Chemical cues from beetle larvae trigger proliferation and putative virulence gene expression of a plant pathogen

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Abstract

Agricultural crop productivity and global forest biomes are coming under increasing threat from insect pests and microbial pathogens. This impact is worsened by inter-kingdom insect-microbe interactions that can increase transmission and disease severity in affected plants. Whilst bacterial chemical cues have been shown to directly influence insect behaviour, the impact of insect-derived compounds on phytopathogens is poorly understood. Here, we investigated the chemical basis for interactions between beetle larvae and bacteria in acute oak decline (AOD), a disease characterised by inner bark necrosis and involving a polymicrobial consortium including *Brenneria goodwinii* and larval galleries of *Agrilus biguttatus*. We found that *A. biguttatus* larval extracts contain chemical elicitors that increase bacterial growth rate and final cell density during *in vitro* culture, and stimulate the differential expression of ~600 genes, including the Type III Secretion System and its effectors, which are major virulence factors in plant pathogens. Chemical compounds from closely related insect species did not have this effect. These findings highlight the importance of inter-kingdom interactions in plant disease and suggests a novel mode-of-action for insect-derived chemical elicitors in facilitating the virulence of phytopathogens.

Introduction (500 words)

Insect-microbe interactions are increasingly important in the context of plant disease owing to increasing incidence of pest and disease outbreaks, driven in part by international trade and climate change (Coolen et al., 2022; Singh et al., 2023). For example, the six-spotted leafhopper *Macrosteles quadrilineatus* is a vector for bacterial phytoplasmas that cause Aster Yellows disease and in-turn benefits from secreted bacterial effectors that targets the plant defence mechanisms (Sugio et al., 2011). Similarly, *Xylella fastidiosa*, which infects a wide range of plant hosts, is transmitted by xylem sap-feeding insects (Sicard et al., 2018). Volatile organic compounds produced by bacteria have been shown to influence insect behaviour (Davis et al., 2013; Weisskopf et al., 2021), which in turn assists in bacterial dispersion (Robacker et al., 2004). A handful of studies have examined the influence of insect metabolites on bacteria, finding inhibitory mechanisms based on the production of antimicrobial compounds (Hall et al., 2011; Teh et al., 2013). However, weather insect-derived compounds have positive positive effects on bacterial activity, increasing growth and/or virulence has not been thoroughly explored. In one example, honeydew produced by aphids has been shown to increase *Pseudomonas syringae* growth (Smee & Hendry, 2022). In addition to serving as a source of nutrition, bacteria use chemical signals as a primary source of information to coordinate their behaviour at the population level (Miller & Bassler, 2001). As such, our limited understanding of the impact of insect-derived compounds on plant-associated bacteria remain a major knowledge gap.Understanding multi-kingdom interactions in

plant disease is therefore crucial, especially in decline diseases such as Acute Oak Decline (AOD), where insects could play a pivotal role. AOD is a complex decline disease affecting native British oak species (*Quercus robur* and *Quercus petraea*) and recently identified in many European countries (Carluccio et al., 2024; Ruffner et al., 2020; Tkaczyk & Sikora, 2024). It involves the cumulative action of multiple biotic and abiotic factors, such as larvae of bark-boring beetles and bacterial phytopathogens (Denman & Webber, 2009). The disease is characterised by inner bark tissue necrosis caused by a polymicrobial consortium composed of at least three bacterial species (*Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana*), and characterised by the presence of larval galleries of the two-spotted oak buprestid, *Agrilus biguttatus* on the phloem–sapwood interface.

B. goodwinii is recognised as the dominant pathogen within necrotic lesions (Denman et al., 2018) and transcriptomic analysis of *B. goodwinii* in oak stem tissue revealed the up-regulation of putative virulence genes (Doonan et al., 2020). Co-infection of *A. biggutatus* larvae significantly increased the expression of these genes including Type III secretion system effectors *srfB* and *avrE*, known to contribute to virulence and suppression of host immunity in other pathosystems (Worley et al., 2000) (Degrave et al., 2015), and *katG* which detoxifies hydrogen peroxide released as part of the host defence response (Jittawuttipoka et al., 2009). The presence of *A. biguttatus* larvae also increased the area of necrotic tissue caused by bacteria (Denman et al., 2018; Doonan et al., 2020). Taken together, these findings suggest that the interaction between *B. goodwinii* and *A.*

79 *biguttatus* larvae in the inner bark enhances bacteria-induced necrosis and facilitates the
80 proliferation and spread of damaging bacteria within infected trees.

81 Although interactions between *B. goodwinii* and *A. biguttatus* larvae have been
82 demonstrated *in planta* (Denman et al., 2018; Doonan et al., 2020), the mechanism
83 underpinning this interaction remains unknown. In this study, we inoculated oak logs
84 with *A. biguttatus* eggs and collected late-instar larvae and gallery wood tissue from the
85 logs. As *A. biguttatus* larval feeding may chemically modify their environment and/or
86 trigger host responses, we extracted chemicals from asymptomatic as well as gallery
87 wood tissue and the bodies of *A. biguttatus* larvae and independently assessed their
88 bioactivity towards *B. goodwinii* in controlled *in vitro* experiments. We tested three
89 hypotheses: (1) *B. goodwinii* proliferation and virulence gene expression are indirect
90 effects of *A. biguttatus* larval activity and are triggered by chemicals associated with
91 gallery wood tissue; (2) *B. goodwinii* proliferation and virulence gene expression are
92 direct effects of *A. biguttatus* larvae and are triggered by larval chemical elicitors; (3) the
93 interaction between *B. goodwinii* and *A. biguttatus* larvae is specific to *A. biguttatus*. We
94 show that chemical elicitors from *A. biguttatus* larvae (hypothesis 2), but not gallery wood
95 tissue (hypothesis 1), directly trigger an increase in *B. goodwinii* growth and the up-
96 regulation of 307 genes, including effectors involved in bacterial Type III secretion
97 system. Such an effect was not observed with extracts from closely related wood-boring
98 beetle species, suggesting some specificity in this interaction (hypothesis 3). Determining
99 the mechanisms of insect-microbiota interactions and their emergent impacts on plant

100 disease outcomes is critical to inform integrated pest management strategies for globally

101 important plant diseases.

