

1 **An *Enterobacteriaceae* bloom in aging animals is restrained by the gut microbiome**

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7

8 **Abstract**

9 The gut microbiome plays important roles in host function and health. Core microbiomes have
10 been described for different species, and imbalances in their composition, known as dysbiosis, are
11 associated with pathology. Changes in the gut microbiome and dysbiosis are common in aging,
12 possibly due to multi-tissue deterioration, which includes metabolic shifts, dysregulated immunity,
13 and disrupted epithelial barriers. However, the characteristics of these changes, as reported in
14 different studies, are varied and sometimes conflicting. Using clonal populations of *C. elegans* to
15 highlight trends shared among individuals, and employing NextGen sequencing, CFU counts and
16 fluorescent imaging to characterize age-dependent changes in worms raised in different microbial
17 environments, we identified an *Enterobacteriaceae* bloom as a common denominator in aging
18 animals. Experiments using *Enterobacter hormachei*, a representative commensal, suggested that
19 the *Enterobacteriaceae* bloom was facilitated by a decline in Sma/BMP immune signaling in aging
20 animals and demonstrated its detrimental potential for increasing susceptibility to infection.
21 However, such detrimental effects were context-dependent, mitigated by competition with
22 commensal communities, highlighting the latter as determinants of healthy versus unhealthy aging,
23 depending on their ability to restrain opportunistic pathogens.

24

25 **Introduction**

26 Aging is a process of multi-tissue deterioration, including muscular atrophy, neurodegeneration,
27 epithelial barrier disruption, immune dysregulation, and metabolic remodeling. Vulnerabilities and
28 pathologies associated with this deterioration directly impact lifespan. In the case of the intestine,
29 age-dependent impairments (immune, barrier, metabolic) further converge to alter the niche that

30 is home to a complex community of microbes, the gut microbiome. However, how such changes
31 affect the gut microbiome is not well understood.

32 The gut microbiome is increasingly appreciated for its contributions to host functions ¹⁻⁴ and
33 imbalances in its composition, or dysbiosis, are associated with pathology, in some cases (e.g.
34 obesity) in a causative role ^{5,6}. Age-dependent dysbiosis was described in flies, mice and humans
35 and was suggested to negatively impact both barrier functions and immune fitness ⁷⁻⁹. Studies in
36 human populations have shown that microbiomes of healthy octogenarians differed from those of
37 unhealthy individuals of similar age ¹⁰. Other studies characterized the trajectory of microbiome
38 changes through aging all the way to semi-supercentenarian (105-110 year old) ¹¹, offering further
39 insights into the relationship between age-dependent changes in microbiome composition and host
40 health. Importantly, transplanting microbiomes from young mice to old reduced markers of aging
41 and ameliorated health ^{12,13}, and a similar transfer in killifish increased lifespan ¹⁴, demonstrating
42 a causal role for age-dependent changes in microbiome composition in host aging. However, for
43 the most part, such studies could not identify common trends in age-dependent changes that may
44 offer points for intervention to ameliorate pathologies. Perhaps the sole exception is the
45 identification of increased abundance of *Proteobacteria* (now re-named *Pseudomonadota*) in
46 aging animals. Bacteria of this phylum are minor constituents of the vertebrate gut microbiome in
47 young individuals, but increase in abundance during aging ¹⁵⁻¹⁷. *Pseudomonadota* comprise a
48 larger part of the gut microbiome in invertebrates, and as seen in fruit flies further increase during
49 aging ⁷. Whether this bloom is a universal signature of the aging gut microbiome, and what its
50 significance may be, is yet unknown.

51 The nematode *Caenorhabditis elegans*, a useful model for aging research, is now gaining
52 momentum as a model for microbiome research, offering the advantage of working with
53 synchronized, initially germ-free, clonal populations, overcoming limitations of inter-individual
54 variation common to vertebrate models to better discern shared patterns in microbiome
55 composition ¹⁸. As a bacterivore, *C. elegans* ingests bacteria from its environment. While some
56 bacteria are digested as food, others persist, giving rise to a characteristic gut microbiome that is
57 surprisingly diverse, distinct from microbial communities in its respective environments, and
58 similar in worms isolated from different geographical locations ¹⁹⁻²¹. As in other organisms, gut
59 commensals were shown to provide diverse benefits to their host, including faster development
60 and resistance to pathogens ²⁰⁻²⁴. The power of *C. elegans* as a genetic model further enabled

61 identification of some of the genes, many of which are immune regulators²⁵⁻²⁷, that help control
62 commensals and their function and shape microbiome composition.

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

71

72 **Results**

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

74 Worms continuously developing and aging in natural-like microcosm environments and analyzed
75 by 16S next generation sequencing (NGS) showed gut microbiomes that were distinct from their
76 environment, as previously described¹⁹. The composition of these gut microbiomes changed as
77 worms aged, but independently of bacterial environmental availability, which remained relatively
78 constant during the experiment. (Fig. 1B, grazed soil). Most prominently, we observed an
79 expansion in gut *Enterobacteriaceae*, particularly in post-reproductive worms (post-gravid, day
80 five of adulthood). This increase could be due to ecological succession, shaped by interbacterial
81 interactions. Alternatively, it could be determined by age-dependent changes in the gut niche. To
82 distinguish between the two possibilities, we carried out microcosm experiments where worms of
83 advancing age were exposed to the complex microcosm community for a fixed amount of time
84 (Fig. 1A, C). While the initial soil microbiome in this experiment showed relatively higher
85 microbial diversity compared to the experiment described in Fig. 1B, the *Enterobacteriaceae*
86 expansion re-emerged, suggesting that this expansion was associated with age-dependent changes
87 in the intestinal niche, rather than with the time of exposure, and highlighting this bloom as a
88 potential hallmark of worm aging (Fig. 1C).

89 Bacterial diversity (alpha diversity) within the gut microbiome demonstrated an overall trend of
90 decline during aging, which was more pronounced in the fixed-colonization-time aging experiment
91 (Fig. 1E), but also seen in post-gravid worms in the continuous aging experiment (Fig. 1D).

92 Declines were observed in worm microbiome diversity both with respect to species richness and
93 evenness, represented by the Shannon Index, as well as with respect to phylogenetic diversity,
94 represented by the Faith Index (Fig. 1D, E). Additionally, worm gut microbiomes of different ages
95 differed from one another. Principal coordinate analysis (PCoA) based on weighted UNIFRAC
96 distances showed that in both experiments worms of a specific age harbored similar gut
97 microbiomes, which were distinct from worm microbiomes in other ages (Fig. 1F, G). This is in
98 agreement with previous studies of the gut microbiome in aging mice ²⁸. Together, these results
99 support a role for the age-modified intestinal niche in driving age-dependent changes in
100 microbiome composition, including a prominent expansion of *Enterobacteriaceae* as well as a
101 general decline in bacterial diversity.

