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

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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 factor shaping microbiota composition in this species, likely due to differences in pH, oxygen availability and redox potential. In addition, *P. japonica* also harbored a stable bacterial community across all developmental stages, consisting of taxa well-known for the degradation of plant material, namely the families Ruminococcacae, Christensenellaceae and Lachnospiraceae. Interestingly, the family Christensenallaceae had so far been observed exclusively in humans. However, the Christensenellaceae OTUs found in *P. japonica* belong to different taxonomic clades within this family.

Key words: *Popillia japonica*, gut microbiota, Ruminococcacae, Christensenellaceae, Lachnospiraceae,

48 **Introduction**

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

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

74 modulation of immune responses, and even extending life span (Daisley et al., 2018; Kim et al., 2016;
75 Obata, Fons, & Gould, 2018; Roh et al., 2008).
76 Several factors can influence the gut microbiota structure and composition. Among these factors, the
77 most important ones are diet and environment, but other factors (e.g., age) can also be at play
78 (Anderson et al., 2018; Montagna, Chouaia, et al., 2015; Montagna, Gómez-Zurita, et al., 2015;
79 Montagna et al., 2016; Sanders et al., 2017; Tiede, Scherber, Mutschler, McMahon, & Gratton, 2017;
80 Vacchini et al., 2017; Wong, Ng, & Douglas, 2011). Although various factors can influence the insect
81 gut microbiota, the existence of a shared core microbial community in some species indicates that at
82 least some members of the gut microbiota can be vertically transmissible. Several studies have
83 investigated this possibility by tracking the changes in gut microbiota composition along the
84 developmental stages of different insect species. These studies showed that the transmission of the
85 gut microbiota throughout the different developmental stages may depend on the usefulness of certain
86 bacteria (Malacrinò, Campolo, Medina, & Palmeri, 2018; Zhukova et al., 2017). For instance, the
87 bacterial communities of fruit flies (Tephritideae) change throughout the insect's developmental
88 stages to respond to the physiological needs of the host (Aharon et al., 2013; Malacrinò et al., 2018).
89 In holometabolous insects, the pupal stage generally represents a bottleneck where most of the larval
90 gut microbiota is lost and adult insects may have to resort to indirect ways (e.g. via environmental
91 transmission) to insure the transfer of beneficial bacteria from larvae to adults (Zhukova et al., 2017).
92 For instance, in certain bee species, certain bacterial taxa are not trans-stadially transmitted but re-
93 acquired from the environment (McFrederick, Mueller, Wcislo, & Hout, 2014). While the gut
94 microbiota is not constant across the developmental stages in most insects, in some cases the
95 microbial community can be relatively stable throughout the developmental stages. This has been
96 observed in some tephretid flies as well as in the Black Soldier Fly *Hermetia illucens* and in the moth
97 *Plodia interpunctella* (De Smet, Wynants, Cos, & Van Campenhout, 2018; Mereghetti, Chouaia,
98 Limonta, Locatelli, & Montagna, 2017; Yong, Song, Chua, & Lim, 2017).

99 In the present study, we focused on the highly polyphagous invasive Japanese beetle *Popillia japonica*
100 Newman (Coleoptera: Scarabaeidae). This invasive insect is listed in the EPPO Annex 2 due to the
101 damages caused to different crops and turfs (EPPO, 2000). Native to Japan and the far east of Russia
102 (Fleming, 1972), this beetle became an established pest in North America in the early 1900's (Switzer,
103 Enstrom, & Schoenick, 2009), in the Azores in the early 1970's (Vieira, 2008) and more recently in
104 continental Europe, where it was recorded for the first time in Italy in 2014 (EPPO, 2014; Pavesi,
105 2014) and in Switzerland in 2017 (EPPO, 2017). Several laboratory and field trials have been carried
106 out to limit the spread of this pest in mainland Europe and to evaluate the environmental resilience of
107 the infested areas (e.g. Marianelli et al., 2018; Mazza et al., 2017; Paoli, Marianelli, Binazzi, et al.,
108 2017; Paoli, Marianelli, Torrini, et al., 2017). The damages to plants are caused by the different
109 developmental stages of the beetle: the larvae, being underground dwellers, feed on the plant roots
110 and soil organic matter while adults, living in an above-ground environment, feed on leaves and floral
111 parts of different plant species (Fleming, 1972; Vieira, 2008).
112 Insect-associated bacteria can potentially contribute to an insect's invasive potential by helping their
113 hosts to adapt to changing environmental conditions. Such symbiotic bacteria can be part of a core
114 microbiota that is stably transmitted throughout the host's life cycle or selectively recruited from the
115 environment at each developmental stage. The aim of this study was to investigate microbiota
116 dynamics in an invasive population of *P. japonica* from Italy. Specifically, we addressed the
117 following questions: i) Does *P. japonica* harbour a stable core microbiota or are the bacteria mainly
118 acquired from the surrounding environment (i.e. rhizospheric soil exploited by larvae and pupae *vs*
119 aerial environment exploited by adults)? ii) Is the gut microbiota maintained across the post-
120 embryonic developmental stages (i.e. larvae, pupae and adults) or is there a major turnover due to
121 insect development? iii) Do different gut micro-environments impact microbial community structure?
122