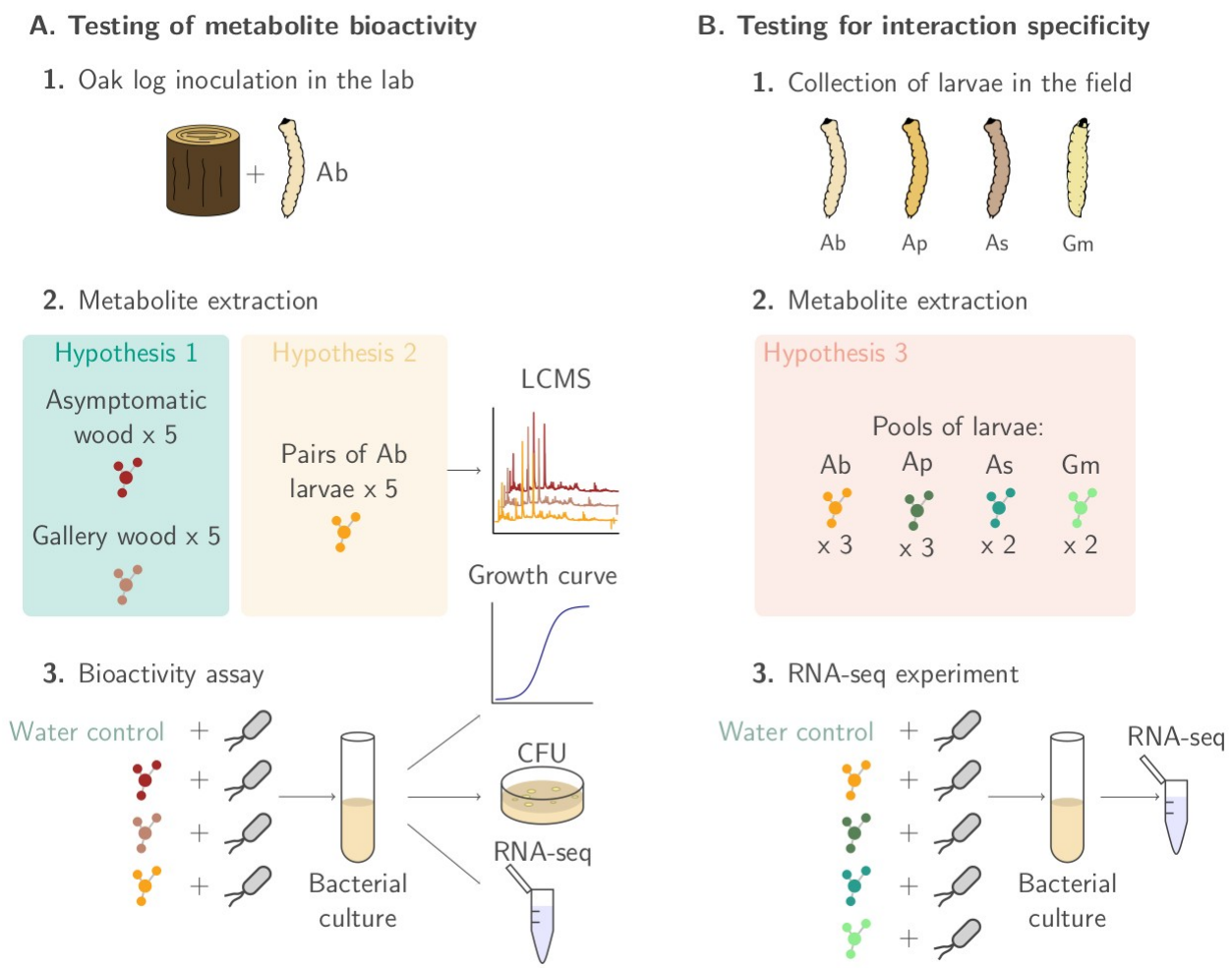

Figure 1: **Summary of the experimental design. A.** For hypotheses 1 and 2, oak logs were inoculated in the lab to yield Ab larvae and wood tissue samples for extraction. **B.** For hypothesis 3, *Agrilus* larvae (Ab, Ap and As) were collected from the field or purchased (Gm). Metabolites from the larvae were extracted and tested for bioactivity on *Brenneria goodwinii* cultures. Ab = *Agrilus biguttatus*; Ap = *A. planipennis*; As = *A. sulcicollis*; Gm = *Galleria mellonella*

102 # Results

103 ## Gallery wood tissue extracts do not increase bacterial proliferation
104 ## compared to healthy wood tissue extracts

105 To test the effect of chemical extracts from galleries made by *A. biguttatus* larvae on *B.*
106 *goodwinii* proliferation (hypothesis 1), we compared bacterial growth in cultures
107 supplemented with asymptomatic wood extract and gallery wood tissue extract
108 (Figure 1A). At 24 hpi (hours post inoculation), cultures reached an OD_{600} of 0.75 ± 0.05,
109 0.78 ± 0.09 and 0.9 ± 0.06 for asymptomatic wood tissue extracts, gallery wood tissue
110 extracts and water control, respectively (Figure 2A). The growth rate of cultures
111 supplemented with gallery wood extracts (0.30 ± 0.04) was significantly lower than
112 those supplemented with asymptomatic wood extracts (0.39 ± 0.03, Figure 2B, pairwise
113 Wilcoxon test, p-value < 0.05). The CFU density at 24 hpi did not differ significantly
114 between gallery wood tissue and asymptomatic wood treatments (12.69 ± 2.5 x 10^8
115 CFU/mL and 12.32 ± 10.0 x 10^8 CFU/mL, respectively Figure 2C) suggesting that *B.*
116 *goodwinii* grows faster with asymptomatic wood extract but the number of living cells in
117 stationary phase is the same for both asymptomatic and gallery wood extracts. These
118 results show that chemicals compounds extracted from gallery wood tissue do not cause
119 more proliferation of *B. goodwinii* than asymptomatic wood chemicals compounds.

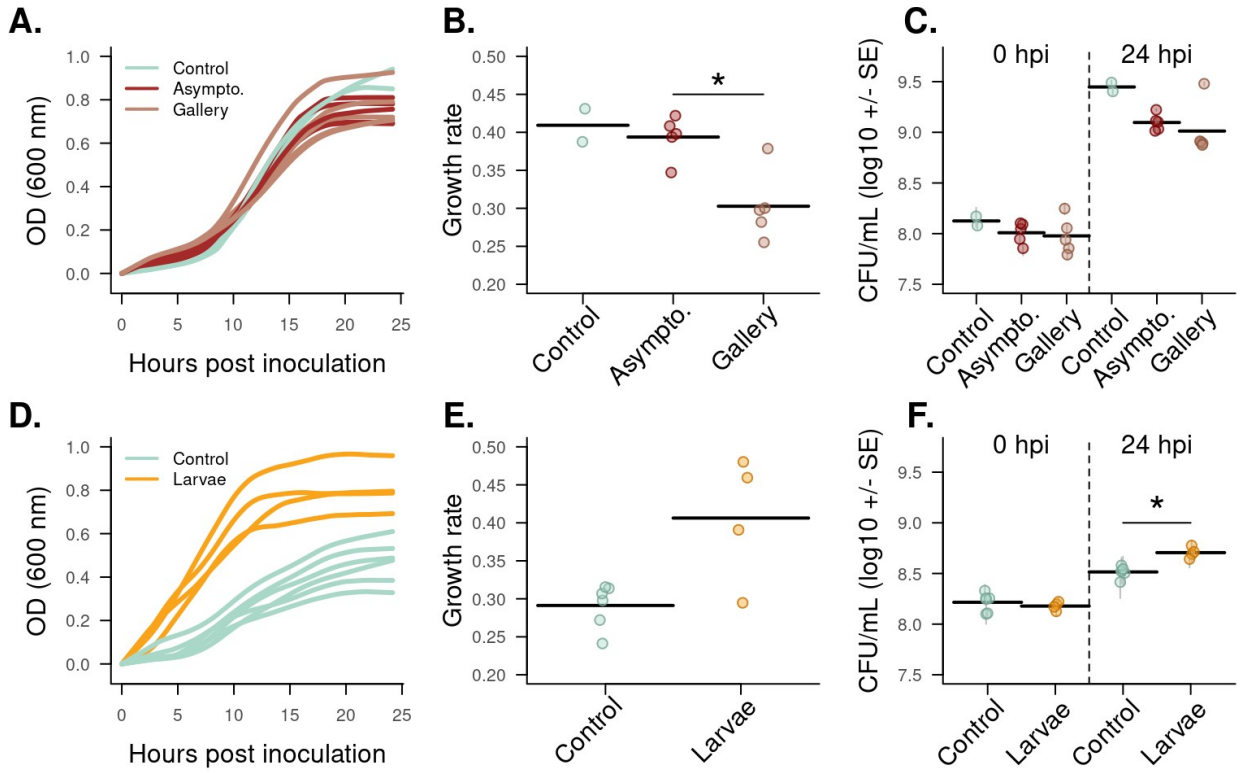

Figure 2: ***B. goodwinii* growth curves (A), growth rates (B), and cell density (C)** obtained in 3 mL liquid cultures supplemented with gallery wood tissue (Gallery) and asymptomatic wood (Asympto.) chemical compounds, compared to chemical compound extraction blanks (Controls). Growth curves (D), growth rates (E), and cell density (F) obtained in 200 µL liquid cultures supplemented with beetle larvae body extracts (Larvae), compared to chemical compound extraction blanks (Controls). The controls were the same in both experiments but the difference in growth rate observed in (A) and (D) are due to the different volumes of cultures. An additional experiment replicating the results of (A) in the same volume as (D) can be found in Figure S1. Stars indicate significant differences (pairwise Wilcoxon test (above) or t-test (below), p-value < 0.05). OD = optical density; CFU = colony-forming unit; SE = standard error

A. biguttatus larval elicitors cause bacterial proliferation

To test the direct effect of *A. biguttatus* (Ab) larval chemical elicitors on *B. goodwinii* proliferation (hypothesis 2), we compared bacterial growth in cultures supplemented with Ab larval extract or water (extraction blank control). At 24 hpi, cultures in a 200 µL volume reached an OD_{600} of 0.75 ± 0.09 and 0.47 ± 0.1 for Ab extracts and water

125 control, respectively (Figure 2D). The growth rate of cultures supplemented with Ab
126 extracts was higher than that of control cultures (0.41 ± 0.07 and 0.30 ± 0.04,
127 respectively; Figure 2E). This trend was confirmed by the Ab larval bulk extract growth
128 curves in Figure 4B. The CFU density at 24 hpi was also significantly higher for cultures
129 supplemented with Ab extract than for control cultures (5.1 ± 0.67 x 10^8 CFU/mL and
130 3.3 ± 0.4 x 10^8 CFU/mL, respectively; Figure 2F). These results indicate that chemical
131 elicitors extracted from *A. biguttatus* larvae stimulate bacterial growth.

