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ORIGINAL ARTICLE

Evidence for a conserved microbiota across the different developmental stages of *Plodia interpunctella*

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Abstract Diversity and composition of lepidopteran microbiotas are poorly investigated, especially across the different developmental stages. To improve this knowledge, we characterize the microbiota among different developmental stages of the Indian meal moth, *Plodia interpunctella*, which is considered one of the major pest of commodities worldwide. Using culture-independent approach based on Illumina 16S rRNA gene sequencing we characterized the microbiota of four developmental stages: eggs, first-, and last-instar larvae, and adult. A total of 1022 bacterial OTUs were obtained, showing a quite diversified microbiota associated to all the analyzed stages. The microbiotas associated with *P. interpunctella* resulted almost constant throughout the developmental stages, with approximately 77% of bacterial OTUs belonging to the phylum of Proteobacteria. The dominant bacterial genus is represented by *Burkholderia* (~64%), followed by *Propionibacterium*, *Delftia*, *Pseudomonas*, and *Stenotrophomonas*. A core bacterial community, composed of 139 OTUs, was detected in all the developmental stages, among which 112 OTUs were assigned to the genus *Burkholderia*. A phylogenetic reconstruction, based on the 16S rRNA, revealed that our *Burkholderia* OTUs clustered with *Burkholderia* cepacia complex, in the same group of those isolated from the hemipterans *Gossyparia spuria* and *Acanthococcus aceris*. The functional profiling, predicted on the base of the bacterial 16S rRNA, indicates differences in the metabolic pathways related to metabolism of amino acids between preimaginal and adult stages. We can hypothesize that bacteria may support the insect host during preimaginal stages.

Key words *Burkholderia*; Illumina sequencing; Indian meal moth; insect symbiosis; Proteobacteria

Introduction

All animals, including insects, harbor a multitude of microorganisms (bacteria, protozoa and fungi) (McFall-Ngai *et al.*, 2013). In particular the successful adaptation of insects reflects the very close and ancient mutualistic association that these groups of animals shared with the microorganisms (Douglas, 2014). In recent years, culture-independent approaches based on high throughput sequencing became a powerful method to investigate the microbial community associated with insects in a wide taxonomic range of hosts (Chakravorty *et al.*, 2007; Huse *et al.*, 2008; Wang & Qian, 2009). In most of the cases, microbiota investigation highlighted interesting host–symbiont interactions, representing key aspects in insect's survival and adaptation, such as development, nutrition, detoxification, and maintaining of the immune system (Dillon & Dillon, 2004; Engel & Moran, 2013). Regarding lepidopterans, the studies on their bacterial communities have increased in the recent years, especially for insect pest moths such as *Ostrinia nubilalis*

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(Belda *et al.*, 2011), *Spodoptera littoralis* (Tang *et al.*, 2012), *Lymantria dispar* (Broderick *et al.*, 2004; Lin *et al.*, 2015), *Helicoverpa armigera* (Xiang *et al.*, 2006), *Plutella xylostella* (Xia *et al.*, 2013), and *Bombyx mori* (Liang *et al.*, 2014). Furthermore, some of these studies revealed the role played by the bacteria in helping the host in a broad range of activities. As a matter of fact, *Enterococcus* sp. and *Staphylococcus* sp. provide their hosts, *Hyles euphorbiae* and *Brithys crini* (two lepidopterans feeding on plants producing alkaloids and latex), resistance to these toxic compounds (Vilanova *et al.*, 2016). A further symbiont-derived benefit regards the production of antimicrobial peptides, which protect the insect host against the invasion of foreign bacteria (e.g., Shao *et al.*, 2017). Almost all the previously reported studies focused on a single developmental stage (e.g., third instar larvae, Hernàndez-Flores *et al.*, 2015; Snyman *et al.*, 2016; the adult stage, Montagna *et al.*, 2016), ignoring the changes that may occur of the microbiota structure along the life cycle of these holometabolous insects. To our knowledge, only two studies have investigated the microbiota's structure across the life cycle in lepidopterans. The first investigation was reported by Hammer *et al.* (2014) and revealed that larvae and adults of the neotropical butterflies *Heliconius erato* harbored a taxonomically different microbiota. A similar result was observed in the cotton leafworm *Spodoptera littoralis* (Chen *et al.*, 2016).

The Indian meal moth (IMM) *Plodia interpunctella* (Hübner) represents one of the major pests of food commodities worldwide (Vick *et al.*, 1986; Cuperus *et al.*, 1990; Rees, 2004). The economic impact of this moth is very high, because it feeds on a wide range of stored products, such as grains, chocolate, nuts, powdered milk, and dry meats (Perez-Mendoza & Aguilera-Pena, 2004); damages are represented by larval silk webs and frass (Fasulo & Knox, 2009). In a previous study the microbiota associated with IMMs reared under different diets was investigated (Montagna *et al.*, 2016). Two distinct entomotypes were discovered associated with IMMs based on their diet, namely protein-rich versus carbohydrate-rich, suggesting the diet as a major driver in shaping the bacterial community (Montagna *et al.*, 2016).

In this study, we used high-throughput sequencing technology to investigate the microbiota of IMMs in different developmental stages, namely eggs, the first- and last-instar larvae, male and female adults. The aims of this study are: (i) estimate the shift in the IMM-associated microbiota during selected stages of its life cycle and in the case of bacterial community changes; (ii) evaluate the presence of a bacterial core throughout the developmental stages, and (iii) predict the functional profiles of the bacterial communities.

