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

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Originality-Significance

In this manuscript, we describe the microbial communities associated with the developmental stages and different gut sections of P. japonica, a highly invasive and resilient pest. Our results show that although an important fraction of the larval gut bacteria are originating from the surrounding environment (i.e. soil), only a small part is maintained throughout development. Furthermore, we show that a functionally important bacterial core composed of cellulose-degrading bacteria is maintained throughout the different life stages of the insect, suggesting the presence of a transmission mechanism from larvae to adults. In addition, we observed several clades belonging to the bacterial family Christensenellaceae, previously known to be associated with humans.

Besides these biological results, the paper also applies a new methodology, namely the adaptation of Gene Enrichment Analyses developed for gene expression data in order to identify enriched taxonomic groups in metabarcoding data.

Abstract

Popillia japonica Newman (Coleoptera: Scarabaeidae) is a highly polyphagous invasive beetle originating from Japan. This insect is highly resilient and able to rapidly adapt to new vegetation. Insect-associated microorganisms can play important roles in insect physiology, helping their hosts to adapt to changing conditions and potentially contributing to an insect's invasive potential. Such symbiotic bacteria can be part of a core microbiota that is stably transmitted throughout the host's life cycle or selectively recruited from the environment at each developmental stage. The aim of this study was to investigate the origin, stability and turnover of the bacterial communities associated with an invasive population of *P. japonica* from Italy. Our results demonstrate that soil microbes represent an important source of gut bacteria for *P. japonica* larvae, but as the insect develops, its gut microbiota richness and diversity decreased substantially, paralleled by changes in community composition. Notably, only 16.75% of the soil bacteria present in larvae are maintained until the adult stage. We further identified the micro-environments of different gut sections as an important

53 factor shaping microbiota composition in this species, likely due to differences in pH, oxygen
54 availability and redox potential. In addition, *P. japonica* also harbored a stable bacterial community
55 across all developmental stages, consisting of taxa well-known for the degradation of plant material,
56 namely the families Ruminococcacae, Christensenellaceae and Lachnospiraceae. Interestingly, the
57 family Christensenallaceae had so far been observed exclusively in humans. However, the
58 Christensenellaceae OTUs found in *P. japonica* belong to different taxonomic clades within this
59 family.
60
61 **Key words**: Gut microbiota, Japanese beetle, Insect-bacteria symbiosis, Invasive Species,
62 Christensenellaceae, Ruminococcacae.

63 **Introduction**

64 Insects are the most diverse and abundant animal clade (Foottit and Adler, 2009). The
65 diversification and evolutionary success of insects have been partially attributed to their ability to
66 establish associations with different beneficial microorganisms (e.g., Douglas, 2014; Corbin *et al.*,
67 2017; Sudakaran *et al.*, 2017; Heddi and Zaidman-Rémy, 2018). These microorganisms can play
68 key roles for different physiological functions such as the supply of essential nutrients missing from
69 unbalanced diets; contributing to the digestion of recalcitrant food components; protection from
70 predators, parasites and pathogens; and controlling mating and reproductive systems (e.g., Leftwich
71 *et al.*, 2017; Muhammad *et al.*, 2017).

72 As for essentially all animals, microbial communities are particularly prominent in the digestive
73 tract (e.g., Douglas, 2015, 2018; Clayton *et al.*, 2018; Münger *et al.*, 2018). The insect gut is
74 generally structured into foregut, midgut and hindgut, presenting a multitude of micro-environments
75 suitable for microbial colonization. Differences in morphology and physico-chemical properties
76 between different gut sections can greatly influence the microbial colonization patterns and
77 community structure depending on the host species. Gut bacteria have the potential to provide many
78 beneficial services to their hosts and insects display a wide range in degree of dependence on gut
79 bacteria for basic functions. Paramount to the evolution of intimate associations with gut
80 microorganisms is the development of secure transmission routes between host individuals and
81 generations. The lack of such mechanism in most insect species may hinder the establishment of
82 such long-term associations. With the exception of social insects, such as termites and ants, where
83 social interactions provide opportunities for the transfer of gut bacteria (Zhukova *et al.*, 2017),
84 insects had to develop original ways in order to transmit the important components of their gut
85 microbiota (Fukatsu and Hosokawa, 2002; Gonella *et al.*, 2012; Hosokawa *et al.*, 2013; Mason *et*
86 *al.*, 2019). These "heritable" gut bacteria have been shown to play crucial roles in the nutrition,
87 protection against different pathogens and xenobiotics, modulation of immune responses, and even
88 extending life span (Roh *et al.*, 2008; Kim *et al.*, 2016; Daisley *et al.*, 2018; Obata *et al.*, 2018).

89 Several factors can influence the gut microbiota structure and composition. Among these factors,
90 the most important ones are diet and environment, but other factors (e.g., age) can also be at play
91 (Wong *et al.*, 2011; Montagna, Chouaia, *et al.*, 2015; Montagna, Gómez-Zurita, *et al.*, 2015;
92 Montagna *et al.*, 2016; Sanders *et al.*, 2017; Tiede *et al.*, 2017; Vacchini *et al.*, 2017; Anderson *et*
93 *al.*, 2018). Although various factors can influence the insect gut microbiota, the existence of a
94 shared core microbial community in some species could indicate that there are mechanisms (e.g.
95 vertical transmission) favoring the presence of certain members of the gut microbiota. Several
96 studies have investigated this possibility by tracking the changes in gut microbiota composition
97 along the developmental stages of different insect species. These studies showed that the
98 transmission of the gut microbiota throughout the different developmental stages may depend on
99 the usefulness of certain bacteria (Zhukova *et al.*, 2017; Malacrinò *et al.*, 2018). For instance, the
100 bacterial communities of fruit flies (Tephritideae) change throughout the insect's developmental
101 stages to respond to the physiological needs of the host (Aharon *et al.*, 2013; Malacrinò *et al.*,
102 2018). In holometabolous insects, the pupal stage generally represents a bottleneck where most of
103 the larval gut microbiota is lost and adult insects may have to resort to indirect ways (e.g. via
104 environmental transmission) to insure the transfer of beneficial bacteria from larvae to adults
105 (Zhukova *et al.*, 2017). For instance, in certain bee species, certain bacterial taxa are not trans-
106 stadially transmitted but re-acquired from the environment (McFrederick *et al.*, 2014). While the
107 gut microbiota is not constant across the developmental stages in most insects, in some cases the
108 microbial community can be relatively stable throughout the developmental stages. This has been
109 observed in some Tephritid flies as well as in the Black Soldier Fly *Hermetia illucens* and in the
110 moth *Plodia interpunctella* (Mereghetti *et al.*, 2017; Yong *et al.*, 2017; De Smet *et al.*, 2018).
111 In the present study, we focused on the highly polyphagous invasive Japanese beetle *Popillia*
112 *japonica* Newman (Coleoptera: Scarabaeidae, Fig. S1a). This invasive insect is listed in the EPPO
113 Annex 2 due to the damages caused to different crops and turfs (EPPO, 2000). Native to Japan and
114 the far east of Russia (Fleming, 1972), this beetle became an established pest in North America in

