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**Developmental stages and gut microenvironments influence gut microbiota dynamics in the invasive beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae).**

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2 **invasive beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae)**

3

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24

25 **Abstract**

26 *Popillia japonica* Newman (Coleoptera: Scarabaeidae) is a highly polyphagous invasive beetle  
27 originating from Japan. This insect is highly resilient and able to rapidly adapt to new vegetation.  
28 Insect-associated microorganisms can play important roles in insect physiology, helping their hosts  
29 to adapt to changing conditions and potentially contributing to an insect's invasive potential. Such  
30 symbiotic bacteria can be part of a core microbiota that is stably transmitted throughout the host's  
31 life cycle or selectively recruited from the environment at each developmental stage. The aim of this  
32 study was to investigate the origin, stability and turnover of the bacterial communities associated with  
33 an invasive population of *P. japonica* from Italy. Our results demonstrate that soil microbes represent  
34 an important source of gut bacteria for *P. japonica* larvae, but as the insect develops, its gut microbiota  
35 richness and diversity decreased substantially, paralleled by changes in community composition.  
36 Notably, only 16.75% of the soil bacteria present in larvae are maintained until the adult stage. We  
37 further identified the micro-environments of different gut sections as an important factor  
38 shaping microbiota composition in this species, likely due to differences in pH, oxygen availability  
39 and redox potential. In addition, *P. japonica* also harboured a stable bacterial community across all  
40 developmental stages, consisting of taxa well known for the degradation of plant material, namely  
41 the families Ruminococcaceae, Christensenellaceae and Lachnospiraceae. Interestingly, the family  
42 Christensenellaceae had so far been observed exclusively in humans. However, the  
43 Christensenellaceae operational taxonomic units found in *P. japonica* belong to different taxonomic  
44 clades within this family.

45

46

47 **Introduction**

48 Insects are the most diverse and abundant animal clade (Footitt and Adler, 2009). The diversification  
49 and evolutionary success of insects have been partially attributed to their ability to establish  
50 associations with different beneficial microorganisms (e.g. Douglas, 2014; Corbin et al., 2017;  
51 Sudakaran et al., 2017; Heddi and Zaidman-Rémy, 2018). These microorganisms can play key roles  
52 for different physiological functions such as the supply of essential nutrients missing from unbalanced  
53 diets; contributing to the digestion of recalcitrant food components; protection from predators,  
54 parasites and pathogens; and controlling mating and reproductive systems (e.g. Leftwich et al., 2017;  
55 Muhammad et al., 2017).

56 As for essentially all animals, microbial communities are particularly prominent in the digestive tract  
57 (e.g., Douglas, 2015; 2018; Clayton et al., 2018; Münger et al., 2018). The insect gut is generally  
58 structured into foregut, midgut and hindgut, presenting a multitude of micro-environments suitable  
59 for microbial colonization. Differences in morphology and physico-chemical properties between  
60 different gut sections can greatly influence the microbial colonization patterns and community  
61 structure depending on the host species. Gut bacteria have the potential to provide many beneficial  
62 services to their hosts and insects display a wide range in degree of dependence on gut bacteria for  
63 basic functions. Paramount to the evolution of intimate associations with gut microorganisms is the  
64 development of secure transmission routes between host individuals and generations. The lack of  
65 such mechanism in most insect species may hinder the establishment of such longterm associations.  
66 With the exception of social insects, such as termites and ants, where social interactions provide  
67 opportunities for the transfer of gut bacteria (Zhukova et al., 2017), insects had to develop original  
68 ways in order to transmit the important components of their gut microbiota (Fukatsu and Hosokawa,  
69 2002; Gonella et al., 2012; Hosokawa et al., 2013; Mason et al., 2019). These ‘heritable’ gut bacteria  
70 have been shown to play crucial roles in the nutrition, protection against different pathogens and

71 xenobiotics, modulation of immune responses and even extending life span (Roh et al., 2008; Kim et  
72 al., 2016; Daisley et al., 2018; Obata et al., 2018).

73 Several factors can influence the gut microbiota structure and composition. Among these factors, the  
74 most important ones are diet and environment, but other factors (e.g. age) can also be at play (Wong  
75 et al., 2011; Montagna et al., 2015a; 2015b; Montagna et al., 2016; Sanders et al., 2017; Tiede et al.,  
76 2017; Vacchini et al., 2017; Anderson et al., 2018). Although various factors can influence the insect  
77 gut microbiota, the existence of a shared core microbial community in some species could indicate  
78 that there are mechanisms (e.g. vertical transmission) favouring the presence of certain members of  
79 the gut microbiota. Several studies have investigated this possibility by tracking the changes in gut  
80 microbiota composition along the developmental stages of different insect species. These studies  
81 showed that the transmission of the gut microbiota throughout the different developmental stages  
82 may depend on the usefulness of certain bacteria (Zhukova et al., 2017; Malacrinò et al., 2018). For  
83 instance, the bacterial communities of fruit flies (Tephritidae) change throughout the insect's  
84 developmental stages to respond to the physiological needs of the host (Aharon et al., 2013; Malacrinò  
85 et al., 2018). In holometabolous insects, the pupal stage generally represents a bottleneck where most  
86 of the larval gut microbiota is lost and adult insects may have to resort to indirect ways (e.g. via  
87 environmental transmission) to insure the transfer of beneficial bacteria from larvae to adults  
88 (Zhukova et al., 2017). For instance, in certain bee species, certain bacterial taxa are not trans-stadially  
89 transmitted but re-acquired from the environment (McFrederick et al., 2014). While the gut  
90 microbiota is not constant across the developmental stages in most insects, in some cases, the  
91 microbial community can be relatively stable throughout the developmental stages. This has been  
92 observed in some Tephritid flies as well as in the Black Soldier Fly *Hermetia illucens* and in the moth  
93 *Plodia interpunctella* (Mereghetti et al., 2019; Yong et al., 2017; De Smet et al., 2018).

94 In the present study, we focused on the highly polyphagous invasive Japanese beetle *Popillia japonica*  
95 Newman (Coleoptera: Scarabaeidae, Supporting Information Fig. S1a). This invasive insect is listed  
96 in the EPPO Annex 2 due to the damages caused to different crops and turfs (EPPO, 2000). Native to

97 Japan and the far east of Russia (Fleming, 1972), this beetle became an established pest in North  
98 America in the early 1900's (Switzer et al., 2009), in the Azores in the early 1970's (Vieira, 2008)  
99 and more recently in continental Europe, where it was recorded for the first time in Italy in 2014  
100 (EPPO, 2014; Pavesi, 2014) and in Switzerland in 2017 (EPPO, 2017). Several laboratory and field  
101 trials have been carried out to limit the spread of this pest in mainland Europe and to evaluate the  
102 environmental resilience of the infested areas (Mazza et al., 2017; Paoli et al., 2017a, 2017b;  
103 Marianelli et al., 2018a, 2018b). The damages to plants are caused by the different developmental  
104 stages of the beetle: the larvae, being underground dwellers, feed on the plant roots and soil organic  
105 matter while adults, living in an above-ground environment, feed on leaves and floral parts of  
106 different plant species (Fleming, 1972; Vieira, 2008).

107 Insect-associated bacteria can potentially contribute to an insect's invasive potential by helping their  
108 hosts to adapt to changing environmental conditions. Such symbiotic bacteria can be part of a core  
109 microbiota that is stably transmitted throughout the host's life cycle or selectively recruited from the  
110 environment at each developmental stage. The aim of this study was to investigate microbiota  
111 dynamics in an invasive population of *P. japonica* from Italy. Specifically, we addressed the  
112 following questions: (i) does *P. japonica* harbour a stable core microbiota or are the bacteria mainly  
113 acquired from the surrounding environment (i.e. rhizospheric soil exploited by larvae and pupae vs  
114 aerial environment exploited by adults)? (ii) is the gut microbiota maintained across the post-  
115 embryonic developmental stages (i.e. larvae, pupae and adults) or is there a major turnover due to  
116 insect development? (iii) do different gut micro-environments impact microbial community  
117 structure?

118

## 119 **Results**

### 120 *Alpha, beta and phylogenetic diversity of the gut microbiota*

121 In this study, we analysed the microbiota associated with three gut sections (foregut, midgut and  
122 hindgut) of the different developmental stages (L1, L2, L3, pupae, adult males and females) of *P.*