132 *A. biguttatus* larval elicitors modify bacterial gene expression profile and 133 induce putative virulence gene up-regulation

134 To test for the impact of chemical elicitors on bacterial gene expression, we compared
135 the gene expression profile of *B. goodwinii* in cultures supplemented with asymptomatic
136 and gallery wood tissue extracts (hypothesis 1), and *A. biguttatus* larval extracts and
137 water control (hypothesis 2). Cultures supplemented with gallery wood tissue or
138 asymptomatic wood extracts did not have a clearly distinct gene expression profile and
139 did not cluster separately based on the correlation matrix of normalised transcript count
140 per million (CPM) (Figure 3A). A total of 12 genes were differentially expressed between
141 the gallery and asymptomatic wood tissue, with 3 up-regulated and 9 down-regulated
142 genes in the gallery wood tissue treatment (Table S1). Two out of the 3 up-regulated
143 genes were linked to flagellum production (*fliA*, *fliF* Figure 4). These results show that
144 chemical compounds extracted from gallery wood tissue have a small impact on the gene
145 expression profile of *B. goodwinii* compared to extracts from asymptomatic wood tissue
146 and mainly impact flagellar genes.

147 In contrast, cultures supplemented with *A. biguttatus* larval extracts had a distinct gene
148 expression profile and formed a separate cluster from the control cultures (no larval
149 extract) based on the correlation matrix of normalised transcript CPM (yellow and light
150 blue samples in Figure 3B). A total of 585 genes were differentially expressed between
151 *B. goodwinii* cultures receiving Ab larval extracts and the control treatment, with 307 up-
152 regulated and 278 down-regulated genes in the Ab larvae treatment (Table S2). The 30
153 most differentially expressed genes and their functional annotation are represented in
154 Figure 3C. Up-regulated genes included the Type III secretion system apparatus and
155 effectors (*avrE*, *hrpN*, Figure 4) which have been found to be expressed during lesion
156 formation *in planta* (Broberg et al., 2018; Doonan et al., 2020). These results show that
157 Ab larval elicitors have a significant impact on bacterial gene expression and trigger the
158 expression of putative bacterial virulence genes.

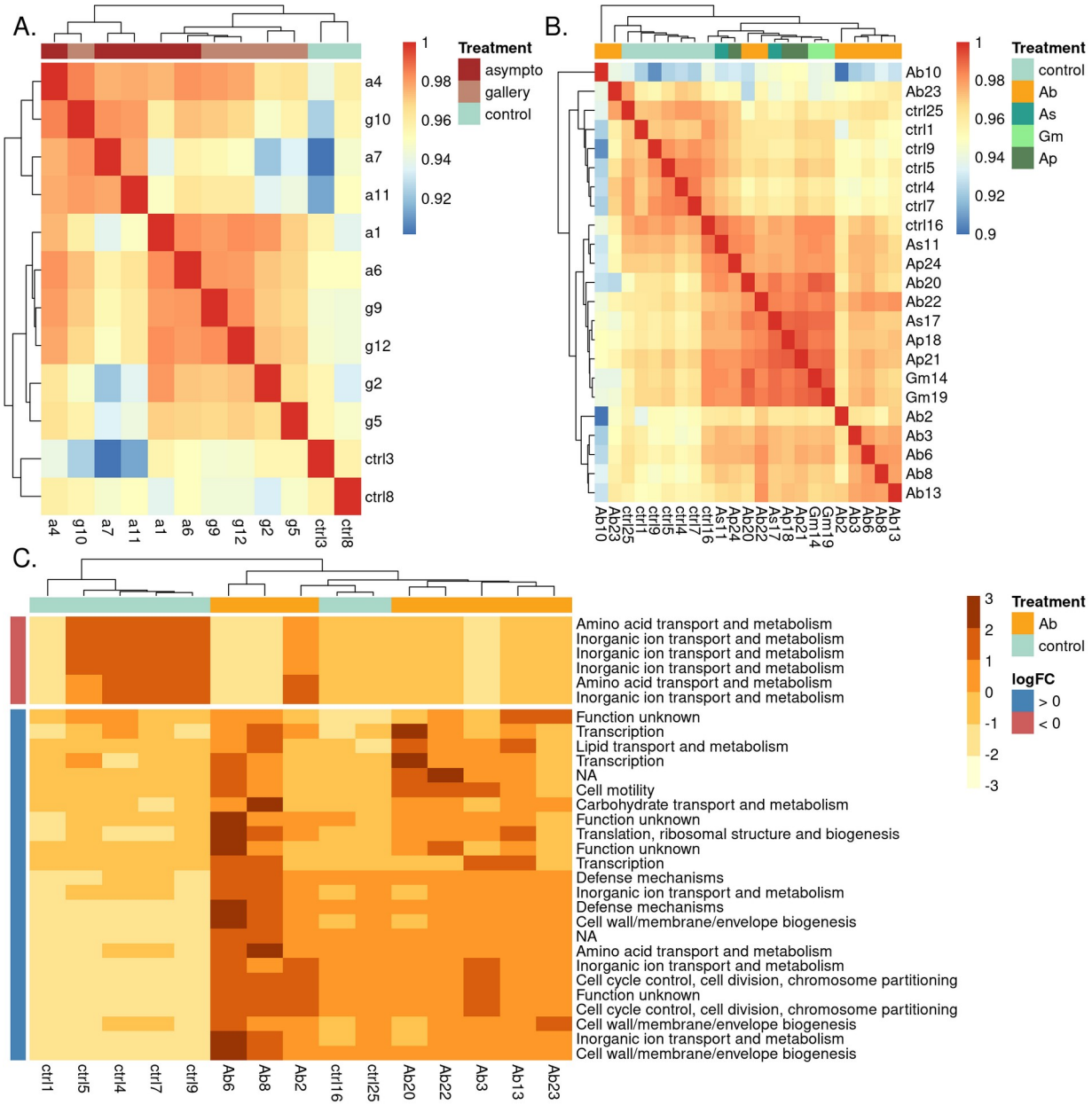

Figure 3: Gene expression profiles obtained from RNAseq data of *B. goodwinii* cultures supplemented with chemical extracts from oak wood tissues, insect larvae or water (control). **A.** and **B.** Heatmaps of the sample correlation matrix based on the normalised transcript counts per million (CPM). Asympto = asymptomatic wood extract, gallery = gallery wood tissue extract, Ab = *A. biguttatus* larval extract, Ap = *A. planipennis* larval extract, Gm = *Galleria mellonella* larval extract. Sample Ab10 had a gene expression profile different from all other samples with low correlation values and was considered as an outlier and removed from the differential expression analysis. **C.** Heatmap of the centred and scaled normalised transcript CPM of the top 30 differentially expressed genes in Ab treatment, compared to control. Row names correspond to the Clusters of Orthologous Groups of proteins (COGs) annotation for each gene.

Extracts from other beetle species influence *B. goodwinii* gene expression but do not induce putative virulence genes up-regulation

To test for the specificity of the bacteria-beetle interaction in AOD, we replicated the RNAseq experiment using two other species of *Agrilus*: the European oak borer *A. sulcicollis* (As) and the emerald ash borer *A. plannipenis* (Ap). We also included in the experiment the wax moth *Galleria mellonella* (Gm) as an outgroup. Because of the limited availability of larvae extract, only one experiment was performed and culture were grown for 10 hpi only to allow sampling for RNAseq analysis. OD_{600} measurements from these 10 first hours of growth were performed on some of the cultures and show a trend of higher growth rates for Ab, Ap, and Gm compared to As and control (Figure S2). However, differences in growth rates were not significant which may be due to the small sample size for these measurements.

B. goodwinii cultures supplemented with larval extracts clustered together and away from the water control based on the correlation matrix of normalised transcript CPM (Figure 3B). However, the different species of larvae did not necessarily form distinct species-specific clusters, indicating some variability in the bacterial response to larval chemical compounds. Nevertheless, most Ab samples clustered separately from the other larval extracts. Cultures supplemented with As and Gm extracts did not show any significant differentially expressed genes compared to the control, although data shows a trend of higher CPM for *hrpN* compared to control. Cultures supplemented with Ap extracts showed 125 differentially expressed genes, including 85 up-regulated and 40 down-regulated genes (Table S3). Most up-regulated genes were related to cell

multiplication, while down-regulated genes contained 9 flagellar production- and motility-related genes. However, the putative virulence genes that were differentially expressed in the presence of Ab larval extracts were not significantly differentially expressed in the presence of Ap larval extracts, although *hrpN* shows a trend of higher CPM compared to the control (Figure 4).