115 the early 1900's (Switzer *et al.*, 2009), in the Azores in the early 1970's (Vieira, 2008) and more
116 recently in continental Europe, where it was recorded for the first time in Italy in 2014 (EPPO,
117 2014; Pavesi, 2014) and in Switzerland in 2017 (EPPO, 2017). Several laboratory and field trials
118 have been carried out to limit the spread of this pest in mainland Europe and to evaluate the
119 environmental resilience of the infested areas (Mazza *et al.*, 2017; Paoli, Marianelli, Binazzi, *et al.*,
120 2017; Paoli, Marianelli, Torrini, *et al.*, 2017; Marianelli, Paoli, Sabbatini Peverieri, *et al.*, 2018;
121 Marianelli, Paoli, Torrini, *et al.*, 2018). The damages to plants are caused by the different
122 developmental stages of the beetle: the larvae, being underground dwellers, feed on the plant roots
123 and soil organic matter while adults, living in an above-ground environment, feed on leaves and
124 floral parts of different plant species (Fleming, 1972; Vieira, 2008).
125 Insect-associated bacteria can potentially contribute to an insect's invasive potential by helping their
126 hosts to adapt to changing environmental conditions. Such symbiotic bacteria can be part of a core
127 microbiota that is stably transmitted throughout the host's life cycle or selectively recruited from the
128 environment at each developmental stage. The aim of this study was to investigate microbiota
129 dynamics in an invasive population of *P. japonica* from Italy. Specifically, we addressed the
130 following questions: i) Does *P. japonica* harbour a stable core microbiota or are the bacteria mainly
131 acquired from the surrounding environment (i.e. rhizospheric soil exploited by larvae and pupae *vs*
132 aerial environment exploited by adults)? ii) Is the gut microbiota maintained across the post-
133 embryonic developmental stages (i.e. larvae, pupae and adults) or is there a major turnover due to
134 insect development? iii) Do different gut micro-environments impact microbial community
135 structure?

136 **Results**

137 *Alpha, beta and phylogenetic diversity of the gut microbiota*

138 In this study, we analyzed the microbiota associated with three gut sections (foregut, midgut,
139 hindgut) of the different developmental stages (L1, L2, L3, pupae, adult males and females) of *P.*
140 *japonica*. For each sample type, 16S rRNA gene amplicons were obtained from three biological

141 replicates, each containing the tissues of five individuals. In addition, we analyzed the microbiota of
142 nine soil samples taken from the same habitat from which the insects were sampled. A total of
143 5175086 high-quality reads longer than 250 bp were kept after quality filtering and chimera
144 removal. These reads clustered into 1612 OTUs. On average, 67299 high-quality reads grouped into
145 336 OTUs were obtained from larvae, 80249 reads/204 OTUs from pupae, 88397 reads/99 OTUs
146 from adults and 148324 reads/1093 OTUs from soil samples (see Table S1a, Supporting
147 Information, for details). Rarefaction curves of the observed OTU richness in 25,000 sub-sampled
148 sequences showed that our sequencing effort was sufficient to capture the major part of the bacterial
149 diversity associated with both insect and soil samples (Fig. S2). OTU richness and diversity (Fig.
150 S2), as determined by the species richness estimator Chao1 and the Shannon Index of diversity,
151 were higher in soil samples than in insect samples (Chao1: all t-tests $P < 0.01$; Shannon: all t-tests P
152 < 0.01; see supplementary Table S1b for more details on the statistics for the different
153 comparisons). Regarding the different developmental stages of *P. japonica*, OTU richness and
154 diversity were the highest in the larvae (Chao 1: all t-tests $P < 0.01$; Shannon: all t-tests $P < 0.01$,
155 see supplementary Table1 and Table S1b for all ecological indices). On the other hand, these
156 indices were the lowest for adults (Chao 1: all t-tests $P < 0.01$; Shannon: all t-tests $P < 0.01$; Table1
157 and Table S1b). The different larval instars had similar richness and diversity with the Chao 1 and
158 Shannon indices of 360.26 ± 52.2 and 4.99 ± 0.77, respectively, for L1 larvae, 313.92 ± 48.44 and
159 5.47 ± 0.28 for L2 larvae and 342.96 ± 43.02 and 5.74 ± 0.27 for L3 larvae (Chao 1: all t-tests p-
160 value > 0.5; Shannon: all t-tests $P > 0.5$, Table S1b). It is noteworthy that the values of Pielou's
161 evenness also followed a similar pattern, with the soil having the highest value (Pielou'J = 0.84;
162 Table1), then larvae (Pielou'J = 0.67; Table1) and with pupae and adults having similar values
163 (Pielou'J = 0.47 and 0.49 respectively; Table 1).
164 The standardized effect size of mean pairwise distance values (SES_MPD) of the bacterial
165 communities associated with the samples ranged from positive values for soil bacterial communities
166 (median value of $\text{SES_MPD}_{\text{SOIL}} = 0.78$ associated with high quantiles, Table S1c) to negative

167 values for bacterial communities associated with the larval and pupal stages (median values
168 SES_MPD_{LARVAE} = -3.38 and SES_MPD_{PUPAE}= -3.9, low quantile values, Table S1c) (Fig. 1C).
169 SES_MDP values were significantly different between sample types (one-way ANOVA, F = 36.75,
170 df1 = 3, df2=21.4, p < 0.001), namely between larvae and soil (Tamhane post-hoc test, p < 0.001)
171 and between larvae and adults (Tamhane post-hoc test, p = 0.001). The positive SES_MPD values
172 for the soil communities indicate a phylogenetic overdispersion, as expected for communities
173 characterized by high species richness and evenness such as those of soil. In contrast, the negative
174 SES_MPD values for the bacterial communities associated with larvae and pupae indicate a
175 phylogenetic clustering of these communities, possibly due to the selection towards certain closely-
176 related bacterial lineages by the insect gut environment or to the adaptation of these bacteria to the
177 gut environment. Interestingly, the bacterial communities associated with adults were characterized
178 by slightly negative SES_MPD values (median value of SES_MPD_{ADULTS}= -0.53; Table S1c),
179 indicating a phylogenetic evenness of these communities (Fig. 1C). This increasing trend of
180 SES_MPD values from larvae and pupae (negative values) towards adults (slightly negative values)
181 contrasted with the trend of decreasing community species richness from larvae to adults (Fig. S3).

182 *Factors affecting gut microbiota composition*

183 Soil was different from the insect samples in terms of bacterial composition (adonis: P < 0.001, R^2
184 = 0.33; anosim: P < 0.001, R = 0.54) with few OTUs shared between soil and the different insect
185 developmental stages (Fig. 1A). Specifically, 891 OTUs out of the 1102 "core OTUs" of the soil
186 were not found in the insect samples (Fig. 1B). On the other hand, only 35 "core OTUs" present in
187 soil were also present in all the insect developmental stages (Fig. 1B). Moreover, the nestedness
188 component of the β-diversity between soil and the different insect developmental stage was very
189 low (0.16 on average) and the turnover was high (0.84 on average) (Fig. S4), indicating that very
190 few "core OTUs" were shared between soil and insect microbiotas while the variable fraction was
191 high.

