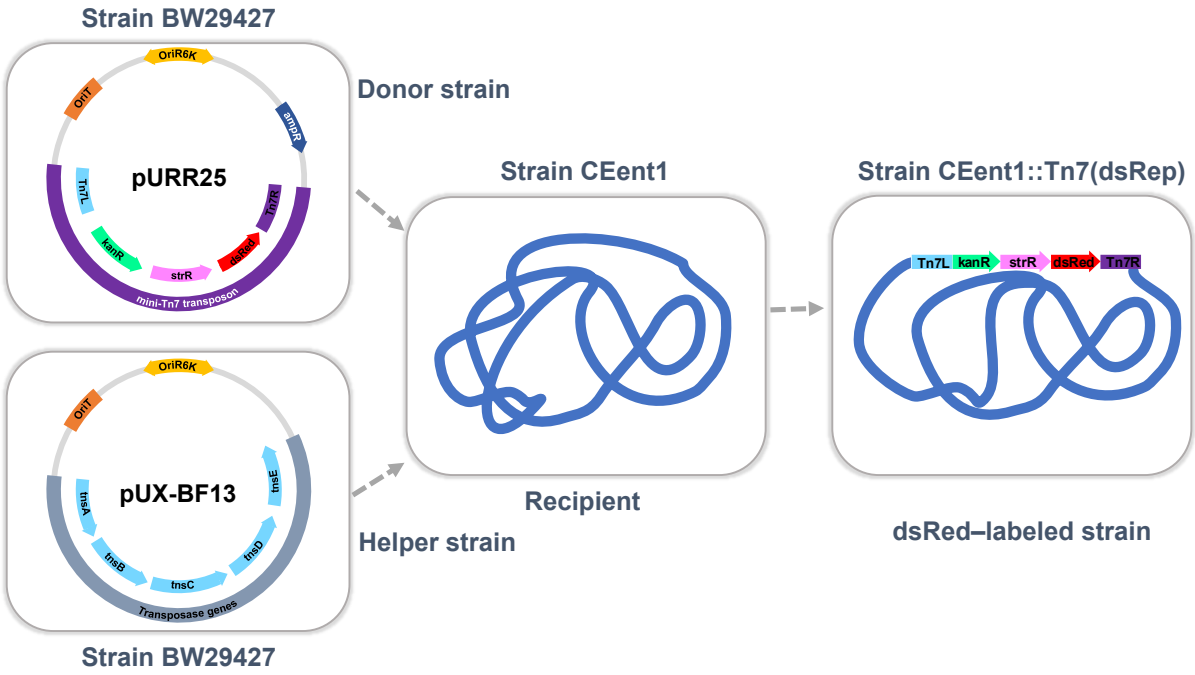


Figure 6: Commensal communities mitigate age-dependent susceptibility to infection. A. Survival of worms raised on the designated strains/communities and shifted to *E. faecalis* at D4; Pan, a community of three *Pantoea* strains; **, $p<0.0001$ (CEent1 compared to each group), log rank test (n=82-90/group); averages ± SDs for three plate replicates. **Inset.** CEent1-colonized dead worms, one day after shift to *E. faecalis.* **B**. Survival of worms raised on designated subset of CeMbio (CB) and shifted to *E. faecalis* at D4; BIGb0393, a *Pantoea* strain **, $p<0.0001$, (n=95-105/group). Shown are results of one representative experiment out of two with similar results. **C.** Lifespan of wildtype worms raised on designated communities. *Entero* stands for *Lelliotia* Jub66 and *Enterobacter* CEent1. Averages ± SDs for three plate replicates (n=67-79/group).



Supplementary Figure 1. Plasmids used in triparental mating to generate dsRed-expressing *Enterobacter hormaechei* CEent1. Shown are maps of the Tn7-gfp donor plasmid (pURR25) and the transposase-carrying helper plasmid (pUX-BF13). Tn7R and Tn7L are the sites recognized and cut by the transposase.

TABLES

Table 1: SC20 members

Strain name	Family	genus	species
CEent1-dsRed	*Enterobacteriaceae*	*Enterobacter*	*hormaechei*
MSPm1	*Pseudomonadaceae*	*Pseudomonas*	*Berkeleyensis*
Cre-5.2	*Enterobacteriaceae*	*Enterobacter*	*NA*
L3-3L	*Sphingobacteriaceae*	*Sphingobacterium*	*puteale*
oak-5.2	*Enterobacteriaceae*	*Buttiauxella*	*NA*
CEent3-GFP	*Enterobacteriaceae*	*Enterobacter*	*ludwigii*
WG-2.2	*Pseudomonadaceae*	*Pseudomonas*	*plecoglossicida*
WG-2.4	*Micrococcaceae*	*Arthrobacter*	*NA*
WG-2.5	*Microbactericeae*	*Microbacterium*	*barkeri*
2.1	*Bacillaceae*	*Bacillus*	*nealsonii*
3.1	*Bacillaceae*	*Bacillus*	*asahii*
3.2	*Bacillaceae*	*Bacillus*	*megaterium*
10.1	*Bacillaceae*	*Lysinibacillus*	*pakistanensis*
10.3	*Bacillaceae*	*Lysinibacillus*	*pakistanensis*
14.1	*Bacillaceae*	*Bacillus*	*subtilis*
19.1.7	*Bacillaceae*	*Lysinibacillus*	*fusiformis*
19.3.3	*Enterobacteriaceae*	*Rahnella*	*victoriana*
19.3.8	*Enterobacteriaceae*	*Buttiauxella*	*agrestis*
CBent2	*Enterobacteriaceae*	*Lelliottia*	*amnigena*
Cbent1	*Enterobacteriaceae*	*Citrobacter*	*fruendii*

CHAPTER 3: The Coastal Redwood leaf microbiome in relation to hydroclimatic gradients and fire

ABSTRACT

Leaf-associated microbial communities have been studied for their roles in plant health and fitness. Bacterial and fungal microorganisms on the surface of (epiphytic) and on the interiors (endophytic) of leaves are exposed to and shaped by environmental abiotic and biotic factors, which can vary greatly across a species range. We examine the leaf microbiome of the Coastal Redwood (*Sequoia sempervirens,* D. Don), the tallest tree in the world, that grows along the central and northwestern coastal zone of the US. The redwood range is characterized by a natural hydroclimate that varies in precipitation levels and summertime fog. Fog-borne water is a major source of water for redwoods and is known to be taken up through their leaves. In redwood-dominated landscapes, wildfires have also increased and are known to severely impact vegetation and forest associated microbiomes. Bacterial communities associated with redwood root systems and microbes associated with leaves have been found to shift with various factors including water availability, geographical location, and tree height. While studies show evidence of shared endophytic fungal communities amongst trees, the presence of a core bacterial community is less clear. Here, we harvested leaf samples and conducted 16S and ITS rRNA sequencing to characterize bacterial and fungal communities associated with epiphytic (on leaves) and endophytic (in leaves) microbiomes. Communities were compared along the different gradients including 1) the well-characterized north and south hydrological gradient, 2) vertically within tree crowns, and 3) in relation to fire severity from recent wildfires. Leaf microbiome compositions differed within and between individual trees and were more similar overall to those within their geographical locations, including sites that experienced fire events. These results show that microclimates, geographically and along a vertical gradient of a single tree, harbor distinct microbiomes that may reflect selective pressures associated with their unique environments. Additionally, endophytic communities differ in composition from their respective epiphytic communities, which are more pronounced in fungal compared to bacterial communities, providing evidence for plant host filtering processes that can be further studied for their plant-microbe interactions.

INTRODUCTION

The phyllosphere microbiome

Plants encounter a vast diversity of microorganisms in their environment, giving rise to a wide range of interactions that can both benefit and harm the host plant. Microbial communities, including both bacteria and fungi, have been increasingly studied for their roles in shaping plant physiology, health, and ecology. At the root-soil interface, or rhizosphere, microbes are known to influence nutrient acquisition, drought resistance, growth, and other physiological characteristics (Marschner and Dell, 1994; Bennett et al., 2009; Hartmann et al., 2009; Lau and Lennon, 2011; Rolli et al., 2015; van der Heijden et al., 2015; Willing et al., 2020; Allsup et al., 2023). Aboveground plant tissues

also host diverse microbiomes that play key functional roles on both the surface (epiphytic) and on the inside of (endophytic) leaves. Plant leaves and their associated microbes are collectively referred to as the phyllosphere and comprise a substantial proportion of biomass in plants. Leaves provide a distinct niche for bacteria and fungi which form microbiomes that can both impact plant health and be shaped by the plant host itself (Lindow and Brandl, 2003; Laforest-Lapointe et al., 2017; Koskella, 2020; Sohrabi et al., 2023). The phyllosphere is exposed to a range of dynamic abiotic factors such as varying temperatures, moisture, and sunlight, which can all impact the composition of both epiphytic and endophytic microbial communities (Vorholt, 2012; Koskella, 2020). Many phyllosphere microbes are commensal, coexisting on plant surfaces being neither beneficial nor detrimental to their host. Some can cause disease while yet others can have positive effects on plant function such as suppressing plant pathogens, enhancing growth, or mitigating drought related stress (Manulis et al., 1998; Lindow and Brandl, 2003; Ritpitakphong et al., 2016; Koskella, 2020; Bechtold et al., 2021). In this study, we examine the microbial compositions associated with the phyllosphere of an ancient tree species within their natural environment to set the foundation of exploring potential roles of microbes in plant health.

The California Coastal Redwoods and their ecological gradients

The aerial plant biomass is greatest in the long-lived California Coastal Redwood, the tallest tree species on Earth (Van Pelt, 2001). In contrast to their surprisingly shallow root systems that rarely extend downward more than a few meters (Noss, 2000), redwoods can grow to well over 100 meters tall and host billions of leaves (Van Pelt et al., 2016). Found along the northwestern coast of the US between southern Oregon and Big Sur in central California, redwood forests thrive in the cool, wet regions in the north with greater annual rainfall and extends into the drier regions in the south. Redwoods are well adapted to their local climates where water resources fluctuate seasonally with abundant wintertime rainfall and summertime fog, which contribute a significant source of water in otherwise dry summers (Dawson, 1998; Burgess and Dawson, 2004; Ewing et al., 2009). Their leaves have adapted structurally to facilitate foliar water uptake to utilize water resources from fog and reduce water loss (Burgess and Dawson, 2004; Limm et al., 2009; Simonin et al., 2009; Chin et al., 2022). Bacteria associated with Coastal Redwood roots have been found to shift with water availability, where specific bacterial groups are enriched in drier conditions providing evidence for selection on specific bacterial morphologies that may be linked to water acquisition (Willing et al., 2020). Recent studies have begun exploring microbial communities in the phyllosphere across their geographical gradient where they experience different climates (Carrell and Frank, 2015; Harrison et al., 2016) and events such as fire, which is also known to impact microbial diversity and function in forests (Dove et al., 2021; Nelson et al., 2022).