123 *japonica*. For each sample type, 16S rRNA gene amplicons were obtained from three biological  
124 replicates, each containing the tissues of five individuals. In addition, we analysed the microbiota of  
125 nine soil samples taken from the same habitat from which the insects were sampled. A total of 5 175  
126 086 high-quality reads longer than 250 bp were kept after quality filtering and chimera removal.  
127 These reads clustered into 1612 operational taxonomic units (OTUs). On average, 67 299 high-quality  
128 reads grouped into 336 OTUs were obtained from larvae, 80 249 reads/204 OTUs from pupae, 88  
129 397 reads/99 OTUs from adults and 148 324 reads/1093 OTUs from soil samples (see Table S1a,  
130 Supporting Information, for details). Rarefaction curves of the observed OTU richness in 25 000  
131 subsampled sequences showed that our sequencing effort was sufficient to capture the major part of  
132 the bacterial diversity associated with both insect and soil samples (Supporting Information Fig. S2).  
133 OTU richness and diversity (Supporting Information Fig. S2), as determined by the species richness  
134 estimator Chao1 and the Shannon Index of diversity, were higher in soil samples than in insect  
135 samples (Chao1: all t-tests  $p < 0.01$ ; Shannon: all t-tests  $p < 0.01$ ; see Supporting Information Table  
136 S1b for more details on the statistics for the different comparisons). Regarding the different  
137 developmental stages of *P. japonica*, OTU richness and diversity were the highest in the larvae (Chao  
138 1: all t-tests  $p < 0.01$ ; Shannon: all t-tests  $p < 0.01$ , see Supporting Information Table 1 and Table  
139 S1b for all ecological indices). On the other hand, these indices were the lowest for adults (Chao 1:  
140 all t-tests  $p < 0.01$ ; Shannon: all t-tests  $p < 0.01$ ; Table 1 and Supporting Information Table S1b). The  
141 different larval instars had similar richness and diversity with the Chao 1 and Shannon indices of  
142  $360.26 \pm 52.2$  and  $4.99 \pm 0.77$ , respectively, for L1 larvae,  $313.92 \pm 48.44$  and  $5.47 \pm 0.28$  for L2  
143 larvae and  $342.96 \pm 43.02$  and  $5.74 \pm 0.27$  for L3 larvae (Chao 1: all t-tests  $p > 0.5$ ; Shannon: all t-  
144 tests  $p > 0.5$ , Supporting Information Table S1b). It is noteworthy that the values of Pielou's evenness  
145 also followed a similar pattern, with the soil having the highest value (Pielou'J = 0.84; Table 1), then  
146 larvae (Pielou'J = 0.67; Table 1) and with pupae and adults having similar values (Pielou'J = 0.47  
147 and 0.49, respectively; Table 1).



148 The standardized effect size of mean pairwise distance values (SES\_MPD) of the bacterial  
149 communities associated with the samples ranged from positive values for soil bacterial communities  
150 (median value of SES\_MPDSOIL = 0.78 associated with high quantiles, Supporting Information  
151 Table S1c) to negative values for bacterial communities associated with the larval and pupal stages  
152 (median values SES\_MPDLARVAE = -3.38 and SES\_MPDPUPAE = -3.9, low quantile values,  
153 Supporting Information Table S1c) (Fig. 1C). SES\_MDP values were significantly different between  
154 sample types (one-way ANOVA,  $F = 36.75$ ,  $df_1 = 3$ ,  $df_2 = 21.4$ ,  $p < 0.001$ ), namely between larvae  
155 and soil (Tamhane post hoc test,  $p < 0.001$ ) and between larvae and adults (Tamhane post hoc test,  $p$   
156 = 0.001). The positive SES\_MPD values for the soil communities indicate a phylogenetic  
157 overdispersion, as expected for communities characterized by high species richness and evenness  
158 such as those of soil. In contrast, the negative SES\_MPD values for the bacterial communities  
159 associated with larvae and pupae indicate a phylogenetic clustering of these communities, possibly  
160 due to the selection toward certain closely related bacterial lineages by the insect gut environment or  
161 to the adaptation of these bacteria to the gut environment. Interestingly, the bacterial communities  
162 associated with adults were characterized by slightly negative SES\_MPD values (median value of  
163 SES\_MPDADULTS = -0.53; Supporting Information Table S1c), indicating a phylogenetic  
164 evenness of these communities (Fig. 1C). This increasing trend of SES\_MPD values from larvae and  
165 pupae (negative values) toward adults (slightly negative values) contrasted with the trend of  
166 decreasing community species richness from larvae to adults (Supporting Information Fig. S3).

#### 167 *Factors affecting gut microbiota composition*

168 Soil was different from the insect samples in terms of bacterial composition (adonis:  $p < 0.001$ ,  $R^2 =$   
169 0.33; ANOISM:  $p < 0.001$ ,  $R = 0.54$ ) with few OTUs shared between soil and the different insect  
170 developmental stages (Fig. 1A). Specifically, 891 OTUs out of the 1102 'core OTUs' of the soil were  
171 not found in the insect samples (Fig. 1B). On the other hand, only 35 'core OTUs' present in soil  
172 were also present in all the insect developmental stages (Fig. 1B). Moreover, the nestedness  
173 component of the  $\beta$ -diversity between soil and the different insect developmental stage was very low

174 (0.16 on average) and the turnover was high (0.84 on average) (Supporting Information Fig. S4),  
175 indicating that very few ‘core OTUs’ were shared between soil and insect microbiotas while the  
176 variable fraction was high.

177 Although more bacterial OTUs were shared between the insect samples (i.e. developmental stages  
178 and gut sections combined) than between insects and soil, these samples still formed distinct clusters  
179 as shown by non-metric multidimensional scaling (NMDS) analysis (Fig. 2A). Specifically, insect  
180 developmental stages segregated along the first axis with the larvae microbiotas being clearly distinct  
181 from adult microbiotas, while pupal microbiotas were intermediate. The second axis further separated  
182 the samples based on gut sections. For larvae and adults, the microbiotas of the different gut sections  
183 formed distinct clusters with the midgut microbiota being more different than the foregut and hindgut  
184 microbiotas. In contrast, the pupal microbiotas showed a different pattern with a clear cluster for the  
185 hindgut, while foregut and midgut microbiotas loosely clustered together.

186 Based on the correlations of the tested factors (i.e. developmental stages and gut sections) with the  
187 NMDS ordinations of the insect-associated bacterial communities, the main factor driving this  
188 segregation was the gut section ( $R^2 = 0.18$ ,  $p = 0.003$ ) and to a lesser extent the developmental stage.  
189 These results were further supported by the Random Forest (RF) analysis which was carried out to  
190 investigate the specificity of the microbiota of each sample category by trying to assign each sample  
191 to its respective category based on its microbiota. The RF analysis (Supporting Information Table  
192 S1d) was able to successfully classify adults and larvae in 100% and 91.7% of the cases, respectively.  
193 Conversely, pupae were successfully identified in only 55.6% of the cases. These results suggest that  
194 the pupal stage represents a transitional step not only in the development of the insect but also for its  
195 associated microbiota. The most important OTUs discriminating between the different developmental  
196 stages belonged to the Firmicutes (Clostridiales and Bacilli), Proteobacteria (Alphaproteobacteria)  
197 and Actinobacteria (see Supporting Information Table S1f). On the other hand, the RF was able to  
198 successfully classify the foregut, midgut and hindgut samples in 80%, 82% and 78% of the cases,  
199 respectively (Supporting Information Table S1e). The most relevant OTUs allowing to discriminate

200 between the different gut sections were identified as Firmicutes (Clostridiales) and Proteobacteria  
201 (Betaproteobacteria). These results indicate that the different gut sections as well as larvae and adults  
202 have distinct microbial communities, whereas the pupal stage has not.

203 In order to further investigate the correlation between the physico-chemical conditions of the gut and  
204 microbial composition, we measured pH, O<sub>2</sub> concentration and redox potential in each gut section  
205 for both male adults and L3 larvae (see Supporting Information Table S1 and Fig. S5). While the  
206 adult gut constituted a niche with a neutral pH (or at most slightly sub-acidic conditions), the pH in  
207 the larval gut increased from neutral in the foregut to alkaline conditions in the midgut and hindgut.  
208 Both larval and adult digestive systems were characterized by anoxic conditions, with the exception  
209 of the the adult foregut where conditions fluctuated from anoxia to microaerophilia. Finally, positive  
210 redox potential values were measured in all gut compartments of both larvae and adults, with the  
211 exception of the larval hindgut where a decrease in redox potential was measured, underlining the  
212 existence of reducing conditions in this region. These three factors were significantly correlated with  
213 the microbial composition in the different gut sections. Notably, pH was significantly correlated with  
214 the microbiota of larvae ( $R^2 = 0.75$ ,  $p = 0.001$ ), while O<sub>2</sub> concentrations ( $R^2 = 0.54$ ,  $p = 0.002$ ) and  
215 redox potential ( $R^2 = 0.74$ ,  $p = 0.001$ ) correlated significantly with the bacterial composition in adult  
216 gut regions (Fig. 2B).

#### 217 *Taxonomic composition of P. japonica gut microbiota*

218 The microbiota associated with different developmental stages of the host and with soil not only  
219 differed in terms of bacterial richness and diversity but also concerning bacterial community  
220 composition (Fig. 3; Fig. 2A, Supporting Information Fig. S6). Although Proteobacteria represented  
221 the most abundant phylum considering all sample types ( $35.9\% \pm SE 4.2\%$ ), followed by Firmicutes  
222 ( $32.9\% \pm SE 5.4\%$ ) and Bacteroidetes ( $15.4\% \pm SE 3.7\%$ ), these proportions changed among the  
223 different sample types. Considering larvae (Fig. 3B, Supporting Information Fig. S6), the most  
224 abundant phylum was Firmicutes with an average of  $49.5\% \pm SE 7.9\%$  (range  $26.5\% \pm SE 5.5\%$  in  
225 L2 larvae to  $74.5\% \pm SE 8.7\%$  in L1 larvae), followed by Proteobacteria ( $31.3\% \pm SE 5.8\%$  on

226 average; range: 13.9%  $\pm$  SE 5.1% in L1 larvae to 50.3%  $\pm$  SE 5.9% in L2 larvae) and Actinobacteria  
227 (9.4%  $\pm$  SE 2.6% on average; range 5%  $\pm$  SE 2.5% in L1 larvae to 13.9%  $\pm$  SE 4% in L3 larvae). On  
228 the other hand, the most abundant taxa in adults were Bacteroidetes (33.7%  $\pm$  SE 7.8% on average;  
229 39%  $\pm$  SE 10.6% in females, 28.3%  $\pm$  SE 12.9% in males) followed by Firmicutes (29.6% on average;  
230 14.5%  $\pm$  SE 1.5% in females, 44.8%  $\pm$  SE 4.1% in males) then Proteobacteria (29.1% on average;  
231 40%  $\pm$  SE 12.6% in females, 18.2%  $\pm$  6.6% SE in males). In pupae, the most abundant phylum was  
232 Proteobacteria with 59.7%  $\pm$  SE 11.5%, followed by Bacteroidetes (19.1%  $\pm$  SE 9.2%) and Firmicutes  
233 (15.4%  $\pm$  SE 9.9%). It is noteworthy that the proportion of Actinobacteria decreased when passing  
234 from soil to adults (going from 24.8%  $\pm$  SE 1.5% in soil to 6.4%  $\pm$  SE 1.9% in adults), while the  
235 proportion of Bacteroidetes followed the opposite trend, going from 8%  $\pm$  SE 1.2% in soil to 33.7%  
236  $\pm$  SE 7.9% in adults (Fig. 3A). Other bacterial taxa present at minor proportions (such as Acidobacteria,  
237 Chloroflexi and Nitrospira) followed a trend similar to Actinobacteria, with their proportions  
238 decreasing from soil to adults.

