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Ingested soil bacteria breach gut epithelia and prime systemic immunity in an insect

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Main Manuscript for

3 **Break through the barrier: Ingested soil bacteria breach gut epithelia and prime**
4 **systemic immunity in an insect**

5

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Main Text

Figures 1 to 6

20

21 **Abstract**

22 Insects lack acquired immunity and were thought to have no immune memory, but recent
23 studies reported a phenomenon called immune priming, wherein sub-lethal dose of pathogens
24 or non-pathogenic microbes stimulate immunity and prevent subsequential pathogen infection.
25 Although the evidence for insect immune priming is accumulating, the underlying
26 mechanisms are still unclear. The bean bug *Riptortus pedestris* acquires its gut microbiota
27 from ambient soil and spatially structures them into a multispecies and variable community in
28 the anterior midgut and a specific, mono-species *Caballeronia* symbiont population in the
29 posterior region. We demonstrate that a particular *Burkholderia* strain colonizing the anterior
30 midgut, stimulates systemic immunity by penetrating gut epithelia and migrating into the
31 hemolymph. The activated immunity, consisting of a humoral and a cellular response, had no
32 negative effect on the host fitness, but on the contrary protected the insect from subsequent
33 infection by pathogenic bacteria. Interruption of contact between the *Burkholderia* strain and
34 epithelia of the gut weakened the host immunity back to pre-infection levels and made the
35 insects more vulnerable to microbial infection, demonstrating that persistent acquisition of
36 environmental bacteria is important to maintain an efficient immunity.

37

38

39 **Significance statement**

40 Immune priming has evolved in memory-deficient invertebrates to combat sudden infections
41 effectively. Although this phenomenon has been reported in diverse insects, understanding of
42 underpinning mechanisms remains limited. We found that a member of the gut microbiota in
43 the bean bug *Riptortus pedestris* can cross the epithelia and be released into the hemolymph,
44 thereby stimulating systemic immunity and preventing subsequent infection of lethal
45 pathogens. In this process, both humoral and cellular immunity play a pivotal role, and there
46 was no trade-off with insect fitness. This finding not only highlights the utilization of
47 environmental microbes by insects for shaping immunity but also provides a new, symbiotic
48 perspective on bacterial intestinal barrier breaching, which has been generally discussed only
49 in terms of pathology.

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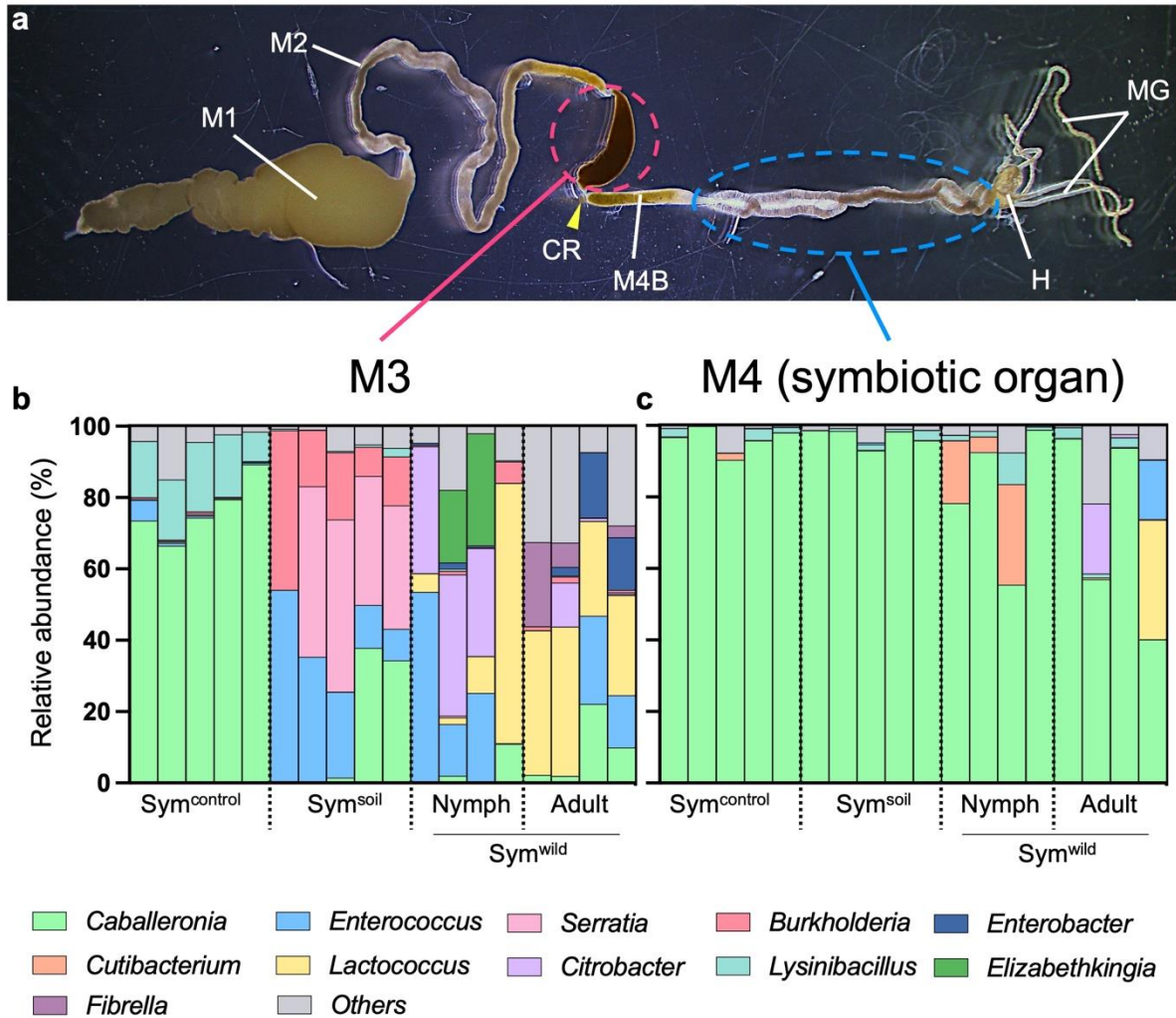
51 **Introduction**

52 To protect themselves from invasion by environmental pathogens, insects are armed with a
53 well-developed innate immunity (1-4). Unlike vertebrates, insects lack acquired immunity and
54 do not possess immune memory against pathogens. However, recent studies have reported
55 that insects exhibit high defensive capabilities against pathogen infection through a
56 phenomenon called immune priming (5-8). Immune priming is triggered when insects are
57 exposed to sublethal doses of pathogenic microbes or their microbe-associated molecular
58 patterns (MAMPs), such as peptidoglycan, eliciting the immune system and enabling
59 immediate defense upon subsequent infection, even against lethal doses of pathogens.
60 Moreover, immune priming can also be induced by non-pathogenic bacteria (9, 10).
61 Experimental evidence confirmed that immune priming can be achieved through injection of
62 microbes into the hemolymph or ingestion of a diet containing microorganisms (8, 11, 12).
63 However, the mechanisms underlying immune priming in insects are not fully understood.
64 A major weapon to kill infecting microbes is the systemic humoral immunity, which is
65 activated from the fat bodies (3). Fat bodies produce massive amounts of antimicrobial
66 peptides (AMPs) upon microbial infection. AMPs are secreted in the hemolymph and kill and
67 rapidly eliminate microbes from the insect's body (3, 13, 14). The humoral immune cascade is
68 generally activated by two different pathways: the Toll pathway and the immune deficiency
69 (IMD) pathways, which are triggered by MAMPs and regulate the expression of the AMPs as
70 well as other effectors (15, 16). In addition to humoral immunity, insects utilize a cellular
71 immune system in the hemolymph mediated by phagocytic immune cells (17). These
72 circulating hemocytes bind infecting microbes via MAMP-recognizing receptors or opsonins,
73 which engage phagocytosis pathways. The activated hemocytes engulf infecting microbes and
74 internalize them into phagosomes where they are lysed (17).
75 Insect guts are continuously exposed to environmental bacteria including pathogens, which
76 are mostly ingested orally from their food and habitat. Orally ingested pathogens are
77 confronted with a local gut immune response, which is dissimilar to the systemic immunity,
78 wherein microbicidal reactive oxygen species (ROS) are generated and secreted in the gut
79 lumen by the NADPH oxidase enzyme dual oxidase (Duox) that is highly activated by
80 microbial infection in the midgut (18-20). When ROS-resistant pathogens colonize the midgut
81 for a long period, AMPs are produced by the midgut epithelial cells via the IMD pathway (3,
82 21, 22), as a fail-safe system to eliminate the ROS-tolerant bacteria, thus maintaining gut
83 homeostasis (21). In addition, most insects possess a physical barrier, the peritrophic
84 membrane that lines the luminal side of the midgut epithelia and prevents direct contact by
85 ingested bacteria with the midgut epithelial cells (2, 23). Despite these physical and

86 immunological barriers, some enteropathogenic bacteria such as *Serratia* and *Pseudomonas*
87 species can nevertheless penetrate the peritrophic membrane and gut epithelial cells, causing
88 sepsis followed by killing of the insect (24, 25).

89 The bean bug *Riptortus pedestris* (Hemiptera: Alydidae) is a notorious pest of legumes and
90 harbors a specific symbiont belonging to the genus *Caballeronia*, within the *Burkholderia*
91 *sensu lato* group (Betaproteobacteria: Burkholderiaceae), in a crypt-bearing, posterior region
92 of its midgut, called M4 (26, 27) (Fig. 1a). *Caballeronia* symbionts are not essential for the
93 survival of the host insect but significantly enhance their growth and fecundity (28, 29). New-
94 born *R. pedestris* nymphs are free of microbiota and acquire the *Caballeronia* gut symbiont
95 together with a large diversity of other bacteria from the soil (28, 30, 31). Thus, the bean bug
96 evolved stringent selection mechanisms including a symbiont sorting organ, the constricted
97 region (CR) located inbetween the anterior midgut and the posterior symbiotic organ, to
98 winnow out most bacteria derived from the soil (32). *Caballeronia* symbionts pass through
99 the CR but other bacteria are mostly filtered out and retained in the M3 midgut region which
100 is located immediately before the CR (32) (Fig. 1a). However, the interactions between the
101 M3-resident bacteria and the host bean bug are poorly understood.

102 In this study, we investigated the interactions in the midgut between soil-borne gut bacteria
103 colonizing the M3 region and the bean bug host. We found that a *Burkholderia sensu stricto*
104 (*s.s.*) species isolated from the M3 region primed the host systemic immunity by penetrating
105 the gut epithelia and entering into the hemolymph, without any pathogenetic effects on the
106 host insect. The activated immunity protected the host against subsequent pathogen infection,
107 demonstrating that, in addition to the M4 crypt-colonizing *Caballeronia* symbionts, some M3-
108 resident bacteria are also beneficial to the bean bug host.



109

Fig. 1. Soil-infected and wild bean bugs have more complex gut microbiota than those solely infected with cultured *Caballeronia* symbiont.

a Intestinal tract of *R. pedestris*, highlighting the five distinct midgut sections: M1 (first section), M2 (second section), M3 (third section), CR (constricted region), M4B (bulbous region preceding M4), and M4 (fourth section containing crypts, symbiotic organ). H, hindgut; MG, malpighian tubes. **b** M3 microbiota of Sym^{control} ($n = 5$), Sym^{soil} ($n = 5$), and Sym^{wild} ($n = 4$ for both nymphs and adults) bean bugs. **c** M4 microbiota of Sym^{control}, Sym^{soil}, and Sym^{wild} bean bugs. The bacterial community composition in **b** and **c** were determined via 16S rRNA amplicon sequencing.

110 Results

111 Diverse bacteria colonize M3

112 Bacterial communities in the M3 and M4 regions from bean bugs infected with cultivated
 113 *Caballeronia insecticola* symbiont (Sym^{control}), reared with environmental soil (Sym^{soil}), or
 114 captured in the field (Sym^{wild}) were analyzed by 16S rRNA gene amplicon sequencing.
 115 Bacterial diversity was higher in the M3 than in the M4 symbiotic organ, and Sym^{soil} and
 116 Sym^{wild} bean bugs possessed a more complex M3 bacterial community than Sym^{control} insects
 117 (Fig. S1a). The M3 bacterial community of Sym^{control} bean bugs was relatively simple and

118 low-abundant since the insects were not fed other bacteria besides the native gut symbiont *C.*
119 *insecticola* (Figs. 1b and S1a and b). Most M3-colonizing bacteria in Sym^{control} bugs belonged
120 to the genus *Caballeronia* ($76.6 \pm 8.5\%$), with a second major genus, *Lysinibacillus* ($15.5 \pm$
121 4.3%), making up the rest of the community (Fig. 1b). The latter probably originated from the
122 rearing conditions such as the soybeans and water they were fed. By comparison, the
123 proportion of *Caballeronia* was much lower in the M3 of Sym^{soil} insects ($14.9 \pm 19.4\%$),
124 whereas the genera *Serratia* ($33.4 \pm 19.7\%$), *Enterococcus* ($26.7 \pm 18.3\%$), and *Burkholderia*
125 ($20.3 \pm 14.2\%$) were more abundant (Fig. 1b). The bacterial community of Sym^{wild} bean bugs
126 was also more diverse than Sym^{control} insects, with only a small quantity of *Caballeronia* in
127 the M3 region ($6.2 \pm 7.7\%$) (Fig. 1b). Instead, a high proportion of other non-symbiotic
128 bacteria, such as *Enterococcus*, *Lactococcus*, *Citrobacter*, *Burkholderia*, *Enterobacter*,
129 *Elizabethkingia*, and *Fibrella*, were detected from the M3 of Sym^{wild} bean bugs (Fig. 1b).

130 In contrast to that of the M3 region, the bacterial community residing in the M4 symbiotic
131 organ was not strongly different across Sym^{control}, Sym^{soil}, and Sym^{wild} bean bugs (Figs. 1c and
132 S1a). Both Sym^{control} and Sym^{soil} insects, which are maintained under laboratory conditions,
133 predominantly harbored *Caballeronia* gut symbiont in the symbiotic organ (Fig. 1c). Sym^{wild}
134 bugs also possessed a high proportion of *Caballeronia* symbionts in the M4, but some
135 individuals had in addition a relatively large amount of other bacteria, belonging to genera
136 such as *Cutibacterium*, *Citrobacter*, *Lactococcus*, *Enterococcus*, and *Lysinibacillus* (Fig. 1c).
137 The presence of bacteria other than *Caballeronia* spp. in the M4 could be due to the re-
138 opening of the symbiont sorting organ. Indeed, the CR is tightly sealed immediately after
139 colonization of the M4 by the *Caballeronia* symbiont (33), but reopens at the late
140 developmental stage of the bean bug due to aging (34). However, it should be noted that the
141 core bacterial taxon in the M4 of these Sym^{wild} insects is still *Caballeronia* (Fig. 1c).

