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Influence of diets and developmental stages on microbiota associated with the Indian Meal Moth *Plodia interpunctella*

PhD thesist

Valeria **MEREGHETTI**

R10838

Scientific Tutor: Matteo **MONTAGNA**, PhD

Co-tutor: Bessem **CHOUAIA**, PhD

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5. ANNEX

1. ABSTRACT

Plodia interpunctella, also named indian meal moth (IMM), is considered a stored product pest due to its high polyphagous activity on a wide range of human food such as nuts, dry fruits and meat. Due to their ecology these pest can also carry a risk for the human health. However, to date, little is known on the microbial communities associated with this pest and the factors that may influence them. Recent studies highlighted the presence of three potential factors that may influence the bacteria community of moth pests, namely, diet, origin of the populations and the developmental stages. Due to scarcity of studies investigating the microbiota associated with *P. interpunctella*, I decided to investigate the bacterial community of this moth pest, using NGS approaches (454-pyrosequencing and Illumina platform). The aim of this thesis was to: *i)* evaluate the impact of different diets on the microbiota associated with *P. interpunctella*; *ii)* characterize the microbial community changes across different ontogenetic stages of *P. interpunctella*. In particular the possible effect of the diet on bacterial community of this pest was investigated by testing different diets (artificial diet, powder of *Moringa oleifera* leaves and *Vicia faba* beans for the laboratory reared population; *Capsicum annuum* chilli and white-black buckwheat for the wild/field populations) and among different life stages as eggs, first and last instar larvae and adults (both males and females). The main result showed that the only factor influencing the bacterial community associated to *P. interpunctella* was the diet, highlighting the presence of two different bacterial communities (entomotypes) in relation to the different nutrient composition of the diet (etomotype *Atopococcus* associated to moths reared on artificial diet and white- black buckewheat, entomotype *Proprionibacterium* associated to IMM reared on *V. faba* beans, *C. annuum* chilli and *M. oleifera* leaves). Moreover, the microbiota do not change among the analysed developmental stages of *P. interpunctella* and was dominated by the genus *Burkholderia*.

The results achieved during the PhD program and reported in the present thesis were published as research articles (Evidence of a bacterial core in the stored products pest *Plodia interpunctella*: the influence of different diets; Evidence for a conserved microbiota across the different developmental

stages of *Plodia interpunctella* and as review (New insights into the microbiota of moth pests).

1. RIASSUNTO

Plodia interpunctella, comunemente chiamata tignola fasciata del grano, è un lepidottero polifago che infesta una grande varietà di derrate alimentari (cereali, frutta secca, noccioline, carne secca, etc..). Data la sua ecologia, *P. interpunctella* è considerata inoltre un insetto vettore di potenziali patogeni umani. Tuttavia, le informazioni riguardanti le comunità batteriche associate a questo insetto e i fattori che possono influenzare tali comunità sono, ad oggi, poco conosciute. Recenti studi hanno evidenziato la presenza di tre possibili fattori in grado di influenzare le comunità batteriche dei lepidotteri che attaccano le derrate alimentari: *i)* la dieta; *ii)* l'origine della popolazione e; *iii)* lo stadio di sviluppo. Data la scarsità di studi riguardanti tale lepidottero, il principale scopo di questa tesi di dottorato è stato quello di investigare il ruolo del microbiota associato a *P. interpunctella* utilizzando le tecnologie di Next Generation Sequencing (pirosequenziamento 454 e piattaforma Illumina). Gli scopi di questa tesi sono stati: *i)* valutare l'impatto di diverse diete sul microbiota associato a *P. interpunctella*; *ii)* caratterizzare eventuali cambiamenti della comunità batterica in diversi stadi di sviluppo di *P. interpunctella*. In particolare, il possibile effetto svolto dalla dieta sulla comunità batterica di *P. interpunctella* è stato investigato testando cinque diversi substrati (dieta artificiale, foglie polverizzate di *Moringa oleifera*, e *Vicia faba* per la popolazione di laboratorio; *Capsicum annuum* e pizzoccheri per la popolazione di campo) e diversi stadi di sviluppo (uova, prima ed ultima età larvale e adulti, maschi e femmine). I risultati hanno mostrato che l'unico fattore in grado di influenzare la comunità batterica associata a *P. interpunctella* è la dieta. In particolare sono stati caratterizzate due distinte comunità batteriche (entomotipi) in relazione alla diversa composizione nutrizionale della dieta (entomotipo *Atopococcus* associato a *P. interpunctella* alimentate su dieta artificiale e pizzoccheri, entomotipo *Propionibacterium* associato agli insetti alimentati su *V. faba*, *C. annuum* e *M. oleifera*). Per quanto riguarda la caratterizzazione delle comunità batteriche attraverso i vari stadi di sviluppo di *P. interpunctella* è stato osservato che il microbiota non varia ed in particolare è dominato dal genere *Burkholderia*.

I risultati riportati nella presente tesi di dottorato sono stati pubblicati sotto forma di articoli scientifici (Evidence of a bacterial core in the stored products pest *Plodia interpunctella*: the influence of different diets; Evidence for a

conserved microbiota across the different developmental stages of *Plodia interpunctella*) e review scientifica (New insights into the microbiota of moth pests).

2. INTRODUCTION

2.1 INSECT-BACTERIA SYMBIOSIS

Insects are widespread colonized by bacteria. They are recognized as multi-habitat organisms because usually, 1-10% of the insect's biomass is represented by bacteria. This biomass percentage was also named microbiota (Douglas, 2015). Despite culture-dependent and culture independent approaches, the bacterial communities associated to insect of several orders were the focus of different studies. Insect bacterial community housed different type of bacteria, from pathogens to symbionts. Symbiosis is extremely important for the insect's functions and fitness. Although the presence of different kinds of symbioses (mutualistic, commensal and antagonistic), mutualism is ubiquitous in all types of ecosystem (Boucher et al., 1982; Bronstein, 2009).

Symbionts could inhabit different insect compartments such as cuticle, gut, hemocoel and were present also within insect cells (Douglas, 2015). Although cuticle was recognised as a physical and chemical barrier against pathogens (Vallet-Gely et al., 2008), insects have evolved some particular structure in which bacteria are growing. As example, ants evolved some glandular invaginations (foveae) that harbour bacteria belonging to the genus *Pseudonocardia* (Currie et al., 2006). Furthermore, the gut possessed extremely different unfavourable conditions (e.g. oxygen content, pH, redox potential) among insect orders and developmental stages, represent a good accession point for some possible symbionts. In particular, Lepidoptera showed a very inhospitable gut lumen, due to the alkalinity of their pH (pH 8-12), but it has been demonstrated that the symbiont *Enterococcus mundtii* persisted in the gut trough metamorphosis of *Spodoptera littoralis* and secreted antimicrobial compound against pathogens (Shao et al., 2016). In others insect species, gut represent a site for the microbial adhesion of pathogens that can be transmitted to plant, human or animals, as in the case of the leafhopper *Graphocephala atropunctata*, that harbour *Xylella fastidiosa* (Newman et al., 2004). Furthermore, primary symbionts (P-symbiont), housed within bacteriocytes, are vertically transmitted from mother to their offspring (Buchner, 1965). These obligate intracellular symbionts are essentials

for the insect, providing some essential compounds that are absent in the diet host (Feldhaar, 2011). Bacteriocytes are widely distributed in some insect orders, such as Blattodea, Phthiraptera, Hemiptera and Coleoptera (Douglas, 1989, Braendle et al., 2003). In contrast, secondary symbionts (S-symbiont), maybe associated to bacteriocytes, are able to colonize different tissue (e.g., salivary glands, malphigian tubules and reproductive organs (Mitsuhashi et al., 2002; Sacchi et al., 2008; Bution et al., 2008; Dobson et al., 1999). S-symbionts are not essential for the insect, and it is not necessarily harboured in all individual of a population (van den Bosch & Welte, 2016). However, S-symbiont can confer important ecological traits, especially in aphids where a mutation in the bacteria of the genus *Buchnera* mediate thermal tolerance (Dunbar et al., 2007), facilitate use of novel hosts (Tsuchida et al., 2011) or *Hamiltonella defensa* and *Regiella insecticola* that defenced aphids against parasitoids wasp and fungal pathogen respectively (Oliver et al., 2003, 2005; Scaraborough et al., 2005).

Symbionts, as well as *Wolbachia*, ensure their transmission through insect developing different mechanism such as, embryonic male-killing, larval male-killing, feminization, parthenogenesis induction and cytoplasmatic incompatibility (Stouthamer et al., 1999, Engelstadter & Hurst, 2009).

Insect symbionts are recognised have a great impact on insect phenotype, helping the host in several essential activities. As example, a lot of studies highlight that symbiont play a key role in insect nutrition. To date, insect nutritional symbionts are related to the class of Gammaproteobacteria, which their genome is characterised by: small genome sizes, low G+C content and increased substitution rates (McCutcheon & Moran, 2007). The capability of symbiotic bacteria to synthetize essential amino acids is well documented. The main example was represented by the genome of the bacterium *Buchnera aphidicola*, belonging to the family of Enterobacteriaceae, which retain genes for the synthesis of some essential amino acids that the black bean aphid (*Aphis fabae*) are not able to produce (Shigenobu et al., 2000; Douglas, 2001). The production of amino acids by symbiont bacteria is confirmed for other insects such as *Periplaneta americana* (Sabree et al., 2009), *Reticulitermes flavipes* (Potrikus & Breznak, 1981) *Camponotus floridanus* (Feldhaar et al., 2007) and *Nilaparvata lugens* (Sasaki et al., 1996). B

vitamins and cofactors are also a crucial nutrients provide by symbionts to the host. *Baumannia cicadellinicola*, harboured by the sharpshooter *Homodalisca coagulata*, is an obligate symbiont that contributes to the synthesis of vitamins of B groups (B_1, B_2, B_3, B_5 an B_6) as well as biotin and folic acid (Wu et al., 2006). Furthermore, the blood-sucking tsetse fly, maybe due to their restricted diet, is not able to synthetize some essential compound, but the endocellular obligate symbiont *Wigglesworthia glossinidia* provide B vitamins and cofactors (Akman et al., 2002). Symbionts also make a critical cotributions in the nitrogen fixation (Morales-Jiménez et al., 2009; Nardi et al., 2002; Newell & Douglas, 2014) processes and in the degradation of plant cell wall polysaccharides such as cellulose and hemicellulose (Calderon-Cortes et al., 2012).

The insect's immune system is helped by symbionts against natural enemies and/or pathogens. Antimicrobial compounds are known to be one of the symbiont mechanisms to overcome infection in insects especially in the case of fungal infestation as in the cases of the wasp European beewolf (*Philanthus triangulum*) that harbour an antibiotic-producing bacterium (*Streptomyces* spp.) (Kaltenpoth et al., 2005) or in the bark beetle *Dentroctonous rufipennis* that contain *Micrococcus luteus* in their oral secretion (Cardoza et al., 2006) that protects insect from fungal infestation.

Talking about symbiont, one particular aspect to take into account, is the symbiont-mediated detoxification or "detoxifying symbiosis" (van den Bosch & Welte, 2016). Although insects showed their detoxification mechanism (e.g. cytochrome P450 monooxygenases, glutathione S-transferases and esterares) (Scott et al., 1998), some symbionts are responsible for the detoxification of plant secondary metabolites or insecticides in several insect orders. Symbiont-degradation of insecticide is well documented in the Japanese legume pest *Riptortus pedestris* and other Heteroptera species by Kikuchi and colleagues (2007, 2011). Furthermore, *Bacillus cereus,* in the Lepidoptera *Plutella xylostella,* is able to utilise the insecticide indoxacarb in their metabolism (Ramya et al., 2016). Insecticides are not the only harmful compounds for insects. As a matter of fact, the plants on which the insects feed may contain secondary metabolites that normally are produced by plants against the insect. Insect overcomes to this toxicity harbouring several symbionts. As

example, the conifers on which the mountain beetle *Dentroctonous ponderosae* feed contain toxic terpenes for protection (Hamberger et al., 2011) and *Pseudomonas* and *Rhanella* genus within insect degraded this compound (Adams et al., 2013). Another forest pest, the gypsy moth Lymantria dispar, harbour in their microbiota some bacteria responsible for the monoterpene degradations (Mason et al., 2014, 2015). Larvae of *Batrocera oleae*, fed on unripe olives that are rich in oleuropein. Ben-Yosef and colleagues (2015) showed that *Candidatus Erwinia dacicola* permitted a healthy growth of the *B. oleae* larvae, while aposymbiotic larvae do not complete their development. Other examples, it has been reported for Delia radicum and Hypothenemus hampei, that harbour symbionts able to degrade isothiocyanates and alkaloids respectively (Welte et al., 2016; Ceja-Navarro et al., 2015).

Symbiosis especially mutualisms, enhance invasions of many alien species. In particular, some symbionts turns insect into a invasive one (Lu et al., 2016). Although many of these interactions are related to fungi-insect associations, *Bemisia tabaci* showed an invasive profile characterized by a faster development and higher offspring proportion, due to their relationship with the symbiont *Rickettsia* sp. near *bellii* (Himler et al., 2011).

In conclusion the multitude of existence symbioses between bacteria and almost all insect order, ranging from "nutritional", "defensive", "detoxifying" or "invasive" symbiosis, helped the insect in their fitness and the adaptation of new possible ecological niche.

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2.2 THE BACTERIAL COMMUNITIES OF MOTH PESTS

2.2.1 ABSTRACT

In recent years, next generation sequencing (NGS) technologies have helped improve our understanding of the bacterial communities associated with insects, shedding light on their wide taxonomic and functional diversity. To date, little is known about the microbiota of lepidopterans, which includes some of the most damaging agricultural and forest pests worldwide. Studying their microbiota could help us better understand their ecology and offer insights into developing new pest control strategies. In this paper, we review the literature pertaining to the microbiota of lepidopterans with a focus on pests, and highlight potential recurrent patterns regarding microbiota structure and composition.

2.2.2 INTRODUCTION

Insects represent the most successful taxa of eukaryotic life, being able to colonize almost all environments, including Antarctica, which is populated by some species of chironomids (e.g., *Belgica antarctica*, *Eretmoptera murphyi*, and *Parochlus steinenii*) (Grimaldi & Engel, 2005; Allegrucci et al., 2005). Many insects are beneficial to plants, playing important roles in seed dispersal, pollination, and plant defense (by feeding upon herbivores, for example) (Dillon & Dillon, 2004). On the other hand, there are also damaging insects that feed on crops, forest and ornamental plants, or stored products, and, for these reasons, are they considered pests. Less than 0.5% of the known species of insects are considered pests, but the severe damage they cause results in losses of billions of dollars annually and represents a great challenge regarding food security (Oliveira et al., 2004; Fitt, 1989). Pest insects belong to various orders, such as Coleoptera (e.g., *Diabrotica virgifera*, *Sithophilus* spp., *Dendroctonus* spp.), Diptera (e.g., *Drosophila suzukii*, *Bactrocera oleae*), Hemiptera (e.g., *Psylla betulae*, *Psylla piri*, *Aphis* spp., *Myzus persicae*, *Chermes viridis*), and Lepidoptera (e.g., *Ostrinia nubilalis*, *Eupoecilia ambiguella*, *Helicoverpa zea*, *Lymantria dispar*). Within Lepidoptera, moths, comprising approximately 160,000 species, are major pests in different parts of

the world (Covell, 1984). The larvae are the primary stage responsible for plant or food damage, as they feed voraciously on leaves, flowers and seeds (Carter, 1984). Nowadays, several techniques are used to control pest insects, such as the sterile insect technique (SIT), chemical insecticides, or biological pest control using predators and parasitoids (Bartlett & Staten, 1996; Wright, 1984; Greathead & Greathead, 1992). More recently, the potential exploitation of symbionts that are associated with insects has emerged as a promising tool in insect pest management (van den Bosch & Welte, 2016). This was made possible by a better understanding of the relationship between bacterial symbionts and their insect hosts, in part due to the advent of next generation sequencing (NGS). Many studies describe the microbial communities associated with insects using classical culture-dependent approaches, but with the advent of NGS approaches, it has been possible to further improve and refine this knowledge. Indeed, recent work suggests that the microbiota plays an important role during insect developmental stages and is involved in many host activities, such as nutrition (Douglas, 1998; Gil et al., 2004; Brune, 2014, Salem et al., 2014), reproduction (Dillon & Dillon, 2004), and protection against insecticides or plant secondary metabolites (e.g., terpenes, caffeine, nicotine, cocaine, isothiocyanates) (Marmulla & Harder, 2014; Summers et al., 2012; Summers et al., 2015; Brandsch, 2006; Fan et al., 2011; Narasimhan et al., 2012). The symbiotic relationship between bacteria and insects is very important, given the evidence that a strict association exists between some groups of insects and a core of commensal bacteria inhabiting the gut (Dillon & Dillon, 2004, Tang et al., 2012; Morrison et al., 2009). The structure of insect bacterial communities can be influenced by a multitude of factors, such as pH, host phylogeny, life stage, host environment, and diet (Montagna et al., 2015a; Montagna et al., 2015b). These different factors are not necessarily exclusive, but recent work has suggested that the microbiota can be altered by diet and host phylogeny (Montagna et al., 2015a; Montagna et al., 2015b; Colman et al., 2012; Brucker et al., 2013). In this review, we summarize the information regarding the bacterial communities of the principal moth pests obtained mainly using NGS approaches, organizing moth pests in two main groups: forest and crop pests. Furthermore, we investigated the possible recursive bacterial core within

each moth group and potential factors that may shape the structure and composition of the moth-associated microbiota.

