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Evidence of a bacterial core in the stored products pest *Plodia interpunctella*: the influence of different diets

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Summary

The potential influence of insects' feeding behaviour on their associated bacterial communities is currently a matter of debate. Using the major pest of commodities, *Plodia interpunctella*, as a model and adopting a culture-independent approach, the impact of different diets on the host-associated microbiota was evaluated. An analysis of similarity showed differences among the microbiotas of moths fed with five substrates and provided evidence that diet represents the only tested factor that explains this dissimilarity. Bacteria shared between food and insects provide evidence for a limited conveyance to the host of the bacteria derived from the diet; more likely, the content of carbohydrates and proteins in the diets promotes changes in the insect's microbiota. Moth microbiotas were characterized by two robust entomotypes, respectively, associated with a carbohydrate-rich diet and a protein-rich diet. These results were also confirmed by the predicted metagenome functional potential. A core microbiota, composed of six taxa, was shared between eggs and adults, regardless of the origin of the population. Finally, the identification of possible human and animal pathogens on chili and associated with the moths that feed on it highlights the possibility that these bacteria may be conveyed by moth frass.

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Introduction

The Indian meal moth (IMM), *Plodia interpunctella* (Hübner 1813) (Lepidoptera: Pyralidae), is a cosmopolitan moth (Rees, 2004) that infests a multitude of stored-products, such as different types of cereals, nuts and dried fruits but also spices and dried meat (Hamlin *et al.*, 1931; Sedlacek *et al.*, 1996; Nansen *et al.*, 2004; Mohandass *et al.*, 2007; Fontenot *et al.*, 2012). Due to the wide spectrum of infested products, *P. interpunctella* is considered a major pest of commodities, causing extensive economic damages by reducing the quality grade of harvested products. Direct damage is represented by the feeding activity through all the larval instars, whereas indirect damage is due to the presence of larval frasses, exuviae and the silk-web (Allotey and Goswami, 1990). The capability of the IMM to efficiently colonize a wide spectrum of commodities poses the question of whether these metabolic capabilities are host constitutive or if they are provided or improved by the host-associated bacterial communities.

The establishment of mutualistic associations between the insect and microorganisms (bacteria, protozoa and fungi) plays an important role in the evolution of many insect lineages, such as termites and cockroaches (e.g., Bandi *et al.*, 1995; Brune, 1998; López-Sánchez *et al.*, 2009; Köhler *et al.*, 2012). Consortia of mutualistic bacteria provide the host with essential compounds, such as vitamins, amino acids and sterols, but they also contribute to the digestion of ingested materials (e.g., Moran *et al.*, 2003; Moran, 2006; Douglas, 2009; López-Sánchez *et al.*, 2009; McCutcheon *et al.*, 2009). In addition, it was recently postulated that a diversified host-associated microbiota may confer selective advantages to their host in changing environments, providing the capability to exploit different food sources, and thus, adapt to new ecological niches (Montagna *et al.*, 2015a; Sudakaran *et al.*, 2015).

Although the importance of bacterial communities on insect physiology is well recognized, the evolutionary and ecological determinants (e.g., diet, life stage, gender and host environment) that shape these communities are not well understood. The intuitive role played by the host's food substrate in shaping the associated bacterial communities has been previously established in several insects,

such as the cockroach *Periplaneta americana*, the cotton bollworm *Helicoverpa armigera*, the mealybug *Planococcus ficus*, and the red palm weevil *Rhynchophorus ferrugineus* (Kane and Breznak, 1991; Priya *et al*., 2012; Iasur-Kruh *et al*., 2015; Montagna *et al*., 2015a). Nevertheless, a limited or even null impact on the host-associated bacterial community was determined in the case of *Spodoptera littoralis* (Tang *et al*., 2012) and in a closely related leaf beetle species (Montagna *et al*., 2015b); in the latter case, the altitude of the sites where the specimens were collected generated the ecological trait that affected the insect's microbiota. These results suggest that the host's microbiota is influenced by a multitude of biological factors, such as the host trophic guilds, and environmental factors, such as the altitude of the collecting site.

In this study, using a culture-independent approach based on 454 pyrosequencing targeting the bacterial 16S rRNA gene, we used the IMM *P. interpunctella* as a study model to address the following biological questions: (i) Are the microbiotas associated with insects reared on five types of diet different? (ii) Is there a core microbiota associated with eggs that is retained until adulthood, regardless of diet and population origin? (iii) Are the microbiotas associated with adults transferred from food *via* ingestion? The presence of bacteria potentially hazardous for human and/or animal health was also evaluated.

Results and discussion

α, β-diversity and community structure

In total, the analysed samples yielded approximately 200,000 16S rRNA reads. After the removal of the low-quality bases, chimeras, mitochondrial and chloroplast sequences, a total of 79,933 16S rRNA bacterial sequences were retained, of which 78,278 of the bacterial reads were associated with *P. interpunctella* specimens (average 3,011) and 1,655 were associated with their food (average 331). The 16S rRNA bacterial gene sequences were clustered into 1611 bacterial operational taxonomic units (OTUs; Table 1). The bacterial communities associated with *P. interpunctella* reared on an artificial diet and pizzoccheri and characterized by the highest carbohydrate content had the highest species diversity (Table 1), with an average number ($\pm$SD) of 195 ± 49 and 192 ± 89.7 detected OTUs. Only 62 OTUs were associated with the insects reared on fava beans (high protein content). A relationship between the protein content of the insect's diet and the bacterial diversity associated with the host was previously observed in different species, such as in *Drosophila* spp., *Lymantria dispar* and *Blattella germanica* (Chandler *et al*., 2011; Mason and Raffa, 2014; Pérez-Cobas *et al*., 2015). The bacterial communities associated with the IMMs analysed in this study showed, on average, a significantly higher number of bacterial OTUs compared with OTU abundance reported in other studies for different caterpillars (Broderick *et al*., 2004; Yu *et al*., 2008; Pinto-Tomás *et al*., 2011). The recovered differences in the OTU richness may be explained by the fact that the IMMs, feeding on a variety of stored products, do not require a selected microbiota but will benefit from the diversified metabolic capabilities provided by a differentiated microbiota, whereas the latter feed on plant tissues, which are potentially rich in secondary compounds or allelochemicals that benefit a selected microbiota to overcome plant constitutive biochemical barriers.

The highest values of the Shannon and Pielou's evenness indices (Table 1) were recovered in the microbiotas associated with specimens feeding on moringa leaves and chili (3.70 and 3.36, 0.77 and 0.75 respectively), whereas the bacterial communities of the *P. interpunctella* feeding

Table 1. Diversity indices estimated for the bacterial communities associated with the analysed samples: eggs, adults and food substrates.

