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

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Summary

***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 harboured 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 Christensenallaceae had so far been observed exclusively in humans. However, the Christensenellaceae operational taxonomic units found in *P. japonica* belong to different taxonomic clades within this family.**

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Introduction

Insects are the most diverse and abundant animal clade (Foottit and 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. Douglas, 2014; Corbin *et al*., 2017; Sudakaran *et al*., 2017; Heddi and Zaidman-Rémy, 2018). 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 *et al*., 2017; Muhammad *et al*., 2017).

As for essentially all animals, microbial communities are particularly prominent in the digestive tract (e.g., Douglas, 2015; 2018; Clayton *et al*., 2018; Münger *et al*., 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 *et al*., 2017), insects had to develop original ways in order to transmit the important components of their gut microbiota (Fukatsu and Hosokawa, 2002; Gonella *et al*., 2012; Hosokawa *et al*., 2013; Mason *et al*., 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 (Roh *et al*., 2008; Kim *et al*., 2016; Daisley *et al*., 2018; Obata *et al*., 2018).

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 (Wong *et al*., 2011; Montagna *et al*., 2015a; 2015b; Montagna *et al*., 2016; Sanders *et al*., 2017; Tiede *et al*., 2017; Vacchini *et al*., 2017; Anderson *et al*., 2018). Although various factors can influence the insect gut microbiota, the existence of a shared core microbial community in some species could indicate that there are mechanisms (e.g. vertical transmission) favouring the presence of certain members of the gut microbiota. 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 (Zhukova *et al*., 2017; Malacrinò *et al*., 2018). For instance, the bacterial communities of fruit flies (Tephritideae) 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 *et al*., 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* (Mereghetti *et al*., 2019; Yong *et al*., 2017; De Smet *et al*., 2018).

In the present study, we focused on the highly polyphagous invasive Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae, Supporting Information Fig. S1a). 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 *et al*., 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 (Mazza *et al*., 2017; Paoli *et al*., 2017a, 2017b; Marianelli *et al*., 2018a, 2018b). 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?

Results

Alpha, beta and phylogenetic diversity of the gut microbiota

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

Table 1. 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

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, Supporting Information Table S1c) 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, Supporting Information Table S1c) (Fig. 1C). SES_MDP values were significantly different between sample types (one-way ANOVA, F = 36.75, df1 = 3, df2 = 21.4, $p < 0.001$), namely between larvae and soil (Tamhane post hoc test, $p < 0.001$) and between larvae and adults (Tamhane post hoc test, $p = 0.001$). 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 toward certain closely related bacterial lineages by the insect gut environment or to the adaptation of these bacteria to the gut environment. Interestingly, the bacterial communities associated with adults were characterized by slightly negative SES_MPD values (median value of $SES\_MPD_{ADULTS} = -0.53$; Supporting Information Table S1c), indicating a phylogenetic evenness of these communities (Fig. 1C). This increasing trend of SES_MPD values from larvae and pupae (negative values) toward adults (slightly negative values) contrasted with the trend of decreasing community species richness from larvae to adults (Supporting Information Fig. S3).

Factors affecting gut microbiota composition

Soil was different from the insect samples in terms of bacterial composition (adonis: $p < 0.001$, $R^2 = 0.33$; ANOISM: $p < 0.001$, $R = 0.54$) 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