2.2.3 MICROBIOTA: AN OVERVIEW

Culture-independent tools, such as NGS technologies, offer the chance to interrogate the high diversity of unculturable microorganisms (bacteria, fungi, and viruses) that are present in a large variety of matrices, such as soil, foods, and animals (including arthropods) (Yergeau et al., 2012; Rogers et al., 2010; Ceuppens et al., 2017; Hubert et al., 2017). Furthermore, these tools provide a more comprehensive view of the host's microbial inhabitants, allowing us to answer the general question: "What types of bacteria are present, and what are they doing"? As an example, the study of the bacterial community associated with humans has become very important for human health, both for developing new therapeutic approaches and for diagnostics (e.g., the use of microbiota signatures as biomarkers of disease presence, antibacterial molecules produced by the microbiota used for therapeutic purposes, etc.) (www.ncbi.nlm.nih.gov/books/NBK154091). A great number of studies and discoveries that successfully applied NGS technologies to study microorganisms in humans, e.g., the Human Microbiome Project (https://commonfund.nih.gov/hmp) and MetaHIT (metahit.eu), advanced questions about the importance of the bacterial community that is associated with animals, and in particular, insects. Insects are colonized by a multitude of microorganisms, comprising bacteria, archaea, and eukaryotes (fungi and unicellular eukaryotes). Regarding bacteria, the main focus of this review, primarily four phyla of bacteria have been found in association with insects, namely Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria (Douglas, 2015). The cuticle and gut of the insect represent the two main habitats for bacteria (Douglas, 2015). The cuticle presents the first barrier against microorganisms (commensals or pathogens) (Yek et al., 2011). However, symbiotic bacteria of the genus Pseudonocardia are present in the exocrine glands in the cuticular crypts of some species of attine ants. These symbionts are able to protect their host by secreting antibiotics against the parasitic fungi of the genus Escovopsis (Currie et al., 2006).

The structure of the gut is very different between disparate insect taxa and across the developmental stages; this organ, more specifically the hindgut, represents the main habitat for bacteria (Douglas, 2015). Most studies have focused on the bacterial communities that are associated with the gut, and several studies have discovered that a number of symbionts in different insect orders play key roles in the fitness of their hosts (Sabree et al., 2009; Adamns et al., 2013; Olivier et al., 2005). These symbiotic associations represent the major driving force of evolutionary innovation by conferring novel phenotypic traits on the host, allowing for the colonization of new ecological niches. An enhanced understanding of the bacterial community of insects is that the resident microbiota may offer new possibilities to improve integrated pest management methods targeting economically important insects. The first step consists of investigating the host-associated bacterial community, targeting symbionts of interest and then developing strategies to use symbiont(s) to control insect pests. Monitoring the symbionts inside the insect and their possible routes of transmission represents a crucial step, because the bacterial community in insects is not always stable (Jaenike et al., 2010; Himler et al., 2011). A possible option could be the use of antibiotics targeting pest symbionts to decrease the host population, but such an approach may induce the appearance and spread of antibiotic resistant bacteria.

Studies have focused on developing new alternatives to antibiotics, such as disrupting the cellular processes underlying vertical transmission (Koga et al., 2012) or the nutrient interaction between the insect and bacterial symbionts (Russell et al., 2013). Such approaches might represent new alternatives for symbiont-based control strategies (Douglas, 2015). The study of the bacterial communities associated with insect/arthropods could also improve our knowledge about the presence of potential pathogens that could be transmitted to humans. As an example, Van Treuren and colleagues (2015) were able to confirm the presence of *Borrelia burgdorferi*, the vector of Lyme disease, in two tick species (*Ixodes scapularis* and *Ixodes affinis*) using 454-pyrosequencing. On the other hand, Epis and colleagues (2012) showed that the strain of *Asaia* harbored by *Anopheles stephensi* was different from the opportunistic human pathogen, as described by Alazeut et al. (2010), allowing for the development of potential symbiont-based control strategies

against malaria. The detection of pathogens in food for human consumption is very important, especially in the case of food consumed raw, such as spices, nuts, and dry meat. Montagna and Mereghetti (2016, 2017) showed the presence of *Staphylococcus*, *Streptococcus*, and *Burkholderia* within *P. interpunctella*, three genera implicated in human and animal disease (Whiley et al., 2009; Schleifer et al., 2009). Furthermore, NGS approaches may also allow for screening insect pathogens. As a matter of fact, the analysis of the *Apis mellifera* microbiome showed that the NGS approach is useful for the detection of bacterial pathogens (*Paenibacillus larvae* and *Melisoccoccus plutonius*) that are the causative agents of American foulbrood (EFB), a quarantine disease of the larvae and pupae of the honeybee (Erban et al., 2017a,b). Interestingly, *M. plutonius* was also found in the enzootic state, suggesting the involvement of other factors in the manifestation of EFB, such as the presence of secondary invaders (e.g., *Enterococcus faecalis*) (Erban et al., 2017b).

2.2.4 MICROBIOTA OF MOTH PESTS

Microbiota of forest and garden pests

Choristoneura fumiferana, (eastern spruce budworm, SBW), cause serious damage to spruce and fir in Canada (McMorran, 1965) (Table 1). The bacterial community of this moth was studied using high-throuput sequencing (Landry et al., 2015). Landry and colleagues (2015) investigated the effects of diets and sampling (laboratory *vs* field populations) on the bacterial gut composition; no significant differences were observed in terms of bacterial composition but *Pseudomonas*, a member of the phylum of Proteobacteria, was the most abundant taxon in all three groups. This result is in agreement with a previous study, that, using culture-dependent approach, identified this bacterium as one of the predominant taxa in a laboratory population of *C. fumiferana* (van Frankenhuyzen et al., 2010). In addition to these results it was found that the presence of the genus *Bradyrhizobium* in the lab-reared populations it was the only difference between laboratory and field

populations of *C. fumiferana* (Landry et al., 2015). To date, the role played by these bacteria has not been investigated.

A similar pattern in the bacterial composition was also observed in the moth *Lymantria dispar*. The gypsy moth *L. dispar* it is considered a highly pholypagous and invasive folivore species in North America. This insect is considered a forest pest because of its ability to attack more than 300 species of deciduous trees, especially oak trees, causing economic damage to forest industries (Liebhold et al., 1995; Montgomery et al., 1989) (Table 1). A first study examined the effect of different diets and insect origin (laboratory or field populations) on the microbiota associated with this moth, using traditional culture-dependent and independent approaches (i.e.; PCR amplification of bacterial 16S rRNA gene and Terminal Restriction Fragment Length Polymorphism analysis or T-RFLP analysis) (Broderick et al., 2004). The results showed that the bacterial communities in *L. dispar* were very simple (only 23 phylotypes) with the predominance of *Enterococcus* sp. and *Enterobacter* sp. (Broderick et al., 2004). This trend of bacterial structure was confirmed by Allen and colleagues (2009), using a metagenomic approach, but they also discovered the presence of antibiotic-resistant bacteria in the midgut of the moth. In particular, they were able to detect the presence of genes of the class of β-lactamases (namely LRG-1 and RamA). These genes were assigned to *Enterobacter* sp., *Pseudomonas* and *Erwinia* sp. (Allen et al., 2009). Another experiment discovered a different bacterial community associated to the same moth species and dominated by Burkholderiales (Mason & Raffa, 2014). They used 454-pyrosequencing to investigate the impact of different factors, such as diet and origin of population, on the bacterial composition of egg masses and the midgut of different developmental stages of *L. dispar* (3rd and 5th instar larvae).

The bacterial community associated with the gut of *Automeris zugana*, a polyphagous caterpillar, and the impact that different diets may have on these communities were explored by Sittenfield and colleagues (Sittenfeld et al., 2002). In particular, they studied the microbiota associated with the three gut regions of the last instar larvae and pupae when the insect fed on eight different plants. Although only a culture dependent approach was used, a total of 19 different species, from 102 isolates, were identified. The

composition of the gut microbiota was different both among caterpillars feeding on different diets but also among individuals of the same feeding group. This study revealed that the most frequently occurring genus was *Enterobacter* (found in 81.1% of the samples), followed by *Micrococcus* (51.5%) and *Bacillus* (33.3%). Further investigation by Pinto-Tomás et al. (2007) showed that bacteria isolated from the caterpillar guts and pupae of *A. zugana* (and *Rothschilda lebeau*) belonging to the family of Enterobacteriaceae had a lipolytic and chitinolytic activities. They hypothesised that these bacteria may help the moth in the digestive activities within the gut.

The sand lily *Pancratium maritimum* (family of Amaryllidaceae), contain toxic compounds such as alkaloids, and the garden pest *Brithys crini*, also named lily borer, feeds only on this plant. In order to understand the possible presence of gut symbionts able to degrade these plant secondary metabolites, the bacterial community of the midgut and hindgut of this moth was investigated by NGS sequencing (Villanova et al., 2016). The results showed that the bacterial community associated to *B. crini* was dominated by Firmicutes, assigning 94-99% of the bacterial Operational Taxonomic Units (OTUs) to *Enterococcus* sp. (Enterococcaceae). Since their lower relative abundance (1% of the total numbers of reads), bacteria belonging to the genus *Pseudomonas* sp., *Bacillus* sp., *Sphingomonas* sp., *Propionibacterium* sp., *Klebsiella* sp. and *Corynebacterium* sp. were also detected in *B. crini* specimens. These bacteria are known to be alkaloid-degraders (Villanova et al., 2016). These functional abilities can be very important in allowing the insect host to overcome the plant defence mechanisms. For example in the coffee borer beetle *Hypothenemus hampei* (the primary coffee bean pest) it was found that the gut symbiont *Pseudomonas fulva* was able to degrade the caffeine inside beans helping insect to overcome the plant's toxicity (Ceja-Navarro et al., 2015).

Thaumetopoea pityocampa is considered a polyphagous pests of pine forest in Europe and North Africa feeding preferably on *Pinus* spp., *Cedrus* spp. and *Larix* spp.. This pest can also be responsible for serious human health problems, such as dermatitis, conjunctivitis and anaphylaxis (EPPO, 2004; Battisti, 2015). The bacterial community of this moth was investigated by

Strano and colleagues (2017) using the Illumina Miseq platform. They analysed 4th instar larvae feeding on three different host plant species: the Aleppo pine (*Pinus halepensis*), the Maritime pine (*Pinus pinaster*) and the Laricio pine (*Pinus nigra* subsp. *laricio*). The bacterial communities of *T. pityocampa* were dominated by Proteobacteria (61 %), followed by Actinobacteria (19%), Bacteroidetes (15%) and Firmicutes (3%). Within Proteobacteria, the highest numbers of OTUs were assigned to the family of Moraxellaceae, Enterobacteriaceae and Pseudomonadaceae. Interestingly, they found a significant difference between the bacterial community of *T. pityocampa* feeding on *P. halepensis* and the other two plants, characterized by higher amount of Actinobacteria, and more specifically *Modestobacter* (Strano et al., 2017).

I performed a comparative analysis of the bacterial communities associated with the different forest moth pests showed in this study. All the data at the level family derived from the studies that used NGS technologies was used as input to investigate the possible presence of bacteria family/families shared across Lepidoptera. Only four species were used: *T. pytyocampa*, *L. dispar*, *B. crini* and *C. fumiferana*. The results showed that only three families were shared among the four lepidopteran species analysed. These families are represented by: Enterobacteriaceae, Enterococcaceae, and Pseudomonadaceae (Figure 1 and 2). I can hypothesise that these families were present in all the species investigated mainly because of their metabolic versatility and ability to help the insect overcome and degrade different complex compounds produced by the plant (Ceja-Navarro et al., 2015; De & Gupta, 2016).

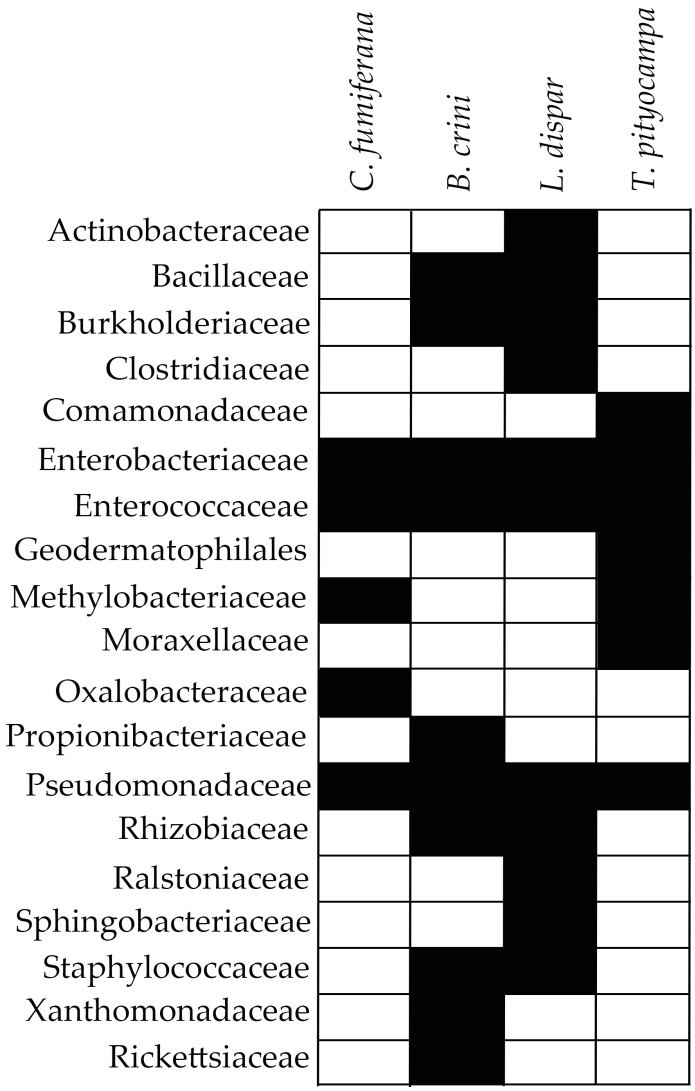

Figure 1. Table representing the family of bacteria shared between the microbiota of forest moths species investigated using NGS technologies. Black-white square indicate the presence-absence of the bacteria family, respectively.

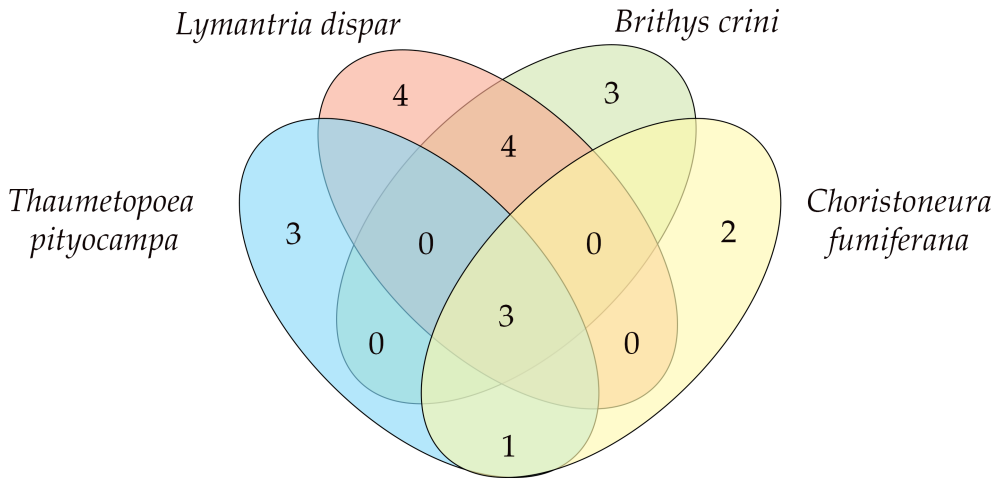

Figure 2. Venn representing the family of bacteria shared between the microbiota of forest moths species investigated using NGS technologies.