Identifier	Gender	Food	$S_{observed}$ [a]	S_{Chao} [b]	Shannon *H'*	Pielou *J'*
Eggs	-	-	121	215.1	3.79	0.79
Pad.1F	♀	Artificial diet	216	368.8	2.63	0.49
Pad.2F	♀	Artificial diet	158	438.1	2.70	0.53
Pad.3F	♀	Artificial diet	264	535.4	2.75	0.49
Pad.1M	♂	Artificial diet	140	217	2.37	0.48
Pad.2M	♂	Artificial diet	196	359.8	2.65	0.50
Ppiz.1F	♀	Pizzoccheri	177	331.1	2.26	0.44
Ppiz.2F	♀	Pizzoccheri	105	181.2	2.34	0.50
Ppiz.3F	♀	Pizzoccheri	162	300.8	2.31	0.45
Ppiz.1M	♂	Pizzoccheri	173	278.6	2.16	0.42
Ppiz.2M	♂	Pizzoccheri	344	552	2.18	0.37
Pchil.1F	♀	Chili	107	197	3.79	0.81
Pchil.2F	♀	Chili	138	207.5	3.74	0.76
Pchil.3F	♀	Chili	87	153	3.44	0.77
Pchil.1M	♂	Chili	44	70.3	2.91	0.77
Pchil.2M	♂	Chili	89	114	2.92	0.65
Pfav.1F	♀	Fava bean	60	90	3.13	0.76
Pfav.2F	♀	Fava bean	68	102.5	3.06	0.73
Pfav.3F	♀	Fava bean	19	21.5	1.98	0.67
Pfav.1M	♂	Fava bean	77	94.3	3.49	0.80
Pfav.2M	♂	Fava bean	86	117	2.94	0.66
Pmor.1F	♀	Moringa	99	162	3.50	0.76
Pmor.2F	♀	Moringa	130	161.7	3.81	0.78
Pmor.3F	♀	Moringa	104	128.5	3.89	0.84
Pmor.1M	♂	Moringa	111	166.5	3.46	0.74
Pmor.2M	♂	Moringa	160	222.7	3.83	0.76
Food_ad		Artificial diet	128	197.8	3.51	0.72
Food_piz		Pizzoccheri	52	98.8	3.35	0.85
Food_chil		Chili	80	111.1	2.32	0.53
Food_fav		Fava bean	1	1	0	-
Food_mor		Moring	2	2	0.64	0.92

a. Number of OTUs observed in the microbiota of each sample.
b. Number of OTUs estimated to be present in the microbiota of each sample.
P: *Plodia interpunctella*; ad: artificial diet; piz: pizzocchero; chil: chili; fav: fava beans; mor: moringa leaves; M: male; F: female.

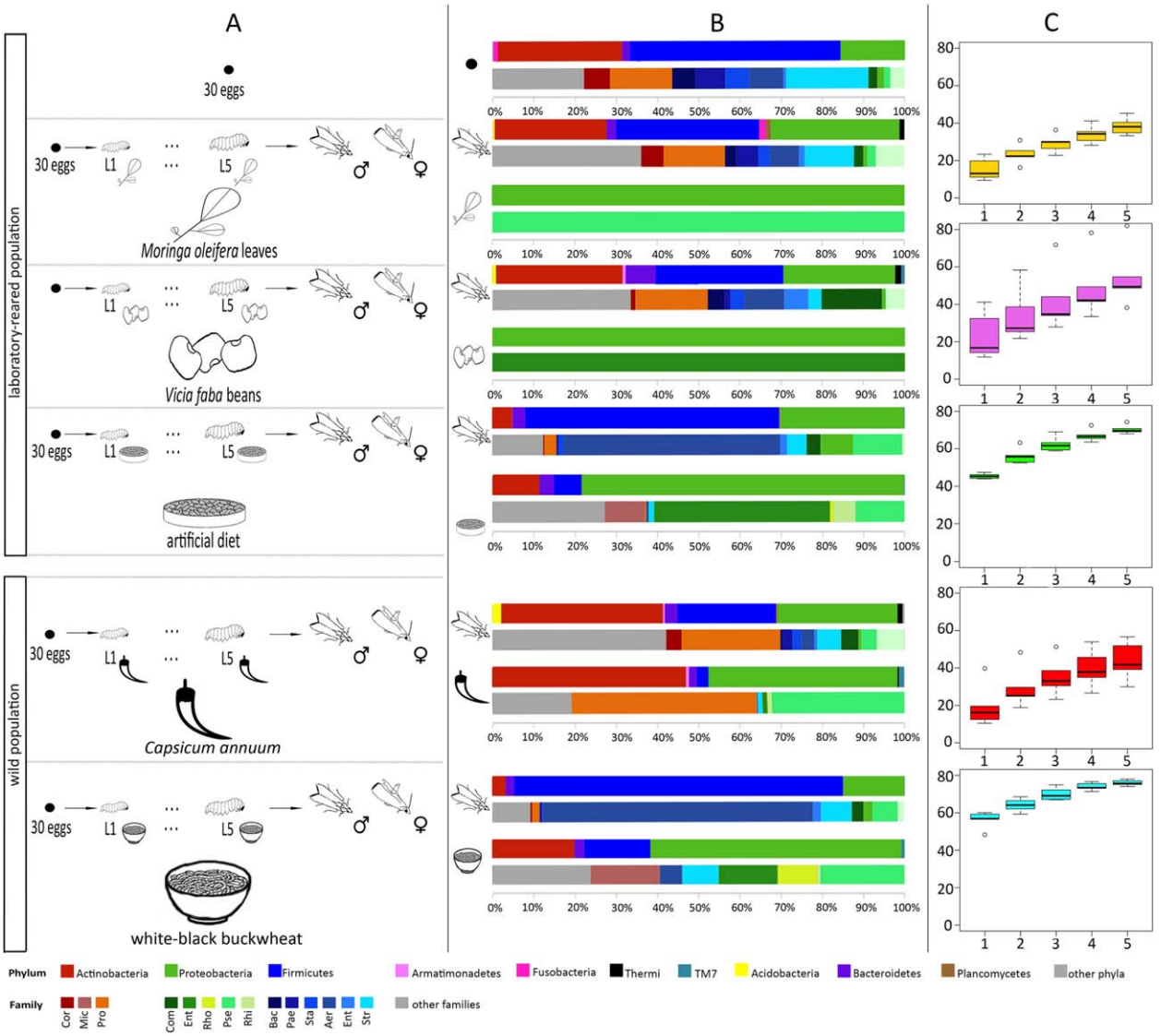

Fig. 1. Experimental design, taxonomic composition of the microbiotas and cumulative abundance of the dominant bacterial OTUs.
A. *Plodia interpunctella* from different populations (laboratory-reared and wild) were reared until adulthood on different substrates (artificial diet, *Moringa oleifera* leaves, *Vicia faba* beans, *Capsicum annum* powder, and white-black buckwheat noodles). DNA was extracted from the eggs of the laboratory-reared population, from five adults (three females and two males) of each group and from the food resources.
B. The taxonomical composition of the microbiotas associated with the analysed samples (eggs, adults and food substrate) reported at the phylum and family levels (upper and lower bars respectively). The average abundance across the five adult specimens is reported; only the bacterial families with an average abundance $\geq$5% in the IMM group are reported, and bacterial families with an abundance lower than 5% are grouped in the category *other families*. Abbreviations: Cor: Corynebacteriaceae; Mic: Microbacteriaceae; Pro: Propionibacteriaceae; Com: Comamonadaceae; Ent: Enterobacteriaceae; Rho: Rhodocyclaceae; Pse: Pseudomonadaceae; Rhi: Rhizobiaceae; Bac: Bacillaceae; Pae: Paenibacillaceae; Sta: Staphylococcaceae; Aer: Aerococcaceae; Ent: Enterococcaceae; Str: Streptococcaceae.
C. Box-plot of the cumulative abundance of the dominant bacterial OTUs (first five) associated with the adult moths reared on different substrates; yellow: moringa leaves; lilac: fava beans; green: artificial diet; red: chili powder; and cyan: white-black buckwheat.

on an artificial diet and pizzoccheri had the lowest values (Table 1). The fact that the bacterial communities associated with these latter samples are unbalanced was also confirmed by the abundance of the most dominant OTU, which, respectively, establishes 45.4% and 57.7% of the total retrieved bacterial 16S rRNA sequences (Fig. 1C). With respect to the microbiotas of the food sources, 128 bacterial OTUs were found in the artificial diet, whereas only one and two OTUs were detected in moringa and chili respectively.