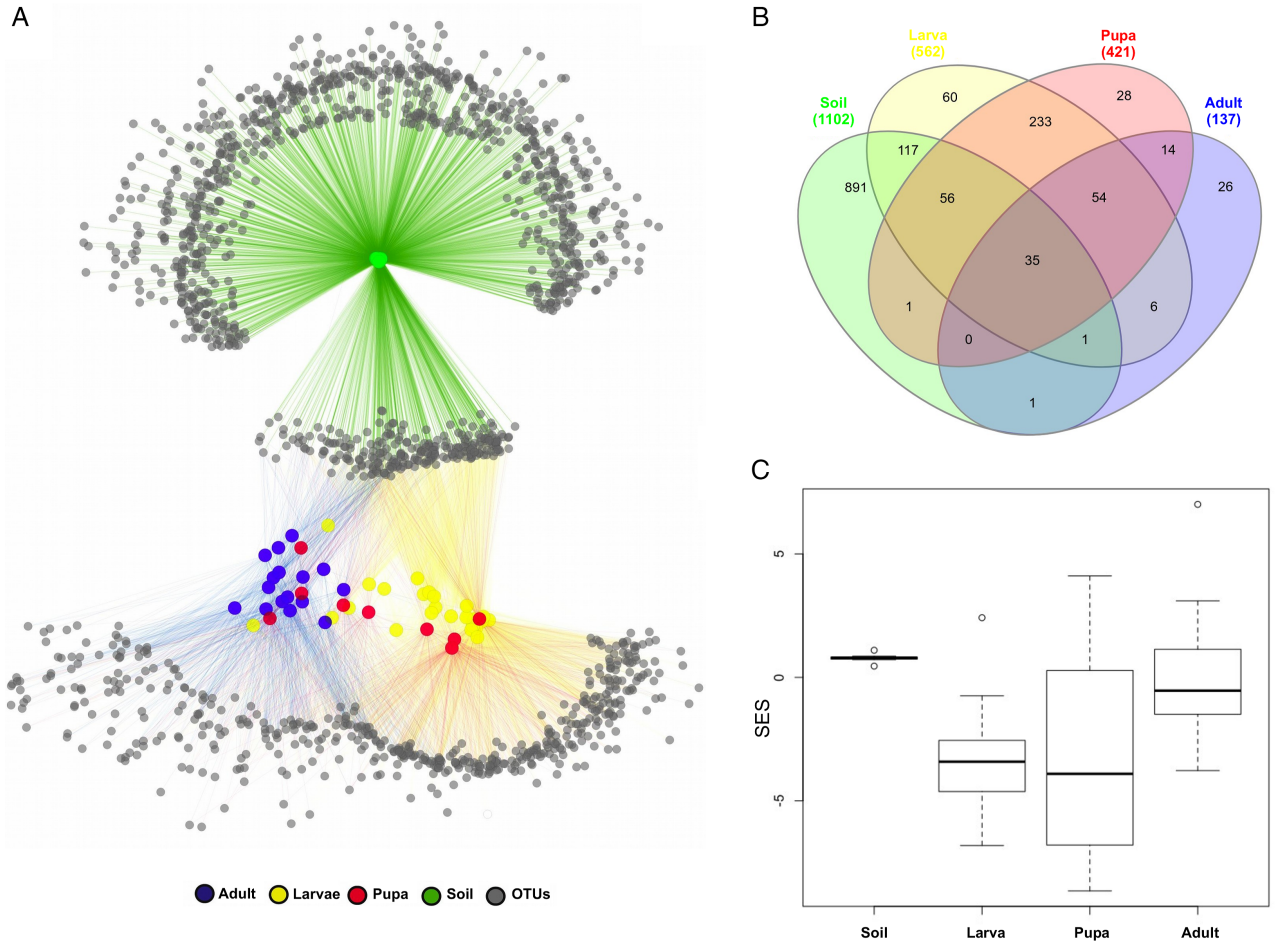

Fig. 1. OTU distribution among the different samples. A. Bacterial community network connecting OTUs (grey circles) to the samples (coloured circles) in which they were observed. B. Venn diagram showing the shared/specific bacterial OTUs (at 97% similarity) between the different developmental stages and soil. C. Box-plots of the estimated standardized phylogenetic diversity (SES_MPD) in the bacterial communities of rhizospheric soil and *P. japonica* developmental stages. [Color figure can be viewed at wileyonlinelibrary.com]

(Fig. 1B). On the other hand, only 35 'core OTUs' present in soil were also present in all the insect developmental stages (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) (Supporting Information Fig. S4), 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 non-metric multidimensional scaling (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.

Based on the correlations of the tested factors (i.e. developmental stages and gut sections) with the NMDS ordinations of the insect-associated bacterial communities, the main factor driving this segregation was the gut section (R^2 = 0.18, p = 0.003) 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 (Supporting Information Table S1d) was able to successfully classify adults and larvae in 100% and 91.7% of the cases, respectively. Conversely, pupae were successfully