Materials and methods

Ethics statement

P. interpunctella are not listed in any national or regional law as protected or endangered species.

Insect rearing and sampling

The specimens used in this study were obtained from a *P. interpunctella* population maintained in the insectary at DEFENS (Department of Food, Environmental and Nutritional Sciences—Università degli Studi di Milano) and reared under controlled conditions of temperature (26 ± 1 °C), humidity (RH = 65% ± 5%) and a 16 h : 8 h light : dark photoperiods. Insects were routinely fed on an artificial diet following Stampini and Locatelli (2007), with some modifications (20% increase of honey content and without beer yeast). In order to obtain eggs, adult female were separated from the main laboratory population and allowed to oviposit for 3 h. Ninety eggs were directly stored at –80 °C without sterilization, while the remaining eggs were allowed to hatch. The first-instar larvae, 3 h after the emergence from the egg, and the last-instar larvae (before pupation) were collected and stored in absolute ethanol at –80 °C. Last-instar larvae were sexed (Hamlin *et al.*, 1931) and then let to molt separating males from females. Once emerged, adults were stored in absolute ethanol at –80 °C.

Sample processing and DNA extraction

Three replicates, for each developmental stage, were processed as described below. Each replicate was composed of five specimens from the same developmental stage. The collected specimens (except the eggs) were surface-sterilized using previously published protocol (Montagna *et al.*, 2015a). Briefly, the sterilization protocol consists of the following steps: (i) washing the samples with distilled water; (ii) washing with a sodium hypochlorite solution at 4% for 3 min; (iii) washing with a solution of PBS and 0.1% Triton-X-100 for 3 min; and (iv) a final washing with sterile distilled water. These steps were repeated three times.

The wings were removed from adults prior to sterilizing the body. Total bacterial DNA was extracted, under sterile conditions, using the classical phenol–chloroform methods (Doyle & Doyle, 1990) with the following modifications: First, 500 μL of 2% CTAB (2% CTAB, 0.2% ascorbic acid, 1.5% PVP, 1.4 mmol/L NaCl, 20 mmol/L EDTA and 100 mmol/L Tris-HCl, pH 8.0) was added to each

sample. Tissues were then disrupted using glass beads (ø 0.1 mm) with the Precellys®24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) and incubated at 65 °C for 15 min to inactivate nucleases. After centrifugation, the supernatant was incubated overnight with 20 μL of proteinase K (20 mg/mL) at 56 °C. In order to purify the DNA, two phenol–chloroform washes (phenol/chloroform/isoamyl alcohol, 25 : 24 : 1, pH 8.0) were performed. DNA was, then, precipitated after addition of 500 μL of isopropanol and incubation for 1 h. Pellet was washed twice with 70% ethanol and eluted in 40 μL of Ultrapure Water (Sigma-Aldrich, Saint Louis, Missouri, USA). Qubit fluorometer with the dsDNA High Sensitivity Assay Kit (Life Technologies Corp., Carlsbad, CA, USA) was used to determine the DNA concentrations. A DNA extraction blank, using the same extraction protocol and molecular biology grade water, was performed as control to monitor for contamination of bacterial DNA. Different volumes of blank sample were used as template for PCR targeting the bacterial 16S rRNA using 27 Fmod and 519 Rmod primers (Lane, 1991); no positive amplifications were obtained.

DNA sequencing

DNA extracted from each sample was used as a template for the amplification of the bacterial 16S rRNA hypervariable V1–V3 region using universal primers (27 Fmod 5′-AGRGTTTGATCMTGGCTCAG-3′ and 519 Rmod 5′-GTNTTACNGCGGCKGCTG-3′) (Lane, 1991; Turner *et al.*, 1999). The resulting amplicon was sequenced using the Illumina Miseq2500 platform at MR DNA laboratory research (Shallowater, Texas, USA).

The data resulting from the sequencing was analyzed using the software package QIIME version 1.9.1 (Caporaso *et al.*, 2010). First, we processed the 16S rRNA reads removing adaptors, filtering low-quality and size selecting reads; keeping only those with Phred score >30 and within the size range 350–500 bp. Uclust (Edgar, 2010) was used to cluster the high-quality 16S rRNA sequences into Operational Taxonomic Units (OTUs) with a similarity cut-off of 97%. Chimeras were then removed using *Chimeraslayer.* The most abundant sequence for each identified OTUs was aligned to *Greengenes* (http://greengenes.lbl.gov/) using Pynast (Caporaso *et al.*, 2010). Finally, taxonomic assignment was performed comparing the representative OTUs with *Greengenes* and *Silva* databases. The sequences obtained in this study were deposited in European Nucleotide Archive with the accession number PRJEB21609.

Bacterial community analysis

The OTUs table obtained by QIIME, representing the bacterial communities associated with each of the analyzed samples, was used as an input for of the diversity indices estimation and following analyses. The package Vegan (Dixon, 2003), implemented under the R software (R Project 3.0.2; http://cran.r-project.org/), was used to estimate, for each developmental stages and replicate, the bias-corrected Chao1 richness index (Chao, 1984), the Shannon H index (Shannon, 1984) and Pielou's evenness (Pielou, 1975). ANOVA (Anderson, 2001) was used to assess statistical differences in richness and diversity values associated with the different developmental stages. Venn diagrams were obtained using the *gplots* package in R with purposes of representing bacterial OTUs shared among the four developmental stages (eggs, first-, and last-instar larvae, adults).

The nonparametric one-way analysis of similarity ANOSIM (Clarke, 1993) was used to investigate the statistically significant difference in the microbiota structure among the developmental stages.