102

103 **An *Enterobacteriaceae* expansion is a recurring theme in aging starting from different initial 104 conditions**

105 Microcosm experiments provide natural-like microbial diversity, and coupled with NGS, offer a
106 view free of culturing biases. However, to investigate the *Enterobacteriaceae* bloom in greater
107 detail we turned to defined communities of characterized gut commensals. Two communities were
108 used, each representing a slightly different paradigm: the publicly available CeMBio community,
109 which includes two *Enterobacteriaceae* strains, *Enterobacter hormachei* strain (CEent1) and
110 *Lelliottia amnigena* (JUB66), but is dominated by *Stenotrophomonas* and *Ochrobactrum*, ²⁹ and
111 SC20, which has a higher proportion of *Enterobacteriaceae* (eight out of 20 species), including
112 CEent1 (Table 1). We used a CEent1-dsRed derivative included in both communities to enable
113 imaging of gut colonization. In both, colonization with CEent1-dsRed increased with age, in line
114 with the *Enterobacteriaceae* bloom observed in natural-like microcosm experiments (Fig. 2A, C).
115 To examine whether the age-dependent bloom was specific to gut *Enterobacteriaceae* or also
116 involved increases in the abundance of other members of the gut microbiome, we used colony
117 forming unit (CFU) counts and quantitative (q)PCR to evaluate gut bacterial load. In worms aging
118 on CeMBio, an increase in total bacterial load (CFUs on non-selective LB) was observed, from
119 10^3 bacterial cells/worm at D0 (L4 larvae) to around 10^5 cells/worm at D12 of adulthood. However,
120 a steeper increase was observed in the *Enterobacteriaceae* load (CFUs on selective VRBG plates)
121 (Fig. 2B), from being barely detectable in D0 to about 5% of the total gut microbiome by old age
122 (~ 5000 cells/worm). A similar trend was observed in worms raised on the *Enterobacteriaceae*-rich

123 SC20 community, as demonstrated with qPCR using universal *Eubacteria* primers or
124 *Enterobacteriaceae*-specific primers and normalized to worm DNA (represented by actin genes).
125 This evaluation showed a much steeper increase in *Enterobacteriaceae* strains compared to the
126 increase in the total bacterial load (Fig. 2D). These results support the notion that an
127 *Enterobacteriaceae* bloom is a hallmark of aging, regardless of the initial conditions such as high
128 or low environmental diversity, or high or low initial proportion of *Enterobacteriaceae*. This
129 bloom involves a large increase in total bacterial load, but a proportionally larger increase in the
130 *Enterobacteriaceae* load per worm.

131

132 **An *Enterobacteriaceae* bloom in aging animals can have detrimental consequences**

133 Previous work showed that CEent1, serving as a representative of gut *Enterobacteriaceae*,
134 protected young animals from infection with the pathogen *Enterococcus faecalis*^{22,30}. However,
135 in worms disrupted for DBL-1/BMP immune signaling, gut abundance of CEent1 increased and
136 the otherwise beneficial commensal became an exacerbating factor in host health outcomes²⁵. We
137 thus used CEent1 to examine the functional significance of the age-dependent *Enterobacteriaceae*
138 bloom. Worms raised on CEent1 and shifted to *E. faecalis* at the end of larval development showed
139 higher pathogen resistance compared to worms raised on the *E. coli* control, as previously shown.
140 In contrast, worms raised on CEent1 to middle age before shifting to *E. faecalis* (day four of
141 adulthood, at which stage worms are well colonized), showed significantly lower pathogen
142 resistance compared to worms raised on *E. coli* controls (Fig. 3A, B). Thus, the *Enterobacteriaceae*
143 bloom has the potential to have detrimental consequences in aging worms.

144

145 **Changes in the intestinal niche associated with an age-dependent decline in DBL-1/BMP 146 signaling may underlie the *Enterobacteriaceae* bloom**

147 What may be the cause for the *Enterobacteriaceae* bloom? Experiments in microcosm
148 environments and with defined communities showed that environmental availability was not a
149 likely cause (Fig. 1B, 2A-D). Ecological succession, driven by accumulating effects of interactions
150 over time, also did not appear to contribute to the expansion (Fig. 1C). This was further supported
151 by a comparison of CEent1-dsRed colonization in worms raised continuously on CEent1-dsRed
152 monocultures versus worms shifted to CEent1-dsRed in different ages for a fixed duration of two
153 days, which showed comparable age-dependent increases in *Enterobacteriaceae* abundance (Fig.

154 4A). We further examined whether age-dependent decline in bacterial uptake may play a role in
155 causing the bloom. To this end, we compared CEent1-dsRed colonization, as part of the SC20
156 community, in wildtype worms, which show an age-dependent decline in pharyngeal pumping
157 (and thus bacterial uptake), and *eat-2* mutants, which lack a pharyngeal receptor for the
158 neurotransmitter acetylcholine resulting in slow pumping rate, which does not change considerably
159 during aging (Fig. 4B inset). In both strains we observed a similar course of age-dependent
160 increases in CEent1-dsRed colonization (Figure 4B), indicating that age-dependent changes in
161 bacterial uptake likely did not contribute to the *Enterobacteriaceae* bloom.

162 DBL-1/BMP signaling is a conserved regulator of development, body size and immunity ³¹.
163 Previous work at the lab identified a role for DBL-1-dependent immune regulation in shaping the
164 worm gut microbiome, particularly affecting *Enterobacter* strains, which bloomed when genes
165 encoding different components of this pathway were disrupted ²⁵. Gene expression data in
166 Wormbase (<https://wormbase.org>) suggested that expression of the pathway's components may
167 decline at the end of larval development. To examine whether this indicated an age-dependent
168 decline in DBL-1 signaling and downstream gene expression, which might affect the gut
169 microbiome, we used a transgenic worm strain expressing GFP from the *spp-9* promoter,
170 previously shown to be negatively regulated by DBL-1 signaling ³². Fluorescent imaging
171 demonstrated age-dependent increase in the expression of the GFP reporter, indicating a decline
172 in DBL-1 signaling in aging worms (Fig. 5A). In line with this, the effects of either disruption or
173 over-expression of the *dbl-1* ligand gene diminished with age. Reduced effects of *dbl-1* disruption
174 were also observed in worm colonization with CEent1, which in middle-aged mutants was
175 comparable to that seen in wildtype animals, indicating a decline in DBL-1 signaling and its
176 involvement in controlling gut *Enterobacteriaceae* abundance during early aging (Fig. 5B).
177 Further support for a decline in DBL-1 control of gut bacteria was provided by experiments with
178 *sma-4(syb2546)* mutants, which carry a gain-of-function (gof) mutation in DBL-1's transcriptional
179 mediator, exhibiting a 20% longer body length compared to wildtype animals (Fig. 5C inset).
180 These mutants showed lower CEent1-dsRed colonization compared to wildtype animals in early
181 days of adulthood, up to day five (blue box compared to dotted line in Fig. 5C), signifying a delay
182 in the CEent1 bloom. The ability to delay the CEent1 bloom had beneficial consequences, as *sma-4(gof)*
183 mutants were partially protected from CEent1's detrimental effects on infection resistance
184 in day four adults (Fig. 5D). Together, these results suggest that an age-dependent decline in DBL-

185 1 signaling alters the intestinal niche, permitting preferential accumulation of *Enterobacteriaceae*,
186 which can be detrimental. Boosting DBL-1 signaling may mitigate the bloom and its
187 consequences, but only partially.