239 Looking at the different gut sections (Fig. 3C), we observed similar trends. Relative abundance of  
240 Actinobacteria and Proteobacteria decreased from soil to hindgut from 24.2% and 39.6%,  
241 respectively, to 1.6% and 17.4% respectively. On the other hand, the relative abundance of Firmicutes  
242 increased from soil to hindgut from 7.3% to 52.3%.

#### 243 *Spatio-temporal changes in the microbiota taxonomic composition*

244 As mentioned earlier, 891 of the 1102 ‘core OTUs’ present in the soil were not found in the insect  
245 samples, while only 35 ‘core OTUs’ were present in both insects and soil (Fig. 1B). These OTUs  
246 belonged predominantly to the Proteobacteria phylum (26 of the 35 OTUs) with Rhizobiales being  
247 the most represented order (eight OTUs). In addition to these 35 OTUs, of the 630 ‘core OTUs’ found  
248 in insects but not in soil, 54 OTUs were shared between all the developmental stages. Proteobacteria,  
249 Bacteroidetes and Firmicutes were the most abundant phyla (28, 10 and 9 OTUs respectively).  
250 Noteworthy, OTUs belonging to the families Rickenellaceae (five OTUs), Lachnospiraceae (three  
251 OTUs) and Ruminococcaceae (one OTU) were among the OTUs shared between the insect

252 developmental stages. These families were identified as taxa specifically enriched in the insect guts  
253 along the different developmental stages.

254 We next performed a (taxon enrichment analysis (TEA) to identify which bacterial families were  
255 consistently enriched in insects compared to soil (Fig. 4). This analysis showed that among the  
256 Firmicutes, the Ruminococcaceae was significantly enriched in larvae compared to soil ( $p < 0.001$ ),  
257 but there were no differences when comparing the different developmental stages. Similarly, other  
258 bacterial families belonging to the Firmicutes and specifically to the order Clostridiales (namely  
259 Christensenellaceae and Lachnospiraceae) resulted to be significantly enriched in larvae and  
260 generally in insects when compared with soil samples. These families were also enriched in the  
261 different compartments of the gut when compared with soil ( $p < 0.001$ ), independent of the insect  
262 developmental stages. Other bacterial families, such as Rikenellaceae (Bacteroidetes) and  
263 Desulfovibrionaceae (Proteobacteria), were also enriched in larvae compared to soil. These bacteria  
264 were also enriched in other portions of the gut but not all of them. Desulfovibrionaceae were also  
265 enriched in the midgut and hindgut, while Rikenellaceae were only enriched in the hindgut.  
266 Interestingly, all enriched families were absent from the soil samples (Supporting Information Table  
267 S3). While these families were not always present in the foregut, Desulfovibrionaceae,  
268 Lachnospiraceae and Ruminococcaceae were present in all midgut and hindgut samples for all  
269 developmental stages. Rikenellaceae, on the other hand, were present in all hindgut samples but  
270 absent from two midgut samples, namely one L1 and one pupal midgut sample (Supporting  
271 Information Table S3).

272 It is noteworthy that the TEA did not evidence any significantly enriched taxonomic group between  
273 the different developmental stages of the insect nor did it evidence enriched taxonomic group between  
274 the different gut sections. This is partly supported by the fact that the nestedness component of the  $\beta$ -  
275 diversity between the different insect developmental stages was relatively high (0.59 on average),  
276 indicating that a higher fraction of the microbiotas is shared between the different insect  
277 developmental stages than between insects and soil.

278 An Indval analysis carried out to identify OTUs specific to a given developmental stage showed that  
279 23 OTUs were unique to larvae, five were associated only with pupae while 13 were specific to adults  
280 (see Table S2a for Supporting Information). Members of the Lachnospiraceae family were the most  
281 represented OTUs among those unique to both larvae and adults (with nine and five OTUs present  
282 respectively).

283 The same analysis carried out on the different gut sections for each developmental stage gave a  
284 different picture. For the pupal stage, there was no OTU specific to a given gut section. For adults,  
285 15 OTUs were found only in the foregut, while 5 OTUs were specific to the hindgut. No OTU was  
286 found to be unique to the midgut. On the other hand, in the larvae, only two OTUs were specific to  
287 the foregut, while the midgut and hindgut had, respectively, 105 and 145 specific OTUs. It is  
288 noteworthy that three out of the five OTUs that were unique to the adult hindgut were also found  
289 specifically associated with the larvae hindgut. These OTUs belonged to the Rikenellaceae  
290 (denovo5575 and denovo143435) and Nitrosomonadaceae (denovo213936) families.

#### 291 *Phylogenetic relationship of Christensenellaceae associated with P. japonica*

292 Bacteria belonging to Christensenellaceae have previously been observed only in humans. To better  
293 understand the phylogenetic relationships between members of the Christensenellaceae associated  
294 with *P. japonica* and those associated with humans, we performed a maximum likelihood phylogeny  
295 using our OTUs and 16S rRNA gene sequences from those isolated from humans (Supporting  
296 Information Fig. S7). The OTUs associated with the insect formed several clusters distinct from the  
297 cluster of human-associated symbionts. Hence, the bacteria associated with *P. japonica* belong to  
298 different taxonomic groups within the Christensenellaceae family.

299

#### 300 **Discussion**

301 In this study, we demonstrate that soil bacteria represent an important source for the gut  
302 microbiota of *P. japonica* larvae, but as the insect develops, the gut bacterial community  
303 experiences important changes in richness, diversity and composition. Specifically, 37% of the

304 OTUs (209 OTUs) present in larvae derived from the soil microbiota and 35 OTUs present in the  
305 soil were maintained throughout all the developmental stages of the insect. In addition, larvae  
306 had a higher OTU richness and diversity compared to adults. This is likely linked to the different  
307 lifestyles of the two stages: larvae are soil-dwelling and similar in OTU numbers to other soil-  
308 dwelling arthropods such as terrestrial isopods (healthy isopods OTUs on average 209; Dittmer  
309 et al., 2016), termites (number of OTUs consistently higher than 400; Su et al., 2016) and ants  
310 (number of OTUs about 400; Vieira et al., 2017; Zhukova et al., 2017), while the OTU numbers  
311 of adults are comparable to those of non-soil-dwelling insects (in 218 insect species, average  
312 OTUs 84; Yun et al., 2014). Pupae are an intermediate state between larvae and adults in terms  
313 of bacterial taxonomic richness and diversity, representing a bottleneck for bacterial transmission  
314 due to metamorphosis. Nonetheless, key bacterial taxa involved in plant material degradation are  
315 still transmitted to adults (see below for a detailed discussion). This reduction in both richness  
316 and diversity at the pupal stage could be due to a combination of factors both random and  
317 deterministic. On the one hand, a reduction of the number of bacterial cells during metamorphosis  
318 could have caused a random reduction in the diversity of the microbiota. On the other hand, the  
319 observed reduction in microbiota diversity throughout host development could be caused by one  
320 (or several) active mechanisms, such as (i) the change of nutrition (or lifestyle) between  
321 soil-dwelling larvae and adults, (ii) specific physico-chemical properties (e.g. the change in gut  
322 pH between larvae and adults), and/or (iii) enzymatic activities, among others. As a matter of  
323 fact, the observed changes (i.e. decrease in richness and diversity) are not a constant in insect  
324 development and other studies monitoring gut microbiota changes throughout development have  
325 shown different trends, such as an increase in species richness (Brucker and Bordenstein, 2012)  
326 or more generally the absence of a clear trend (Oliveira et al., 2018; Gao et al., 2019; Huang et  
327 al., 2019). The trend that we observe in *P. japonica* could be explained by its ecology, because  
328 soil dwelling arthropods such as termites and woodlice consistently present higher microbiota  
329 richness and diversity (Dittmer et al., 2016; Su et al., 2016; Vieira et al., 2017; Zhukova et al.,

330 2017) due to their proximity to a microbially rich and diverse environment (i.e. soil). On the  
331 other, arthropods living in ‘aerial’ ecosystems (i.e. plants and leaves) tend to have a less rich and  
332 diverse gut microbiota (Yun et al., 2014; Mereghetti et al., 2019).