Existing studies examine plant driven microbial selection at the rhizosphere in agricultural or experimental plant species (Berg, 2009; Lau and Lennon, 2011; Rolli et al., 2015; Tkacz and Poole, 2015; Schmidt et al., 2019). However, these studies are limited to crops that have undergone repeated cycles of human-driven selection and exposed to agricultural practices that can disrupt plant-microbe interactions (Porter and

Sachs, 2020). Therefore, the ancient redwood species and their microbial interactions offer a natural study system shaped over millennia by various factors like their natural ecological gradients, long lifespans, and plant chemical characteristics.

Redwoods and their leaf associated microbes

Redwood tree leaf microbiomes have been found to vary in composition with respect to height, a trend observed in individual trees from across their geographical range (Harrison et al., 2016). Endophytic fungal communities have demonstrated some evidence of a shared community amongst trees with recurring fungal species present in multiple trees in central California (Espinosa-Garcia and Langenheim, 1990). However, endophytic bacterial communities have been found to have less of a clear core community between trees and show similarities with other plant and lichen species (Carrell and Frank, 2015). In this study, we examined both the bacterial and fungal communities associated with the external, epiphytic as well as the internal, endophytic leaf surfaces in redwoods. We hypothesized that marked gradients would make a significant impact on the microbiome composition because of the associated environmental changes that they can represent. We therefore characterized the leaf microbiome comparing communities along the 1) north-south geographical gradient, 2) the vertical gradient of an old growth tree, and 3) trees that have experienced a range of fire severity. Both epiphytic and endophytic leaf microbiomes are hypothesized to shift according to different ecological pressures associated with geography, height, and fire disturbance that pose filtering effect on the phyllosphere microbiome.

METHODS

Redwood sites and sample leaf collection

Leaf samples were collected from five unique sites within California and collected between October 2023 and February 2024 (Fig.1). Locations included wetter regions with greater annual rainfall at the northern edge of the range in the Klamath Mountains and drier regions at the southern edge of the range in Big Sur, California. Site details are summarized in Table 1. Portions of the forest at three of our study sites experienced fire events that varied in severity since 2017. Leaves and branchlets collected at ground level were cut with 70% ethanol sterilized scissors or 3.5 m long pole pruner into new 3.8-liter Ziploc bags. Samples were then stored on ice or 4°C overnight before processing in the lab or frozen for long-term storage at -20°C. Microbial communities associated with the both the leaf surface (epiphytic) and internal (endophytic) surfaces were harvested from ground level leaf cuttings (with the exception of samples from the Humboldt location which were only sampled for endophytic communities).

At the Humboldt location, samples were collected along the vertical gradient including the canopy. Samples were obtained from a single tree (#51) at 86 meters (crown), 77 meters, 71 meters, and 51 meters with garden shears. Ground level samples were collected from saplings (<15 years old) adjacent to the base of the tree. Crown samples were also obtained from a second, mature redwood tree on private land in Kneeland, California (40°46'06"N 124°00'00"W, 602 m). Leaf cuttings were stored on ice or at 4°C

for up to 2 days before processing in the lab or storage at -20°C. Leaves obtained along the vertical gradient were surface sterilized and harvested for DNA from only endophytic surfaces.

Microbial community collection

Approximately 1-5 grams of leaves were picked or cut with sterilized tweezers and razor blades into 50 ml falcon tubes. To collect epiphytic samples, leaves were sonicated for 10 min 2X in 10mM MgCl2 0.002% Tween 20 detergent (Smets et al., 2023) to release external leaf microbes and dirt debris. The resulting sonicate was filtered through autoclaved coffee filters (Meyer et al., 2022) to remove excess soil and debris and the remaining solution was centrifuged to pellet epiphytic microbial material for DNA extractions. Endophytic microbial material was obtained by surface sterilizing the sonicated leaves. This was achieved by submerging leaves in tubes containing 70% ethanol solution, rinsing 3 times with sterile water, submerging in bleach solution (2% sodium hypochlorite), and rinsing an additional 3 times with sterile water, before resuspending in sterile phosphate buffer saline (PBS). Each sample was homogenized in 15 ml falcon tubes using a FastPrep-24 Bead Beating Grinder and Lysis System (MP Biomedicals) containing sterile ¼ inch ceramic beads (MP Biomedicals) to release internal leaf microbial material and then centrifuged to pellet endophytic material for DNA extractions.

DNA extraction

DNA from pelleted microbial material for both epiphytic and endophytic communities were extracted alongside an extraction kit control using the DNeasy PowerSoil Pro Kit (Qiagen Cat. No. 47014). Extractions were performed following the manufacturer's instructions with modifications: heated CD1 buffer at 60°C for approximately 10 minutes before homogenizing samples in a PowerLyzer at 2000 RPM for 30 seconds 2 times. Samples were eluted in 45µl of C6 elution buffer. DNA concentrations ranged from 0.1-15ng/µl.

NGS sequencing and analysis

DNA samples were sent to the UC Davis Host Microbe Systems Biology Core for sequencing on the Aviti sequencing system. Each sample was sequenced for paired-end 16S V3-V4 bacterial region (CCTAYGGGRBGCASCAG and GGACTACNNGGGTATCTAAT) and the ITS1F-ITS2 fungal region (CTTGGTCATTTAGAGGAAGTAA and GCTGCGTTCTTCATCGATGC). Data analysis was performed using the Dada2 pipeline (Callahan et al., 2016) and phyloseq (McMurdie and Holmes, 2013) package in R. Bacterial 16S rRNA was analyzed using the SILVA rRNA database (v138.2) (Quast et al., 2013) and fungal ITS results were analyzed using the UNITE database (v10.0) (Abarenkov et al., 2025). Total number of unique Amplicon Sequence Variants (ASV) corresponding to unique reads from each sampled condition from ITS and 16S amplicon sequencing after denoising and filtering steps are summarized in Table 2. Bacterial ASVs were filtered to remove mitochondrial and chloroplast sequences and both bacterial and fungal ASVs were processed with R package, decontam (Davis et al., 2018) to remove potential contaminant ASVs.

RESULTS

Redwood leaf epiphytic and endophytic microbiomes from a northern and southern site have distinct compositions

The leaf microbiome of trees from Angelo Coast Range Reserve (AR), a northern site, and Landels-Hill Big Creek (BC), a southern site, were harvested and analyzed for their external, epiphytic and their respective internal, endophytic microbial communities. The relative abundance of leaf-associated bacterial (4 individual trees) and fungal (8 individual trees) communities at the Order level exhibit greater compositional similarities respective to their geographical locations, which vary greatly in annual precipitation (Fig. 2A and B). Microbiome composition did not differ in relative diversity both in total observed number of unique ASVs in Figure 3A and when accounting for richness and evenness with the Shannon index in Figure 3C. Most of the epiphytic leaf bacterial communities at BC (near the ocean) host Salinisphaerales, a bacterial Order (with members in the *Salinisphaeraceae* family) associated with marine environments (Vetriani et al., 2014). Acetobacterales, largely composed of the *Acetobacteracaeae* family which include nitrogen fixing bacteria (Reis and Teixeira, 2015), is notably found in trees at both sites with greater abundance in epiphytic leaf samples compared to their endophytic counterparts. Endophytic bacterial communities in BC trees include a larger proportion of bacterial orders Bacteroidales and Erysipelotrichales (Fig. 3A). These orders include obligate anaerobic bacteria in genera *Bacteroides* and *Faecalibaculum,* respectively, which were interestingly present in the internal leaf surfaces that are expected to be oxygen rich. Epiphytic and endophytic bacterial communities did not have significant differences in diversity scores when accounting for richness and evenness between leaf surfaces (Fig. 3B).

Fungal epiphytic communities share several Orders including Mycosphaerellales, Tremellales, and Cladosporiales, suggesting fungi that are found ubiquitously across environments when associated with redwood leaves. These specific fungal members are also less abundant in their respective endophytic communities (Fig. 2B) that were overall significantly less diverse compared to their external leaf surface communities (Fig. 3D). Fungal communities have a much higher proportion overall in unassigned (NA), ASVs at the Order level, 60-95% of sequences were identified at the Phylum level, dominated by Ascomycota and Basidiomycota (Supplementary Fig. 1). The high proportion of unassigned sequence variants is likely due to limited identification at lower classification levels with existing databases. At both northern and southern sites, Saccharomycetales is largely present in endophytic communities, which dominates relative abundance in specific trees in the northern site.

Leaf-associated microbiomes were compositionally similar to trees within their geographical location when plotted by Bray-Curtis NMDS distances (Fig. 4). The location-dependent clusters were also evident when accounting for phylogenetic lineages in bacterial communities weighted UNIFRAC distances (Fig. 4B). Endophytic fungal communities were clearly distinct from their epiphytic communities providing

evidence for both geographic- and leaf location-dependent microbial compositions (Fig. 4C). Combined with the significant differences in ASV abundances between leaf surfaces in Figure 3D, the data suggests that fungal microbiomes experience a host filtering effect.

Endophytic leaf community compositions vary along the vertical gradient of trees
We also examined within tree, endophytic leaf microbiomes along the vertical gradient of an old growth tree (approx. 90m tall) in the Redwood Experimental Forest (REF) in Arcata, California within Humboldt County. Many bacterial orders are consistently present in internal leaf structures regardless of height within the redwood canopy (Fig. 5A), however, specific orders are found in greater proportion in the mid-canopy and crown leaves. For example, Rhizobiales increases in abundance relatively in the crown compared to the mid- and lower canopy. Rhizobiales is composed of members in the *Beijerinckiaceae* family, known to be aerobic nitrogen-fixing bacteria and were also present in previous studies of endophytic leaf bacteria (Carrell and Frank, 2015). Relatively small proportions of Rickettsiales are present in the crown associated leaf samples, which include members in the *Wolbachia* family, a parasitic bacterium. The bacterial order Acetobacterales and Sphingomonadales are found in greater proportions in crown leaves. Sphingomonadales are also found in epiphytic leaf samples from the other, relatively northern site, AR in Figure 2A.