192 Although more bacterial OTUs were shared between the insect samples (i.e. developmental stages
193 and gut sections combined) than between insects and soil, these samples still formed distinct
194 clusters as shown by NMDS analysis (Fig 2A). Specifically, insect developmental stages segregated
195 along the first axis with the larvae microbiotas being clearly distinct from adult microbiotas, while
196 pupal microbiotas were intermediate. The second axis further separated the samples based on gut
197 sections. For larvae and adults, the microbiotas of the different gut sections formed distinct clusters
198 with the midgut microbiota being more different than the foregut and hindgut microbiotas. In
199 contrast, the pupal microbiotas showed a different pattern with a clear cluster for the hindgut, while
200 foregut and midgut microbiotas loosely clustered together.
201 Based on the correlations of the tested factors (i.e., developmental stages and gut sections) with the
202 NMDS ordinations of the insect-associated bacterial communities, the main factor driving this
203 segregation was the gut section (R^2 = 0.18, p-value = 0.003) and to a lesser extent the
204 developmental stage. These results were further supported by the Random Forest (RF) analysis
205 which was carried out to investigate the specificity of the microbiota of each sample category by
206 trying to assign each sample to its respective category based on its microbiota. The RF analysis
207 (Supplementary Table S1d) was able to successfully classify adults and larvae in 100% and 91.7%
208 of the cases, respectively. Conversely, pupae were successfully identified in only 55.6% of the
209 cases. These results suggest that the pupal stage represents a transitional step not only in the
210 development of the insect but also for its associated microbiota. The most important OTUs
211 discriminating between the different developmental stages belonged to the Firmicutes (Clostridiales
212 and Bacilli), Proteobacteria (Alphaproteobacteria) and Actinobacteria (see Supplementary Table
213 S1f). On the other hand, the RF was able to successfully classify the foregut, midgut and hindgut
214 samples in 80%, 82% and 78% of the cases, respectively (supplementary Table S1e). The most
215 relevant OTUs allowing to discriminate between the different gut sections were identified as
216 Firmicutes (Clostridiales) and Proteobacteria (Betaproteobacteria). These results indicate that the

217 different gut sections as well as larvae and adults have distinct microbial communities, whereas the
218 pupal stage has not.
219 In order to further investigate the correlation between the physico-chemical conditions of the gut
220 and microbial composition, we measured pH, O_2 concentration and Redox potential in each gut
221 section for both male adults and L3 larvae (see Supplementary table S1; supplementary Fig. S5).
222 While the adult gut constituted a niche with a neutral pH (or at most slightly sub-acidic conditions),
223 the pH in the larval gut increased from neutral in the foregut to alkaline conditions in the midgut
224 and hindgut. Both larval and adult digestive systems were characterized by anoxic conditions, with
225 the exception of the the adult foregut where conditions fluctuated from anoxia to microaerophilia.
226 Finally, positive redox potential values were measured in all gut compartments of both larvae and
227 adults, with the exception of the larval hindgut where a decrease in redox potential was measured,
228 underlining the existence of reducing conditions in this region. These three factors were
229 significantly correlated with the microbial composition in the different gut sections. Notably, pH
230 was significantly correlated with the microbiota of larvae (R^2 = 0.75, p-value = 0.001), while O_2
231 concentrations (R^2 = 0.54, p-value = 0.002) and redox potential (R^2 = 0.74, p-value = 0.001)
232 correlated significantly with the bacterial composition in adult gut regions (Fig 2B)..

233 *Taxonomic composition of* P. japonica *gut microbiota*

234 The microbiota associated with different developmental stages of the host and with soil not only
235 differed in terms of bacterial richness and diversity, but also concerning bacterial community
236 composition (Fig 3; Fig. 2A, supplementary Fig. S6). Although Proteobacteria represented the most
237 abundant phylum considering all sample types (35.9% ± SE 4.2%), followed by Firmicutes (32.9%
238 ± SE 5.4%) and Bacteroidetes (15.4% ± SE 3.7%), these proportions changed among the different
239 sample types. Considering larvae (Fig 3B, supplementary Fig. S6), the most abundant phylum was
240 Firmicutes with an average of 49.5% ± SE 7.9% (range 26.5% ± SE 5.5% in L2 larvae to 74.5% ±
241 SE 8.7% in L1 larvae), followed by Proteobacteria (31.3% ± SE 5.8% on average; range: 13.9% ±
242 SE 5.1% in L1 larvae to 50.3% ± SE 5.9% in L2 larvae) and Actinobacteria (9.4% ± SE 2.6% on

243 average; range 5% ± SE 2.5% in L1 larvae to 13.9% ± SE 4% in L3 larvae). On the other hand, the
244 most abundant taxa in adults were Bacteroidetes (33.7% ± SE 7.8% on average; 39% ± SE 10.6%
245 in females, 28.3% ± SE 12.9% in males) followed by Firmicutes (29.6% on average; 14.5% ± SE
246 1.5% in females, 44.8% ± SE 4.1% in males) then Proteobacteria (29.1% on average; 40% ± SE
247 12.6% in females, 18.2% ± 6.6% SE in males). In pupae, the most abundant phylum was
248 Proteobacteria with 59.7% ± SE 11.5%, followed by Bacteroidetes (19.1% ± SE 9.2%) and
249 Firmicutes (15.4% ± SE 9.9%). It is noteworthy that the proportion of Actinobacteria decreased
250 when passing from soil to adults (going from 24.8% ± SE 1.5% in soil to 6.4% ± SE 1.9% in
251 adults), while the proportion of Bacteroidetes followed the opposite trend, going from 8% ± SE
252 1.2% in soil to 33.7% ± SE 7.9% in adults (Fig. 3A). Other bacterial taxa present at minor
253 proportions (such as Acidobactria, Chloroflexi and Nitrospira) followed a trend similar to
254 Actinobacteria, with their proportions decreasing from soil to adults.
255 Looking at the different gut sections (Fig. 3C), we observed similar trends. Relative abundance of
256 Actinobacteria and Proteobacteria decreased from soil to hindgut from 24.2% and 39.6%,
257 respectively, to 1.6% and 17.4%, respectively. On the other hand, the relative abundance of
258 Firmicutes increased from soil to hindgut from 7.3% to 52.3%.
259 *Spatio-temporal changes in the microbiota taxonomic composition*
260 As mentioned above, 891 OTUs out of the 1,102 "core OTUs" present in the soil were not found in
261 the insect samples, while only 35 "core OTUs" were present in both insects and soil (Fig. 1B).
262 These OTUs belonged predominantly to the Proteobacteria phylum (26 out of the 35 OTUs) with
263 Rhizobiales being the most represented order (8 OTUs). In addition to these 35 OTUs, out of the
264 630 "core OTUs" found in insects but not in soil, 54 OTUs were shared between all the
265 developmental stages. Proteobacteria, Bacteroidetes and Firmicutes were the most abundant phyla
266 (28, 10 and 9 OTUs, respectively). Noteworthy, OTUs belonging to the families Rickenellaceae (5
267 OTUs), Lachnospiraceae (3 OTUs) and Ruminococcaceae (1 OTU) were among the OTUs shared

268 between the insect developmental stages. These families were identified as taxa specifically
269 enriched in the insect guts along the different developmental stages.
270 We next performed a TEA (Taxon Enrichment Analysis) to identify which bacterial families were
271 consistently enriched in insects compared to soil (Fig. 4). This analysis showed that among the
272 Firmicutes, the Ruminococcacae was significantly enriched in larvae compared to soil ($P < 0.001$)
273 but there were no differences when comparing the different developmental stages. Similarly, other
274 bacterial families belonging to the Firmicutes and specifically to the order Clostridiales (namely
275 Christensenellaceae and Lachnospiraceae) resulted to be significantly enriched in larvae and
276 generally in insects when compared to soil samples. These families were also enriched in the
277 different compartments of the gut when compared to soil ($P < 0.001$), independent of the insect
278 developmental stages. Other bacterial families, such as Rikenellaceae (Bacteroidetes) and
279 Desulfovibrionaceae (Proteobacteria), were also enriched in larvae compared to soil. These bacteria
280 were also enriched in other portions of the gut but not all of them. Desulfovibrionaceae were also
281 enriched in the midgut and hindgut, while Rikenellaceae were only enriched in the hindgut.
282 Interestingly, all enriched families were absent from the soil samples (supplementary table S3).
283 While these family were not always present in the foregut, Desulfovibrionaceae, Lachnospiraceae
284 and Ruminococcaceae were present in all midgut and hindgut samples for all developmental stages.
285 Rikenellaceae, on the other hand, were present in all hindgut samples but absent from two midgut
286 samples, namely one L1 and one pupal midgut sample (supplementary table S3)
287 It is noteworthy that the TEA did not evidence any significantly enriched taxonomic group between
288 the different developmental stages of the insect nor did it evidence enriched taxonomic group
289 between the different gut sections. This is partly supported by the fact that the nestedness
290 component of the β-diversity between the different insect developmental stages was relatively high
291 (0.59 on average), indicating that a higher fraction of the microbiotas is shared between the
292 different insect developmental stages than between insects and soil.