The β-diversity, measured as Sørensen's dissimilarity, was estimated over the three groups of IMMs from the laboratory population sharing a common origin and genotype. The values obtained for the two components of the β-

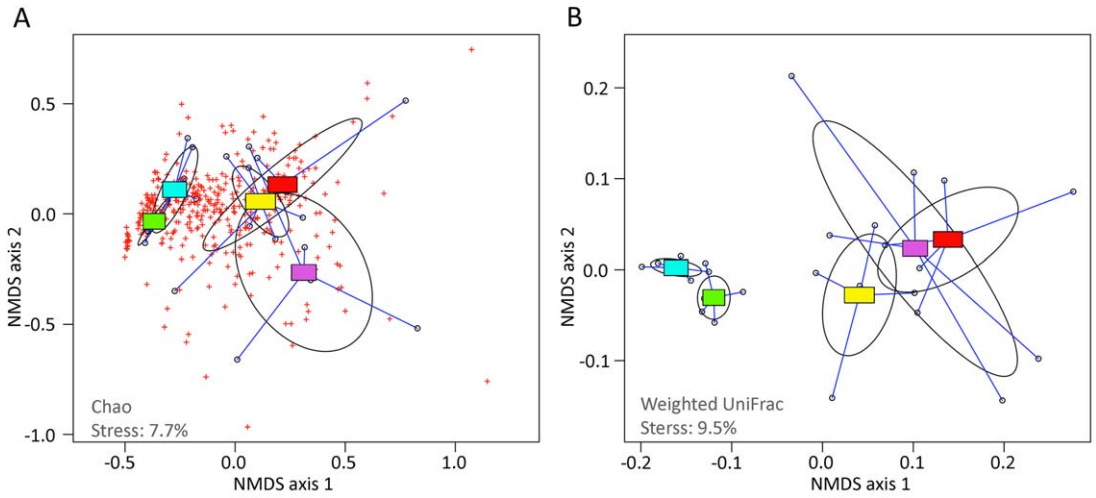

Fig. 2. Non-metric multidimensional scaling analysis of the bacterial community structure using the Chao (A) and Weighted Unifrac (B) metrics. The colour of rectangles indicates the different food resources; yellow: moringa leaves; lilac: fava beans; green: artificial diet; red: chili powder; and cyan: white-black buckwheat. The circular open dots indicate the single organism while the crosses, where present, represent the identified OTUs. The blue lines connect the individual microbiotas to the centroid values of each group. The ellipses represent a 95% confidence area around the mean of the group.

diversity, the turnover and the nestedness (respectively, $\beta_{SIM} = 0.56$ and $\beta_{NES} = 0.2$), indicate that only approximately 20% of the bacterial OTUs is shared among the three analysed groups. The differences in the bacterial communities associated with all five groups of *P. interpunctella* were confirmed by an ANOSIM analysis ($P < 0.001$).

Factors affecting the bacterial community structure

The non-metric multi-dimensional scaling analyses, performed on the moth-associated bacterial OTUs (Chao and Weighted UniFrac distance matrices), were fitted with factors potentially affecting the microbiota composition and structure as follows: (i) the diet; (ii) the IMM population of origin and (iii) the IMM gender (Fig. 2). The diet was the only tested trait able to explain the dissimilarity among the moth-associated bacterial communities ($NMDS_{Chao}$ $R^2 = 0.49$, $P = 0.0002$, stress = 0.08; $NMDS_{UniFrac}$ $R^2 = 0.63$, $P = 0.0001$, stress = 0.09), whereas the gender and population origin did not significantly affect the insect microbiotas. This result is in agreement with those recently achieved in the case of higher termites, *Blattella germanica* and in the larvae of *Melitaea cinxia* (Mikaelyan *et al.*, 2015; Pérez-Cobas *et al.*, 2015; Ruokolainen *et al.*, 2016) and provides further evidence for the relevant role of the host's diet in moulding the associated bacterial community. A possible hypothesis, supported by published studies and that should be tested in a rigorous framework, is that the microbiota associated with a generalist insect, in terms of exploitation of food resources, seems to be more influenced by diet typologies compared to those that are more specialized. An exemplary case of the latter is represented by the pine weevil (*Hylobius abietis*), where its microbiota remains almost unchanged across Europe, and interestingly, it differs from closely related weevil not associated with conifers but is highly similar to that of bark beetles that feed on conifers (Berasategui *et al.*, 2016).

On the basis of the Calinski-Harabasz index and a silhouette score of 0.38, two consistent groups of bacteria (hereafter, entomotypes from the Greek *entomon* meaning insect and referred to as consistent groups of bacteria present within the volume delimited by the exoskeleton), were identified within the IMM microbiotas. The two groups were clearly segregated by the first NMDS axis and were independent of the moth population of origin (Fig. 2). The first group is associated with the specimens that fed on an artificial diet and pizzoccheri, whereas, in the second group, the specimens fed on moringa, fava beans and chili. The taxonomic composition and the relative taxa abundance of the two groups are reported in the next section.

Bacterial taxonomic composition of the IMM and food microbiotas

The microbiotas associated with the IMMs fed an artificial diet and pizzoccheri as well as the bacterial community associated with the eggs were dominated by taxa belonging to Firmicutes (61.6%, 77% and 51% respectively; Fig. 1B and Table S2), whereas the microbiotas associated with the specimens fed chili, fava beans and moringa did not show a dominant taxonomic phylum because the relative abundances of Proteobateria, Firmicutes and Actinobacteria were roughly equivalent (Fig. 1B and Table