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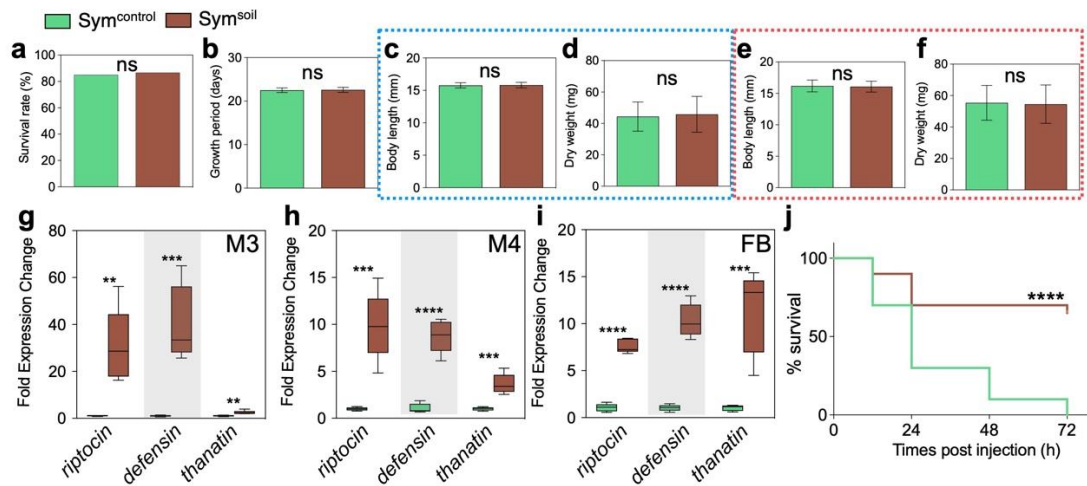


Fig. 2. Soil-borne microbes do not impair bean bug physiology.

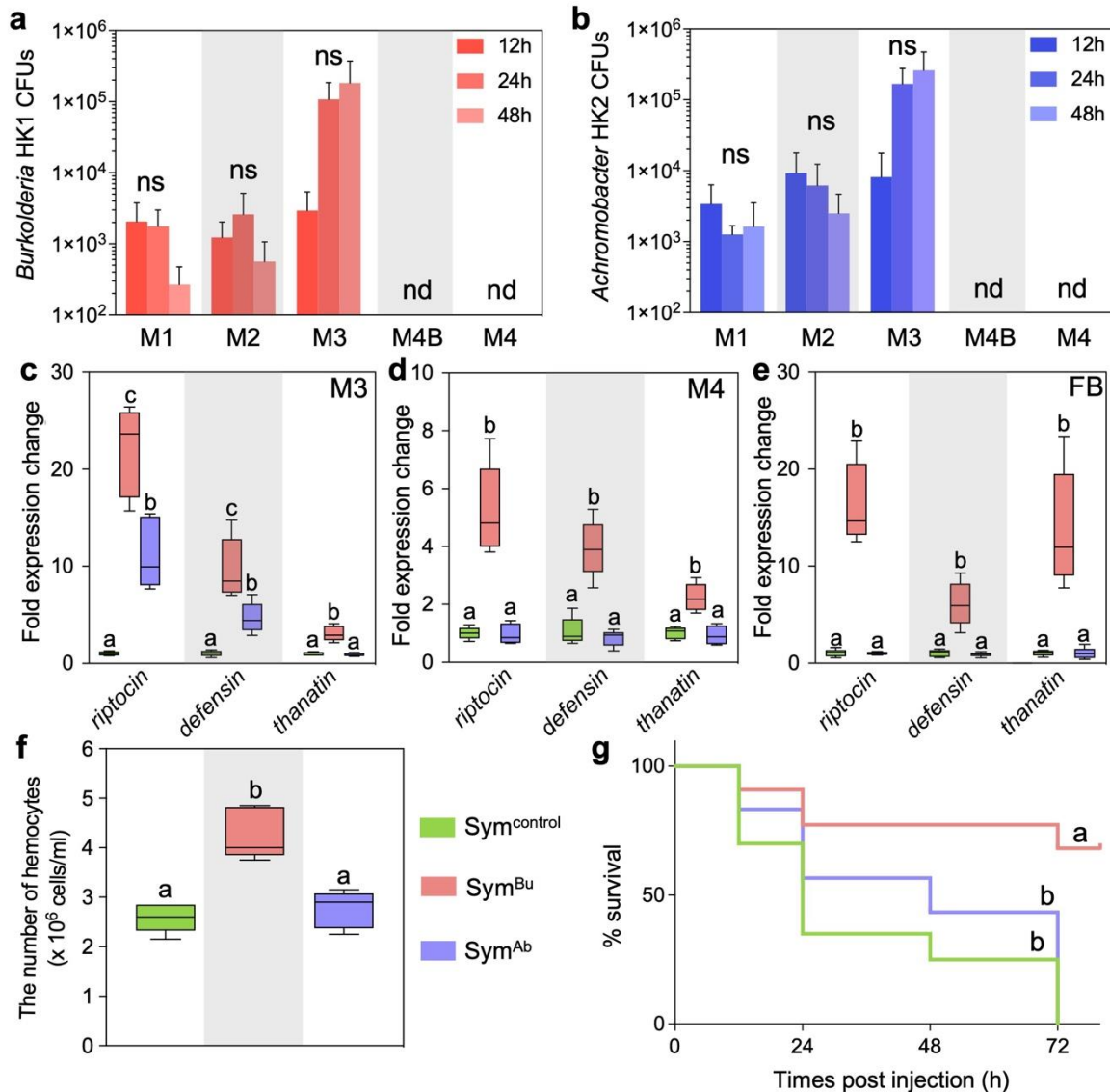
a Host survival rate and **b** time to adulthood of Sym^{control} and Sym^{soil} bean bugs ($n = 51$ and 52 , respectively). **c**, **d** Body length (**c**) and dry body weight (**d**) of adult male bean bugs ($n = 20$). **e**, **f** Body length (**e**) and dry body weight (**f**) of adult female bean bugs ($n = 20$). **g-i** Expression levels of three AMPs (*riptide*, *defensin*, and *thanasin*) in M3 (**g**), M4 (**h**), and fat body (**i**) after oral infection with cultured *Caballeronia* or environmental soil ($n = 5$, respectively). **j** Survival rate of Sym^{control} and Sym^{soil} bean bugs after injection of entomopathogen *P. entomophila* into the hemocoel ($n = 20$). The statistical significances were analyzed by the Mann-Whitney *U* test with Bonferroni correction (**a-i**) or Log-rank test (**j**). Error bars indicate SDs; ns, not statistically significant; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.0001$.

144

145 **Soil-derived gut bacteria enhance immunity and protect the bean bug against the**
 146 **pathogen *Pseudomonas entomophila***

147 To investigate the effects M3-colonizing gut bacteria acquired from the soil on the host bean
 148 bug, the survival rate, developmental period, and fitness parameters (body length and dry
 149 body weight) of Sym^{control} and Sym^{soil} insects were measured (Fig. S2a). Sym^{soil} bean bugs
 150 showed similar survival rate, growth rate, and body size to Sym^{control} insects (Figs. 2a-f),
 151 indicating that orally acquired commensal gut microbes did not show harmful effects on the
 152 host bean bug. Gut-colonizing bacteria in insects are known to activate local gut immune
 153 responses, such as the production of AMPs (14, 35, 36). The enhanced expression level of
 154 three known AMPs (*riptide*, *defensin*, and *thanasin*) (37) in the M3 of Sym^{soil} bean bugs
 155 compared to Sym^{control} demonstrated that ingested M3-colonizing bacteria similarly induces
 156 the local immune response in *R. pedestris* (Fig. 2g). Interestingly, the expression levels of the
 157 AMPs were also up-regulated in the M4 symbiotic organ as well as in the fat bodies although
 158 M3-colonizing bacteria generally do not colonize those organs (Figs. 2h and i), indicating that
 159 the orally acquired soil-derived bacteria stimulated not only the local gut immunity, but also a
 160 systemic immune response in the bean bugs.

161 To determine if the systemic immune activation by M3-colonizing gut bacteria protects the
162 bean bugs against pathogen infection, we first tested the pathogenicity of Gram-negative and
163 Gram-positive bacteria by injecting 10^6 cells of four bacterial species into the hemolymph of
164 Sym^{control} insects. This infection route mimicks natural infections occurring by wounding or
165 by natural parasites. The entomopathogenic Gram-negative bacterium *Pseudomonas*
166 *entomophila* exhibited high pathogenicity, but the bean bugs showed resilience against Gram-
167 positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*) and non-pathogenic Gram-
168 negative bacteria *Escherichia coli* (Fig. S3). Since a previous study demonstrated the
169 insecticidal activity of *P. entomophila* against the bean bug (38), we used *P. entomophila* as
170 the pathogen in further experiments. A total of 10^6 cells of *P. entomophila* were injected in the
171 hemolymph of Sym^{control} and Sym^{soil} insects, and the insects' survival rates were measured. In
172 agreement with previous results showing that *P. entomophila* is pathogenic for bean bugs (Fig.
173 S3), Sym^{control} insects died within 72 hours after bacterial injection (Fig. 2j). In contrast,
174 Sym^{soil} bean bugs showed high survival after *P. entomophila* systemic infection (65%
175 survivability) (Fig. 2j), suggesting that the systemic immunity up-regulated by M3-colonizing
176 bacteria protects the host bean bugs against an infecting pathogen.
177



178
179

Fig. 3. M3-colonizing bacteria elicit systemic immunity, protecting the host from pathogen invasion.

a, b The number of *Burkholderia* HK1 (**a**) and *Achromobacter* HK2 (**b**) cells colonizing the gut of aposymbiotic second instar nymphs was measured in each midgut region at 12, 24, and 48 hours after oral administration ($n = 3$). nd, not detected. **c-e** Relative expression levels of three AMPs (*riptidecin*, *defensin*, and *thanatin*) in M3 (**c**), M4 (**d**), and fat body (**e**) in the *Burkholderia* HK1 or *Achromobacter* HK2 treatments relative to the control treatment ($n = 5$, respectively). *Burkholderia* HK1 elicited the induction of systemic AMPs, while *Achromobacter* HK2 triggered an immune response only in M3. **f** Total number of hemocytes in the hemolymph of bean bugs after oral infection with either *Burkholderia* HK1 or *Achromobacter* HK2 cells ($n = 5$, respectively). **g** Survival rate of Sym^{control}, Sym^{Bu}, and Sym^{Ab} bean bugs post systemic infection with the pathogen *P. entomophila* ($n = 20$, respectively). Different letters indicate statistically significant differences ($P < 0.05$). The statistical significance was analyzed by the Mann-Whitney *U* test with Bonferroni correction (a-f) or Log-rank test with Bonferroni correction (g). Error bars indicate SDs; ns, not significant.

180 **Isolation of M3-colonizing bacteria**

181 To identify bacterial taxa that can enhance host systemic immunity, we isolated M3-
182 colonizing bacteria from the M3 of Sym^{soil} bean bugs by spreading homogenized M3 lysate
183 on nutrient broth agar plates (Fig. S1a). A total of four different bacterial species were
184 identified by 16S rRNA sequencing, three of them belonged to the genus *Burkholderia s.s.*
185 and one to the genus *Achromobacter* (Table S1). One of the *Burkholderia* (*Burkholderia* sp.
186 HK1; Fig. S4) and *Achromobacter* (*Achromobacter* sp. HK2) isolates were orally
187 administered to aposymbiotic (free of *Caballeronia* M4 symbionts) second instar bean bug
188 nymphs, and bacterial titers were measured from each midgut section. Both, *Burkholderia*
189 HK1 and *Achromobacter* HK2, were detected in the M1, M2, and M3 midgut regions, and
190 their number increased over time in the M3 (Figs. 3a and b). Despite their presence in M3,
191 neither bacterial strains were able to colonize the M4B and M4 regions, in concordance with
192 the selective colonization of the symbiotic organ by *Caballeronia* (29). In addition,
193 fluorescence microscopy showed that red fluorescence protein (RFP)-expressing
194 *Burkholderia* HK1 and *Achromobacter* HK2 cells did not pass the CR and were stuck in the
195 M3, confirming that both isolated M3-colonizing bacteria are not M4 crypt symbionts (Fig.
196 S5).

197

198 ***Burkholderia* HK1 protects the bean bug from pathogens by priming host systemic** 199 **immunity**

200 Since infection with soil microbes activated the immune responses of the bean bugs compared
201 to Sym^{control} insects (Figs. 2g-i), we measured the expression levels of AMPs in the M3, M4,
202 and fat body of Sym^{control} insects colonized by *C. insecticola*, symbiotic insects colonized by
203 *C. insecticola* and infected with *Burkholderia* HK1 (Sym^{Bu}), or with *Achromobacter* HK2
204 (Sym^{Ab}) to determine if isolated commensal gut bacteria indeed enhance host immunity. Both
205 *Burkholderia* HK1 and *Achromobacter* HK2 stimulated local gut immunity in the M3 they
206 inhabit, although the expression level of *thanatin* was only up-regulated in Sym^{Bu} and not in
207 Sym^{Ab} insects (Fig. 3c). This could be attributed to the inherently low expression of *thanatin*
208 in the M3 even during bacterial systemic infection (39, 40). Similar to Sym^{soil} bean bugs,
209 infection with *Burkholderia* HK1 but not *Achromobacter* HK2 increased the expression of
210 AMPs in the M4 and the fat body, indicating that commensal *Burkholderia* HK1 can enhance
211 systemic host immunity after oral ingestion (Figs. 3d and e). Since *Burkholderia* HK1
212 activated systemic immune responses, the cellular immunity was subsequently investigated by
213 measuring the number of hemocytes in the hemolymph. The total number of hemocytes in
214 Sym^{Bu} insects but not in Sym^{Ab} was higher than that of Sym^{control} bean bugs, suggesting that
215 *Burkholderia* HK1 also activates cellular immune responses (Fig. 3f). The enhanced systemic

216 immunity of Sym^{Bu} bean bugs was correlated with a protective effect on the host against
217 infection with *P. entomophila*, while Sym^{Ab} bean bugs succumbed to the pathogen infection
218 within 72 hours similarly to Sym^{control} bean bugs (Fig. 3g). These results demonstrate that
219 certain M3-resident bacteria such as *Burkholderia* HK, but not all bacterial species, can prime
220 the host immunity, conferring protection against infection by pathogens.