333 Interestingly, the decrease in microbiota richness and diversity throughout the host  
334 developmental stages is accompanied by a shift in the phylogenetic community structure.  
335 Specifically, larvae and pupae harbour phylogenetically clustered bacterial communities, i.e.  
336 consisting of closely related bacterial taxa. In contrast, the adult microbiota is phylogenetically  
337 overdispersed, similarly to rhizospheric soil communities. The observation that larvae  
338 microbiotas are phylogenetically clustered and at the same time taxonomically rich compared to  
339 adults could be explained by a selection of certain taxonomic groups through the gut  
340 environment. The phylogenetic overdispersion of the adult gut microbiotas suggests that the  
341 pupal stage represents a crucial bottleneck for the gut microbiota in terms of species richness.  
342 This might be due to the random survival of bacterial taxa present in the larvae throughout  
343 metamorphosis (and its associated gut tissue restructuring) at the pupal stage. However, the fact  
344 that a certain number of taxa are maintained throughout the development from larvae to adult but  
345 are absent from soil, suggests the existence of a mechanism to specifically maintain essential  
346 bacterial partners (e.g. Ruminococcaceae, Lachnospiraceae). In other words, the survival of  
347 certain bacterial taxa may not be entirely random. Another possible explanation might be that the  
348 adult gut microbiota is renewed by feeding on leaves and flowers in contrast to rhizospheric soil  
349 and/or that the physico-chemical properties of the adult gut are more stable than in larvae (see  
350 Supporting Information Fig. S5). Hence, despite the potential existence of a mechanism to  
351 maintain and transmit a fraction of the microbiota, other bacterial taxa could still be transient and  
352 dependent on the food source (e.g. different parts of the plant, different plant species), as  
353 observed in *Drosophila melanogaster* where acetic acid bacteria are always associated with the  
354 fly, but the presence of other bacterial taxa is dependent on the environment (Adair et al., 2018;  
355 Wong et al., 2015).



356 This study allowed us to identify several factors potentially shaping microbiota composition in  
357 *P. japonica*. Specifically, we demonstrate that among the tested factors, microbiota composition  
358 varied significantly between different gut sections as well as between insect developmental  
359 stages. This strong correlation between different gut sections and microbiota diversity and  
360 composition is most likely due to (i) differences in the physico-chemical conditions prevailing in  
361 each gut section (Supporting Information Fig. S5) as well as (ii) biotic factors such as host  
362 enzymatic potential and immune response. It is noteworthy that the pupae represent a transitional  
363 stage with a reshuffling of the microbiota between the larval and adult stages. In other words, the  
364 larvae and adult microbiotas formed clearly distinct clusters, while the pupae microbiota was  
365 more dispersed between the larvae and adult clusters. This may have had an impact on the  
366 statistical analyses, leading to an apparently weaker effect of the developmental stages on  
367 microbiota composition.

368 Regarding the physico-chemical factors, oxygen availability was the most strongly correlated  
369 with differences in bacterial community structure between the different gut sections in adults,  
370 while intestinal pH was the most strongly correlated factor in larvae. Although both the midgut  
371 and hindgut compartments were largely anoxic in adults, the oxygen concentration in the midgut  
372 showed a higher degree of variation compared to the more anoxic hindgut. This is likely due to  
373 a considerably larger influx of oxygen via the gut epithelium in the case of the midgut, as  
374 observed in *Pachnoda ephippiata* (Lemke et al., 2003). This variability in oxygen availability  
375 between the different gut compartments may favour bacteria that are more tolerant towards such  
376 fluctuations. In larvae, the pH in the midgut and hindgut was alkaline, while the foregut had a  
377 neutral pH. It is important to note that the larvae are soil-dwellers feeding on fresh roots and  
378 decaying soil organic matter (SOM) (Fleming, 1972). In this regard, they are similar to other  
379 soil-dwelling macroinvertebrates, including many coleopterans, which feed on SOM and play an  
380 important role in its degradation and stabilization (Lavelle et al., 1997; Wolters, 2000). It has  
381 been shown that the conditions in the anterior hindgut of the humivorous termite *Cubitermes* spp.

382 (i.e. high alkalinity and oxygen influx) lead to a decrease of the molecular weight of the organic  
383 matter (Kappler and Brune, 1999), rendering it more soluble and thus more accessible for  
384 digestion in subsequent lessalkaline compartments (Ji et al., 2000; Kappler et al., 2000; Ji and  
385 Brune, 2001). Although the complex microbial communities in the guts of humivorous  
386 macroinvertebrates are thought to participate in the transformation of ingested SOM (Cazemier  
387 et al., 1997; Kane, 1997), detailed information on the composition and activities of the gut  
388 microbiota is lacking. In view of the high midgut alkalinity in *P. japonica*, it is reasonable to  
389 assume that at least some of the bacteria in the midgut are tolerant towards high pH conditions,  
390 because most bacterial taxa are also found in the more neutral gut sections of adults.

391 We further observed differences in microbiota composition at different taxonomic levels (from  
392 order to OTU) between the different developmental stages of *P. japonica*. For instance,  
393 Actinobacteria decreased in abundance from larvae to adults, while Bacteroidetes increased in  
394 abundance. However, no particular taxa were found to be specifically enriched in any of the  
395 developmental stages. A similar pattern was observed for the microbiota associated with different  
396 gut compartments (foregut, midgut and hindgut): no particular taxon was specifically enriched  
397 in any of the compartments. Nonetheless, Proteobacteria decreased from foregut to hindgut,  
398 while Firmicutes increased. Actinobacteria were relatively stable between foregut and midgut  
399 but decreased in the hindgut.

400 In contrast, several taxa were found to be significantly enriched between soil and insect gut.  
401 Those belonged mainly to the families Ruminococcaceae, Christensenellaceae and  
402 Lachnospiraceae. Members of these families are known to degrade cellulose (Flint et al., 2012;  
403 Biddle et al., 2013). The fact of finding them enriched in the insect gut may suggest a possible  
404 symbiotic relationship where these bacteria help their host degrade and metabolize cellulose, as  
405 in the case of the symbiotic association between termites, protists and bacteria (Liu et al., 2013)  
406 or woodlice and certain bacterial taxa (Bredon et al., 2018). These bacteria could be important in  
407 helping their host metabolize plant roots and leaves and might thus contribute to its success as a

408 polyphagous invasive insect. The bacterial taxa that were enriched in the gut of *P. japonica* have  
409 been previously reported in association with various insects but more importantly with ruminants  
410 and humans. *Anaerostipes* spp., *Coprococcus* spp. and *Dorea* spp. (members of the  
411 Lachnospiraceae family) have all been previously described in association with the human gut  
412 (Rainey, 2009) where they are hypothesized to be involved in pectin fermentation. Other  
413 members of the Lachnospiraceae family have also been described in association with other  
414 insects (Huang and Zhang, 2013; Bourguignon et al., 2018). The Ruminococcaceae family,  
415 represented by *Ruminococcus* spp. and *Oscillospira* spp. in *P. japonica*, has also been described  
416 in association with humans, ruminants, coleopterans and termites (Kamagata, 2011; Huang and  
417 Zhang, 2013; Bourguignon et al., 2018). *Ruminococcus*, in addition to *Bacteroides* spp., plays an  
418 important role in the fermentation of hemicellulose and the degradation of different plant material  
419 through the production of Carbohydrate- Active enZymes (CAZymes) (Jose et al., 2017).  
420 CAZymes are very important for the break-down of the different components of lignocellulose  
421 (i.e. cellulose, lignin, hemicellulose; Bredon et al., 2018). It is noteworthy that although some  
422 insects are able to express some of these enzymes, most of them heavily rely on their associated  
423 microorganisms to degrade lignocellulose (Bredon et al., 2018). On the other hand, the role of  
424 *Oscillospira* is still unknown and it is hypothesized that it may be involved in lignocellulose  
425 degradation (Kamagata, 2011). Rickenellaceae, with the genus *Alistipes*, and  
426 Desulfovibrionaceae have also been described in association with the guts of different animals  
427 (Koneru et al., 2016; Ruengsomwong et al., 2016), especially termites (Reid et al., 2014;  
428 Makonde et al., 2015), where they play an important role in the degradation of cellulose polymers  
429 (Ozbayram et al., 2018).

430 The taxa found to be enriched in insect samples could be preferentially present in insects due to  
431 favourable conditions in the gut environment without an actual effect of these bacteria on the  
432 insect host. However, the fact that these bacteria were not detected in soil suggests the presence  
433 of a more direct transmission mechanism independent of the environmental route. In addition,

434 the consistent presence of these bacteria in the gut regions where plant material is degraded  
435 further argues in favour of an active role of these bacteria and not just their presence as transient  
436 passengers.

437 In contrast to the above-mentioned bacterial families which have been observed not only in  
438 mammals but also in insects, the family Christensenellaceae had so far been observed exclusively  
439 in humans. Although its role in the degradation of nutrients is not yet understood, members of  
440 this family (i.e. *Christensenella minuta*) have been shown to play a central role in controlling the  
441 Body Mass Index and in helping to shape a ‘healthy’ microbiota in humans and transfected mice  
442 (Goodrich et al., 2014). Increased titers of *C. minuta* have also been correlated with longevity in  
443 humans (Biagi et al., 2016), while decreased titers were observed during different human diseases  
444 (Petrov et al., 2017; Yu et al., 2017). In addition, other bacteria belonging to the genus  
445 *Christensenella* have been isolated from diseased humans, although no causality has been  
446 established yet (Ndongo et al., 2016). The partial 16S rRNA gene-based phylogeny showed that  
447 the Christensenellaceae OTUs found in association with *P. japonica* do not cluster with the taxa  
448 associated with humans but rather form different clusters, suggesting that they belong to different  
449 taxonomic groups within the Christensenellaceae family (Supporting Information Fig. S7).