293 An Indval analysis carried out to identify OTUs specific to a given developmental stage showed
294 that 23 OTUs were unique to larvae, five were associated only with pupae while 13 were specific to
295 adults (See table S2a for supporting information). Members of the Lachnospiraceae family were the
296 most represented OTUs among those unique to both larvae and adults (with nine and five OTUs
297 present respectively).
298 The same analysis carried out on the different gut sections for each developmental stage gave a
299 different picture. For the pupal stage, there was no OTU specific to a given gut section. For adults,
300 15 OTUs were found only in the foregut, while 5 OTUs were specific to the hindgut. No OTU was
301 found to be unique to the midgut. On the other hand, in the larvae, only two OTUs were specific to
302 the foregut, while the midgut and hindgut had respectively 105 and 145 specific OTUs. It is
303 noteworthy that three out of the five OTUs that were unique to the adult hindgut were also found
304 specifically associated with the larvae hindgut. These OTUs belonged to the Rikenellaceae
305 (denovo5575 and denovo143435) and Nitrosomonadaceae (denovo213936) families.

306 *Phylogenetic relationship of Christensenellaceae associated with* P. japonica

307 Bacteria belonging to Christensenellaceae have previously been observed only in humans. To better
308 understand the phylogenetic relationships between members of the Christensenellaceae associated
309 with *P. japonica* and those associated with humans, we performed a Maximum Likelihood
310 phylogeny using our OTUs and 16S rRNA gene sequences from those isolated from humans
311 (Figure S7). The OTUs associated with the insect formed several clusters distinct from the cluster of
312 human-associated symbionts. Hence the bacteria associated with *P. japonica* belong to different
313 taxonomic groups within the Christensenellaceae family.

314 **Discussion**

315 In this study, we demonstrate that soil bacteria represent an important source for the gut microbiota
316 of *P. japonica* larvae, but as the insect develops, the gut bacterial community experiences important
317 changes in richness, diversity and composition. Specifically, 37% of the OTUs (209 OTUs) present
318 in larvae derived from the soil microbiota and 35 OTUs present in the soil were maintained

319 throughout all the developmental stages of the insect. In addition, larvae had a higher OTU richness
320 and diversity compared to adults. This is likely linked to the different lifestyles of the two stages:
321 larvae are soil-dwelling and similar in OTU numbers to other soil-dwelling arthropods such as
322 terrestrial isopods (healthy isopods OTUs on average 209; Dittmer *et al.*, 2016), termites (number
323 of OTUs consistently higher than 400; Su *et al.*, 2016) and ants (number of OTUs about 400; Vieira
324 *et al.*, 2017; Zhukova *et al.*, 2017), while the OTU numbers of adults are comparable to those of
325 non-soil-dwelling insects (in 218 insect species, average OTUs 84; Yun *et al.*, 2014). Pupae are an
326 intermediate state between larvae and adults in terms of bacterial taxonomic richness and diversity,
327 representing a bottleneck for bacterial transmission due to metamorphosis. Nonetheless, key
328 bacterial taxa involved in plant material degradation are still transmitted to adults (see below for a
329 detailed discussion). This reduction in both richness and diversity at the pupal stage could be due to
330 a combination of factors both random and deterministic. On the one hand, a reduction of the number
331 of bacterial cells during metamorphosis could have caused a random reduction in the diversity of
332 the microbiota. On the other hand, the observed reduction in microbiota diversity throughout host
333 development could be caused by one (or several) active mechanisms, such as (i) the change of
334 nutrition (or lifestyle) between soil-dwelling larvae and adults, (ii) specific physico-chemical
335 properties (e.g., the change in gut pH between larvae and adults), and/or (iii) enzymatic activities,
336 among others. As a matter of fact, the observed changes (i.e. decrease in richness and diversity) are
337 not a constant in insect development and other studies monitoring gut microbiota changes
338 throughout development have shown different trends, such as an increase in species richness
339 (Brucker and Bordenstein 2012) or more generally the absence of a clear trend (Oliveira *et al.*,
340 2018; Gao et *al.*, 2019; Huang *et al.*, 2019). The trend that we observe in *P. japonica* could be
341 explained by its ecology, since soil dwelling arthropods such as termites and woodlice consistently
342 present higher microbiota richness and diversity (Dittmer *et al.*, 2016; Su *et al.*, 2016; Vieira *et al.*,
343 2017; Zhukova *et al.*, 2017) due to their proximity to a microbially rich and diverse environment

344 (i.e. soil). On the other, arthropods living in "aerial" ecosystems (i.e. plants and leaves) tend to have
345 a less rich and diverse gut microbiota (Yun *et al.*, 2014; Mereghetti et al., 2019).
346 Interestingly, the decrease in microbiota richness and diversity throughout the host developmental
347 stages is accompanied by a shift in the phylogenetic community structure. Specifically, larvae and
348 pupae harbor phylogenetically clustered bacterial communities, i.e. consisting of closely-related
349 bacterial taxa. In contrast, the adult microbiota is phylogenetically overdispersed, similarly to
350 rhizospheric soil communities. The observation that larvae microbiotas are phylogenetically
351 clustered and at the same time taxonomically rich compared to adults could be explained by a
352 selection of certain taxonomic groups through the gut environment. The phylogenetic
353 overdispersion of the adult gut microbiotas suggests that the pupal stage represents a crucial
354 bottleneck for the gut microbiota in terms of species richness. This might be due to the random
355 survival of bacterial taxa present in the larvae throughout metamorphosis (and its associated gut
356 tissue restructuring) at the pupal stage. However, the fact that a certain number of taxa are
357 maintained throughout the development from larvae to adult but are absent from soil, suggests the
358 existence of a mechanism to specifically maintain essential bacterial partners (e.g.,
359 Ruminococcaceae, Lachnospiraceae). In other words, the survival of certain bacterial taxa may not
360 be entirely random. Another possible explanation might be that the adult gut microbiota is renewed
361 by feeding on leaves and flowers in contrast to rhizospheric soil and/or that the physico-chemical
362 properties of the adult gut are more stable than in larvae (see Fig. S5). Hence, despite the potential
363 existence of a mechanism to maintain and transmit a fraction of the microbiota, other bacterial taxa
364 could still be transient and dependent on the food source (e.g., different parts of the plant, different
365 plant species), as observed in *D. melanogaster* where acetic acid bacteria are always associated with
366 the fly but the presence of other bacterial taxa is dependent on the environment (Adair et al., 2018;
367 Wong et al., 2015).
368 This study allowed us to identify several factors potentially shaping microbiota composition in *P.*
369 *japonica*. Specifically, we demonstrate that among the tested factors, microbiota composition varied