Fungal communities were dominated by Ascomycota and Basidiomycota phyla which accounted for at least 75% of reads (Supplementary Fig. 2); however, a majority remain unidentified at the Order level (Fig. 5A). Cladosporiales and Pleosporales are present across all samples may represent fungal orders that successfully colonize the leaves. Hypocreales is found throughout the height of the old growth tree and more prevalent in ground and lower leaf samples. Phaeomoniellales and Mycosphaerales are found in the crown and have been associated with endophytic plant pathogens (Crous et al., 2009; Chen et al., 2015). Xylariales, known to colonize conifers as an endophyte (Becker and Stadler, 2021), are found in great abundance in the nearby ground saplings with less prevalence in the old growth tree. Overall diversity along the vertical gradient varied with no significant differences in fungal communities (Fig. 6B). Ground leaf samples from adjacent saplings at the base of tree 51 had the lowest relative bacterial diversity with significant differences from the lower crown samples (77m) (Fig. 6A).

Microbiomes varied along the height of the tree for both bacterial and fungal communities, consistent with previous studies reporting fungal community stratification along the vertical gradient (Harrison et al., 2016). Ground samples from young saplings (<15 years) at the base of tree 51 differed the most compositionally in both bacterial and fungal communities shown in Bray NMDS (Fig. 7A, C) and PCoA beta diversity plots (Fig. 7B). Crown associated fungal communities dispersed more widely compared to mid- to lower canopy leaf samples demonstrating a potential shift along the vertical gradient of the tree in a directional manner.

Fire as a filter on the epiphytic microbial phyllosphere: communities shift with the geographical gradient

Three of the sampled locations experienced severe fire events since 2017 from the more central regions at Pepperwood Preserve (P) of the range to southern edges at Big Creek (BC). BC samples were also previously represented in Figures 2-4. The burn sites were sampled to examine epiphytic phyllosphere microbiomes representing post-burn recovery. Sprouting leaves at the base of old growth and recovering secondary growth trees in the mid-Southern site, at Big Basin (BB), and new leaf growth from young (<50 years old) trees the central, Eastern site, P, shared greater similarities compared to BC for both bacterial and fungal communities. P and BB both shared a high proportion of Bacillales and Enterobacterales bacterial orders while BC epiphytic communities had high proportions of Salinisphaerales, an order associated with marine systems (Fig. 8A). The majority of identified fungal ASVs were assigned to Ascomycota and Basidiomycota (Supplementary Fig. 3). Fungal communities were well characterized at the order level relative to other samples and reflected similar trends with P and BB hosting a larger proportion of Cladosporiales compared to a more different BC community composition (Fig. 8B). Additionally, fungal orders including Cladosporiales, Mycosphaerellales, and Tremallales were shared amongst the epiphytic communities across all three locations.

Overall microbial alpha diversity across the three locations is similar (Fig. 9A, C). Epiphytic leaf surfaces, which only include endophytic comparisons for BC samples, show little differences in bacterial diversity and a greater number of observed ASVs in fungal communities that then loses significance when accounting for both richness and evenness (Fig. 9D). Samples from each location are compositionally similar to one another in both bacterial and fungal communities (Fig. 10). Bacterial beta diversity plots demonstrate location dependent clusters that are relatively more prominent when considering phylogenetic diversity in weighted UNIFRAC plots (Fig. 10B). Fungal diversity plots based on Bray NMDS also show distinct epiphytic clusters that align geographically with the most southern, BC cluster farthest from the more northeastern site, P (Fig. 10C). The geographically-dependent clusters therefore suggest that hydroclimate and climate remain a prominent factor associated with microbial composition.

DISCUSSION

Geographically distinct phyllosphere microbiomes

The phyllosphere microbiome in redwood trees exhibit great diversity along their geographical range characterized in-part by marked gradients of water availability with wetter, cooler climates in the north and drier, warmer conditions towards the southern and sometimes eastern edges. Across all sampled locations, we observe location dependent microbial communities both in fungal and to a lesser degree in bacterial communities. While specific bacterial and fungal orders and families were found across many locations, a shared core microbiome was not clear across the trees sampled in this study, in line with previous findings in endophytic bacterial communities (Carrell and

Frank, 2015). Therefore, geographically distinct climates likely play a strong role in shaping microbial community compositions as seen in bacterial communities associated with redwood roots (Willing et al., 2020). Differences in composition between geographically distant locations were also observed in Poplar tree leaves from varying climates (Firrincieli et al., 2020). Furthermore, seasonal shifts and climate related factors such as temperature and wind have been found to shape epiphytic leaf fungal communities in Mediterranean tree species, while other factors like rainfall and plant tissue type were major contributors to endophytic community composition (Gomes et al., 2018).

Host leaf has a stronger filtering effect on fungal communities

Outer, epiphytic fungal community composition differed from their respective internal, endophytic microbiomes as observed in a systematic shift from one surface to the other when plotted by compositional similarities (Bray-Curtis NMDS) in Figure 4C. Epiphytic leaf surfaces also contained a higher relative diversity of unique fungal members (represented by unique ASVs) compared to endophytic surfaces, suggesting a filtering of outside environmental microbes into internal surfaces. Bacterial communities, however, lack a similar shift in member abundance between leaf surfaces in AR and BC and have a less distinct difference in community composition. The distinct fungal compositional difference between leaf surfaces and lack thereof in bacterial communities potentially highlights unique functional or physical properties of fungi that enable specific types to enter internal leaf surfaces. Leaf surface and structure can influence phyllosphere microbiomes (Doan et al., 2020) and likely impact how microorganisms assemble on and inside of leaves. Fungal hyphae have been documented in redwood leaves to enter from the exterior of a leaf surfaces into stomatal openings, hypothesized to serve as a “water wick” to facilitate water uptake (Burgess and Dawson, 2004). Fungal counterparts, therefore, may play a role in water retention and may even support bacterial communities by reducing moisture loss (Hestrin et al., 2022). Although bacterial communities were not as distinct in composition between leaf surfaces, we find the presence of obligate anaerobic bacterial genera in higher proportions in endophytic communities, a curious finding at the site of oxygen production in the leaf. While we cannot definitively rule out potential sample contamination, other factors such as fungal networks or bacterial biofilm production (Bogino et al., 2013), which *Bacteroides* is known to form, may support conditions that allow obligate anaerobes to establish. The distinct endophytic fungal communities may suggest that the leaf has a more profound filtering effect on fungal communities systematically, compared to bacteria.

Microbiome composition shifts are observed along the vertical gradient

Redwoods host different leaf types along the vertical gradient of individual trees (Chin et al., 2022) that shift in structural features related to branch hydraulics and gas exchange (Ambrose et al., 2009) and can likely impact phyllosphere microbiome composition and assembly. Fungal community compositions were similar amongst leaves at their respective heights along the vertical tree gradient, in line with previous studies (Harrison et al., 2016) Overall diversity in richness and evenness (alpha diversity based on ASVs)

was similar along the individual tree's height. Only the adjacent ground sapling exhibited significantly lower diversity in bacterial community and is distinct in composition from the old growth tree, a trend not observed in fungal communities. Therefore, tree leaf shape and location in the vertical gradient may contribute differently to bacterial and fungal microorganism compositions and assembly. Future studies expanding the sampling efforts across the vertical gradient across the geographical gradient and comparing the epiphytic and endophytic leaf surface communities will be important to establish more definitive trends.

Post-fire epiphytic microbiomes shift along geographical gradient

Epiphytic microbiomes from fire-exposed redwood forest locations remained distinct in composition by geographical location with a proportion of shared bacterial and fungal orders. Leaves were collected from young sprouts at the base of trees or from second growth tree leaves post-fire and may reflect groups that are abundant and able to establish on leaves. The two sites, BB and P are relatively similar in composition and are in more central regions of the redwood range compared to the most southern edge site, BC. The epiphytic communities share large proportions of bacterial orders including Bacillales and Enterobacterales. It is important to note that the control in Figure 8A exhibits a similar composition to both BB and P samples. Despite being a control not specifically associated with those respective leaf samples, the bacterial BB and P samples may reflect kit control microbiomes or contamination. Fungal epiphytes included a significant proportion of Cladosporiales, largely composed of *Cladosporiaceae* family, across P and BB. Capnodiales and Polyporales, which contains *Fomitospidaceae*, both known to be pathogenic and parasitic in plants, were found in P and BB, respectively. This suggests that redwood leaves are not only exposed to potentially pathogenic microbes, but that these microbes can establish on external leaf surfaces.

Further research examining fire as a filter on leaf-associated microbiomes will reveal more consistent trends post-fire in redwood forests. Fire can impact forest susceptibility to infections including those from fungi as seen in other coniferous forests (Parker et al., 2006). While redwoods are known to have antimicrobial properties, pathogenic microbes can indeed establish on redwood leaf surfaces, highlighting the importance of understanding the assembly of and microbiomes over the course of post-fire recovery. Fire is also known to impact microbial diversity and function within forests (Nelson et al., 2022) and have been found to alter relative dominance of microbes and parent to sapling transfer of phyllosphere communities in young aspen trees (Dove et al., 2021). How microbial compositions may shift in response to fire, especially considering the growth of new, young leaves, may reveal unique trends attributed to post-fire disturbances.

The use of fire management by indigenous peoples historically in redwood forests has changed dramatically in the modern age, where forests experience fires of greater severity and duration (Lorimer et al., 2009; Katuna et al., 2024). Understanding the effects of fire disturbance on microbial assembly including re-colonization on tree leaves

based on fire events which have increased in western US (Higuera and Abatzoglou, 2021; Keeley and Syphard, 2021) will be important in examining their effects on host physiology.