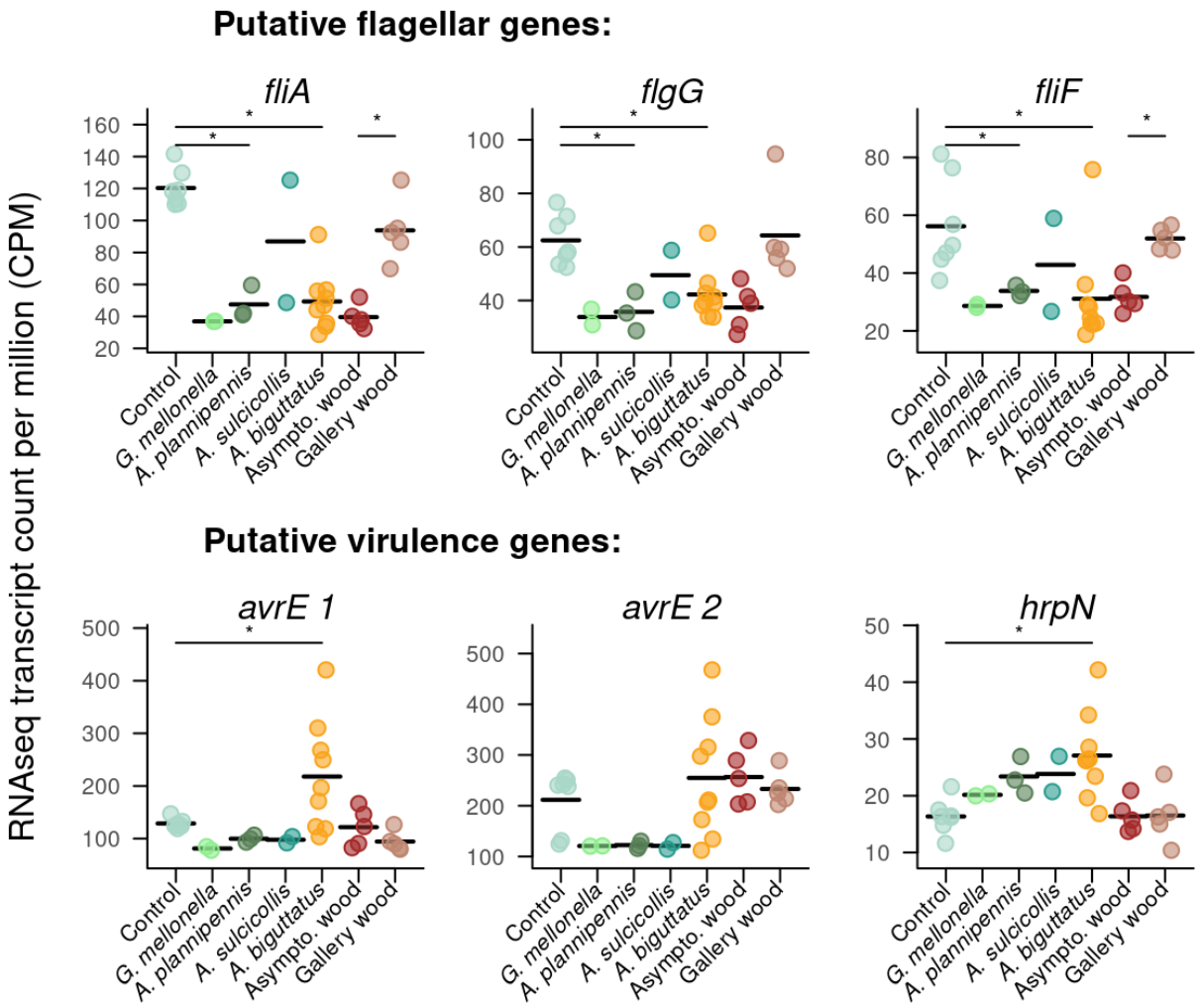

Figure 4: **RNAseq transcipt CPM for genes of interest.** These include putative flagellar and virulence genes. Bars with stars represent significantly differentially expressed genes based on the edgeR differential expression analysis.

186 ## Bacterial proliferation is triggered by a fraction of the *A. biguttatus* larval
187 elicitor extract

188 To narrow down identification of the bioactive fraction(s) of the *A. biguttatus* larval
189 extracts, we compared *B. goodwinii* growth in cultures supplemented with the bulk larval
190 extract with that of cultures supplemented with four fractions of the bulk larval extract
191 (Fraction 1 (F1) to Fraction 4 (F4); Figure 1B and 5A). At 24 hpi, cultures supplemented
192 with the bulk larval extract, F1 or F4 reached greater OD_{600} (0.50±0.04, 0.51±0.05 and
193 0.48±0.01, respectively; Figure 5B) compared to cultures supplemented with water
194 (control), F2 or F3 (0.36±0.03, 0.38±0.03 and 0.40±0.05, respectively; Figure 5B). The
195 growth rate of *B. goodwinii* in cultures supplemented with F1 was significantly higher
196 than any other treatments, including the bulk larval extract. The growth rate of cultures
197 supplemented with F2, F3 and F4 did not significantly differ from the water control
198 (Figure 5C). The cell density at 24 hpi was significantly reduced for *B. goodwinii* in
199 cultures supplemented with F1 and F4 compared to the other treatments (Figure 5D).
200 Altogether, these results suggest that F1 contains the chemical elicitors responsible for
201 the proliferation (higher growth rate) of *B. goodwinii*, but that these chemical compounds,
202 when separated from the other fractions, reduce cell density in the stationary phase of
203 growth.

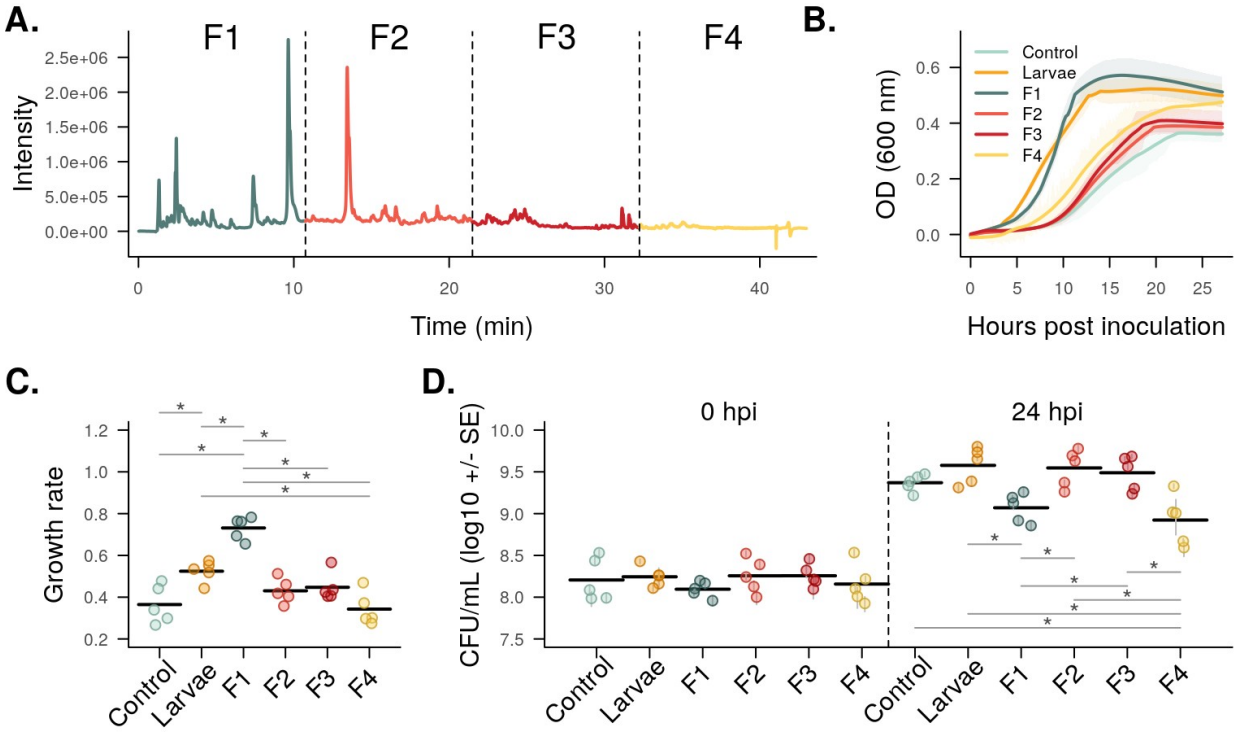

Figure 5: Bioactivity of different fractions of the *A. biguttatus* larval extract. A. LC-MS chromatogram of the bulk larval extract ("Larvae" treatment on panel B to D). A part of it was then fractionated in four fractions (fraction F1 to F4 on panel B to D). **B.** Growth curves, **C.** growth rates, and **D.** cell density were obtained in 200 µL cultures supplemented with the bulk larval extract (Larvae), the four larval extract fractions (F1 to F4) and water (Control). Bars with stars indicate significant differences (Tukey HSD, p-value < 0.05). OD = optical density; CFU = colony-forming unit; SE = standard error

Discussion

Investigations of insect-bacteria interactions in plant diseases have largely focussed on the role of bacterial volatile organic compounds (VOCs) in modulating insect behaviour (Davis et al., 2013; Leroy et al., 2011; Weisskopf et al., 2021). Less attention has been paid to the influence of insect-derived chemical cues on bacterial growth and virulence. In this study, we investigated the impact of insect chemical elicitors on a bacterial

species associated with Acute Oak Decline (AOD). We found that *A. biguttatus* larval elicitors, but not gallery wood tissue extracts, increase *B. goodwinii* proliferation and virulence gene expression. Elicitors from some other insect species influenced bacterial gene expression but did not trigger the expression of T3SS effector genes.

