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 Ruminococcaceae, Christensenellaceae and Lachnospiraceae. Interestingly, the family Christensenellaceae 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, Ruminococcaceae, Christensenellaceae, Lachnospiraceae,
Introduction

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

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

Several factors can influence the gut microbiota structure and composition. Among these factors, the most important ones are diet and environment, but other factors (e.g., age) can also be at play (Anderson et al., 2018; Montagna, Chouaia, et al., 2015; Montagna, Gómez-Zurita, et al., 2015; Montagna et al., 2016; Sanders et al., 2017; Tiede, Scherber, Mutschler, McMahon, & Gratton, 2017; Vacchini et al., 2017; Wong, Ng, & Douglas, 2011). Although various factors can influence the insect gut microbiota, the existence of a shared core microbial community in some species indicates that at least some members of the gut microbiota can be vertically transmissible. Several studies have investigated this possibility by tracking the changes in gut microbiota composition along the developmental stages of different insect species. These studies showed that the transmission of the gut microbiota throughout the different developmental stages may depend on the usefulness of certain bacteria (Malacrinò, Campolo, Medina, & Palmeri, 2018; Zhukova et al., 2017). For instance, the bacterial communities of fruit flies (Tephritidae) change throughout the insect's developmental stages to respond to the physiological needs of the host (Aharon et al., 2013; Malacrinò et al., 2018).

In holometabolous insects, the pupal stage generally represents a bottleneck where most of the larval gut microbiota is lost and adult insects may have to resort to indirect ways (e.g. via environmental transmission) to insure the transfer of beneficial bacteria from larvae to adults (Zhukova et al., 2017). For instance, in certain bee species, certain bacterial taxa are not trans-stadially transmitted but re-acquired from the environment (McFrederick, Mueller, Wcislo, & Hout, 2014). While the gut microbiota is not constant across the developmental stages in most insects, in some cases the microbial community can be relatively stable throughout the developmental stages. This has been observed in some tephritid flies as well as in the Black Soldier Fly Hermetia illucens and in the moth Plodia interpunctella (De Smet, Wynants, Cos, & Van Campenhout, 2018; Mereghetti, Chouaia, Limonta, Locatelli, & Montagna, 2017; Yong, Song, Chua, & Lim, 2017).
In the present study, we focused on the highly polyphagous invasive Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae). This invasive insect is listed in the EPPO Annex 2 due to the damages caused to different crops and turfs (EPPO, 2000). Native to Japan and the far east of Russia (Fleming, 1972), this beetle became an established pest in North America in the early 1900's (Switzer, Enstrom, & Schoenick, 2009), in the Azores in the early 1970's (Vieira, 2008) and more recently in continental Europe, where it was recorded for the first time in Italy in 2014 (EPPO, 2014; Pavesi, 2014) and in Switzerland in 2017 (EPPO, 2017). Several laboratory and field trials have been carried out to limit the spread of this pest in mainland Europe and to evaluate the environmental resilience of the infested areas (e.g. Marianelli et al., 2018; Mazza et al., 2017; Paoli, Marianelli, Binazzi, et al., 2017; Paoli, Marianelli, Torrini, et al., 2017). The damages to plants are caused by the different developmental stages of the beetle: the larvae, being underground dwellers, feed on the plant roots and soil organic matter while adults, living in an above-ground environment, feed on leaves and floral parts of different plant species (Fleming, 1972; Vieira, 2008).

Insect-associated bacteria can potentially contribute to an insect’s invasive potential by helping their hosts to adapt to changing environmental conditions. 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 microbiota dynamics in an invasive population of *P. japonica* from Italy. Specifically, we addressed the following questions: i) Does *P. japonica* harbour a stable core microbiota or are the bacteria mainly acquired from the surrounding environment (i.e. rhizospheric soil exploited by larvae and pupae vs aerial environment exploited by adults)? ii) Is the gut microbiota maintained across the post-embryonic developmental stages (i.e. larvae, pupae and adults) or is there a major turnover due to insect development? iii) Do different gut micro-environments impact microbial community structure?