123 **Materials and methods**

124 *Collection and processing of insect and soil samples*

125 Four campaigns were organized from June to September 2017 to collect insect samples at different
126 developmental stages of the insect. The different stages and instars (in the case of larvae: larval instar
127 1 – L1; larval instar 2 – L2; larval instar 3 – L3) of the insects were collected in Oleggio (Novara,
128 Italy; 45°36′ N, 08°38′ E, altitude ca. 230 m a.s.l.). Simultaneously, at each sampling expedition, 10
129 soil samples were taken from the sampled area and combined into a single sample representative of
130 the area, leading to the collection of three soil samples. Insects were preserved in absolute ethanol
131 while soil samples in 50 ml vials, kept refrigerated on the field and then stored at -20°C before
132 processing. All insects were surface sterilized before dissection using the protocol described in
133 Montagna and colleagues (Montagna, Chouaia, et al., 2015). Individuals were dissected under sterile
134 conditions, and gut removed in sterile Ringer solution. The insect alimentary canal was then separated
135 in its three compartments (i.e. foregut, midgut and hindgut). A total of 90 individuals were dissected.
136 For each developmental stages and larval instar, five homologous gut compartments were pooled
137 together in a single sample resulting in three biological replicates for each sample category. These
138 samples were used for DNA extraction.
139 Additionally, male adults and L3 larvae were collected and immediately processed in order to
140 measure physicochemical properties (pH level, redox potential, oxygen concentration) of different
141 gut regions. Specimens were anesthetized at 4°C for 3' before their dissection.

142 *DNA extraction, amplicon library preparation, sequencing and bioinformatics*

143 The DNA was extracted from each sample (consisting of five homologous gut compartments for a
144 defined insect instar and developmental stage) using the phenol–chloroform methods (Doyle &
145 Doyle, 1990) with the modifications described in Mereghetti and colleages (Mereghetti et al., 2017).
146 The DNA was then eluted in 50 µl of sterile water (Sigma-Aldrich, Saint Louis, Missouri, USA). A
147 DNA extraction blank was performed as control to monitor for contamination of environmental
148 bacteria DNA. DNA from soils was extracted using PowerSoil DNA Isolation Kit (MO BIO
149 Laboratories Inc., Carlsbad, CA) following manufacturer's instructions. Three independent DNA
150 extractions were performed for each of the three representative soil samples. All the extracted DNAs

151 were used as template for PCR targeting the bacterial 16S rRNA gene using 27 Fmod and 519 Rmod
152 primers (Lane, 1991). All samples except to the blank extraction resulted positive.
153 The extracted DNA were used as template for the amplification of V4 hypervariable region of the
154 16S rRNA by PCR primers 515F (Caporaso et al., 2011), 802R (Claesson et al., 2009) and 806R
155 (Caporaso et al., 2011) tailed with two different GC rich sequences enabling barcoding with a second
156 amplification. Each sample was amplified in 20 µl volume reaction containing 8 µl HotMasterMix 5
157 Prime 2.5X (Quanta Bio), 0.4 µl BSA (20 µg/µl) (Sigma-Aldrich), 1 µl EvaGreen™ 20X (Biotium),
158 0.8µl 515 F (10 µM) (- 5' modified with unitail 1 5'-CAGGACCAGGGTACGGTG-3'), 0.4 µl 802 R
159 (10 µM) (- 5' modified with unitail 2 5'-CGCAGAGAGGCTCCGTG-3'), 0.4 µl 806 R (10 µM) (- 5'
160 modified with unitail 2 5'-CGCAGAGAGGCTCCGTG-3'), and 1 µl (50 ng) of DNA template. The
161 PCR amplifications were performed in a CFX 96™ PCR System (Bio-Rad) with 34 cycles of 94°C
162 for 20 s, 52°C for 20 s, 65°C for 40 s and a final extension of 65°C for 2 min.
163 After labeling each sample with a specific Ion Torrent (Ion Express) DNA barcode, each single library
164 was quality checked with agarose gel electrophoresis, quantified with Qubit Fluorometer (Thermo
165 Fisher Scientific) then pooled with the other libraries in equimolar amounts. The final product was
166 then sequenced using the Ion Torrent PGM System. Libraries preparation and sequencing were
167 performed at the Life Sciences Department of Trieste University, Italy.
168 The obtained reads were analyzed using QIIME version 1.9.1 (Caporaso et al., 2010). In detail,
169 adapters were removed, and low-quality reads filtered (Phred < 20, read length < 250pb). Uclust
170 (Edgar, 2010) was used to cluster the 16S rRNA sequences into Operational Taxonomic Units (OTUs)
171 with a similarity cut-off of 97%. Chimeras were removed using Chimeraslayer. A representative
172 sequence for each identified OTUs was aligned to Green-genes (http://greengenes.lbl.gov/) using
173 Pynast (Caporaso et al., 2010). Taxonomic assignment was performed comparing the representative
174 OTUs to Green-genes (release 13.8). Rare OTUs (i.e., singletons and OTUs < 10) and OTUs
175 identified as chloroplast were discarded. The resulting OTU table was then used for the subsequent
176 analyses.

177 The sequences obtained in this study have been deposited in the European Nucleotide Archive and
178 are waiting for the assignment of an accession number.

179 *Diversity analyses*

180 Bacterial OTU richness, diversity and evenness were calculated using the package Vegan (Dixon,
181 2003; Oksanen et al., 2018), implemented under the R software (R Project 3.0.2; http://cran.r-
182 project.org/) adopting the species richness estimator Chao 1 (Chao, 1984), the Shannon H' index
183 (Shannon, 1948) and the Pielou's evenness (Pielou, 1975), after sub-sampling the OTU table to obtain
184 a total of 25,000 sequences per sample. Alpha diversity indices were compared between different
185 groups (i.e. tissues, developmental stages) using two-sample t-tests with 999 Monte Carlo
186 permutations.