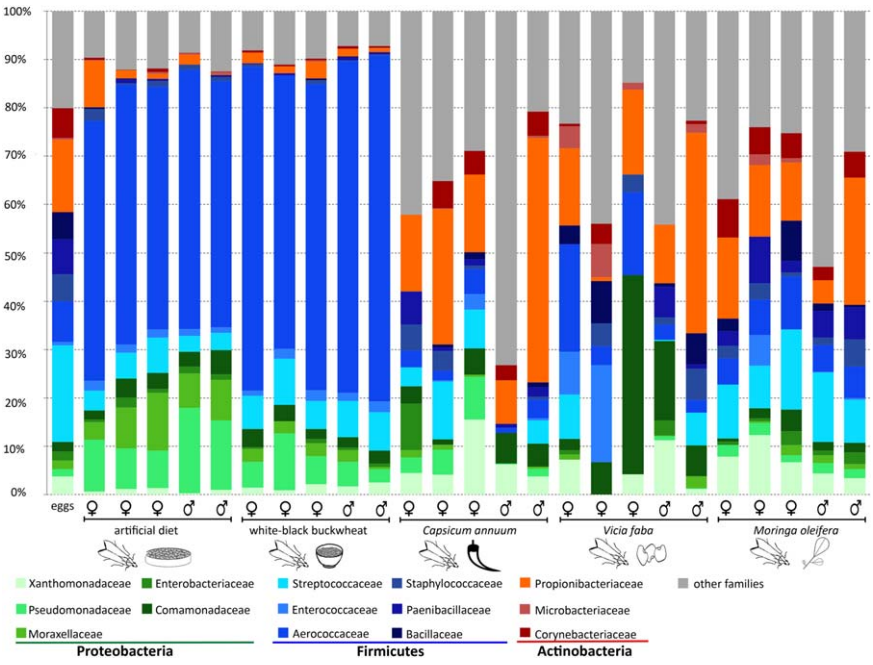

Fig. 3. Histogram representing the taxonomic assignment of the 16S rRNA gene sequences to the bacterial family for the IMM eggs and adults. Only the bacterial families with an abundance ≥5% in at least one moth specimen are reported, and bacterial families with an abundance lower than 5% are grouped in the category *other families*. Each bar corresponds to a single individual.

S2). Firmicutes was the dominant taxon and was also in the microbiota associated with *Spodoptera littoralis* (Tang *et al.*, 2012), whereas their abundance is limited in the bacterial communities associated with tropical saturniid caterpillars and in the cabbage root fly larvae (Pinto-Tomás *et al.*, 2011; Welte *et al.*, 2016).

Members of the family Aerococcaceae were the dominant component of the microbiotas associated with the moths reared on an artificial diet and the wild population that fed on pizzoccheri (52.5% ± 1.77% and 65.4% ± 5.76% respectively; Fig. 3 and Table S3). On the contrary, the bacterial communities associated with the *P. interpunctella* feeding on chili, fava bean and moringa were dominated by members of Propionibacteriaceae (20.7%, 17.6%, and 15% respectively; Fig. 3). A second major component in the microbiota of the moths feeding on chili was represented by Comamonadaceae (14.6%; Fig. 3). Streptococcaceae (19.9%) and Propionibacteriaceae (8.34%), which were the dominant families in the egg microbiotas (Fig. 3).

The genus *Atopococcus* (family Carnobacteriaceae) characterized the entomotype of the *P. interpunctella* reared on an artificial diet and pizzoccheri, representing 45.4% ± 1.42% and 56.2% ± 4.67%, respectively, of the 16S rRNA gene reads (Fig. 4A and Tables 2 and S4). The relative abundance of *Atopococcus* in the moths fed chili, moringa, fava beans and eggs was lower than that in the insects reared on an artificial diet and pizzoccheri (Tables 2 and S4); nevertheless, the presence of this bacterial genus in the majority of the analysed samples allows us to speculate that *Atopococcus* might represent a potential symbiont of *P. interpunctella*.

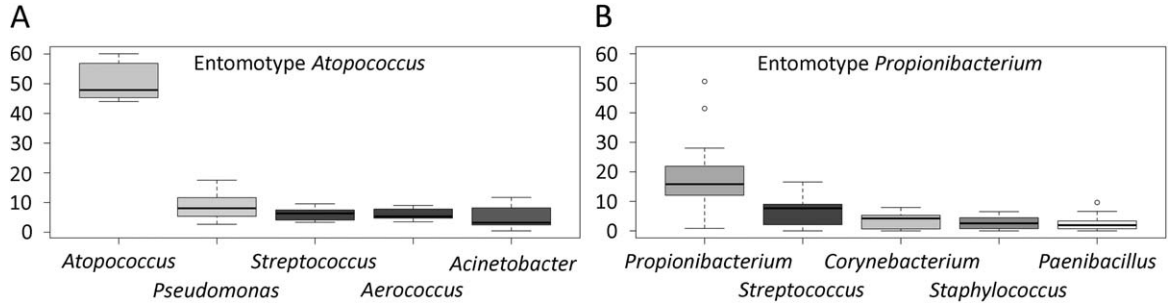

Fig. 4. Boxplots of the bacterial entomotypes characterising the two groups of IMMs identified by the NMDS and clustering analyses.
A. *Atopococcus* entomotype characterising the *P. interpunctella* specimens reared on an artificial diet and pizzoccheri.
B. *Propionibacterium* entomotype associated with the moths fed chili, fava beans and moringa.

Table 2. Genera of bacteria identified in the bacterial communities associated with the analysed samples with their relative abundance expressed as a percentage. Only taxa with an abundance ≥3% in at least one sample are reported.