Table 1. Summary of the moth pests analysed.

Moth species	Family	Feeding behaviour	Common host plants	Type of pest
Agrotis ipsilon	Noctuidae	polyphagous	*Zea mays*, *Gossypium* sp., *Beta vulgaris*, *Solanum lycopersicum*	crop pest
Automeris zugana	Saturniidae	polyphagous	*Quercus* sp., *Salix* sp.	forest pest
Brhitys crini	Noctuidae	monophagous	*Pancratium maritimum*	forest pest
Busseola fusca	Noctuidae	polyphagous	*Zea mays*, *Sorghum* sp.	crop pest
Calyptra thalictri	Noctuidae	polyphagous	*Citrus* sp., *Thalictrum minus*, blood from ungulates (cattle, tapirs, zebu, etc)	crop/ blood-feeding pest
Choristoneura fumiferana	Tortricidae	polyphagous	*Abies balsamea*, *Picea mariana*	forest pest
Helicoverpa armigera	Noctuidae	polyphagous	*Cucurbita pepo*, *Senecio vulgaris*, *Zea*	crop pest

			mays, Lycopesicon esculentum, Medicago sativa, Pisum sativum, Cannabis sativa, Hyoscyamus niger	
Heliothis virescens	Noctuidae	polyphagous	*Gossypium hirsutum*, *Nicotiana tabacum*, *Cicer arietinum*	crop pest
Lymantria dispar	Erebidae	polyphagous	*Acer negundo, Acacia* sp., *Alnus alnobetula, Betula alleghaniensis*	forest pest
Manduca sexta	Sphingidae	polyphagous	*Solanum lycopersicum, Solanum tuberosum, Nicotiana tabacum*	crop pest
Mythimna separata	Noctuidae	polyphagous	*Avena sativa, Beta vulgaris, Brassica rapa* subsp., *Cannabis sativa*	crop pest
Ostrinia nubilalis	Crambidae	polyphagous	*Zea mays*, *Sorghum sp.*, *Pennisetum glaucum*, *Avena sativa*, *Capsicum annuum*, *Amaranthus sp.*, *Gossypium sp.*	crop pest
Plodia interpunctella	Pyralidae	polyphagous	Cereal grains, dried fruits, dried fish, dried meats, nuts	stored-product pest
Plutella xylostella	Yponomeutidae	monophagous	*Brassica sp.*	crop pest
Rothschidia lebeau	Saturnidae	polyphagous	*Zanthoxylum fagara, Fraxinus berlandieriana, Salix sp., Prunus*	crop pest

			persica, *Citrus sp.*	
Spodoptera frugiperda	Noctuidae	polyphagous	*Agrostis gigantean*, *Alcea rosea*, *Allium cepa*	crop pest
Spodoptera littoralis	Noctuidae	polyphagous	*Beta vulgaris*, *Albemoschus esculentus*, *Asparagus officinalis*	crop pest
Spodoptera litura	Noctuidae	polyphagous	*Allium cepa*, *Annona squamosal*, *Arachis hypogaea*, *Beta vulgaris*	crop pest
Thaumatopoea pytiocampa	Thaumetopoeidae	polyphagous	*Pinus spp.*, *Cedrus spp.*, *Larix spp.*	forest pest

Microbiota of crop pests

Moths of the genus *Spodoptera* (Table 1), also called armyworm moth, is represented by roughly 30 species. 15 out of 30 are recognized as agricultural pest due to their highly polyphagous activity on different crop species, causing economic losses annually (Pongue, 2000). Within 15 known pest species, the bacterial communities associated with only four species have been investigated. Only for *Spodoptera littoralis* (cotton leafworm), the bacterial community was investigated using NGS approach. Microbiota of this pest was firstly investigated using a classical 16S rRNA gene sequencing and microarray analysis (Tang et al., 2012). This study showed that the genus *Clostridium* and two species of *Enteroccocus* (in particular *Enterococcus casseliflavus* and *Enterococcus mundtii*) represented the most abundant genera in the gut of *S. littoralis* (Tang et al., 2012). Recent study using *in vivo* Pyro-SIP (coupling pyrosequencing and SIP – Stable Isotope Probing tecnique) discovered that the family of Clostridiaceae, Enterococcaceae and Enterobacteriaceae represent the functional core inside the gut of *S. littoralis* (Shao et al., 2017). Interestingly, *E. casseliflavus* was also found in *Spodoptera litura* (Takur et al., 2015; Takur et al., 2016) and other Lepidoptera such as

Manduca sexta, *Peridroma saucia*, *B. mori*, *Heliothis virescens*, *Hyles euphorbiae* and *Helicoverpa armigera* (Brinkmann et al., 2008; Ping et al., 2007; Madhusan et al., 2011; Staudacher et al., 2016; Xiang et al., 2006) and in 16 other insects species (Martin & Mundt, 1972). The association of this bacterium with a broad range of insect taxa, suggests the question of the possible role of *E. casseliflavus* in insects. A possible explanation is that this bacterium plays an important role in detoxification. For example, in the gut of *S. litura*, *E. casseliflavus*, was able to crystallize some toxic compounds produced by their host plant *Phaseolus lunatus* (lima bean*)* (in particular alpha- and beta-carotenoids), forming a biofilm-like structure. This process helps the insect adaptation to toxic compound produced by their hosts plants (Martin & Mundt, 1972). The presence of *E. casseliflavus* was also detected in the moth *H. euphorbiae*, where investigations using scanning electron microscopy and specific PCR showed the presence of a biofilm ring at the level of the pyloric valve of the moth hindgut, suggesting the role of *E. casseliflavus* in the immobilization of the toxic molecules of the host plants on which this moth feeds (*Euphorbia* sp.) (Villanova et al., 2016). Accordingly, I can suggest that *E. casseliflavus* plays an important role in the protection of different Lepidoptera species against secondary metabolites contained in their host plants. Furthermore, *E. mundtii* represents another dominant *Enterococcus* species in the gut of *S. littoralis*. This bacterium was shown to become dominant starting from 6-day-old larvae of *S. littoralis* (Tang et al., 2012). Using Fluorescent In Situ Hybridization (FISH), it has been observed that *E. mundtii was also able* to form a biofilm-like structure inside the gut of its insect host (Shao et al., 2014; Tang et al., 2012). Similar to the other *Enterococcus* species, *E. mundtii* was also detected in *Anticarsia gemmatalis*, *Choristoneura fumiferana, Mythimna separata, Plutella xylostella* where it displayed an esterase activity (van Frankenhuyzen et al., 2010; Pilon et al., 2013; He et al., 2013; Ramya et al., 2016). In particular, in *S. littoralis*, this bacterial species persists in the gut independently from the diet and across all developmental stages, suggesting an active role in the physiology of this moth. On this basis, Chen and colleagues (2017) can hypothesised a clearly symbiotic relationship between *E. mundtii* and *S. littoralis*. Furthermore, Shao and colleagues (2017) found that *E. mundtii*, present in the gut lumen,

produced an antimicrobials peptide (AMP) composed by 43 amino acids residues, called munditicin KS. This AMP was able to create an additional chemical barrier against pathogens, increasing the host fitness (Shao et al., 2017).

E. casseliflavus is discovered in another important crop pest, the polyphagous moth *Heliothis virescens* (Tobacco budworm). This pest is present in North and South America and is able to feed on over 80 plant species, including 19 crops species (especially clover, cantaloupe, flax, soybean and tobacco) (Fitt, 1989; Blanco et al., 2007) (Table 1). High throughput analysis of its gut microbiota showed that the laboratory-reared population was dominated by Enterococcaceae (73,7 %). In contrast, the field population, do not present a dominant taxon but a higher bacterial diversity (compared to laboratory populations) present, more or less, in the same proportions (Staudacher et al., 2016). Bacterial communities of both *S. littoralis* and *H. virescens* were also investigated across different developmental stages. In both cases the microbiota changed across the life cycle and were characterized by a simple communitiy.

S. litura, also called Oriental leafworm moth, is another important pest of crops, feeding on cruciferous vegetables, cucurbits, groundnuts, cotton, maize, potato, soybean and tobacco (Qui et al., 2004). Using culture-dependent approach, it has been discovered that, in addition to *E. casseliflavus*, a second species was interesting associated with this moth, namely *Enterobacter cloacae* (Takur et al., 2015). Depending on its titer, *E. cloacae* showed an insecticidal activity causing between 30 and 70% mortality in larvae (Takur et al., 2015). This activity has also been observed in other insects such as *Bemisia argentifolii*, *Chrysoperla rufilabris* and *Oberea linearis* (Davidson et al., 2000; Sandra et al., 2004; Campos et al., 2007), suggesting that this bacterium has the potential to become a promising biological control agent due to its ability to influence the immune system of *S. litura* and other insects. *E. cloacae* was also detected in these moth by an independent study where it was observed that in larvae feeding on diet supplemented with the antibiotic streptomycin sulphate, *E. cloacae* disappeared and the larval mortality of *S. litura* was reduced approximately to 20% (Takur et al., 2016).

Manfredi and colleagues (2015), using culturing and enzymatic assay (Filter paper enzymatic activity and β 1-4 endoglucanase activity), founded bacteria with an enzymatic hydrolysis of lignocellulosic material was detected in the gut of *S. frugiperda* (fall armyworm), a leaf eater constituting a corn plague in Argentina [68] (Manfredi et al., 2015). In particular, similarly to other *Spodoptera* species, Firmicutes represented the most abundant phylum (62.2%) with a predominance of *Bacillus* spp., followed by Proteobacteria (24.4%), Actinobacteria (6.7%) and Bacteroidetes (6.7%).

The polyphagous *Helicoverpa armigera* (cotton bollworm or tomato fruit borer) is able to attack many types of crops such as cotton, sunflower, corn and tomato all over the world causing high economic losses (Carter, 1992; Tewari & Krishnamoorthy, 1984). The microbiota of this moth was investigated in both laboratory and field populations feeding on cotton, cabbage and corn. Independently from the diet, the genus *Enterococcus* dominated the host-associated microbiota. This data was confirmed in different studies using both culture-dependent and culture-independent approaches (16S rRNA sequencing and NGS approach) (Tang et al., 2012; Madhusudan et al., 2011; Xiang et al., 2006; Prya et al., 2012). On the contrary, the study by Ranjith and colleagues (2016) showed that, using NGS approach to investigate the microbiota of field populations of *H. armigera* feeding on tomato plants, the major bacteria belonging to the phylum of Actinobacteria followed by Proteobacteria and Firmicutes. The microbiota of the diamondback moth (DBM) *Plutella xylostella* was also investigated using NGS approach. This pest, showed a worldwide distribution, infest *Brassica* spp. crops and it has evolved a resistance to a broad spectrum of insecticides (e.g., pyrethroids, organo-phosphorous compounds) (Shelton, 2004; Baxter et al., 2005). Xia and colleagues (2013) and Ramya and colleagues (2016) focused their attention on the bacterial communities of *P. xylostella*, with the aim of investigating possible implications of the microbiota in the insecticide resistance (Xia et al., 2013; Ramya et al., 2016). Using high-throughput sequencing targeting the V6 region of the bacterial 16S rRNA, three different lines of DBM 3rd instar larvae (two lines resistant to chlorpyrifos and fipronil respectively and the last one susceptible to the insecticide action) were analysed by Xia and colleagues (2013). In all the cases, the bacterial communities were composed of

Enterobacteriales and Lactobacillales (belonging to the phylum of Proteobacteria and Firmicutes respectively), but the insecticide resistant lines exhibited higher proportion of Lactobacillales comparing the susceptible strain, suggesting the possible role of this group of bacteria in the resistant mechanism against insecticide (Xia et al., 2013). The diversity of gut bacteria of DBM larvae and adults was also investigated by Ramya and colleagues (2016), but studying the effects of another insecticide belonging to the oxadiazione group, Indoxacarb. The results showed that *Bacillus cereus*, belonging to the family of Bacillaceae: *i)* displayed an esterase activity; *ii)* was able to metabolise Indoxacarb and; *iii)* was able to grow in presence of this insecticide. The carboxylesterase activity against insecticide was also confirmed in other bacteria (e.g. *Alicyclobacillus tengchongensis*, Zhenrong et al., 2013) and in human it was also found that this enzyme is a important mediator of drug metabolism (Laizure et al., 2013). The bacterial composition of *P. xylostella* was further investigated by Xia and colleagues (2017) using a metagenomic approach. The study showed that *Enterobacter cloacae* and *Enterobacter asburiae*, members of the Proteobacteria phylum, were the dominant taxa, followed by Firmicutes. Gene enrichment followed by functional identification, discovered that these two bacteria helped their insect host by providing a series of enzymes that participated in food digestion (enzymes with a specific brassicaceae cell wall degradation activity), nutrition (enzymes that synthetize histidine and threonine amino acids) and in the detoxification against secondary metabolites produced by Brassicaceae family (particularly phenolic compounds) (Xia et al., 2017).

Similar to other agricultural pest analysed with NGS techniques, *Ostrinia nubilalis* (ECB- European Corn Borer), where *Zea mays* is known to be the primary host, harboured a high proportion of bacteria belonging to the phylum of Firmicutes (98.5%) in the analysed laboratory-population. On the other hand, field populations were dominated by Proteobacteria (62%) followed by Bacteroidetes (29%) and Firmicutes (3%) (Belda et al., 2011). Furthermore, laboratory populations of ECB, harboured several bacteria belonging to the phylum Proteobacteria (*Micrococcus* sp., *Microbacterium paraoxydans* and *Acinetobacter lwoffi*) that potentially possessed enzymes for cellulose degradation (Vilanova et al., 2012). *Busseola fusca* is another

moth that primarily attacks maize (*Z. mays*) in Africa (Calatayud et al., 2014). Their microbiota was investigated applying culture-dependent and -independent approaches. The study investigated the bacterial communities associated to larvae from 30 different maize field (Snyman et al., 2016) and showed a predominance of Firmicutes (48.7%), Proteobacteria (34.6%) and Actinobacteria (16.7%). *Bacillus* and *Enterococcus* was discovered in all samples, suggesting that these two genera may play a role in the fitness of *B. fusca*.

DGGE and 16S rRNA clone library, in addition to culture-dependent techniques, were used to study the bacterial community of the moth *Mythimna separata*, also called Oriental armyworm. *M. separata* showed a high feeding activity on different crop plants such as *Zea mays*, *Sorghum bicolor* and *Oryza sativa* (Kuramitsu et al., 1982). This study revealed that, between the three approaches, the 16S rRNA clone library was the most sensitive approach, identifying a high number of bacterial taxa within *M. separata*. *Escherichia* sp. represented the predominant genus (nine clones), followed by *Ralstonia pickettii* (four clones), *Ochrobactrum anthropic* (four clones), *Enterococcus mundtii* (one clone) and *Frigobacterium* sp. (one clone) (He et al., 2013).

Calyptra thalictri also known as the fruit-piercing moth is able to feed normally by sucking the juice of different fruits such as raspberry, peaches, plums and citrus (Bazinger et al., 1982), but in some particular conditions the male of this moth represent also an opportunistic blood feeder, especially for ungulates (cattle, tapirs and others), and, occasionally for humans (Bazinger et al., 2005). The bacterial community of this moth showed the presence of bacteria belonging to the genera *Klebsiella*, *Sinorhizobium*, *Alcalignes* (*Acrhomobacter*) and *Rhizobium* in the abdomen of this moth (Azambuja et al., 2005) but no further investigation regarding the microbiota was carried out.

Similar to the forest moths, a comparative analysis of the taxa harboured by the agricultural moth pests was carried out. The information at family level obtained from the NGS studies of the microbiota associated with these pests was collected. In this case more insect species were analysed compared to the forest pests, namely: *S. littoralis, H. armigera, H. virescens, B. fusca, A.*

ipsilon, O. nubilalis, P. xylostella and *M. sexta*. The results showed that, also, in the case of the crop pests, the families of Enterobacteriaceae and Enterococcaceae were shared across the different lepidopteran species analysed (Figure 3, 4), suggesting the importance of these two families in the fitness of the moth pests.