187 In order to evaluate if the structures of the bacterial communities associated with soil and the different
188 developmental stages of *P. japonica* were driven by species competition or by environmental factors,
189 thus resulting in a community dominated by closely related species (Mouquet et al., 2012; O'Dwyer,
190 Kembel, & Green, 2012; Webb, Ackerly, McPeek, & Donoghue, 2002), the mean pairwise distance
191 between all taxa in the bacterial communities (MPD; (Webb et al., 2002) was used as metric for
192 phylogenetic structure. To allow the comparison between the bacterial communities of the different
193 types, null models maintaining species occurrence frequency constant were estimated. Standard effect
194 size and relative position of each bacterial community with respect to the null MDP distribution,
195 generated by 999 randomizations of the null model, were calculated using the *ses.mpd* function
196 implemented in the Rpackage *picante* (Kembel et al., 2010). This standardized metric quantifies the
197 relative excess or deficit in the phylogenetic diversity for each community with respect to the entire
198 species pool. Negative values reflect a relative phylogenetic clustering of the species, while positive
199 values indicate a relative phylogenetic evenness (or overdispersion). SES_{MDP} values were visualized
200 as box-plots, grouping the values according to the sample type (i.e. soil, larvae, pupae, adults), and
201 the differences among sample types were assessed using the non-parametric Kruskal–Wallis test

202 (Kruskal & Wallis, 1952) after assessing the homogeneity of variances among the groups using the
203 Levene test (Levene, 1960).
204 The spatial (across the three gut regions) and temporal shifts (across developmental stages) of the *P.*
205 *japonica* bacterial community (presence/absence) were estimated using the Sørensen-based multiple-
206 site dissimilarity (β_{SOR}; (Baselga, 2010) implemented in the R package *betapart* (Baselga & Orme,
207 2012). The turnover and nestedness components of this β-diversity were calculated using Simpson-
208 based multiple-site dissimilarity (β_{SIM}; (Baselga, 2010) and nestedness-resultant multiple-site
209 dissimilarity (β_{NES}; (Baselga, 2010), respectively. In addition, for each β-diversity component, the
210 pairwise dissimilarity values among the microbiotas of all analysed groups (i.e. soil, larvae, pupae
211 and adults) were calculated using the *betapair* function of the R package *betapart* (Baselga & Orme,
212 2012) and visualized through heatmaps using heatmap.2 from the R package gplots.
213 In order to assess the difference in the microbiota structure among soil and insect samples, the sub-
214 sampled OTU table was subjected to a nonparametric one-way analysis of similarity ANOSIM
215 (Clarke, 1993) (Clarke, 1993), implemented in the vegan library and based on the Bray-Curtis
216 dissimilarity (999 permutations permuting within gut samples of the same individuals in order to
217 account for the non-independence of the observations; (Bray & Curtis, 1957).
218 The sub-sampled OTU table, after the removal of soil community samples, was used as input for a
219 Nonmetric Multi-Dimensional Scaling (NMDS; (Kruskal, 1964) biplot based on the Bray–Curtis
220 dissimilarity (Bray & Curtis, 1957), in order to graphically ordinate samples and assess the
221 differences among: i) the developmental stages (i.e. larvae, pupae and adults); ii) the three gut regions,
222 and iii) to evaluate the impact of the gut physicochemical properties on the microbiotas associated
223 with third instar larvae and adults. NMDS analyses were performed using the *metaMDS* function
224 implemented in the R package Vegan (Dixon, 2003; Oksanen et al., 2018). The correlation between
225 the microbiota composition and the tested factors (i.e. developmental stages, gut sections, gut
226 physicochemical properties) was investigated by fitting the NMDS ordination scores with the *envfit*
227 Vegan function (Dixon, 2003; Oksanen et al., 2018). The permutation of the community composition-

228 based dissimilarity matrix (taking into account the non-independence of the different gut samples of
229 the same individuals) allowed assessment of the significance of the fitted factors and vectors, and a
230 squared correlation coefficient (R^2) was calculated.

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

237 *Changes in microbiota composition*

238 In order to identify OTUs shared between the different insect developmental stages and the soil, we
239 only focused on OTUs that were typical for a given sample type (i.e. larvae, pupae, adults, soil). To
240 this end, an OTU was considered "present" in a given sample type only when it occurred in at least
241 66% of the biological replicates of that sample type (in most cases, 2 out of 3 biological replicates).
242 These OTUs are hereafter referred to as "core OTUs". The "core OTUs" specific to or shared among
243 the different developmental stages and the soil were visualized through a Venn diagram. In addition,
244 a bipartite network analysis (Dormann, Gruber, & Fründ, 2008) of the bacterial community associated
245 with the *P. japonica* (larvae, pupae and adults) and the bulk soil was performed using the pairwise
246 dissimilarity matrix generated from the OTU table adopting the Bray-Curtis dissimilarity index (Bray
247 & Curtis, 1957). Cytoscape (Shannon et al., 2003) was used to visualize the network.

248 Differentially abundant taxa were determined after data normalization of the OTU table using the
249 EdgeR package (version 3.16.5 with R (version 3.4.4). Differentially abundant OTUs were then
250 ranked by their $\log_2$ fold change from the most differentially abundant to the least differentially
251 abundant. Ranked OTUs were used to determine enriched families between different groups using
252 the tmod package (version 0.36; (Weiner 3rd & Domaszewska, 2016) with the CERNO test
253 (Yamaguchi et al., 2008) and the Benjamini-Hochberg correction. The position of the OTUs