Phylogenetic placement of the most abundant Burkholderia *OTUs*

Consensus sequences for *Burkholderia* OTUs with abundance >1000 reads were selected to perform a phylogenetic assignment using a selected dataset of Burkholderiales 16S rRNA sequences, as in previous studies (e.g., Kikuchi *et al.*, 2011; Boucias *et al.*, 2012; Michalik *et al.*, 2016). A total of 22 partial 16S rRNA sequences belonging to *Burkholderia* were downloaded from GenBank (Benson *et al.*, 2013) (Table S4), in addition 16S rRNA sequences from representatives of other Burkholderiales genera and *Escherichia coli* (as outgroup) were retrieved (Table S4). The sequences were aligned using MAFFT (Katoh & Standley, 2013) and Q-iNS. Nucleotide substitution model was estimated using jModelTest 2 (Darriba *et al.*, 2012). The model best-fitting the sequence was selected according to Akaike Information Criterion. Phylogenetic reconstructions were performed using Maximum Likelihood and Bayesian inference. Maximum likelihood tree was inferred adopting the General Time Reversible (*GTR*; Lanave *et al.*, 1984) with gamma distribution (Γ) and proportion of invariable sites (I) as models of nucleotide evolution, as obtained by model selection analysis, and approximate likelihood ratio test as node support (aLRT) (Anisimova & Gascuel, 2006), by using PhyML (Guindon *et al.*, 2010). Bayesian inference was performed using MrBayes 3.2 (Ronquist *et al.*,

2012) in two independent runs of 2×10^7 generations that were sampled every 100 generations. The model of nucleotide evolution was settled as $GTR + I + \Gamma$. The potential scale reduction factor value of each parameter of the model was checked and the convergence of the two runs (standard deviation of split frequencies, effective sample size) was visually inspected using MrBayes 3.2 (Ronquist *et al.*, 2012) and TRACER (Drummond *et al.*, 2012). Twenty percent of the trees were discarded as burn-in and then a majority-rule consensus tree was obtained.

Metabolic functional profiles

Using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; Langille *et al.*, 2013) we inferred the metagenomic functional profile of the bacterial community associated with different developmental stages of *P. interpunctella*. The resulting table, with the predicted metabolic potential of each bacterial community associated with a given developmental stage, was analyzed. The bacterial function was annotated according to KEGG Orthology (Kanehisa *et al.*, 2012) and only those with a meaningful interest for the insect physiology were retained. After retaining the selected meaningful functions, the normality distribution of the data and the homoscedasticity among groups were tested using Shapiro and Levene test, respectively (*lawstat* package in R). In order to determine a significant difference (significant level $P < 0.05$) among the IMM life stages the Kruskal–Wallis test (*agricolae* package in R software) and the Bonferroni correction were applied.

Results

Bacterial diversity

The microbiota associated with the selected developmental stages of *Plodia interpunctella* were analyzed using Illumina sequencing. A total of 890 453 raw reads of the V1–V3 region of the bacterial 16S rRNA were obtained. One sample corresponding to the last-instar larvae was removed from the analysis due to the low number of reads (<1000 reads). After quality filtering, 568 229 high-quality reads were retained (median number of sequences per sample = 38 712 SD ± 25.4). The clustering of the reads at 97% of similarity threshold let to a total of 1022 bacterial OTUs. Rarefaction curves based on Shannon index showed that the sampling depth was high enough to have a reliable representation of the actual diversity (Table S1). OTUs richness was also estimated using both the observed OTUs and Chao-1 indices (Table 1). The results showed that the sequencing approach covered approximately the 70% of total bacterial diversity. The highest species diversity was associated with the eggs, with a median value through the three replicates of 663 OTUs (SD ± 323), while the lowest value was recovered in the last larval instar (382 OTUs, SD ± 141); microbiotas of first larval instar, male and female adults were characterized by an intermediate number of OTUs (Table 1). The Shannon index H' ranged from 1.58 to 2.15, in the case of adult females and last-instar larvae; while the values of Pielou's evenness *J'* ranged from 0.24 to 0.36 (Table 1). The low values of Pielou's evenness indicate unbalanced bacterial communities with a low number of OTUs present at high relative abundance. In the case of adult females ($J' = 0.24$), the first OTU in term of abundance accounted for the 60% of the bacterial community. No statistical differences were recovered among the IMM developmental stages for the number of detected OTUs as well as for the considered diversity indices (Shannon H 0.24), the *J* and Chao-1).

Taxonomic composition through the developmental stages

The microbiotas associated with all the considered developmental stages were dominated by Proteobacteria, with an increasing relative abundance from eggs to adulthood (77% ± 2.9% in eggs, 96.5% ± 3.6% and 89.9% ± 5.6% in the case of adult females and males,

Table 1 Median values of reads, observed OTUs, and diversity indices for the analyzed stages of *Plodia interpunctella*.

Developmental stage	Reads	Observed OTUs	Shannon H'	Pielou's *J'*	Chao-1
Eggs	68 567	663	2.08	0.32	822.3
Early-instar larvae	43 087	534	1.99	0.32	779.3
Last-instar larvae	17 813	382	2.15	0.36	614.4
Adult female	46 499	619	1.58	0.24	816.6
Adult male	22 254	452	1.70	0.29	655.2