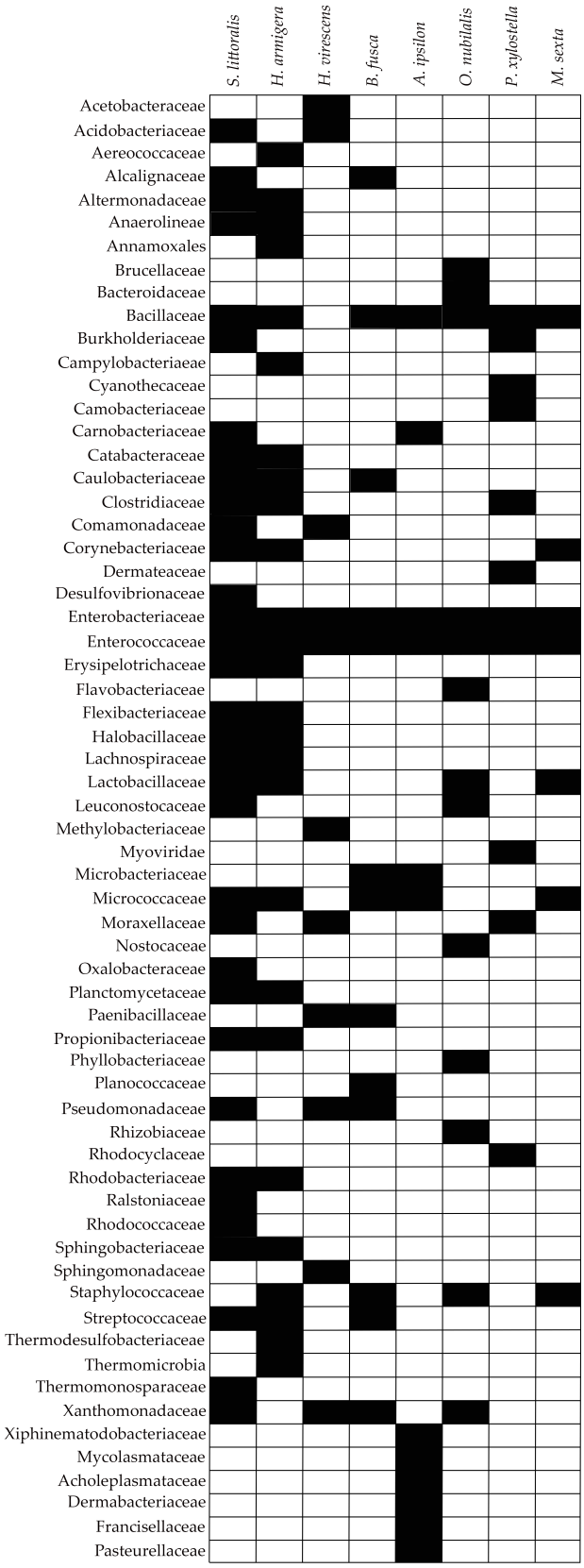

Figure 3. Table representing the family of bacteria shared between the microbiota of crop moths species investigated using NGS technologies. Black square indicate the presence of the bacteria family.

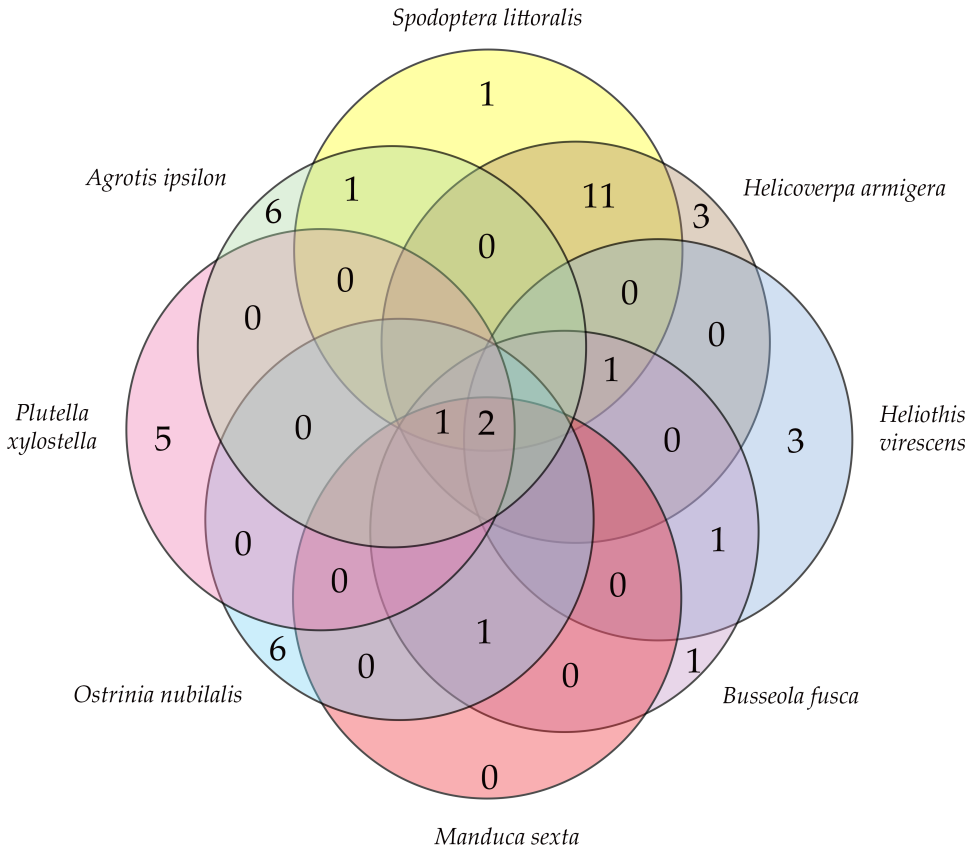

Figure 4. Venn representing the family of bacteria shared between the microbiota of crop moths species investigated using NGS technologies.

2.2.5 FACTORS AFFECTING THE MOTH'S MICROBIOTA

Diet

The impact of the host diet on host-associated microbiota has been demonstrated in a plethora of studies and in different insect orders, such as Diptera (e.g. *Drosophila* sp.) (Chandler et al., 2011)(Iasur-Kruh et al., 2015), Coleoptera (e.g. red palm weevil, *Cryptocephalus marginellus* species complex) (Montagna et al., 2015a,b), Hemiptera (e.g. wine mealybug *Planococcus ficus*) (Iasur-Kruh et al., 2015) (Kane & Breznak, 1991)[119] and Blattodea (e.g. cockroach) (Kane & Breznak, 1991; Pérez-Cobas et al., 2015).

Moths are globally distributed and are able to feed on different substrates, such as leaves, flowers, seed and human stored products (Futuyma, 1976). Furthermore, they display a wide range of phenotypes from monophagous to

highly polyphagous; such habits have been linked to their solar activity (e.g. nocturnal or diurnal species) (Altermatt & Pearse, 2011).

The avaiable literature regarding moth pests indicate that bacterial gut communities are greatly influenced by diet. These results have been observed using both culture-dependent and culture-independent approaches based on NGS technology (Tang et al., 2012; Broderick et al., 2004; Sittenfeld et al., 2002; De & Gupta, 2016; Staudacher et al., 2016; Ranjith et al., 2016; Vilanova et al., 2012). In *Thaumetopoea pityocampa*, eight OTUs of the genus *Modestobacter* were present in the microbiota of all the studied insects, but their relative abundances changed when fed on *Pinus halepensis* rather than *Pinus nigra* subsp. *laricio* and *Pinus pinaster* (Strano et al., 2017). A possible explanation of these results was the difference in altitude at which these three different pine species were found (300-500 m a.s.l. for *P. halepensis*, and 700 m a.s.l. for *P. pinaster* and *P. nigra*) (Strano et al., 2017). The presence of *Modestobacter* was also correlated with the main compound found in the needles, represented by myrcene for *P. halepensis* and a-pinene for *P. pinaster* and *P. nigra*. *Modestobacter* is hypothesized to play a role in *T. pityocampa* adaptation to these species of *Pinus*. Notably, this was the first report of *Modestobacter* in Lepidoptera (Strano et al., 2017).

The bacterial community of *Automeris zugana*, a forest pest, was investigated using a culture-dependent approach (Sittenfeld et al., 2002). *A. zugana* was reared on eight different diets (*Cydista heterophylla*, *Trigonia rugosa*, *Calycophyllum candidissimum*, *Annona purpurea*, *Inga vera*, *Quercus oleoides*, *Paullinia cururu* and *Cordia alliodora*). The results showed that diet not only influenced the bacterial community of these moths, but also had an impact on body mass. In fact, larvae feeding on four out of eight tested diets (*C. heterophylla*, *T. rugosa*, *C. candidissimum*, *A. purpurea*) grew normally compared to the other four tested diets. The microbiota associated with moths reared on all eight diets differed from each other, but *Serratia marcescens* was the only bacterial genus present in the moths that fed on the four plants on which *A. zugana* grows normally. These results prompted further investigations in order to understand if this bacterium plays a role in the digestion of some specific/toxic compounds within these plants. Furthermore, *S. marcescens* was described in association with other lepidopteran pests

such as *Lymantria dispar*, *Helithios virescens*, *Helicorverpa zea* and *Ostrinia nubilalis* (Broderick et al., 2004; Sikorowski et al., 1998, 2001; Bell, 1969; Lynch et al., 1976) and other insects (Sikorowski et al., 2001). This bacterium is not commonly pathogenic to insects, but there are reports that it may display entomopathogenic activity (Sikorowski, 1985).

Two studies investigated if and how the bacterial community changes when *L. dispar* fed on different diets (i.e., commercial sterilized artificial diet, *Populus tremuloides*, *Larix laricina*, *Quercus alba* and *Salix fragilis* in [x and *Betula papyrifera*, *P. tremuloides* and *Q. alba* in (Mason & Raffa, 2002)]. Although the bacterial community appeared to differ between the different *L. dispar* specimens feeding on the different substrates analyzed by Broderick and colleagues (2004), all of the specimens in the study by Mason and Raffa (2002) appeared to be dominated by Burkholderiales. A possible explanation for the different results observed by Broderick and colleagues (2004) and Mason and Raffa (2002) could be the use of different approaches in the two studies (culture-dependent approach, 16S rRNA gene sequencing and T-RFLP technique *vs.* 454-pyrosequencing). Notably, bacteria belonging to the genus *Acinetobacter* were present among *L. dispar* specimens feeding on *P. tremuloides* in both studies (Broderick et al., 2004; Mason & Raffa, 2002). Further investigation revealed that this genus was acquired by *L. dispar* from the diet (*P. tremuloides*) (Mason et al., 2014) and that it is able to degrade phenolic glycosides, a secondary metabolic compound produced by aspen trees (Mason et al., 2015). Interestingly, *Acinetobacter* sp. was found as the predominant bacteria in the microbiota associated with *T. pityocampa* feeding on *P. pinaster* and *P. nigra* (Strano et al., 2017), suggesting the involvement of these bacteria in the degradation of a wide spectrum of toxic compounds within the coniferous species.

The bacterial community of *Choristoneura fumiferana* was investigated and moths fed on three different diets (artificial diet, *Abies balsamea* and *Picea mariana*) were dominated by *Pseudomonas* spp., and in particular the proportion of this genus increased in the bacterial community of moths fed on *A. balsamea* and *P. mariana* (>80%) compared to moths feeding on an artificial diet. Interestingly, in *Bombyx mori,* it has been demonstrated that

bacteria belonging to this genus are involved in the degradation of pectin, one of the components of plant cell walls (Mason et al., 2015).

In *Spodoptera littoralis*, differences were observed when the moths fed on an artificial diet, showing a predominance of *Enterococcus* (Tang et al., 2012; Shao et al., 2014) compared to when these insects fed on toxic lima beans, where the main genus was *Clostridium* (Tang et al., 2012). Studies on the effect of the diet on the microbial composition of the pest *Heliothis virescens* showed that the microbiota of different populations fed on different diets (*Gossypium hirsutum*, *Cicer arietinum* and *Nicotiana* sp.) were different, but it also showed that different populations feeding on the same diet had different microbiota, thus indicating that multiple factors help in shaping the microbial community of this pest.

Only one study has investigated the bacterial community associated with *Helicoverpa armigera* using NGS techniques (Ranjith et al., 2016); unfortunately, they did not analyze how the bacterial community changed when the moths fed on different substrates. Interestingly, Prya and colleagues (Priya et al., 2012), using T-RFLP analysis, investigated the microbiota associated with leaf crops and the corresponding groups of larvae feeding on them (castor, chickpea, cotton, ladyfinger, redgram, sorghum, sunflower and tomato), discovering that the bacteria associated with the leaf phyllospere was shared with the microbiota of the different moth groups. Furthermore, a difference in the bacterial community was detected in moths collected from different localities (Bangalore, Delhi, Pachora). As reported for *T. pityocampa* (Strano et al., 2016), but also for another insect order [(e.g. *Cryptocephalus* sp. (Montagna et al., 2015b)], the insect-associated bacterial community was influenced by the plant's geographical location, thereby enabling the host to adapt to the environment.

In conclusion, from these studies, we can affirm that the bacterial community associated with moth pests (or insects in general) can be influenced by the diet in two different ways: *i)* a direct effect, consisting in directly conveying bacteria, more or less consistently, from the substrate to the insect; *ii)* an indirect effect, by shaping the pre-existing bacterial community associated with the moth.

Interestingly, a contrasting study investigating the microbial diversity associated with 31 species of butterfly, both carnivorous and herbivorous, showed that bacterial communities do not change with different diets (Whitaker et al., 2016). In order to test if there was a factor influencing moth microbiota, we used the data on the moth microbiota provided by the available studies. A non-metric multi-dimensional scaling analysis (NMDS) (Kruskal et al., 1964) was performed and fitted with factors related to moth trophism that might affect the structure of the associated bacterial communities. The factors used in this analysis were: *i*) feeding behavior (i.e., monophagous, oligophagous or polyphagous); *ii*) the damaged plants, (gymnosperms or angiosperms); *iii*) the type of food (leaves or seeds). The only diet factor that affected the bacterial communities (family level) of moth pests was the plant group (gymnosperms and angiosperms); p-value = 0.041. One group was composed of moths that feed on gymnosperms, namely *Lymantra dispar*, *Brithys crini*, *Choristoneura fumiferana* and *Thaumatopoea pityocampa,* while the second group was composed of moth pests feeding on angiosperms: *Ostrinia nubilalis*, *Plutella xylostella*, *Manduca sexta*, *Helicoverpa armigera*, *Spodoptera littoralis*, *Busseola fusca*, *Heliothis virescens* and *Agrotis ipsilon* (Figure 5). The fact that *Plodia interpunctella* feeds on stored products made it impossible to assign it specifically to one of these two groups, but based on the analysis, we can confirm that the bacterial community of this moth is more similar to that of moths that feed on angiosperms than to the other group (Figure 5). This result can be explained by the fact that forest moths generally feed on conifers, which contain a high variety of compounds such as terpenes (the main component of resins in conifers) (Furstenberg-Hagg et al., 2013). In addition, the host plants of *P. pityocampa* (*Pinus halepensis*, *Pinus nigra* and *Pinus pinaster*) contain some specific essential oils such as the monoterpenes myrcene and a-pinene ((Macchioni et al., 2003), while *Pancratium maritimum*, the host plant of *B. crini*, contains potent alkaloids (Hetta & Shafei, 2013). Monoterpenes are known to have toxic effects on insects, such as *Spodoptera litura* or several beetle species (Trapp & Croteau, 2001; Hummelbrunner & Isman, 2001). As for other insects (Ceja-navarro et al., 2015), we can hypothesize that the microbiota of the moths feeding on these conifers may play a role in the

detoxification of such toxic compounds. Although insects have detoxification mechanisms (e.g. cytochrome P450 monooxygenases, glutathione S-transferases and esterases) (Scott et al., 1998), some symbionts within the host-associated bacterial community are responsible for the detoxification of plant secondary metabolites. As example, the gypsy moth *L. dispar* harbors in its microbiota some bacteria responsible for monoterpene degradation (Mason et al., 2014, 2015). In other insects, such as *Dentroctonous ponderosea* (mountain pine beetle), some bacteria belonging to the genus *Pseudomonas* and *Rahnella* are able to degrade terpenes, allowing *D. ponderosea* to feed on terpene-rich trees (Adamns et al., 2013).