221 To distinguish between the possibilities that protection arises from enhanced resistance or
222 alternatively from acquired tolerance to the pathogen, the number of *P. entomophila* cells in
223 the hemolymph was monitored after their injection in the hemolymph of Sym^{Bu} and Sym^{control}
224 insects. This experiment showed that the pathogen was significantly decreased in Sym^{Bu}
225 insects and dropped more than 100-fold 12 h after *P. entomophila* injection or more than
226 1,000-fold 24 h after injection, contrary to Sym^{control} insects where the pathogen stayed at high
227 level in the hemolymph (Fig. S6). This observation suggests that colonization of the M3 by
228 *Burkholderia* HK1 confers resistance against pathogen infection.

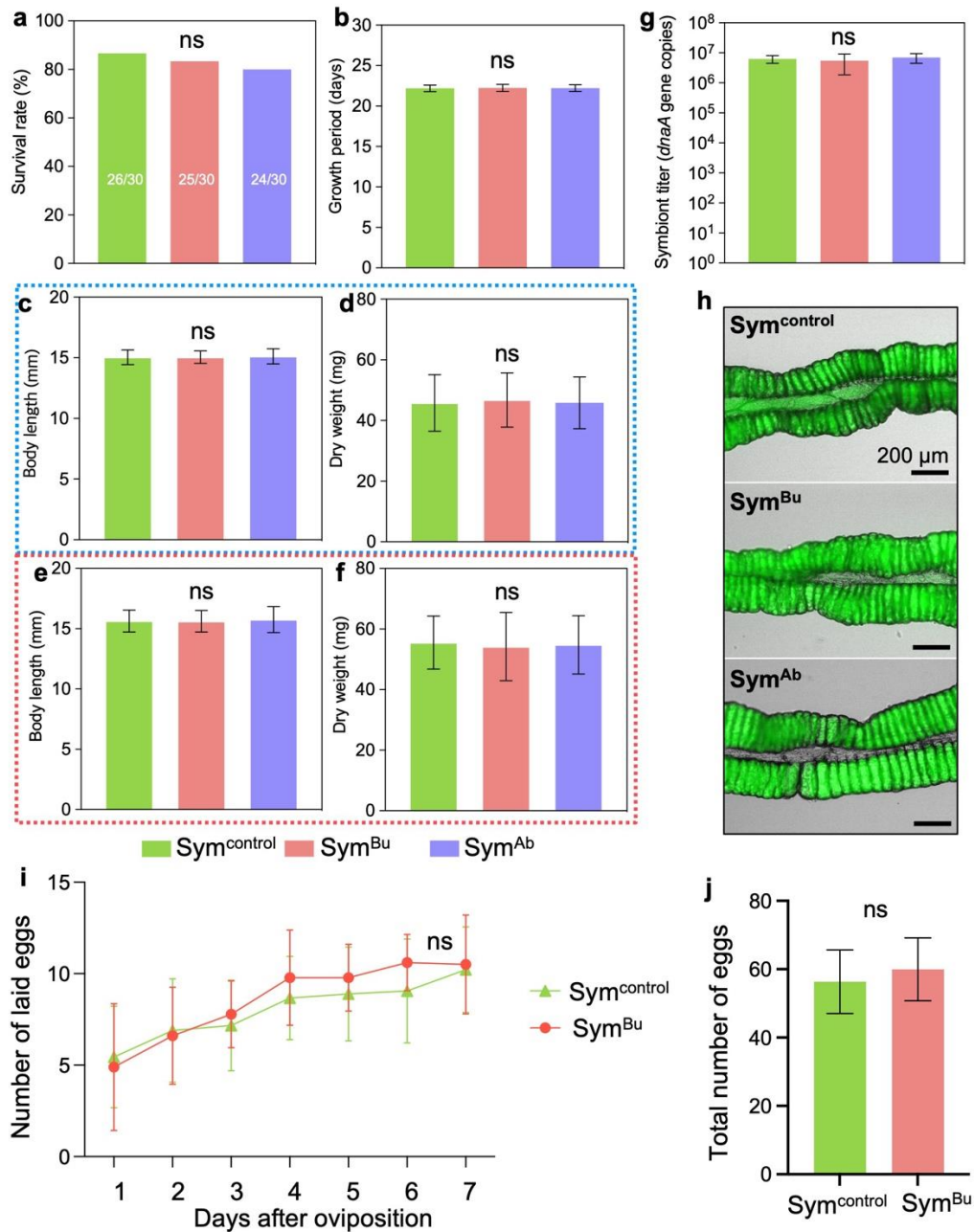


Fig. 4. M3-colonizing symbiont do not affect host physiology and M4 gut symbiosis.

a Survival rate and **b** growth period to adulthood of Sym^{control}, Sym^{Bu}, and Sym^{Ab} bean bugs ($n = 26, 25,$ and $24,$ respectively). **c,d** Body length (**c**) and dry body weight (**d**) of adult male bean bugs ($n = 20,$ respectively). **e,f** Body weight (**e**) and dry body weight (**f**) of female bean bugs ($n = 20,$ respectively). **g** Copy number of the *dnaA* gene of *Caballeronia* symbiont in the M4 region ($n = 5,$ respectively), as measured by RT-PCR. **h** Fluorescence microscopic images of isolated M4. Green indicates the symbiont-derived GFP signal. **i** Number of eggs laid since the beginning of oviposition ($n = 18,$ respectively). **j** Total number of eggs during a week of oviposition ($n = 18,$ respectively). The statistical significance was analyzed by the Mann-Whitney U test with Bonferroni correction. Error bars indicate SDs; ns, not statistically significant.

229

230

231

232 **Immune priming by M3-colonizing *Burkholderia* has no obvious negative effects on the**
233 **bean bug host**

234 We next checked the effects of isolated *Burkholderia* HK1 and *Achromobacter* HK2 on the
235 bean bug fitness by measuring the insect survival rate, growth period, and biomass parameters.
236 *Burkholderia* or *Achromobacter* cells were orally administered following infection with the
237 gut symbiont *C. insecticola* in order to compare all parameters with Sym^{control} bean bugs (Fig.
238 S2b). Both *Burkholderia* HK1 and *Achromobacter* HK2 showed no negative effects on the
239 survival rate, development, and fitness parameters of the bean bugs compared with Sym^{control}
240 insects (Figs. 4a-f). Additionally, the population of *C. insecticola* symbionts in the symbiotic
241 organ was similar between Sym^{control}, Sym^{Bu}, and Sym^{Ab} insects, demonstrating that the
242 infection by both M3-colonizing bacteria did not affect the M4 symbiotic association in the
243 bean bug (Figs. 4g and h). Taken together, the gut commensal bacteria derived from the soil
244 exhibit no negative impact on the host bean bug. In many insects, there is a physiological
245 trade-off between immune activation and reproduction; an increased immunity due to the
246 presence of bacteria leads to a decrease in fecundity (41, 42). We thus investigated the
247 fecundity of Sym^{control} and Sym^{Bu} bean bugs by determining the time at which they start
248 laying eggs, and counting the number of eggs produced over a week. In each replicate, two
249 males and one female bean bug were reared together to promote mating. The time before egg
250 laying was not significantly different between Sym^{control} and Sym^{Bu} bean bugs (6-7 days after
251 molting to the adult stage), and the number of laid eggs was unchanged in Sym^{Bu} insects
252 when compared to Sym^{control} insects (Fig. 4i and j). These results thus demonstrate that
253 immune priming did not affect fecundity in *R. pedestris* and are in accordance with prior
254 studies in beetles (43, 44).

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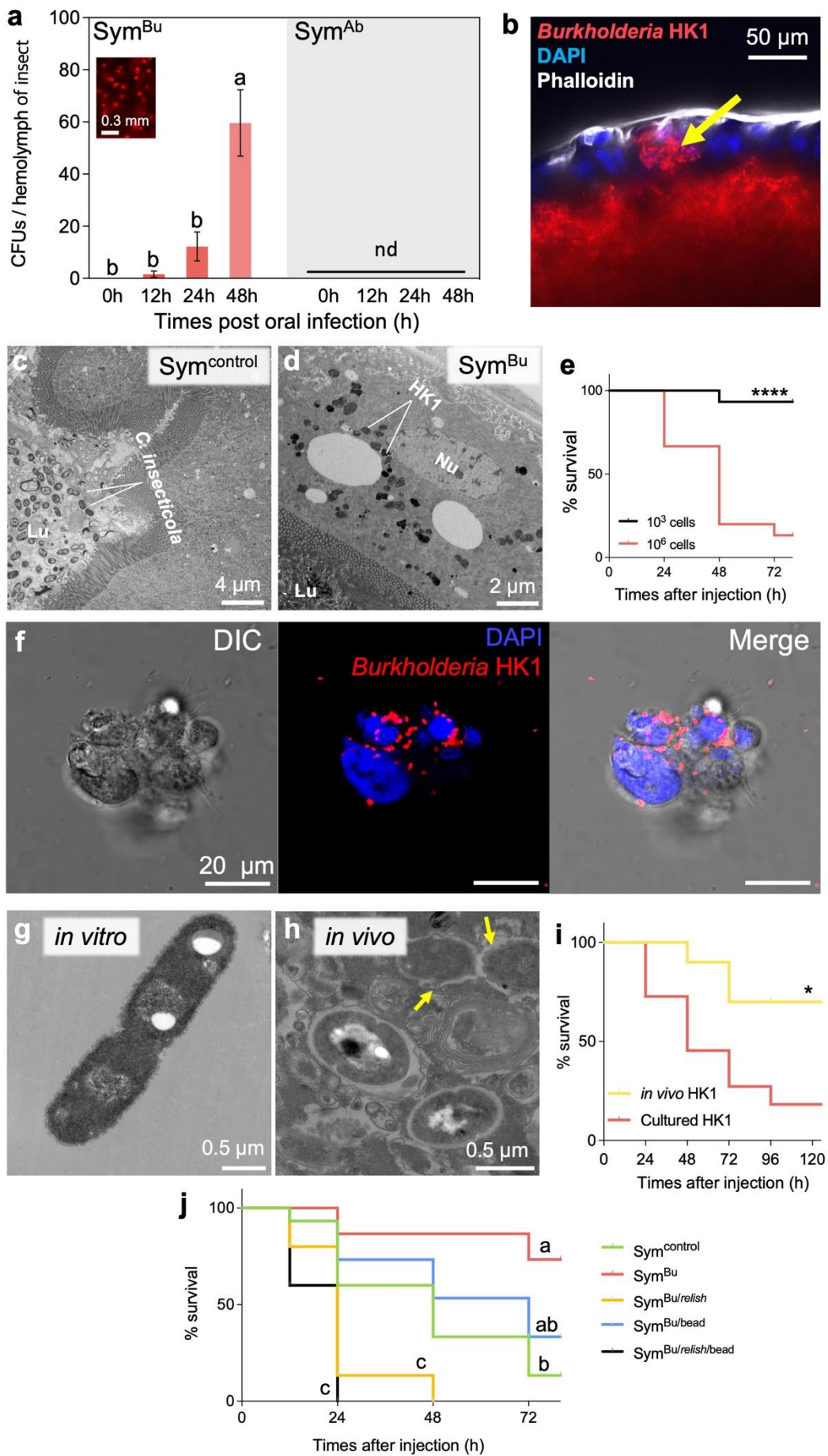


Fig 5. *Burkholderia* HK1 breaches gut epithelium, migrates into the hemolymph, and stimulates host systemic immunity.

a Bacterial titers in the hemolymph after oral infection of *Burkholderia* HK1 or *Achromobacter* HK2 cells ($n = 5$, respectively). The inset shows the RFP signal derived from *dsRed*-expressing *Burkholderia* HK1 cells by direct fluorescence microscopy observation of a droplet of hemolymph. **b** Confocal microscopy image of gut-breaching *Burkholderia* HK1. A few HK1 cells are located between M3 epithelial cells. Red, RFP-labelled *Burkholderia* HK1 cells; blue, host nuclei; White, host cytoskeleton. **c** and **d** TEM images of M3 in $\text{Sym}^{\text{control}}$ and Sym^{Bu} bean bugs. In the gut lumen of the $\text{Sym}^{\text{control}}$ insect, numerous *C. insecticola* symbiont cells are present (**c**). *Burkholderia* HK1 cells are situated in the M3 epithelial layer, migrating towards the hemolymph side (**d**). Nu; nuclei of host gut epithelium, Lu; luminal side of the midgut. **e** Pathogenicity of *Burkholderia* HK1. 10^3 or 10^6 cells of *Burkholderia* HK1 were injected into the hemocoel, and the survival rate of bean bugs was measured ($n = 15$, respectively). **f** Bright field, fluorescence microscopy, and merged images of phagocytosed *Burkholderia* HK1 cells by the host hemocytes. In the fluorescence image, red represents RFP-labelled *Burkholderia* HK1 cells, and blue indicates nuclei of hemocytes. **g,h** TEM images of *in vitro* cultured (**g**) and *in vivo* M3-colonizing (**h**) *Burkholderia* HK1 cells. White granules in bacterial cells represent polyhydroxyalkanoate (PHA) granules. Yellow arrows point to lysed cells in the midgut. **i** Pathogenicity of *in vitro* cultured and *in vivo* M3-colonizing *Burkholderia* HK1 ($n = 15$, respectively). The survival rate of bean bugs was measured after injecting each type of bacterial cells into the host hemocoel. **j** Survival rate of $\text{Sym}^{\text{control}}$, Sym^{Bu} , and immune-suppressed Sym^{Bu} insects after *P. entomophila* injection ($n = 15$, respectively). *Relish* is the key gene for bean bug's humoral immunity. $\text{Sym}^{\text{Bu/relish}}$, *relish*-silenced Sym^{Bu} insects by RNAi; $\text{Sym}^{\text{Bu/bead}}$, Sym^{Bu} insects with suppressed cellular immunity; $\text{Sym}^{\text{Bu/relish/bead}}$, both cellular and humoral immunity-suppressed Sym^{Bu} insects. Different letters of figure **a** indicate statically significant differences ($P < 0.05$). The statistical significance was analyzed by the Mann-Whitney *U* test with Bonferroni correction (a), Log-rank test (e and i) or Log-rank test with Bonferroni correction (j). Error bars indicate SDs; nd, not detected.