Extracts from larval gallery wood tissue did not cause bacterial proliferation as compared to healthy wood controls, suggesting that proliferation of the bacteria around *A. biguttatus* galleries *in planta* (Denman et al., 2018) is not mediated by the tree host chemicals. The growth rate of cultures was in fact lower for extracts from gallery wood tissue compared to healthy wood tissue; potentially owing to tree host defence compounds produced in response to larval invasion (Broberg et al., 2018). However, the final cell density was not significantly different between gallery and healthy wood tissue extract treatments, suggesting that the extracted chemicals only affect the speed of proliferation but not final cell density. In addition to a reduction of growth rate, the wood gallery extracts triggered the expression of flagellum-associated genes, which are mainly involved in bacterial motility (Thomas et al., 2001; Wilkinson et al., 2011) and could represent a mechanism for the bacteria to move away from an unsuitable environment. Some flagellar genes are involved in virulence and plant wall adherence in certain plant pathogens (Jahn et al., 2008; Malamud et al., 2011) which could indicate the activation of some virulence process components. However, any such indirect effect of *A. biguttatus* larvae on *B. goodwinii* virulence gene expression through the modification of the wood tissue environment is thus less powerful than the direct effect of larval extracts but cannot be completely excluded.

232 *A. biguttatus* larval extracts increased bacterial proliferation *in vitro*, suggesting that the
233 interaction between insect and bacteria observed *in planta* is at least in part directly
234 mediated by larval chemical compounds. Previous studies conducted on artificially
235 infected oak logs showed that the lesion size caused by AOD bacteria significantly
236 increased in the presence of beetle larvae (Denman et al., 2018). In addition, AOD
237 lesions are observed in close proximity to beetle galleries *in natura*, which suggests a
238 bacteria-beetle interaction, and/or the dissemination of the bacteria within wood tissues
239 by the beetle larvae. In the light of our results, we hypothesise that elicitors are released
240 by *A. biguttatus* larvae as they form galleries, and that these chemical compounds trigger
241 the proliferation of *B. goodwinii*, which starts degrading the wood. However, we cannot
242 exclude the possibility that elicitors may be metabolised by the bacteria and may act as a
243 food source in our experiment.

244 The link between larval elicitor production and bacterial lesion formation is further
245 supported by the fact that *A. biguttatus* larval extracts also triggered the expression of
246 putative virulence genes in *B. goodwinii*. Among the 307 significantly up-regulated genes
247 induced by larval extracts were some potential virulence genes associated with a Type III
248 secretion system (T3SS), which is well reported in other plant pathogens. These include
249 the *hrpN* gene encoding for the harpin component of the needle structure of the T3SS in
250 *Pseudomonas syringae* (Collmer et al., 2000) and has been found over-expressed in AOD
251 lesions *in situ* (Broberg et al., 2018). This T3SS belongs to the Hrc-Hrp1 family only
252 present in plant pathogens and is distinctive in that it possesses a long, flexible pilus able
253 to traverse the thick plant cell wall, suggesting that the target of this T3SS is the plant

host (Troisfontaines & Cornelis, 2005). In addition, we found a*vrE* genes up-regulated in the presence of *A. biguttatus* larval elicitors, which were previously reported to be up-regulated in the presence of *A. biguttatus* larvae *in planta* (Doonan et al., 2020). The a*vrE* gene encodes for a T3SS effector involved in the suppression of plant immunity by *P. syringae* and other plant pathogens (Degrave et al., 2015) which induce water flux into host cells resulting in cell swelling and bursting (Nomura et al., 2023). These results give another strong indication that beetle larvae are involved in triggering the virulence of *B. goodwinii.* It should be noted, however, that some bacteria have also been reported to possess different T3SS effectors to allow interactions both with plant hosts and insect vectors (Correa et al., 2012). The response of *B. goodwinii* to *A. biguttatus* chemical compounds could thus potentially be involved in the colonisation of larvae by the bacteria; a*vrE* gene knock-outs could confirm its function in the AOD system.

We investigated the specificity of this insect-bacterium interaction by extracting chemical compounds from the European oak borer *A. sulcicollis*, the emerald ash borer *A. planipennis*, and the wax moth *Galleria melonella.* We did not find any significant differentially expressed genes in *B. goodwinii* cultures supplemented with *A. sulcicollis* and *G. mellonella* compared to the control, which could be explained by the lower sample size we had for these insect species. However, *B. goodwinii* cultures supplemented with *A. planipennis* extracts showed 125 differentially expressed genes compared to the water control. Among these, numerous motility-related genes were down-regulated, which is similar to the response of *B. goodwinii* to *A. biguttatus* extracts and indicate a change of phenotype in response to insect-derived chemical compounds. However, none of the

276 T3SS-related genes found up-regulated in the presence of *A. biguttatus* were
277 differentially expressed in response to *A. planipennis* extracts. These results suggest that
278 chemical compounds from different insect species can show bioactivity towards
279 *B. goodwinii*, but the trigger of virulence gene expression is specific to *A. biguttatus* under
280 the conditions of our experiment.

281 One fraction of the *A. biguttatus* larval extract produced the same growth curves as the
282 bulk larval extract when added to a culture of *B. goodwinii*. The growth rate obtained
283 with this fraction was in fact higher than with the bulk larval extract, while the final cell
284 density at 24 hpi was lower. This indicates that several chemical compounds are
285 necessary to reproduce the full phenotype observed when supplementing cultures with
286 the bulk larval extract, which may be present in several of the four fractions. Identifying
287 the bioactive chemical compounds should be a future priority but it is hindered by the
288 limited availability and seasonality of *A. biguttatus* larvae and the predicted significant
289 challenges associated with the identification work. However, we successfully narrowed
290 down the activity range of separate fractions of larval extracts responsible for the
291 proliferation and up-regulation of effector gene expression in *B. goodwinii*, which will aid
292 chemical compound characterisation efforts.

293 The strong effects of *A. biguttatus* extracts on *B. goodwinii* in comparison to those from
294 gallery wood tissue strengthens the argument that *A. biguttatus* acts as a synergistic
295 factor with *B. goodwinii*, the major pathogen found in AOD lesions. Whether the bacteria
296 are present in the tree in a non-pathogenic state before beetle invasion, or if they are

vectored by the beetle to the tree remains to be answered, but our study emphasises the necessity of integrating *A. biguttatus* population survey and management into programs tackling AOD. The potential benefits this interaction provides to each partner need to be further evaluated to understand their evolutionary consequences. It is possible that this interaction results from co-adaptation and provides a benefit to *A. biguttatus* beetle larvae, as *B. goodwinii* expresses detoxification genes when infecting the tree (Denman et al., 2018; Doonan et al., 2020). This may provide protection for the larvae against plant defences as well as weakening the tree, thus increasing larval survival and overall reproductive success. For the bacteria, supporting the growth and emergence of beetle larvae could potentially lead to facilitated multiplication and transmission between trees. On the other hand, this interaction could equally be incidental and not a result of any evolutionary relationship, with no impact on the bacteria or beetle fitness.

Although *A. sulcicollis* shares its oak hosts with *A. biguttatus*, the same interaction with *B. goodwinii* was not observed in our experiment. Anecdotally, *A. sulcicollis* colonises very poorly defended or recently dead oak tissues, which are drier and more degraded than those colonised by *A. biguttatus* (K. Reed, personal observation). Perhaps it therefore does not have the same need for protection from host defences as *A. biguttatus*, which is able to colonise stressed, but still living host tissue. On the other hand, *A. planipennis*, a highly aggressive species, which is able to kill healthy ash trees, did provoke differential gene expression in *B. goodwinii.* Given the economic cost of damage due to *Agrilus* pests, future studies should explore the interaction between Agrilus species and microbial communities in trees.