**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, Chouaia, et al., 2015). Individuals were dissected under sterile conditions, and gut removed in sterile Ringer solution. The insect alimentary canal was then separated in its three compartments (i.e. foregut, midgut and hindgut). A total of 90 individuals were dissected. For each developmental stages 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.

Additionally, male adults and L3 larvae were collected and immediately processed in order to measure physicochemical properties (pH level, redox potential, oxygen concentration) of different gut regions. Specimens were anesthetized 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 & Doyle, 1990) with the modifications described in Mereghetti and colleagues (Mereghetti et al., 2017). 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 bacteria 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. All the extracted DNAs
were used as template for PCR targeting the bacterial 16S rRNA gene using 27 Fmod and 519 Rmod primers (Lane, 1991). All samples except to the blank extraction resulted positive.

The extracted DNA were used as template for the amplification of V4 hypervariable region of the 16S rRNA by PCR primers 515F (Caporaso et al., 2011), 802R (Claesson et al., 2009) and 806R (Caporaso et al., 2011) tailed with two different GC rich sequences enabling barcoding with a second amplification. Each sample was amplified in 20 µl volume reaction containing 8 µl HotMasterMix 5 Prime 2.5X (Quanta Bio), 0.4 µl BSA (20 µg/µl) (Sigma-Aldrich), 1 µl EvaGreen™ 20X (Biotium), 0.8µl 515 F (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.

After labeling 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.

The obtained reads were analyzed 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 Operational Taxonomic Units (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/) 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.
The sequences obtained in this study have been deposited in the European Nucleotide Archive and are waiting for the assignment of an accession number.

**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/](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, developmental stages) using two-sample t-tests with 999 Monte Carlo permutations.

In order to evaluate if 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 (Mouquet et al., 2012; O’Dwyer, Kembel, & Green, 2012; Webb, Ackerly, McPeek, & Donoghue, 2002), 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 Rpackage *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, grouping the values according to the sample type (i.e. soil, larvae, pupae, adults), and the differences among sample types were assessed using the non-parametric Kruskal–Wallis test.
(Kruskal & Wallis, 1952) after assessing the homogeneity of variances among the groups using the Levene test (Levene, 1960).

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 ($\beta_{\text{SOR}}$; (Baselga, 2010) implemented in the R package *betapart* (Baselga & Orme, 2012). The turnover and nestedness components of this $\beta$-diversity were calculated using Simpson-based multiple-site dissimilarity ($\beta_{\text{SIM}}$; (Baselga, 2010) and nestedness-resultant multiple-site dissimilarity ($\beta_{\text{NES}}$; (Baselga, 2010), respectively. In addition, for each $\beta$-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 & 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 nonparametric one-way analysis of similarity ANOSIM (Clarke, 1993) (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 non-independence of the observations; (Bray & Curtis, 1957).

The sub-sampled OTU table, after the removal of soil community samples, was used as input for a Nonmetric Multi-Dimensional Scaling (NMDS; (Kruskal, 1964) biplot based on the Bray–Curtis dissimilarity (Bray & 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 Random Forest regressors based on the relative abundance OTU table (Knights, Costello, & Knight, 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, 2 out of 3 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, Gruber, & Fründ, 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 & 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 log$_2$ 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; (Weiner 3rd & Domaszewska, 2016) 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 ROC curves (Receiver Operating Characteristic curves).

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, Stecher, Li, Knyaz, & Tamura, 2018).

In order to detect OTUs that are specific for a given gut section within the same developmental stage, the indicator value (Dufrêne & Legendre, 1997) was calculated using the R package indicspecies (De Cáceres & 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, Stingl, & Brune, 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, Aarhus, Denmark), 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
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 (Ri > 1014 Ω). Unisense microsensor multimeter allowed to measure the current and data were recorded by using SensorTracePRO software (Unisense, Aarhus, Denmark). Microsensors were positioned using a motorized micromanipulator (Unisense, Aarhus, Denmark). Measurements were carried out at room temperature.