188

189 **Commensal communities can effectively mitigate the detrimental consequences of the**
190 ***Enterobacteriaceae* bloom**

191 The ability of the *sma-4(gof)* mutation to partially mitigate the detrimental effects of CEent1
192 expansion suggested that protection from an *Enterobacteriaceae* bloom is possible. Considering
193 that the decline in DBL-1 signaling also increased abundance of non-*Enterobacteriaceae* bacteria
194 (Fig. 2), we examined whether other bacteria could compete with CEent1 and help prevent its
195 detrimental effects. Recent work identified members of the genus *Pantoea* as common worm
196 commensals effectively colonizing the gut and capable of competing with an invading pathogen
197 ²³. Wildtype worms raised on a community consisting of three such *Pantoea* commensals in
198 addition to CEent1-dsRed (in equal parts) and shifted to *E. faecalis* in middle-age were as resistant
199 as worms raised on *E. coli* alone, and significantly more resistant than worms raised on a similar
200 inoculum of CEent1-dsRed mixed with *E. coli* (Fig. 6A). While mortality on *E. faecalis* plates was
201 attributed to the pathogen, 96.7% of the worms raised on the CEent1-dsRed/*E. coli* mix, which
202 died in any of the days of the infection assay, were heavily colonized with CEent1-dsRed (Fig. 6A
203 inset), indicating proliferation alongside *E. faecalis*. In contrast, only 62.5% of the worms who
204 were initially raised on the CEent1-dsRed/*Pantoea* mix were colonized, together indicating that
205 the *Pantoea* community was able to mitigate CEent1 proliferation in some of the worms and to
206 reduce mortality in the population. To examine whether mitigating the detrimental effects of
207 CEent1 proliferation was unique to *Pantoea*, worms were raised on a subset of seven members of
208 CeMBio (see Methods), with or without BIGb393 (one of the protective *Pantoea* strains, which is
209 also a member of CeMBio) and with an excess of CEent1-dsRed (50% of total) and shifted at
210 middle age to *E. faecalis*. Raising worms on the CeMBio subsets, with or without BIGb0303,
211 conferred significantly higher resistance to infection than in worms raised on CEent1-dsRed alone
212 (Fig. 6B). Again, fewer of the dead worms were colonized with CEent1-dsRed among those raised
213 on CeMBio/CEent1-dsRed (51.4%), compared to those raised on CEent1-dsRed alone (84.6%).
214 These experiments demonstrate that over proliferation of *Enterobacteriaceae* and its detrimental
215 consequences in aging worms can be mitigated with more than one combination of gut

216 commensals. Lastly, in the context of such a community, CEent1, or other *Enterobacteriaceae*,
217 did not compromise the lifespan of their host, as worms grown on CeMBio with or without its
218 *Enterobacteriaceae* members (CEent1 and JUB66) had a comparable lifespan (Fig. 6C).

219 **Discussion**

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

234 It is accepted that aging is accompanied by gut dysbiosis ^{7-9,14,33}. Human studies have documented
235 diverse changes in gut microbiome composition. Among those, increased abundance of
236 *Proteobacteria/Pseudomonadota*, and specifically of *Enterobacteriaceae*, is a recurring theme
237 ^{17,34}. In line with this, our results show a replicable age-associated expansion of
238 *Enterobacteriaceae*, suggesting that it may be an evolutionarily conserved signature of aging.
239 What causes this bloom is not clear. A study in fruit flies describes gut dysbiosis characterized by
240 a biphasic change in the microbiome of aging flies, in which a midlife bloom of γ -*proteobacteria*
241 led to intestinal barrier dysfunction, and a subsequent increase in α -*proteobacteria* ⁷. Whereas
242 barrier dysfunction was suggested as the cause for gut dysbiosis ³⁵, what initiated the γ -
243 *proteobacteria* bloom was not clear. The *Enterobacteriaceae* bloom we observed in worms may
244 be analogous to the initial phase of dysbiosis in flies. In worms, initiation of the
245 *Enterobacteriaceae* bloom was associated with a decline in DBL-1/BMP signaling. DBL-1

246 signaling was previously shown to play a central role in controlling gut *Enterobacteriaceae* ²⁵.
247 Thus, its age-dependent decline may change the host intestinal niche making it more permissible
248 for *Enterobacteriaceae* expansion. Supporting a causal role for DBL-1 signaling in the
249 *Enterobacteriaceae* bloom, gain-of-function mutants for the SMA-4 BMP mediator showed a
250 delay in *Enterobacter* colonization as well as attenuated infection susceptibility. These results
251 suggest that decline of immune signaling during aging is an important factor in initiating dysbiosis.
252 However, at least in the case of SMA-4, revamping the immune pathway to mitigate the
253 detrimental effects of the bloom was only partially successful, suggesting that additional changes
254 in the gut niche may take place to promote the *Enterobacteriaceae* bloom, and further limiting the
255 potential of DBL-1/BMP reactivation in aging worms as an intervention to alleviate gut dysbiosis.

256 *Enterobacteriaceae* blooms are associated with increased susceptibility to infection ³⁶⁻³⁹. In
257 agreement with this, expansion of *E. hormachei* CEent1 in aging worms compromised infection
258 resistance and survival. Continued gut accumulation of CEent1 following a shift to pathogen plates
259 further suggests that in old worms CEent1 (and perhaps additional *Enterobacteriaceae* members)
260 was an opportunistic pathogen, or a pathobiont. Previous results support the notion that CEent1
261 was a pathobiont, as worms growing on CEent1 alone have a shorter lifespan compared to those
262 raised on an *E. coli* diet ²². However, in the context of a community (CeMBio), inclusion or
263 removal of CEent1 did not have any effect on lifespan, supporting the importance of a diverse
264 microbiome in keeping pathobionts in check as the host ages.

265 The genetic tractability and short lifespan of *C. elegans* has made it a useful model for aging
266 research. Its more recent establishment as a model for microbiome research adds to that the
267 advantages of longitudinal microbiome analysis in clonal host populations, while having greater
268 control over bacterial availability, to facilitate studies of host-microbiome interactions during
269 aging. Using this model, we identified what seems to be an evolutionary conserved signature of
270 dysbiosis in aging animals and have begun to dissect its causes as well as its consequences. As
271 often seen in different scenarios of gut dysbiosis, the *Enterobacteriaceae* bloom that we identified
272 is associated with pathology. However, this pathology can be circumvented by manipulating the
273 gut microbiome using various commensal communities. Thus, while an *Enterobacteriaceae* bloom
274 seems to be an inevitable consequence of aging, its extent and outcomes can be restrained by other

275 members of the gut microbiome. What differentiates between communities that can or cannot
276 achieve this remains to be seen.

277 **Materials and methods**

278 **Worm strains**

279 *C. elegans* strains included wildtype N2, *dbl-1(nk3)*, and the *dbl-1* overexpressing strain BW1940,
280 obtained from the *Caenorhabditis* Genome Center (CGC); transgenic strains expressing GFP from
281 the *spp-9* promoter: TLG690, texIs127[*spp-9p::GFP*], TLG707, texIs127;*dbl-1(nk3)* and TLG708,
282 texIs127;texIs100[*dbl-1p::GFP::dbl-1*], were gratefully received from Tina Gumienny^{32,40}; gain
283 of function (gof) *sma-4(syb2546)* mutants were gratefully received from Cathy Savage-Dunn; and
284 *eat-2(ad1116)* mutants were gratefully received from Andrew Dillin.