	Food					*Plodia interpunctella*																									
Genus/samples ID	a	p	c	f	m	Artificial diet					Pizzoccheri					Chili					Fava bean					Moringa				
Deinococcus	-	-	-	-	-	-	-	-	-	-	-	0.3	-	0.2	-	2.6	0.6	-	3.9	0.1	2.8	-	-	-	-	-	-	-	-	-
Meiothermus	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.8	0.1	5.4	-	-	-	-
Brevibacterium	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	3.5	-	-	-	-	-	-	-	-	-	-	-	1.1
Corynebacterium	-	-	0.7	-	-	0.5	0.1	0.8	-	-	0.5	0.4	0.4	0.6	0.3	-	5.7	4.9	3.1	5	0.6	4.2	-	-	0.8	7.9	5.6	5.2	2.8	5.4
Microbacterium	0.6	-	-	-	-	0.2	-	-	-	-	-	-	0.2	-	0.1	-	-	-	-	0.4	1.6	6.8	-	-	0.1	-	-	0.9	0.1	-
Kocuria	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	2.2	0.4	-	-
Propionibacterium	0.5	-	44.9	-	-	9.7	1.7	1	2	0.2	2.2	1.4	3.5	1.6	0.8	15.8	28	15.6	9.1	50.6	15.9	0.8	17.6	12.1	41.5	16.8	14.8	12	4.8	26.2
Prevotella	-	-	-	-	-	-	-	0.4	-	0.8	0.3	0.5	0.6	0.1	0.3	-	0.2	-	-	3.7	-	-	-	-	-	-	-	0.4	-	-
Cloacibacterium	-	-	-	-	-	0.3	2.1	2.9	1.2	1.5	0.7	1	0.8	0.8	0.4	-	-	1.4	-	1.5	3.6	13.1	-	-	-	0.3	-	1.6	0.2	0.3
Exiguobacterium	-	-	-	-	-	-	0.1	0.2	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	0.3	5.1	-	-
Thermicanus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.1	-	-	-	-	-	1.3	-	-
Kenstanbolensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	-	-	-	3.8	8.7	-	-	-	-	-	8.2	-	0.5
Geobacillus	-	-	-	-	-	0.3	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	5.3	-	-	0.1	-	-
Brevibacillus	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	3.2	-	-	-	-	-	-	-	3.6	-	-	-	-	-	-
Paenibacillus	0.3	-	0.1	-	-	-	0.8	0.4	-	0.3	0.2	0.4	-	0.7	0.4	3.7	0.7	1.3	0.8	1.9	0.1	-	-	2.7	0.9	3.1	9.6	2.5	5.5	6.6
Staphylococcus	0.6	-	0.7	-	-	2.4	0.4	1.2	1	0.8	0.5	-	0.8	0.2	0.2	5.3	4.1	0.8	-	0.6	-	4.8	3.7	1.5	6.5	2.6	3.4	0.8	1.5	5.7
Atopococcus	0.3	4	-	-	-	46.2	45.3	44.2	47.4	44	56.6	48.3	56.9	59.3	60.1	1	1.8	0.2	1.1	2.1	7.4	-	-	-	-	1.7	3.7	6.5	4.8	1
Aerococcus	-	-	0.1	-	-	4.8	5.1	3.6	3.5	5.3	8.1	6.2	5.4	7.8	9	0.8	0.1	4.9	-	0.4	-	-	-	-	2.5	3.7	0.2	3.6	0.4	4.5
Facklamia	-	-	-	-	-	1.2	1.5	1.1	1.6	0.2	0.5	-	-	0.6	1	-	-	-	-	-	-	-	-	3.2	-	-	2.8	-	-	-
Enterococcus	-	-	0.2	-	-	2	1.6	1.7	1.1	0.2	1.1	1.6	2.2	1.5	1.9	-	-	1.8	-	0.4	8.8	20.1	-	-	-	-	5.9	-	-	0.4
Lactococcus	3.7	10.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.4	-	-	-	1.3
Streptococcus	-	-	1	-	-	4.1	5.4	7.3	3.4	3.5	6.9	9.6	5.8	7.5	8	4	12	8.1	-	4.8	9.2	-	-	0.2	6.8	8.8	8.8	16.6	14.4	7.7
Finegoldia	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	0.4	-	16.5	1	-	-	-	-	-	-	0.3	0.3	0.1	1.1
Fusobacterium	-	-	-	-	-	-	0.6	-	-	0.3	-	-	0.4	-	-	-	-	-	-	0.1	-	-	-	-	-	-	1.2	-	6.2	0.3
Agrobacterium	4.4	0.8	0.9	-	-	-	-	-	-	0.6	-	-	-	0.2	-	-	-	-	-	0.5	-	-	-	-	-	-	-	0.4	0.1	-
Burkholderia	0.5	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	5.5	-	-	-	-	1.8	0.1	-	-	-	-	-
Comomonas	-	-	0.2	-	-	1	2.1	0.3	1.8	1.2	1.8	0.4	0.2	0.6	1	-	0.9	3.3	-	-	-	-	-	0.8	5.7	0.6	0.1	0.5	-	0.9
Tepidimonas	-	-	0.3	-	-	-	0.2	-	-	0.1	-	-	0.5	-	0.1	2.9	-	2.1	6.3	2.8	1.4	-	-	12	0.1	-	0.6	-	-	0.2
Haemophilus	-	-	-	-	-	0.1	-	-	-	-	-	-	0.1	0.1	0.2	0.1	0.2	-	-	-	-	-	-	-	-	1	-	0.3	24.3	0.4
Acinetobacter	0.3	-	0.6	-	-	3.7	8.2	11.7	7	8.4	2.5	2.5	2.7	2.4	0.5	-	-	0.4	-	0.4	1.1	-	-	-	2.5	-	0.4	1.9	-	0.1
Pseudomonas	11.9	20.6	32.4	-	10	10.6	8.4	7.7	17.5	14.3	5.4	11.6	5.6	5	2.7	3.3	5.1	8.8	0.2	1.6	-	-	-	0.8	0.1	2.5	2.5	1.3	2.1	2

A: artificial diet, p: pizzochero, c: chili powder, f: fava beans, m: moringa leaves.