Results

1. Alpha, beta and phylogenetic diversity of the gut microbiota

A total of 5175086 high-quality reads longer than 250 bp were kept after quality filtering and chimera removal. These reads clustered into 1612 OTUs. On average, 67299 high-quality reads grouped into 336 OTUs were obtained from larvae, 80249 reads/204 OTUs from pupae, 88397 reads/99 OTUs from adults and 148324 reads/1093 OTUs from soil samples (see Table S1a, Supporting Information, for details). Rarefaction curves of the observed OTU richness in 25,000 sub-sampled sequences showed that our sequencing effort was sufficient to capture the major part of the bacterial diversity associated with the different developmental stages. On the other hand, a higher sequencing depth would probably have added new taxa to the bacterial communities present in soil (Fig. S1). OTU richness and diversity (Fig. S1), as determined by the species richness estimator Chao1 and the Shannon Index of diversity, were higher in soil samples than in insect samples (Chao1: all t-tests P < 0.01; Shannon: all t-test P < 0.01; Table1). Regarding the different developmental stages of *P. japonica*, OTU richness and diversity were the highest in the larvae (Chao1: all t-tests P < 0.01; Shannon: all t-tests P < 0.01, see supplementary Table1 and Table S1a for all ecological indices). On the other hand, these indices were the lowest for adults (Chao1: all t-tests P < 0.01; Shannon: all t-tests P < 0.01; Table1). The different larval instars had similar richness and diversity (Chao1: all t-tests P = 1; Shannon: all t-tests P =1). Noteworthy, the values of Pielou’s evenness also followed a
similar pattern with the soil having the highest value (Pielou’J = 0.84; Table1), then larvae (Pielou’J = 0.67; Table1) and with pupae and adults having similar values (Pielou’J = 0.47 and 0.49 respectively; Table1).

The standardized effect size of mean pairwise distance values (SES_MPD) of the bacterial communities associated with the samples ranged from positive values for soil bacterial communities (median value of SES_MPD_SOIL = 0.78 associated with high quantiles, Table S1b) to negative values for bacterial communities associated with the larval and pupal stages (median values SES_MPD_LARVAE = -3.38 and SES_MPD_PUPAE = -3.9, low quantile values, Table S1b) (Fig. 1C).

The positive SES_MPD values for the soil communities indicate a phylogenetic overdispersion, as expected for communities characterized by high species richness and evenness such as those of soil. In contrast, the negative SES_MPD values for the bacterial communities associated with larvae and pupae indicate a phylogenetic clustering of these communities, possibly due to the selection towards certain closely-related bacterial lineages by the insect gut environment, characterized by peculiar features such as by high enzymatic activities and host immune response. Interestingly, the bacterial communities associated with adults were characterized by slightly negative SES_MPD values (median value of SES_MPD_ADULTS = -0.53; Table S1b), indicating a phylogenetic evenness of these communities (Fig. 1C). This increasing trend of SES_MPD values from larvae and pupae (negative values) towards adults (slightly negative values) contrasted with the trend of decreasing community species richness from larvae to adults (Fig. S2).

2. Factors affecting gut microbiota composition

Soil was different from the insect samples in terms of bacterial composition (adonis: P < 0.001, R² = 0.33; anosim: P < 0.001, R = 0.54) with few OTUs shared between soil and the different insect developmental stages (Fig. 1A). Specifically, 891 OTUs out of the 1102 “core OTUs” of the soil were not found in the insect samples (Fig. 1B). On the other hand, only 35 “core OTUs” were present in both insects and soil (Fig. 1B). Moreover, the nestedness component of the β-diversity between soil and the different insect developmental stage was very low (0.16 on average) and the turnover was...
high (0.84 on average) (Fig. S3), indicating that very few “core OTUs” were shared between soil and insect microbiotas while the variable fraction was high.

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

The main factor driving this segregation was the gut section (P < 0.001) and to a lesser extent the developmental stage. These results were further supported by the Random Forest (RF) analysis which was carried out to investigate the specificity of the microbiota of each sample category by trying to assign each sample to its respective category based on its microbiota. The RF analysis (Supplementary Table S1c) carried out in order to classify the different developmental stages was able to successfully classify adults and larvae in 100% and 91.7% of the cases, respectively. Conversely, pupae were successfully identified in only 55.6% of the cases. These results suggest that the pupal stage represents a transitional step not only in the development of the insect but also for its associated microbiota. On the other hand, the RF carried out in order to classify the different insect gut sections was able to successfully classify the foregut, midgut and hindgut samples in 80%, 82% and 78% of the cases, respectively. These results indicate that the different gut sections as well as larvae and adults have distinct microbial communities, whereas the pupal stage has not.