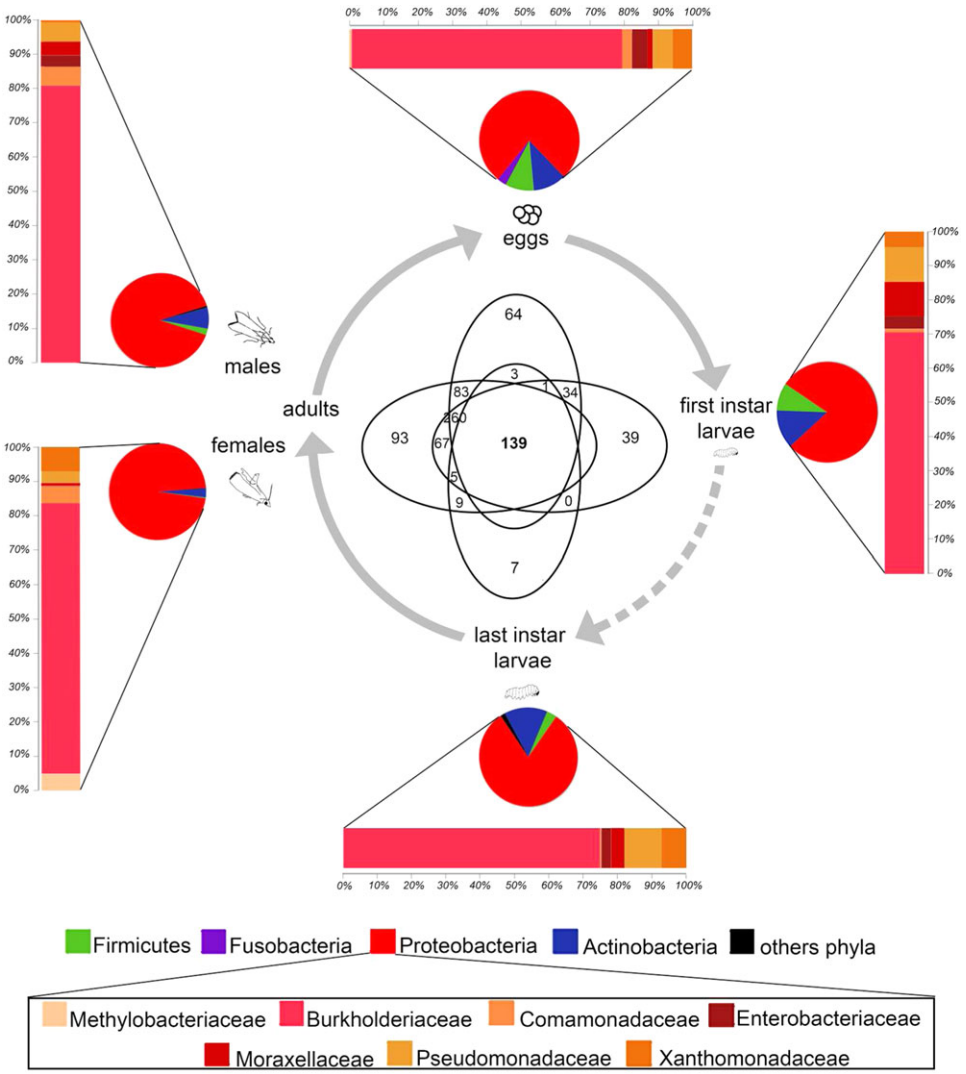

Fig. 1 Taxonomic composition and bacterial core of the microbiotas associated with the analyzed developmental stages of *Plodia interpunctella* (eggs, first-instar, last-instar, adult females and males) at phylum (pie charts) and family levels (bars). Only the bacteria families with an average abundance ≥ 5% are reported. In the middle of the figure, the Venn diagrams report bacterial OTUs shared among the four developmental stages (the tip of each oval corresponds to the developmental stage reported in the outer schematic representation of the *P. interpunctella* life cycle).

respectively; Fig. 1; Table S1). Actinobacteria resulted the second most abundant phylum, followed by bacteria belonging to Firmicutes (Fig. 1; Table S1). Burkholderiaceae resulted the most abundant bacterial family in all the insect semaphoronts (Fig. 1; Fig. 2; Table S2); within this family *Burkholderia* resulted the genus with the highest abundance in all of the *P. interpunctella* developmental stages, ranging from ~45% to ~80% in the case of last-instar larvae and adult females (Table 2). Others important genera composing the *P. interpunctella* microbiota were represented by *Propionibacterium*, *Delftia*, *Pseudomonas* and *Stenotrophomonas* (Table 2; Table S3).

Core microbiota and β-diversity

A core microbiota composed of 139 OTUs was identified after comparing the four developmental stages (Fig. 1). Noteworthy, 112 OTUs out of a total of 139 were assigned to the genus *Burkholderia*, the remaining 27 OTUs were assigned to the following 12 genera: *Bacillus* and *Pseudomonas* with four OTUs each, *Acinetobacter*

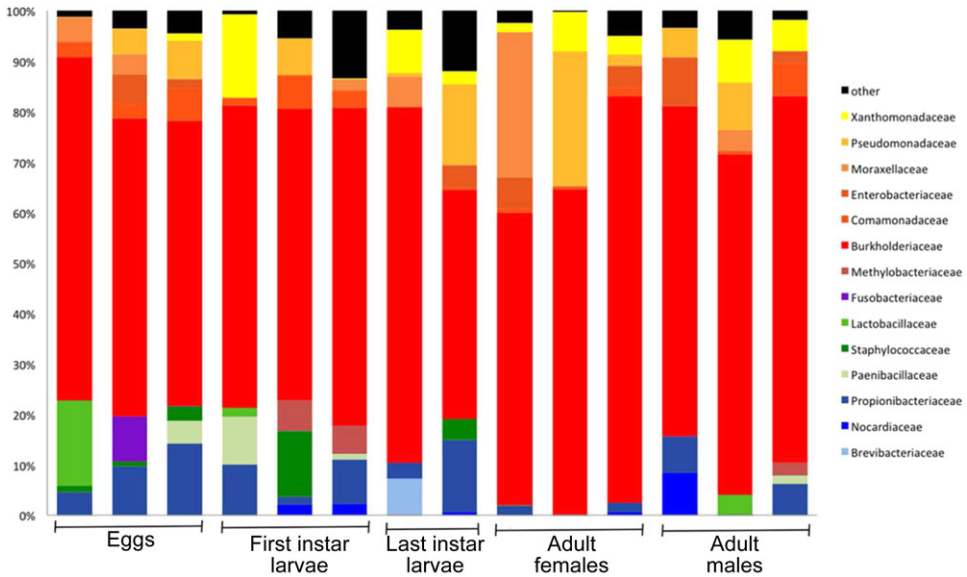

Fig. 2 Histogram representing the bacterial composition, at family level, for the analyzed developmental stages. In the histograms are reported only taxa with a relative abundance ≥ 5%.