450 Although three biological replicates containing homologous gut regions from five individuals  
451 might be limiting, based on the results obtained in this study, we can conclude that the gut  
452 microbiota of *P. japonica* is highly dynamic across the developmental stages of the insect and  
453 changes in microbiota composition strongly correlated with the physico-chemical properties of  
454 the gut. Despite the microbiota high variability, 89 OTUs were maintained from larvae to adults,  
455 including 35 OTUs originating from the soil environment. As a future perspective, it would be  
456 interesting to investigate if these OTUs represent a stable core microbiota present in all *P.*  
457 *japonica* populations in different parts of the world or if they are subject to change in different  
458 environments. In the first case, this might indicate a more intimate symbiotic relationship  
459 potentially maintained via vertical transmission. In the latter case, the variable microbiota would

460 provide a means to investigate the origin of new invasions of this beetle, via a comparative  
461 analysis of the local soil and insect gut microbiotas.

462

## 463 **Materials and methods**

### 464 *Collection and processing of insect and soil samples*

465 Four campaigns were organized from June to September 2017 to collect insect samples at  
466 different developmental stages of the insect. The different stages and instars (in the case of larvae:  
467 larval instar 1 – L1; larval instar 2 – L2; larval instar 3 – L3) of the insects were collected in  
468 Oleggio (Novara, Italy; 45°36' N, 08°38' E, altitude ca. 230 m a.s.l.). Simultaneously, at each  
469 sampling expedition, 10 soil samples were taken from the sampled area and combined into a  
470 single sample representative of the area, leading to the collection of three soil samples. Insects  
471 were preserved in absolute ethanol while soil samples in 50 ml vials, kept refrigerated on the  
472 field and then stored at  $-20^{\circ}\text{C}$  before processing. All insects were surface sterilized before  
473 dissection using the protocol described in Montagna and colleagues (Montagna et al., 2015a).  
474 Ninety individuals (i.e. 15 individuals of each larval instar, 15 pupae, 15 males, 15 females) were  
475 dissected under sterile conditions, and the gut (Supporting Information Fig. S1b) was removed  
476 in sterile Ringer solution. The insect alimentary canal was then aseptically separated into its three  
477 compartments (i.e. foregut, midgut and hindgut). For each developmental stage and larval instar,  
478 five homologous gut compartments were pooled together in a single sample, resulting in three  
479 biological replicates for each sample category. These samples were used for DNA extraction (see  
480 Supporting Information Table S1 for a detail on the samples).

481 Additionally, male adults (N = 9) and L3 larvae (N = 6) were collected and immediately  
482 processed in order to measure physicochemical properties (pH level, redox potential, oxygen  
483 concentration) of different gut regions. Specimens were anaesthetised at  $4^{\circ}\text{C}$  for 3' before their  
484 dissection.

### 485 *DNA extraction, amplicon library preparation, sequencing and bioinformatics*

486 The DNA was extracted from each sample (consisting of five homologous gut compartments for  
487 a defined insect instar and developmental stage) using the phenol– chloroform methods (Doyle  
488 and Doyle, 1990) with the modifications described in Mereghetti and colleagues (Mereghetti et  
489 al., 2019). The DNA was then eluted in 50 µl of sterile water (Sigma-Aldrich, Saint Louis,  
490 Missouri, USA). A DNA extraction blank was performed as control to monitor for contamination  
491 of environmental bacterial DNA. DNA from soils was extracted using PowerSoil DNA Isolation  
492 Kit (MO BIO Laboratories Inc., Carlsbad, CA) following manufacturer’s instructions. Three  
493 independent DNA extractions were performed for each of the three representative soil samples.  
494 The extracted DNA was used as template for the amplification of the V4 hypervariable region of  
495 the 16S rRNA gene using the PCR primers 515F (Caporaso et al., 2011) and a blend of reverse  
496 primers 802R (Claesson et al., 2009) and 806R (Caporaso et al., 2011) in order to reduce  
497 amplification bias. Forward and reverse primers were tailed with two different GC rich  
498 sequences, enabling barcoding with a second amplification. Each sample was first amplified in  
499 20 µl reaction volume containing 8 µl HotMasterMix 5 Prime 2.5X (Quanta Bio), 0.4 µl BSA  
500 (20 µg µl<sup>-1</sup>) (Sigma-Aldrich), 1 µl EvaGreen™20X (Biotium), 0.8 µl 515F (10 µM) (– 5’  
501 modified with unitail 1 5’- CAGGACCAGGGTACGGTG-3’), 0.4 µl 802 R (10 µM) (– 5’  
502 modified with unitail 2 5’-CGCAGAGAGGCTCCGTG-3’), 0.4 µl 806 R (10 µM) (– 5’ modified  
503 with unitail 2 5’- CGCAGAGAGGCTCCGTG-3’), and 1 µl (50 ng) of DNA template. The PCR  
504 amplifications were performed in a CFX 96™PCR System (Bio-Rad) with 34 cycles of 94°C for  
505 20 s, 52°C for 20 s, 65°C for 40 s and a final extension of 65°C for 2 min. The second PCR  
506 amplification was performed in 25 µl reaction volume containing the same reagents as the first  
507 PCR but with 1.5 µl barcoded/TrP1 primers (10 µM) and with 1 µl of the first PCR amplification  
508 in the following conditions: 8 cycles of 94°C for 10 s, 60°C for 10 s, 65°C for 40 s, and a final  
509 extension of 72°C for 3min.

510 After labelling each sample with a specific Ion Torrent (Ion Express) DNA barcode, each single  
511 library was quality checked with agarose gel electrophoresis, quantified with Qubit Fluorometer

512 (Thermo Fisher Scientific) then pooled with the other libraries in equimolar amounts. The final  
513 product was then sequenced using the Ion Torrent PGM System. Libraries preparation and  
514 sequencing were performed at the Life Sciences Department of Trieste University, Italy.

515 Four samples (see Supporting Information Table S1a for details) were excluded from the  
516 following analyses because they did not have enough reads (<200). The reads of the remaining  
517 samples were analysed using QIIME version 1.9.1 (Caporaso et al., 2010). In detail, adapters  
518 were removed, and low-quality reads filtered (Phred <20, read length < 250pb). Uclust (Edgar,  
519 2010) was used to cluster the 16S rRNA sequences into OTUs with a similarity cut-off of 97%.  
520 Chimeras were removed using ChimeraSlayer. A representative sequence for each identified  
521 OTUs was aligned to Green-genes ([http:// greengenes.lbl.gov/](http://greengenes.lbl.gov/)) using Pynast (Caporaso et al.,  
522 2010). Taxonomic assignment was performed comparing the representative OTUs to Green-  
523 genes (release 13.8). Rare OTUs (i.e. singletons and OTUs <10) and OTUs identified as  
524 chloroplast were discarded. The resulting OTU table was then used for the subsequent analyses.

#### 525 *Diversity analyses*

526 Bacterial OTU richness, diversity and evenness were calculated using the package Vegan (Dixon,  
527 2003; Oksanen et al., 2018), implemented under the R software (R Project 3.0.2; [http://cran.r-](http://cran.r-project.org/)  
528 [project.org/](http://cran.r-project.org/)) adopting the species richness estimator Chao 1 (Chao, 1984), the Shannon H' index  
529 (Shannon, 1948) and the Pielou's evenness (Pielou, 1975), after sub-sampling the OTU table to  
530 obtain a total of 25 000 sequences per sample. Alpha diversity indices were compared between  
531 different groups (i.e. tissues and developmental stages) using two-sample t-tests with 999 Monte  
532 Carlo permutations.

533 In order to evaluate whether the structures of the bacterial communities associated with soil and  
534 the different developmental stages of *P. japonica* were driven by species competition or by  
535 environmental factors, thus resulting in a community dominated by closely related species (Webb  
536 et al., 2002; Mouquet et al., 2012; O'Dwyer et al., 2012), the mean pairwise distance between all  
537 taxa in the bacterial communities (MPD; Webb et al., 2002) was used as metric for phylogenetic

538 structure. To allow the comparison between the bacterial communities of the different types, null  
539 models maintaining species occurrence frequency constant were estimated. Standard effect size  
540 and relative position of each bacterial community with respect to the null MDP distribution,  
541 generated by 999 randomizations of the null model, were calculated using the `ses.mpd` function  
542 implemented in the R package `picante` (Kembel et al., 2010). This standardized metric quantifies  
543 the relative excess or deficit in the phylogenetic diversity for each community with respect to the  
544 entire species pool. Negative values reflect a relative phylogenetic clustering of the species, while  
545 positive values indicate a relative phylogenetic evenness (or overdispersion). `SES_MDP` values  
546 were visualized as box-plots based on sample type (i.e. soil, larvae, pupae, adults) and statistical  
547 differences among sample types were assessed using Welch's oneway ANOVA (Welch, 1951),  
548 because `SES_MDP` values were normally distributed based on Shapiro–Wilk test (Royston 1982)  
549 ( $p > 0.05$ ), but the variance between groups was not homogeneous based on Levene test (Levene,  
550 1960) ( $p < 0.001$ ). Hence, we used the Tamhane post hoc test for multiple comparisons without  
551 homoscedasticity.