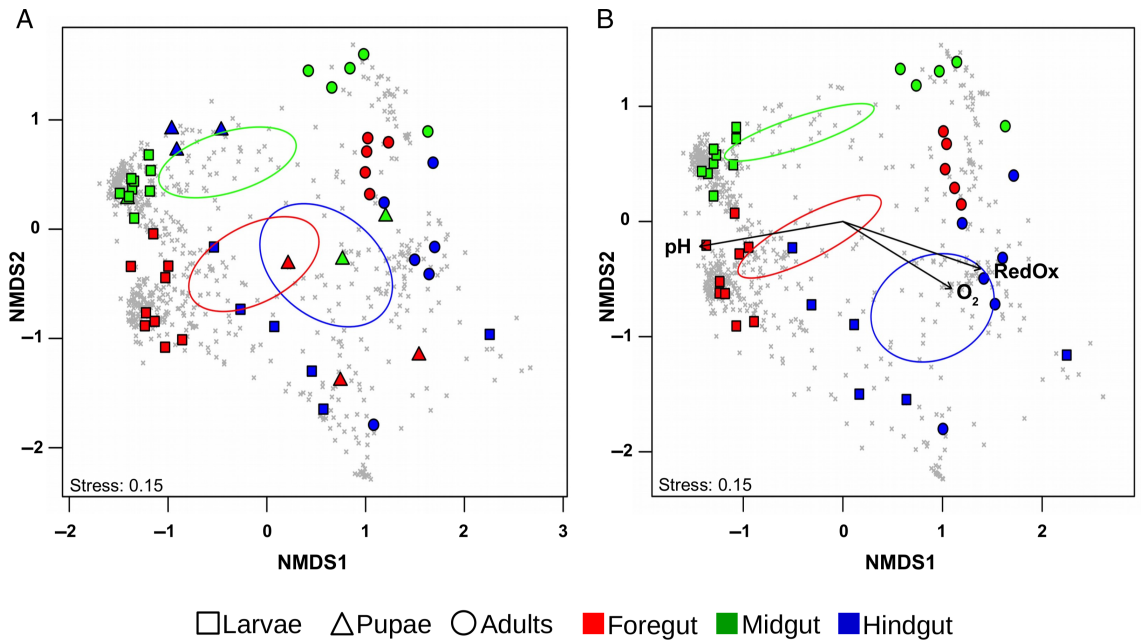

Fig. 2. NMDS analysis plots displaying sample β-diversity inferred from the OTU table. A. Biplot of the first two axes for the NMDS representing correlations between the OTUs abundance in all insect samples and ecological and ontological factors (i.e. developmental stage and gut section). B. NMDS plots 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 ($p < 0.05$). In both figures, shapes indicate the different developmental stages (i.e. square for larvae, triangle for pupae, circle for adults) while colours indicate the gut region (i.e. red for foregut, green for midgut, blue for hindgut). [Color figure can be viewed at wileyonlinelibrary.com]

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. The most important OTUs discriminating between the different developmental stages belonged to the Firmicutes (Clostridiales and Bacilli), Proteobacteria (Alphaproteobacteria) and Actinobacteria (see Supporting Information Table S1f). On the other hand, the RF was able to successfully classify the foregut, midgut and hindgut samples in 80%, 82% and 78% of the cases, respectively (Supporting Information Table S1e). The most relevant OTUs allowing to discriminate between the different gut sections were identified as Firmicutes (Clostridiales) and Proteobacteria (Betaproteobacteria). 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 correlation between the physico-chemical conditions of the gut and microbial composition, we measured pH, O_2 concentration and redox potential in each gut section for both male adults and L3 larvae (see Supporting Information Table S1 and Fig. S5). While the adult gut constituted a niche with a neutral pH (or at most slightly sub-acidic conditions), the pH in the larval gut increased from neutral in the foregut to alkaline conditions in the midgut and hindgut. Both larval and adult digestive systems were characterized by anoxic conditions, with the exception of the the adult foregut where conditions fluctuated from anoxia to microaerophilia. Finally, positive redox potential values were measured in all gut compartments of both larvae and adults, with the exception of the larval hindgut where a decrease in redox potential was measured, underlining the existence of reducing conditions in this region. These three factors were significantly correlated with the microbial composition in the different gut sections. Notably, pH was significantly correlated with the microbiota of larvae ($R^2 = 0.75$, $p = 0.001$), while O_2 concentrations ($R^2 = 0.54$, $p = 0.002$) and redox potential ($R^2 = 0.74$, $p = 0.001$) correlated significantly with the bacterial composition in adult gut regions (Fig. 2B).

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, Supporting Information Fig. S6). Although Proteobacteria represented the most abundant phylum considering all sample types (35.9% ± SE 4.2%), followed by Firmicutes (32.9% ± SE 5.4%) and Bacteroidetes (15.4% ± SE 3.7%), these proportions changed among the different sample types. Considering larvae (Fig. 3B, Supporting Information Fig. S6), the most abundant phylum was Firmicutes with an average of 49.5% ± SE 7.9% (range 26.5% ± SE 5.5% in L2

larvae to 74.5% ± SE 8.7% in L1 larvae), followed by Proteobacteria (31.3% ± SE 5.8% on average; range: 13.9% ± SE 5.1% in L1 larvae to 50.3% ± SE 5.9% in L2 larvae) and Actinobacteria (9.4% ± SE 2.6% on average; range 5% ± SE 2.5% in L1 larvae to 13.9% ± SE 4% in L3 larvae). On the other hand, the most abundant taxa in adults were Bacteroidetes (33.7% ± SE 7.8% on average; 39% ± SE 10.6% in females, 28.3% ± SE 12.9% in males) followed by Firmicutes (29.6% on average; 14.5% ± SE 1.5% in females, 44.8% ± SE 4.1% in males) then Proteobacteria (29.1% on average; 40% ± SE 12.6% in females, 18.2% ± 6.6% SE in males). In pupae, the most abundant phylum was Proteobacteria with 59.7% ± SE 11.5%, followed by Bacteroidetes (19.1% ± SE 9.2%) and Firmicutes (15.4% ± SE 9.9%). It is noteworthy that the proportion of Actinobacteria decreased when passing from soil to adults (going from 24.8% ± SE 1.5% in soil to 6.4% ± SE 1.9% in adults), while the proportion of Bacteroidetes followed the opposite trend, going from 8% ± SE 1.2% in soil to 33.7% ± SE 7.9% 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%.