254 belonging to enriched families along the continuum of ranked OTUs was also assessed visually using
255 ROC curves (Receiver Operating Characteristic curves).
256 The OTU sequences of enriched taxa of interest (i.e. Christensenellaceae) were retrieved from the
257 OTU file then aligned to complete or near complete 16S rRNA sequences downloaded from the NCBI
258 website (www.ncbi.nlm.nih.gov) using Clustal W. After gap removal, the evolution model was
259 estimated using jModeltest according to the Akaike Information Criterion (AIC) parameter (Akaike,
260 1976). The phylogenetic tree was reconstructed using maximum likelihood with the Kimura 2
261 parameters model and 500 bootstraps. The phylogenetic tree was reconstructed and visualized using
262 Mega X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).
263 In order to detect OTUs that are specific for a given gut section within the same developmental stage,
264 the indicator value (Dufrêne & Legendre, 1997) was calculated using the R package *indicspecies* (De
265 Cáceres & Legendre, 2009). Briefly, the indicator value of an OTU varies from 0 to 1 and attains its
266 maximum value when all reads of an OTU occur in all samples of only one specific gut section. We
267 tested the significance of the indicator value for each OTU with a Monte Carlo randomization
268 procedure with 999 permutations.
269 *Measurement of the gut physicochemical properties*
270 Physico-chemical parameters of oxygen partial pressure (pO_2), pH and redox potential were measured
271 in the different sections of *P. japonica* gut (foregut, midgut and hindgut) with microsensors and
272 microelectrodes (Unisense, Aarhus, Denmark). Freshly dissected guts from both L3 larvae and males
273 were placed on a layer of 2% (Low Melting Point) agarose prepared with Ringer's solution (7.2 g/L
274 NaCl; 0.37 g/L KCl; 0.17 g/L $CaCl_2$, pH 7.3-7.4) and immediately covered with a second layer of
275 0.5% agarose prepared with Ringer's solution (Šustr, Stingl, & Brune, 2014). Oxygen microsensors
276 (OX-50), with a tip diameter of 50 µm, were calibrated after an overnight polarization in water
277 saturated with air and in 0.1 M sodium dithionite anoxic solution by using the CAL 300 calibration
278 chamber (Unisense, Aarhus, Denmark), following an overnight polarization. pH microelectrodes
279 (PH-50), with a tip diameter of 50 µm, were calibrated with standard solutions at pH 4.0, 7.0 and

280 10.0. Redox potential microelectrodes (RD-50) had a tip diameter of 50 µm and were calibrated using
281 saturated quinhydrone solutions at pH 4.0 and 7.0. Electrode potentials for microelectrodes were
282 measured against Ag-AgCl reference electrodes by using a high-impedance voltmeter (Ri > 1014 Ω).
283 Unisense microsensor multimeter allowed to measure the current and data were recorded by using
284 SensorTracePRO software (Unisense, Aarhus, Denmark). Microsensors were positioned using a
285 motorized micromanipulator (Unisense, Aarhus, Denmark). Measurements were carried out at room
286 temperature.
287
288 **Results**
289 *1. Alpha, beta and phylogenetic diversity of the gut microbiota*
290 A total of 5175086 high-quality reads longer than 250 bp were kept after quality filtering and chimera
291 removal. These reads clustered into 1612 OTUs. On average, 67299 high-quality reads grouped into
292 336 OTUs were obtained from larvae, 80249 reads/204 OTUs from pupae, 88397 reads/99 OTUs
293 from adults and 148324 reads/1093 OTUs from soil samples (see Table S1a, Supporting Information,
294 for details). Rarefaction curves of the observed OTU richness in 25,000 sub-sampled sequences
295 showed that our sequencing effort was sufficient to capture the major part of the bacterial diversity
296 associated with the different developmental stages. On the other hand, a higher sequencing depth
297 would probably have added new taxa to the bacterial communities present in soil (Fig. S1). OTU
298 richness and diversity (Fig. S1), as determined by the species richness estimator Chao1 and the
299 Shannon Index of diversity, were higher in soil samples than in insect samples (Chao1: all t-tests $P <$
300 0.01; Shannon: all t-test $P < 0.01$; Table1). Regarding the different developmental stages of *P.*
301 *japonica*, OTU richness and diversity were the highest in the larvae (Chao 1: all t-tests $P < 0.01$;
302 Shannon: all t-tests $P < 0.01$, see supplementary Table1 and Table S1a for all ecological indices). On
303 the other hand, these indices were the lowest for adults (Chao 1: all t-tests $P < 0.01$; Shannon: all t-
304 tests $P < 0.01$; Table1). The different larval instars had similar richness and diversity (Chao 1: all t-
305 tests $P = 1$; Shannon: all t-tests $P =1$). Noteworthy, the values of Pielou's evenness also followed a

306 similar pattern with the soil having the highest value (Pielou'J = 0.84; Table1), then larvae (Pielou'J
307 = 0.67; Table1) and with pupae and adults having similar values (Pielou'J = 0.47 and 0.49
308 respectively; Table1).
309 The standardized effect size of mean pairwise distance values (SES_MPD) of the bacterial
310 communities associated with the samples ranged from positive values for soil bacterial communities
311 (median value of SES_MPD_{SOIL} = 0.78 associated with high quantiles, Table S1b) to negative values
312 for bacterial communities associated with the larval and pupal stages (median values
313 SES_MPD_{LARVAE} = -3.38 and SES_MPD_{PUPAE}= -3.9, low quantile values, Table S1b) (Fig. 1C).
314 The positive SES_MPD values for the soil communities indicate a phylogenetic overdispersion, as
315 expected for communities characterized by high species richness and evenness such as those of soil.
316 In contrast, the negative SES_MPD values for the bacterial communities associated with larvae and
317 pupae indicate a phylogenetic clustering of these communities, possibly due to the selection towards
318 certain closely-related bacterial lineages by the insect gut environment, characterized by peculiar
319 features such as by high enzymatic activities and host immune response. Interestingly, the bacterial
320 communities associated with adults were characterized by slightly negative SES_MPD values
321 (median value of SES_MPD_{ADULTS}= -0.53; Table S1b), indicating a phylogenetic evenness of these
322 communities (Fig. 1C). This increasing trend of SES_MPD values from larvae and pupae (negative
323 values) towards adults (slightly negative values) contrasted with the trend of decreasing community
324 species richness from larvae to adults (Fig. S2).