285 **Bacterial strains and communities**

286 *Escherichia coli* strain OP50 and the Gram-positive pathogen, *Enterococcus faecalis* strain V583
287 were obtained from the CGC. Two defined communities of worm gut commensals were used:
288 CeMBio, with twelve strains, represents the worm core gut microbiome²⁹ and SC20, a subset of
289 twenty strains of the previously described SC1²⁵, with eight species out of the 20 of the
290 *Enterobacteriaceae* family (Table 1). In addition, a subset of CeMBio strains was used in
291 experiments testing effects on susceptibility to *Enterococcus faecalis* infection, including only
292 strains that are sensitive to gentamycin, which is used in *E. faecalis* plates, to prevent enrichment
293 of gut commensals through the environment. Additional commensals, of the genus *Pantoea*, family
294 *Erwiniaceae* (a recent splinter off *Enterobacteriaceae*), included BIGB0393 (also in CeMBio) and
295 the recently characterized *Pantoea cypripedii* strains V8 and T16²³.

296 Bacterial communities were prepared for experiments by growing individual strains in LB at 28°C
297 for two days, adjusting cultures to 1 OD, concentrating 10-fold and mixing equal volumes from
298 each culture. 100-200 µL aliquots of the mix were plated on either minimal nematode growth
299 medium (NGM) or on peptone-free medium (PFM), which further limits bacterial growth²⁹, as
300 described, and air-dried for 2 to 12 hours prior to the addition of worms.

301 **Construction of fluorescently-tagged *Enterobacter hormaechei* CEent1-dsRed**

302 *E. hormaechei* CEent1, previously misidentified as *E. cloacae*²², is a member in both the CeMBio
303 and SC20 communities. The construction of its dsRed-expressing derivative was achieved by
304 integrating the *dsRed* gene into the functionally neutral *attTn7* site in the CEent1 genome using

305 the site-specific *Tn7*-mini transposon system⁴¹ (Supplementary Fig. 1). Transposon insertion was
306 achieved through triparental mating with a donor strain and a transposase-expressing helper strain,
307 both based on the DAP-auxotrophic and *pir1*-positive *E. coli* strain BW29427, containing the
308 conjugative RP4 mating system as a chromosomal insert. This strain, as well as other *E. coli* strains
309 and plasmids used in constructing the fluorescently-tagged strains, were gratefully received from
310 the Goodrich-Blair lab, University of Tennessee Knoxville. Briefly, the original *GFP* in the mini-
311 transposon, carried on plasmid pURR25 (Supplementary Fig. 1), was replaced with *dsRed* by
312 digesting the plasmid with BseRI and NheI to cleave out the *GFP* coding sequence⁴², amplifying
313 the *dsRed* gene from pBK-miniTn7-ΩGm-DsRed⁴³ using primers 5'-TAC GTG CAA GCA GAT
314 TAC GG-3' and 5'-ATC CAG TGA TTT TCT CCAT-3,' and ligating the amplified *dsRed*
315 to the linearized pURR25 vector. The modified pURR25 plasmid, carrying the *pir1*-dependent
316 *oriR6K*, as well as *dsRed* and the antibiotic resistance genes *KanR* and *StrR*, was re-introduced by
317 electroporation into its original host strain, constituting the donor strain. The transposase plasmid,
318 pUX-BF13, in the helper strain, also included the *pir1*-dependent *oriR6K* and *AmpR* antibiotic
319 resistance, along with the *Tn7*-transposase⁴⁴.

320 Recipient strain CEent1 (*pir1*-negative), donor, and helper strains were each cultured until they
321 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.
322 The mixture was spread on LB DAP plates for an additional 24-hour incubation to promote
323 conjugation. Bacteria subsequently underwent several rounds of re-streaking on Kan⁺/DAP⁻ plates
324 to select for integrant CEent1 cells and to dilute-out the *pir1*-negative plasmids which cannot be
325 replicated in CEent1 (Supplementary Fig. 1). Integrant clones were verified as CEent1 by
326 sequencing a 200bp fragment of the CEent1 gene for *gyrB* using the primers 5'-GCA AGC AGG
327 AAC AGT ACA TT-3' and 5'-TCG GCT GAT AAA TCA GCT CTT TC-3'.

328 **Microcosm experiments**

329 Compost microcosms harboring diverse microbial communities were prepared from local soil
330 composted with produce for up to two weeks essentially as previously described^{19,45}. Briefly, local
331 soils were supplemented with banana peels or chopped apple, composted soils were split into two
332 parts: one part (6 gr in a glass vial) autoclaved to eliminate native nematodes and the other (10 gr
333 soil) suspended in M9 buffer to obtain a microbial extract which was concentrated and added to
334 the autoclaved samples to reconstitute the original microbial community.

335 In continuous aging experiments, synchronized populations of germ-free L1 worms were raised at
336 20°C in separate vials containing the same compost and harvested at advancing ages up to day five
337 of adulthood (D5) (Fig. 1A). The final time point was determined by the need to distinguish
338 between the original cohort (post-gravid at D5) and progeny (mid-stage gravids), which could not
339 be achieved in subsequent time points. In experiments with fixed time colonization, worms were
340 raised on live *E. coli* until the L4 stage to ensure proper development, then transferred to
341 kanamycin-killed *E. coli*⁴⁶ from which worms were further transferred at advancing ages to
342 microcosm environments for two days before harvesting for analysis. For the earliest time point
343 (gravids, day zero of adulthood), worms were raised on live *E. coli* from L1 to the L4, then shifted
344 to dead *E. coli* for 4 hours to minimize carry over of live *E. coli*, before transferring to microcosms.
345 Worm harvesting was carried out using a Baermann funnel as described⁴⁵.
346 Soil samples (1g) were taken from microcosms of the same compost batch used to grow worms
347 (“soil”), or from the same microcosm from which worms were harvested (“grazed soil”).

348 **Experiments with defined bacterial communities**

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

354 **DNA extraction**

355 Worms harvested in microcosm experiments (100-500 per group) were extensively washed,
356 surface-sterilized by letting them crawl for an hour on plates with 100 µg/mL gentamycin and
357 used for DNA extraction with the QIAGEN PowerSoil DNA isolation kit (Cat. #12888) as
358 previously described²⁵.

359 Worms harvested from plates with defined communities (100-150 per group) were washed three
360 times with M9, paralyzed with 25mM levamisole (Acros Organics) to close their intestine, and
361 surface-sterilized with 2% bleach in M9, as described⁴⁵. DNA was extracted using the DNeasy
362 PowerSoil Pro Kit (Qiagen Cat. # 47016) according to manufacturer instructions, with the
363 following modification: to break open worms prior to the first step of the protocol, worms were
364 incubated in the kit’s buffer for 10 minutes at 60°C, then crushed with added zirconium beads
365 using a PowerLyzer (2000 RPM 2 x 30 seconds).