Table 2 Genera of bacteria identified in the analyzed *Plodia interpunctella* stages with abundance >5% in at least one specimens.

Genus/sample ID	Eggs			Early-instar larvae			Last-instar larvae		Adult females			Adult males		
	1†	2	3	1	2	3	1	2	1	2	3	1	2	3
Brevibacterium	–	–	–	–	–	–	7.3	–	–	–	–	–	–	–
Rhodococcus	–	–	–	–	2.1	2.1	–	0.6	–	–	0.6	8.4	–	–
Propionibacterium	4.5	9.6	14.1	10.0	1.6	8.8	3.0	14.3	1.9	–	1.9	7.2	–	6.2
Brevibacillus	–	–	4.6	9.6	–	1.2	–	–	–	–	–	–	–	1.7
Staphylococcus	1.3	1.0	2.8	–	13.0	–	–	4.0	–	–	–	–	–	–
Lactobacillus	16.8	–	–	1.6	–	–	–	–	–	–	–	–	3.9	–
Fusobacterium	–	8.9	–	–	–	–	–	–	–	–	–	–	–	–
Methylobacterium	–	–	–	–	6.0	5.4	–	–	–	–	–	–	–	2.3
Burkholderia	68.0	59.0	56.5	59.9	57.8	62.9	70.4	45.3	58.0	64.4	80.5	65.4	67.5	72.6
Delftia	1.6	2.8	4.9	1.4	5.1	3.4	–	–	0.8	–	–	–	0.6	1.3
Hydrogenophaga	–	–	–	–	–	–	–	–	–	–	–	–	–	5.3
Pantoea	–	5.7	1.7	–	–	–	–	1.8	4.6	–	4.3	9.5		2.3
Acinetobacter	4.7	–	0.1	–	–	2.2	5.9	–	–	–	–	–	4.0	–
Enhydrobacter	–	4.1	–	–	–	–	–	–	28.8	–	–	–	–	–
Pseudomonas	0.1	5.0	7.6	–	7.2	–	0.7	15.9	–	26.7	2.2	5.7	9.4	0.1
Stenotrophomonas	–	–	1.4	16.4	–	–	8.6	2.7	1.8	7.8	3.6	–	8.6	6.1

†The numbers stand for replicates.

and *Propinebacterium* with three OTUs, *Enterobacter* (two OTUs), and one OTU for the genera *Corynebacterium*, *Rhodococcus*, *Brevibacillus*, *Staphylococcus*, *Fusobacterium*, *Achromobacter*, *Delftia*, *Enhydrobacter*, *Micrococcus*, *Brevibacterium*, and *Defluvibacter*.

The ANOSIM analysis performed on the OTU table, using as grouping factor the developmental stages, confirmed the absence of differences among the bacterial communities associated with *P. interpunctella* ($R = 0.2$; $P = 0.34$).