325 *2. Factors affecting gut microbiota composition*

326 Soil was different from the insect samples in terms of bacterial composition (adonis: $P < 0.001$, R^2 =
327 0.33; anosim: $P < 0.001$, R = 0.54) with few OTUs shared between soil and the different insect
328 developmental stages (Fig. 1A). Specifically, 891 OTUs out of the 1102 "core OTUs" of the soil were
329 not found in the insect samples (Fig. 1B). On the other hand, only 35 "core OTUs" were present in
330 both insects and soil (Fig. 1B). Moreover, the nestedness component of the β-diversity between soil
331 and the different insect developmental stage was very low (0.16 on average) and the turnover was

332 high (0.84 on average) (Fig. S3), indicating that very few “core OTUs” were shared between soil and
333 insect microbiotas while the variable fraction was high.
334 Although more bacterial OTUs were shared between the insect samples (i.e. developmental stages
335 and gut sections combined) than between insects and soil, these samples still formed distinct clusters
336 as shown by NMDS analysis (Fig 2A). Specifically, insect developmental stages segregated along
337 the first axis with the larvae microbiotas being clearly distinct from adult microbiotas, while pupal
338 microbiotas were intermediate. The second axis further separated the samples based on gut sections.
339 For larvae and adults, the microbiotas of the different gut sections formed distinct clusters with the
340 midgut microbiota being more different than the foregut and hindgut microbiotas. In contrast, the
341 pupal microbiotas showed a different pattern with a clear cluster for the hindgut, while foregut and
342 midgut microbiotas loosely clustered together.
343 The main factor driving this segregation was the gut section ($P < 0.001$) and to a lesser extent the
344 developmental stage. These results were further supported by the Random Forest (RF) analysis which
345 was carried out to investigate the specificity of the microbiota of each sample category by trying to
346 assign each sample to its respective category based on its microbiota. The RF analysis
347 (Supplementary Table S1c) carried out in order to classify the different developmental stages was
348 able to successfully classify adults and larvae in 100% and 91.7% of the cases, respectively.
349 Conversely, pupae were successfully identified in only 55.6% of the cases. These results suggest that
350 the pupal stage represents a transitional step not only in the development of the insect but also for its
351 associated microbiota. On the other hand, the RF carried out in order to classify the different insect
352 gut sections was able to successfully classify the foregut, midgut and hindgut samples in 80%, 82%
353 and 78% of the cases, respectively. These results indicate that the different gut sections as well as
354 larvae and adults have distinct microbial communities, whereas the pupal stage has not.
355 In order to further investigate the impact of the physico-chemical conditions inside the gut on
356 microbial composition, we measured pH, O_2 concentration and Redox potential in each gut section
357 for both male adults and L3 larvae (see Supplementary table S1; supplementary Fig. S4). These three

358 factors had a significant impact on the microbial composition. pH had a more significant impact on
359 the microbiota of larvae ($p < 0.01$) while O_2 concentrations and redox potential impacted more the
360 bacterial gut composition in adult gut regions (Fig 2B). Positive redox potential values were measured
361 in all the different gut compartments for both larvae and adults with the exception of larval hindgut
362 where a decrease in redox potential was reported, underlining the existence of reducing conditions in
363 this regions.

364 *3. Taxonomic composition of* P. japonica *gut microbiota*

365 The microbiota associated with different developmental stages of the host and with soil not only
366 differed in terms of bacterial richness and diversity, but also concerning bacterial community
367 composition (Fig 3; Fig. 2A). Even though Proteobacteria represented the most abundant phylum
368 considering all sample types (39.7%), followed by Firmicutes (24.9%) and Bacteroidetes (16.7%),
369 these proportions changed among the different sample types. Considering larvae (Fig 3B), the most
370 abundant phylum was Firmicutes with 44.3% followed by Proteobacteria (33.9%) and Actinobacteria
371 (11.5%). On the other hand, the main taxa in adults were Bacteroidetes (35.2%) followed by
372 Firmicutes (31.7%) then Proteobacteria (25.7%). It is noteworthy that the proportion of
373 Actinobacteria decreased when passing from soil to adults going from 24.2% to 6.4 while the
374 proportion of Bacteroidetes followed the opposite trend going from 8.4% in soil to 35.2% in adults
375 (Fig. 3A). Other bacterial taxa present at minor proportions (such as Acidobactria, Chloroflexi and
376 Nitrospira) followed a trend similar to Actinobacteria with their proportions decreasing from soil to
377 adults.