366 **16S Next Generation Sequencing (NGS) and analysis**

367 DNA samples from worms and their respective environments were used to generate sequencing
368 libraries of the 16S V4 region. Libraries from microcosms experiments were prepared using
369 tailed primers, as previously described ¹⁹, and sent for 150 paired-end sequencing to the UC
370 Davis sequencing facility. Analysis of bacterial 16S amplicon data was carried out using the
371 QIIME2 software pipeline ⁴⁷. Sequence reads were demultiplexed and filtered for quality control,
372 with 85.6% of all reads passing quality filtering, providing an average read of 121,053 reads per
373 sample. Sequences were aligned and clustered into operational taxonomic units (OTU) based on
374 the closed reference OTU picking algorithm using the QIIME2 implementation of UCLUST ⁴⁸,
375 and the taxonomy of each OTU was assigned based on 99% similarity to reference sequences
376 based on Greengenes release 13_8. Prior to diversity analysis, all communities were rarefied to
377 116,543 sequences per sample. Shannon's diversity and Faith's phylogenetic diversity were
378 calculated to assess *alpha* diversity of soil and worms gut microbiotas ^{49,50}. Shannon diversity is
379 a composite measure of both richness and evenness, while Faith method takes the phylogenetic
380 distance of species into account. Weighted UniFrac distances were calculated to assess *beta*
381 diversity and used in principal coordinates analysis (PCoA). Raw data and metadata can be
382 accessed at <https://www.ncbi.nlm.nih.gov/sra> with accession no.: PRJNA982115.

383 **Fluorescence imaging**

384 For each time point examined, 30-40 worms were picked off CEent1-dsRed, or CEent3-GFP
385 containing plates (with the single strain, or with the fluorescent strains as part of a community),
386 washed with M9, paralyzed with 10 mM Levamisole, and mounted on a slide with a 2-4%
387 agarose pad. Imaging was performed with a Leica MZ16F equipped with a QImaging
388 MicroPublisher 5.0 camera. Quantification of fluorescent signal was conducted using the Fiji
389 plugin within the ImageJ package v2.10/1.53c or 1.53f51 ⁵¹. Integrated Density values were
390 measured per worm after subtracting background mean gray values and autofluorescence and
391 normalized for worm area.

392 **Quantifying bacterial load by colony forming unit (CFU) counting.**

393 For each time point examined 10-15 worms were washed three times in M9-T (M9/0.0125%
394 TritonX-100), paralyzed with 25 mM Levamisole, and surface sterilized by a three-minute
395 incubation in 2% bleach ²⁹. After two washes of 1mL of PBS-T (PBS/0.0125% Triton-X 100),
396 worms were collected in a final volume of 100 μ L. Samples of final wash were plated and

397 incubated at 28°C for 2 days to verify removal of external bacteria. To release bacteria from the
398 worm gut, worms were crushed with 10-15 zirconium beads in 100µL of PBS in a PowerLyzer at
399 4000 RPM for 30 to 45 seconds. Released bacteria were diluted and plated either on non-
400 selective LB agar plates (for all bacteria) or *Enterobacteriaceae*-selective VRBG plates. CFUs
401 were counted after 1-2 days of incubation at 28°C. CFUs for *E. hormaechei* and *Lelliottia*
402 *amnigena* could be distinguished on VRBG plates based on morphology.

403 **Quantifying bacterial load via qPCR**

404 Relative bacterial load was measured with real-time quantitative PCR using the eubacterial 16S
405 rDNA primers 806f (5'-AGATACCCCGGTAGTCC-3') and 895r (5'-
406 CYGYACTCCCCAGGYG-3'), and the *Enterobacteriaceae*-specific 16S specific primers
407 Ent_MB_F (5'-ACCTGAGCGTCAGTCTTGTC-3') and R (5'-
408 GTAGCGGTGAAATGCGTAGAGA-3')²⁵. qPCR was performed using Bio-Rad SsoAdvanced
409 Universal SYBR Green qPCR Supermix and an Applied Biosystems StepOne Plus real-time
410 PCR system. Cycling (eubacterial primers): 95°C for 5 min, 45 x [95°C for 15 sec, 60°C for 30
411 sec, 72°C for 15 sec], 72°C for 5 min; and for the *Enterobacteriaceae* specific primers: 95°C for
412 5 minutes, 40 x [95°C for 15 sec, 60°C for 30 sec], 72°C for 5 minutes.

413 Ct values for bacterial 16S were normalized to worm material by subtracting Ct values obtained
414 for worm actin using the pan-actin primers^{52,53}.

415 **Survival assays**

416 For infection resistance experiments, synchronized worm populations were raised from L1 on PFM
417 plates with *E. coli* OP50, CEent1, or designated communities, and shifted, at L4, or at day 4 of
418 adulthood, to *E. faecalis* plates prepared with Brain Heart Infusion Agar containing 25 µg/µL
419 gentamicin and seeded with bacteria a day before the transfer of worms. Assays were carried out
420 at 25°C and dead or live worms were counted every day³⁰. For lifespan assays, worms were raised,
421 on designated strains or communities in PFM plates at 20°C and scored daily for survival
422 beginning at L4 (t₀).

423 **Statistical analyses**

424 Statistical tests were conducted in R (v 3.6.3). Survival curves were statistically compared using
425 Kaplan-Meier analysis and log-rank tests using the survdiff R package⁵⁴ and all graphs were
426 created with the ggplot R package⁵⁵.

427 **Table 1**

428 **SC20 members**

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

429

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437 **Contributions**

438 R.C. M.B. and M.S. conceived the project, and M.S. supervised it; M.B. conducted microcosm
439 experiments which were analyzed by D.K. R.C., R.B., B.P., D.M., E.D. and J.C. carried out

440 experiments and analyses. V.N. generated the CEent1-dsRed strain. R.C. and M.S. compiled the
441 results and wrote the manuscript.

442 **Ethics declarations**

443 The authors declare that the research was conducted in the absence of any commercial or financial
444 relationships that could be construed as a potential conflict of interest.