Spatio-temporal changes in the microbiota taxonomic composition

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

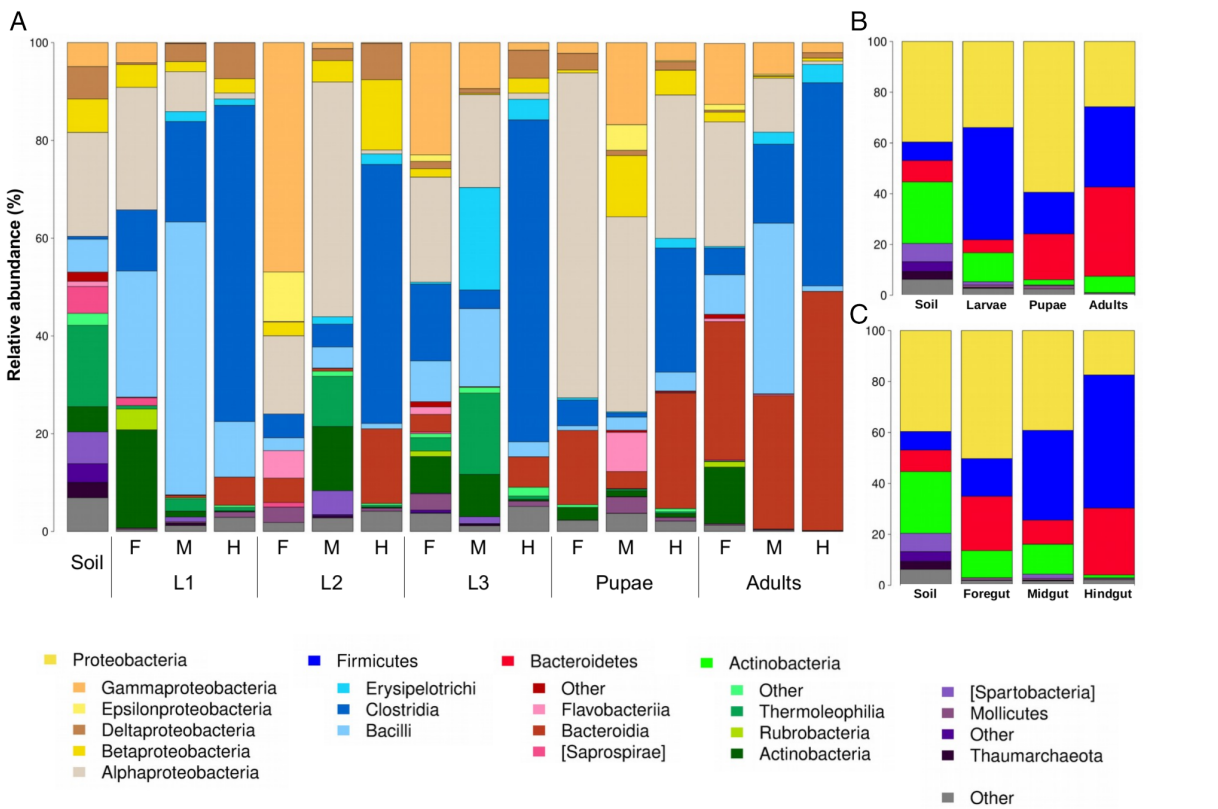

Fig. 3. Histograms summarizing the bacterial composition at different taxonomic levels. The different histograms report only taxa with a relative abundance ≥3%. A. The taxa summary at the order level for the different samples grouped by category. F indicates foregut, M indicates midgut and H indicates 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). [Color figure can be viewed at wileyonlinelibrary.com]