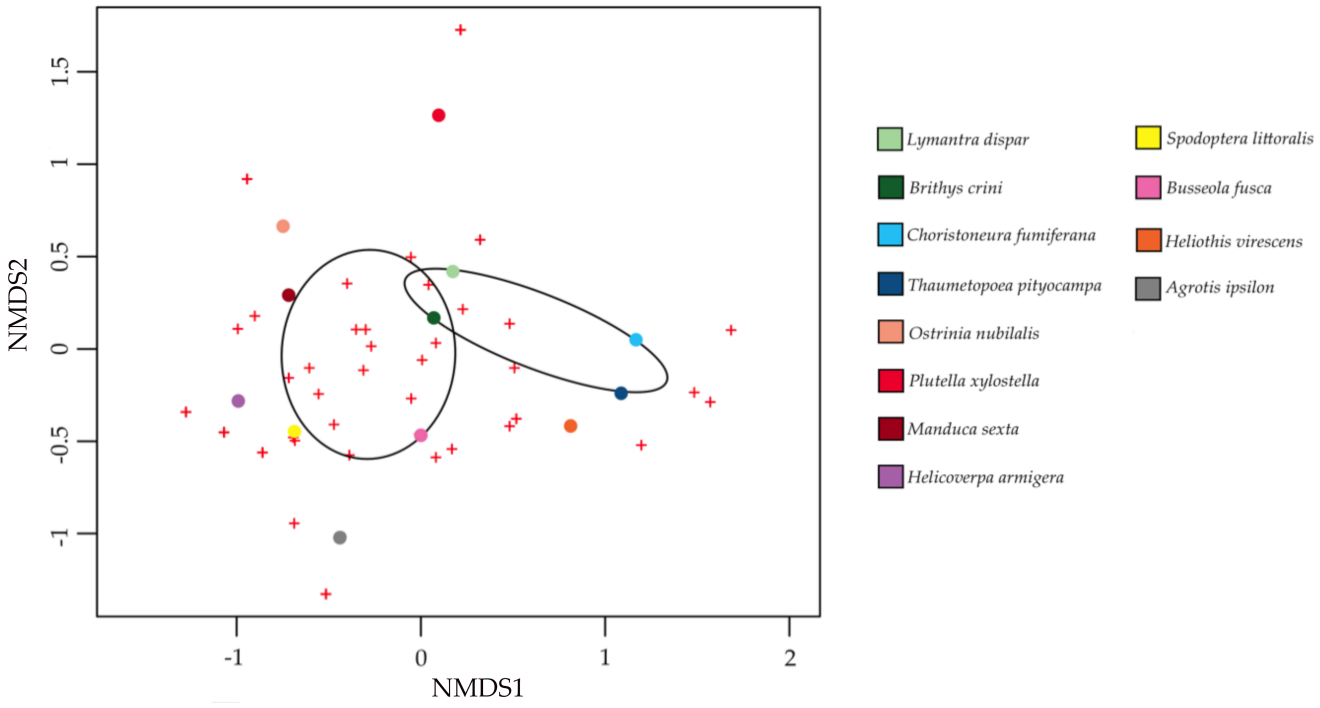

Figure 5. NMDS plots showing the correlations between the bacterial families associated with the moths and different diets factors. Circular points indicate the moths coloured according to the different species, the crosses represent the identified OTUs at family level. The ellipses represent a 95% confidence area around the mean of the group.

Laboratory vs field populations

In studies focused on Lepidoptera, but also on other insect orders, the general finding is that bacterial communities in laboratory reared insects are very simple and dominated by one or a few bacterial strain(s), while communities of field populations tend to have greater diversity and evenness ((Xiang et al., 2006; Belda et al., 2011; Mead et al., 1988). A clear example is represented by *Heliothis virescens*, where the microbiota of two populations, i.e. a laboratory strain fed on *Nicotiana attenuata* and a field population feeding on whole cotton (*Gossypium hirsutum*), chickpea (*Cicer arietinum*) and tobacco plants (*Nicotiana tabacum*), were compared; the results showed that no OTUs were shared between the two groups (Staudacher et al., 2016). The laboratory population was dominated by Enterococcaceae, while field populations feeding on cotton, chickpea and tobacco showed a richer microbiota composed of different bacterial families with more or less the same abundance (Staudacher et al., 2016). A similar pattern was observed in the case of *Helicoverpa armigera, Ostrinia nubilalis* and *Rothschildia lebeau*, where the microbiota associated with field populations were more diverse than those of laboratory populations (Pinto-Tomas et al., 2007; Brinkmann et al., 2008; Xiang et al., 2006; Ranjith et al., 2016). A possible explanation for this trend is that, under field conditions, eggs, larvae, pupae and adults are laid/feed on different substrates such as the ground parts of plants, leaves, flowers or soil, and thus are continually subjected to an enormous influx of a variety of bacterial strains. Furthermore, when field populations ingest their food, exposure to a wide range of phytochemicals or insecticides may confer more plasticity to gut microbiota, in order to help with the digestion of these substances (Patankar et al., 2001). In contrast, laboratory moths are caged in a stable environment with the same controlled artificial diet containing the same bacterial community (Mead et a., 1988).

Rybanska and colleagues (2016) highlighted differences in four different populations of the mite *Tyrophagus putrescentiae*. They analyzed differences in both location (Bustehrad, Zvoleneves, The Netherlands, USA) and origin (laboratory and field populations) (Rybanska et al., 2016). The bacterial community was different between the laboratory and the field populations, and was also associated with a difference in the growth rate between the

two populations (Rybanska et al., 2016). Such evidence suggests that the bacterial community can influence growth and development in moths (e.g. *A. zugana* (Sittenfeld et al., 2002), but without clear evidence, further investigations are needed to test this hypothesis.

This pattern of differentiation between the microbiota of field population insects and their lab-reared counterparts was not observed for *C. fumiferana* in which populations from the field and the laboratory showed a similar bacterial composition dominated by Proteobacteria (Pinto-Tomas et al., 2007). In addition, the microbiota of lab-reared populations harbored *Bradirizhobium*, which was absent from field populations. Furthermore, the number of OTUs present on the artificial diet was higher than the number of OTUs observed in the field population. For *Lymantria dispar*, despite different bacterial communities in the starting egg masses, both laboratory and field populations harbored a similarly diverse bacterial community, dominated by Burkholderiales (Mason & Raffa, 2014).

Developmental stages

Lepidoptera are holometabolous insects characterized by different life stages (i.e., egg, larva, pupa and adult). The first stage is represented by eggs that hatch into larvae (Chen et al., 2016). Larvae represent the most active stages, in which these insects feed, molt and grow larger, until the pupal stage. Thereafter, winged adults emerge from the pupae. In general, the gut of Lepidoptera (moths and butterflies) is characterized by less compartmentalization than the gut of many other insect orders (Dali et al., 1998). The pH is generally very alkaline and, during metamorphosis, the gut structure changes, potentially compromising stable colonization of the bacterial community (Dali et al., 1998). Concerning Lepidoptera, and in particular moth pests, no clear pattern has been observed with regard to changes in microbiota structure or composition. In *Plutella xylostella*, developmental stages do not affect the bacterial community, while in other species, bacterial communities change across the different developmental stages (e.g. *Heliothis virescens* and *Spodoptera littoralis*). For *H. virescens*, the bacterial community changes drastically, such that no OTUs are shared across the different analyzed developmental stages (Staudacher et al., 2016).

In *S. littoralis*, the egg microbiota was dominated by the genus *Pantoea*, and early-instar larvae were similar to eggs, but *Pantoea* was replaced by *Enterococcus* in last-instar larvae and pupae. Furthermore, the microbiota of adults differed between genders. Males were dominated by the genus *Klebsiella*, while females showed the presence of Proteobacteria and Firmicutes in the same proportion (Chen et al., 2016). While in *S. littoralis* the microbiota changed over development, interestingly, species of the genus *Enterococcus*, especially *Enterococcus mundtii*, were maintained during metamorphosis (Chen et al., 2016). *Galleria mellonella* also retained *E. mundtii* throughout metamorphosis. This bacterium has been shown to play an important role in the maintenance of a "healthy" gut microbiota, conferring a protection against pathogens (Jonston et al., 2015). The bacterial community of *H. virescens* changed drastically throughout metamorphosis.

In *Plutella xylostella,* the bacterial communities did not change across the analyzed developmental stages (3rd instar larvae, pupae and adults for *P. xylostella*). *Enterobacter cloacae* and *Burkholderia* sp., belonging to the phyla Proteobacteria, dominated the microbiota throughout the life cycle of *P. xylostella* (Xia et al., 2013).

In *Lymantria dispar*, a study on the microbial community across developmental stages (namely eggs, 3rd and 5th instar larvae) showed that, in these different stages, Burkholderiales was the dominant taxa in the microbiota (Mason & Raffa, 2014). Only one study investigated the bacterial community of the butterfly *Heliconius erato* using Illumina sequencing, targeting the V4 region of the 16S rRNA gene. *H. erato*, also called the red postman, harbored a microbiota that changed across the life stages of this neotropical butterfly (Hammer et al., 2014). In particular, larvae were dominated by *Acinetobacter*, while adults showed a microbiota dominated by *Asaia* and *Lactococcus*.

No signatures of vertical transmission from females to their offspring have been detected in these studies. Despite some similarity between the microbiota associated with females and eggs of *H. virescens* (Staudacher et al., 2016), no clear evidence supports the vertical transmission of bacteria. However, a previous study found that *Serratia marcescens* was vertically transmitted in a laboratory population of *H. virescens* (Sikorowoski and

Lawrence, 1998). Furthermore, studying the immune system of *G. mellonella*, Freitak and colleagues (2015) used fluorescently-labeled *E. coli* to show that this bacterium penetrated the ovary and was subsequently found in the chorion of ovipositioned eggs (Freitak et al., 2015).

The intracellular bacterium *Wolbachia* infects many arthropods (Higenboecker et al., 2008; Muhammad et al., 2015). In Lepidoptera, the vertical transmission of this bacterium has been confirmed, with an estimated incidence of infection of 84% in lepidopteran species (Narita et al., 2009). The presence of *Wolbachia* has been found in different lepidopteran pests, such as *Colias erate*, *Ephestia cautella*, *Ephestia kuehniella*, *Ostrinia furnacalis*, *Spodoptera exempta* and *Phyllonorycter blancardella (Muhammad et al., 2015; Sasaki et al., 1999; Kageyama et al., 2002; Kaiser et al., 2010; Graham et al., 2012)*. In particular, in *S. exempta*, *Wolbachia* increases the susceptibility of its host to baculovirus, making it a potential biological control agent against this crop pest (Kaiser et al., 2010).

2.2.6 CONCLUSION AND OUTLOOK

Here I reported the current knowledge on bacterial communities associated with forest and agricultural moth pests, with a focus on bacterial community changes related with the major factor analysed during these studies such as diet, developmental stages and the origin of the population (mainly laboratory *versus* field populations). It has been observed that diet and the origin of population have a great impact in shaping the bacterial communities associated with moth pests. Regarding the developmental stages, it has been observed that for some species the bacterial community changes with the life cycle, while for others no. For this reason and because of the few studies investigated that considered the developmental stage as a potential factor effecting microbiota, it is hard to determine the effective impact of the developmental stage in influencing the bacterial communities of the moth pests. Furthemore, two groups of bacteria belonging to the family of Enterobacteriaceae and Enterococcaceae, were present both in forest and agricultural moth pests. The advent of the NGS technologies, in combinations with the culture-dependent approach, will help deeping the

knowledge regarding the microbiota. In particular, these NGS-based studies will improve our understanding of the ecology of the bacterial communities associated with these insects helping us to understand the different factors that influence it while culture-dependent could help unveil the role played by these bacteria inside their host by allowing a glimpse into their physiology with regard to the hosts fitness. A better understanding of such interaction could be the first step in developing possible new ecologically-friendly symbiont-based biocontrol strategies.

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3. RESULTS

3.1 THE INFLUENCE OF DIFFERENT DIETS ON MICROBIOTA ASSOCIATED WITH THE STORED PRODUCTS PEST *PLODIA INTERPUNCTELLA* (LEPIDOPTERA, PYRALIDAE)

3.1.1 ABSTRACT

The potential influence of insect's feeding behaviour on their associated bacterial communities is currently a matter of debate. *Plodia interpunctella*, the major pest of commodities, was used as a model for studying the impact of different diets on their microbiota using a culture-independent approach. An analysis of similarity showed differences among the microbiotas of moths reared on 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 different entomotypes, respectively, associated with a carbohydrate-rich diet (entomotype *Atopococcus*) and a protein-rich diet (entomotype *Propionibacterium*). These results were also confirmed by the predicted functional profile. A core microbiota, composed of six taxa, was shared between eggs and adults, independently from 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.

3.1.2 INTRODUCTION

Plodia interpunctella (Hübner 1813), also named Indian meal moth (IMM), is a cosmopolitan moth (Rees, 2004) of the family Pyralidae. This Lepidoptera 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). For this reason, *P. interpunctella* is considered a major pest of commodities, causing extensive economic damages. The quality grade of harvested products is reduced both by direct damage, represented by the feeding

activity through all the larval instars, and indirect damage for the presence of larval frasses, exuviae and the silkweb (Allotey and Goswami, 1990). The capability of *P. interpunctella* 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 (e.g., Bandi et al., 1995; Brune, 1998; Lopez-Sanchez et al., 2009; Kohler 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 (e.g., Moran et al., 2003; Moran, 2006; Douglas, 2009; L_opez-S_anchez et al., 2009; McCutcheon et al., 2009). Furthermore, 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 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 communities was determined in the case of *Spodoptera littoralis* (Tang et al., 2012) and in a closely related leaf beetle species; where, the altitude of the sites where the specimens were collected generated the ecological trait that affected the insect's microbiota (Montagna et al., 2015b). 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, IMM was used as a study model to address the following biological questions: *(i)* Are the microbiotas associated with insects feeding on five types of diet different? *(ii)* Is there a core microbiota associated with eggs shared until adulthood? *(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.

3.1.3 MATERIALS & METHODS

Sampling

The *Plodia interpunctella* specimens used as a laboratory-reared populations 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). This population derived from a line maintained since 1980 in the rearing facilities established at the DEFENS (University of Milan). In order to understand the impact of the diet in shaping the bacterial communities associated with the IMM, 120 eggs were collected randomly from the same cohort of the laboratory-reared population. Then 90 out of 120 eggs 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 understand 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 moths. 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 was extracted as described by Montagna and colleagues (2015b). In order also to characterize the chorion (the first meal of the newborn larvae), the eggs were not surface sterilized. 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 investigated the bacteria associated with the five food substrates, 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 moths were developed.

Pyrosequencing

The microbiotas associated with the *P. interpunctella* specimens and their 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 NGS assays were deposited in the European Nucleotide Archive (Accession No. PRJEB14361), mapping file is provided in Table S1 (see Annex). Using QIIME platform (Caporaso et al., 2010), the obtained raw 16S rRNA sequences were quality filtered 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. 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 16S rRNA bacterial 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 analysis of different diversity indices and further 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 insects 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). Change 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 following the online protocol. The functional profile was predicted for the bacterial communities associated with the IMM adults. The table with the predicted L3-functions counts persamples, 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 (SD50.04), 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.

3.1.4 RESULTS & DISCUSSION

α, β-diversity and community structure

In total, we obtained approximately 200,000 16S rRNA reads. Quality filtering permitted to obtain a total of 79,933 16S rRNA bacterial sequences, 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). After clustering 1611 bacterial operational taxonomic units (OTUs) were obtained (Table 1). The substrate represented by the artificial diet and pizzoccheri are characterized by the highest carbohydrate content and the bacterial communities associated to *P. interpunctella* reared on these substrate showed the highest species diversity (Table 1), with an average number (±SD) of 195 ± 49 and 192 ± 89.7 detected OTUs. Only 62 OTUs were associated with the insects reared on *Vicia faba* 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 insect's species, such as in *Drosophila* spp., *Lymantria dispar* and *Blattella germanica* (Chandler et al., 2011; Mason and Raffa, 2014; Perez-Cobas et al., 2015). The microbiotas associated with the moths analysed in this study showed, on average, a significantly higher number of bacterial OTUs compared with other studies that analysed the bacterial communities associated to different caterpillars (Broderick et al., 2004; Yu et al., 2008; Pinto-Tomas et al., 2011). The resulted differences in the OTUs richness could be explained due to the IMMs, feeding on a variety of stored products, do not needs 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 bacterial communities associated with specimens reared on moringa leaves and chili showed the highest values of

the Shannon and Pielou's evenness (3.70 and 3.36, 0.77 and 0.75 respectively) (Table 1). Furthermore, the lowest values of these indices were recovered in the bacterial communities of *P. interpunctella* feeding on an artificial diet and pizzoccheri (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 16S rRNA sequences (Fig. 1C). Concerning 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 oleifera* leaves and chili respectively. The Sørensen's dissimilarity (β-diversity), was estimated among 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-diversity, the turnover and the nestedness (respectively, β_{SIM}=0.56 and β_{NES}=0.2), indicate that only approximately 20% of the bacterial OTUs is shared among the three analysed groups. The ANOSIM analysis (P<0.001) confirmed the differences in the bacterial communities associated with all five groups of *P. interpunctella*.