552 The spatial (across the three gut regions) and temporal shifts (across developmental stages) of  
553 the *P. japonica* bacterial community (presence/absence) were estimated using the Sørensen-  
554 based multiple-site dissimilarity ( $\beta$ SOR; Baselga, 2010) implemented in the R package `betapart`  
555 (Baselga and Orme, 2012). The turnover and nestedness components of this  $\beta$ -diversity were  
556 calculated using Simpson-based multiple-site dissimilarity ( $\beta$ SIM; Baselga, 2010) and  
557 nestedness-resultant multiple-site dissimilarity ( $\beta$ NES; Baselga, 2010) respectively. In addition,  
558 for each  $\beta$ -diversity component, the pairwise dissimilarity values among the microbiotas of all  
559 analysed groups (i.e. soil, larvae, pupae and adults) were calculated using the `betapair` function  
560 of the R package `betapart` (Baselga and Orme, 2012) and visualized through heatmaps using  
561 `heatmap.2` from the R package `gplots`.

562 In order to assess the difference in the microbiota structure among soil and insect samples, the  
563 sub-sampled OTU table was subjected to a non-parametric one-way analysis of similarity



564 ANOSIM (Clarke, 1993), implemented in the vegan library and based on the Bray–Curtis  
565 dissimilarity (999 permutations permuting within gut samples of the same individuals in order to  
566 account for the nonindependence of the observations (Bray and Curtis, 1957).

567 The sub-sampled OTU table, after the removal of soil community samples, was used as input for  
568 a NMDS (Kruskal, 1964) biplot based on the Bray–Curtis dissimilarity (Bray and Curtis, 1957),  
569 in order to graphically ordinate samples and assess the differences among: (i) the developmental  
570 stages (i.e. larvae, pupae and adults), (ii) the three gut regions and (iii) to evaluate the impact of  
571 the gut physicochemical properties on the microbiotas associated with third instar larvae and  
572 adults. NMDS analyses were performed using the metaMDS function implemented in the R  
573 package Vegan (Dixon, 2003; Oksanen et al., 2018). The correlation between the microbiota  
574 composition and the tested factors (i.e. developmental stages, gut sections, gut physicochemical  
575 properties) was investigated by fitting the NMDS ordination scores with the envfit Vegan  
576 function (Dixon, 2003; Oksanen et al., 2018). The permutation of the community composition-  
577 based dissimilarity matrix (taking into account the non-independence of the different gut samples  
578 of the same individuals) allowed assessment of the significance of the fitted factors and vectors,  
579 and a squared correlation coefficient ( $R^2$ ) was calculated.

580 To determine the level of specificity of the microbiota composition associated with each  
581 developmental stage or gut region, model predictions were generated using RF regressors based  
582 on the relative abundance OTU table (Knights et al., 2011). In order to classify the microbiota  
583 samples based on host developmental stage or gut region, the supervised\_learning.py script from  
584 the QIIME pipeline was used. cv10 was used as error correction method with 999 replicate trees.

#### 585 *Changes in microbiota composition*

586 In order to identify OTUs shared between the different insect developmental stages and the soil,  
587 we only focused on OTUs that were typical for a given sample type (i.e. larvae, pupae, adults,  
588 soil). To this end, an OTU was considered ‘present’ in a given sample type only when it occurred  
589 in at least 66% of the biological replicates of that sample type (in most cases, two of the three

590 biological replicates). These OTUs are hereafter referred to as ‘core OTUs’. The ‘core OTUs’  
591 specific to or shared among the different developmental stages and the soil were visualized  
592 through a Venn diagram. In addition, a bipartite network analysis (Dormann et al., 2008) of the  
593 bacterial community associated with the *P. japonica* (larvae, pupae and adults) and the bulk soil  
594 was performed using the pairwise dissimilarity matrix generated from the OTU table adopting  
595 the Bray–Curtis dissimilarity index (Bray and Curtis, 1957). Cytoscape (Shannon et al., 2003)  
596 was used to visualize the network.

597 Differentially abundant taxa were determined after data normalization of the OTU table using  
598 the EdgeR package (version 3.16.5) with R (version 3.4.4). Differentially abundant OTUs were  
599 then ranked by their log<sub>2</sub> fold change from the most differentially abundant to the least  
600 differentially abundant. Ranked OTUs were used to determine enriched families between  
601 different groups using the tmod package (version 0.36) with the CERNO test (Yamaguchi et al.,  
602 2008) and the Benjamini–Hochberg correction. The position of the OTUs belonging to enriched  
603 families along the continuum of ranked OTUs was also assessed visually using receiver operating  
604 characteristic (ROC) curves. The enriched families were then tested for their presence in all  
605 samples (Supporting Information Table S3).

606 The OTU sequences of enriched taxa of interest (i.e. Christensenellaceae) were retrieved from  
607 the OTU file then aligned to complete or near complete 16S rRNA sequences downloaded from  
608 the NCBI website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using Clustal W. After gap removal, the evolution  
609 model was estimated using jModeltest according to the Akaike information criterion (AIC)  
610 parameter (Akaike, 1976). The phylogenetic tree was reconstructed using maximum likelihood  
611 with the Kimura 2 parameters model and 500 bootstraps. The phylogenetic tree was reconstructed  
612 and visualized using Mega X (Kumar et al., 2018).

613 In order to detect OTUs that are specific for a given gut section within the same developmental  
614 stage, the indicator value (Dufrêne and Legendre, 1997) was calculated using the R package  
615 *indicspecies* (De Cáceres and Legendre, 2009). Briefly, the indicator value of an OTU varies

616 from 0 to 1 and attains its maximum value when all reads of an OTU occur in all samples of only  
617 one specific gut section. We tested the significance of the indicator value for each OTU with a  
618 Monte Carlo randomization procedure with 999 permutations.

619 *Measurement of the gut physicochemical properties*

620 Physico-chemical parameters of oxygen partial pressure (pO<sub>2</sub>), pH and redox potential were  
621 measured in the different sections of *P. japonica* gut (foregut, midgut and hindgut) with  
622 microsensors and microelectrodes (Unisense, Aarhus, Denmark). Freshly dissected guts from  
623 both L3 larvae and males were placed on a layer of 2% (low melting point) agarose prepared  
624 with Ringer's solution (7.2 g l NaCl; 0.37 g l KCl; 0.17 g l CaCl<sub>2</sub>, pH 7.3–7.4) and immediately  
625 covered with a second layer of 0.5% agarose prepared with Ringer's solution (Šustr et al., 2014).  
626 Oxygen microsensors (OX-50), with a tip diameter of 50 μm, were calibrated after an overnight  
627 polarization in water saturated with air and in 0.1 M sodium dithionite anoxic solution by using  
628 the CAL 300 calibration chamber (Unisense), following an overnight polarization. pH  
629 microelectrodes (PH-50), with a tip diameter of 50 μm, were calibrated with standard solutions  
630 at pH 4.0, 7.0 and 10.0. Redox potential microelectrodes (RD-50) had a tip diameter of 50 μm  
631 and were calibrated using saturated quinhydrone solutions at pH 4.0 and 7.0. Electrode potentials  
632 for microelectrodes were measured against Ag-AgCl reference electrodes by using a high-  
633 impedance voltmeter (R<sub>i</sub> > 10<sup>14</sup> Ω). Unisense microsensor multimeter allowed to measure the  
634 current and data were recorded by using SensorTracePRO software (Unisense). Microsensors  
635 were positioned using a motorized micromanipulator (Unisense). Measurements were carried out  
636 at room temperature.

637 *Data accessibility.* The raw reads obtained in this work have been submitted to the Short  
638 Reads Archive (SRA) under the specifically created bioproject PRJNA526430. The data are  
639 already publicly available and will be linked to this paper once the manuscript is accepted. In  
640 addition to the sequencing data, all other data produced for this manuscript are provided as excel  
641 files in the Supporting Information.

642

643 **Author Contributions**

644 BC, MM and LM designed the experiments. BC performed the microbiota and enrichment  
645 analyses. MM, GMg and NG performed the statistical analyses. SA performed the network  
646 analyses. GMz, EG, FP, LM, PFR and AA performed the sampling. NG dissected the insects and  
647 extracted the DNA. FF and FG performed the sequencing. MC, MF, EC and DD performed the  
648 physicochemical analyses. BC and MM wrote the manuscript. All authors read and commented  
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650