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

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

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

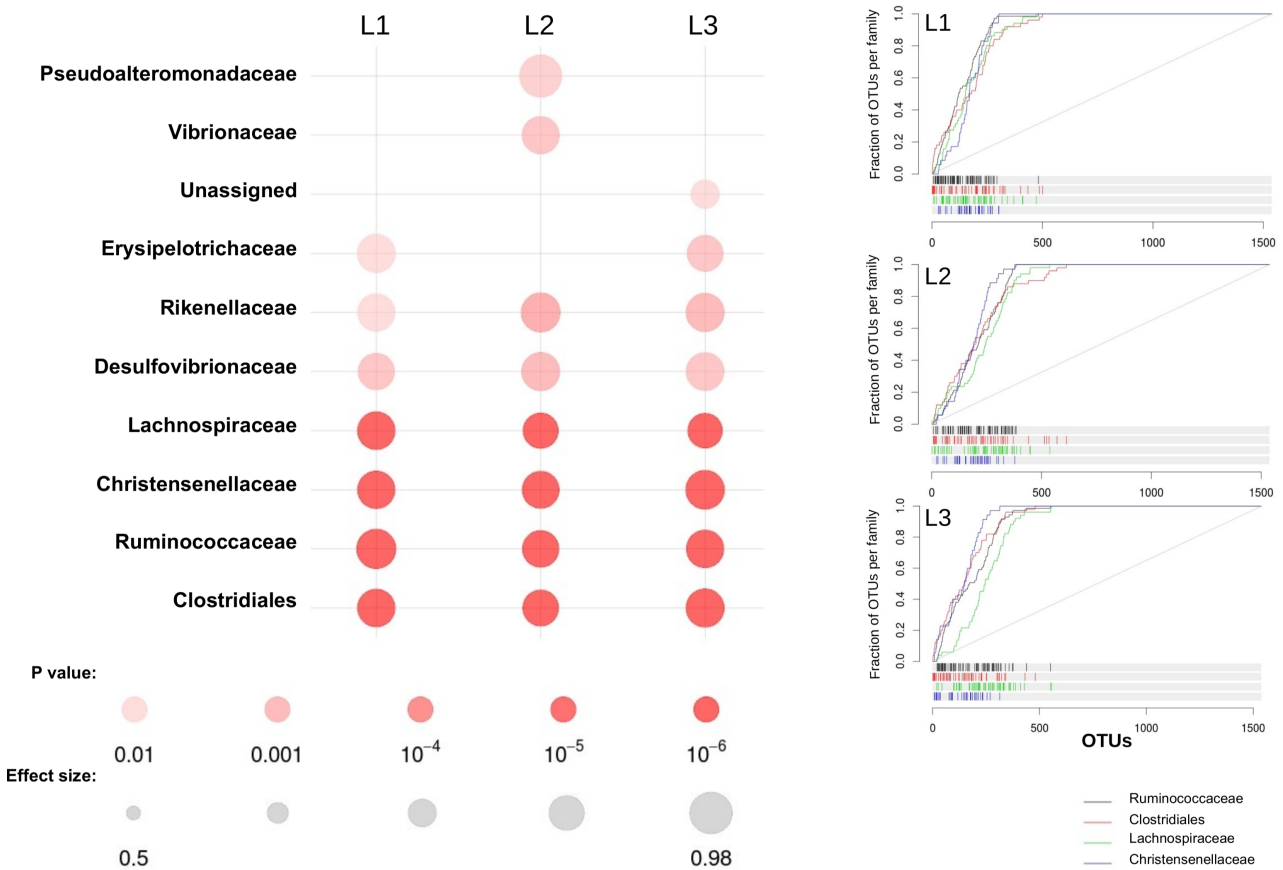

Fig. 4. TEA carried out on the different larval stages using soil as reference. The main figure indicates the families that were enriched in the different larval stages compared to soil. The colour intensity of the circles indicates the *p* value while its size indicates the effect size. The panels on the right-hand side are the ROC curves, plotting the ranked OTUs belonging to the enriches families against the totality of the ranked OTUs, represent the rank of the different OTUs belonging to the families Lachnospiraceae (green), Christensenellaceae (blue), Ruminococcaceae (black) and the order Clostridiales (red) in general. [Color figure can be viewed at wileyonlinelibrary.com]

An Indval analysis carried out to identify OTUs specific to a given developmental stage 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 sections for each developmental stage gave a different picture. For the pupal stage, there was no OTU specific to a given gut section. 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 specific to the foregut, while the midgut and hindgut had, respectively, 105 and 145 specific OTUs. It is noteworthy that three out of the five OTUs that were unique to the adult hindgut were also found specifically associated with the larvae hindgut. These OTUs belonged to the Rikenellaceae (denovo5575 and denovo143435) and Nitrosomonadaceae (denovo213936) families.