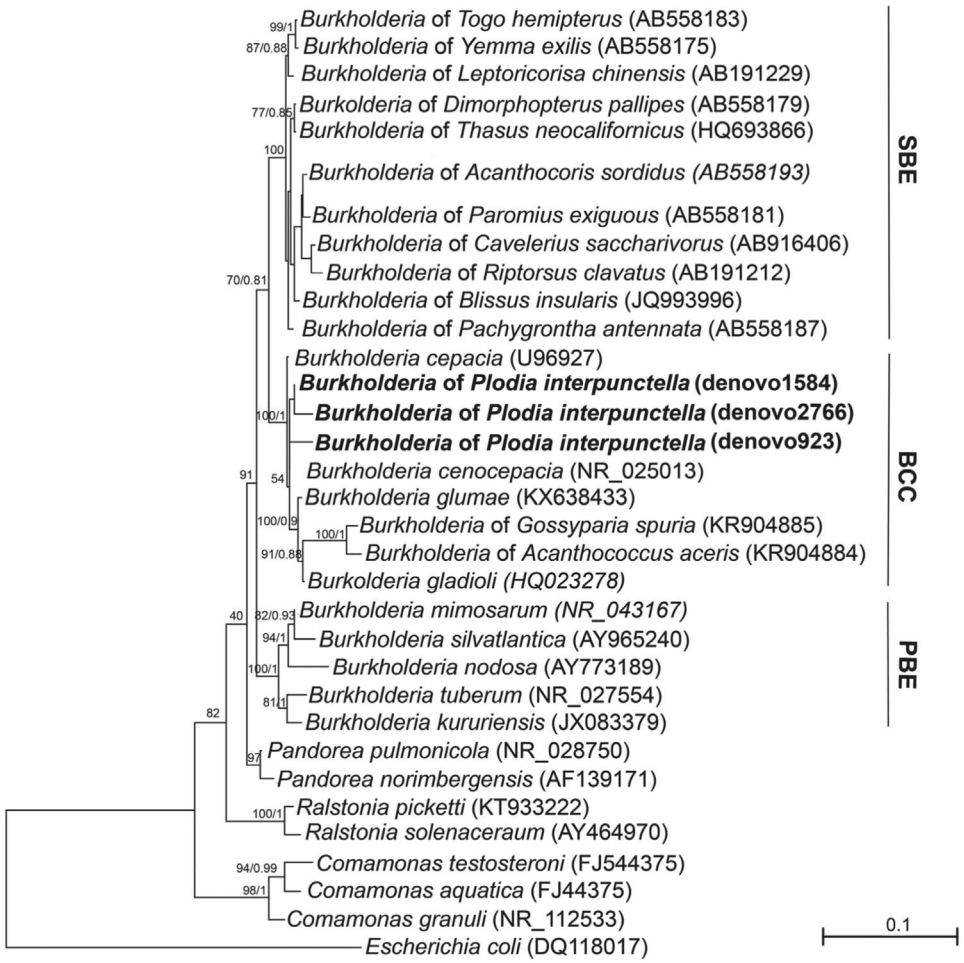

Fig. 3 Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences. Vertical dashes lines indicate the three different *Burkholderia* groups. SBE: stinkbug associated beneficial and environmental *Burkholderia*; BCC: *Burkholderia cepacia* complex; PBE: plant-associated beneficial and environmental *Burkholderia*. On the nodes of the main the lineages the support values, expressed as aLRT (first value) and Bayesian posterior probability (second value) of the Bayesian inferred tree (Fig. S2), are reported. The scale bar at the bottom indicates the distance in nucleotide substitution per site. The numbers in brackets represent the GeneBank accession number of the 16S rRNA gene sequences or the numbers that identify the *de novo* OTUs.

Phylogenetic placement of the most abundant Burkholderia OTUs

Three *Burkholderia* OTUs, with a number of reads >1000, were selected for the phylogenetic inference, namely denovo923 with 1685 reads, denovo1584 with 323 731 reads, and denovo2766 with 1016 reads. The three selected OTUs were present in each of the three replicates of all the analyzed developmental stages of *P. interpunctella*. The topology resulting from the 16S rRNA-based phylogenetic analysis confirmed the results previously obtained (Fig. 3; Fig. S2) (Coeyne *et al.*, 2001; Kikuchi *et al.*, 2011; Suarez-Moreno *et al.*, 2012; Itoh *et al.*, 2014). Three well-supported clades of *Burkholderia* were recovered: (i) a group composed of the stinkbug associated and environmental *Burkholderia* (SBE); (ii) the *Burkholderia cepacia* complex (BCC); and (iii) a group with the plant-associated and environmental *Burkholderia* (PBE). Interestingly the *Burkholderia* of the *P. interpunctella* microbiota clustered within the BCC group, as those associated with the two hemipterans *Gossyparia spuria* and *Acanthococcus aceris* (Michalik *et al.*, 2016).

Metabolic potential

The metagenomic functional potential of the insect-associated bacterial communities was predicted on the

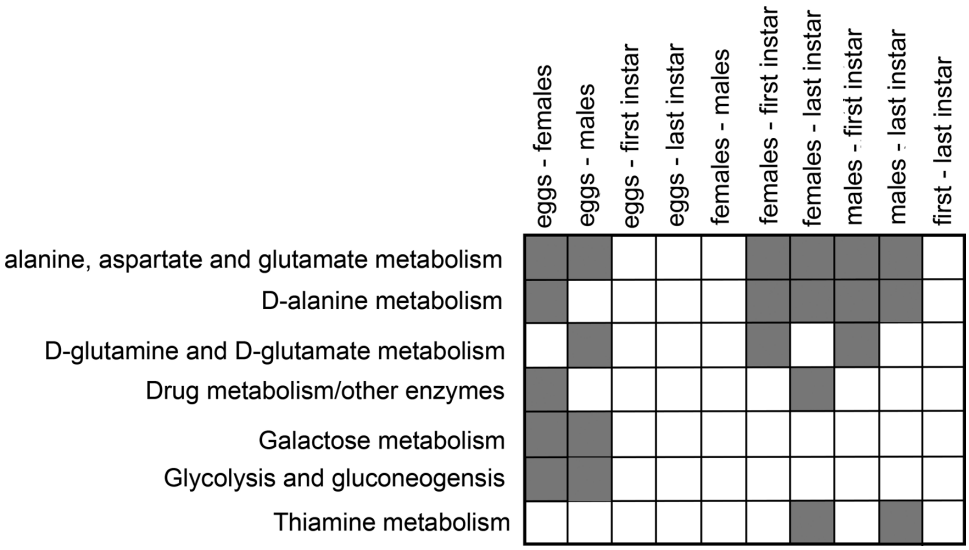

Fig. 4 Table reporting the metabolic predicted function inferred from the bacterial 16S rRNA gene sequences for which differences among the developmental stages were recovered by Kruskal–Wallis test with the Bonferroni correction. White square: $P > 0.05$; grey squares: $P < 0.05$.

basis of 16S rRNA sequences (Table S5). The relative abundance of seven functional categories, which can be linked to the insect physiology, was significantly different among the microbiotas associated with the developmental stages of *P. interpunctella* (Fig. 4). All these functions were metabolism related. In particular, three out of seven were related to the metabolism of amino acids (i.e., alanine, aspartate and glutamate, $\chi^2 = 9.65$, df $= 4$, $P = 0.047$; D-alanine and D-glutamine, $\chi^2 = 8.85$, df $= 4$, $P = 0.037$; D-glutamate metabolism, $\chi^2 = 9.54$, df $= 4$, $P = 0.048$). Two functional categories were associated to carbohydrate metabolism; namely, the metabolism of galactose ($\chi^2 = 9.08$, df $= 4$, $P = 0.031$) and the glycolysis and gluconeogenesis ($\chi^2 = 9.16$, df $= 4$, $P = 0.047$). The two last functional categories belong to cofactors metabolism (thiamine metabolism, $\chi^2 = 9.84$, df $= 4$, $P = 0.043$) and to the vitamins and xenobiotics biodegradations metabolism (drug metabolism, $\chi^2 = 9.31$, df $= 4$, $P = 0.041$).