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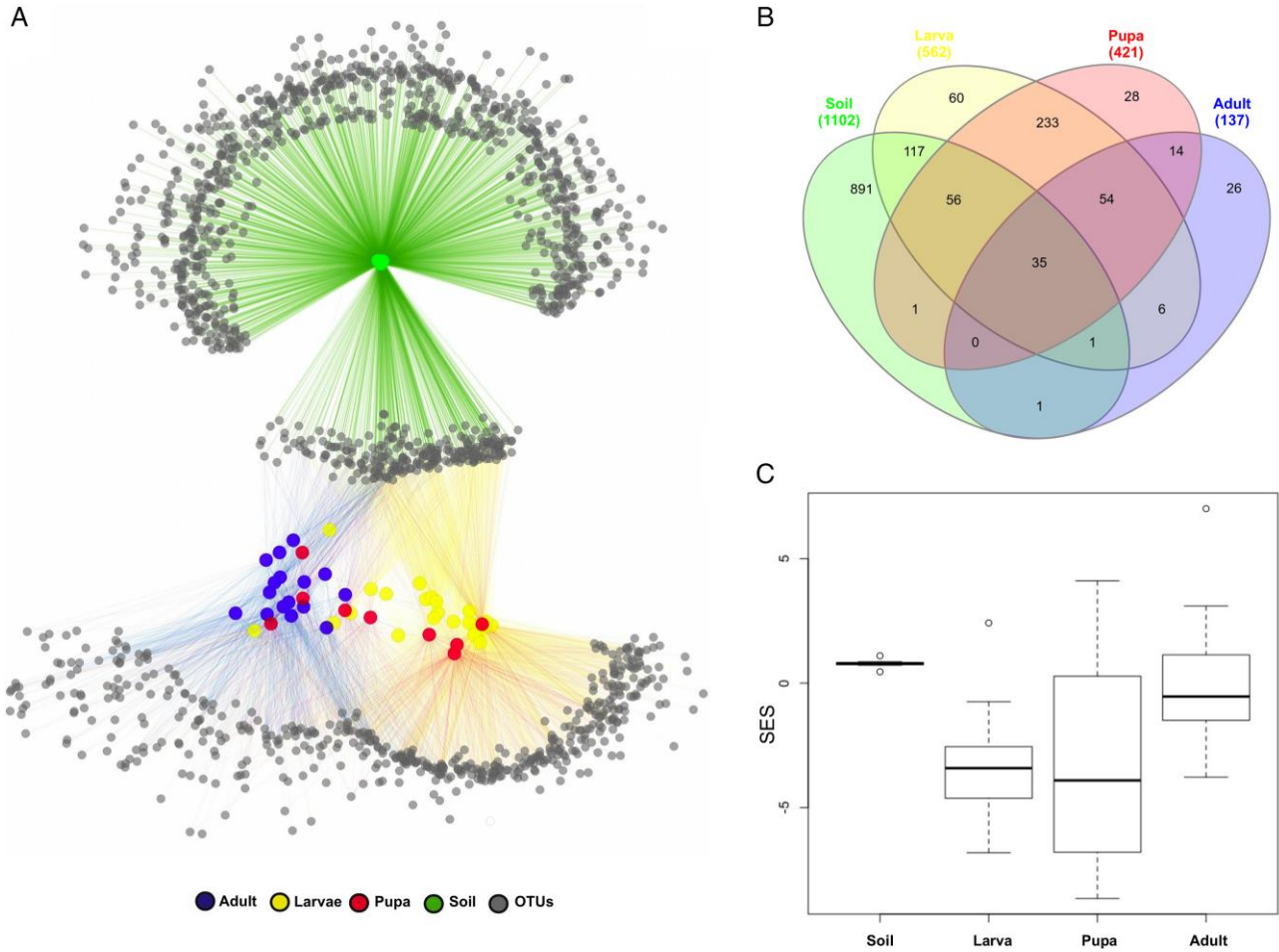


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974 **Tables**975 **Table1:** Ecological indices by developmental stage (mean  $\pm$  SE)

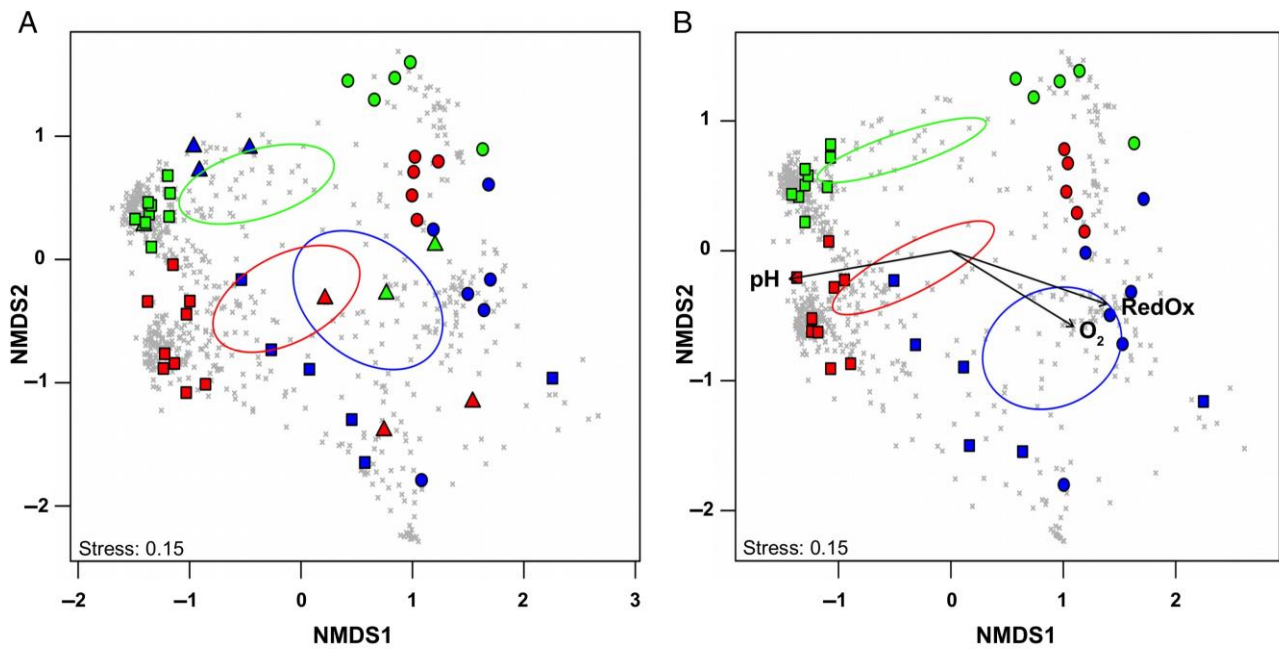
	Richness (Chao1)	Diversity (Shannon)	Evenness (Pielou)
Soil	1099 $\pm$ 1.35	5.88 $\pm$ 0.03	0.84 $\pm$ 0.00
Larvae	369.93 $\pm$ 28.95	3.77 $\pm$ 0.19	0.67 $\pm$ 0.03
Pupae	241.12 $\pm$ 43.51	2.49 $\pm$ 0.39	0.47 $\pm$ 0.06
Adults	129.65 $\pm$ 7.33	2.22 $\pm$ 0.18	0.49 $\pm$ 0.04

976 **Figures**



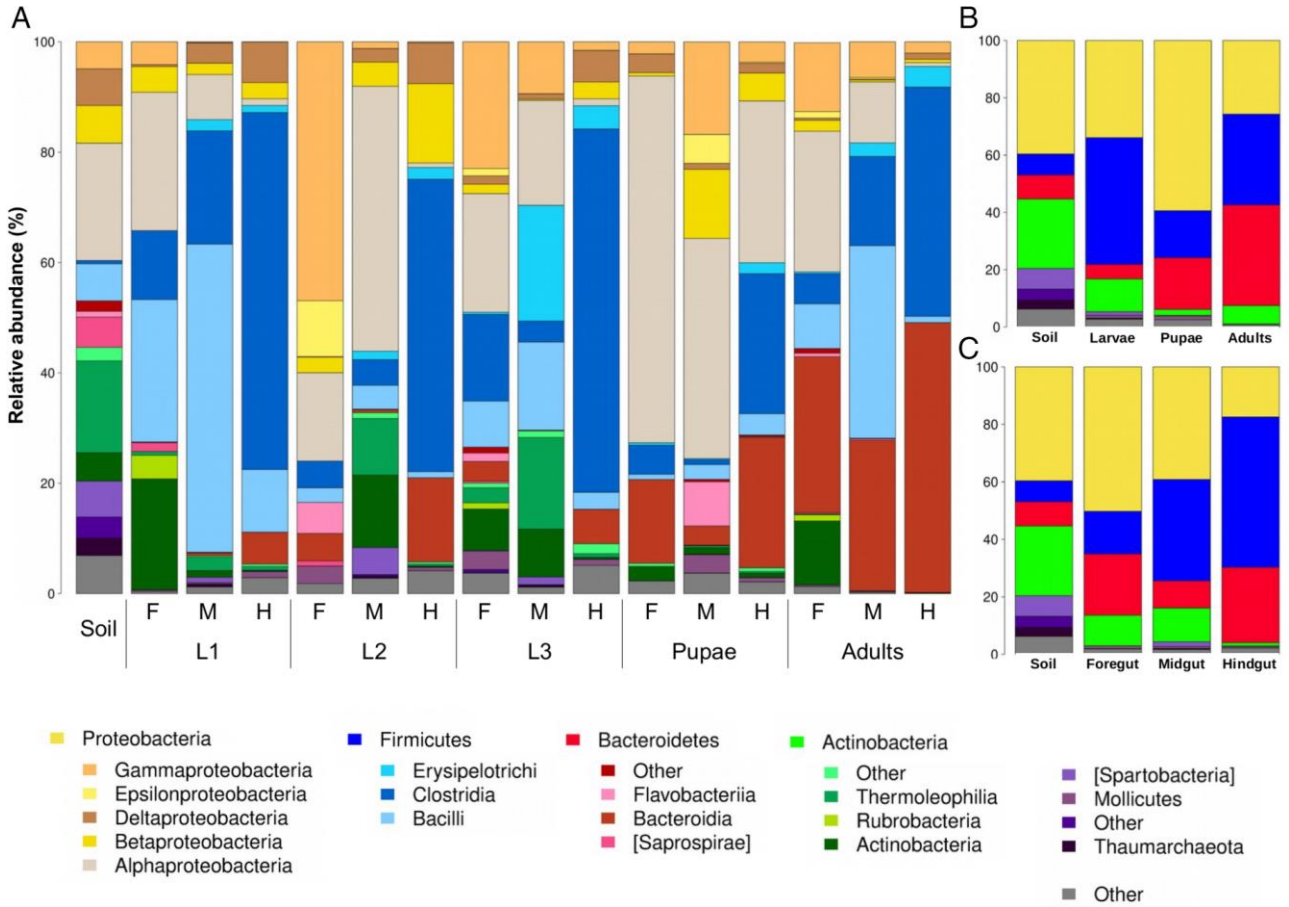
977

978 **Figure 1:** OTU distribution among the different samples. A. Bacterial community network  
 979 connecting OTUs (grey circles) to the samples (coloured circles) in which they were observed. B.  
 980 Venn diagram showing the shared/specific bacterial OTUs (at 97% similarity) between the different  
 981 developmental stages and soil. C. Box-plots of the estimated standardized phylogenetic diversity  
 982 (SES\_MPD) in the bacterial communities of rhizospheric soil and *P. japonica* developmental stages.