Conclusions

As an extremely long-lived species, aging centuries and evolving over millennia, the Redwoods provide an opportunity to study early age plant hosts where microbe interactions are initially formed, to more mature, established communities that reflect a relationship evolving and co-adapting over time. We find that microbiomes are largely geographically dependent, however, bacterial and fungal communities shift in composition and diversity in different ways. Fungi show more distinct compositions respective to location and leaf surface compared to bacteria and further studies are needed to parse out how these microorganisms interact with one another and their host. Understanding these interactions in a natural system is essential in redwood forest management as species that supports biodiversity as a habitat for many epiphytic species (Sillett and Pelt, 2007) and as major carbon sequesters in the ecosystem (Jones and O'hara, 2012; Watt and Kimberley, 2022). In the face of changing global climate patterns that include increased frequencies of severe fires and droughts, plant species ranges can and will shift. Plant interactions with their phyllosphere microbiome will no doubt influence host performance, fitness, and survival in relation to their dynamic environment (Busby et al., 2022; Perreault and Laforest-Lapointe, 2022).

FIGURES

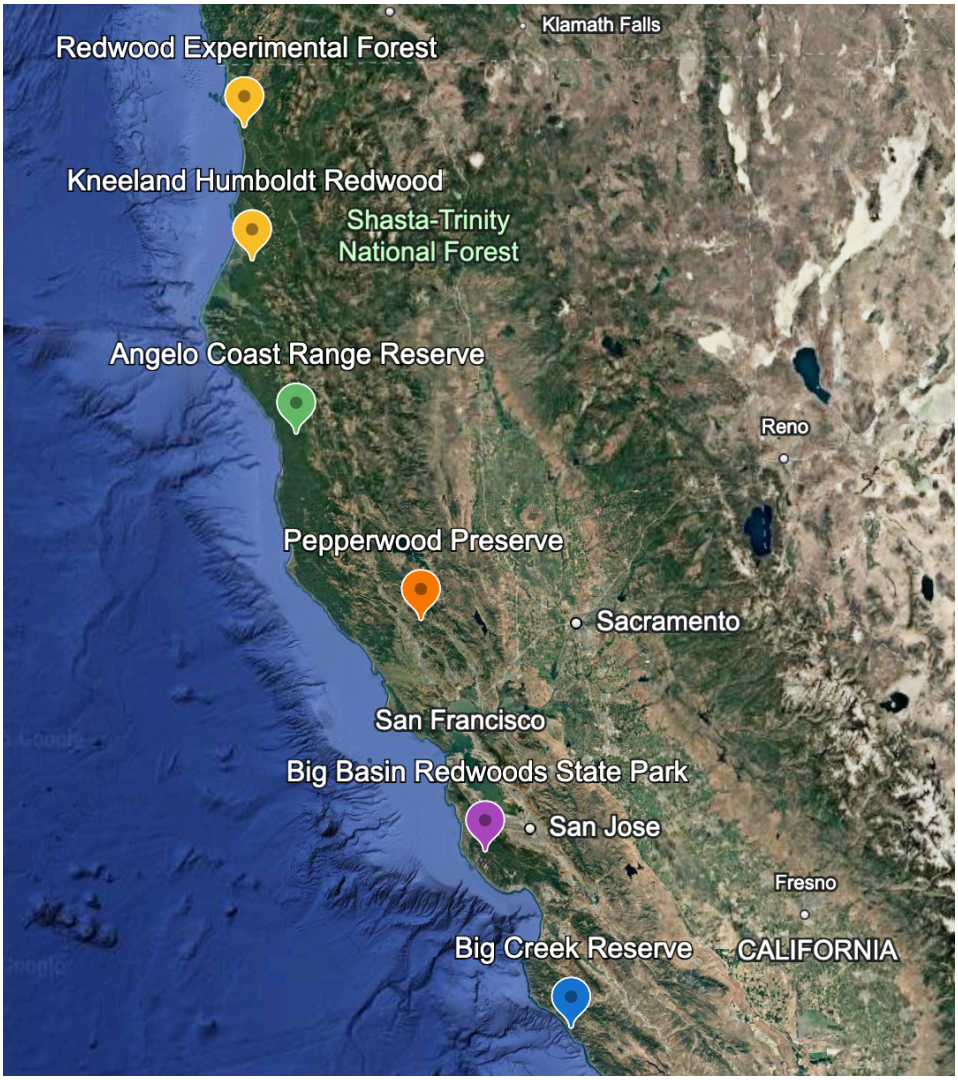
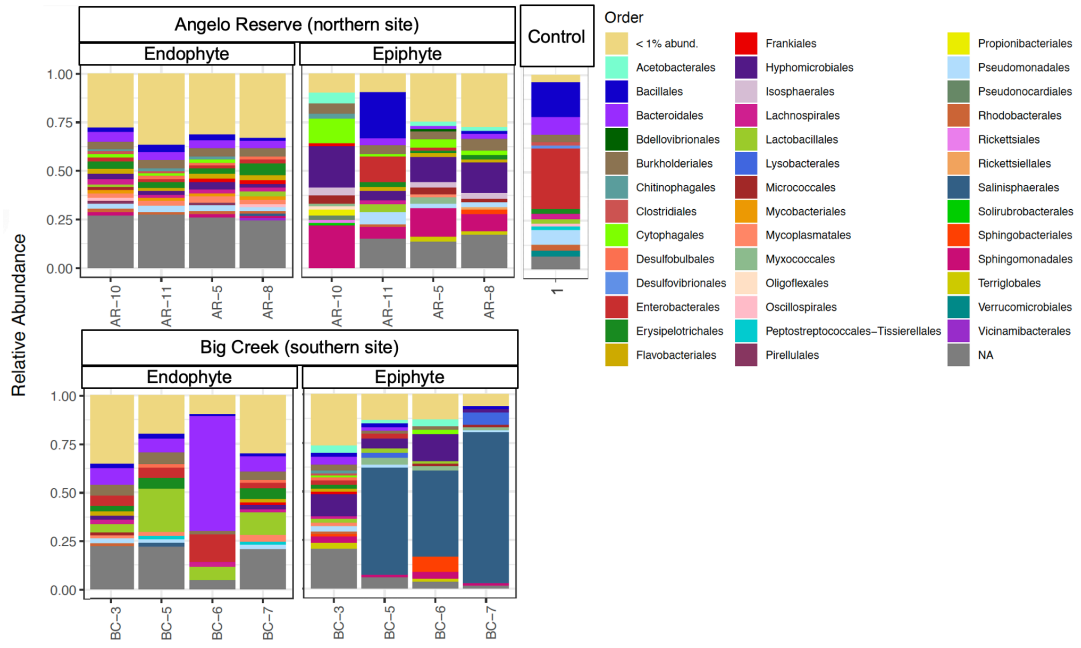


Figure 1: Sampling locations along the Redwood range.

A.



B.

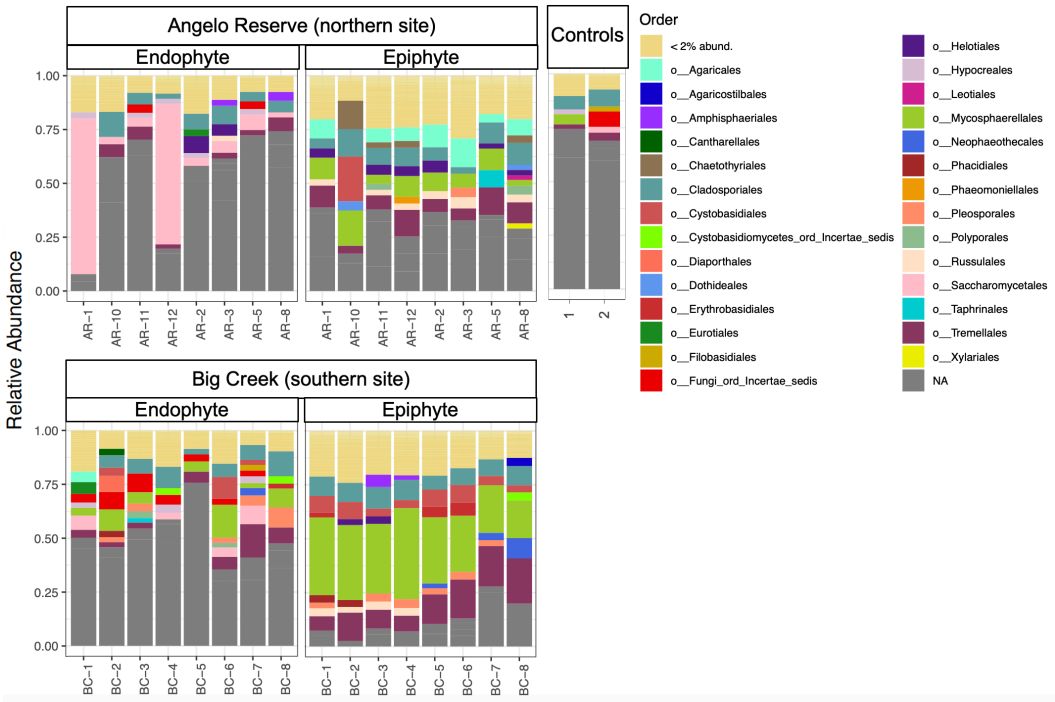


Figure 2: Bacterial 16S (A) and fungal ITS (B) relative abundance plots. Bars represent relative abundance of communities harvested at the Order level from leaves sampled at the Northern (Angelo Range Reserve) and Southern (Landels-Hill Big Creek) location for both endophytic and epiphytic samples (16S n = 4 trees/location; ITS n = 8 trees/location, coded with alphanumeric value corresponding to individual tree). NA represents unassigned ASV at specified level.