In order to further investigate the impact of the physico-chemical conditions inside the gut on microbial composition, we measured pH, O2 concentration and Redox potential in each gut section for both male adults and L3 larvae (see Supplementary table S1; supplementary Fig. S4). These three
factors had a significant impact on the microbial composition. pH had a more significant impact on the microbiota of larvae (p < 0.01) while O₂ concentrations and redox potential impacted more the bacterial gut composition in adult gut regions (Fig 2B). Positive redox potential values were measured in all the different gut compartments for both larvae and adults with the exception of larval hindgut where a decrease in redox potential was reported, underlining the existence of reducing conditions in this regions.

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

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

4. Spatio-temporal changes in the microbiota taxonomic composition
As mentioned above, 891 OTUs out of the 1,102 “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 out of the 35 OTUs) with Rhizobiales being the most represented order (8 OTUs). In addition to these 35 OTUs, out 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 (5 OTUs), Lachnospiraceae (3 OTUs) and Ruminococcaceae (1 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 TEA (Taxon Enrichment Analysis) 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 to soil samples. These families were also enriched in the different compartments of the gut when compared to 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 was also enriched in the midgut and hindgut while Rikenellaceae were only enriched in the hindgut.

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.

The Indval analysis carried out on the different developmental stages showed that 23 OTUs were unique to larvae, five were associated only with pupae while 13 were specific to adults (See table S2a for supporting information). Members of the Lachnospiraceae family were the most represented OTUs among those unique to both larvae and adults (with nine and five OTUs present respectively).

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

5. Phylogenetic relationship of Christensenellaceae associated with P. japonica

Bacteria belonging to Christensenellaceae have previously been observed only in humans. To better understand the phylogenetic relationships between members of the Christensenellaceae associated with P. japonica and those associated with humans, we performed a Maximum Likelihood phylogeny using our OTUs and 16S rRNA gene sequences from those isolated from humans (Figure S5). The OTUs associated with the insect formed several clusters distinct from the cluster of human-associated symbionts. Hence the bacteria associated with P. japonica belong to different taxonomic groups within the Christensenellaceae family.

Discussion
In this study, we demonstrate that soil bacteria represent an important source for the gut microbiota of *P. japonica* larvae, but as the insect develops, the gut bacterial community experiences important changes in richness, diversity and composition. Specifically, 37% of the OTUs present in larvae derived from the soil microbiota and 35 OTUs present in the soil were transmitted 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, Lesobre, Moumen, & Bouchon, 2016), termites (number of OTUs consistently higher than 400; Su et al., 2016) and ants (number of OTUs about 400; Vieira, Ramalho, Martins, Martins, & Bueno, 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). Interestingly, the decrease of the 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, due to its specific physico-chemical properties and enzymatic activities, as well as the insect immune system. The phylogenetic overdispersion of the adult gut microbiotas suggests that the pupal stage represents a crucial bottleneck for the gut microbiota. 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. Other possible explanations 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 Fig. S4).