370 significantly between different gut sections as well as between insect developmental stages. This
371 strong correlation between different gut sections and microbiota diversity and composition is most
372 likely due to: i) differences in the physico-chemical conditions prevailing in each gut section
373 (supplementary Fig S5) as well as ii) biotic factors such as host enzymatic potential and immune
374 response. It is noteworthy that the pupae represent a transitional stage with a reshuffling of the
375 microbiota between the larval and adult stages. In other words, the larvae and adult microbiotas
376 formed clearly distinct clusters, while the pupae microbiota was more dispersed between the larvae
377 and adult clusters. This may have had an impact on the statistical analyses, leading to an apparently
378 weaker effect of the developmental stages on microbiota composition.
379 Regarding the physico-chemical factors, oxygen availability was the most strongly correlated with
380 differences in bacterial community structure between the different gut sections in adults, while
381 intestinal pH was the most strongly correlated factor in larvae. Although both the midgut and
382 hindgut compartments were largely anoxic in adults, the oxygen concentration in the midgut
383 showed a higher degree of variation compared to the more anoxic hindgut. This is likely due to a
384 considerably larger influx of oxygen via the gut epithelium in the case of the midgut, as observed in
385 *Pachnoda ephippiata* (Lemke *et al.*, 2003). This variability in oxygen availability between the
386 different gut compartments may favour bacteria that are more tolerant towards such fluctuations. In
387 larvae, the pH in the midgut and hindgut was alkaline, while the foregut had a neutral pH. It is
388 important to note that the larvae are soil-dwellers feeding on fresh roots and decaying soil organic
389 matter (SOM) (Fleming, 1972). In this regard, they are similar to other soil-dwelling
390 macroinvertebrates, including many coleopterans, which feed on SOM and play an important role in
391 its degradation and stabilization (Lavelle *et al.*, 1997; Wolters, 2000). It has been shown that the
392 conditions in the anterior hindgut of the humivorous termite *Cubitermes* spp. (i.e. high alkalinity
393 and oxygen influx) lead to a decrease of the molecular weight of the organic matter (Kappler and
394 Brune, 1999), rendering it more soluble and thus more accessible for digestion in subsequent less-
395 alkaline compartments (Ji *et al.*, 2000; Kappler *et al.*, 2000; Ji and Brune, 2001). Although the

396 complex microbial communities in the guts of humivorous macroinvertebrates are thought to
397 participate in the transformation of ingested SOM (Cazemier *et al.*, 1997; Kane, 1997), detailed
398 information on the composition and activities of the gut microbiota is lacking. In view of the high
399 midgut alkalinity in *P. japonica*, it is reasonable to assume that at least some of the bacteria in the
400 midgut are tolerant towards high pH conditions, since most bacterial taxa are also found in the more
401 neutral gut sections of adults.
402 We further observed differences in microbiota composition at different taxonomic levels (from
403 order to OTU) between the different developmental stages of *P. japonica*. For instance,
404 Actinobacteria decreased in abundance from larvae to adults, while Bacteroidetes increased in
405 abundance. However, no particular taxa were found to be specifically enriched in any of the
406 developmental stages. A similar pattern was observed for the microbiota associated with different
407 gut compartments (foregut, midgut, hindgut): no particular taxon was specifically enriched in any of
408 the compartments. Nonetheless, Proteobacteria decreased from foregut to hindgut, while Firmicutes
409 increased. Actinobacteria were relatively stable between foregut and midgut but decreased in the
410 hindgut.
411 In contrast, several taxa were found to be significantly enriched between soil and insect gut. Those
412 belonged mainly to the families Ruminococcacae, Christensenellaceae and Lachnospiraceae.
413 Members of these families are known to degrade cellulose (Flint *et al.*, 2012; Biddle *et al.*, 2013).
414 The fact of finding them enriched in the insect gut may suggest a possible symbiotic relationship
415 where these bacteria help their host degrade and metabolise cellulose, as in the case of the
416 symbiotic association between termites, protists and bacteria (Liu *et al.*, 2013) or woodlice and
417 certain bacterial taxa (Bredon *et al.*, 2018). These bacteria could be important in helping their host
418 metabolise plant roots and leaves and might thus contribute to its success as a polyphagous invasive
419 insect. The bacterial taxa that were enriched in the gut of *P. japonica* have been previously reported
420 in association with various insects but more importantly with ruminants and humans. *Anaerostipes*
421 spp., *Coprococcus* spp. and *Dorea* spp. (members of the Lachnospiraceae family) have all been

422 previously described in association with the human gut (Rainey, 2009) where they are hypothesized
423 to be involved in pectin fermentation. Other members of the Lachnospiraceae family have also been
424 described in association with other insects (Huang and Zhang, 2013; Bourguignon *et al.*, 2018). The
425 Ruminococcaceae family, represented by *Ruminococcus* spp. and *Oscillospira* spp. in *P. japonica*,
426 has also been described in association with humans, ruminants, coleopterans and termites
427 (Kamagata, 2011; Huang and Zhang, 2013; Bourguignon *et al.*, 2018). *Ruminococcus*, in addition to
428 *Bacteroides* spp., plays an important role in the fermentation of hemicellulose and the degradation
429 of different plant material through the production of Carbohydrate-Active enZymes (CAZymes)
430 (Jose *et al.*, 2017). CAZymes are very important for the break-down of the different components of
431 lignocellulose (i.e. cellulose, lignin, hemicellulose; Bredon *et al.*, 2018). It is noteworthy that
432 although some insects are able to express some of these enzymes, most of them heavily rely on their
433 associated microorganisms to degrade lignocellulose (Bredon *et al.*, 2018). On the other hand, the
434 role of *Oscillospira* is still unknown and it is hypothesized that it may be involved in lignocellulose
435 degradation (Kamagata, 2011). Rickenellaceae, with the genus *Alistipes*, and Desulfovibrionaceae
436 have also been described in association with the guts of different animals (Koneru *et al.*, 2016;
437 Ruengsomwong *et al.*, 2016), especially termites (Reid *et al.*, 2014; Makonde *et al.*, 2015), where
438 they play an important role in the degradation of cellulose polymers (Ozbayram *et al.*, 2018).
439 The taxa found to be enriched in insect samples could be preferentially present in insects due to
440 favorable conditions in the gut environment without an actual effect of these bacteria on the insect
441 host. However, the fact that these bacteria were not detected in soil suggests the presence of a more
442 direct transmission mechanism independent of the environmental route. In addition, the consistent
443 presence of these bacteria in the gut regions where plant material is degraded further argues in favor
444 of an active role of these bacteria and not just their presence as transient passengers.
445 In contrast to the above-mentioned bacterial families which have been observed not only in
446 mammals but also in insects, the family Christensenallaceae had so far been observed exclusively in
447 humans. Although its role in the degradation of nutrients is not yet understood, members of this