Table 1. Diversity indices estimated for the bacterial communities associated with the analyzed 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
PA.1F	♀	artificial diet	216	368.8	2.63	0.49
PA.2F	♀	artificial diet	158	438.1	2.70	0.53
PA.3F	♀	artificial diet	264	535.4	2.75	0.49
PA.1M	♂	artificial diet	140	217	2.37	0.48
PA.2M	♂	artificial diet	196	359.8	2.65	0.50
PW.1F	♀	pizzoccheri	177	331.1	2.26	0.44
PW.2F	♀	pizzoccheri	105	181.2	2.34	0.50
PW.3F	♀	pizzoccheri	162	300.8	2.31	0.45
PW.1M	♂	pizzoccheri	173	278.6	2.16	0.42
PW.2M	♂	pizzoccheri	344	552	2.18	0.37
PC.1F	♀	chili	107	197	3.79	0.81
PC.2F	♀	chili	138	207.5	3.74	0.76
PC.3F	♀	chili	87	153	3.44	0.77
PC.1M	♂	chili	44	70.3	2.91	0.77
PC.2M	♂	chili	89	114	2.92	0.65
PF.1F	♀	*Vicia faba*	60	90	3.13	0.76
PF.2F	♀	*Vicia faba*	68	102.5	3.06	0.73

PF.3F	♀	*Vicia faba*	19	21.5	1.98	0.67
PF.1M	♂	*Vicia faba*	77	94.3	3.49	0.80
PF.2M	♂	*Vicia faba*	86	117	2.94	0.66
PL.1F	♀	moringa	99	162	3.50	0.76
PL.2F	♀	moringa	130	161.7	3.81	0.78
PL.3F	♀	moringa	104	128.5	3.89	0.84
PL.1M	♂	moringa	111	166.5	3.46	0.74
PL.2M	♂	moringa	160	222.7	3.83	0.76
F_ad		artificial diet	128	197.8	3.51	0.72
F_piz		pizzoccheri	52	98.8	3.35	0.85
F_chil		chili	80	111.1	2.32	0.53
F_fav		*Vicia faba*	1	1	0	-
F_mor		moringa	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: Vicia vaba beans; mor: Moringa oleifera leaves; M: male; F: female.

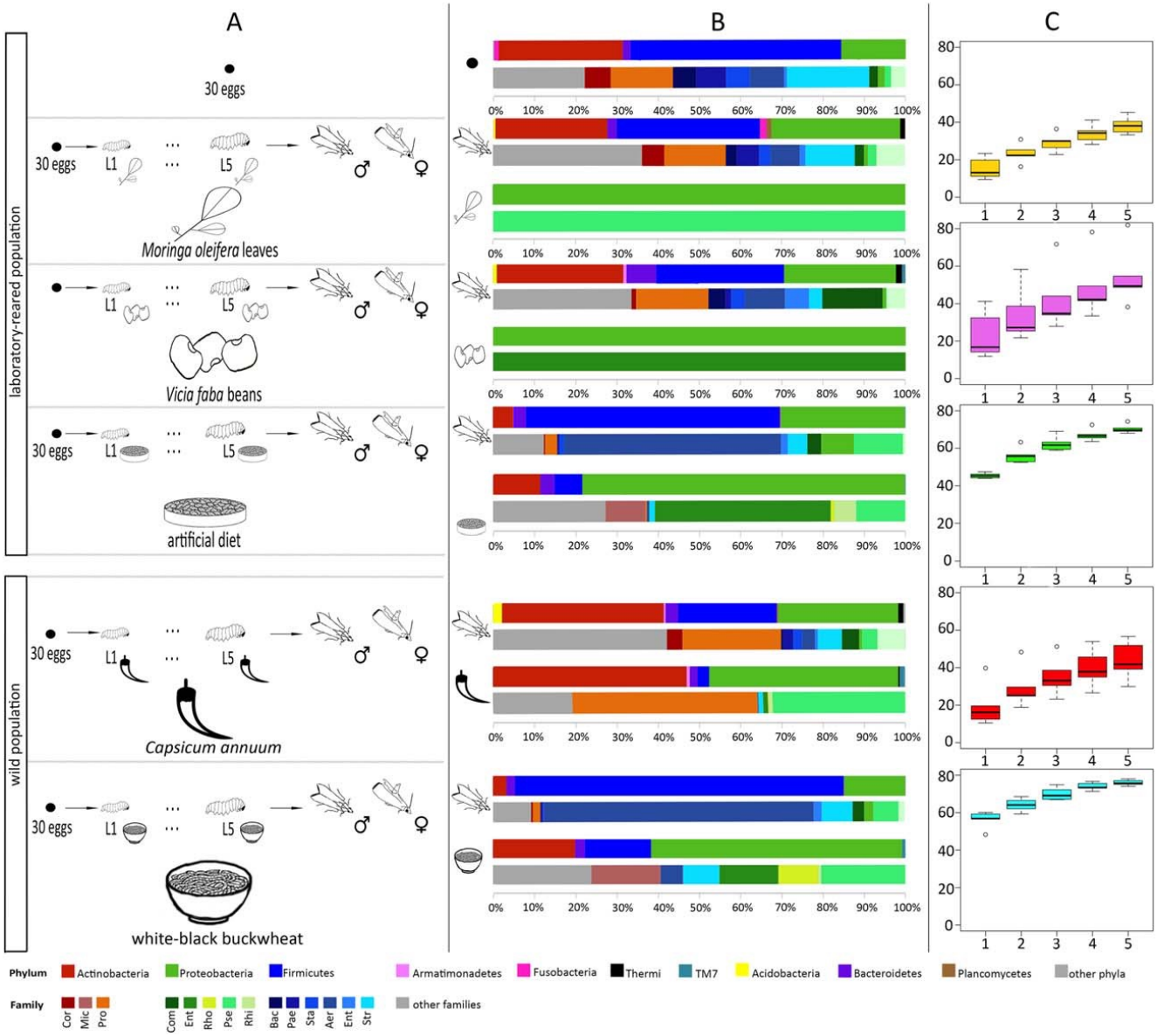

Figure 1. Experimental design, taxonomic composition of the bacterial communities and cumulative abundance of the dominant bacterial OTUs. A) *Plodia interpunctella* from different populations (laboratory-reared and wild) were reared until adulthood on different substrates (artificial diet, *Moringa oleifera* leaves, *Vicia faba* beans, *Capsicum annuum* powder, white-black buckwheat noodles). DNA were extracted from eggs of the laboratory-reared population, from five adults (three females and two males) of each group and from the food substrate. B) Taxonomical composition of microbiotas associated with the analyzed samples (eggs, adults and food resources) reported at phylum level and family level (upper and lower bars respectively). The average abundance across the five adult specimens is reported; only the bacterial families with an average abundance ≥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 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; cyan: white-black buckwheat.

Factors affecting the bacterial community structure

The non-metric multi-dimensional scaling analyse (NMDS), performed on the moth-associated bacterial OTUs, using both Chao and Weighted UniFrac distance matrices, were fitted with fac- tors potentially are able to affect the microbiota composition and structure. These factors are represented by: *i)* the diet; *ii)* the IMM population of origin and; *iii)* the IMM gender (Fig. 2). The diet was the only tested factor 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). On the contrary the moth's 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. As example, the bacterial communiy associated to the pine weevil (*Hylobius abietis*), 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 (), were identified within the IMM microbiotas. Hereafter, these two groups of bacteria as called entomotypes from the Greek entomon meaning insect and referred to as consistent groups of bacteria present within the volume delimited by the exoskeleton. These two entomotypes 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.

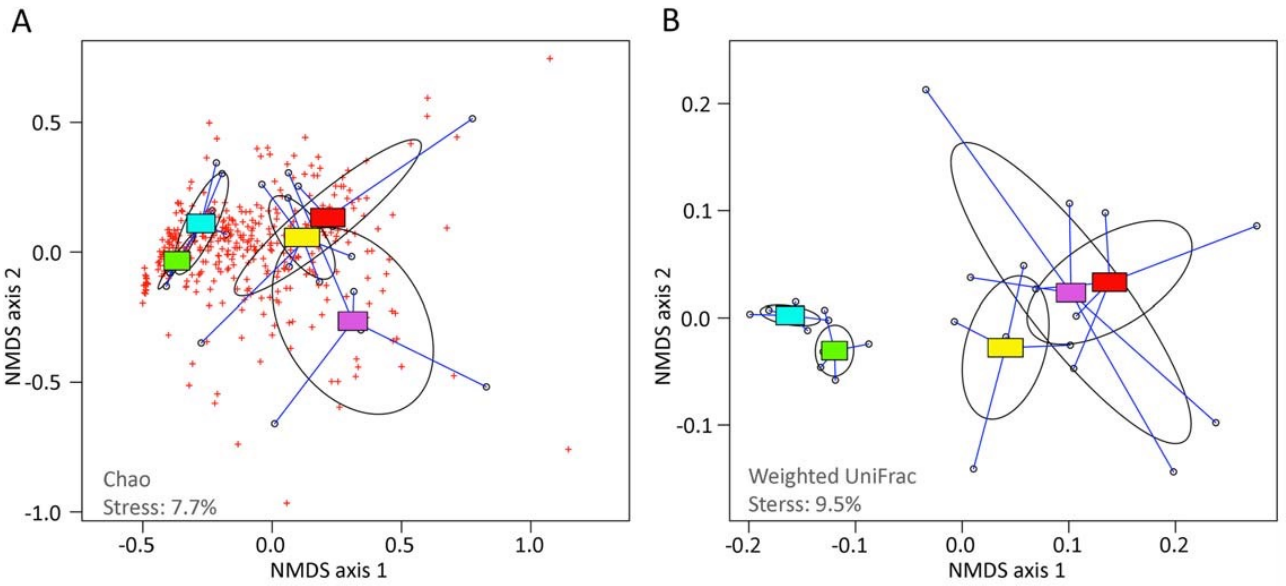

Figure. 2. Non metric multidimensional scaling analysis of the bacterial community structure using Chao (A) and Weighted Unifrac (B) metrics. The colour of rectangles indicates the different food resources; yellow: *Moringa oleifera* leaves; lilac: *Vicia faba* beans; green: artificial diet; red: chili powder; and cyan: pizzocchero. The circular open dots indicate the single organism while the crosses 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.

Bacterial taxonomic composition of the IMM and food microbiotas

The microbiotas associated with the IMMs fed on artificial diet and pizzoccheri and also 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 in annex), whereas the microbiotas associated with the specimens reared on chili, fava beans and moringa did not present a dominant taxonomic phylum because the number of OTUs of Proteobateria, Firmicutes and Actinobacteria were roughly equivalent (Fig. 1B and Table S2 in annex). 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-Tomàs et al., 2011; Welte et al., 2016).

The dominant component of the microbiotas associated with the moths feeding on an artificial diet and the wild population that fed on pizzoccheri belonging to the family of Aerococcaceae (52.5%61.77% and 65.4%6 5.76% respectively; Fig. 3 and Table S3 in annex). On the contrary, the bacterial communities associated with *P. interpunctella* feeding on chili, fava bean and moringa were dominated by members of Propionibacteriaceae (20.7%, 17.6%, and 15% respectively; Fig. 3). Comamonadaceae represent the second major component in the microbiota of the moths feeding on chili (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% 6 1.42% and 56.2% 6 4.67%, respectively, of the 16S rRNA gene sequence (Fig. 4A and Tables 2 and S4 in annex). 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 in annex). Furthermore, the genus *Atopococcus* is present in the majority of the analysed samples allows us to speculate that this genus might represent a potential symbiont of *P. interpunctella*. Lactic acid bacteria (LAB) tipically inhabits the insect gut (especially the foregut) of different insects , such as the wood- and soil-feeding termites (Bauer et al., 2000), cockroaches (Kane and Breznak, 1991) and honeybees (Apis spp.; Vasquez et al., 2009; Olofsson et al., 2011). The biological role of LAB correlated to insect physiology remains, at present, poorly investigated. In cockroaches, lactate produced by LAB is used to support the animal's respiratory requirements (Kane and Breznak, 1991) or plays a crucial 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.

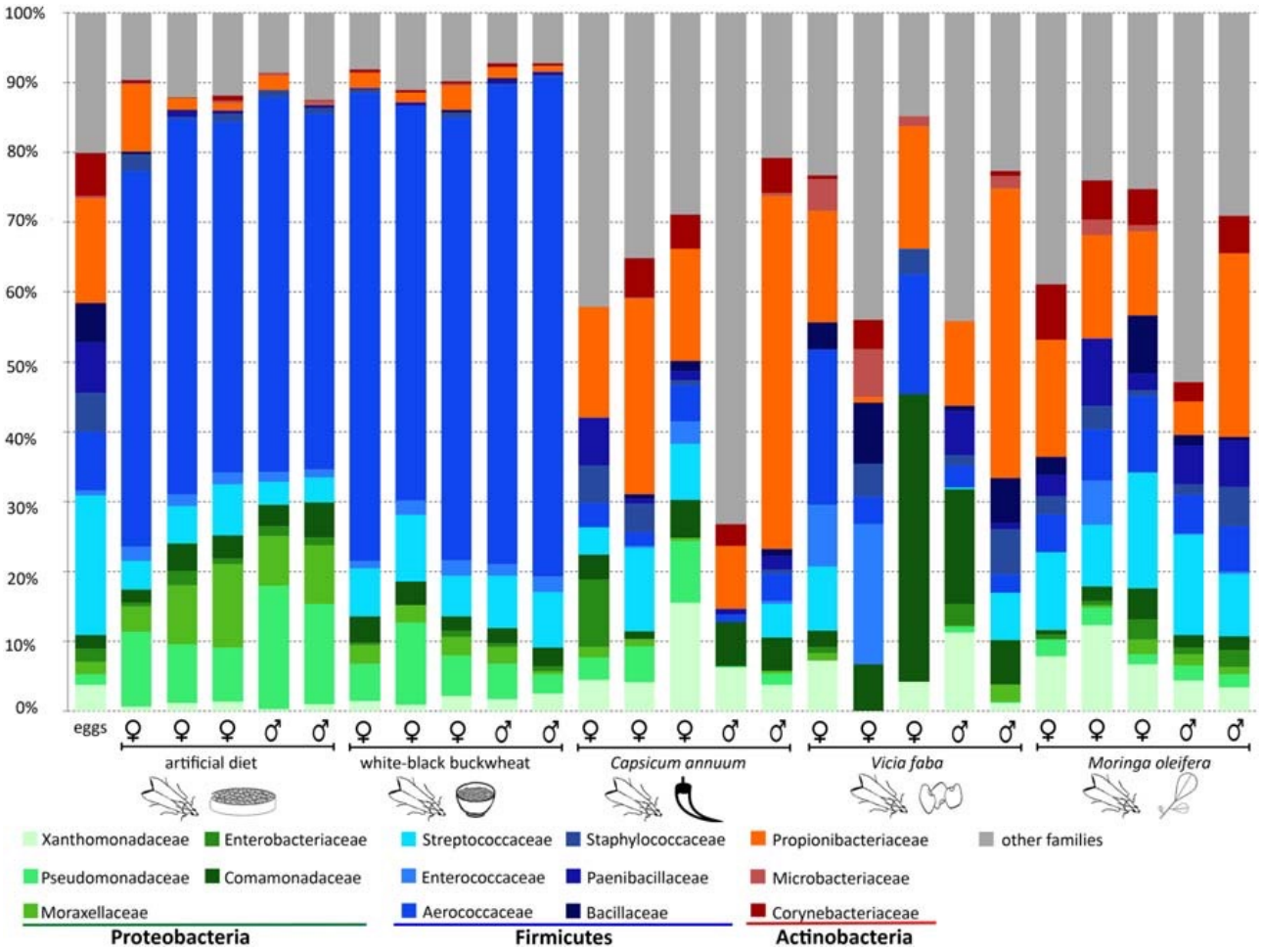

Figure 3. Histogram representing the taxonomic assignment of the 16S rRNA gene sequences to the bacterial family for the IMM eggs and adults. Only the bacterial families with an abundance ≥5% in at least one moth specimen are reported, and bacterial families with an abundance lower than 5% are grouped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

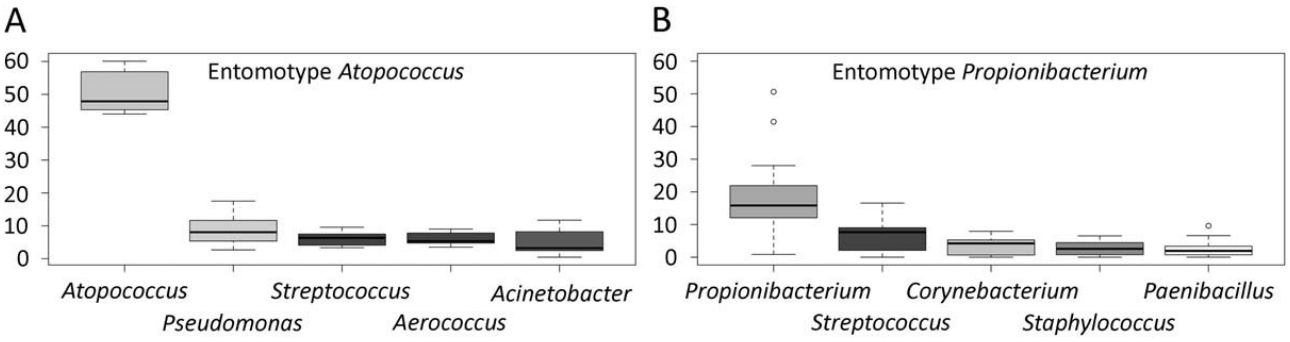

Figure 4. Boxplots of the bacterial entomotypes characterising the two groups of IMMs identified by the NMDS and clustering analyses. A. Atopococcus entomotype characterising the P. interpunctella specimens reared on an artificial diet and pizzoccheri. B. Propionibacterium

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 abun- dance 3% in at least one sample are reported.

	food[a]					*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

Metabolic potential

The predictive functional profile 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 in annex). A total of 38 functional categories showed significant differences using Kruskal–Wallis test ($P<0.05$) among the five groups of IMMs (Fig. 5 and Table S6 in annex). 31 categories are related to metabolism, while only three and one functions were assigned to genetic and environmental information processing respectively (Fig. 5). Within the functional categories related to metabolism, the majority were related 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 in annex). 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 moths reared on an artificial diet and pizzoccheri. These two groups were 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 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 in annex) with a low protein content (high C:N ratio; Table S8 in annex), 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 in annex).