257

258 ***Burkholderia* HK1 enhances systemic immunity by breaking through gut epithelia and**
259 **migrating into hemolymph**

260 To reveal how gut-colonizing *Burkholderia* HK1 stimulates host systemic immune responses,
261 the bacterial titer in the hemolymph was measured after oral administration of *Burkholderia*
262 HK1. Two μl of hemolymph were collected from insects by removing a leg or antenna and
263 collecting the pooled hemolymph (Fig. S7). A few colonies of *Burkholderia* HK1 were
264 detected in the hemolymph 12 hours after oral infection and the population increased over
265 time until 48 hours (Fig. 5a). The migration of *Burkholderia* HK1 cells to the hemolymph was
266 confirmed through fluorescence microscopy using the RFP-expressing *Burkholderia* HK1
267 (Fig. 5a). On the other hand, *Achromobacter* HK2, which stimulates only local gut immunity
268 (Fig. 3c), was not detected from the host hemolymph, indicating that migration to the
269 hemolymph from the M3 is required to enhance systemic immunity (Fig. 5a). Confocal
270 microscopy confirmed the penetration of the M3 epithelia by *Burkholderia* HK1. Twelve

271 hours after oral infection by *Burkholderia* HK1, bacterial cells entered the M3 region and
272 attached to the gut epithelial cells (Fig. S8). After 24 hours, *Burkholderia* HK1 was detected
273 in-between the M3 gut epithelial cells (Figs. 5b and S8). Transmission electron microscope
274 (TEM) images also showed that *Burkholderia* HK1 cells penetrated the M3 gut epithelia
275 while there was no detectable bacterial penetration in Sym^{control} bean bugs (Fig. 5c and d). The
276 *C. insecticola* gut symbiont, which is temporarily present in the M3 before entering the
277 symbiotic organ, only colonized the luminal side of the M3 (Fig. 5c).

278 Since bacterial presence in the hemolymph could kill the host bean bug (38, 45), we then
279 tested the pathogenicity of *Burkholderia* HK1 by injecting bacterial cells directly into the
280 hemocoel. A high dose of bacteria led to high mortality in bean bugs, but a low number of
281 *Burkholderia* HK1 cells were not lethal, suggesting that a small number of *Burkholderia*
282 HK1 cells migrating from the M3 into the hemolymph will not kill the host (Fig. 5e).
283 Indeed, host bean bugs rapidly eliminated the migrated *Burkholderia* HK1 cells in the
284 hemolymph by phagocytosis (Fig. 5f). Moreover, comparison of the morphology of
285 *Burkholderia* HK1 cells between cultured and M3-colonizing cells showed that the cell
286 membrane of *in vivo* colonizing *Burkholderia* HK1 is abnormal (Fig. 5g and h). This
287 membrane alteration could fragilize the bacteria and we observed indeed that the gut-
288 colonizing *Burkholderia* HK1 cells were more susceptible to various stresses compared to
289 *in vitro* cultured *Burkholderia* HK1 cells (Fig. S9). This weakening of the bacteria in the
290 M3 could facilitate the rapid elimination of *Burkholderia* HK1 cells from the hemolymph
291 by the enhanced humoral and cellular immunity. Accordingly, the pathogenicity of gut-
292 colonizing *Burkholderia* HK1 cells to the bean bugs was attenuated compared to normally
293 cultured HK1 cells (Fig. 5i).

294 Next, we determined if bacterial breaching of the gut epithelia is also taking place, and by
295 which bacterial species, in the immune-primed insects reared on soil. These insects carry a
296 more diverse microbiota than gnotobiotic insects inoculated with specific microbes (Fig. 1b).
297 Hemolymph was extracted from third instar Sym^{soil} nymphs and plated on agar medium.
298 Fifteen colonies were randomly selected, and the bacterial species were identified using 16S
299 rRNA gene sequencing. Different bacteria including *Curpiavidus* and *Paraburkholderia* spp.
300 were detected in addition to a majority of *Burkholderia s.s.* spp., indicating that taxonomically
301 diverse bacteria and not only *Burkholderia s.s.* can breach the gut epithelial wall (Table S2).
302 Nevertheless, it is striking that all the identified species are closely related and belong to the
303 *Burkholderiaceae* family. Since the taxonomic diversity in the M3 microbiota of Sym^{soil}
304 insects includes members of several other bacterial families (Fig. 1b), our data suggest that
305 the capacity to breach the gut epithelia is mostly restricted to species of the *Burkholderiaceae*.

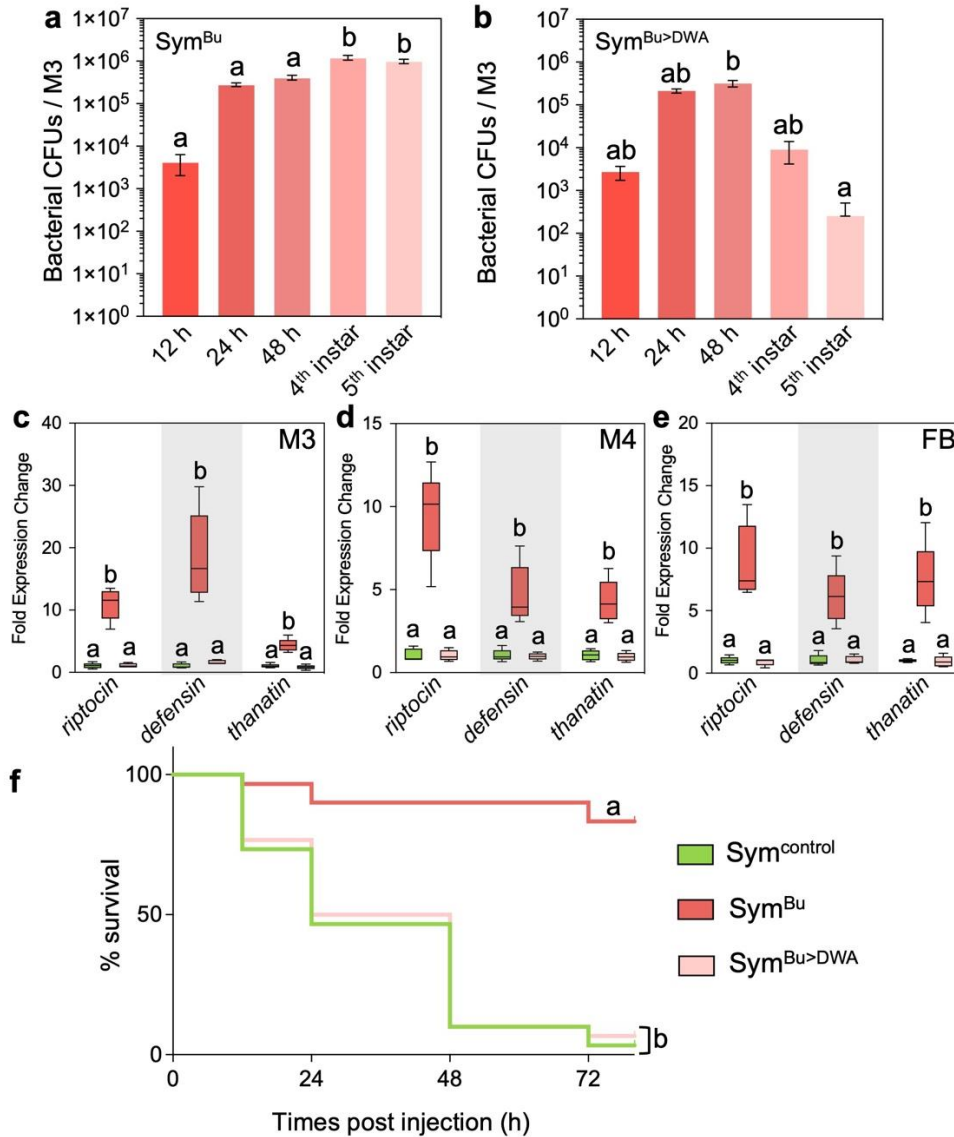
306 Thus, the immune priming observed in Sym^{soil} insects (Fig. 2g-j) is probably triggered by a
307 similar mechanism as in Sym^{Bu} insects, involving infection of the hemolymph by gut
308 commensal bacteria belonging to the *Burkholderiaceae*.

309

310 **Systemic immunity primed by M3-colonizing *Burkholderia* plays a pivotal defense role**

311 To investigate whether induced host systemic immunity is a main factor protecting host bean
312 bugs against pathogenic infection, we suppressed host humoral and cellular immunity by
313 RNAi of the *relish* gene to suppress humoral immunity or pre-injecting latex beads to induce
314 saturation of phagocytosis of the hemocytes in Sym^{Bu} bean bugs (Fig. S2c). Then, *P.*
315 *entomophila* was injected into the bean bug hemocoel and the insect survival rates were
316 measured (Fig. 5j). Cellular immunity-repressed insects (Sym^{Bu/bead}) succumbed to the
317 pathogen faster than the Sym^{Bu} insects, at rates similar to Sym^{control} insects, but humoral
318 immunity-repressed insects (Sym^{Bu/relish}) died even more rapidly. Accordingly, bean bugs
319 lacking both cellular and humoral immunities (Sym^{Bu/relish/bead}) showed the fastest mortality.
320 Together, these observations demonstrate that both cellular and host humoral immune priming
321 by gut-colonizing *Burkholderia* HK1 is critical to protect host bean bugs against pathogen
322 infection, although the contribution of humoral immunity seems to be higher than the cellular
323 immunity in our experimental conditions.

324



325

Fig 6. Spontaneous ingestion of commensal bacteria is important to immunopotentialiation.

a Bacterial titers in the M3 region of Sym^{Bu} and Sym^{Bu>DWA} bean bugs over time. Sym^{Bu>DWA}, HK1 cells were supplied only once to bean bugs and then changed to DWA ($n = 3$, respectively). **c-e** The expression levels of three AMPs (*riptocin*, *defensin*, and *thanatin*) in M3 (**c**), M4 (**d**), and fat body (**e**) of Sym^{control}, Sym^{Bu}, and Sym^{Bu>DWA} insects ($n = 5$, respectively). **f** Survival rate of Sym^{control}, Sym^{Bu}, and Sym^{Bu>DWA} insects after pathogen *P. entomophila* septic infection ($n = 15$, respectively). Different letters indicate statically significant differences ($P < 0.05$). The statistical significance was analyzed by the Mann-Whitney *U* test with Bonferroni correction (a-e) or Log-rank test with Bonferroni correction (f). Error bars indicate SDs.

326

327 **Persistent colonization of *Burkholderia* HK1 in the M3 is important to continuous**
 328 **immune enhancement**

329 Since M3-colonizing *Burkholderia* HK1 cells are fragilized, the enhanced immunity in Sym^{Bu}
 330 insects could rapidly eliminate the bacteria from the midgut. To test this, we divided bean

331 bugs into two groups: one group was continuously supplied with *Burkholderia* HK1 (Sym^{Bu}),
332 and the other group was provided *Burkholderia* HK1 only once, after which the bacterial
333 suspension was changed to sterile drinking water (DWA) (Sym^{Bu>DWA}) (Fig S2d). In the M3
334 of bean bugs continuously infected with *Burkholderia* HK1, a high number of bacterial cells
335 was continuously detected until the fifth instar (Fig. 6a). However, the bacterial titer gradually
336 decreased from third until the fifth instar nymph when the supply of *Burkholderia* HK1 was
337 halted (Fig. 6b). Additionally, the up-regulated local gut immunity as well as the systemic
338 immunity returned to a lower level in Sym^{Bu>DWA} bean bugs, reaching similar levels as those
339 of Sym^{control} insects (Fig. 6c-e). Consequently, Sym^{Bu>DWA} bean bugs, which lost their
340 upregulated immunity, also lost the protective effect against infection by pathogen *P.*
341 *entomophila* (Fig. 6f). Taken together, these results indicate that continuous oral ingestion of
342 *Burkholderia* HK1 is required to maintain enhanced systemic immunity in the bean bug *R.*
343 *pedestris* and is important to protect the host against pathogens.

344

345 **Discussion**

346 Immune priming is thought of as a mechanism analogous to vertebrate immune memory, and
347 plays a crucial role enabling insects, which only possess an innate immunity, to combat lethal
348 pathogenic infections (6, 7). However, the mechanisms underlying immune priming in natural
349 environments remain poorly understood. In this study, we discovered that in *R. pedestris*, the
350 anterior midgut M3-colonizing bacterium *Burkholderia* HK1, acquired from soil, penetrates
351 the gut epithelia, subsequently migrates to the hemocoel and triggers systemic immunity,
352 which ultimately confers resistance against a lethal dose of pathogens. We previously revealed
353 that the M4-colonizing *Caballeronia* symbionts strongly contribute to the activation of the
354 host immunity (45). Thus, in addition to the M4 symbionts, also M3-colonizing bacteria boost
355 immunity in an unexpected way, by penetrating the intestinal wall. Cells of the M3-colonizing
356 *Burkholderia* HK1 exhibited lower stress tolerance compared to cells cultured *in vitro* in
357 nutrient rich media (Fig. S9), suggesting their inactive or less-active state in the M3. Indeed,
358 although a few *Burkholderia* HK1 cells were detected in the hemolymph of infected insects
359 (Fig. 5a), no pathogenic properties were observed. Notably, immune priming induced by
360 *Burkholderia* HK1 had no discernible adverse effects on host fitness (Fig. 4). Previous studies
361 have reported a trade-off between immune activation and overall fitness (42, 46, 47).
362 However, the immune priming elicited by commensal gut bacteria in nature, as observed in
363 this study, could provide significant immune benefits with minimal costs and therefore,
364 *Burkholderia* HK1 as well as other M3-colonizing and hemolymph-infecting
365 *Burkholderiaceae* can be considered as symbionts.

366 The midgut of insects is constantly exposed to a large number of ingested gut microbes, thus,
367 insects have to be armed with a well-developed immunity to maintain gut homeostasis. In
368 *Drosophila*, many orally infected bacteria, including food-borne pathogens, are eliminated
369 from the midgut by local immunity. ROS, generated by the Duox enzyme upon microbial
370 infection in the midgut, results in the elimination of invading pathogens (18, 19, 48). Genetic
371 blocking of the *duox* gene actually increases the load of midgut-colonizing bacteria and *duox*-
372 deficient flies succumb to oral microbial infection (18). In contrast to *Drosophila*, the Duox is
373 not important in the midgut mucosal immunity of the bean bug. RNAi of *duox* neither altered
374 the survival rate of the bean bugs upon pathogen infection nor increased bacterial population
375 in the M3 (49). Additionally, the production of AMPs by bacterial infection in gut epithelia of
376 *Drosophila* complementarily kills ROS-resistant pathogens (21). Besides these immune
377 reactions, *Drosophila* possesses the acellular chitinous and proteinaceous peritrophic
378 membrane that lines the midgut epithelium. This matrix physically protects midgut cells from
379 direct contact with gut microbes thereby avoiding immune activation (50). It should be noted
380 that, while peritrophic membranes are found in most of insect groups, they are completely
381 absent in many hemipteran insects (51), including the bean bug *R. pedestris* (49). The M3-
382 colonizing bacteria are thus potentially in more direct contact with M3 epithelia of the host
383 compared to other insects and can more readily activate gut immunity that could immediately
384 affect gut microbes. Indeed, the M3 symbiont *Burkholderia* HK1 or *Achromobacter* HK2,
385 highly induced locally the expression of AMPs in the M3 region (Fig. 3).