448 family (i.e. *Christensenella minuta*) have been shown to play a central role in controlling the Body
449 Mass Index and in helping to shape a "healthy" microbiota in humans and transfected mice
450 (Goodrich *et al.*, 2014). Increased titers of *C. minuta* have also been correlated with longevity in
451 humans (Biagi *et al.*, 2016), while decreased titers were observed during different human diseases
452 (Petrov *et al.*, 2017; Yu *et al.*, 2017). In addition, other bacteria belonging to the genus
453 *Christensenella* have been isolated from diseased humans, although no causality has been
454 established yet (Ndongo *et al.*, 2016). The partial 16S rRNA gene-based phylogeny showed that the
455 Christensenellaceae OTUs found in association with *P. japonica* do not cluster with the taxa
456 associated with humans but rather form different clusters, suggesting that they belong to different
457 taxonomic groups within the Christensenellaceae family (Fig. S7).
458 Although three biological replicates containing homologous gut regions from five individuals might
459 be limiting, based on the results obtained in this study we can conclude that the gut microbiota of *P.*
460 *japonica* is highly dynamic across the developmental stages of the insect and changes in microbiota
461 composition strongly correlated with the physico-chemical properties of the gut. Despite the
462 microbiota high variability, 89 OTUs were maintained from larvae to adults, including 35 OTUs
463 originating from the soil environment. As a future perspective, it would be interesting to investigate
464 if these OTUs represent a stable core microbiota present in all *P. japonica* populations in different
465 parts of the world or if they are subject to change in different environments. In the first case, this
466 might indicate a more intimate symbiotic relationship potentially maintained via vertical
467 transmission. In the latter case, the variable microbiota would provide a means to investigate the
468 origin of new invasions of this beetle, via a comparative analysis of the local soil and insect gut
469 microbiotas.
470

471 **Materials and methods**

472 *Collection and processing of insect and soil samples*

473 Four campaigns were organized from June to September 2017 to collect insect samples at different
474 developmental stages of the insect. The different stages and instars (in the case of larvae: larval
475 instar 1 – L1; larval instar 2 – L2; larval instar 3 – L3) of the insects were collected in Oleggio
476 (Novara, Italy; 45°36′ N, 08°38′ E, altitude ca. 230 m a.s.l.). Simultaneously, at each sampling
477 expedition, 10 soil samples were taken from the sampled area and combined into a single sample
478 representative of the area, leading to the collection of three soil samples. Insects were preserved in
479 absolute ethanol while soil samples in 50 ml vials, kept refrigerated on the field and then stored at -
480 20°C before processing. All insects were surface sterilized before dissection using the protocol
481 described in Montagna and colleagues (Montagna, Chouaia, *et al.*, 2015). 90 individuals (i.e. 15
482 individuals of each larval instar, 15 pupae, 15 males, 15 females) were dissected under sterile
483 conditions, and the gut (Fig. S1b) was removed in sterile Ringer solution. The insect alimentary
484 canal was then aseptically separated into its three compartments (i.e. foregut, midgut and hindgut).
485 For each developmental stage and larval instar, five homologous gut compartments were pooled
486 together in a single sample, resulting in three biological replicates for each sample category. These
487 samples were used for DNA extraction (see table S1 for a detail on the samples).
488 Additionally, male adults (N=9) and L3 larvae (N=6) were collected and immediately processed in
489 order to measure physicochemical properties (pH level, redox potential, oxygen concentration) of
490 different gut regions. Specimens were anesthetized at 4°C for 3’ before their dissection.
491 *DNA extraction, amplicon library preparation, sequencing and bioinformatics*
492 The DNA was extracted from each sample (consisting of five homologous gut compartments for a
493 defined insect instar and developmental stage) using the phenol–chloroform methods (Doyle and
494 Doyle, 1990) with the modifications described in Mereghetti and colleagues (Mereghetti *et al.*,
495 2017). The DNA was then eluted in 50 μl of sterile water (Sigma-Aldrich, Saint Louis, Missouri,
496 USA). A DNA extraction blank was performed as control to monitor for contamination of
497 environmental bacterial DNA. DNA from soils was extracted using PowerSoil DNA Isolation Kit

498 (MO BIO Laboratories Inc., Carlsbad, CA) following manufacturer's instructions. Three
499 independent DNA extractions were performed for each of the three representative soil samples.
500 The extracted DNA was used as template for the amplification of the V4 hypervariable region of the
501 16S rRNA gene using the PCR primers 515F (Caporaso *et al.*, 2011) and a blend of reverse primers
502 802R (Claesson *et al.*, 2009) and 806R (Caporaso *et al.*, 2011) in order to reduce amplification bias.
503 Forward and reverse primers were tailed with two different GC rich sequences, enabling barcoding
504 with a second amplification. Each sample was first amplified in 20 µl reaction volume containing 8
505 µl HotMasterMix 5 Prime 2.5X (Quanta Bio), 0.4 µl BSA (20 µg/µl) (Sigma-Aldrich), 1 µl
506 EvaGreen™ 20X (Biotium), 0.8µl 515 F (10 µM) (- 5' modified with unitail 1 5'-
507 CAGGACCAGGGTACGGTG-3'), 0.4 µl 802 R (10 µM) (- 5' modified with unitail 2 5'-
508 CGCAGAGAGGCTCCGTG-3'), 0.4 µl 806 R (10 µM) (- 5' modified with unitail 2 5'-
509 CGCAGAGAGGCTCCGTG-3'), and 1 µl (50 ng) of DNA template. The PCR amplifications were
510 performed in a CFX 96™ PCR System (Bio-Rad) with 34 cycles of 94°C for 20 s, 52°C for 20 s,
511 65°C for 40 s and a final extension of 65°C for 2 min. The second PCR amplification was
512 performed in 25 µl reaction volume containing the same reagents as the first PCR but with 1.5 µl
513 barcoded/TrP1 primers (10 µM) and with 1 µl of the first PCR amplification in the following
514 conditions: 8 cycles of 94°C for 10 s, 60°C for 10 s, 65°C for 40 s and a final extension of 72°C for
515 3 min.
516 After labeling each sample with a specific Ion Torrent (Ion Express) DNA barcode, each single
517 library was quality checked with agarose gel electrophoresis, quantified with Qubit Fluorometer
518 (Thermo Fisher Scientific) then pooled with the other libraries in equimolar amounts. The final
519 product was then sequenced using the Ion Torrent PGM System. Libraries preparation and
520 sequencing were performed at the Life Sciences Department of Trieste University, Italy.
521 Four samples (see table S1a for details) were excluded from the following analyses since they did
522 not have enough reads (less than 200). The reads of the remaining samples were analyzed using
523 QIIME version 1.9.1 (Caporaso *et al.*, 2010). In detail, adapters were removed, and low-quality

524 reads filtered (Phred < 20, read length < 250pb). Uclust (Edgar, 2010) was used to cluster the 16S
525 rRNA sequences into Operational Taxonomic Units (OTUs) with a similarity cut-off of 97%.
526 Chimeras were removed using Chimeraslayer. A representative sequence for each identified OTUs
527 was aligned to Green-genes (http://greengenes.lbl.gov/) using Pynast (Caporaso *et al.*, 2010).
528 Taxonomic assignment was performed comparing the representative OTUs to Green-genes (release
529 13.8). Rare OTUs (i.e., singletons and OTUs < 10) and OTUs identified as chloroplast were
530 discarded. The resulting OTU table was then used for the subsequent analyses.

531 *Diversity analyses*

532 Bacterial OTU richness, diversity and evenness were calculated using the package Vegan (Dixon,
533 2003; Oksanen et al., 2018), implemented under the R software (R Project 3.0.2; http://cran.r-
534 project.org/) adopting the species richness estimator Chao 1 (Chao, 1984), the Shannon H' index
535 (Shannon, 1948) and the Pielou's evenness (Pielou, 1975), after sub-sampling the OTU table to
536 obtain a total of 25,000 sequences per sample. Alpha diversity indices were compared between
537 different groups (i.e. tissues, developmental stages) using two-sample t-tests with 999 Monte Carlo
538 permutations.