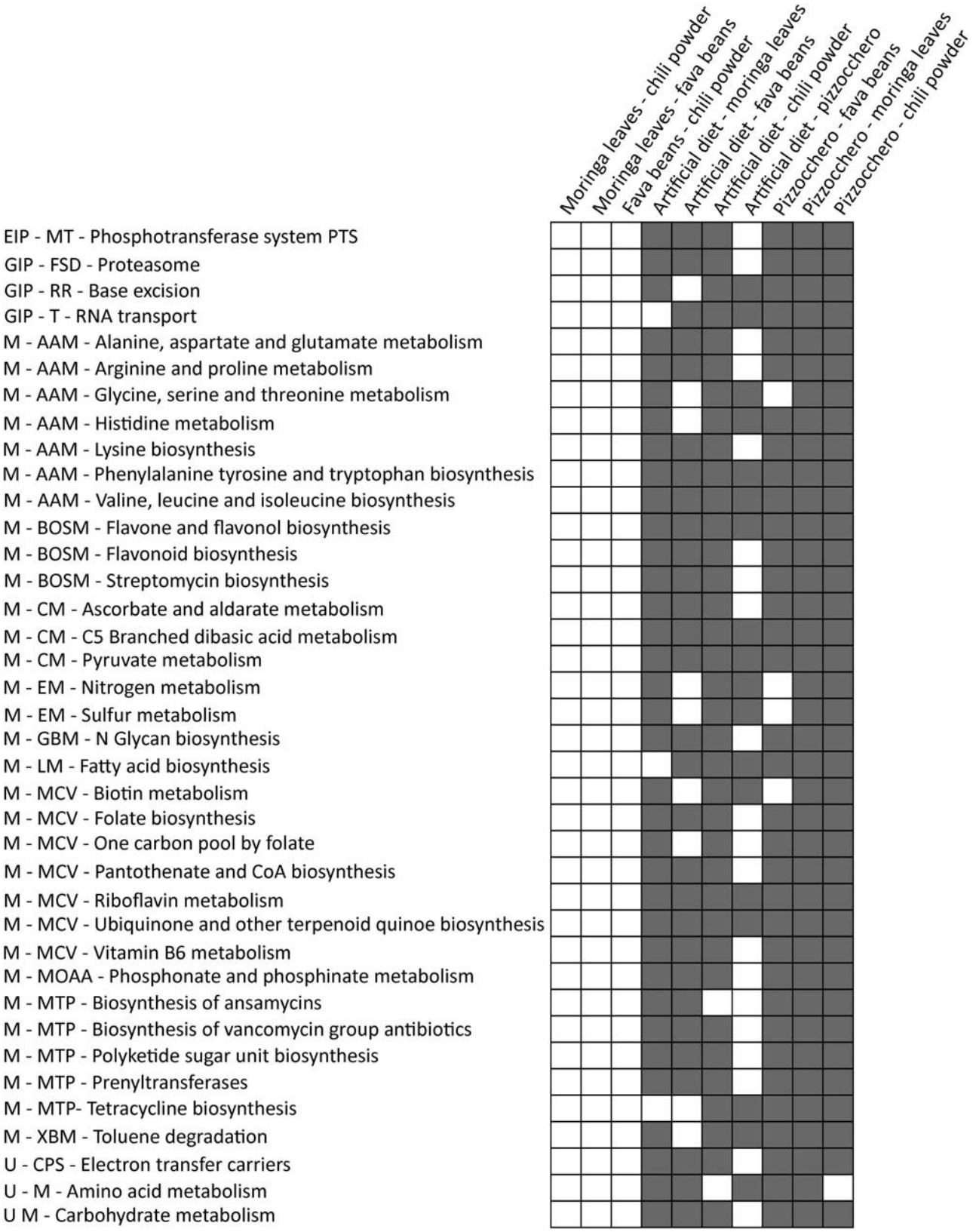

Figure 5. Mann–Whitney– Wilcoxon table reporting the categories of functional potential, inferred from the bacterial 16S rRNA gene sequences for which differences among the fivegroup were recovered by the Kruskal–Wallis test. Mann– Whitney–Wilcoxon test were performed to assess the differences in the pairwise comparisons of IMM groups: the white squares indicate a P_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.

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 cooccurrence table (Fig. 6 and Tables S9 and S10 in annex). The moths feeding 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 annex); 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 an average number of OTU reads per sample of 102; Figs 6, S1 and S2 in annex). A different result was detected in the case of the moths reared on chili powder, where the same number of OTUs (15 OTUs), even if with taxonomic differences, was shared between the eggs (average number of OTU reads per sample of 42) and the substrate 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. In conclusion, it is reasonable to think that 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 *P. interpunctella* specimens 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. We believe that further investigations are required to isolate and identify these bacteria, identifing the possible pathogenic implications. 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- Tom_as 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 in annex). This number includes the core microbiota shared with the eggs plus 36 further bacterial OTUs.

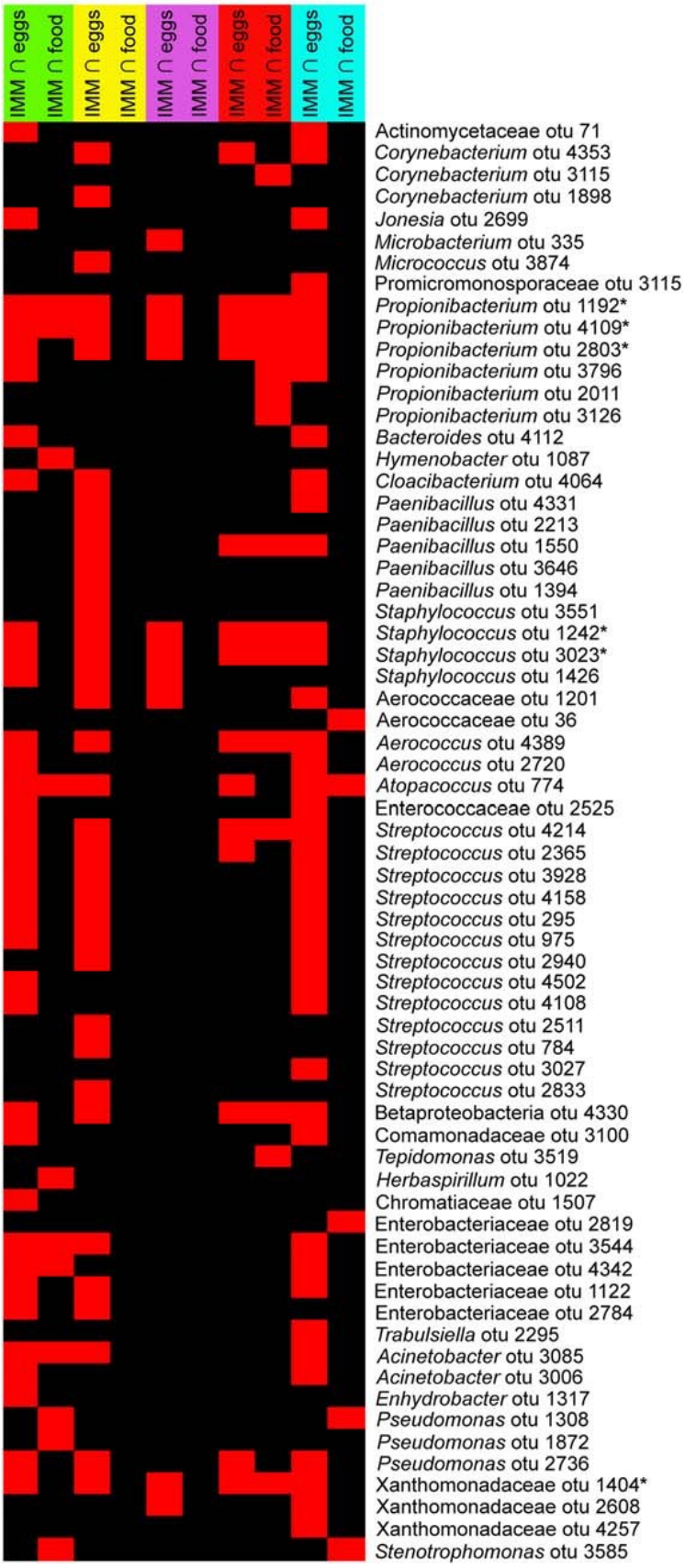

Figure 6. Co-occurrence OTUs table. For each IMM group, the co-occurrence table reports the IMM bacterial OTUs shared between the eggs from the laboratory population (left) and the food resource used for larval development (right). The colour of the rectangles indicates the different IMM groups: yellow: 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.

3.1.5 CONCLUSION

The microbiotas associated with the analysed Indian meal moths, reared on different food source (both laboratory and field populations), 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 bacterial communities associated to *P. interpunctella* specimens 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-Tom_as 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 associated to 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 the substrate 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. A six-OTU bacterial core was shared between eggs from the laboratory population and the adults from different populations. Future studies, with the inclusion of specimens from different geographic areas, could confirm the presence of the identified bacterial core and 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 profile 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 differences between groups are related to metabolism (Fig. 5), and in particular, with the metabolism of amino acids, cofactors and vitamins. The discovered 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.

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3.2 THE MICROBIOTA ACROSS THE DIFFERENT DEVELOPMENTAL STAGES OF *PLODIA INTERPUNCTELLA*

3.2.1 ABSTRACT

Diversity and composition of lepidopteran microbiotas are poorly investigated, in particular across the different developmental stages. To improve this knowledge, we characterize the microbiota among different developmental stages of *Plodia interpunctella* that 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, were 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.

3.2.2 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, Wang *et al.*, 2009, Huse *et al.*, 2008). 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 (Engel & Moran, 2013; Dillon & Dillon, 2004). Regarding lepidopterans, the studies on their bacterial communities have increased in the recent years, especially for insect pest moths such as *Ostrinia nubilalis* (Belda *et al.*, 2011), *Spodoptera littoralis* (Tang *et al.*, 2012), *Lymantria dispar* (Broderick *et al.*, 2004, Lin *et al.*, 2015), *Helicoverpa harmigera* (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., 3rdinstar larvae Snyman *et al.,* 2016; Hernàndez-Flores *et al.,* 2015, 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 and colleagues (2014) and revealed that larvae and adults of the neotropical butterflies *Heliconius erato* harboured 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 (Rees, 2004; Cuperus *et al.*, 1990; Vick *et al.*, 1986). 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 and 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, *viz* protein-rich *vs* carbohydrate-rich, suggesting the diet as a major driver in shaping the bacterial community (Montagna *et al.*, 2016).

In the present 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; *iii)* predict the functional profiles of the bacterial communities.

3.2.3 MATERIALS & METHODS

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 (half-double content of honey and without beer yeast). In order to obtain eggs, adults females were separated from the main laboratory population and allowed to oviposit for three hours. 90 eggs were directly stored at -80°C without sterilization, while the remaining eggs were allowed to hatch. The first instar larvae, three hours 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 4% solution of sodium hypochlorite for 3 min; *iii*) washing with PBS with 0.1% Triton-X-100 for 3 min, and *iv*) a final washing with sterile distilled water. The previous 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 CTAB 2% (2% CTAB, 0.2% ascorbic acid, 1.5% PVP, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added to each sample. Tissues were, then, disrupted using glass beads (ø 0.1 mm) using the Precellys®24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), then 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 hour. 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) 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 27Fmod and 519Rmod primers (Lane et al., 1991); no positive amplifications were obtained.

DNA sequencing and bacterial community analysis

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 (27Fmod 5'-AGRGTTTGATCMTGGCTCAG-3' and 519Rmod 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. U.S.).

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 a Phred score <30 and within the size range 350 - 500 bp. Uclust (Edgar *et al.*, 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.

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, 1948) 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. Weighed Unifrac distance matrix was created using QIIME software (script: *beta_diversity.py*) and used for generating the Principal Coordinates Analysis (PCoA) plots. 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 > 1,000 reads were selected to perform a phylogenetic assignment using a selected dataset of Burkholderiales 16S rRNA sequences, as in previous studies (e.g., Michalik *et al.*, 2016; Boucias *et al.*, 2012; Kikuchi *et al.*, 2011). A total of 22 partial 16S rRNA sequences belonging to *Burkholderia* were downloaded from GenBank (Benson *et al.*, 2013) (Table S4 in annex), in addition 16S rRNA sequences from representatives of other Burkholderiales genera and *Escherichia coli* (as outgroup) were retrieved (Table S4 in annex). 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 *et al.*, 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 + Γ. 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 stagewas analyzed. The bacterial functiona were 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-value* < 0.05) among the IMM life stages the Kruskal-Wallis test (*agricolae* package in R software) and the Bonferroni correction were applied.

3.2.4 RESULTS

Bacterial diversity

The microbiota associated with the selected developmental stages of *Plodia interpunctella* were analyzed using Illumina sequencing. 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 (< 1,000 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 in annex). 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', Pielou's J' and Chao-1).

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

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, respectively; Fig. 1; Table S1 in annex). Actinobacteria resulted the second most abundant phylum, followed by bacteria belonging to Firmicutes (Fig. 1; Table S1 in annex). Burkholderiaceae resulted the most abundant bacterial family in all the insect semaphoronts (Fig. 1; Fig. 2; Table S2 in annex); 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 in annex).