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 (Supporting Information Fig. S7). 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 (209 OTUs) present in larvae derived from the soil microbiota and 35 OTUs present in the soil were maintained throughout all the developmental stages of the insect. In addition, larvae had a higher OTU richness and diversity compared to adults. This is likely linked to the different lifestyles of the two stages: larvae are soil-dwelling and similar in OTU numbers to other soil-dwelling arthropods such as terrestrial isopods (healthy isopods OTUs on average 209; Dittmer *et al*., 2016), termites (number of OTUs consistently higher than 400; Su *et al*., 2016) and ants (number of OTUs about 400; Vieira *et al*., 2017; Zhukova *et al*., 2017), while the OTU numbers of adults are comparable to those of non–soil-dwelling insects (in 218 insect species, average OTUs 84; Yun *et al*., 2014). Pupae are an intermediate state between larvae and adults in terms of bacterial taxonomic richness and diversity, representing a bottleneck for bacterial transmission due to metamorphosis. Nonetheless, key bacterial taxa involved in plant material degradation are still transmitted to adults (see below for a detailed discussion). This reduction in both richness and diversity at the pupal stage could be due to a combination of factors both random and deterministic. On the one hand, a reduction of the number of bacterial cells during metamorphosis could have caused a random reduction in the diversity of the microbiota. On the other hand, the observed reduction in microbiota diversity throughout host development could be caused by one (or several) active mechanisms, such as (i) the change of nutrition (or lifestyle) between soil-dwelling larvae and adults, (ii) specific physico-chemical properties (e.g. the change in gut pH between larvae and adults), and/or (iii) enzymatic activities, among others. As a matter of fact, the observed changes (i.e. decrease in richness and diversity) are not a constant in insect development and other studies monitoring gut microbiota changes throughout development have shown different trends, such as an increase in species richness (Brucker and Bordenstein, 2012) or more generally the absence of a clear trend (Oliveira *et al*., 2018; Gao *et al*., 2019; Huang *et al*., 2019). The trend that we observe in *P. japonica* could be explained by its ecology, because soil dwelling arthropods such as termites and woodlice consistently present higher microbiota richness and diversity (Dittmer *et al*., 2016; Su *et al*., 2016; Vieira *et al*., 2017; Zhukova *et al*., 2017) due to their proximity to a microbially rich and diverse environment (i.e. soil). On the other, arthropods living in 'aerial' ecosystems (i.e. plants and leaves) tend to have a less rich and diverse gut microbiota (Yun *et al*., 2014; Mereghetti *et al*., 2019).

Interestingly, the decrease in microbiota richness and diversity throughout the host developmental stages is accompanied by a shift in the phylogenetic community structure. Specifically, larvae and pupae harbour phylogenetically clustered bacterial communities, i.e. consisting of closely related bacterial taxa. In contrast, the adult microbiota is phylogenetically overdispersed, similarly to rhizospheric soil communities. The observation that larvae microbiotas are phylogenetically clustered and at the same time taxonomically rich compared to adults could be explained by a selection of certain taxonomic groups through the gut environment. The phylogenetic overdispersion of the adult gut microbiotas suggests that the

pupal stage represents a crucial bottleneck for the gut microbiota in terms of species richness. This might be due to the random survival of bacterial taxa present in the larvae throughout metamorphosis (and its associated gut tissue restructuring) at the pupal stage. However, the fact that a certain number of taxa are maintained throughout the development from larvae to adult but are absent from soil, suggests the existence of a mechanism to specifically maintain essential bacterial partners (e.g. Ruminococcaceae, Lachnospiraceae). In other words, the survival of certain bacterial taxa may not be entirely random. Another possible explanation might be that the adult gut microbiota is renewed by feeding on leaves and flowers in contrast to rhizospheric soil and/or that the physico-chemical properties of the adult gut are more stable than in larvae (see Supporting Information Fig. S5). Hence, despite the potential existence of a mechanism to maintain and transmit a fraction of the microbiota, other bacterial taxa could still be transient and dependent on the food source (e.g. different parts of the plant, different plant species), as observed in *Drosophila melanogaster* where acetic acid bacteria are always associated with the fly, but the presence of other bacterial taxa is dependent on the environment (Adair *et al*., 2018; Wong *et al*., 2015).