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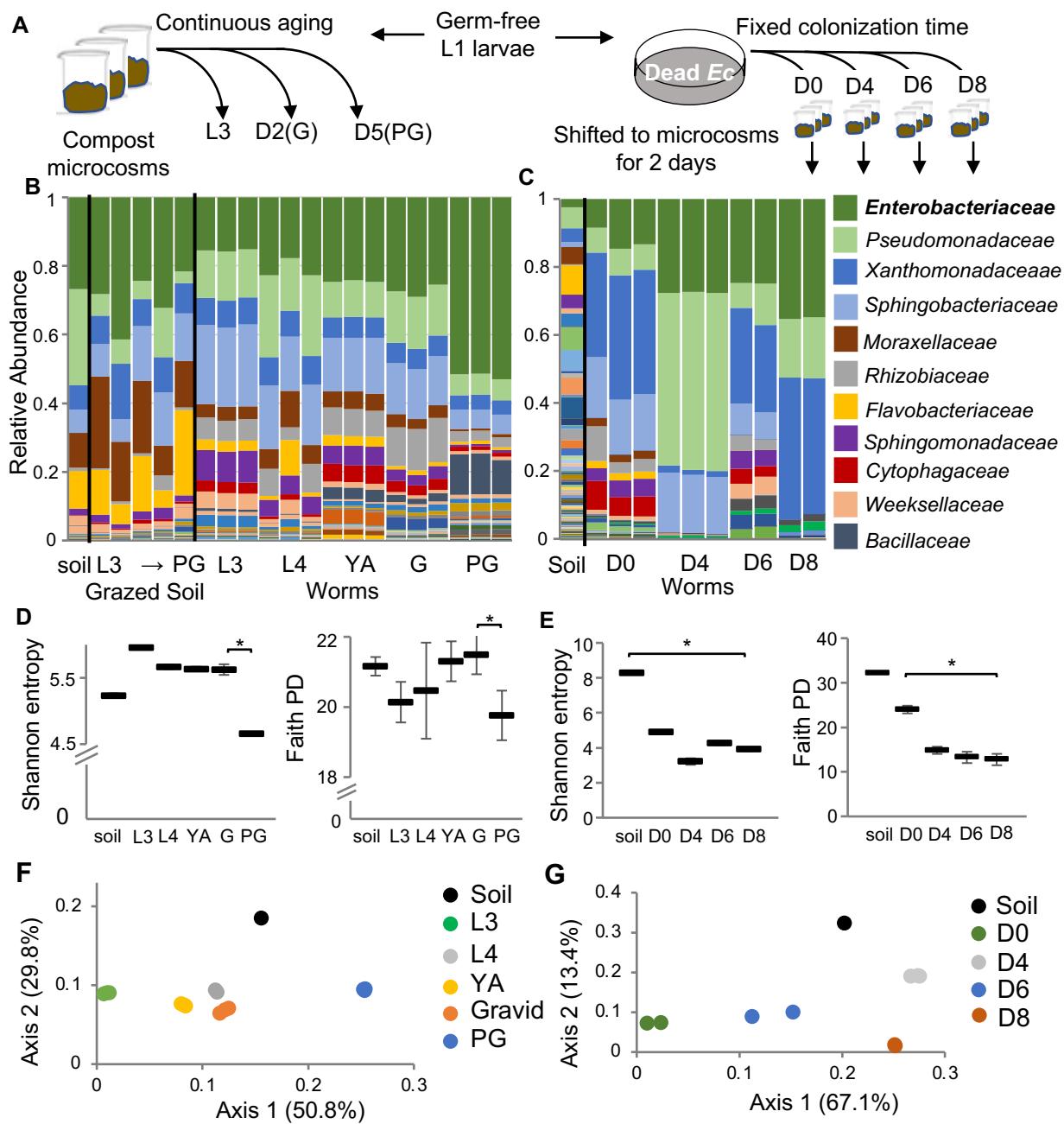


Figure 1. A gut *Enterobacteriaceae* bloom in worms aging in natural-like environments. A. Two sampling schemes for worm microbiome analysis during late development and early aging. **B,C.** Microbiome composition in worms raised continuously in microcosm environments (B) or in worms of advancing ages, shifted for two days to microcosm environments (C). L3, L4, larval stages; G, gravids (D2, second day of adulthood); PG, post-gravid (D5); D0 = early gravids. 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. **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.

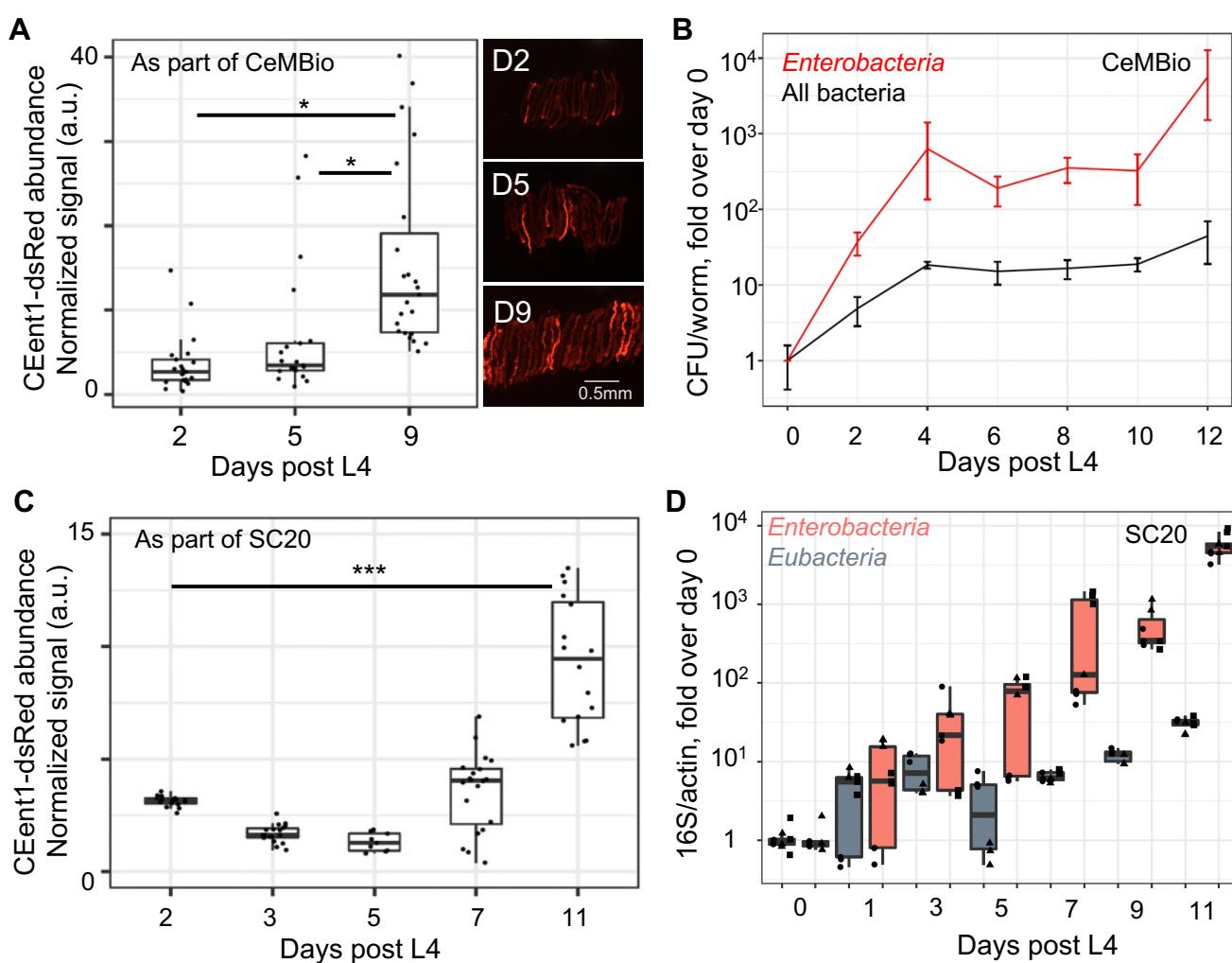


Figure 2: An *Enterobacteriaceae* bloom is a common denominator of aging worms raised in different microbial environments. **A.** Colonization of individual aging worms raised on CeMBio with CEent1dsRed, n=21-23/group; $p < 0.01$, pairwise t-tests; **B.** Bacterial load in aging worms raised on CeMBio, based on CFU counts of *Enterobacteriaceae* on VRBG plates and of total bacteria on LB plates. Shown are averages \pm SDs for 3 plates per time point (n=4-12 worms/time point). **C.** CEent1 colonization in aging worms raised on SC20 with CEent1dsRed (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).