In particular, statistically significant differences were observed when comparing the metabolic functions of the preimaginal stages and adults (males and females) (Fig. 4).

In terms of relative abundance, the highlighted metabolic functions were higher in the preimaginal stages in respect to adult females and males (Table S5). No significant differences in the metabolic functions were observed when comparing microbiotas associated with preimaginal stages (e.g., eggs vs. first-instar larvae) as well as comparing adult males versus adult females (Fig. 4).

Discussion

Bacterial diversity and taxonomic composition

In this study, we investigated the microbiota associated with the developmental stages, from eggs to adulthood, of the holometabolous insect *Plodia interpunctella*. In detail, we focused our attention on the following stages: egg, first-, last-instar larvae (e.g., the first stage soon upon hatching and the last stage before pupation), and adults. In addition, adult females and males were analyzed separately in order to investigate gender-associated shift in the bacterial community.

In this study the highest number of bacterial OTUs was recovered in eggs, followed by adult females, first-instar larvae, adult males, and by the last-instar larvae. In a previous study (Montagna *et al.*, 2016), addressed to estimate the impact of the insect's diet on the associated bacterial communities, the values of observed bacterial OTUs resulted significantly lower than those obtained in this study (on average approximately 220 OTUs compared with ~510 OTUs). This result seems to support the role of the diet in shaping the bacterial communities associated with the IMM. A further possible explanation may rely on the different sequencing technologies adopted here in respect to Montagna *et al.* (2016), namely Illumina sequencing versus 454 pyrosequencing.

Compared to previous studies, where changes in the bacterial communities were observed across the different developmental stages (Hammer *et al.*, 2014; Chen

et al., 2016), in this study no notable differences in the microbiotas were detected. Our results provide evidence for the existence of a stable microbiota throughout the ontogenetic development of *P. interpunctella*. A possible explanation for the contrasting results of our study respect to Hammer *et al.* (2014) and Chen *et al.* (2016) might rely in the different adults feeding behavior. *Heliconius erato* and *Spodoptera littoralis*, where major changes in the microbiotas of pupae and adults were observed (Hammer *et al.*, 2014; Chen *et al.*, 2016), possess a nectivorous adult life-style, whereas *P. interpunctella* does not feed at the adult stage.

Considering the structure of the bacterial communities across the developmental stages, low values of Pielou's evenness ($J'_{AVERAGE} = 0.25 \pm 0.03$, Table 1) indicate highly imbalanced microbiotas, contrasting with previously obtained values ($J'_{AVERAGE} = 0.64 \pm 0.15$; Montagna *et al.*, 2016). An imbalanced bacterial community has also been observed in insect belonging to other groups, such beetles (e.g., Montagna *et al.*, 2015a, b) or Diptera (Muturi *et al.*, 2016). The low evenness is reflected on the taxonomic composition (e.g., at phylum and at genus levels; Fig. 1, Tables 2, S3, and S4), with Proteobacteria accounting, on average, for ~77% of the communities and, at the genus level, with *Burkholderia* accounting for ~64%. Similar results (i.e., an imbalanced bacterial community) were obtained also for other lepidopterans (Broderick *et al.*, 2004; Xiang *et al.*, 2006; Brinkmann *et al.*, 2008; Hernandez-Flores *et al.*, 2015), in particular, in the case of *S. littoralis* (Chen *et al.*, 2016) and of lab-reared *Ostrinia nubilalis* (Belda *et al.*, 2011), the associated bacterial communities were dominated by Firmicutes (>80%).

The bacterial core shared among the analyzed developmental stages was composed by more than 100 OTUs, of which the majority belongs to the genus *Burkholderia* (Fig. 1; Table 2). Two different entomotypes were previously recovered associated with *P. interpunctella*, depending on carbohydrate and protein-rich diets (Montagna *et al.*, 2016). The artificial diet on which IMMs of this study were reared was different respect those used by Montagna *et al.* (2016). We hypothesize that due to the different diets, *Burkholderia* replaced *Atopococcus* as the dominant taxa (Montagna *et al.*, 2016). However, apart from *Atopococcus*, 5 out of the 10 genera of the core identified in this study are shared with the previously described entomotypes (i.e., *Pseudomonas*, *Acinetobacter*, *Propionibacterium*, *Corynebacterium*, and *Staphylococcus*; Montagna *et al.*, 2016).

Burkholderia is found in different environments such as water, soil, and the plant rhizosphere (Woods & Sokol, 2005). It was first described as a plant pathogen (Yabuuchi *et al.*, 1992), but it can also be hazardous to humans and animals (Coenye & Vandamme, 2003). However, *Burkholderia* has also been described as an insect symbiont; notably *Burkholderia* has been described in association with *Riptortus pedestris* (Kikuchi *et al.*, 2005) and other heteropterans (Kikuchi *et al.*, 2005; Kikuchi *et al.*, 2011) where it inhabits gut crypts. In *R. pedestris* the environmental acquisition of this bacterium was demonstrated (Kikuchi *et al.*, 2007). Furthermore, it was observed that after acquisition by second instar nymphs, the bacterium is retained by the insect for the entire life cycle (Kim *et al.*, 2014). This bacterium plays an important role in *R. pedestris* growth (Kikuchi *et al.*, 2007) but is also able to confer insecticide resistance (Kikuchi *et al.*, 2012) to its host. In *Caevelerius saccharivorus*, *Burkholderia* showed a mixed strategy for its transmission (i.e., vertical transmission and environmental acquisition; Itoh *et al.*, 2014) while it was only vertically transmitted in *Acanthococcus aceris* and *Gossyparia spuria*, where it inhabits the fat body cells (Michalik *et al.*, 2016).