This study allowed us to identify the main drivers of microbiota composition in *P. japonica*. Specifically, we demonstrate that gut section is the main factor shaping microbiota composition, while insect developmental stages are secondary drivers. This importance of the gut section as driver of microbiota diversity and composition is most likely due to: i) differences in the physico-chemical conditions prevailing in each gut section (supplementary Fig S4) 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 microorganisms 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 conditions, oxygen availability was the most influential factor determining differences in bacterial community structure between the different gut sections in adults, while intestinal pH was the most influential factor in larvae. Although both the midgut and hindgut compartments are 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, Stingl, Egert, Friedrich, & Brune, 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 & Brune, 1999), rendering it more soluble and thus more accessible for digestion in subsequent less-alkaline compartments (Ji & Brune, 2001; Ji, Kappler, & Brune, 2000; Kappler, Ji, & Brune, 2000). Although the complex microbial communities in the guts of humivorous macroinvertebrates are thought to participate in the transformation of ingested SOM (Cazemier, Hackstein, Camp, Rosenberg, & van der Drift, 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, since 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, 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 (Biddle, Stewart, Blanchard, & Leschine, 2013; Flint, Scott, Duncan, Louis, & Forano, 2012). The fact of finding them enriched in the insect gut may suggest a possible symbiotic relationship where these bacteria help their host degrade and metabolise 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, Dittmer, Noël, Moumen, & Bouchon, 2018). These bacteria could be important in helping their host metabolise 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 (Bourguignon et al., 2018; Huang & Zhang, 2013). 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 (Bourguignon et al., 2018; Huang & Zhang, 2013; Kamagata, 2011). *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, Appoothy, More, & Arun, 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). Rikenellaceae, with the genus *Alistipes*, and Desulfovibrionaceae have also been described in association with the guts of different animals (Koneru, Salinas, Flores, & Hong, 2016; Ruengsomwong et al., 2016), especially termites (Makonde, Mwirichia, Osiemo, Boga, & Klenk, 2015; Reid, Addison, West, & Lloyd-Jones, 2014), where they play an important role in the degradation of cellulose polymers (Ozbayram, Akyol, Ince, Karakoç, & Ince, 2018).

In contrast to the above-mentioned bacterial families which have been observed not only in mammals but also in insects, the family Christensenallaceae 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 transfected 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, Khelaifia, Fournier, & Raoult, 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 (Fig. S5).

In conclusion, the gut microbiota of *P. japonica* is highly dynamic across the developmental stages and influenced by physico-chemical properties of the gut. Nonetheless, 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.

**References**


Kim, J. K., Lee, J. B., Jang, H. A., Han, Y. S., Fukatsu, T., & Lee, B. L. (2016). Understanding regulation of the host-mediated gut symbiont population and the symbiont-mediated host immunity in the Riptortus-
Burkholderia symbiosis system. Developmental & Comparative Immunology, 64, 75–81. doi:https://doi.org/10.1016/j.dci.2016.01.005


**Tables**

**Table 1:** Ecological indices by developmental stage (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>Richness (Chao1)</th>
<th>Diversity (Shannon)</th>
<th>Evenness (Pielou)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>1099 ± 1.35</td>
<td>5.88 ± 0.03</td>
<td>0.84 ± 0.00</td>
</tr>
<tr>
<td>Larvae</td>
<td>369.93 ± 28.95</td>
<td>3.77 ± 0.19</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>Pupae</td>
<td>241.12 ± 43.51</td>
<td>2.49 ± 0.39</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>Adults</td>
<td>129.65 ± 7.33</td>
<td>2.22 ± 0.18</td>
<td>0.49 ± 0.04</td>
</tr>
</tbody>
</table>
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 parameters 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 summary 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
index for all the samples. E: Shannon index reported by gut section. F: Shannon index reported by developmental stage.

**Figure S2**: Biplot of the estimated standardized phylogenetic diversity (SES-MPD) and OTUs richness of each community. The dashed grey line represents the linear regression, for the bacterial communities associated with insect samples, of the SES-MPD onto the OTUs richness.

**Figure S3**: Heatmaps showing the relative pairwise nestedness and turnover values for the different developmental stages and soil

**Figure S4**: Box-plots displaying the value ranges of the different physico-chemical properties measured for the different gut sections for both adults and larvae. A: pH, B: Oxygen concentration; C: RedOx potential.

**Figure S5**: Maximum likelihood phylogenetic tree based on the partial 16S rRNA gene sequences. The blue circle indicates the Christensenellaceae group of bacteria associated with the human gut. All other taxa were detected in the present study in association with P. japonica gut sections. The scale bar at the bottom indicates the distance in nucleotide substitution per site. The alphanumeric sequence at each node either the GeneBank accession number or the *de novo* OTUs.
Author Contribution: BC, MM, LM and PFR designed the experiments. BC performed the microbiota and enrichment analyses. MM and NG performed the statistical analyses. GM, EG, FP, LM and AA performed the sampling. NG dissected the insects and extracted the DNA. FF and FF performed the sequencing. MC, EC and DD performed the physico-chemical analyses. BC and MM wrote the manuscript. All the authors red and commented on the manuscript.