386
387 Even if most gut-dwelling microbes in *Drosophila* are confined to the gut lumen due to the
388 peritrophic membrane and thus come not in contact with gut epithelial cells, some pathogens
389 (e.g. *Serratia marcescens* and *P. entomophila*) nevertheless can cross the peritrophic and
390 epithelial barriers by secreting proteases (24, 25). However, *S. marcescens*, which had
391 traversed the *Drosophila* midgut barriers and migrated to the hemolymph, did not induce
392 systemic immunity since most bacterial cells were directly engulfed by hemocytes (24). In
393 contrast, the M3 symbiont *Burkholderia* HK1 that migrated to the hemolymph in *R. pedestris*,
394 activated a systemic immune response of the fat body despite the fact that these *Burkholderia*
395 HK1 cells were also phagocytosed by hemocytes (Fig. 5f). Then, what accounts for this
396 difference in the immune response against gut-breaching bacteria between *Drosophila* and *R.*
397 *pedestris*? One possibility is variations in the velocity and efficiency of phagocytosis by
398 hemocytes between the insect species. Additionally, it is plausible that *Burkholderia* HK1
399 possess some mechanisms enabling their survival within the hemocoel, evading complete
400 elimination through phagocytosis and then priming systemic immunity of the bean bug.

401 Another possibility is that *Burkholderia* HK1 penetrates the gut epithelia with greater
402 frequency and quantity, thereby consistently priming the fat bodies. Indeed, since *R. pedestris*
403 lacks a peritrophic membrane, gut-colonizing bacteria could more easily penetrate the
404 intestinal epithelium than in other insects with a peritrophic membrane. Alternatively, the
405 difference could be caused by different immune regulations between holometabolous and
406 hemimetabolous insects. The immunity of hemimetabolous insects has been poorly studied,
407 but it is clear that it is distinct from that of holometabolous insects (52, 53). In *Drosophila*,
408 Gram-negative and Gram-positive bacteria are recognized by the IMD and Toll pathways,
409 respectively (3, 54). However, many hemimetabolous insects lack key components of the
410 IMD pathway, such as Fadd, Relish and Imd (52, 53). A recent study of a hemimetabolous
411 insect, the brown-winged green stinkbug *Plautia stali* (Hemiptera: Pentatomidae), revealed
412 that the IMD and Toll pathways are functionally intertwined to possibly create an immune
413 pathway with new functionalities (55). In this stinkbug species, Gram-negative and Gram-
414 positive bacteria are recognized by both the IMD and Toll pathway. Therefore, the different
415 immune response regulation pathway in the bean bug could account for the activated systemic
416 immunity by phagocytosed *Burkholderia* HK1 cells. Despite these differences, it is important
417 to note that immune priming is also frequently observed in insects that possess a peritrophic
418 membrane. In these insects, immune priming may not result from bacteria directly entering
419 the hemolymph but could instead be initiated by other immune-stimulating molecules, such as
420 peptidoglycan (56). However, the molecular mechanism of how these molecules can prime
421 insect immunity should be elucidated in future studies.

422 Wild populations of the bean bug necessarily acquire a large number of non-symbiotic
423 bacteria from the environment when the insect obtains its *Caballeronia* M4 symbiont from the
424 soil. Indeed, the proportion of bacteria belonging to the genus *Caballeronia* in field-collected
425 soil was less than 0.1% (57). In other words, more than 99.9% of soil-living bacteria are not
426 M4 symbionts. Despite the high proportion in the soil of bacteria that are not M4 symbionts,
427 *R. pedestris* winnows out bacteria through the highly efficient CR that functions as a
428 symbiont sorting organ (32). Since most bacteria can't pass the CR, they are stuck in the M3
429 region located anteriorly to the CR, making up the microbiome of the M3. Insect gut
430 microbiota generally have diverse beneficial effects on the host such as assisting food
431 digestion, providing nutrients, detoxification, enhancing immunity, and many others (58-63).
432 The composition of insect gut microbiota is immensely affected by conditions such as host
433 habitats and food source (64). The M3-microbiota of *R. pedestris* is also different across
434 $\text{Sym}^{\text{control}}$, Sym^{soil} , and Sym^{wild} insects (Fig. 1b). Although the composition of bacteria in the
435 M3 at the genus level differed between the three groups of bean bugs, in each case a few

436 specific genera seemed to dominate the population. Even though the specific microbiota of
437 the soil in this study has not been characterized, the M3-microbiota associated with the bean
438 bug, irrespective of the source of infection, is relatively simple when compared to the known
439 complexity and diversity of general soil microbiota (65) (Fig. 1b), because of multiple
440 selection layers inside the gut, including nutrients, pH, oxygen level, and antimicrobial
441 peptides (49, 66-68). Another notable point is that, in Sym^{soil} bean bugs, diverse bacteria
442 including *Burkholderia s.s.*, *Paraburkholderia*, and *Cupriavidus* were isolated from
443 hemolymph in soil-reared nymphs (Table S2), strongly suggesting that these bacteria breach
444 gut epithelia and thereby prime immunity. Although further inspections are still needed,
445 diverse bacterial species may play a pivotal role in immunity in field bean bug populations as
446 well. Bean bugs are constantly exposed to bacteria that could potentially prime their
447 immunity throughout their lives. For example, bean bugs routinely interact with environments
448 teeming with microbes, including soil and plant surfaces, and they consume microbial-
449 contaminated foods.

450 Insect guts are generally inhabited with more or less complex microbiota, which provide
451 numerous and diverse services to their host, such as synthesizing nutritional supplements,
452 facilitating the degradation of food, detoxifying environmental toxins and providing
453 colonization resistance (69). Although the penetration of gut epithelia has been reported for
454 some enteropathogenic bacteria (24, 25), our study provides an example of gut-breaching by a
455 member of the soil-derived gut microbiota, whereby the bacterial species primes host
456 immunity without obvious harmful effects to the host insect. This type of gut symbiotic
457 bacteria has not yet been well studied and further molecular, immunological and histological
458 studies remain needed in the future. Our study not only highlights how insects employ soil
459 bacteria to cope with pathogens but also provides a new, symbiotic perspective on bacterial
460 intestinal barrier breaching, which has been generally discussed only in terms of pathology.

461

462 **Materials and methods**

463 **Insect rearing and symbiont infection**

464 The bean bug *R. pedestris* was originally collected from a soybean field of Tsukuba, Ibaraki,
465 Japan, and reared in laboratory conditions for over ten years. The bean bugs were maintained
466 under a long-day regimen (16 h light and 8 h dark) at 25 °C in Petri dishes (90 mm diameter
467 and 20 mm height). They were supplied soybean seeds and distilled water containing 0.05%
468 ascorbic acid (DWA) which were periodically renewed. The bean bugs were infected with gut
469 symbiont *C. insecticola* through diluted bacterial solution in DWA (10^7 cells/ml) at the second
470 instar nymphal stage. To produce soil-infected Sym^{soil} insects, second instar nymphs of the

471 bean bug were infected by a suspension of soil collected from Sapporo, Japan. A total of 1
472 gram of soil was placed on the cotton pads, which was moistened by adding distilled water
473 (DWA) to the soil-covered pads.

474

475 **Measurement of host survival rate, development, and fitness parameters**

476 The survival rate of insects was assessed by daily counts of carcasses. Growth rate was
477 determined by the time taken to reach adulthood. Fitness parameters, specifically dry body
478 length and weight, were measured in adult insects. Detailed information for these
479 measurements and for determining the survival rate following bacterial injection can be found
480 in *SI Appendix, Materials and Methods*.

481

482 **Construction of RFP-labelled bacteria**

483 *Burkholderia* HK1 and *Achromobacter* HK2 were genetically engineered to express RFP by
484 introducing the pIN29 plasmid, which carries the *dsRed* gene. The construction process is
485 detailed in *SI Appendix, Materials and Methods*.

486

487 **16S rRNA amplicon sequencing**

488 The gut microbiota of the M3 and M4 regions of Sym^{control}, Sym^{soil}, and Sym^{wild} bean bugs
489 were analyzed through 16S rRNA sequencing, specifically focusing on the variable region 4
490 (V4), using an Illumina iSeq 100 sequencer. Detailed information for sequencing and
491 microbiota analyses are available in *SI Appendix, Materials and Methods*.

492

493 **CFU assays from host midgut and hemolymph**

494 The quantity of M3-colonizing *Burkholderia* HK1 or *Achromobacter* HK2 cells was
495 determined by spreading the M3 lysate of insects infected with RFP-expressing bacteria onto
496 yeast-glucose (YG) agar plates supplemented with 15 µg/ml of chloramphenicol and counting
497 the colonies after 2 days incubation at 30 °C. To measure the number of *Burkholderia* HK1
498 and *Achromobacter* HK2 cells in the hemolymph, 10⁷ cells/ml of RFP-expressing bacterial
499 cells were orally administered to the fifth instar nymph of Sym^{control} bean bugs. Following 12
500 h, 24 h, or 48 h of oral infection, 2 µl of the hemolymph was extracted by cutting antenna and
501 forelegs and collecting leaking hemolymph with a micropipette. The obtained hemolymph
502 was immediately mixed with 100 µl of phosphate-buffered saline (PBS), and this suspension
503 was spread on YG agar plates with 15 µg/ml of chloramphenicol. After 2 days of incubation
504 at 30 °C, the number of colonies were counted.

505 To isolate gut-breached bacteria, the hemolymph of Sym^{soil} third instar nymphs was collected.

506 The collected hemolymph was suspended in 100 µl of PBS, spread on YG agar, and incubated
507 at 30 °C for two days. Bacterial colonies were then subjected to colony PCR using universal
508 primers for the 16S rRNA gene, and approximately 1,400 bp were sequenced for bacterial
509 identification. As a control, Sym^{Control} insects fed only sterilized DWA were examined in the
510 same manner to confirm that no bacterial colonies were detected in their hemolymph.

511

512 **Stress-sensitivity tests**

513 Different concentrations of chemical reagents (EtOH, NaCl, SDS, polymyxin B, proteinase K,
514 and Twen 20) were diluted in PBS and mixed with approximately 10⁵ cells of either *in vitro*
515 cultured or *in vivo* M3-colonizing *Burkholderia* HK1 cells. After 1 h incubation, the number
516 of viable bacteria was counted by CFUs assay. Detailed information is provided in [SI](#)
517 [Appendix, Materials and Methods](#).

518

519 **Quantitative PCR**

520 The number of M4-colonizing *C. insecticola* symbionts in Sym^{control}, Sym^{Bu}, and Sym^{Ab}
521 insects were quantified through qPCR. This was performed using the BSdnaA-F and
522 BSdnaA-R primer set (Table S3). In addition, the expression levels of three AMPs (*riptocin*,
523 *defencin*, and *thanatin*) were measured by qPCR. Detailed information is available in [SI](#)
524 [Appendix, Materials and Methods](#).

525

526 **Immune suppression**

527 To suppress the humoral and cellular immunity in bean bugs, two methods were employed:
528 RNAi of the *relish* gene was used to inhibit humoral immunity, and the saturation of
529 hemocytes with injected microbeads was implemented to suppress cellular immunity.
530 Detailed information is available in [SI Appendix, Materials and Methods](#).

531

532 **Infection of *Burkholderia* HK1 and *Achromobacter* HK2 cells**

533 To make *Burkholderia* HK1-infected (Sym^{Bu}) and *Achromobacter* HK2-infected (Sym^{Ab})
534 insects, second instar nymph *R. pedestris* were initially infected with cultured *C. insecticola*
535 gut symbiont. Two days post symbiont infection, these insects were subsequently infected
536 with 10⁷ cells of either cultured *Burkholderia* HK1 or *Achromobacter* HK1 cells. The
537 bacterial cells were supplied to the insects through their water source by suspending them in
538 DWA. To make Sym^{Bu>DWA} bean bugs, the bacterial solution was replaced with DWA, which
539 did not contain any bacteria, two days after the infection with *Burkholderia* HK1 or
540 *Achromobacter* HK2 cells.

541

542 **Microscopy analyses**

543 i) Fluorescence microscopy

544 The midgut colonized by bacterial cells was isolated in PBS, promptly transferred to a glass-
545 bottom dish (Matsunami), and subsequently covered with a cover glass. The GFP or RFP
546 signal, originating from the gut-colonizing bacteria, was observed with an epifluorescence
547 microscope (DMI4000B; Leica). To observe the FITC-derived fluorescence of beads engulfed
548 by host hemocytes, the hemolymph was collected as described above and mixed with 100 ul
549 of PBS. Hemocytes were then harvested by centrifuging the solution at 300 rpm for 10 mins
550 at 4 °C. The supernatant was carefully discarded, and the pellet was resuspended in 20 ul of
551 PBS. The prepared samples were deposited onto a glass slide, covered with a cover slip, and
552 the phagocytosed beads were then observed with an epifluorescence microscope.

553 ii) Confocal microscopy

554 The M3 region of *R. pedestris* was dissected in PBS 12 h or 24 h post-infection with
555 *Burkholderia* HK1 and transferred into a 2 mL microcentrifuge tube. The midgut samples
556 were fixed by 4% paraformaldehyde in PBS for 15 min at room temperature (RT) and then
557 permeabilized using PBS containing 0.5% Triton X-100 (PBST) for 10 minutes at RT.
558 Subsequently, the samples were stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo
559 Fisher Scientific) for host nuclei and Alexa Fluor™ 647 Phalloidin (Thermo Fisher Scientific)
560 for the host cytoskeleton, for 20 minutes at RT. To observe hemocytes and phagocytosed
561 *Burkholderia* HK1 cells, hemolymph was extracted as described above from the HK1-
562 infected insects and immediately fixed and stained with DAPI for 10 min. After staining, the
563 samples were rinsed three times with fresh PBS. The prepared samples were then placed on a
564 glass-bottom dish, mounted by ProLong Gold Antifade Mountant (Thermo Fisher Scientific),
565 and covered with a cover slip. Observations were made using a confocal microscope with a
566 40x magnification oil objective (x40/1.3 HC PL Apo CS oil).

567 iii) Transmission electron microscopy

568 To observe bacterial morphology, *in vitro* *Burkholderia* HK1 cells were prepared by
569 inoculating pre-cultured bacteria, which had been cultivated overnight, into fresh YG liquid
570 media and then cultivating it an additional 8 hours. *In vivo* *Burkholderia* HK1 cells were
571 prepared by dissecting the M3 of fifth instar Sym^{Bu} bean bugs in PBS, homogenizing the
572 sample with a pestle, and subsequently filtering it through a 5 µm pore size filter to eliminate
573 gut debris. To observe gut-colonizing bacteria as well as epithelia-breaching bacteria, M3
574 samples of Sym^{control} and Sym^{Bu} bean bugs were prepared by isolating the midgut region in a
575 fixative solution (0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde, pH 7.4).