319 Although the importance of interactions between insects and bacteria is well established
320 in the case of symbiosis (Douglas, 2009) or insect behaviour manipulation by bacterial
321 VOCs (Davis et al., 2013), our suggests another form of insect-bacteria interaction,
322 where insect-derived chemicals modulate bacterial gene expression potentially
323 influencing virulence. The implications of this finding are of particular importance in the
324 context of recent increases in plant disease outbreaks involving pest-pathogen interactions.
325 Our study together with previous work strongly suggest that an insect herbivore affects bacterial
326 behaviour by triggering the expression of virulent phenotypes and facilitating lesion formation in
327 live bark tissue, thus contributing to tree decline (Broberg et al., 2018; Doonan et al., 2020). Such
328 overlooked interactions warrant future investigation to safeguard forest biomes.

329 Material and methods

330 Gallery wood tissue and larvae sample production

331 Sections of bark and sapwood cut from *A. biguttatus* (Ab) infested oak trees were stored
332 in purpose-built, walk-in emergence cages at Forest Research (Farnham, UK). After the
333 adults emerged, they were collected and maintained in the lab as described by (Reed et
334 al., 2018). A maximum of 8 beetles were housed per 2 L cage, and the beetles were
335 provided with freshly-cut oak leaves and 20 % sugar solution and water on cotton wool.
336 Beetle eggs, laid on paper towels placed underneath the cages, were collected twice
337 weekly and incubated in 15 mL Falcon tubes at 17.5 °C until 1 or 2 days before the
338 hatch time predicted by the egg hatching model (Reed et al., 2018). Oak billets were cut
339 to a length of approximately 90-100 cm and allowed to stand in water for 2 weeks prior

340 to inoculation. 10-15 eggs were placed in wounds made in the phloem with a 5 mm cork
341 borer (4 wounds per billet). Infested billets were incubated at room temperature for 3
342 months before harvesting of Ab larvae from the billets. Ab larvae and wood tissue
343 samples were collected by removing the outer bark with a wood knife to expose larval
344 galleries. Undamaged Ab larvae were collected in 1.5 mL tubes and frozen into liquid
345 Nitrogen. Asymptomatic wood tissue and gallery wood tissue were collected in 15 mL
346 tubes and frozen in liquid Nitrogen. Samples were stored at -80 °C until chemical
347 extraction.

348 Final instar larvae of and *Agrilus sulcicollis* (As) were collected from the phloem of
349 recently-dead, infested large oak branches (~15cm diameter) and stems at Richmond
350 Park, Greater London. They were morphologically differentiated from *A. biguttatus*,
351 which is the only other species found in large oak branches and stems at Richmond Park,
352 using the key in (Volkovitsh et al., 2020).

353 Third and fourth instar *Agrilus planipennis* (Ap) larvae were produced on *Fraxinus excelsior*
354 billets within the Holt Containment Facility at Forest Research, Farnham, as follows.
355 Eggs, laid onto filter paper, were couriered from the USDA APHIS PPQ EAB Biocontrol
356 Facility (Brighton, UK). 8 eggs per billet were cut out from the filter paper and wrapped
357 with Parafilm to hold them snugly against the bark. The billets were kept standing in
358 trays of water at 25 °C for 3-4 weeks before the bark was peeled and the larvae were
359 harvested. After collection, undamaged Ab, As and Ap larvae were frozen in 15 mL tubes

360 at -80 °C, where they were stored until processing. Finally, *Galleria melonella* (Gm) larvae
361 were commercially purchased (Livefood UK Ltd) and stored at -80 °C until processing.

362 Larval and wood tissue chemical compound extraction and analysis

363 Chemical extraction

364 Wood tissue chemical compounds were extracted from 2 x 2 cm (64±20 mg) sections of
365 inner bark material (either asymptomatic or containing larval galleries) in 5 mL 100 %
366 MeOH for 2 h. To remove cellular debris, extracts were filtered through a plug of
367 silanized glass wool held within a Pasteur pipette, into pre-weighed glass vials. Resultant
368 extracts were dried to completion under a gentle stream of N_2. Five biological replicates
369 were performed per treatment, and an extraction blank containing only 100 % methanol
370 was also included.

371 Ten snap-frozen Ab larvae collected from the oak log trial were weighed and individually
372 extracted in 5 mL of 100 % methanol for 2 h. To remove cellular debris, extracts were
373 filtered through a plug of silanized glass wool held within a Pasteur pipette, into pre-
374 weighed glass vials. Five extraction blanks containing only 100 % methanol were also
375 included. 400 µL of each extract were kept for LC-MS (liquid chromatography and mass
376 spectrometry) analysis. The remaining of the extracts were dried to completion under a
377 gentle stream of N_2. Dried extracts were resuspended in Milli-Q water to a 3 mg/mL
378 concentration and filter sterilised through a 0.22 µm pore size filter, for bioactivity tests.
379 The 10 larval extracts were then pooled in pairs in equal volume to obtain sufficient

380 amount of chemical compounds for bioactivity tests, representing five biological
381 replicates, and stored at -20 °C until processing.

382 Chemical compounds were extracted from As larvae, Ap larvae and Gm larvae following the
383 same protocol. Due to different quantities of chemical compounds extracted from different
384 species, larval extracts were pooled, e.g. two or three individual larvae were pooled to obtain at
385 least 3 biological replicates per insect species at 3 mg/mL.

386 **LC-MS conditions**

387 LC-MS analysis was carried out using an Acquity ultra-high-pressure liquid
388 chromatography (UPLC) system coupled to a Synapt G2Si Q-Tof mass spectrometer with
389 an electrospray ionisation source (Waters, UK). The system was controlled through
390 Masslynx 4.1 software (Waters). Chromatographic separation was carried out at a flow
391 rate of 0.21 $mL.min^{-1}$ using a UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm, Waters)
392 coupled to a C18 Vanguard pre-column (2.1 x 5 mm, 1.7 µm, Waters). The mobile phase
393 consisted of solvent A (0.01% formic acid v/v, water) and solvent B (0.01% formic acid
394 v/v in methanol) with the following gradient: 0 min, 95:5 (A:B), to 85:15 at 7 min, 75:25
395 at 11 min, 70:30 at 13 min, 50:50 at 18 min; 25:75 at 25 min; 0:100 at 30 min, and 95:5
396 at 39.1 min.

397 An Acquity photodiode array (PDA) detector was used to monitor the UV trace (range
398 200-450nm), sampling rate of 10 points s-1 with resolution set to 2.4 nm. The Synapt
399 was operated in sensitivity mode and set to a mass range of 50 – 1200 Da and scan

400 time = 0.1 s, in both ionisation modes. The system was operated in MS1 mode with the
401 following conditions: capillary voltage – 2.5 KV, sample cone voltage 30 V, sample offset
402 80 V, source temperature 100 °C, desolvation temperature 300 °C, desolvation gas flow
403 800 $L.h^{-1}$, cone gas flow 57 $L.h^{-1}$.

404 Preparative HPLC conditions

405 To locate active fractions within the larval extracts, preparative HPLC was performed by
406 repeated injections on a Hichrom ACE 5 C18 column (250 × 4.6 mm i.d.) connected
407 to Shimadzu Nexera UC SFC-HPLC hybrid system. The mobile phase consisted of
408 solvent A (water) and solvent B (methanol) with the following gradient: 0 min, 95:5 (A:B),
409 to 85:15 at 7 min, 75:25 at 11 min, 70:30 at 13 min, 50:50 at 18 min; 25:75 at 25 min;
410 0:100 at 30 min, and 95:5 at 39.1 min. The injection volume was 100 µL (five successive
411 injections), and the separation was carried out over 43 min with a flow rate of 1 $mL.min^{-}$
412 ^{1}. Fractions were collected every 10.75 min, yielding four individual fractions. The four
413 fractions collected using prep-HPLC were evaporated to 1 ml using a rotary evaporator,
414 after which the fractions were dried under a gentle stream of nitrogen.

415 Chemical compound bioactivity analysis

416 Bacterial strain and medium

417 Bioactivity assays were conducted on the type strain *Brenneria goodwinii* FRB141 kept in
418 glycerol stocks at -80 °C. Before the experiment, the strain was streaked onto Nutrient
419 Agar (LAB M Limited) and incubated for 48 h at 28 °C. Starter cultures were then
420 prepared by picking a single colony and growing it overnight in 10 mL of Nutrient Broth

421 n°2 (Oxoid) at 28 °C and 150 rpm shaking. Starter cultures were rinsed twice by
422 pelleting the cells for 5 min at 3000 g, discarding the supernatant and adding 10 mL of ¼
423 strength Ringers solution (LAB M Limited). Cells were then pelleted again, and OD
424 (Optical Density) at 600 nm was adjusted to 0.5 in ¼ strength Ringers solution.

