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

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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 ± 52.2 and 4.99 ± 0.77 , respectively, for L1 larvae, 313.92 ± 48.44 and 5.47 ± 0.28 for L2
 143 larvae and 342.96 ± 43.02 and 5.74 ± 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's $J = 0.84$; Table 1), then
 146 larvae (Pielou's $J = 0.67$; Table 1) and with pupae and adults having similar values (Pielou's $J = 0.47$
 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_MPD_{SOIL} = 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_MPD_{LARVAE} = -3.38 and SES_MPD_{PUPAE} = -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_MPD_{ADULTS} = -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 β -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, O₂ 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 O₂ 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 ($35.9\% \pm \text{SE } 4.2\%$), followed by Firmicutes
222 ($32.9\% \pm \text{SE } 5.4\%$) and Bacteroidetes ($15.4\% \pm \text{SE } 3.7\%$), 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 $49.5\% \pm \text{SE } 7.9\%$ (range $26.5\% \pm \text{SE } 5.5\%$ in
225 L2 larvae to $74.5\% \pm \text{SE } 8.7\%$ in L1 larvae), followed by Proteobacteria ($31.3\% \pm \text{SE } 5.8\%$ on

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

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

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

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

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

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

It is noteworthy that the TEA did not evidence any significantly enriched taxonomic group between the different developmental stages of the insect nor did it evidence enriched taxonomic group between the different gut sections. This is partly supported by the fact that the nestedness component of the β -diversity between the different insect developmental stages was relatively high (0.59 on average), indicating that a higher fraction of the microbiotas is shared between the different insect 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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Materials and methods

Collection and processing of insect and soil samples

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

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

DNA extraction, amplicon library preparation, sequencing and bioinformatics

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

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

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

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

Diversity analyses

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

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

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

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

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

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

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

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

Changes in microbiota composition

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

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

Differentially abundant taxa were determined after data normalization of the OTU table using the EdgeR package (version 3.16.5) with R (version 3.4.4). Differentially abundant OTUs were then ranked by their log₂ fold change from the most differentially abundant to the least differentially abundant. Ranked OTUs were used to determine enriched families between different groups using the tmod package (version 0.36) with the CERNO test (Yamaguchi et al., 2008) and the Benjamini–Hochberg correction. The position of the OTUs belonging to enriched families along the continuum of ranked OTUs was also assessed visually using receiver operating characteristic (ROC) curves. The enriched families were then tested for their presence in all samples (Supporting Information Table S3).

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

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

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

Measurement of the gut physicochemical properties

Physico-chemical parameters of oxygen partial pressure (pO₂), pH and redox potential were measured in the different sections of *P. japonica* gut (foregut, midgut and hindgut) with microsensors and microelectrodes (Unisense, Aarhus, Denmark). Freshly dissected guts from both L3 larvae and males were placed on a layer of 2% (low melting point) agarose prepared with Ringer's solution (7.2 g l NaCl; 0.37 g l KCl; 0.17 g l CaCl₂, pH 7.3–7.4) and immediately covered with a second layer of 0.5% agarose prepared with Ringer's solution (Šustr et al., 2014). Oxygen microsensors (OX-50), with a tip diameter of 50 µm, were calibrated after an overnight polarization in water saturated with air and in 0.1 M sodium dithionite anoxic solution by using the CAL 300 calibration chamber (Unisense), following an overnight polarization. pH microelectrodes (PH-50), with a tip diameter of 50 µm, were calibrated with standard solutions at pH 4.0, 7.0 and 10.0. Redox potential microelectrodes (RD-50) had a tip diameter of 50 µm and were calibrated using saturated quinhydrone solutions at pH 4.0 and 7.0. Electrode potentials for microelectrodes were measured against Ag-AgCl reference electrodes by using a high-impedance voltmeter (R_i > 10¹⁴ Ω). Unisense microsensor multimeter allowed to measure the current and data were recorded by using SensorTracePRO software (Unisense). Microsensors were positioned using a motorized micromanipulator (Unisense). Measurements were carried out at room temperature.