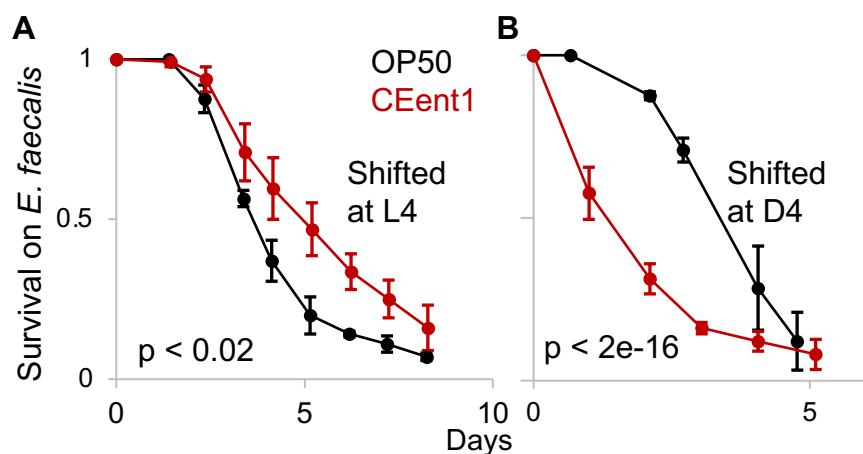


Figure 3: *Enterobacter hormachei* CEent1 bloom in aging worms is associated with increased susceptibility to infection. Survival curves for wildtype worms raised on designated monocultures and shifted to to plates with the pathogen, *E. faecalis* at L4 (A, n = 85-99/group), or at day four of adulthood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

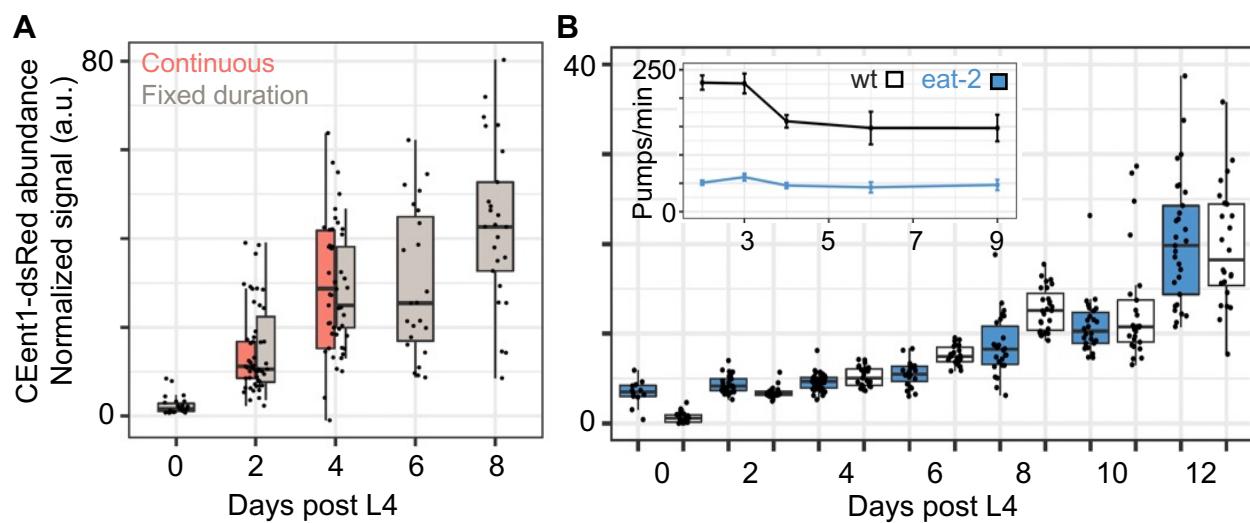


Figure 4: Neither duration of exposure to bacteria nor their rate of uptake contributes to the *Enterobacteriaceae* bloom. **A.** Colonization of aging worms by *CEent1-dsRed* following continuous exposure from larval stages or shifting worms for two days prior to the designated time points (fixed duration) (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.** *CEent1dsRed* 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.