425 Subsequent growth measurements were performed in M9+glu minimal medium
426 composed of M9 salt (Na_2HPO_4 42 mM; KH_2PO_4 22 mM; NaCl 8.55 mM; NH_4Cl
427 18.6 mM; $MgSO_4 \bullet 7H_2O$ 2 mM; $CaCl_2$ 0.1 mM) and 20% glucose, with or without the
428 addition of chemical compounds tested for bioactivity.

429 **Growth experiment**

430 The bioactivity test of gallery wood tissue chemical compounds was performed in
431 culture broth composed of 1 mL of gallery wood tissue or asymptomatic wood tissue
432 extract (3 mg/mL), 0.66 mL of M9+glu minimum medium (4.5 x concentration), 1.04 mL
433 of water, and 0.3 mL of starter culture (starting OD_{600} of 0.05), for a final volume of
434 3 mL. For controls, wood tissue extracts were replaced by chemical compound
435 extraction blanks. Chemical compounds from 5 gallery wood tissue samples and 5
436 asymptomatic wood samples were tested (5 biological replicates per group and 1
437 technical replicate) and cultures were placed in a 12-well plate. To allow for comparison
438 with hypothesis 2 which could not be performed in a 3 mL volume due to the lower
439 quantity of larval chemical compounds obtained from the extraction step, we replicated
440 the growth experiment in a 200 µL volume (Figure S1).

441 Because the number of Ab larvae available for the experiment was limited, the quantity
442 of chemical extract available for the experiments was small. Consequently, the test of Ab
443 larval chemical compounds on bacterial growth was performed in a smaller culture
444 volume. The broth was composed of 60 µL of larval extract (3 mg/mL), 40 µL of M9+glu
445 minimum medium (4.5X), 80 µL of water, and 20 µL of starter culture (starting OD_{600} of
446 0.05), for a final volume of 200 µL. For controls, Ab larval extracts were replaced by
447 chemical compound extraction blanks. Chemical compounds from 5 pairs of Ab larvae
448 were tested with 6 technical replicates to account for the higher noise in OD
449 measurement when performed in small culture volumes and cultures were placed in a
450 96-well plate.

451 For each growth experiment, a replicate plate containing the same liquid growth
452 medium, but without bacterial inoculation, was performed to get OD background
453 correction with sterile medium and check for medium contamination. Plates were placed
454 into the Stratus plate reader (Cerillo) for OD_{600} measurement every 3 minutes over the
455 course of the experiment. The plate reader was incubated at 28 °C and shaken at
456 150 rpm for 24 h. A 20 µL aliquot of each culture was collected at 0 hpi (hours post
457 inoculation) and 24 hpi for Colony Forming Unit (CFU) quantification.

458 The number of viable cells in the cultures was estimated at 0 hpi and 24 hpi by adding
459 20 µL of culture to 180 µL of sterile ¼ strength Ringers solution and completing a serial
460 dilution in the same way up to 10^{-8} in a 96-well microplate. Six replicates of 10 µL of
461 each dilution were then pipetted onto Nutrient Agar and incubated for 24 h at 28 °C.

The number of CFUs was counted from the appropriate dilution, and the total CFU/mL of culture was estimated as $\frac{CFU \times 10^{dilution}}{10} \times 1000$. CFU concentration from the 6 replicate 10 µL drops were averaged and standard error was calculated.

Transcriptome experiments

To ensure sufficient biomass for RNA-seq analysis, cultures were set up in a final volume of 3 mL as described above, and larval extracts had to be pooled in pairs to obtain enough material for RNA extraction. Three separate RNA-seq experiments were performed with the following sample sizes: Experiment 1: extracts from 5 gallery wood tissue samples and 5 asymptomatic wood tissue samples; Experiment 2: extracts from 5 pairs of Ab larvae and 5 extraction blanks; Experiment 3: extracts from 3 pairs of Ab larvae, 3 pools of Ap larvae, 2 pools of As larvae, 2 pools of Gm larvae and 3 extraction blanks. Extracts were added to culture broth as described above and culture plates were incubated for 10 hours to obtain cultures in mid-exponential phase of growth. Cultures were transferred into 2 mL screw cap tubes, centrifuged for 5 min at 3000 rcf to discard the supernatant, and immediately frozen into liquid nitrogen. Cell pellets were then stored at -80 °C until RNA extraction.

The total RNA was extracted from cultures using a phenol-chloroform extraction adapted from (Griffiths et al., 2000). Briefly, cells were mechanically lysed by adding to the frozen cells 0.5 g of 452-600 µm acid-washed glass beads (Sigma), 500 µL of 5 % CTAB in potassium phosphate buffer 120 mM pH 8 and 500 µL Phenol:Chloroform:Isoamyl-alcohol (25:24:1) pH 8 (Thermofisher). Tubes were then

483 shaken for 30 s at 2500 rps with the PowerLyzer 24 (MO BIO Laboratories Inc) and
484 centrifuged at 22,000 rcf for 10 min at 4 °C. The aqueous phase was washed with
485 500 µL of Chloroform:Isoamyl-alcohol (24:1) and centrifuged at 22,000 rcf for 10 min at
486 4 °C. 1 mL of 30% Polyethylen Glycol 6000 solution was added to the aqueous phase
487 and incubated overnight at 4 °C to precipitate nucleic acids. Tubes were then
488 centrifuged at 22,000 rcf for 30 min at 4 °C and pellets were washed with ice-cold 70%
489 ethanol. Pellets were dried for 20 min at room temperature and re-suspended in 50 µL
490 water. Genomic DNA was removed using the TURBO DNAse (Invitrogen) following the
491 manufacturer's instructions. RNA was precipitated for 2 hours at -20 °C with 25 µL of
492 NaCl (6 M), 5 µL of Sodium Acetate (3 M) and 50 µL of ice-cold 2-propanol. Tubes were
493 centrifuged, pellets were washed with ethanol and air-dried as previously described.
494 RNA was re-suspended in a final volume of 20 µL of nuclease-free water. RNA integrity
495 was observed using agarose gel electrophoresis and the concentration and purity were
496 assessed by spectrophotometry (ND-1000, Nanodrop) and fluorometry (Qubit RNA High
497 Sensitivity kit and Qubit 3, Invitrogen). None of the extraction negative controls
498 contained detectable RNA and were thus not sent for sequencing.

499 Samples from the 3 RNA-seq experiments were sequenced separately. RNA extract
500 Quality Control (QC) and sequencing were performed by Novogene (Cambridge).
501 Quantity, purity, and integrity of the samples were assessed using Nanodrop
502 (ThermoFisher), Agarose Gel Electrophoresis and Bioanalyzer 2100 (Agilent). Library
503 preparation steps included rRNA depletion, RNA fragmentation, reverse transcription,

504 Illumina adapter ligation, size selection and PCR amplification. The libraries were
505 sequenced on the Illumina NovaSeq platform with the PE150 strategy.

506 Data analysis

507 Data analyses were performed on R version 4.3.3 (R Core Team, 2024). All scripts used for
508 the analysis are available as a Quarto notebook at
509 https://doi.org/10.6084/m9.figshare.27331281.

510 *In vitro* growth data

511 Raw OD_{600} values were averaged between technical replicates and the noise in OD
512 measurement was reduced with a locally weighted scatterplot smoothing regression
513 using the lowess function from the stats package v4.3.2 (R Core Team, 2024). Growth
514 rates were estimated from these averaged growth curves using the growthcurver
515 package v0.3.1 (Sprouffske & Wagner, 2016). Statistical differences between treatments
516 for growth rates and CFU number were assessed using either a t-test, with the t.test
517 function, or a Wilcoxon pairwise comparison when the condition of validity for the t-test
518 were not met.

519 RNA-seq data

520 The bioinformatic analysis was conducted on the Supercomputing Wales cluster. The
521 reference genome and annotations from the FRB141 strain were downloaded from
522 Genbank under the accession GCF_002291445.1 (BioProject PRJNA308225). A
523 transcript FASTA file was produced from the reference genome and annotations using

524 gffread v0.11.5 (Pertea & Pertea, 2020). The RNA-seq data were then analysed with the
525 nf-core/rnaseq nextflow pipeline v3.10.1 (10.5281/zenodo.1400710; (Ewels et al.,
526 2020) with raw fastq read, the reference genome, the reference annotation, and the
527 reference transcript FASTA as input. Briefly, raw read quality was assessed with fastQC.
528 Genome index was generated with STAR (Dobin et al., 2013) using the CDS entries from
529 the annotation and a transcript index was generated using Salmon (Patro et al., 2017).
530 The transcriptome reads were then aligned against the genome index using STAR and
531 gene transcripts were quantified with Salmon and count tables were generated.