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 are 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 Supporting Information.

Author Contributions

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 FG performed the sequencing. MC, MF, EC and DD performed the physicochemical analyses. BC and MM wrote the manuscript. All authors read and commented on the manuscript.

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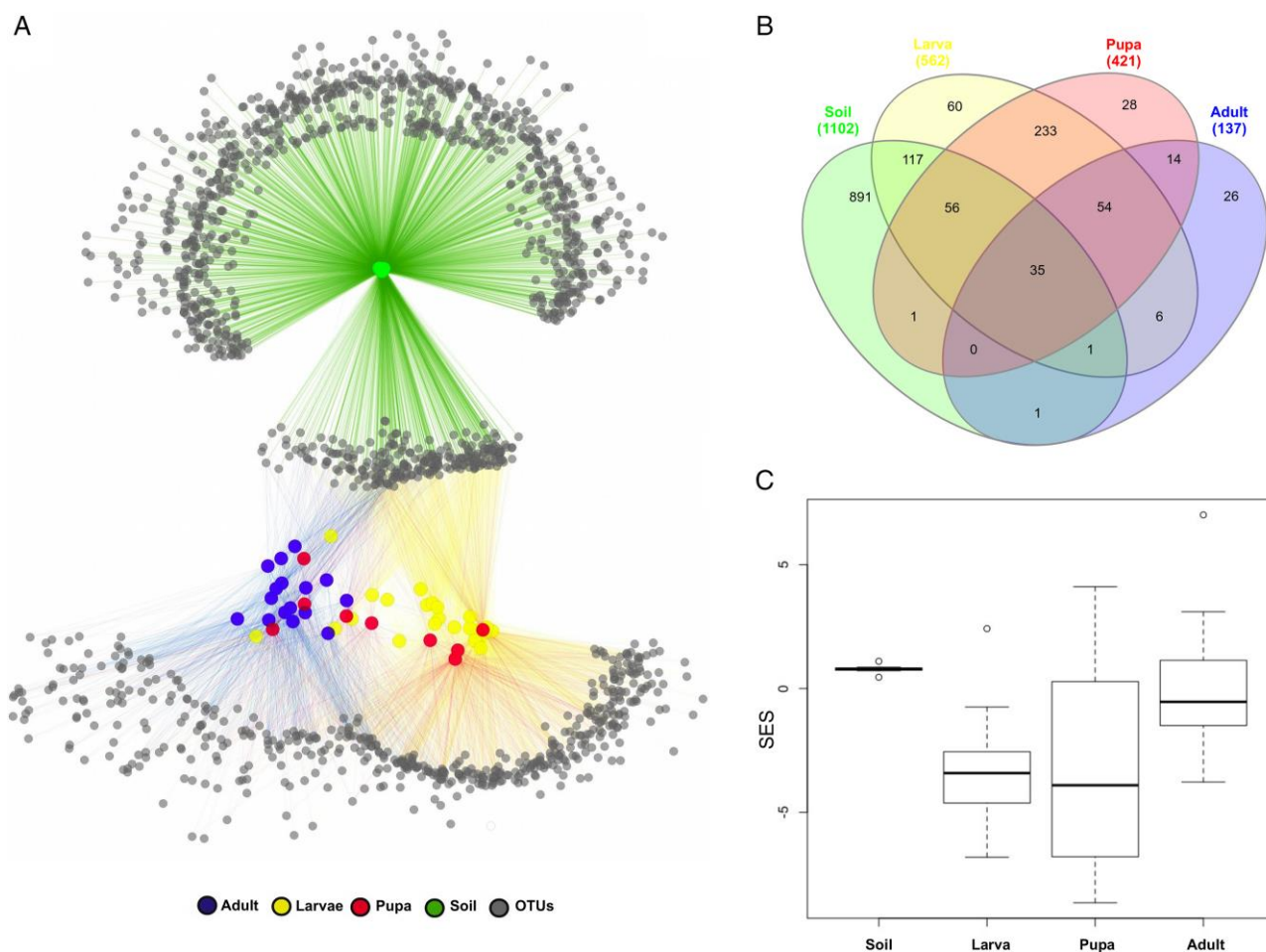
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974 **Tables**