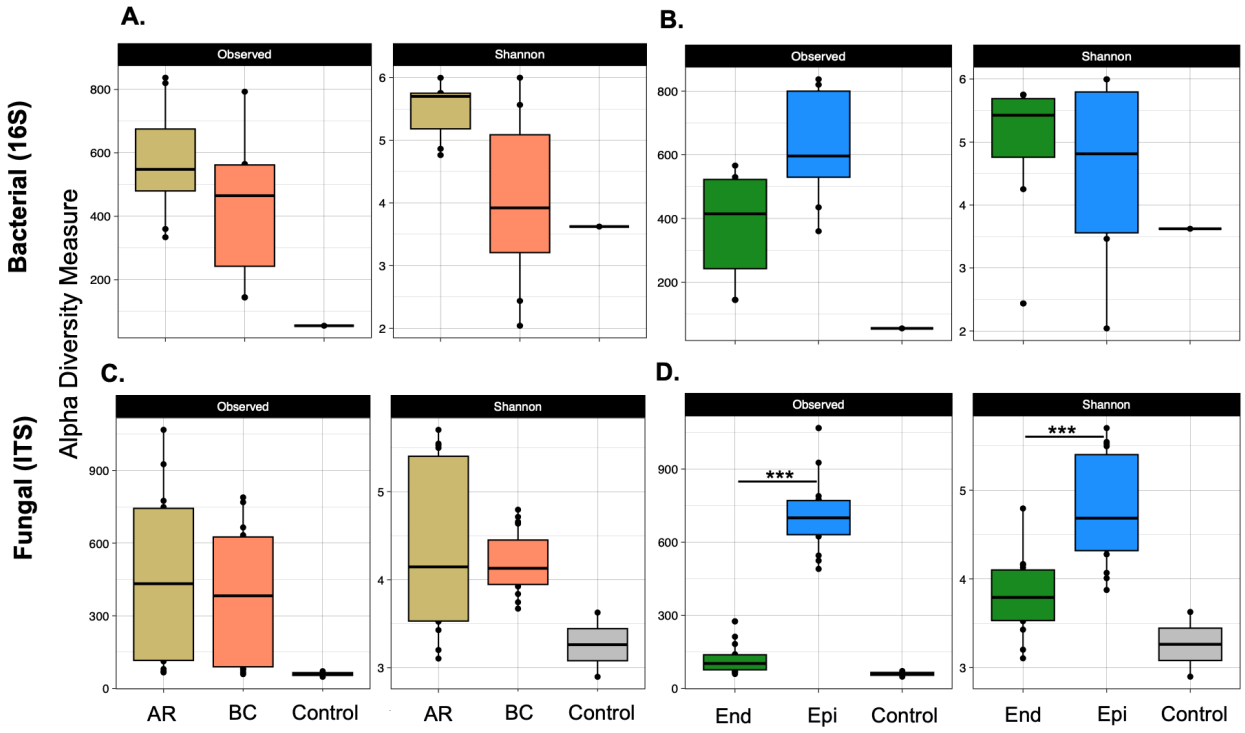


Figure 3: Alpha diversity plots comparing location and leaf surface communities. Observed and Shannon diversity index means for bacterial communities (n = 4 trees) by location (A) and leaf surface (B) and fungal communities (n = 8 trees) by location (C) and leaf surface (D). Kruskal Wallis Dunn Test post hoc Bonferroni corrected p-adj. *** $p < 0.001$. P-value between conditions and control not shown (control, n = 1-2).

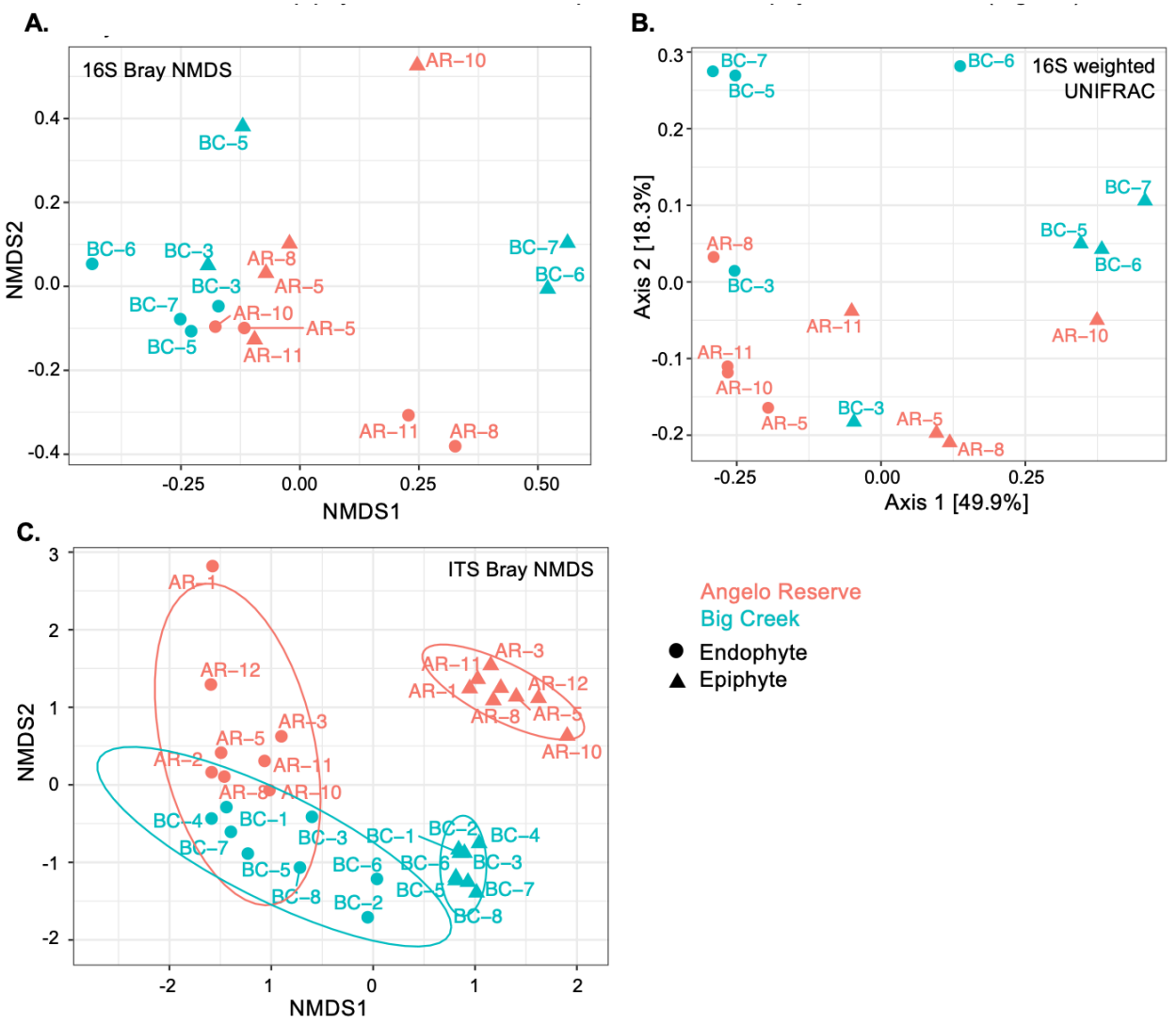


Figure 4: Beta diversity plots for bacterial and fungal comparing location and leaf surfaces. Microbial communities plotted comparing location and leaf surface by Bray NMDS in 16S bacterial (A) and ITS fungal (C). PCoA plots of bacterial communities based on weighted UNIFRAC distances (2000 ASVS out of 7851 total filtered reads) (B).

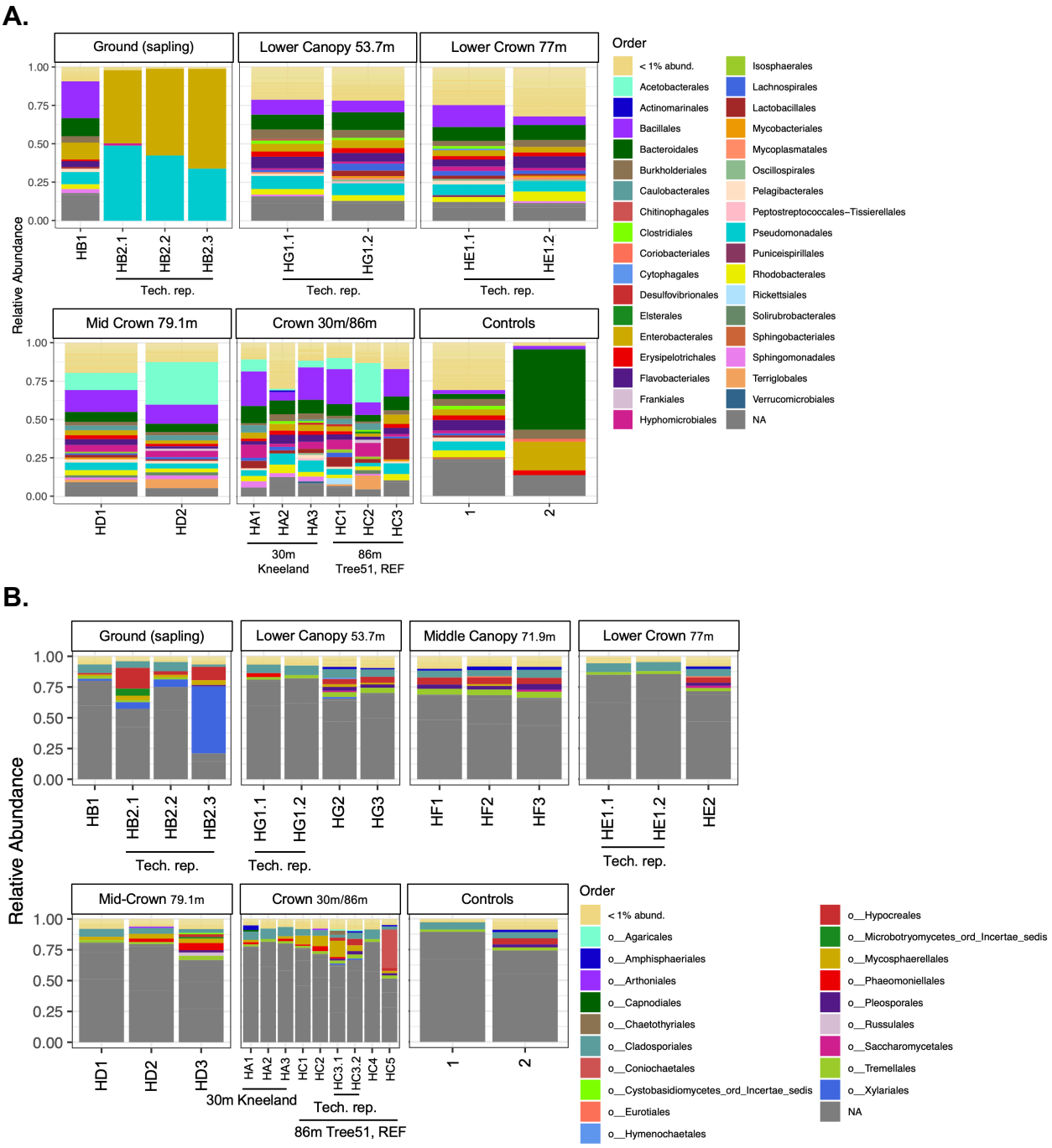


Figure 5: Bacterial 16S (A) and fungal ITS (B) relative abundance plots. Bars represent relative abundance of communities harvested at the Order level from leaves sampled along the vertical gradient of Tree 51 in the Redwood Experimental Forest (REF) (approx. 90m tall) and Kneeland tree (approx. 30m tall). Ground samples obtained from sapling near base of Tree 51. Technical replicates noted with (Tech. rep.) NA represents unassigned ASV at specified level.