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

Regarding the physico-chemical factors, oxygen availability was the most strongly correlated with differences in bacterial community structure between the different gut sections in adults, while intestinal pH was the most strongly correlated factor in larvae. Although both the midgut and hindgut compartments were largely anoxic in adults, the oxygen concentration in the midgut showed a higher degree of variation compared to the more anoxic hindgut. This is likely due to a considerably larger influx of oxygen via the gut epithelium in the case of the midgut, as observed in *Pachnoda ephippiata* (Lemke *et al*., 2003). This variability in oxygen availability between the different gut compartments may favour bacteria that are more tolerant towards such fluctuations. In larvae, the pH in the midgut and hindgut was alkaline, while the foregut had a neutral pH. It is important to note that the larvae are soil-dwellers feeding on fresh roots and decaying soil organic matter (SOM) (Fleming, 1972). In this regard, they are similar to other soil-dwelling macroinvertebrates, including many coleopterans, which feed on SOM and play an important role in its degradation and stabilization (Lavelle *et al*., 1997; Wolters, 2000). It has been shown that the conditions in the anterior hindgut of the humivorous termite *Cubitermes* spp. (i.e. high alkalinity and oxygen influx) lead to a decrease of the molecular weight of the organic matter (Kappler and Brune, 1999), rendering it more soluble and thus more accessible for digestion in subsequent less-alkaline compartments (Ji *et al*., 2000; Kappler *et al*., 2000; Ji and Brune, 2001). Although the complex microbial communities in the guts of humivorous macroinvertebrates are thought to participate in the transformation of ingested SOM (Cazemier *et al*., 1997; Kane, 1997), detailed information on the composition and activities of the gut microbiota is lacking. In view of the high midgut alkalinity in *P. japonica*, it is reasonable to assume that at least some of the bacteria in the midgut are tolerant towards high pH conditions, because most bacterial taxa are also found in the more neutral gut sections of adults.

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

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

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

The taxa found to be enriched in insect samples could be preferentially present in insects due to favourable conditions in the gut environment without an actual effect of these bacteria on the insect host. However, the fact that these bacteria were not detected in soil suggests the presence of a more direct transmission mechanism independent of the environmental route. In addition, the consistent presence of these bacteria in the gut regions where plant material is degraded further argues in favour of an active role of these bacteria and not just their presence as transient passengers.

In contrast to the above-mentioned bacterial families which have been observed not only in mammals but also in insects, the family 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 *et al*., 2016). The partial 16S rRNA gene-based phylogeny showed that the Christensenellaceae OTUs found in association with *P. japonica* do not cluster with the taxa associated with humans but rather form different clusters, suggesting that they belong to different taxonomic groups within the Christensenellaceae family (Supporting Information Fig. S7).

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

Materials and methods

Collection and processing of insect and soil samples

Four campaigns were organized from June to September 2017 to collect insect samples at different developmental stages of the insect. The different stages and instars (in the case of larvae: larval instar 1 – L1; larval instar 2 – L2; larval instar 3 – L3) of the insects were collected in Oleggio (Novara, Italy; 45°36′ N, 08°38′ E, altitude ca. 230 m a.s.l.). Simultaneously, at each sampling expedition, 10 soil samples were taken from the sampled area and combined into a single sample representative of the area, leading to the collection of three soil samples. Insects were preserved in absolute ethanol while soil samples in 50 ml vials, kept refrigerated on the field and then stored at −20°C before processing. All insects were surface sterilized before dissection using the protocol described in Montagna and colleagues (Montagna *et al*.,

2015a). Ninety individuals (i.e. 15 individuals of each larval instar, 15 pupae, 15 males, 15 females) were dissected under sterile conditions, and the gut (Supporting Information Fig. S1b) was removed in sterile Ringer solution. The insect alimentary canal was then aseptically separated into its three compartments (i.e. foregut, midgut and hindgut). For each developmental stage and larval instar, five homologous gut compartments were pooled together in a single sample, resulting in three biological replicates for each sample category. These samples were used for DNA extraction (see Supporting Information Table S1 for a detail on the samples).

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

DNA extraction, amplicon library preparation, sequencing and bioinformatics

The DNA was extracted from each sample (consisting of five homologous gut compartments for a defined insect instar and developmental stage) using the phenol–chloroform methods (Doyle and Doyle, 1990) with the modifications described in Mereghetti and colleagues (Mereghetti *et al*., 2019). The DNA was then eluted in 50 μl of sterile water (Sigma-Aldrich, Saint Louis, Missouri, USA). A DNA extraction blank was performed as control to monitor for contamination of environmental bacterial DNA. DNA from soils was extracted using PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) following manufacturer's instructions. Three independent DNA extractions were performed for each of the three representative soil samples.

The extracted DNA was used as template for the amplification of the V4 hypervariable region of the 16S rRNA gene using the PCR primers 515F (Caporaso *et al*., 2011) and a blend of reverse primers 802R (Claesson *et al*., 2009) and 806R (Caporaso *et al*., 2011) in order to reduce amplification bias. Forward and reverse primers were tailed with two different GC rich sequences, enabling barcoding with a second amplification. Each sample was first amplified in 20 μl reaction volume containing 8 μl HotMasterMix 5 Prime 2.5X (Quanta Bio), 0.4 μl BSA (20 μg μl^{-1}) (Sigma-Aldrich), 1 μl EvaGreen™ 20X (Biotium), 0.8 μl 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. The second PCR amplification was performed in 25 μl reaction volume containing the same reagents as the first PCR but with 1.5 μl barcoded/TrP1 primers (10 μM) and with 1 μl of the first PCR amplification in the following conditions: 8 cycles of 94°C for 10 s, 60°C for 10 s, 65°C for 40 s, and a final extension of 72°C for 3 min.