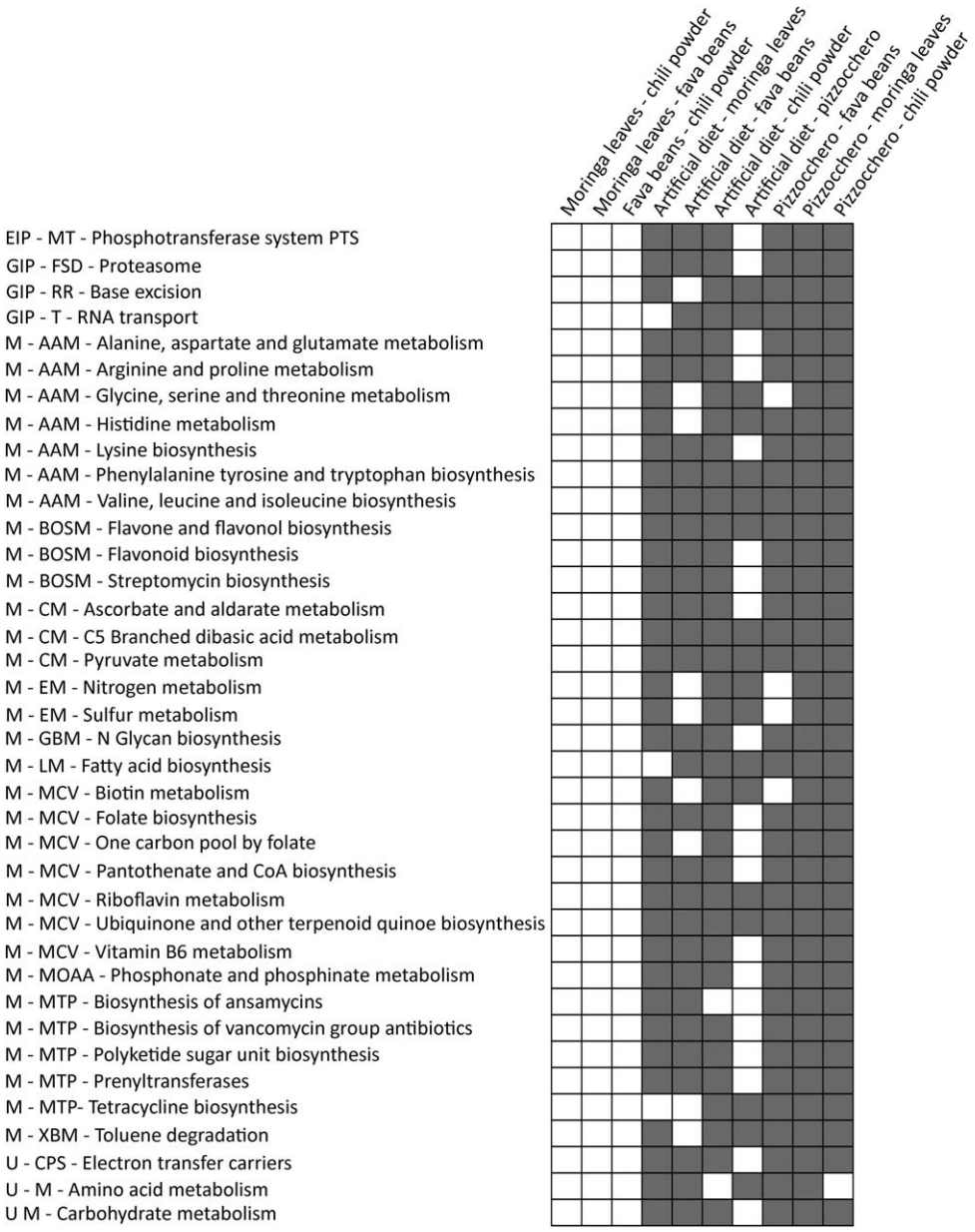

Fig. 5. Mann–Whitney–Wilcoxon table reporting the categories of functional potential, inferred from the bacterial 16S rRNA gene sequences for which differences among the five-group were recovered by the Kruskal–Wallis test. Mann–Whitney–Wilcoxon test were performed to assess the differences in the pairwise comparisons of IMM groups: the white squares indicate a $P \geq 0.05$, while the grey squares indicate a $P < 0.05$. EIP: environmental information processing; GIP: genetic information processing; M: metabolism; U: unclassified; MT: membrane transport; FSD: folding sorting and degradation; RR: replication and repair; T: translation; AAM: amino acid metabolism; BOSM: biosynthesis of other secondary metabolites; CM: carbohydrate metabolism; EM: energy metabolism; GBM: glycan biosynthesis and metabolism; LM: lipid metabolism; MCV: metabolism of cofactors and vitamins; MOAA: metabolism of other amino acids; MTP: metabolism of terpenoids and polyketides; XBM: xenobiotics biodegradation and metabolism; CPS: cellular processes and signalling.

Lactic acid bacteria (LAB) is found as a typical inhabitant in the insect gut (especially the foregut), such as in the wood- and soil-feeding termites (Bauer *et al*., 2000), cockroaches (Kane and Breznak, 1991) and honeybees (*Apis* spp.; Vásquez *et al*., 2009; Olofsson *et al*., 2011). The biological role of LAB with respect to insect physiology remains, at present, poorly investigated and understood. In cockroaches, lactate produced by LAB is used to support the animal's respiratory requirements (Kane and Breznak, 1991) or plays a fundamental role in the insect's aggregative behaviour (McFarlane and Alli, 1986); in honeybees, LAB is involved in the defense against the insect's pathogens (Vásquez *et al*., 2012). Based on previous evidence, we hypothesize that LAB-mediated pathogen protection represents a useful adaptation for the generalist trophic behaviour of IMM; the involvement of LAB lactate in the IMM aggregating semiochemicals cannot be excluded.

Metabolic potential

The metagenomic functional potential of the bacterial communities associated with the IMMs fed from different resources was inferred from the bacterial 16S rRNA sequences using PICRUSt (Langille *et al*., 2013; Table S5). Among the five groups of IMMs significant differences were recovered by the Kruskal–Wallis test ($P < 0.05$) in a

total of 38 functional categories (Fig. 5 and Table S6). The majority of these categories are related to metabolism (31), while only few cases were observed for genetic (3) and environmental (1) information processing (Fig. 5). Within the functional categories related to metabolism, the majority relapsed to the metabolism of amino acids, cofactors and vitamins, including both biosynthesis and catabolism (Fig. 5).

Interestingly, no differences in functional potential were assessed by the Mann–Whitney–Wilcoxon test with Hochberg correction for pairwise comparisons between the IMMs reared on moringa leaves, fava beans and chili pepper powder (Fig. 5 and Table S7). This result represents a metabolic confirmation of the pattern achieved by the NMDS analyses (Figs 2 and 4), where these three groups of insects belong to the same cluster and are characterized by the *Propionibacterium* entomotype. Similar results, with no differences in a total of 20 of 38 functional categories, were obtained for comparisons between the IMMs reared on an artificial diet and pizzoccheri and the two groups characterized by the *Atopococcus* entomotype and recovered in the same cluster by NMDS analyses (Figs 2 and 4). All of the remaining pairwise comparisons possessed the highest number of differences in the functional categories.

The achieved pattern is congruent with the detected bacterial entomotypes (Fig. 4) as well as with the differences in the nutrient composition of the food resources. An artificial diet and pizzoccheri possessed the highest amount of carbohydrates (more than 50%; Table S8) with a low protein content (high C:N ratio; Table S8), whereas, on the contrary, the moringa leaves, chili powder and fava beans are characterized by low carbohydrates and a high protein content (low C:N ratio; Table S8).

Impact of the eggs and diet microbiotas on the adult bacterial communities

The bacterial OTUs associated with the eggs/food resources and retained by a majority of the adult specimens (i.e., in at least three out of five specimens) were inferred from the OTU table and were then visualized through a co-occurrence table (Fig. 6 and Tables S9 and S10). The moths reared on an artificial diet, moringa leaves and fava beans, which came from the same population, retained, until adulthood, 35, 37 and 10 bacterial OTUs of the eggs microbiota, respectively (average number of OTU reads per sample of 109, 26 and 33; Figs 6, S1 and S2); in contrast, only a few (11 in the case of artificial diet) or none (in moringa and fava beans) of the bacterial OTUs associated with food resources were recovered in the insect microbiotas. Similar results were also achieved in the case of the IMMs from the population fed with pizzoccheri (41 OTUs were retained from the eggs and five from the food, with

Fig. 6. Co-occurrence OTUs table. For each IMM group, the co-occurrence table reports the IMM bacterial OTUs shared between the eggs from the laboratory-established population (left) and the food resource used for larval development (right). The colour of the rectangles indicates the different IMM groups: yellow: moringa leaves; lilac: fava beans; green: artificial diet; red: chili powder; and cyan: white-black buckwheat. The red squares of the table indicate the OTU co-occurrence, while the black squares indicate no co-occurrence of the bacterial OTU.

an average number of OTU reads per sample of 102; Figs 6, S1 and S2). A slightly different result was achieved in the case of the moths reared on chili powder, where the same number of OTUs (15), even if with taxonomic differences, was shared between the eggs (average number of

OTU reads per sample of 42) and the diet microbiotas. The co-occurrence of diet-associated bacteria in the host could be the result of two effects that are not easily distinguishable: (i) the inoculation of the insect with food-associated bacteria through ingestion, or alternatively, (ii) the contamination of food resources by insect frass conveying host bacteria and/or their DNA. Even if conclusive results could not be reached, it is reasonable to interpret our findings as a result of the second effect because digestive enzymes, pH and reactive oxygen species present in the insect gut usually kill most of the bacteria ingested with food (Vallet-Gely *et al.*, 2008; Garcia *et al.*, 2010). For this reason, the IMMs may convey bacteria to humans or animals on the infested harvested products, especially in the case of food consumed raw, such as spices, nuts and dry meat. In the present study, the OTUs identified as *Staphylococcus* (OTU 1242 and 3023; Fig. 6) and *Streptococcus* (OTU 4214; Fig. 6), which are two genera that include species pathogenic to humans and animals (Schleifer and Bell, 2009; Whiley and Hardie, 2009), were found in association with the infested chili pepper. Because our results are based only on a fragment of approximately 400 bp of the 16S rRNA and this topic is outside of the scope of our research, we believe that further investigations are required to isolate and identify these bacteria.