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

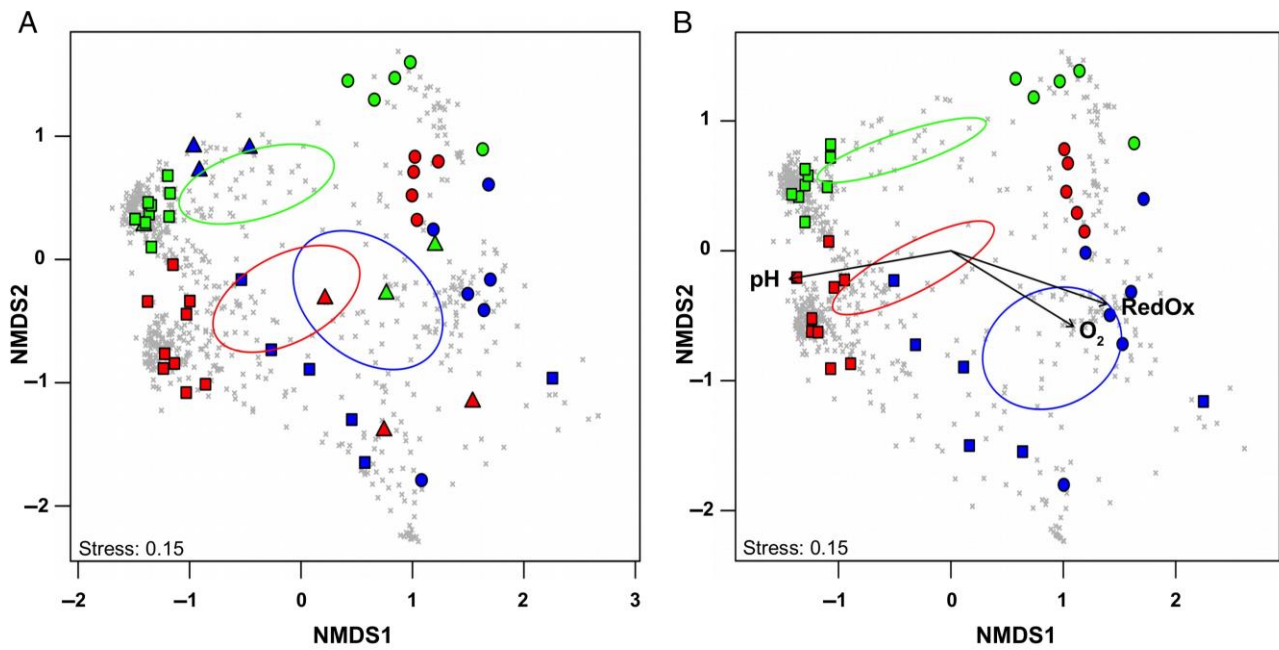
	Richness (Chao1)	Diversity (Shannon)	Evenness (Pielou)
Soil	1099 \pm 1.35	5.88 \pm 0.03	0.84 \pm 0.00
Larvae	369.93 \pm 28.95	3.77 \pm 0.19	0.67 \pm 0.03
Pupae	241.12 \pm 43.51	2.49 \pm 0.39	0.47 \pm 0.06
Adults	129.65 \pm 7.33	2.22 \pm 0.18	0.49 \pm 0.04