576 Each of these prepared samples was prefixed in the fixative solution at 4 °C overnight and
577 then postfixed in 2% osmium tetroxide at 4 °C for 1 h. After undergoing a series of
578 dehydration steps with ethanol, each sample was embedded in Epon812 resin (TAAB).
579 Ultrathin sections were made using an ultramicrotome (EM UC7; Leica), placed onto a
580 copper mesh, stained with uranyl acetate and lead citrate, and observed under a transmission
581 electron microscope (H-7600; Hitachi).

582

583 **Statistical analyses**

584 Statistical significance for host natural survival, growth period, fitness parameters, gene
585 expression levels of AMPs, the count of bacteria and host hemocytes, and the number of eggs
586 laid was determined using the Mann-Whitney *U* test with Bonferroni correction. For the host
587 survival rate after bacterial injection into the hemolymph, the Log-rank test was used for two
588 samples, and the Log-rank test with Bonferroni correction was applied for more than three
589 samples.

590

591 **Data availability**

592 The 16S rRNA nucleotide sequence of gut commensal bacterium, *Burkholderia* HK1, have
593 been deposited in the GenBank nucleotide database with accession number OR451220. Raw
594 Illumina sequencing data of M3 and M4 microbiome from *R. pedestris* have been deposited in
595 the DDBJ Sequence Read Archive (DRA) with accession codes SAMD00637910 to
596 SAMD00637945.

597

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605

606 **Author contributions**

607 SJ and YK designed the study. SJ, PM, and YK developed the study. SJ and KI performed
608 experiments. SJ analyzed the sequencing data. SJ, PM and YK wrote and revised the
609 manuscript. All authors checked and edited the manuscript before submission.

610

611 **Competing interest statement**

612 The authors declare that they have no competing interests.

613

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Main Manuscript for

Break through the barrier: Ingested soil bacteria breach gut epithelia and prime systemic immunity in an insect

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Tables S1 to S3

Supplementary text

Supporting Materials and Methods

Measurement of host survival rate, development, and fitness parameters

The survival rate of bean bugs was evaluated from the second instar to the adult stage by counting the number of dead insects every day. The growth rate was determined by measuring the duration required to reach adulthood post hatching. Five days after molting to the adult stage, the bean bugs were immersed in acetone and thoroughly dried; their dry weight and body length were subsequently measured using an electronic scale and ruler respectively. Regarding the survival rate of bean bugs upon infection by *P. entomophila*, the number of dead insects were counted daily. Host fecundity was assessed by counting the eggs laid by female Sym^{control} and Sym^{Bu} insects. Two male and one female bean bugs were paired together to encourage mating. Mated females begin oviposition 6 to 7 days after molting to adulthood. Upon commencement of egg-laying, the egg count was recorded daily for a week.

Construction of RFP-labelled bacteria

Burkholderia HK1 and *Achromobacter* HK2 were engineered to express the RFP by introducing the pIN29 plasmid, which contains the *dsRed* gene. This introduction was facilitated through electroporation using the Ec2 setting of a MicroPulser Electroporator (Bio-Rad). As the pIN29 has a marker gene for chloramphenicol resistance, successfully transformed bacterial cells were identified by culturing the electroporated cells on YG agar media with 15 µg/ml of chloramphenicol. Due to the regulation of the *dsRed* gene by the potent pTAC promoter in pIN29, pronounced *dsRed* expression was observed, leading to a distinct red coloration to the bacterial cells. The presence of the red fluorescence in the transformed HK1 or HK2 colonies was confirmed using the LED-EXTRA equipped with a red filter (OptoCode).

16S rRNA amplicon sequencing and analysis of gut microbiota

DNA was extracted from the M3 or M4 midgut regions of Sym^{control}, Sym^{soil}, and Sym^{wild} bean bugs by the QIAamp DNA Mini Kit. The variable region 4 (V4) of bacterial 16S rRNA gene was amplified by PCR using universal primer set 515F-806R and Q5 High-Fidelity DNA polymerase. The PCR product was amplified again by using 515F primer extended with Illumina P5 sequences, 806R primer extended with 6-bases indexes

and Illumina P7 sequences (1), and Q5 High-Fidelity DNA polymerase (New England BioLabs). The PCR product was purified by AMPure XP beads (Beckman Coulter), the target DNA was confirmed by gel electrophoresis, and then purified again using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA libraries containing all tagged amplicons and internal controls (phiX) were prepared for paired-end sequencing using iSeq 100 i1 Reagent v2 (Illumina) and sequenced on an Illumina iSeq 100 sequencer.

The paired-end (2 x 151 bp) reads obtained from Illumina iSeq 100 were analyzed by USEARCH (2). Consensus forward and reverse reads were assembled using the '-fastq_mergepairs' command and low-quality sequences (Q score < 30) were filtered out with the '-fastq_filter' command. Unique sequences were identified by the '-fastx_uniques' command and then clustered into operational taxonomic units (OTUs) using the '-cluster_otus' option. Incorrectly joined chimeric sequences were then removed and biologically meaningful amplicon sequence variants (ASVs) were identified using the UNOISE3 algorithm (3). Bacterial taxonomy of ASVs was assigned by investigating the similarity of the V4 region of 16S rRNA sequences with the EzBioCloud database (4) using the amplicon sequence as a query.

Stress-sensitivity tests

Various concentrations of chemical reagents (EtOH, NaCl, SDS, polymyxin B, proteinase K, and Tween 20) were diluted in PBS and mixed with approximately 10^5 cells of *in vitro* cultured or *in vivo* M3-colonizing *Burkholderia* HK1 cells. In the case of *in vivo* cells, bacteria were extracted from the M3 of 10 individual Sym^{Bu} bean bugs. Since the colonization rate of *Burkholderia* HK1 cells in each individual insect can vary, the number of *in vivo* cells used for sensitivity test differed from sample to sample. The bacteria-chemical mixtures were incubated at 30 °C for 1 h, and 10 µl of the serially diluted suspension was spotted onto YG agar plates supplemented with 15 µg/ml of chloramphenicol. The number of bacterial colonies was counted, and the relative bacterial growth was calculated by normalizing the number of reagent-treated cells to the number of untreated bacterial cells.

Quantitative PCR

To quantify the number of *C. insecticola* symbionts from the M4 region of Sym^{control}, Sym^{Bu}, and Sym^{Ab} insects, the bean bugs were dissected in PBS under a stereomicroscope and their M4 was isolated. Total DNA was extracted from the isolated M4 using QIAamp DNA mini kit (Qiagen Inc.). A real-time quantitative PCR was carried out using the primer set of BSdnaA-F and BSdnaA-R (Table S3), the KAPA SYBR fast qPCR master mix kit, and the Roche LightCycler 96 system to amplify the single-copy *dnaA* gene of *C. insecticola* gut symbiont. The number of gut-colonizing *C. insecticola* symbionts was determined based on a standard curve for the *dnaA* gene containing 10, 10², 10³, 10⁴, 10⁵, 10⁶, and 10⁷ copies per reaction of the target PCR product.

To assess the expression levels of AMPs (*riptocin*, *defencin*, and *thanatin*), bean bugs were dissected in PBS, and their midgut M3, M4, and fat bodies were collected and transferred into 1.5 ml microcentrifuge tubes containing 200 µl of RNAiso Plus (Takara Bio Inc.). The transferred organs were then homogenized using a pestle, and an additional 300 µl of RNAiso and 100 µl of chloroform were sequentially added to the tubes. Total RNA was extracted using RNeasy mini kit (Qiagen Inc.) and cDNA was synthesized using PrimeScript™ RT reagent kit with gDNA erase (Takara Bio Inc.). RT-qPCR was conducted using the primers listed in Table S3 to detect relative expression levels of the three AMPs. The gene expression of each AMP was normalized to the expression level of the housekeeping gene elongation factor 1 alpha (*ef1a*).

Immune suppression

i) Suppression of humoral immunity by RNAi

To silence *relish*, total RNA was extracted from the fat bodies of fifth instar nymphs by using RNAiso Plus (Takara Bio.) and the RNeasy Mini Kit (Qiagen) as described above. Then cDNA was synthesized from 1 µg of the extracted total RNA with the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio.). A 525 bp of the *relish* gene was amplified from the cDNA template by primers listed in Table S3. The PCR product was subsequently cloned to the pT7Blue-T-vector and transformed into *E. coli* DH5α competent cells (Invitrogen). Positive clones were verified by colony PCR and a cloned plasmid was extracted from *E. coli* cells. Target DNA was amplified by PCR from

cloned plasmid using specific upstream and downstream primers, each extended with the T7 promoter sequences (Table S3) to create the template for RNA polymerization. The MEGAscript RNAi kit (Thermo Fisher Scientific) was then used to synthesize the dsRNA targeting *relish*. In total, 100 ng of the synthesized dsRNA was injected directly into the hemocoel of fourth instar nymphs via a glass capillary and the Femtojet microinjector (Eppendorf) to suppress *relish* mRNA expression.

ii) Suppression of cellular immunity

Host cellular immunity was suppressed by saturating hemocytes through the injection of approximately 10^8 fluorescence latex beads/ μl into the hemolymph prior to the bacterial injection. The injected beads are rapidly engulfed by host hemocytes, leading to a notable decrease in immunologically active hemocytes within the hemolymph.

Bacterial pathogenicity test

All bacterial strains examined in this study were injected into the fifth instar nymphs. The protective effects of gut commensal bacteria were investigated by injecting 10^6 cells/ $2 \mu\text{l}$ of *P. entomophila* into the hemocoel of bean bugs. The insecticidal effect of *Burkholderia* HK1 cells was evaluated following the injection of 10^3 or $10^6/2 \mu\text{l}$ bacterial cells. The pathogenicity of other Gram-negative (*E. coli* strain JM109) and Gram-positive bacteria (*M. luteus* and *S. aureus*) was assessed by injecting 10^6 cells/ $2 \mu\text{l}$ of bacterial cells into the host's hemolymph. We tracked host survival rates by counting dead individuals daily.

To determine whether Sym^{Bu} insects are resistant or tolerant against the pathogen infection, changes in the number of *P. entomophila* in hemolymph after injection were measured by counting CFUs. The antennae and legs of the fifth instar Sym^{Bu} nymphs were removed 2, 12, and 24 hours after the injection of 10^6 cells of the *P. entomophila*, and $2 \mu\text{l}$ of hemolymph was collected. The hemolymph was suspended in $100 \mu\text{l}$ of PBS, spread on YG agar, and incubated at $30 \text{ }^\circ\text{C}$ for two days. Then the number of grown colonies was counted. As a control, the pathogen reduction in Sym^{control} insects was measured.

Phylogenetic analysis

The 16S rRNA sequences of the isolated *Burkholderia* HK1 and other bacteria belonging to related genera (*Burkholderia* s.s., *Caballeronia*, and *Paraburkholderia*)

were aligned using the MAFFT program. The maximum likelihood phylogenies of these sequences were inferred using the MEGA X software, with *Pandoraea pulmonicota* serving as an outgroup bacterial species. Bootstrap tests were carried out with 1000 replications. The resulting phylogenetic tree was visualized using the iTOL tool.

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Supplementary figures

Figure S1

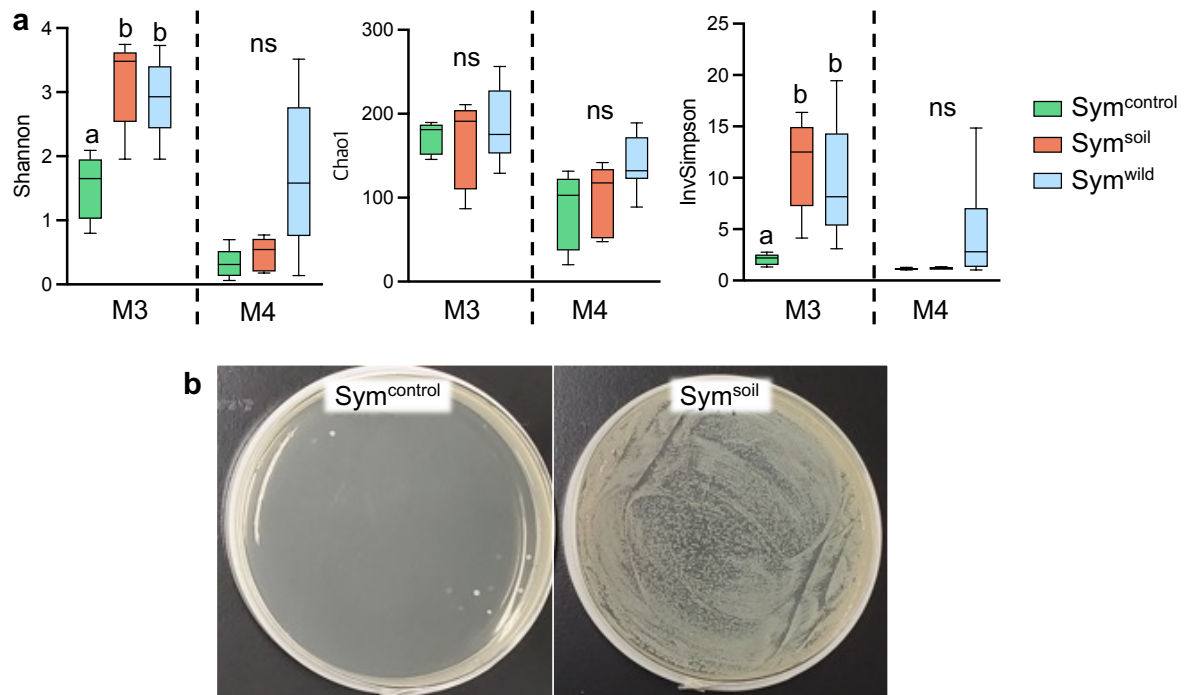


Fig S1. Diversity of M3 and M4 microbiota.

a Alpha diversity of M3 and M4 microbiota is analyzed by Shannon, Chao1, and InvSimpson indices. The statistical significance of was analyzed by the Mann-Whitney U test with Bonferroni correction. **b** Colony formation on nutrient agar by spreading homogenized M3 lysate of Sym^{control} and Sym^{soil} bean bugs.