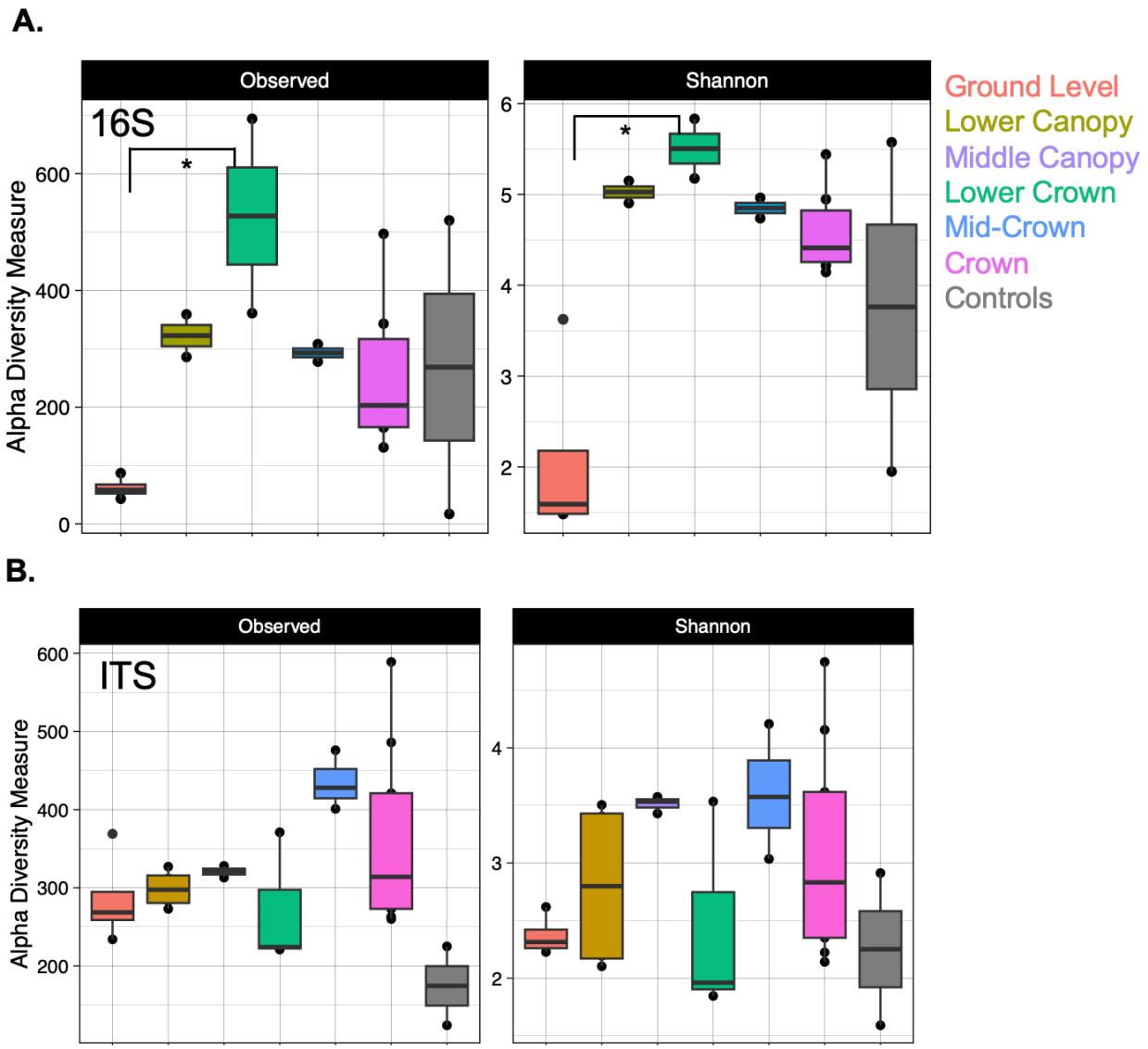


Figure 6: Alpha diversity plots comparing leaf communities along vertical tree gradient. Observed and Shannon diversity means in bacterial (A) and fungal communities (B). Kruskal Wallis Dunn Test post hoc Bonferroni corrected p-adj. *p <0.05.

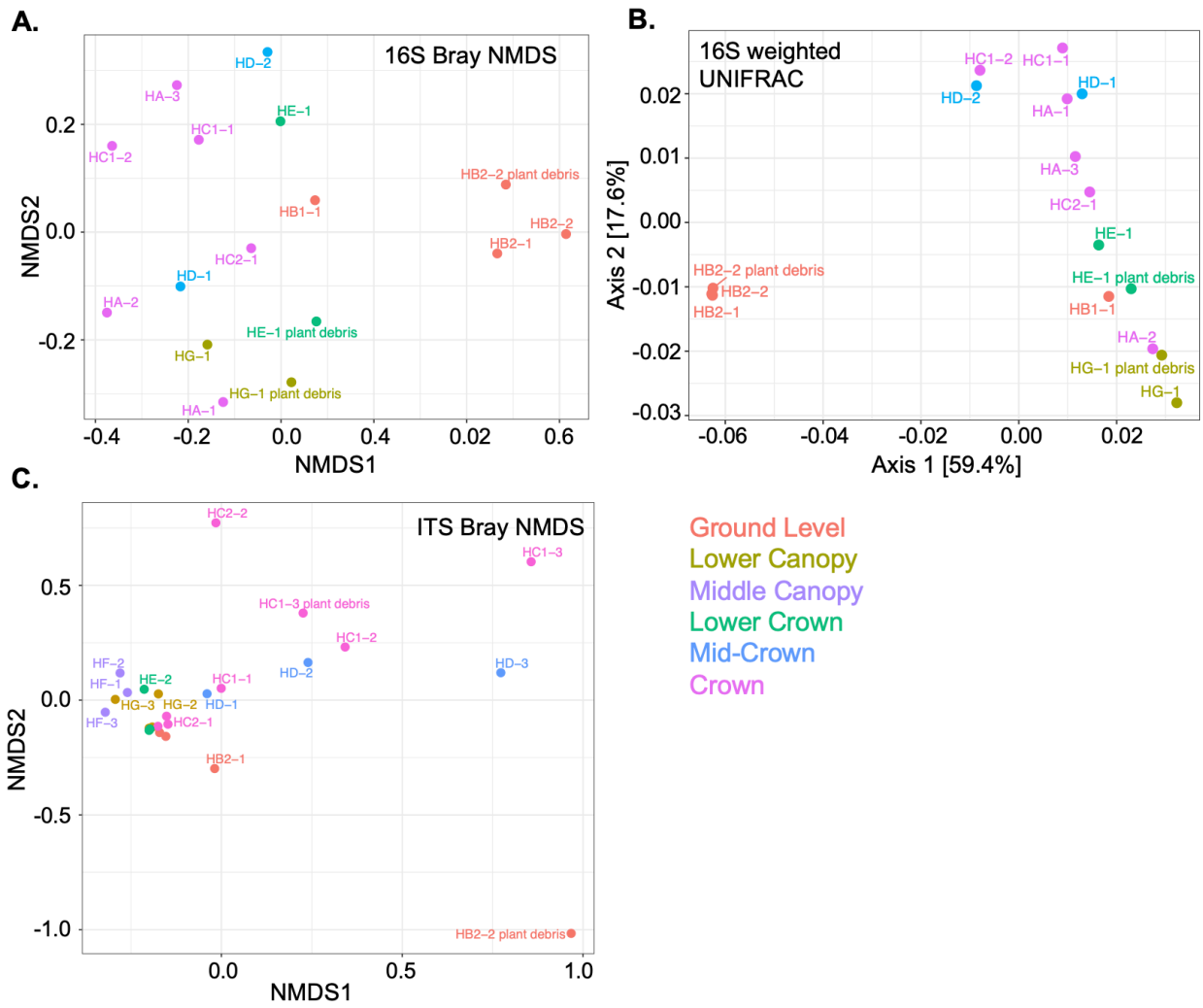


Figure 7: Beta diversity plots comparing leaf communities along vertical gradient. Microbial communities plotted by Bray NMDS in 16S bacterial (A) and ITS fungal (C). PCoA plots of bacterial communities based on weighted UNIFRAC distances (2000 ASVS out of 7900 total filtered reads) (B).