532 Samples from the wood tissue extract experiment (experiment 1) were then analysed on
533 their own, and samples from the larval extract experiments (experiment 2 and 3) were
534 analysed together. Count data were imported in R using the tximport package v1.28.01
535 (Soneson et al., 2016). Subsequent analyses were performed using the edgeR package
536 v4.0.16 (Robinson et al., 2010). Transcripts with less than 10 reads in at least two
537 samples were filtered out, read distributions were normalised using the read trimmed
538 mean of M-values method (Robinson & Oshlack, 2010). The data was transformed for
539 linear modelling using the voom function from the limma package v3.58.1 (Ritchie et al.,
540 2015) and the model was fitted with the treatment variable (i.e. the identity of chemical
541 compounds added to the culture) and the experiment batch for the larvae chemical
542 compound experiments. Coefficients were then computed for the following contrasts:
543 gallery wood tissue vs asymptomatic wood tissue, Ab vs water control, Ap vs water
544 control, As vs water control, Gm vs water control, Ab vs Ap, Ab vs As, Ab vs Gm. p-
545 values were computed and adjusted for multiple testing using the Benjamini-Hochberg

546 method. Differentially expressed genes were determined considering a 5 % false rate
547 discovery (adjusted p-value < 0.05) and at least a 1.5-fold change between treatment
548 (log2 fold change (LFC) > 0.58).

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560 Authors contributions

561 SD, JV and JM had the original idea and secured funding for the study. MCC and GT
562 designed the experiments. MC and KR performed beetle collection and rearing and set
563 up the oak billets infestation. GT performed chemical compounds extractions and
564 analysis, with feedback and support from JC and JV. MCC developed protocols and
565 performed the bioactivity assays and RNA seq experiment with support and feedback

566 from JM, JD and UH. MCC analysed the data, created the figures and wrote the first
567 draft of the manuscript. All authors reviewed the manuscript.

568 Data availability

569 The raw RNAseq data are available on the European Nucleotide Archive under the
570 project accession PRJEB69286. All scripts used for the analysis are available as a
571 computational notebook at https://doi.org/10.6084/m9.figshare.27331281, and raw
572 data and intermediate files to reproduce the analysis are available at
573 https://doi.org/10.6084/m9.figshare.27331488.

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577 Supplementary material

578 **Table S1:** Differentially expressed genes in *B. goodwinii* cultures supplemented with
579 gallery wood tissue extracts compared to asymptomatic wood extracts.

580 **Table S2:** Differentially expressed genes in *B. goodwinii* cultures supplemented with Ab
581 larval extracts compared to water control.

582 **Table S3:** Differentially expressed genes in *B. goodwinii* cultures supplemented with Ap
583 larval extracts compared to water control.

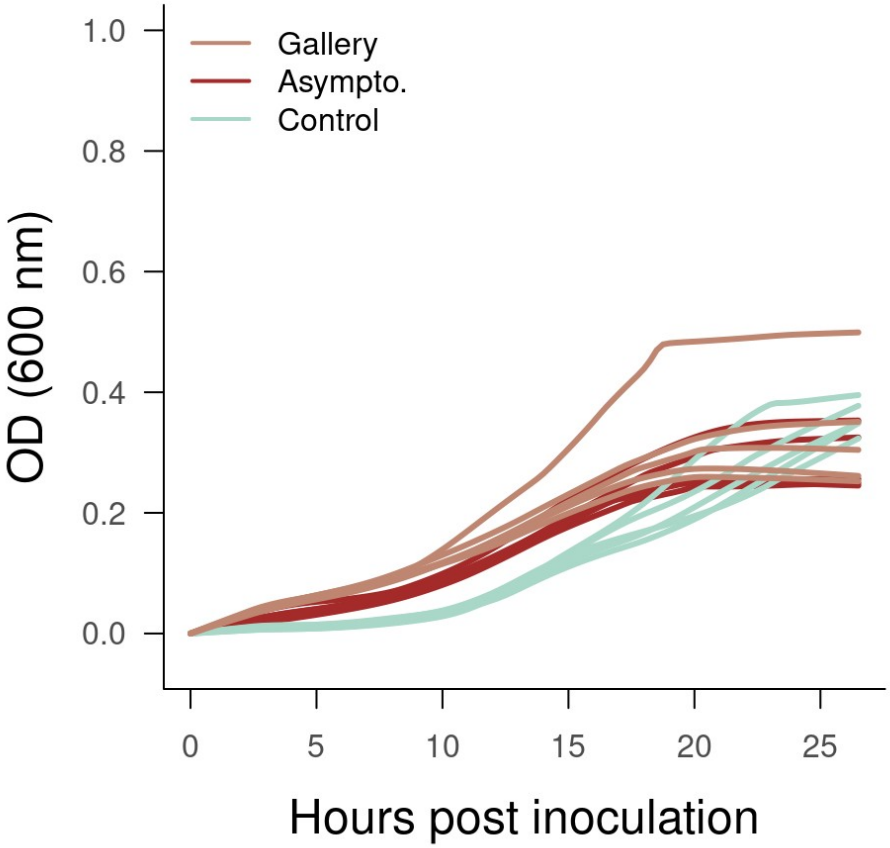

Figure S1: Growth curves obtained in 200 µL liquid cultures supplemented with gallery wood tissue (Gallery) and asymptomatic wood tissue (Asympto.) chemical compounds, compared to chemical compound extraction blanks (Controls).

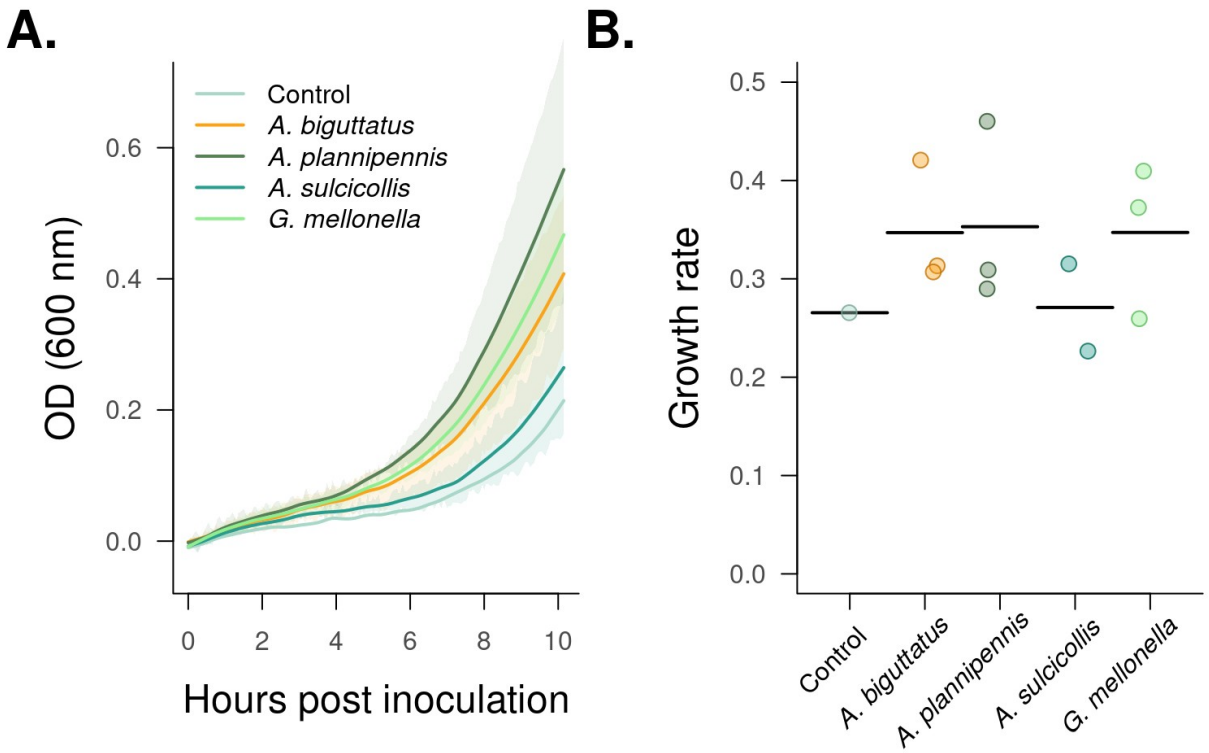

Figure S2: Growth of B. goodwinii cultures supplemented with insect larvae extracts. Due to the limited amount of extract, only one experiment was conducted with these larvae species and cultures were only grown for 10 hpi and collected for RNAseq analysis. **A.** OD_{600} of culture supplemented with *A. biguttatus*, *A. sulcicollis*, *A. plannipennis* and *G. melonella*. Control cultures were supplemented with extraction blanks (water). Growth was stopped at 1 hpi. **B.** Growth rates of cultures calculated on the 10 hours of growth. Differences between treatment were not significant.

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