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

382 *4. Spatio-temporal changes in the microbiota taxonomic composition*

383 As mentioned above, 891 OTUs out of the 1,102 “core OTUs” present in the soil were not found in
384 the insect samples, while only 35 “core OTUs” were present in both insects and soil (Fig. 1B). These
385 OTUs belonged predominantly to the Proteobacteria phylum (26 out of the 35 OTUs) with
386 Rhizobiales being the most represented order (8 OTUs). In addition to these 35 OTUs, out of the 630
387 “core OTUs” found in insects but not in soil, 54 OTUs were shared between all the developmental
388 stages. Proteobacteria, Bacteroidetes and Firmicutes were the most abundant phyla (28, 10 and 9
389 OTUs, respectively). Noteworthy, OTUs belonging to the families Rickenellaceae (5 OTUs),
390 Lachnospiraceae (3 OTUs) and Ruminococcaceae (1 OTU) were among the OTUs shared between
391 the insect developmental stages. These families were identified as taxa specifically enriched in the
392 insect guts along the different developmental stages.
393 We next performed a TEA (Taxon Enrichment Analysis) to identify which bacterial families were
394 consistently enriched in insects compared to soil (Fig. 4). This analysis showed that among the
395 Firmicutes, the Ruminococcacae was significantly enriched in larvae compared to soil ($P < 0.001$)
396 but there were no differences when comparing the different developmental stages. Similarly, other
397 bacterial families belonging to the Firmicutes and specifically to the order Clostridiales (namely
398 Christensenellaceae and Lachnospiraceae) resulted to be significantly enriched in larvae and
399 generally in insects when compared to soil samples. These families were also enriched in the different
400 compartments of the gut when compared to soil ($P < 0.001$), independent of the insect developmental
401 stages. Other bacterial families, such as Rikenellaceae (Bacteroidetes) and Desulfovibrionaceae
402 (Proteobacteria), were also enriched in larvae compared to soil. These bacteria were also enriched in
403 other portions of the gut but not all of them. Desulfovibrionaceae was also enriched in the midgut and
404 hindgut while Rikenellaceae were only enriched in the hindgut.
405 It is noteworthy that the TEA did not evidence any significantly enriched taxonomic group between
406 the different developmental stages of the insect nor did it evidence enriched taxonomic group between
407 the different gut sections. This is partly supported by the fact that the nestedness component of the β-
408 diversity between the different insect developmental stages was relatively high (0.59 on average),

409 indicating that a higher fraction of the microbiotas is shared between the different insect
410 developmental stages than between insects and soil.
411 The Indval analysis carried out on the different developmental stages showed that 23 OTUs were
412 unique to larvae, five were associated only with pupae while 13 were specific to adults (See table S2a
413 for supporting information). Members of the Lachnospiraceae family were the most represented
414 OTUs among those unique to both larvae and adults (with nine and five OTUs present respectively).
415 The same analysis carried out on the different gut portions for each developmental stage gave a
416 different picture. For the pupal stage, there was no OTU specific to a given gut section. As for adults
417 15 OTUs were found only in the foregut, while 5 OTUs were specific to the hindgut. No OTU was
418 found to be unique to the midgut. On the other hand, in the larvae, only two OTUs were found to be
419 specific to the foregut while the midgut and hindgut had respectively 105 and 145 OTUs that were
420 specifically associated with them. It is noteworthy that three out of the five OTUs that were unique
421 to the adult hindgut were also found specifically associated to the larvae hindgut. These OTUs
422 belonged to the Rikenellaceae (denovo5575 and denovo143435) and Nitrosomonadaceae
423 (denovo213936) families.
424 *5. Phylogenetic relationship of Christensenellaceae associated with* P. japonica
425 Bacteria belonging to Christensenellaceae have previously been observed only in humans. To better
426 understand the phylogenetic relationships between members of the Christensenellaceae associated
427 with *P. japonica* and those associated with humans, we performed a Maximum Likelihood phylogeny
428 using our OTUs and 16S rRNA gene sequences from those isolated from humans (Figure S5). The
429 OTUs associated with the insect formed several clusters distinct from the cluster of human-associated
430 symbionts. Hence the bacteria associated with *P. japonica* belong to different taxonomic groups
431 within the Christensenellaceae family.
432
433 **Discussion**

434 In this study, we demonstrate that soil bacteria represent an important source for the gut microbiota
435 of *P. japonica* larvae, but as the insect develops, the gut bacterial community experiences important
436 changes in richness, diversity and composition. Specifically, 37% of the OTUs present in larvae
437 derived from the soil microbiota and 35 OTUs present in the soil were transmitted throughout all the
438 developmental stages of the insect. In addition, larvae had a higher OTU richness and diversity
439 compared to adults. This is likely linked to the different lifestyles of the two stages: larvae are soil-
440 dwelling and similar in OTU numbers to other soil-dwelling arthropods such as terrestrial isopods
441 (healthy isopods OTUs on average 209; Dittmer, Lesobre, Moumen, & Bouchon, 2016), termites
442 (number of OTUs consistently higher than 400; Su et al., 2016) and ants (number of OTUs about 400;
443 Vieira, Ramalho, Martins, Martins, & Bueno, 2017; Zhukova et al., 2017), while the OTU numbers
444 of adults are comparable to those of non-soil-dwelling insects (in 218 insect species, average OTUs
445 84; Yun et al., 2014). Pupae are an intermediate state between larvae and adults in terms of bacterial
446 taxonomic richness and diversity, representing a bottleneck for bacterial transmission due to
447 metamorphosis. Nonetheless, key bacterial taxa involved in plant material degradation are still
448 transmitted to adults (see below for a detailed discussion). Interestingly, the decrease of the
449 microbiota richness and diversity throughout the host developmental stages is accompanied by a shift
450 in the phylogenetic community structure. Specifically, larvae and pupae harbour phylogenetically
451 clustered bacterial communities, i.e. consisting of closely-related bacterial taxa. In contrast, the adult
452 microbiota is phylogenetically overdispersed, similarly to rhizospheric soil communities. The
453 observation that larvae microbiotas are phylogenetically clustered and at the same time taxonomically
454 rich compared to adults could be explained by a selection of certain taxonomic groups through the
455 gut environment, due to its specific physico-chemical properties and enzymatic activities, as well as
456 the insect immune system. The phylogenetic overdispersion of the adult gut microbiotas suggests that
457 the pupal stage represents a crucial bottleneck for the gut microbiota. This might be due to the random
458 survival of bacterial taxa present in the larvae throughout metamorphosis (and its associated gut tissue
459 restructuring) at the pupal stage. Other possible explanations might be that the adult gut microbiota