539 In order to evaluate if the structures of the bacterial communities associated with soil and the
540 different developmental stages of *P. japonica* were driven by species competition or by
541 environmental factors, thus resulting in a community dominated by closely related species (Webb *et*
542 *al.*, 2002; Mouquet *et al.*, 2012; O'Dwyer *et al.*, 2012), the mean pairwise distance between all taxa
543 in the bacterial communities (MPD; Webb *et al.*, 2002) was used as metric for phylogenetic
544 structure. To allow the comparison between the bacterial communities of the different types, null
545 models maintaining species occurrence frequency constant were estimated. Standard effect size and
546 relative position of each bacterial community with respect to the null MDP distribution, generated
547 by 999 randomizations of the null model, were calculated using the *ses.mpd* function implemented
548 in the Rpackage *picante* (Kembel *et al.*, 2010). This standardized metric quantifies the relative
549 excess or deficit in the phylogenetic diversity for each community with respect to the entire species

550 pool. Negative values reflect a relative phylogenetic clustering of the species, while positive values
551 indicate a relative phylogenetic evenness (or overdispersion). SES_{MDP} values were visualized as
552 box-plots based on sample type (i.e., soil, larvae, pupae, adults) and statistical differences among
553 sample types were assessed using Welch's one-way ANOVA (Welch, 1951), since SESMDP values
554 were normally distributed based on Shapiro-Wilk test (Royston 1982) (p-value > 0.05), but the
555 variance between groups was not homogeneous based on Levene test (Levene, 1960) (p-value <
556 0.001). Hence, we used the Tamhane post-hoc test for multiple comparisons without
557 homoscedasticity.
558 The spatial (across the three gut regions) and temporal shifts (across developmental stages) of the *P.*
559 *japonica* bacterial community (presence/absence) were estimated using the Sørensen-based
560 multiple-site dissimilarity (β_{SOR}; Baselga, 2010) implemented in the R package *betapart* (Baselga
561 and Orme, 2012). The turnover and nestedness components of this β-diversity were calculated using
562 Simpson-based multiple-site dissimilarity (β_{SIM}; Baselga, 2010) and nestedness-resultant multiple-
563 site dissimilarity (β_{NES}; Baselga, 2010), respectively. In addition, for each β-diversity component,
564 the pairwise dissimilarity values among the microbiotas of all analysed groups (i.e. soil, larvae,
565 pupae and adults) were calculated using the *betapair* function of the R package *betapart* (Baselga
566 and Orme, 2012) and visualized through heatmaps using heatmap.2 from the R package gplots.
567 In order to assess the difference in the microbiota structure among soil and insect samples, the sub-
568 sampled OTU table was subjected to a nonparametric one-way analysis of similarity ANOSIM
569 (Clarke, 1993), implemented in the vegan library and based on the Bray-Curtis dissimilarity (999
570 permutations permuting within gut samples of the same individuals in order to account for the non-
571 independence of the observations (Bray and Curtis, 1957).
572 The sub-sampled OTU table, after the removal of soil community samples, was used as input for a
573 Nonmetric Multi-Dimensional Scaling (NMDS; Kruskal, 1964) biplot based on the Bray–Curtis
574 dissimilarity (Bray and Curtis, 1957), in order to graphically ordinate samples and assess the
575 differences among: i) the developmental stages (i.e. larvae, pupae and adults); ii) the three gut

576 regions, and iii) to evaluate the impact of the gut physicochemical properties on the microbiotas
577 associated with third instar larvae and adults. NMDS analyses were performed using the *metaMDS*
578 function implemented in the R package Vegan (Dixon, 2003; Oksanen *et al.*, 2018). The correlation
579 between the microbiota composition and the tested factors (i.e. developmental stages, gut sections,
580 gut physicochemical properties) was investigated by fitting the NMDS ordination scores with the
581 *envfit* Vegan function (Dixon, 2003; Oksanen *et al.*, 2018). The permutation of the community
582 composition-based dissimilarity matrix (taking into account the non-independence of the different
583 gut samples of the same individuals) allowed assessment of the significance of the fitted factors and
584 vectors, and a squared correlation coefficient (R^2) was calculated.
585 To determine the level of specificity of the microbiota composition associated with each
586 developmental stage or gut region, model predictions were generated using Random Forest
587 regressors based on the relative abundance OTU table (Knights *et al.*, 2011). In order to classify the
588 microbiota samples based on host developmental stage or gut region, the supervised_learning.py
589 script from the QIIME pipeline was used. cv10 was used as error correction method with 999
590 replicate trees.
591 *Changes in microbiota composition*
592 In order to identify OTUs shared between the different insect developmental stages and the soil, we
593 only focused on OTUs that were typical for a given sample type (i.e. larvae, pupae, adults, soil). To
594 this end, an OTU was considered "present" in a given sample type only when it occurred in at least
595 66% of the biological replicates of that sample type (in most cases, 2 out of 3 biological replicates).
596 These OTUs are hereafter referred to as "core OTUs". The "core OTUs" specific to or shared
597 among the different developmental stages and the soil were visualized through a Venn diagram. In
598 addition, a bipartite network analysis (Dormann *et al.*, 2008) of the bacterial community associated
599 with the *P. japonica* (larvae, pupae and adults) and the bulk soil was performed using the pairwise
600 dissimilarity matrix generated from the OTU table adopting the Bray-Curtis dissimilarity index
601 (Bray and Curtis, 1957). Cytoscape (Shannon *et al.*, 2003) was used to visualize the network.

602 Differentially abundant taxa were determined after data normalization of the OTU table using the
603 EdgeR package (version 3.16.5) with R (version 3.4.4). Differentially abundant OTUs were then
604 ranked by their log_2 fold change from the most differentially abundant to the least differentially
605 abundant. Ranked OTUs were used to determine enriched families between different groups using
606 the tmod package (version 0.36) with the CERNO test (Yamaguchi *et al.*, 2008) and the Benjamini-
607 Hochberg correction. The position of the OTUs belonging to enriched families along the continuum
608 of ranked OTUs was also assessed visually using ROC curves (Receiver Operating Characteristic
609 curves). The enriched families were then tested for their presence in all samples (supplementary
610 table S3).

611 The OTU sequences of enriched taxa of interest (i.e. Christensenellaceae) were retrieved from the
612 OTU file then aligned to complete or near complete 16S rRNA sequences downloaded from the
613 NCBI website (www.ncbi.nlm.nih.gov) using Clustal W. After gap removal, the evolution model
614 was estimated using jModeltest according to the Akaike Information Criterion (AIC) parameter
615 (Akaike, 1976). The phylogenetic tree was reconstructed using maximum likelihood with the
616 Kimura 2 parameters model and 500 bootstraps. The phylogenetic tree was reconstructed and
617 visualized using Mega X (Kumar *et al.*, 2018).

618 In order to detect OTUs that are specific for a given gut section within the same developmental
619 stage, the indicator value (Dufrêne and Legendre, 1997) was calculated using the R package
620 *indicspecies* (De Cáceres and Legendre, 2009). Briefly, the indicator value of an OTU varies from 0
621 to 1 and attains its maximum value when all reads of an OTU occur in all samples of only one
622 specific gut section. We tested the significance of the indicator value for each OTU with a Monte
623 Carlo randomization procedure with 999 permutations.