To our knowledge, this is the first time that bacteria belonging to the genus *Burkholderia* have been recovered within holometabolous insects with high abundance and persistence across the developmental stages, suggesting that this bacterium can maintain stable association with *P. interpunctella*. Noteworthy, the majority of the identified *Burkholderia* OTUs are part of the core of OTUs shared among the analyzed developmental stages. We can thus conclude that, in addition to the previously identified entomotypes (i.e., entomotype *Atopococcus* and entomotype *Propionibacterium*; Montagna *et al.*, 2016), a new *P. interpunctella* entomotype is here observed, the entomotypes *Burkholderia*.

The phylogenetic analysis performed to investigate the phylogenetic position of *Burkholderia* OTUs obtained by this study (Fig. 3) suggested that these are closely related to the BCC group. Within this group, *Burkholderia cepacia* has been reported to cause serious diseases in cystic fibrosis patients and individuals with compromised immune systems (Lipuma, 2005). Furthermore, other *Burkholderia* strains within the BCC group have been described as plant pathogens, such as *Burkholderia gladioli* (Segonds *et al.*, 2009) and *Burkholderia glumae* (Ham *et al.*, 2011). Other strains have been isolated from insects, as in the case of the two Hemiptera *Acanthococcus aceris* and *Gossyparia spuria* (Michalik *et al.*, 2016). Based on these findings and on the previously reported information on the microbiotas of adults *P. interpunctella* (Montagna *et al.*, 2016), we can hypothesize that this bacterium was conveyed in the gut of *P. interpunctella* through the food ingestion or, more speculatively, that in the analyzed population of *P. interpunctella*, it is

vertically transmitted due to its dominance in the eggs microbiota (abundance of approximately 80%). *Ad hoc* experiments such as FISH and immunostaining assays targeting *Burkholderia* are required to test the last hypothesis.

Metabolic potential

The seven predicted metabolic functions, where differences among the developmental stages were recovered, are almost all connected with the amino acid metabolism (Fig. 4). Interestingly, the differences were recovered among adults and preimaginal stages; while no differences were detected comparing the different preimaginal semaphoronts, nor between adult males and females (Fig. 4). These results are compatible with the different physiological requirements of these stages (Klowden, 2007a). As example, among the selected seven metabolic functions, are present alanine and glutamine metabolisms. These two amino acids are involved in many physiological processes such as the chemistry of the cuticle (Andersen *et al.*, 1995). In particular, alanine represents the second major component of the resilin, a rubber-like proteins that constitute the insects cuticle; while glutamine, involved in the amination of the glucose-6-phosphate in glucosamine-6-phosphate, an important metabolite for the biosynthesis of the cuticle (Klowden, 2007b). These two amino acids are also implicated in Krebs cycle (Nation, 2016). In fact, although the principal source of pyruvate entering the Krebs cycle comes from glycolysis, insects are also able to use alanine as a source of pyruvate (Sacktor & Wormser-Shavit, 1966). Glutamate, on the other hand, represents an intermediate metabolite fundamental in the Krebs cycle, since it is transaminated with pyruvate to form α-ketoglutarate (Sacktor & Wormser-Shavit, 1966). Based on the fact that the relative abundance of the metabolic functions was higher in the preimmaginal stages respect to adults, we can hypothesize higher requirements for these amino acids during the high energy-demanding molting processes (Nation, 2016). However, the high alanine and glutamine metabolism recovered in the bacterial communities associated with preimaginal stages can be the result of the different feeding behavior between larvae and adults, which have an impact on the gut physiology (Altermatt & Pearse, 2011). As a matter of facts, *P. interpunctella* do not feed at the adult stage (Fasulo & Knox, 2009).

The ability of some bacteria to provide essentials amino acids is well documented in insects (Douglas, 2006), however no cases of nutritional symbiosis have been recorded so far among lepidopterans (McCutcheon & Moran, 2007; Ayayee *et al.*, 2016). The results achieved in this study may pave the way for future studies to investigate the role of *Burkholderia* and other bacteria of the *P. interpunctella* microbiota in supporting the host development.

To this day studies on lepidopteran microbiota remain largely scarce. This is the first report that analyses the bacterial composition in different developmental stages of the Indian meal moth, *P. interpunctella* (eggs, first-, and last-instar larvae, and both adult females and males). Our study shows that: (i) the microbiota do not exhibit a great variation across the analyzed developmental stages; (ii) the bacterial communities resulted dominated by few taxa; (iii) bacteria of the genus *Burkholderia* dominate all the developmental stages of *P*. interpunctella; (iv) a core of bacteria (dominated by Burkholderia) is shared across eggs, larvae and adults; and (v) since the microbiota described in this study differs from that of a previous study, the role of the insect's diet in shaping its bacterial community is further supported.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Rarefaction curves obtained using Shannon Index.

Fig. S2 Bayesian phylogram based on 16S rRNA gene sequences. Vertical dashes lines indicate the three different *Burkholderia* groups. SBE: stinkbug associated beneficial and environmental *Burkholderia*; BCC: *Burkholderia cepacia* complex; PBE: plant-associated beneficial and environmental *Burkholderia*. On the nodes of the main the lineages the support values, expressed as Bayesian posterior probability, are reported. The scale bar at the bottom indicates the distance in nucleotide substitution per site. The numbers in brackets represent the GeneBank accession number of the 16S rRNA gene sequences or the numbers that identify the *de novo* OTUs.

Table S1. Taxonomic composition at phylum level.

Table S2. Taxonomic composition at family level.

Table S3. Taxonomic composition at genus level.

Table S4. Predicted functional profiles inferred using bacterial 16S rRNA sequences, table with the abundance of the predicted L3-functions.