Figure S2

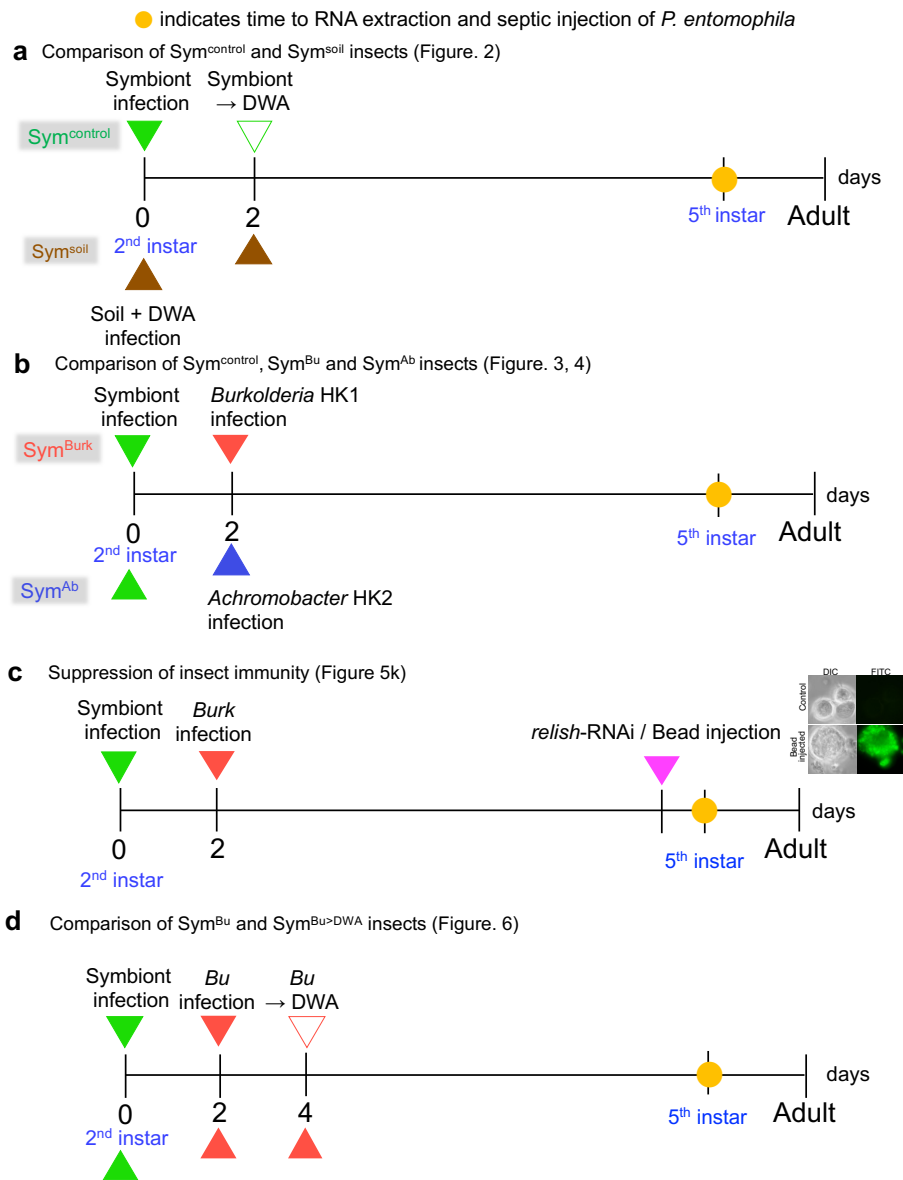


Fig S2. Experiment design of bacterial oral infection and pathogen injection used in this study.

a Experiment design for comparison of $Sym^{control}$ and Sym^{soil} insects (see Fig. 2). **b** Experiment design for comparison of $Sym^{control}$, Sym^{Bu} , and Sym^{Ab} insects (see Fig. 3, 4). **c** Timing of immune suppression by *relish*-RNAi or microbeads injection (see Fig. 5k). **d** Experiment design for comparison Sym^{Bu} and $Sym^{Bu>DWA}$ insects (see Fig. 6). Yellow circle indicates timepoint of RNA extraction or septic injection of *P. entomophila*.

Figure S3

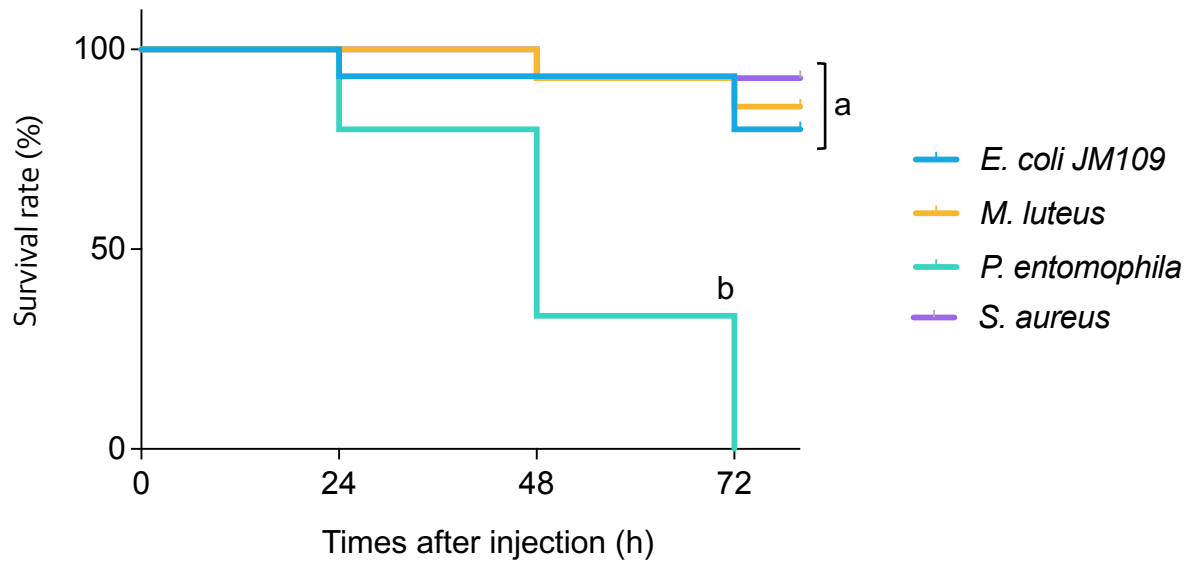


Fig S3. Pathogenicity of Gram-negative and Gram-positive bacteria to bean bug.

The 10^6 cells of Gram-negative bacteria (*E. coli* JM109 and *P. entomophila*) and Gram-positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus* RN4220) were injected to the hemocoel of bean bugs and the survival rate of insects were measured. Different letters indicate statically significant differences ($P < 0.05$). The statistical significance was analyzed by the Log-rank test with Bonferroni correction.

Figure S4

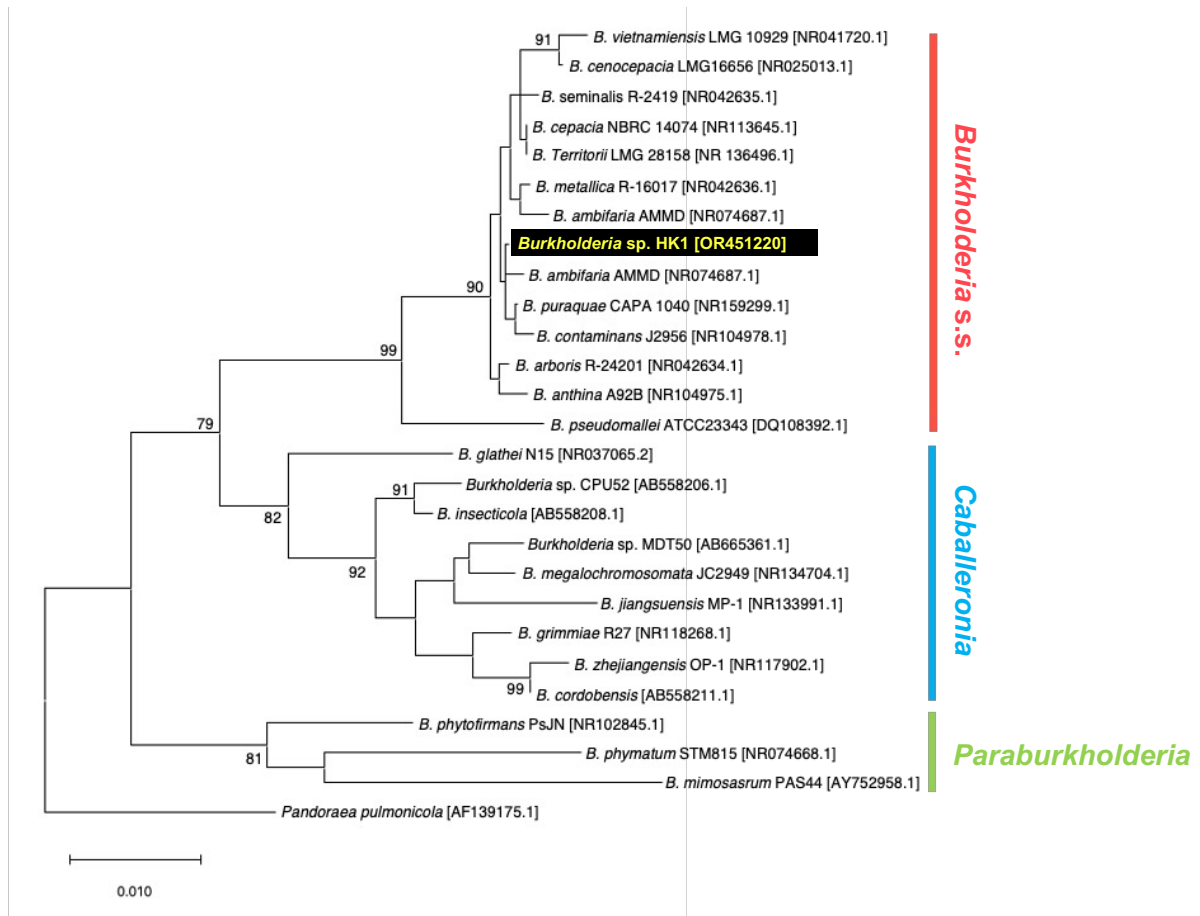


Fig S4. Phylogenetic position of *Burkholderia* HK1 in the *Burkholderia sensu lato* clade

Burkholderia sp. HK1, isolated from M3 of soil reared *R. pedestris*, belongs to the genus *Burkholderia sensu stricto*. A maximum likelihood tree was constructed based on 16S rRNA gene sequences of HK1 and phylogenetically close bacteria. Bootstrap values (1,000 repetitions) are shown at the nodes.

Figure S5

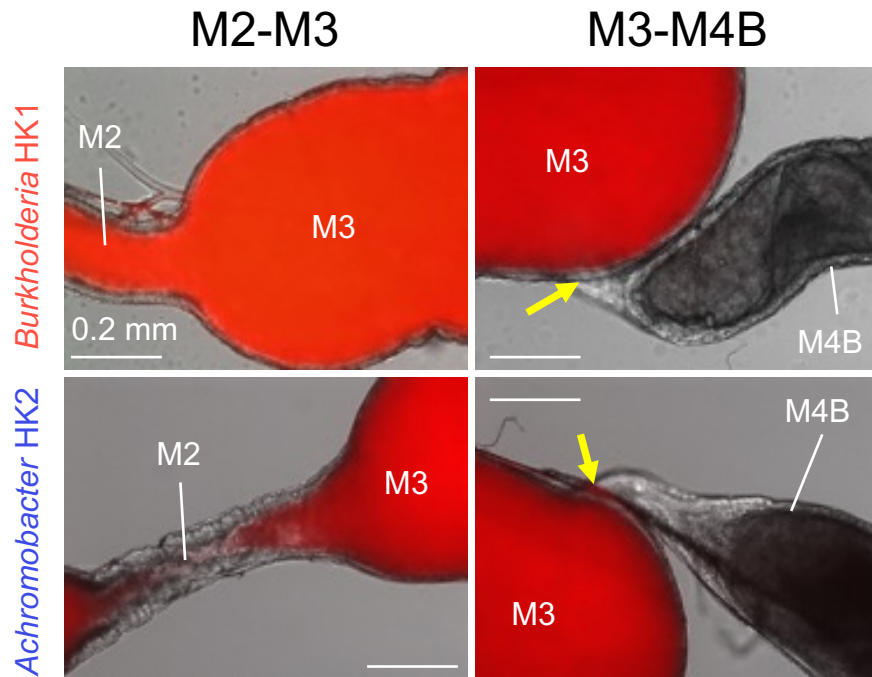


Fig S5. Localization of *Burkholderia* HK1 and *Achromobacter* HK2 in the midgut of *R. pedestris*.

RFP-labelled *Burkholderia* HK1 and *Achromobacter* HK2 were orally administered to aposymbiotic second instar nymphs of *R. pedestris*. After 72 hours, red fluorescent signal was observed in the midgut under an epifluorescence microscope. Both M3 commensal bacteria can not pass the CR and accumulate in the M3 region. Yellow arrows indicate the CR.

Figure S6

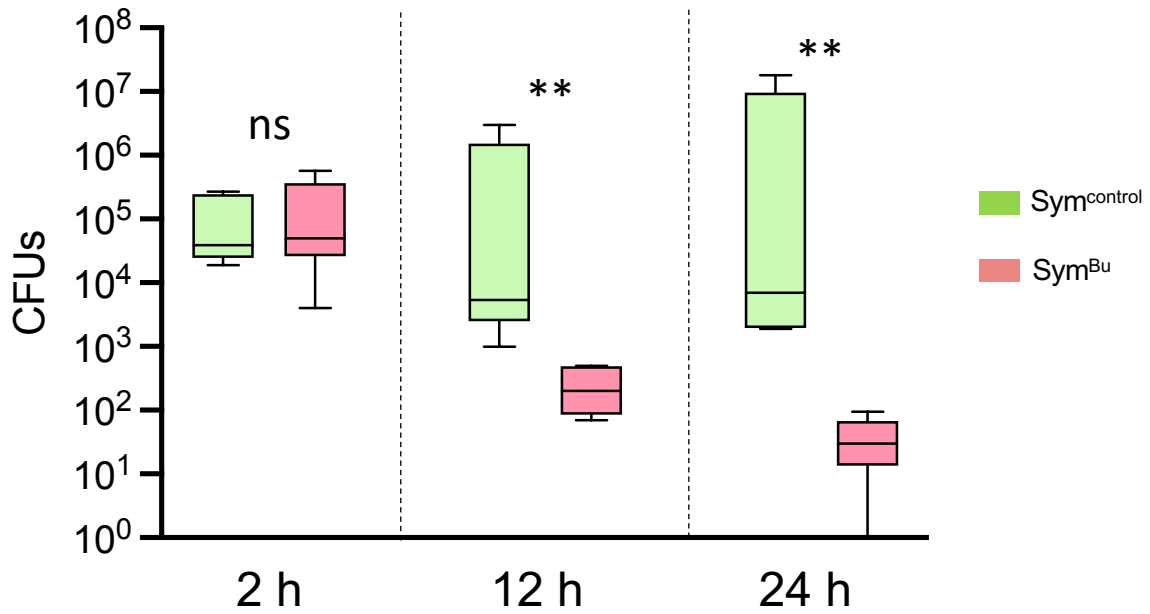


Fig S6. M3 *Burkholderia* symbiont confers resistance against pathogen infection. CFUs of entomopathogen *P. entomophila* were measured by spreading the collected hemolymph from *Sym*^{control} and *Sym*^{Bu} insects after injection of *P. entomophila*. After 12 hours of bacterial injection, a significant reduction in the number of *P. entomophila* in the hemolymph of *Sym*^{Bu} insects was observed compared to *Sym*^{control} insects.