After labelling each sample with a specific Ion Torrent (Ion Express) DNA barcode, each single library was quality checked with agarose gel electrophoresis, quantified with Qubit Fluorometer (Thermo Fisher Scientific) then pooled with the other libraries in equimolar amounts. The final product was then sequenced using the Ion Torrent PGM System. Libraries preparation and sequencing were performed at the Life Sciences Department of Trieste University, Italy.

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

Diversity analyses

Bacterial OTU richness, diversity and evenness were calculated using the package Vegan (Dixon, 2003; Oksanen *et al*., 2018), implemented under the R software (R Project 3.0.2; http://cran.r-project.org/) adopting the species richness estimator Chao 1 (Chao, 1984), the Shannon H′ index (Shannon, 1948) and the Pielou′s evenness (Pielou, 1975), after sub-sampling the OTU table to obtain a total of 25 000 sequences per sample. Alpha diversity indices were compared between different groups (i.e. tissues and developmental stages) using two-sample t-tests with 999 Monte Carlo permutations.

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

in the bacterial communities (MPD; Webb *et al*., 2002) was used as metric for phylogenetic structure. To allow the comparison between the bacterial communities of the different types, null models maintaining species occurrence frequency constant were estimated. Standard effect size and relative position of each bacterial community with respect to the null MDP distribution, generated by 999 randomizations of the null model, were calculated using the *ses.mpd* function implemented in the 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 based on sample type (i.e. soil, larvae, pupae, adults) and statistical differences among sample types were assessed using Welch's one-way ANOVA (Welch, 1951), because SES_MDP values were normally distributed based on Shapiro–Wilk test (Royston 1982) (p >0.05), but the variance between groups was not homogeneous based on Levene test (Levene, 1960) (p <0.001). Hence, we used the Tamhane post hoc test for multiple comparisons without homoscedasticity.

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

In order to assess the difference in the microbiota structure among soil and insect samples, the sub-sampled OTU table was subjected to a non-parametric one-way analysis of similarity ANOSIM (Clarke, 1993), implemented in the vegan library and based on the Bray–Curtis dissimilarity (999 permutations permuting within gut samples of the same individuals in order to account for the non-independence of the observations (Bray and Curtis, 1957).

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

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

Changes in microbiota composition

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

Differentially abundant taxa were determined after data normalization of the OTU table using the EdgeR package (version 3.16.5) with R (version 3.4.4). Differentially abundant OTUs were then ranked by their $\log_2$ 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) with the CERNO test (Yamaguchi *et al*., 2008) and the Benjamini–

Hochberg correction. The position of the OTUs belonging to enriched families along the continuum of ranked OTUs was also assessed visually using receiver operating characteristic (ROC) curves. The enriched families were then tested for their presence in all samples (Supporting Information Table S3).

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

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

Measurement of the gut physicochemical properties

Physico-chemical parameters of oxygen partial pressure (pO_2), 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 $CaCl_2$, pH 7.3–7.4) and immediately covered with a second layer of 0.5% agarose prepared with Ringer's solution (Šustr *et al*., 2014). Oxygen microsensors (OX-50), with a tip diameter of 50 μm, were calibrated after an overnight polarization in water saturated with air and in 0.1 M sodium dithionite anoxic solution by using the CAL 300 calibration chamber (Unisense), following an overnight polarization. pH microelectrodes (PH-50), with a tip diameter of 50 μm, were calibrated with standard solutions at pH 4.0, 7.0 and 10.0. Redox potential microelectrodes (RD-50) had a tip diameter of 50 μm and were calibrated using saturated quinhydrone solutions at pH 4.0 and 7.0. Electrode potentials for microelectrodes were measured against Ag-AgCl reference electrodes by using a high-impedance voltmeter (Ri > 1014 Ω). Unisense microsensor multimeter allowed to measure the current and data were recorded by using SensorTracePRO software (Unisense). Microsensors were positioned using a motorized micromanipulator (Unisense). Measurements were carried out at room temperature.

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

Author Contributions

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

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Supporting Information

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

Table S2 Indval 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.

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

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

Figure S2 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 S3 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 S4 Heatmaps showing the relative pairwise nestedness and turnover values for the different developmental stages and soil

Figure S5 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 S6 Histograms summarizing the bacterial composition at the order level. The different histograms report only taxa with a relative abundance ≥3%. A: The taxa summary at the order level for the different samples. F indicates foregut, M indicates midgut and H indicates hindgut. B the taxa summary at the order level for the different samples grouped by individual pools. Namely each column correspond to the samples (foregut, midgut and hindgut) from the same pooled individuals.

Figure S7 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.