All of the groups of insects, at least in three specimens out of the analysed five, retained, until adulthood, six bacterial OTUs of the eggs microbiota (average number of OTU reads per sample of 53 and 49, in the adults and eggs respectively). These taxa, represented by *Propionibacterium* (three OTUs), *Staphylococcus* (two OTUs) and one OTU assigned to the family Xanthomonadaceae, are regarded as the core microbiota, i.e., the number and identity of the bacteria associated with the eggs and retained until insect adulthood independently from the population of origin (Fig. 6). The co-occurrence of bacterial phylotypes between sterilized eggs and the midgut of caterpillars was also observed in the case of *Rothschildia lebeau* (Pinto-Tomás *et al.*, 2011). The mechanisms behind the transmission of the core microbiota from egg to adult were not elucidated in this study; however, we can hypothesize that the newborn larvae, using the egg corion as the first meal after hatching, acquires the associated bacterial community from which partially derives the adult microbiota.

Venn diagrams, reporting the bacterial OTUs shared among the five groups of IMMs, highlighted the presence of 42 OTUs shared among the five groups of samples (Fig. S3). This number includes the core microbiota shared with the eggs plus 36 further bacterial OTUs.

Conclusion

The bacterial communities associated with the analysed Indian meal moths were relatively complex and diversified (see Table 1), with the maximum number of bacterial OTUs recovered in the moths reared on an artificial diet ($OTUs_{avg} = 195$) and the minimum in the case of the moths fed with fava beans ($OTUs_{avg} = 62$). Notably, the microbiotas associated with *P. interpunctella* were characterized by a relatively high species richness if compared with that of phytophagous caterpillars (e.g., Broderick *et al.*, 2004; Yu *et al.*, 2008; Pinto-Tomás *et al.*, 2011; Tang *et al.*, 2012). These results are in agreement with the hypothesis that diversified host-associated microbiotas may confer essential metabolic capabilities in exploiting highly different food sources, as in the case of *P. interpunctella*.

As reported for other insects (e.g., cockroaches, the cotton bollworm and the red palm weevil), and also in the case of *P. interpunctella*, the diet on which larvae have developed significantly contributes to the shaping of the structure and taxonomy of the associated bacterial communities. Nonetheless, considering the bacteria associated with food and shared with the insect, our findings provide evidence for a limited role of the diet in the active conveying of bacteria to the host. Conversely, the data from this study support the alternative hypothesis that diets with different compositions indirectly promote changes in the host-associated bacterial communities. The comparison of the microbiotas associated with eggs from the laboratory population with those associated with adults from different populations, including those from the laboratory, highlights the presence of a six-OTU bacterial core. Future studies, with the inclusion of specimens from different geographic areas and developed on different substrates, are required to confirm the presence of the identified bacterial core and, if confirmed, to elucidate the possible mechanisms of its transmission through generations (i.e., by feeding the egg corion or by transovarian transmission).

Interestingly, the five groups of IMMs were characterized by two different entomotypes (Figs 2 and 4). The first was dominated by bacteria of the genus *Atopococcus*, and the second was dominated by *Propionibacterium* and *Streptococcus*. The identification of LAB in the microbiota of *P. interpunctella* represent an interesting discovery because it was demonstrated that these bacteria could play an important role in insect biology. Apart from the possible biological role played by the LAB, these bacteria represent a possible target to control IMMs by developing symbiotic control strategies.

The functional potential of the IMMs microbiotas, predicted on the basis of 16S rRNA gene sequences, confirmed, from a metabolic point of view, the separation of bacterial communities associated with the IMM in two distinct groups (Figs 2, 4 and 5) and characterized by two different entomotypes. The majority of the between-group differences are related to metabolism (Fig. 5), and in particular, with the metabolism of amino acids, cofactors and vitamins. The achieved pattern is also congruent with the

differences of the food resources in nutrient composition, with the artificial diet and pizzoccheri (high carbohydrates and low proteins) on one side and moringa leaves, chili and fava beans (low carbohydrates and high protein) on the other.

Further investigations are required to understand the possible involvement of IMMs in conveying bacterial pathogens to the food substrates used for development.

Experimental procedures

Experimental design

All of the *Plodia interpunctella* specimens used in the assays were laboratory reared on different substrates under controlled conditions of light (light-dark cycle 16:8), humidity (RH = 70% ± 5%) and temperature (27 ± 1°C). In order to evaluate the impact of the diet in shaping the microbiotas associated with the IMM, 90 out of 120 eggs collected randomly from the same cohort of the laboratory-reared population, from a line maintained since 1980 in the rearing facilities established at the DEFENS, were reared until adulthood on the following different substrates: (i) 30 eggs on an artificial diet prepared according to Stampini and Locatelli (2007); (ii) 30 eggs on dried *Moringa oleifera* leaves (hereafter named moringa) and (iii) 30 eggs on *Vicia faba* beans (hereafter fava bean). The remaining 30 eggs were directly analysed in order to provide information on the native microbiotas associated with the laboratory-reared population. Newborn adults of two distinct wild populations collected in Italy and living, respectively, on dried *Capsicum annuum* (chili pepper; hereafter named chili) and on noodles of white-black buckwheat flour (named pizzoccheri) were processed for DNA extraction to better investigate the variability of the bacterial communities associated with the IMM. In order to avoid possible shifts in the composition and structure of the insect-associated microbiotas due to a seasonal variability (Jia *et al.*, 2013), all of the analysed moth specimens were collected during the first twenty days of May 2014.

DNA extraction

A total of five newborn adults (three females and two males) from each population were surface sterilized and DNA extraction was performed on the whole body (Montagna *et al.*, 2015b). In order to characterize their internal and external microbiotas, the eggs were not surface sterilized because the outer shell of an insect's egg, called the chorion, is the first meal of the newborn larvae. The DNA was extracted from each sample using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. The concentration and purity of the extracted DNA were determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). In order to characterize the bacteria associated with the five substrates used as food sources, DNA was extracted using the DNeasy Plant Kit (Qiagen) starting from 0.5 g of food after homogenisation with liquid nitrogen of the whole amount of substrate on which the IMMs were developed.

Pyrosequencing

The bacterial communities associated with the insects and food samples were characterized by targeting the V1-V3 variable regions of the 16S rRNA gene using universal primers for bacteria as reported in a previous studies (Mazza *et al.*, 2014; Montagna *et al.*, 2015a,b). A commercial service performed the PCR reactions and the 454 pyrosequencing by Roche 454 GS FLX Titanium (MR DNA, Shallowater, TX). The 16S rRNA gene sequences obtained by the 454 pyrosequencing assays were deposited in the European Nucleotide Archive (Accession No. PRJEB14361), mapping file is provided in Table S1.