460 is renewed by feeding on leaves and flowers in contrast to rhizospheric soil and/or that the physico-
461 chemical properties of the adult gut are more stable than in larvae (see Fig. S4).
462 This study allowed us to identify the main drivers of microbiota composition in *P. japonica*.
463 Specifically, we demonstrate that gut section is the main factor shaping microbiota composition,
464 while insect developmental stages are secondary drivers. This importance of the gut section as driver
465 of microbiota diversity and composition is most likely due to: i) differences in the physico-chemical
466 conditions prevailing in each gut section (supplementary Fig S4) as well as; ii) biotic factors such as
467 host enzymatic potential and immune response. It is noteworthy that the pupae represent a transitional
468 stage with a reshuffling of the microbiota between the larval and adult stages. In other words, the
469 larvae and adult microbiotas formed clearly distinct clusters, while the pupae microbiota was more
470 dispersed between the larvae and adult clusters. This may have had an impact on the statistical
471 analyses, leading to an apparently weaker effect of the developmental stages on microbiota
472 composition.
473 Regarding the physico-chemical conditions, oxygen availability was the most influential factor
474 determining differences in bacterial community structure between the different gut sections in adults,
475 while intestinal pH was the most influential factor in larvae. Although both the midgut and hindgut
476 compartments are largely anoxic in adults, the oxygen concentration in the midgut showed a higher
477 degree of variation compared to the more anoxic hindgut. This is likely due to a considerably larger
478 influx of oxygen via the gut epithelium in the case of the midgut, as observed in *Pachnoda ephippiata*
479 (Lemke, Stingl, Egert, Friedrich, & Brune, 2003). This variability in oxygen availability between the
480 different gut compartments may favour bacteria that are more tolerant towards such fluctuations. In
481 larvae, the pH in the midgut and hindgut was alkaline, while the foregut had a neutral pH. It is
482 important to note that the larvae are soil-dwellers feeding on fresh roots and decaying soil organic
483 matter (SOM) (Fleming, 1972). In this regard, they are similar to other soil-dwelling
484 macroinvertebrates, including many coleopterans, which feed on SOM and play an important role in
485 its degradation and stabilization (Lavelle et al., 1997; Wolters, 2000). It has been shown that the

486 conditions in the anterior hindgut of the humivorous termite *Cubitermes* spp. (i.e. high alkalinity and
487 oxygen influx) lead to a decrease of the molecular weight of the organic matter (Kappler & Brune,
488 1999), rendering it more soluble and thus more accessible for digestion in subsequent less-alkaline
489 compartments (Ji & Brune, 2001; Ji, Kappler, & Brune, 2000; Kappler, Ji, & Brune, 2000). Although
490 the complex microbial communities in the guts of humivorous macroinvertebrates are thought to
491 participate in the transformation of ingested SOM (Cazemier, Hackstein, Camp, Rosenberg, & van
492 der Drift, 1997; Kane, 1997), detailed information on the composition and activities of the gut
493 microbiota is lacking. In view of the high midgut alkalinity in *P. japonica*, it is reasonable to assume
494 that at least some of the bacteria in the midgut are tolerant towards high pH conditions, since most
495 bacterial taxa are also found in the more neutral gut sections of adults.

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

504 In contrast, several taxa were found to be significantly enriched between soil and insect gut. Those
505 belonged mainly to the families Ruminococcacae, Christensenellaceae and Lachnospiraceae.
506 Members of these families are known to degrade cellulose (Biddle, Stewart, Blanchard, & Leschine,
507 2013; Flint, Scott, Duncan, Louis, & Forano, 2012). The fact of finding them enriched in the insect
508 gut may suggest a possible symbiotic relationship where these bacteria help their host degrade and
509 metabolise cellulose, as in the case of the symbiotic association between termites, protists and bacteria
510 (Liu et al., 2013) or woodlice and certain bacterial taxa (Bredon, Dittmer, Noël, Moumen, &
511 Bouchon, 2018). These bacteria could be important in helping their host metabolise plant roots and

512 leaves and might thus contribute to its success as a polyphagous invasive insect. The bacterial taxa
513 that were enriched in the gut of *P. japonica* have been previously reported in association with various
514 insects but more importantly with ruminants and humans. *Anaerostipes* spp., *Coprococcus* spp. and
515 *Dorea* spp. (members of the Lachnospiraceae family) have all been previously described in
516 association with the human gut (Rainey, 2009) where they are hypothesized to be involved in pectin
517 fermentation. Other members of the Lachnospiraceae family have also been described in association
518 with other insects (Bourguignon et al., 2018; Huang & Zhang, 2013). The Ruminococcaceae family,
519 represented by *Ruminococcus* spp. and *Oscillospira* spp. in *P. japonica*, has also been described in
520 association with humans, ruminants, coleopterans and termites (Bourguignon et al., 2018; Huang &
521 Zhang, 2013; Kamagata, 2011). *Ruminococcus*, in addition to *Bacteroides* spp., plays an important
522 role in the fermentation of hemicellulose and the degradation of different plant material through the
523 production of Carbohydrate-Active enZymes (CAZymes) (Jose, Appoothy, More, & Arun, 2017).
524 CAZymes are very important for the break-down of the different components of lignocellulose (i.e.
525 cellulose, lignin, hemicellulose; (Bredon et al., 2018). It is noteworthy that although some insects are
526 able to express some of these enzymes, most of them heavily rely on their associated microorganisms
527 to degrade lignocellulose (Bredon et al., 2018). On the other hand, the role of *Oscillospira* is still
528 unknown and it is hypothesized that it may be involved in lignocellulose degradation (Kamagata,
529 2011). Rickenellaceae, with the genus *Alistipes*, and Desulfovibrionaceae have also been described
530 in association with the guts of different animals (Koneru, Salinas, Flores, & Hong, 2016;
531 Ruengsomwong et al., 2016), especially termites (Makonde, Mwirichia, Osiemo, Boga, & Klenk,
532 2015; Reid, Addison, West, & Lloyd-Jones, 2014), where they play an important role in the
533 degradation of cellulose polymers (Ozbayram, Akyol, Ince, Karakoç, & Ince, 2018).
534 In contrast to the above-mentioned bacterial families which have been observed not only in mammals
535 but also in insects, the family Christensenallaceae had so far been observed exclusively in humans.
536 Although its role in the degradation of nutrients is not yet understood, members of this family (i.e.
537 *Christensenella minuta*) have been shown to play a central role in controlling the Body Mass Index