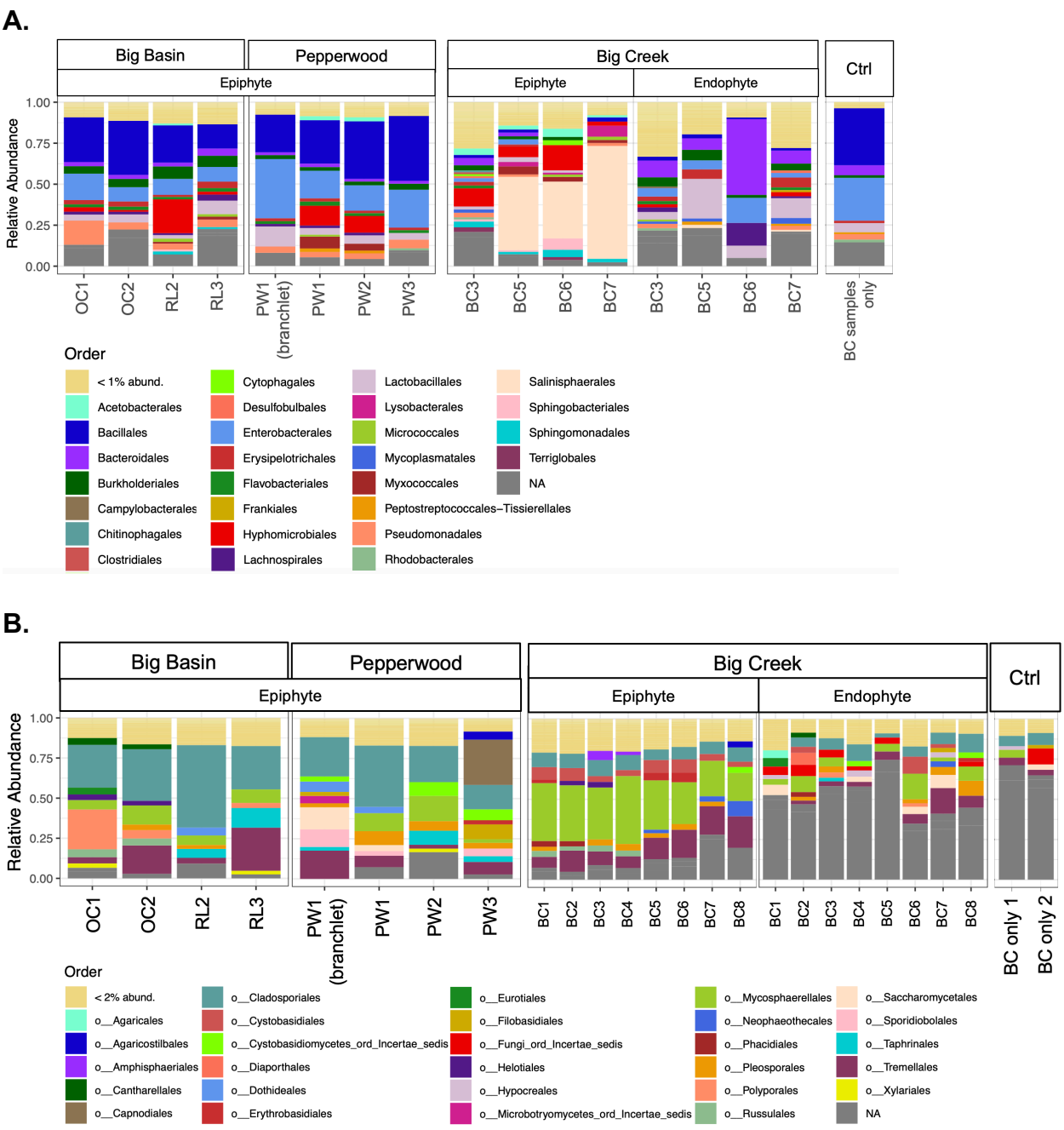


Figure 8: Bacterial 16S (A) and fungal ITS (B) relative abundance plots. Bars represent relative abundance of epiphytic and endophytic (at one location) communities harvested from locations with a major fire event since 2017 (n = 11 bacterial and n = 12 fungal, coded with alphanumeric value corresponding to individual tree; end = Endophyte, epi = Epiphyte). NA represents unassigned ASV at specified level.

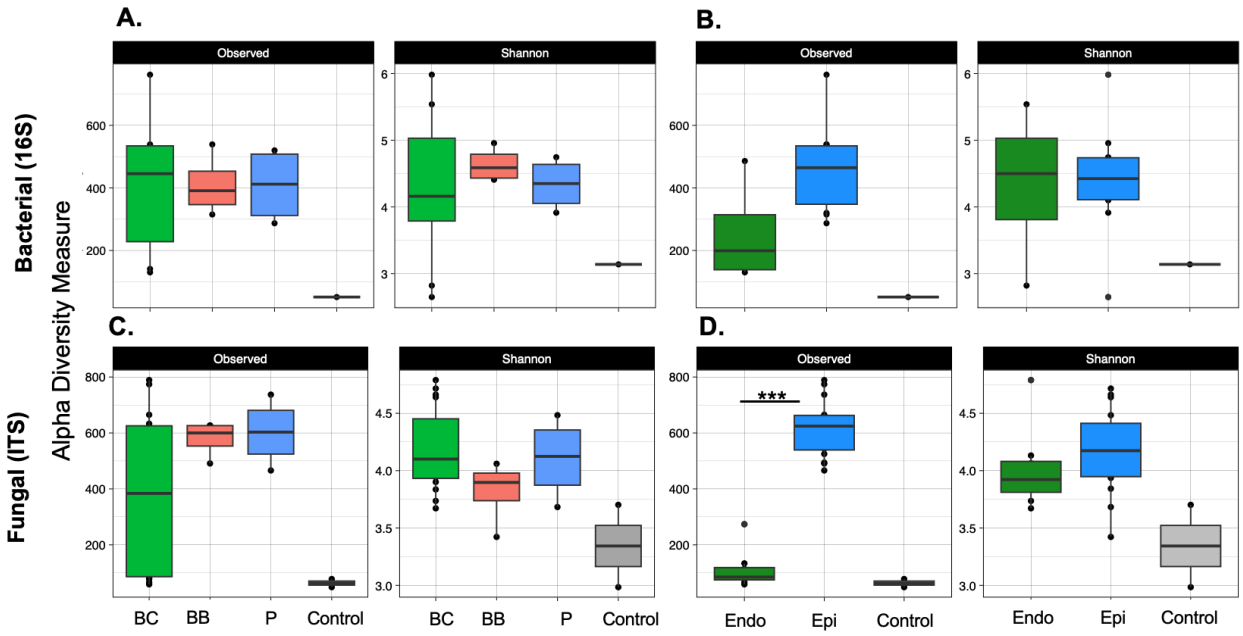


Figure 9: Alpha diversity plots comparing location and leaf surface communities of locations with fire event. Observed and Shannon diversity index means for bacterial communities (n = 11 trees) by location (A) and leaf surface (B) and fungal communities (n = 12 trees) by location (C) and leaf surface. Kruskal Wallis Dunn Test post hoc Bonferroni corrected p-adj. *** $p < 0.001$. P-value between conditions and control not shown (control, n = 1-2).