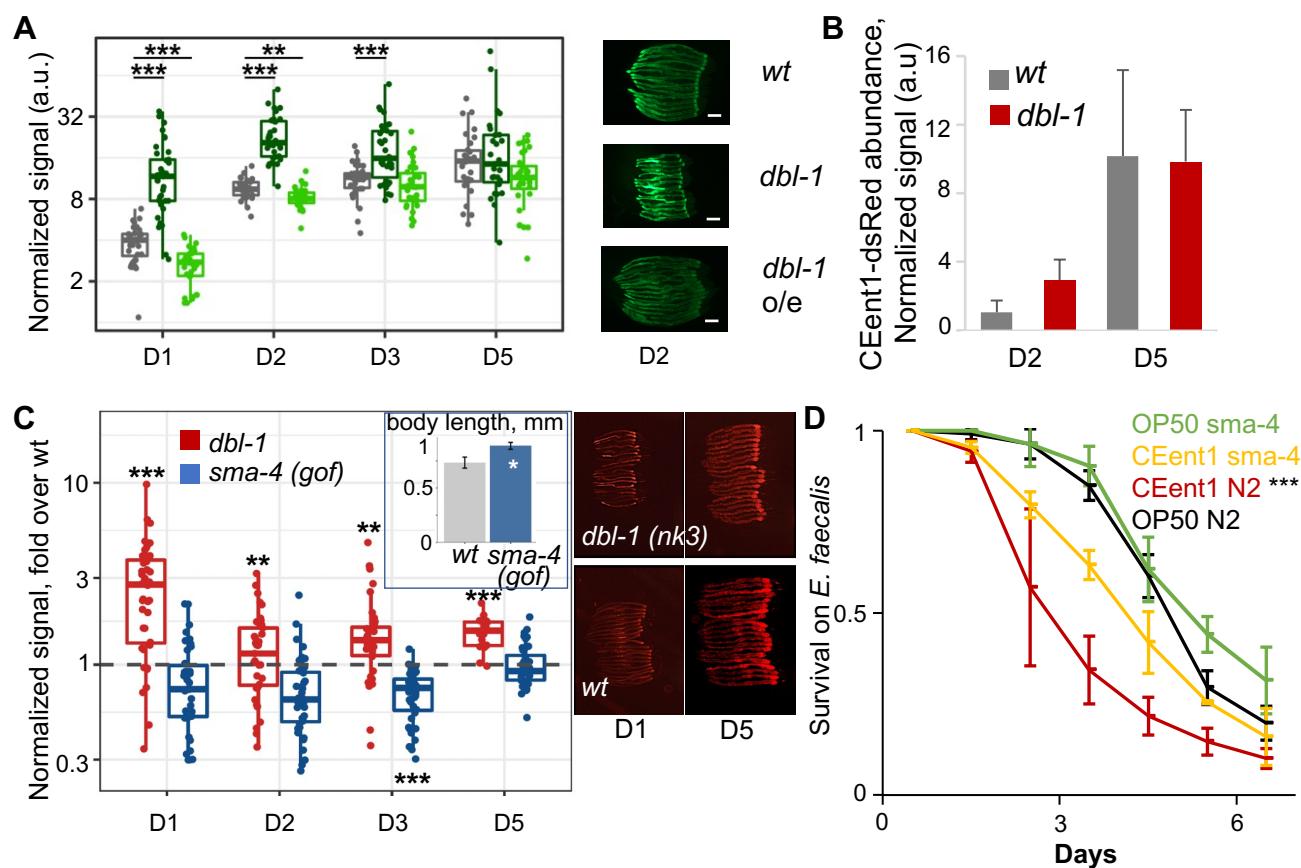


Figure 5: The *Enterobacteriaceae* bloom is associated with an age-dependent decline in DBL-1/BMP signaling. **A.** GFP expression from the *spp-9* promoter in designated strains; representative images (scale bar = 200 μ M) and quantification ($n = 27-37$ /group/time point); ** $p < 0.01$, and *** $p < 0.001$, Kruskal-Wallis rank sum test and *post hoc* Wilcoxon test (mutant vs wt), Bonferroni corrected. **B.** CEent1dsRed colonization in worms of designated strains at designated days of adulthood. **C.** *dbl-1* null mutants, and *sma-4* gof mutants aging on CeMBio containing CEent1-dsRed ($n = 39-40$ /group/time point); fold over wt median. **Inset.** Body length of designated strains ($n=9-12$, $p < 0.0001$) **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); ***, $p < 0.001$, logrank test.

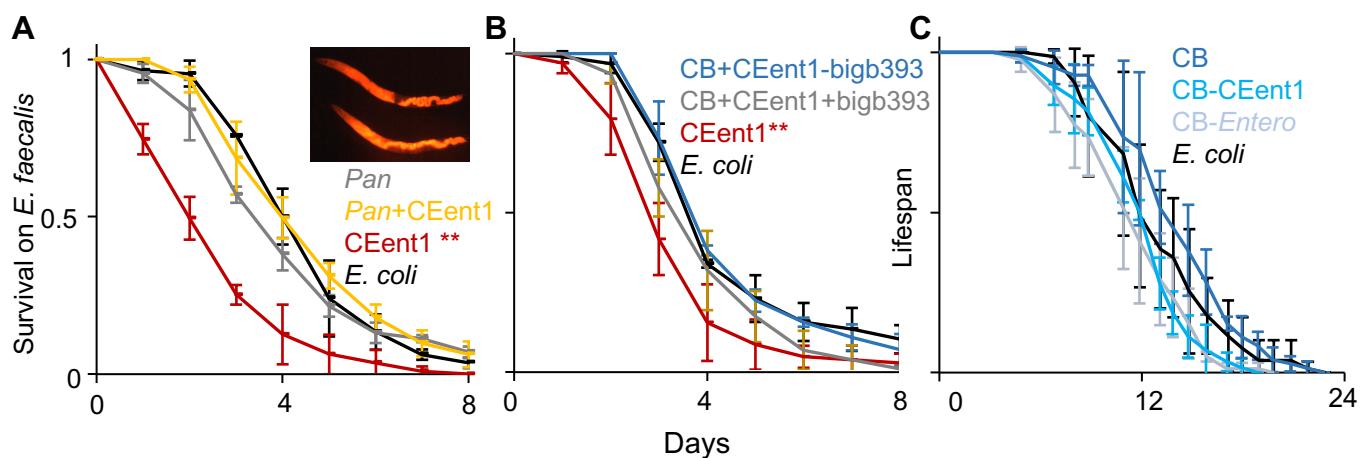
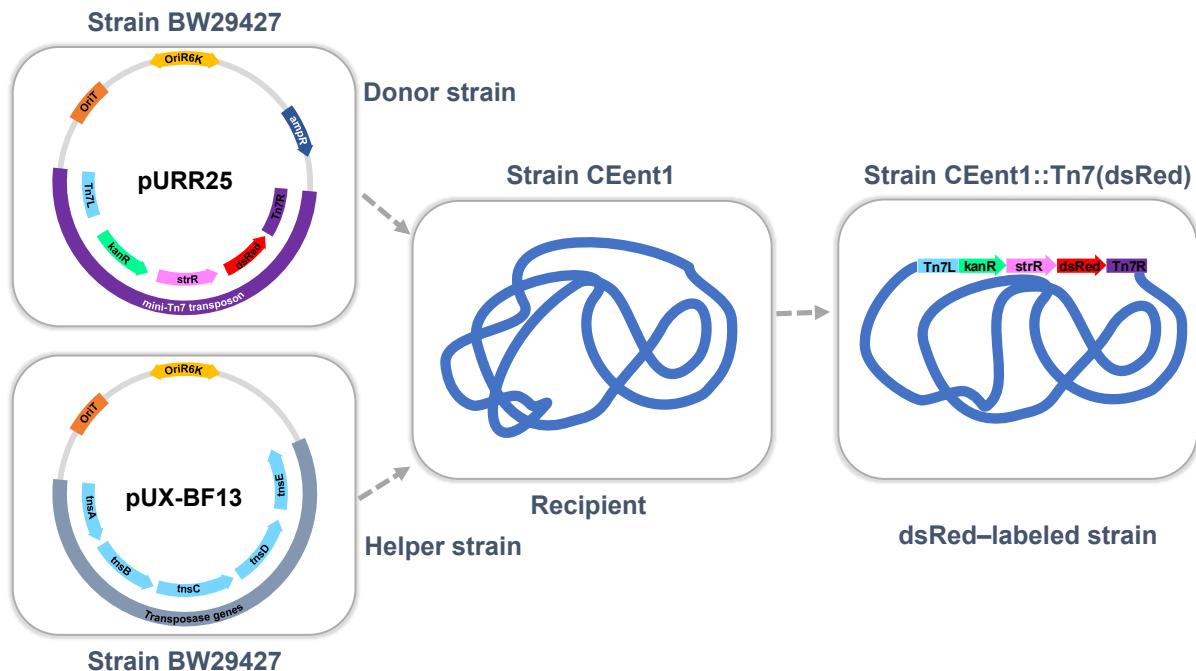


Figure 6: Commensal communities mitigate age-dependent susceptibility to infection. **A.** Survival of worms raised on the designated strains/communities and shifted to *E. faecalis* at D4; *Pan*, a community of three *Pantoea* strains; **, p < 0.0001, log rank test (n = 82-90/group); averages ± SDs for three plate replicates. **Inset.** CEent1-colonized dead worms, one day after shift to *E. faecalis*. **B.** BIGb393, a *Pantoea* strain in CeMBio.(CB) **, 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. *Enterobacter* 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.