538 and in helping to shape a "healthy" microbiota in humans and transfected mice (Goodrich et al., 539 2014). Increased titers of *C. minuta* have also been correlated with longevity in humans (Biagi et al., 540 2016), while decreased titers were observed during different human diseases (Petrov et al., 2017; Yu 541 et al., 2017). In addition, other bacteria belonging to the genus *Christensenella* have been isolated 542 from diseased humans, although no causality has been established yet (Ndongo, Khelaifia, Fournier, 543 & Raoult, 2016). The partial 16S rRNA gene-based phylogeny showed that the Christensenellaceae 544 OTUs found in association with *P. japonica* do not cluster with the taxa associated with humans but 545 rather form different clusters, suggesting that they belong to different taxonomic groups within the 546 Christensenellaceae family (Fig. S5).

547 In conclusion, the gut microbiota of *P. japonica* is highly dynamic across the developmental stages 548 and influenced by physico-chemical properties of the gut. Nonetheless, 89 OTUs were maintained 549 from larvae to adults, including 35 OTUs originating from the soil environment. As a future 550 perspective, it would be interesting to investigate if these OTUs represent a stable core microbiota 551 present in all *P. japonica* populations in different parts of the world or if they are subject to change 552 in different environments. In the first case, this might indicate a more intimate symbiotic relationship 553 potentially maintained via vertical transmission. In the latter case, the variable microbiota would 554 provide a means to investigate the origin of new invasions of this beetle, via a comparative analysis 555 of the local soil and insect gut microbiotas.

556

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865

866 **Tables**

867 **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

Tables and figures

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

Figure 2: Non-metric multi-dimensional scaling analysis (NMDS) plots displaying sample β-diversity inferred from the OTU table. A: Biplot of the first 2 axes for the NMDS representing correlations between the OTUs abundance in all insect samples and ecological and ontological factors (i.e. developmental stage and gut section). B: NMDS plots showing the correlation between the bacterial OTUs of Adults and larvae and the different physico-chemical properties (pH, O2 concentration and RedOx potential) of the different gut regions (foregut, midgut and hindgut). The vectors represent the mean direction and strength of correlation of the different parameteres measured (p-value < 0.05). In both figures, shapes indicate the different developmental stages (i.e. square for larvae, triangle for pupae, circle for adults) while colors indicate the gut region (i.e. red for foregut, green for midgut, blue for hindgut).

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

Figure 4: Taxa Enrichment analysis (TEA) carried out on the different larval stages using soil as reference. The panels on the right-hand side are the ROC curves representing the rank of the different OTUs belonging to the families Lachnospiraceae, Christensenellaceae, Ruminococcaceae and the order Clostridiales in general.

Table S1: Summary of the different ecological indices and Random Forest results for each sample. 1a: Ecological indices summary for the different samples. 1b: Standardized phylogenetic evenness results for all the samples. 1c: Results of the Random Forest goodness of prediction for the developmental stages. 1d: Results of the Random Forest goodness of prediction for the gut section.

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

Figure S1: Alpha diversity parameters by sample or sample type. A: Chao1 index for all the samples. B: Chao1 index reported by gut section. C: Chao1 index reported by developmental stage. D: Shannon

903 index for all the samples. E: Shannon index reported by gut section. F: Shannon index reported by
904 developmental stage.
905 **Figure S2**: Biplot of the estimated standardized phylogenetic diversity (SES-MPD) and OTUs
906 richness of each community. The dashed grey line represents the linear regression, for the bacterial
907 communities associated with insect samples, of the SES-MPD onto the OTUs richness.
908 **Figure S3**: Heatmaps showing the relative pairwise nestedness and turnover values for the different
909 developmental stages and soil
910 **Figure S4**: Box-plots displaying the value ranges of the different physico-chemical properties
911 measured for the different gut sections for both adults and larvae. A: pH, B: Oxygen concentration;
912 C: RedOx potential.
913 **Figure S5**: Maximum likelihood phylogenetic tree based on the partial 16S rRNA gene sequences.
914 The blue circle indicates the Christensenellaceae group of bacteria associated with the human gut. All
915 other taxa were detected in the present study in association with P. japonica gut sections. The scale
916 bar at the bottom indicates the distance in nucleotide substitution per site. The alphanumeric sequence
917 at each node either the GeneBank accession number or the *de novo* OTUs.
918

919 **Author Contribution**: BC, MM, LM and PFR designed the experiments. BC performed the
920 microbiota and enrichment analyses. MM and NG performed the statistical analyses. GM, EG, FP,
921 LM and AA performed the sampling. NG dissected the insects and extracted the DNA. FF and FF
922 performed the sequencing. MC, EC and DD performed the physico-chemical analyses. BC and MM
923 wrote the manuscript. All the authors red and commented on the manuscript.