624 *Measurement of the gut physicochemical properties*

625 Physico-chemical parameters of oxygen partial pressure (pO_2), pH and redox potential were
626 measured in the different sections of *P. japonica* gut (foregut, midgut and hindgut) with
627 microsensors and microelectrodes (Unisense, Aarhus, Denmark). Freshly dissected guts from both

628 L3 larvae and males were placed on a layer of 2% (Low Melting Point) agarose prepared with
629 Ringer's solution (7.2 g/L NaCl; 0.37 g/L KCl; 0.17 g/L $CaCl_2$, pH 7.3-7.4) and immediately
630 covered with a second layer of 0.5% agarose prepared with Ringer's solution (Šustr *et al.*, 2014).
631 Oxygen microsensors (OX-50), with a tip diameter of 50 µm, were calibrated after an overnight
632 polarization in water saturated with air and in 0.1 M sodium dithionite anoxic solution by using the
633 CAL 300 calibration chamber (Unisense, Aarhus, Denmark), following an overnight polarization.
634 pH microelectrodes (PH-50), with a tip diameter of 50 µm, were calibrated with standard solutions
635 at pH 4.0, 7.0 and 10.0. Redox potential microelectrodes (RD-50) had a tip diameter of 50 µm and
636 were calibrated using saturated quinhydrone solutions at pH 4.0 and 7.0. Electrode potentials for
637 microelectrodes were measured against Ag-AgCl reference electrodes by using a high-impedance
638 voltmeter (Ri > 1014 Ω). Unisense microsensor multimeter allowed to measure the current and data
639 were recorded by using SensorTracePRO software (Unisense, Aarhus, Denmark). Microsensors
640 were positioned using a motorized micromanipulator (Unisense, Aarhus, Denmark). Measurements
641 were carried out at room temperature.

642

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Data Accessibility

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

Author Contribution

BC, MM and LM designed the experiments. BC performed the microbiota and enrichment analyses. MM, GMg and NG performed the statistical analyses. SA performed the network analyses. GMz, EG, FP, LM, PFR and AA performed the sampling. NG dissected the insects and extracted the DNA. FF and FF performed the sequencing. MC, MF, EC and DD performed the physico-chemical analyses. BC and MM wrote the manuscript. All authors read and commented on the manuscript.

922 **Tables**

923 **Table1:** Ecological indices by developmental stage (mean ± SE)

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

924 **Tables and figures**

925 **Figure 1**: OTU distribution among the different samples. A: Bacterial community network
926 connecting OTUs (grey circles) to the samples (colored circles) in which they were observed. B:
927 Venn diagram showing the shared/specific bacterial OTUs (at 97% similarity) between the different
928 developmental stages and soil. C: Box-plots of the estimated standardized phylogenetic diversity
929 (SES-MPD) in the bacterial communities of rhizospheric soil and *Popillia japonica* developmental
930 stages.
931 **Figure 2**: Non-metric multi-dimensional scaling analysis (NMDS) plots displaying sample β-
932 diversity inferred from the OTU table. A: Biplot of the first 2 axes for the NMDS representing
933 correlations between the OTUs abundance in all insect samples and ecological and ontological
934 factors (i.e. developmental stage and gut section). B: NMDS plots showing the correlation between
935 the bacterial OTUs of Adults and larvae and the different physico-chemical properties (pH, O2
936 concentration and RedOx potential) of the different gut regions (foregut, midgut and hindgut). The
937 vectors represent the mean direction and strength of correlation of the different parameteres
938 measured (p-value < 0.05). In both figures, shapes indicate the different developmental stages (i.e.
939 square for larvae, triangle for pupae, circle for adults) while colors indicate the gut region (i.e. red
940 for foregut, green for midgut, blue for hindgut).
941 **Figure 3**: Histograms summarizing the bacterial composition at different taxonomic levels. the
942 different histograms report only taxa with a relative abundance ≥ 3%. A: The taxa summary at the
943 order level for the different samples grouped by category. F indicates foregut, M indicates midgut
944 and H indicates hindgut. B and C the taxa summary at the phylum level for the different samples
945 grouped by developmental stages (B) and by gut section (C).
946 **Figure 4**: Taxa Enrichment analysis (TEA) carried out on the different larval stages using soil as
947 reference. The main figure indicates the families that were enriched in the different larval stages
948 compared to soil. The color intensity of the circles indicates the p value while its size indicates the
949 effect size. The panels on the right-hand side are the ROC curves, plotting the ranked OTUs
950 belonging to the enriches families against the totality of the ranked OTUs, represent the rank of the
951 different OTUs belonging to the families Lachnospiraceae (green), Christensenellaceae (blue),
952 Ruminococcaceae (black) and the order Clostridiales (red) in general.
953 **Table S1**: Summary of the different ecological indices and Random Forest results for each sample.
954 1a: Ecological indices summary for the different samples. 1b: summary statistics of the comparison
955 of the different alpha diversity values between the different developemental stages. 1c:
956 Standardized phylogenetic evenness results for all the samples. 1d: Results of the Random Forest
957 goodness of prediction for the developmental stages. 1e: Results of the Random Forest goodness of
958 prediction for the gut section. 1f: Top 10 OTU predictors of the Random Forest prediction for the

959 developmental stages. 1g: Top 10 OTU predictors of the Random Forest prediction for the gut
960 sections.
961 **Table S2**: Indval results indicating the OTUs specific for each developmental stage and gut section.
962 2a: Indval report for the specific OTUs per each developmental stage 2b: Indval report for the
963 specific OTUs per each gut section for each developmental stage.
964 **Table S3**: presence-absence matrix of the enriched families for each sample.
965 **Figure S1**: 1a. Male adult specimen of *Popillia japonica*. 1b. Gut of an adult *P. japonica* with the
966 different sections delimited.
967 **Figure S2**: Alpha diversity parameters by sample or sample type. A: Chao1 index for all the
968 samples. B: Chao1 index reported by gut section. C: Chao1 index reported by developmental stage.
969 D: Shannon index for all the samples. E: Shannon index reported by gut section. F: Shannon index
970 reported by developmental stage.
971 **Figure S3**: Biplot of the estimated standardized phylogenetic diversity (SES-MPD) and OTUs
972 richness of each community. The dashed grey line represents the linear regression, for the bacterial
973 communities associated with insect samples, of the SES-MPD onto the OTUs richness.
974 **Figure S4**: Heatmaps showing the relative pairwise nestedness and turnover values for the different
975 developmental stages and soil
976 **Figure S5**: Box-plots displaying the value ranges of the different physico-chemical properties
977 measured for the different gut sections for both adults and larvae. A: pH, B: Oxygen concentration;
978 C: RedOx potential.
979 **Figure S6**: Histograms summarizing the bacterial composition at the order level. the different
980 histograms report only taxa with a relative abundance ≥ 3%. A: The taxa summary at the order level
981 for the different samples. F indicates foregut, M indicates midgut and H indicates hindgut. B the
982 taxa summary at the order level for the different samples grouped by individual pools. Namely each
983 column correspond to the samples (foregut, midgut and hindgut) from the same pooled individuals.
984 **Figure S7**: Maximum likelihood phylogenetic tree based on the partial 16S rRNA gene sequences.
985 The blue circle indicates the Christensenellaceae group of bacteria associated with the human gut.
986 All other taxa were detected in the present study in association with P. japonica gut sections. The
987 scale bar at the bottom indicates the distance in nucleotide substitution per site. The alphanumeric
988 sequence at each node either the GeneBank accession number or the *de novo* OTUs.
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