976 Figures



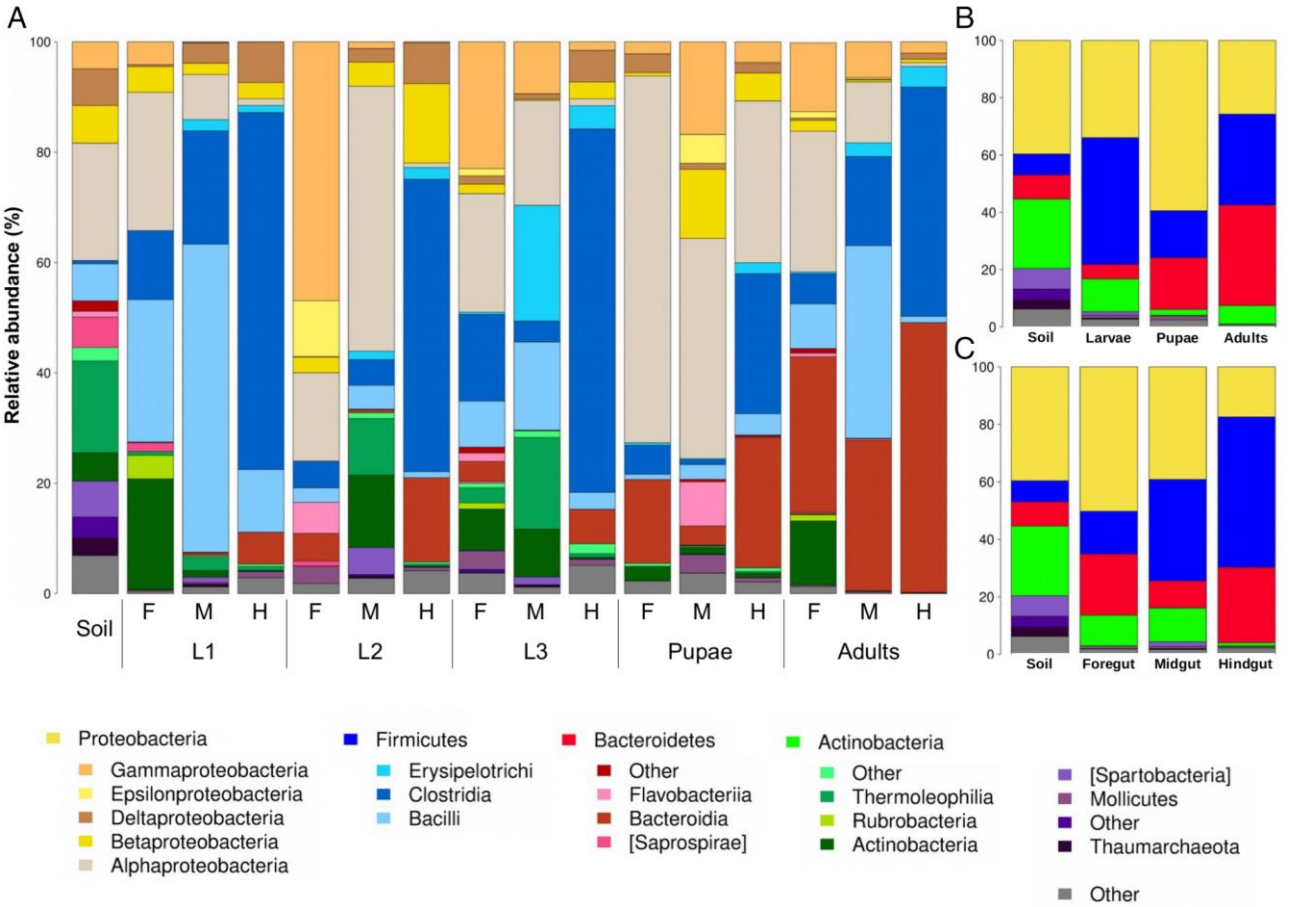
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978 **Figure 1:** OTU distribution among the different samples. A. Bacterial community network
 979 connecting OTUs (grey circles) to the samples (coloured circles) in which they were observed. B.
 980 Venn diagram showing the shared/specific bacterial 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.