Figure S7

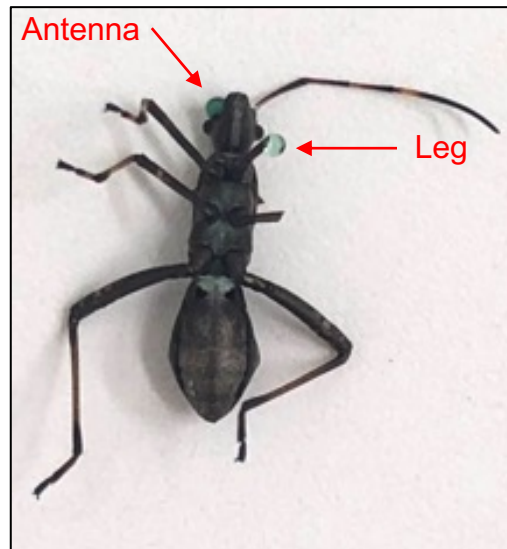
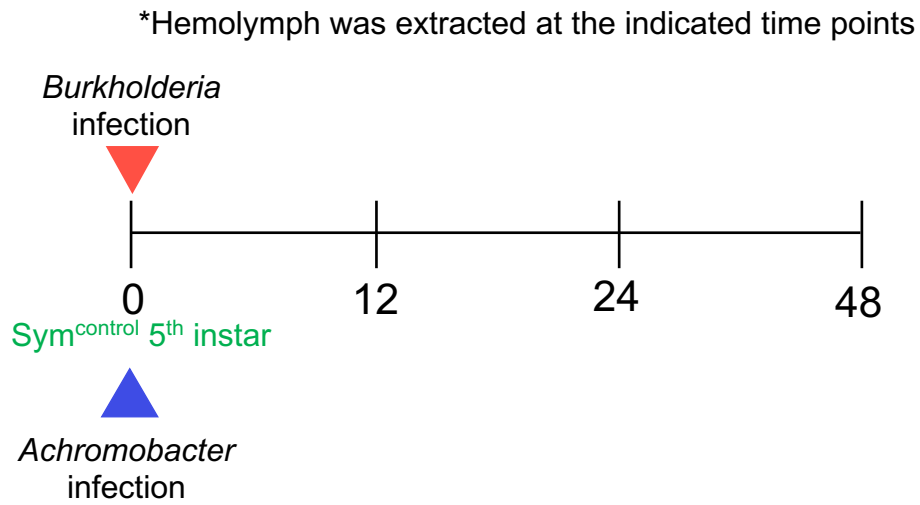


Fig S7. Method for hemolymph collection.

Hemolymph was collected 12, 24, and 48 hours after oral bacterial infection. To extract hemolymph from the body of bean bugs, antenna and forelegs were cut by scissors and leaking hemolymph was collected by a pipette and transferred to a microcentrifuge tube. (See Fig. 5)

Figure S8

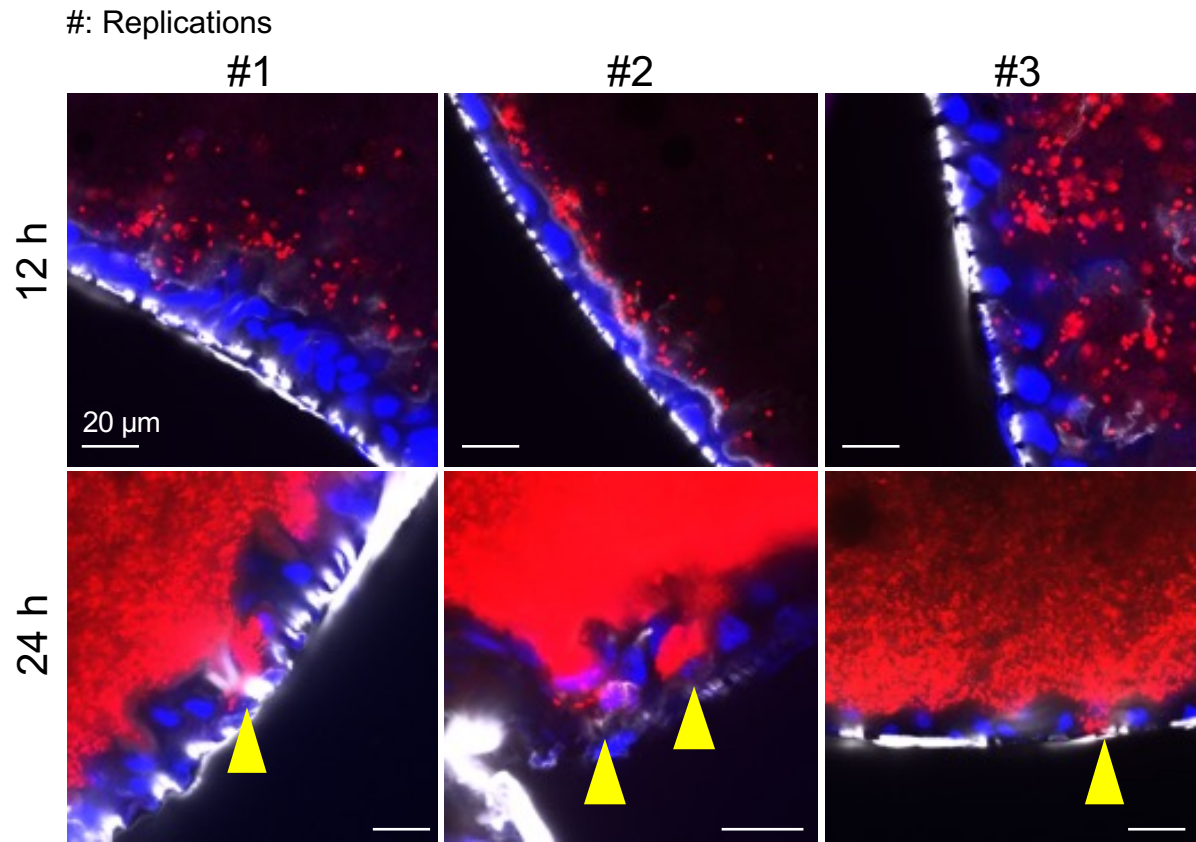


Fig S8. Confocal microscope images of M3-breaching *Burkholderia* HK1 cells (see also Fig. 5b).

RFP-labelled *Burkholderia* HK1 cells were orally administered to the 5th instar of bean bugs. After 12 and 24 hours, the M3 region was isolated through dissection, and the midgut was observed under a confocal microscope. Red, RFP-labelled *Burkholderia* HK1 cells; blue, host nuclei. Yellow arrows indicate bacterial cells located in-between the M3 gut epithelial cells.

Figure S9

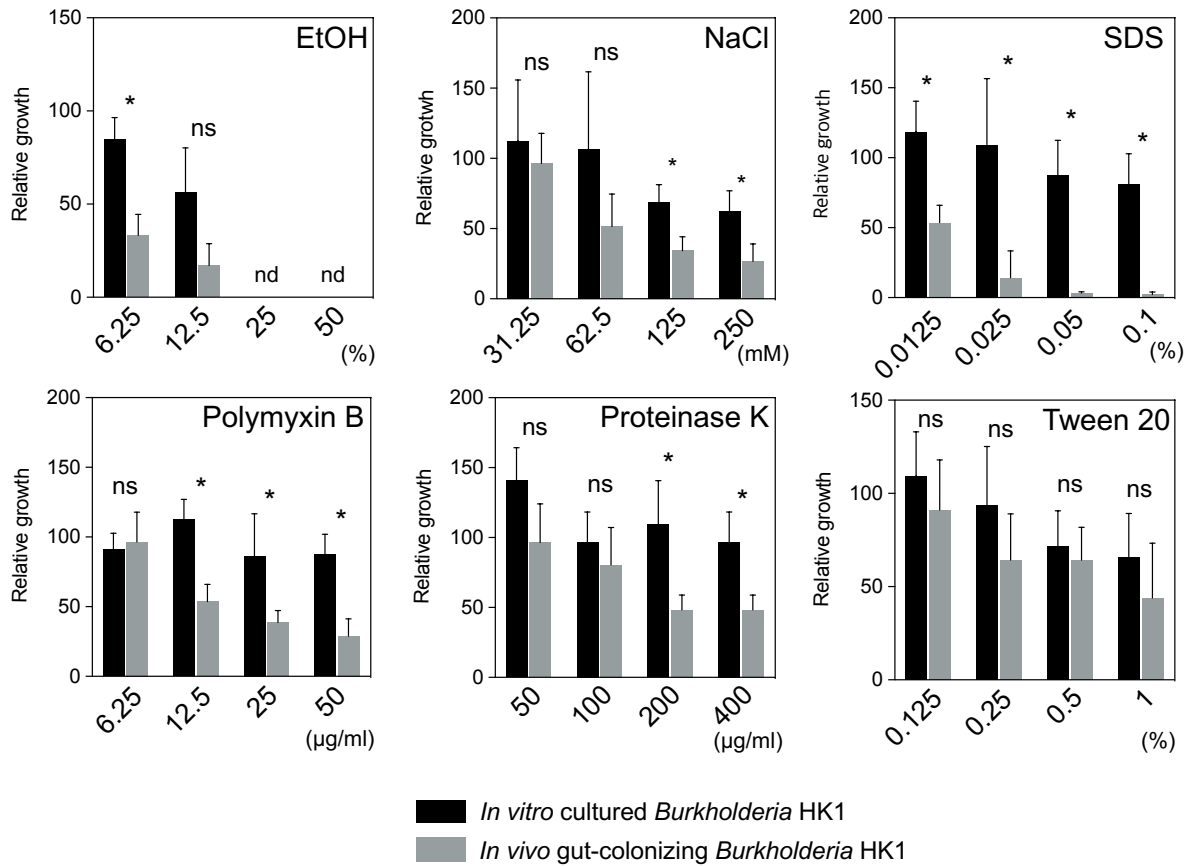


Fig S9. Stress sensitivity test of *in vitro* cultured and *in vivo* M3-colonizing *Burkholderia HK1*.

10^5 cells of *In vitro* cultured and *in vivo* *Burkholderia HK1* were incubated with various concentrations of EtOH, NaCl, SDS, polymyxin B, proteinase K, and Tween 20 for 1 hour. After incubation, bacteria-chemical mixtures were serially diluted, and each solution was spotted on YG agar plates. The number of colonies that appeared was counted, and the relative number of colonies, compared to non-treated control cells, was calculated. The statistical significance was analyzed by the Mann-Whitney *U* test with Bonferroni correction.

Supplementary tables

Table S1

Strain name	Sequence completeness*	Closest species	Query Cover	Identity (%)
HK1	Full (1,428 bp)	<i>Burkholderia contaminans</i> strain J2956 16S ribosomal RNA, partial sequence	100%	98.04%
HK2	Full (1,361 bp)	<i>Achromobacter spanius</i> strain LMG 5911 16S ribosomal RNA, partial sequence	100%	99.63%
HK3	Partial (783 bp)	<i>Burkholderia ambifaria</i> strain AMMD 16S ribosomal RNA, partial sequence	100%	99.87%
HK4	Partial (778 bp)	<i>Burkholderia ambifaria</i> strain AMMD 16S ribosomal RNA, partial sequence	100%	99.61%

*Full 16S rRNA sequence was obtained using the universal primer set 27F and 1492R, while the partial sequence was obtained with the primer set 518F and 1492R.

Table S2

# Colony	Bacterial species	Similarity (%)	Completeness ^a (%)
1	<i>Burkholderia stabilis</i>	99.93	95.4
2	<i>Burkholderia orbicola</i>	99.7	96.2
3	<i>Burkholderia ambifaria</i>	99.93	92.4
4	<i>Cupriavidus basilensis</i>	99.79	96.1
5	<i>Cupriavidus basilensis</i>	99.85	92.6
6	<i>Paraburkholderia strydomiana</i>	99.78	96.2
7	<i>Burkholderia stabilis</i>	99.93	96.4
8	<i>Burkholderia stabilis</i>	99.71	96.3
9	<i>Burkholderia ambifaria</i>	99.93	96
10	<i>Burkholderia ambifaria</i>	99.79	96.5
11	<i>Burkholderia stabilis</i>	99.93	96.3
12	<i>Burkholderia ambifaria</i>	99.71	96.2
13	<i>Burkholderia stabilis</i>	99.93	95.8
14	<i>Cupriavidus basilensis</i>	98.98	95.5
15	<i>Cupriavidus basilensis</i>	99.64	96.1

^aCompleteness (%) indicates the extent to which the length of a 16S rRNA sequence matches that of a reference bacterial sequence

Table S3**Table S3. Primers**

Primer name	Sequence (5' - 3')
27F	AGAGTTTGATCCTGGCTCAG
515F	GTGYCAGCMGCCGCGGTAA
806R	GGACTACNVGGGTWTCTAAT
1492R	GGTTACCTTGTTACGACTT
515F-P5	AATGATACGGCGACCACCGAGTGYCAGCMGCCGCGGTAA
806R-P7	CAAGCAGAAGACGGCATACGAGGACTACNVGGGTWTCTAAT
Ef1a_F	CCTGCATCCGTTGCTTTTGT
Ef1a_R	GGCATCGAGGGCTTCAATAA
Riptocin_F	TCCGAAGCTGAGGGTCTTCCCG
Riptocin_R	TCCGCATCCAAGTTCGCGTCC
Defensin_F	TCGGTCGGACTGAGACTGAA
Defensin_R	TTGCCGCCTTTGTATCCCTT
Thanatin_F	GTCTGCCTTCGTTGAAGACG
Thanatin_R	ATTCGCTTGCAAACGCCG
Relish_dsF	GCTGGCACGAAAACCTACCTAA
Relish_dsR	TGTACATTCTGAGGGCCAAAG
Relish_qF	GCCAGATGAATATTGTCTGAATG
Relish_qR	TAACAGCCGACTGCGAGAG
T7_pT7bR20	TAATACGACTCACTATAGGGGATCTACTAGTCATATGGAT
T7_pT7bU19_2	TAATACGACTCACTATAGGGGACGGCCAGTGAAT
bsDnaA_F	AGCGCGAGATCAGACGGTCGTCTGAT
bsDnaA_R	TCCGGCAAGTCGCGCACGCA