Using a dedicated filtering script working under the QIIME platform (Caporaso *et al.*, 2010), the obtained raw 16S rRNA V1-V3 gene sequences were trimmed to remove adaptors, low quality base calls (<30 Phred score) and short-sized sequences (retained sequences between 350 and 500 bp). Chimeras were removed with *Chimeraslayer*, and the high quality non-chimeric sequences were clustered into operational taxonomic units (OTUs), based on a sequence identity threshold of 97%, using Uclust (Edgar 2010). PyNast (Caporaso *et al.*, 2010) was used to align a representative sequence from each OTU with Greengenes (http://greengenes.lbl.gov/); these representative sequences were then taxonomically classified by BLASTn-based comparisons to the Greengenes and Silva databases. After a first analysis, the OTUs identified as plant chloroplast and mitochondria were removed using an ad hoc perl script. This output was used for a second run of QIIME analyses.

Diversity and statistical analyses

The resulting set of OTUs was used as input for the diversity analyses carried out with different R packages (R Project 3.0.2; http://cran.r-project.org/).

The diversity indices and the following analysis (exceptions are specified) were estimated using the R package *vegan* (Dixon, 2003). The Shannon H index (Shannon, 1948), Pielou's evenness (Pielou, 1975) and the total species richness index Chao 1 (Chao and Lee, 1992; Chao, 1984) were estimated. The bacterial communities associated with the moth samples were ordinated, according to OTU composition similarity, using the distance-based non-metric multi-dimensional scaling (NMDS; Kruskal, 1964) with the Chao probabilistic distance (Chao *et al.*, 2005) as described in a previous work (Montagna *et al.*, 2015a,b). Correlations between the diet typologies (i.e., artificial diet, moringa, fava bean, chili and pizzoccheri), the origin of the samples (i.e., lab reared specimens versus wild populations) and the specimen's gender with the insect-associated bacterial communities were tested by fitting the NMDS scores using the *envfit* function. The significance of the fitted factors was assessed by permutations (9999) of the dissimilarity OTUs matrix. The same analyses were also performed on the pairwise UniFrac distance matrix (rarefaction on the minimum samples size) calculated using the taxonomy-independent weighted UniFrac metric (Lozupone *et al.*, 2007), which accounts for similarities in the phylogenetic structure of the communities associated to each moth samples. The best number of clusters identified by the NMDS analyses was evaluated using the Calinski-Harabasz criterion

(Calinski and Harabasz, 1974), and their consistency was evaluated using silhouette scores (Rousseeuw, 1987).

The differences among the bacterial communities associated with the IMMs fed with different substrates were estimated by a nonparametric one-way analysis of similarity (ANOSIM; Clarke, 1993). Shifts in the bacterial community structure associated with the three groups of moths reared in laboratory on different substrates (i.e., artificial diet, moringa leaves and fava beans) were estimated with the R package *betapart* (Baselga and Orme, 2012) using Simpson's dissimilarity index as described in Montagna *et al*. (2015b).

In order to evaluate the bacterial OTUs associated with the eggs and those retained until adulthood, as well as those shared between the insects and their food, a co-occurrence (i.e., eggs-IMM and IMM-food microbiotas) table was obtained. The OTU table was filtered using the following criterion: the co-occurrence was assigned when a specific bacterial OTU of the eggs or food microbiotas was present in the microbiota of at least three insects feeding on the same resources. Venn diagrams, acquired with the R package *gplots*, provide a visualisation of the bacteria OTUs numbers, establishing the IMM bacterial core and the bacterial community shared among the adult insects, its food substrates and the eggs. In the Venn diagrams, the presence of a bacterial OTU is assigned to the group of insects feeding on the same resources when: (i) the OTU is reported for at least one specimen of the group and (ii) the OTU is reported for at least three specimens of the group, as for the co-occurrence table.

Predictive functional profiling

The functional profiles of the bacterial communities were investigated using PICRUSt (Langille *et al*., 2013). The OTUs were closed-reference picked against Greengenes (GG version 13.5) using QIIME v 1.9 according to the online protocol. The bacterial metagenome was predicted for the bacterial communities associated with the IMM adults.

The table with the predicted L3-functions counts per-samples, according to the Cluster of Orthologous Groups (Tatusov *et al*., 1997) and identifiers adopted by KEGG Orthology (Kanehisa *et al*., 2012), was cleaned up by removing the categories not related to the bacterial physiology/metabolism and the null categories. The nearest sequenced taxon index median value for the PICRUSt predictions was 0.08 (SD = 0.04), reflecting good availability for the reference genomes on which the metagenome function predictions were based. The overall differences in the amount of counts were estimated by the non-parametric Kruskal–Wallis test (Kruskal and Wallis, 1952) after assessing the homogeneity of the variance among the groups through the Levene test (Levene, 1960). If the *P*-value of Kruskal–Wallis test resulted in a significance level that was less than a 0.05, then a Mann–Whitney–Wilcoxon test with a Hochberg correction (Mann and Whitney, 1947) was applied to compare the pairs of groups. These tests were performed using the lawstat-package (Hui *et al*., 2008) in the R software, and the results are visualized by a table.

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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. Venn diagram pairs showing the bacterial OTUs (at 97% similarity) shared by eggs and moth groups. The presence of a bacterial OTU is assigned to the group of insects feeding on the same resources when it is reported for at least three specimens of the group.

Fig. S2. Venn diagrams showing the bacterial OTUs (at 97% similarity) shared by eggs and the three groups of adult moths from the laboratory population. The presence of a bacterial OTU is assigned to the group of insects feeding on the same resources when it is reported for at least one specimen of the group (A) and at least in three specimens of the group (B).

Fig. S3. Venn diagrams showing the bacterial OTUs (at 97% similarity) shared by the five groups of adult IMMs. The presence of a bacterial OTU is assigned to the group of insects feeding on the same resources when it is reported for at least three specimens of the group (A) and one specimen of the group (B).

Table S1. Mapping file.

Table S2. OTUs abundance. OTUs identification has been performed at Phylum level.

Table S3. OTUs abundance. OTUs identification has been performed at Family level.

Table S4. OTUs abundance. OTUs identification has been performed at Genus level.

Table S5. Predicted functional profiles infereed on the base of the bacterial 16S rRNA, table with the abundance of the predicted L3-functions counts per-samples.

Table S6. P-values obtained with Kruskal–Wallis test for the predicted L3 functional potential.

Table S7. P-values obtained by pairwise Wilcoxon–Mann–Withney test for the predicted L3 functional potential.

Table S8. Macronutrient composition of different substrates.

Table S9. Heat map of the IMM bacterial OTUs shared with their diet and with the eggs from laboratory population. Blue squares indicate OTU co-occurrence, while the black squares indicate no co-occurrence of the bacterial OTU. The OTU presence is assigned to the corresponding group of Plodia when it has been recovered in at least one specimens belonging to the group.

Table S10. Heat map of the IMM bacterial OTUs shared with their diet and with the eggs from laboratory population. Yellow squares indicate OTU co-occurrence, while the black squares indicate no co-occurrence of the bacterial OTU. The OTU presence is assigned to the corresponding group of Plodia when it has been recovered in at least two specimens belonging to the group.