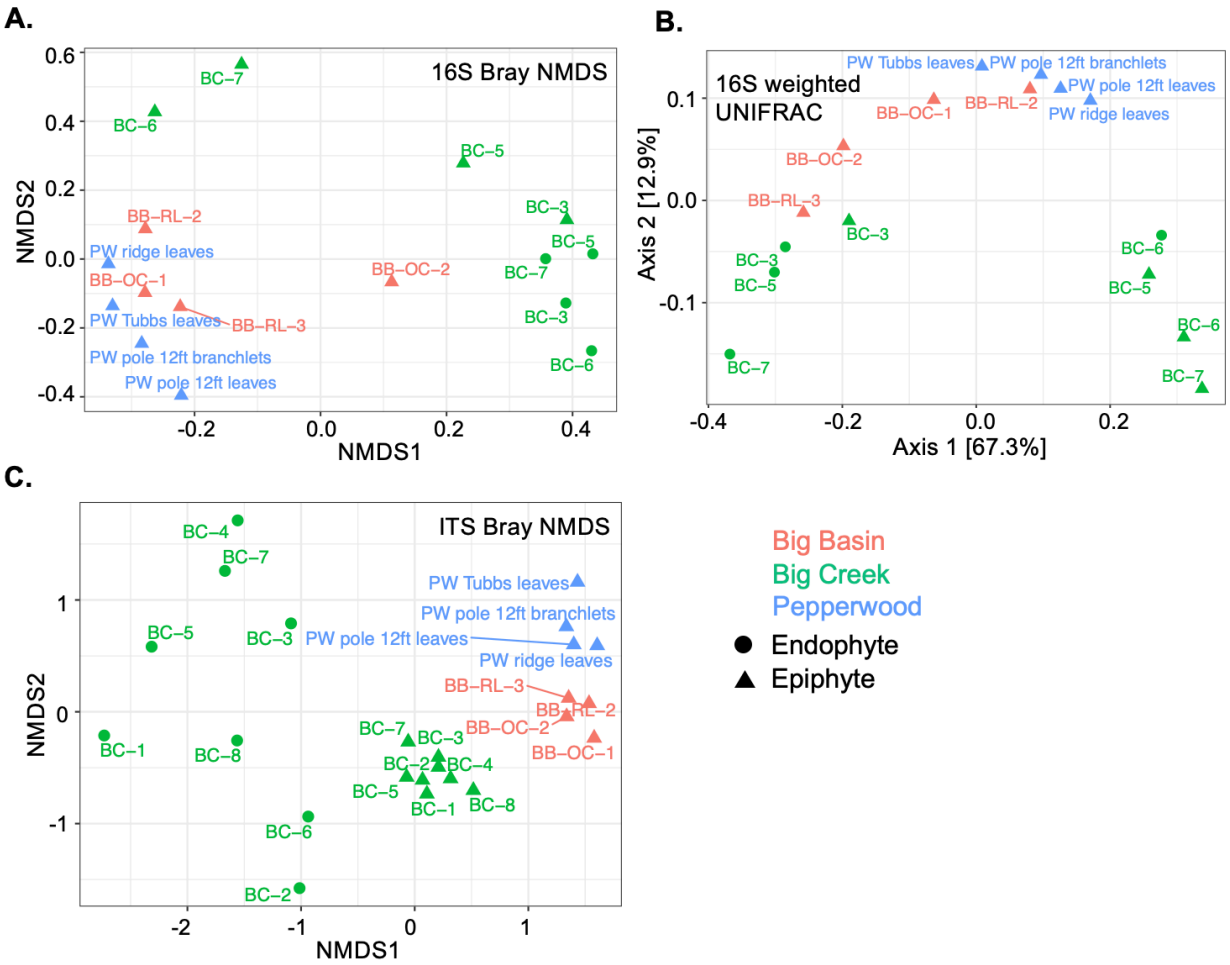
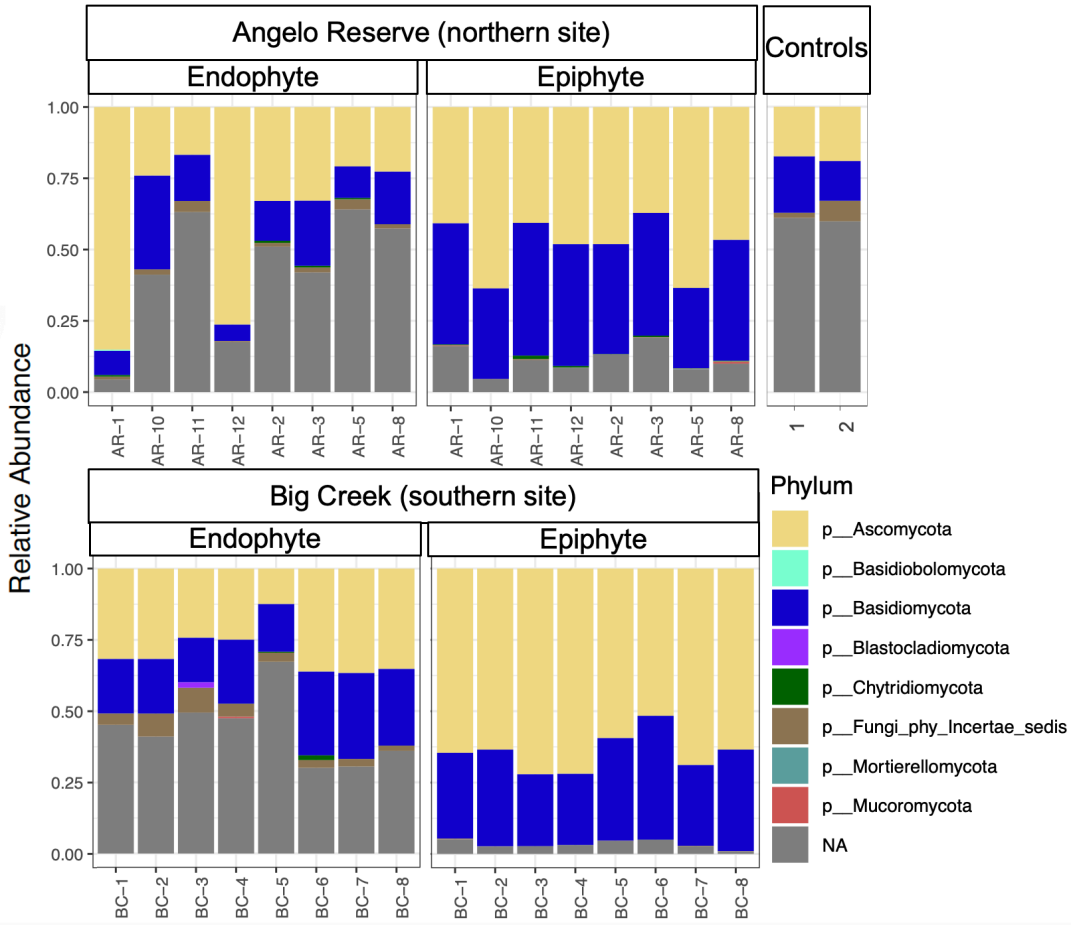
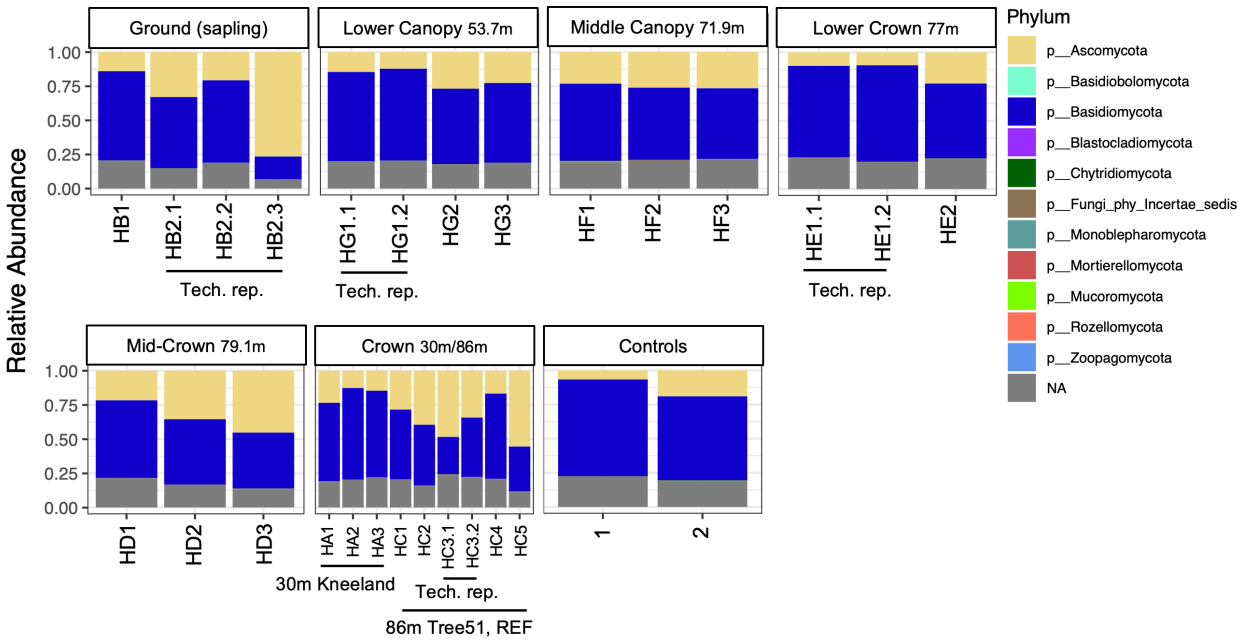


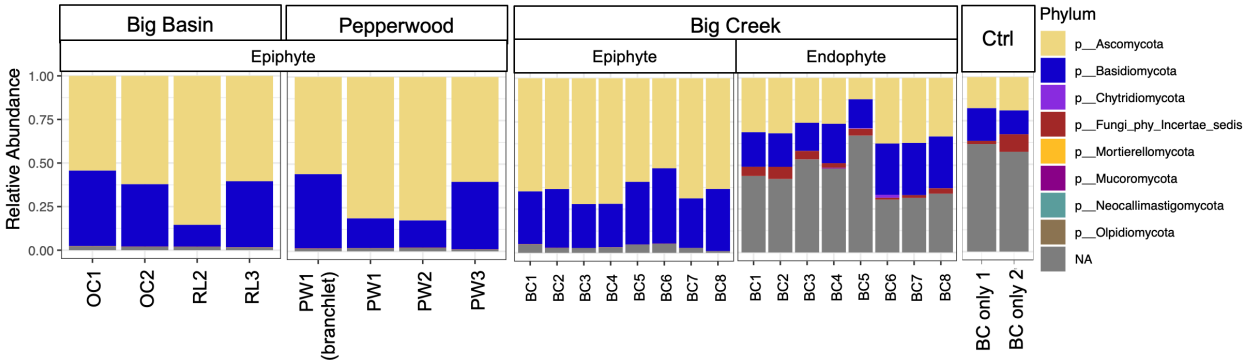
Figure 10: Beta diversity plots comparing leaf communities across fire event locations. Microbial communities plotted by Bray NMDS in 16S bacterial (A) and ITS fungal (C). PCoA plots of bacterial communities based on weighted UNIFRAC distances (1000 ASVS out of 8154 total filtered reads) (B).



Supplementary Figure 1: Relative abundance plots comparing locations and leaf surface communities at the phylum level. Individual trees are labeled alphanumerically corresponding to those sampled at the northern site, Angelo Reserve (AR) and southern site, Big Creek (BC). NA represents unassigned ASV at specified level.



Supplementary Figure 2: Relative abundance plots comparing leaves harvested along the height gradient of trees in Humboldt County, including an old growth tree, at the phylum level. Individual leaf samples from the same tree at different heights are labeled alphanumerically, unless noted as a separate tree. NA represents unassigned ASV at specified level.



Supplementary Figure 3: Relative abundance plots comparing epiphytic leaf surfaces from three fire-exposed locations at the phylum level. Individual trees are labeled alphanumerically corresponding to those sampled at Big Basin (BB), Pepperwood (P), and Big Creek (BC). NA represents unassigned ASV at specified level.

TABLES

Table 1: Sampled locations and their respective estimated annual rainfall, recent fire event (when present), and harvested leaf surface.

Sample location	Appx. Annual rainfall	Recent wildfire events	Epiphyte sample	Endophyte sample
Redwood Experimental Forest (REF)	193cm [1]			x
Angelo Coast Range Reserve	203cm [2]		x	x
Pepperwood Preserve	86.4cm [3]	Tubbs Fire 2017	x	
Big Basin Redwoods State Park	77.8cm (Santa Cruz) [4]	CZA lightening complex 2020	x	
Landels-Hill Big Creek Reserve	62-102cm [5]	CZA lightening complex 2020	x	x

[1] https://www.fs.usda.gov/research/psw/forestsandranges/locations/redwood
[2] https://ucnrs.org/reserves/angelo-coast-range-reserve/
[3] https://www.pepperwoodpreserve.org/about-us/ecology/
[4] https://www.ncei.noaa.gov/access/us-climate-normals/#dataset=normals-annualseasonal&timeframe=30&station=USC00048351
[5] https://ucnrs.org/reserves/landels-hill-big-creek-reserve/

Table 2: Sequencing read counts by three gradients: 1) north and south hydrological gradient, 2) vertically within tree crowns, and 3) fire exposed sites for bacterial 16S rRNA and ITS fungi. The Filtered reads column includes counts after removing chimeras and the ASVs (post-taxa) column includes reads after taxonomic assignments and decontamination (R package, decontam) and pruning.

	16S				ITS			
Gradient	**Sample counts**	**Raw reads**	**Filtered reads**	**ASVs (post-taxa)**	**Sample counts**	**Raw reads**	**Filtered reads**	**ASVs (post-taxa)**
North-South	4 epi-, 4 endophytes (4 trees)	2,336,750	1,313,944	702,778	8 epi-, 8 endophytes (8 trees)	2,756,736	2,117,285	2,114,688
Vertical	12 endophytes (3 trees)	2,255,152	1,833,187	464,891	21 endophytes (3 trees)	3,899,080	3,073,874	2,871,763
Fire	24 epiphytes (11 trees)	2,087,900	1,206,403	619,335	24 epiphytes (15 trees)	3,132,014	2,436,541	2,434,339

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