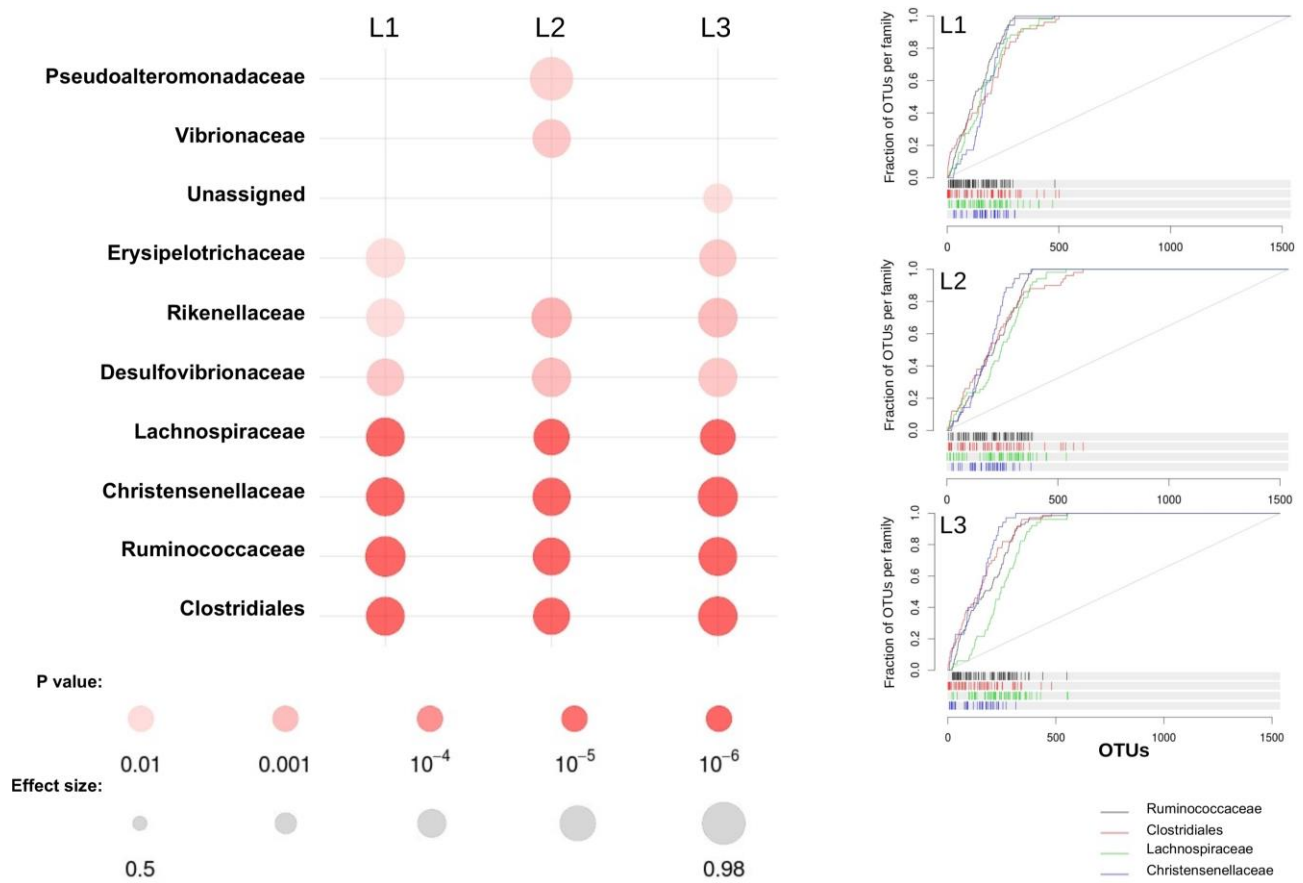
983                    □ Larvae    △ Pupae    ○ Adults    ■ Foregut    ■ Midgut    ■ Hindgut

984 **Figure 2:** NMDS analysis plots displaying sample  $\beta$ -diversity inferred from the OTU table. A. Biplot  
 985 of the first two axes for the NMDS representing correlations between the OTUs abundance in all  
 986 insect samples and ecological and ontological factors (i.e. developmental stage and gut section). B.  
 987 NMDS plots showing the correlation between the bacterial OTUs of Adults and larvae and the  
 988 different physico-chemical properties (pH, O<sub>2</sub> concentration and RedOx potential) of the different  
 989 gut regions (foregut, midgut and hindgut). The vectors represent the mean direction and strength of  
 990 correlation of the different parameters measured ( $p < 0.05$ ). In both figures, shapes indicate the  
 991 different developmental stages (i.e. square for larvae, triangle for pupae, circle for adults) while  
 992 colours indicate the gut region (i.e. red for foregut, green for midgut, blue for hindgut).



994

995 **Figure 3:** Histograms summarizing the bacterial composition at different taxonomic levels. The  
 996 different histograms report only taxa with a relative abundance  $\geq 3\%$ . A. The taxa summary at the  
 997 order level for the different samples grouped by category. F indicates foregut, M indicates midgut  
 998 and H indicates hindgut. B and C. The taxa summary at the phylum level for the different samples  
 999 grouped by developmental stages (B) and by gut section (C).



1000

1001 **Figure 4:** TEA carried out on the different larval stages using soil as reference. The main figure  
 1002 indicates the families that were enriched in the different larval stages compared to soil. The colour  
 1003 intensity of the circles indicates the p value while its size indicates the effect size. The panels on the  
 1004 right-hand side are the ROC curves, plotting the ranked OTUs belonging to the enriches families  
 1005 against the totality of the ranked OTUs, represent the rank of the different OTUs belonging to the  
 1006 families Lachnospiraceae (green), Christensenellaceae (blue), Ruminococcaceae (black) and the  
 1007 order Clostridiales (red) in general.

1008

1009 **Supporting Information**

1010 **Table S1** Summary of the different ecological indices and Random Forest results for each sample. 1a:  
1011 Ecological indices summary for the different samples. 1b: summary statistics of the comparison of the  
1012 different alpha diversity values between the different developmental stages. 1c: Standardized  
1013 phylogenetic evenness results for all the samples. 1d: Results of the Random Forest goodness of prediction  
1014 for the developmental stages. 1e: Results of the Random Forest goodness of prediction for the gut section.  
1015 1f: Top 10 OTU predictors of the Random Forest prediction for the developmental stages. 1 g: Top 10  
1016 OTU predictors of the Random Forest prediction for the gut sections.

1017 **Table S2** Indval results indicating the OTUs specific for each developmental stage and gut section. 2a:  
1018 Indval report for the specific OTUs per each developmental stage 2b: Indval report for the specific OTUs  
1019 per each gut section for each developmental stage.

1020 **Table S3** presence-absence matrix of the enriched families for each sample.

1021 **Figure S1** 1a. Male adult specimen of *Popillia japonica*. 1b. Gut of an adult *P. japonica* with the different  
1022 sections delimited.

1023 **Figure S2** Alpha diversity parameters by sample or sample type. A: Chao1 index for all the samples. B:  
1024 Chao1 index reported by gut section. C: Chao1 index reported by developmental stage. D: Shannon index  
1025 for all the samples. E: Shannon index reported by gut section. F: Shannon index reported by  
1026 developmental stage.

1027 **Figure S3** Biplot of the estimated standardized phylogenetic diversity (SES-MPD) and OTUs richness of  
1028 each community. The dashed grey line represents the linear regression, for the bacterial communities  
1029 associated with insect samples, of the SES-MPD onto the OTUs richness.

1030 **Figure S4** Heatmaps showing the relative pairwise nestedness and turnover values for the different  
1031 developmental stages and soil

1032 **Figure S5** Box-plots displaying the value ranges of the different physico-chemical properties measured  
1033 for the different gut sections for both adults and larvae. A: pH, B: Oxygen concentration; C: RedOx  
1034 potential.

1035 **Figure S6** Histograms summarizing the bacterial composition at the order level. The different histograms  
1036 report only taxa with a relative abundance  $\geq 3\%$ . A: The taxa summary at the order level for the different  
1037 samples. F indicates foregut, M indicates midgut and H indicates hindgut. B the taxa summary at the order

1038 level for the different samples grouped by individual pools. Namely each column correspond to the  
1039 samples (foregut, midgut and hindgut) from the same pooled individuals.

1040 **Figure S7** Maximum likelihood phylogenetic tree based on the partial 16S rRNA gene sequences. The  
1041 blue circle indicates the Christensenellaceae group of bacteria associated with the human gut. All other  
1042 taxa were detected in the present study in association with *P. japonica* gut sections. The scale bar at the  
1043 bottom indicates the distance in nucleotide substitution per site. The alphanumeric sequence at each node  
1044 either the GeneBank accession number or the de novo OTUs.