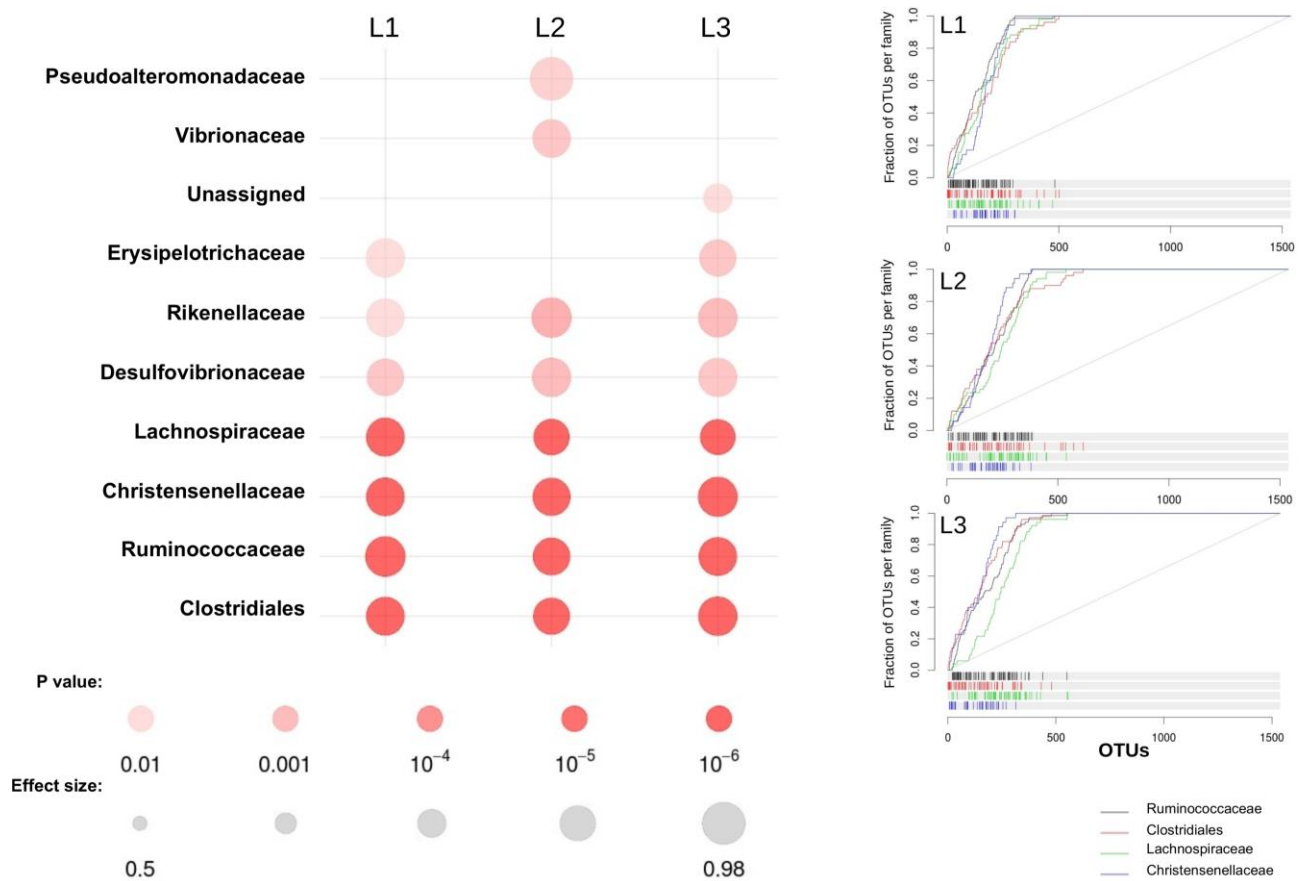


983 □ Larvae △ Pupae ○ Adults ■ Foregut ■ Midgut ■ Hindgut

984 **Figure 2:** NMDS analysis plots displaying sample β -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, O₂ 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).



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.