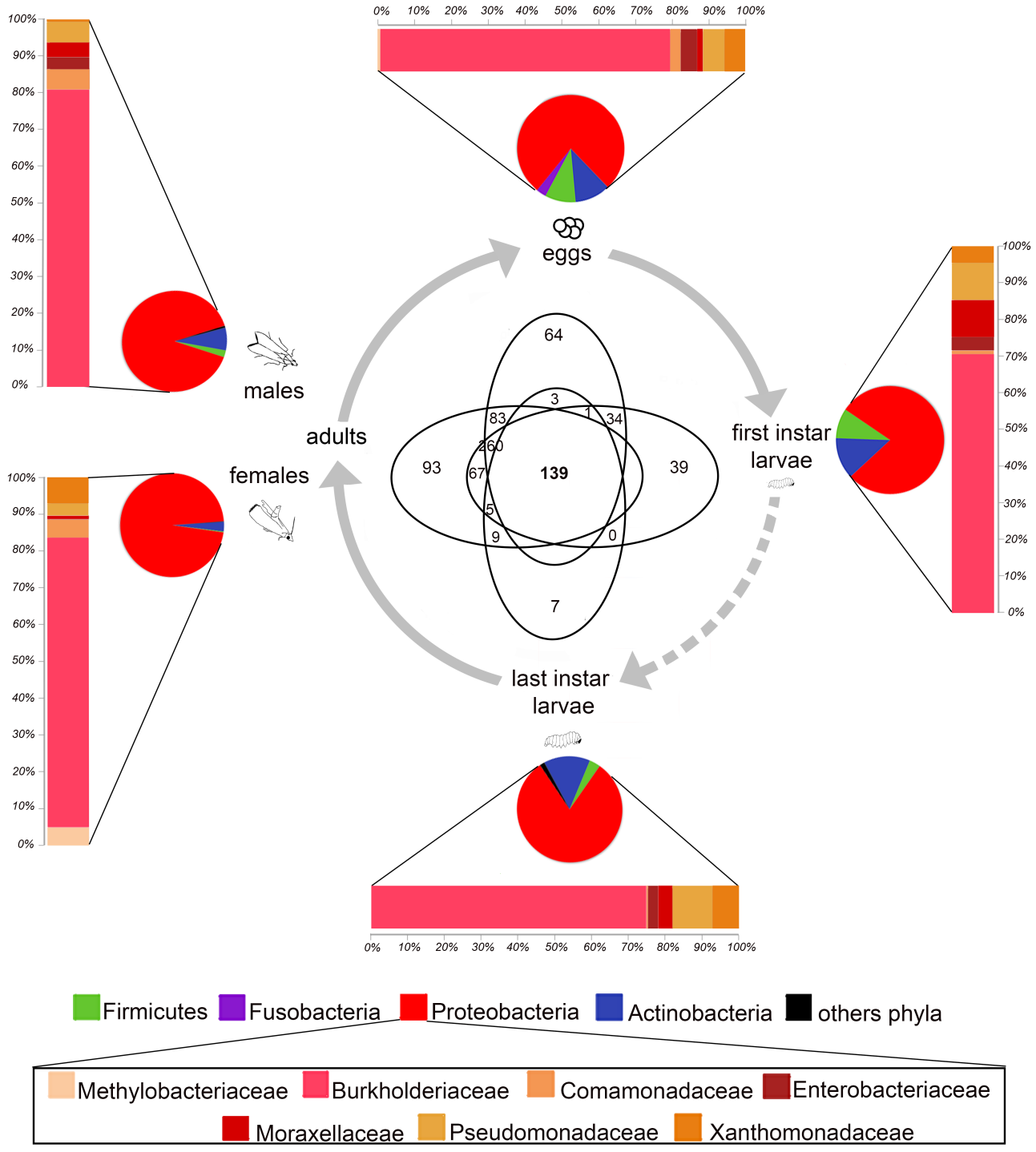

Figure 1. Taxonomic composition and bacterial core of the microbiotas associated with the analyzed developmental stages of *Plodia interpunctella* (eggs, first instar, last instar, adult females and males) at phylum (pie charts) and family levels (bars). Only the bacteria families with an average abundance ≥ 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).

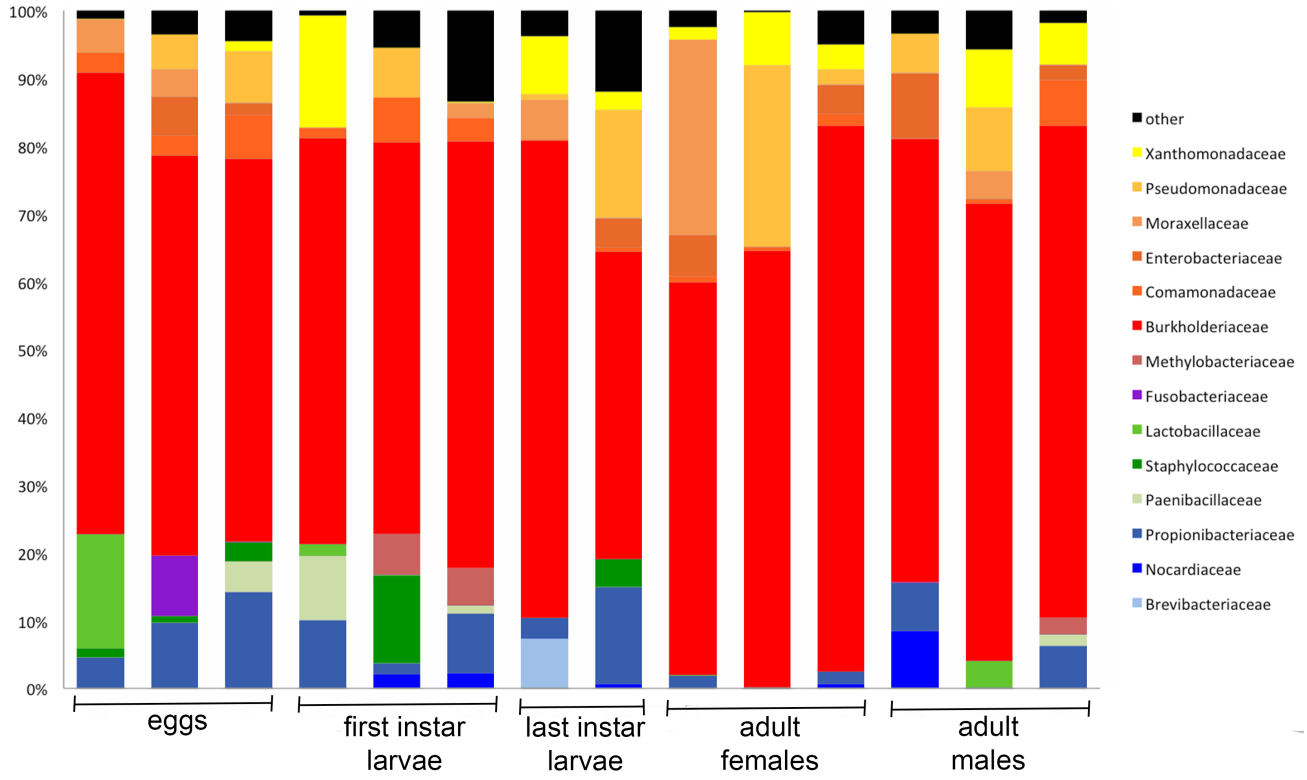

Figure 2. Histogram representing the bacterial composition, at family level, for the analyzed developmental stages. In the histograms are reported only taxa with a relative abundance ≥ 5%.

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

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* and *Propinebacterium* with three OTUs, *Enterobacter* (2 OTUs), and one OTU for the genera *Corynebacterium, Rhodococcus*, *Brevibacillus*, *Staphylococcus*, *Fusobacterium*, *Achromobacter*, *Delftia*, *Enhydrobacter, Micrococcus, Brevibacterium* and *Defluvibacter*.

The β-diversity was investigated using weighted Unifrac distance matrix and visualized with Principal Coordinate Analysis (PCoA) (Fig. S2 in annex). After the analysis of the eigenvalues and of the scree plot, the first two principal components were considered. These components explain approximately ~60% of the variation (1st component 40.8% and 2nd component 19.1%). On axis 1 the bacterial communities associated with the analyzed samples appear to be segregated into two groups, however no clear pattern is identifiable considering the developmental stages. On axis 2 the bacterial communities are dispersed.

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).

Phylogenetic placement of the most abundant Burkholderia OTUs

Three *Burkholderia* OTUs, with a number of reads > 1,000, were selected for the phylogenetic inference, namely denovo923 with 1,685 reads, denovo1584 with 323,731 reads and denovo2766 with 1,016 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. S3 in annex) (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).

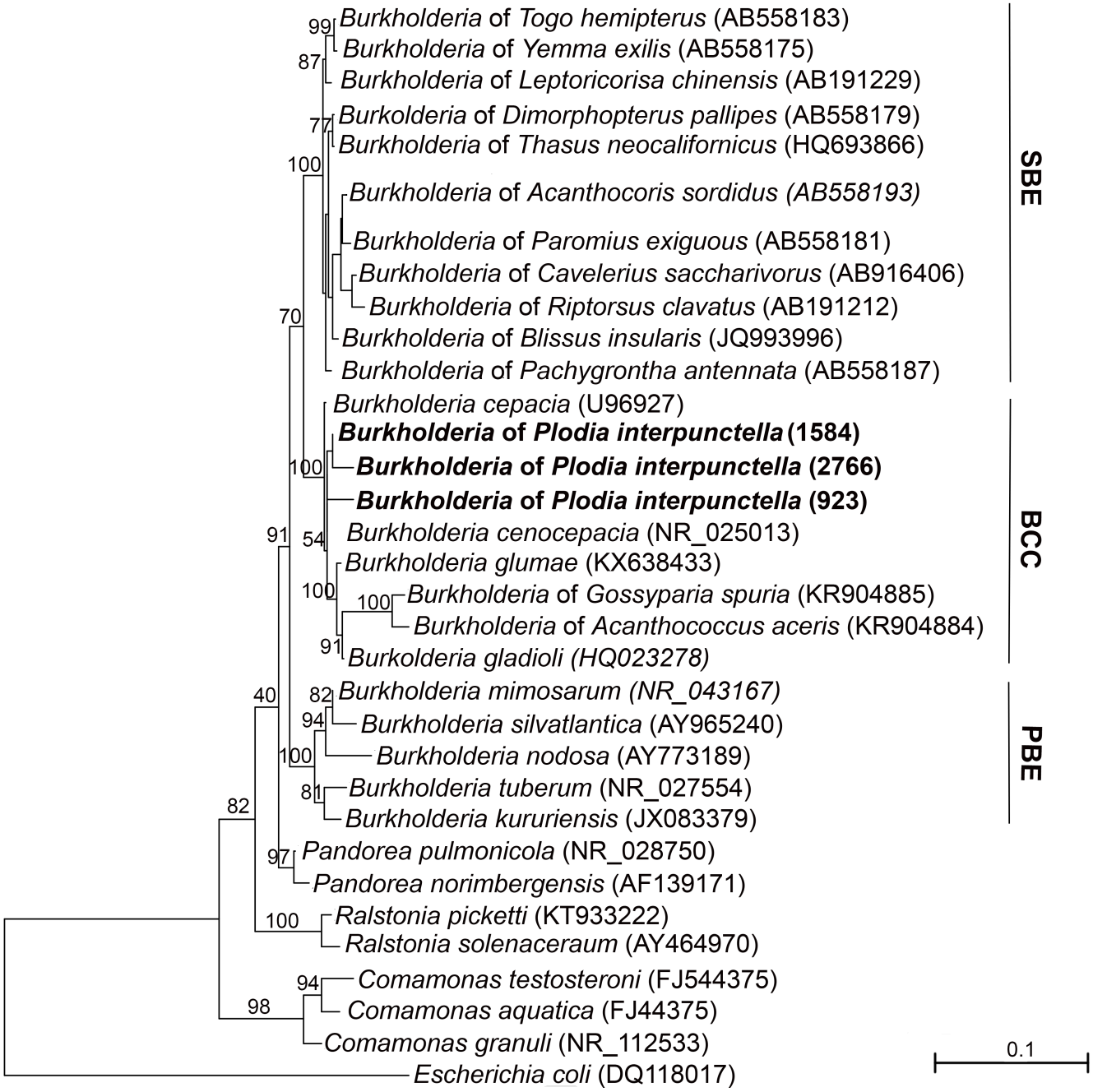

Figure 3. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences. Vertical dashes lines indicate the three different *Burkholderia* groups. SBE: stinkbug associated beneficial and environmental *Burkholderia*; BCC: *Burkholderia cepacia* complex; PBE: plant-associated beneficial and environmental *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 in annex), 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 denovo OTUs.

Metabolic potential

The metagenomic functional potential of the insect-associated bacterial communities were predicted on the basis of 16S rRNA sequences (Table S5 in annex). The relative abundance of seven functional categories, that can be linked to the insect physiology, were 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-value* = 0.047; D-alanine and D-glutamine, $\chi^2 = 8.85$, df =4, *p-value* = 0.037; D-glutamate metabolism, $\chi^2 = 9.54$, df =4, *p-value* = 0.048). Two functional categories were associated to carbohydrate metabolism; namely, the metabolism of galactose ($\chi^2 = 9.08$, df =4, *p-value* = 0.031) and the glycolysis and gluconeogenesis ($\chi^2 = 9.16$, df =4, *p-value* = 0.047). The two last functional categories belong to cofactors metabolism (thiamine metabolism, $\chi^2 = 9.84$, df =4, *p-value* = 0.043) and to the vitamins and xenobiotics biodegradations metabolism (drug metabolism, $\chi^2 = 9.31$, df =4, *p-value* = 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 in annex). 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 *vs* adult females (Fig. 4).

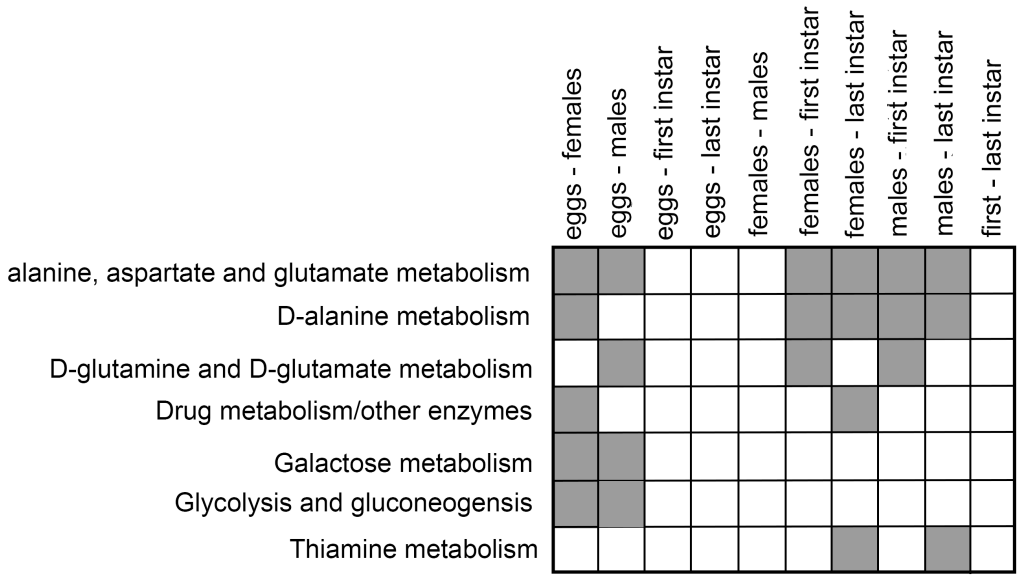

Figure 4. Table reporting the metabolic predicted function inferred from the bacterial 16S rRNA gene sequences for which differences among the developmental stages were recovered by Kruskal-Wallis test with the Bonferroni correction. White square: p-value > 0.05; grey squares: p-value < 0.05.

3.2.5 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 and last larval instar (i.e., 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 the present 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 *vs* 454 pyrosequencing.

Compared to previous studies, where changes in the bacterial communities were observed across the different developmental stages (Chen *et al.*, 2016; Hammer *et al.*, 2014), in the present 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 behaviour. *Heliconius erato* and *Spodoptera littoralis*, where major changes in the microbiotas of pupae and adults were observed (Chen *et al.*, 2016; Hammer *et al.*, 2014), 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 ± 0.03, Table 1) indicate highly imbalanced microbiotas, contrasting with previously obtained values ($J'_{AVERAGE}$ = 0.64 ± 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, Table 2, S3 and S4 in annex), with Proteobacteria accounting, on overage, 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 (Xiang *et al.*, 2006, Broderick *et al.*, 2004, 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 carbhoydrate and protein-rich diets (Montagna *et al.*, 2016). The artificial diet on which IMMs of the present 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*, five out of the ten genera of the core identified in the present study are shared with the previously described entomotypes (i.e., *Pseudomonas*, *Acinetobacter*, *Propionibacterium*, *Corynebacterium, Staphylococcus;* Montagna *et al.*, 2016).

Burkholderia is found in different environments such as water, soil and the plant rhizosphere (Woods & Sokol, 2000). It was first described as a plant pathogen (Yabuuchi *et al.*, 1992) but it can also be hazardous to humans and animals (Coenye *et al.*, 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, as example 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 glycolisis, 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 a-ketoglutarate (Sacktor & Wormser-Shavit, 1966). Based on the fact that the relative abundance of the metabolic functions were 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 of larvae and adults, which have an impact on the gut physiology (Altermatt & Pearse, 2011). In fact, *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 (Ayayee *et al.*, 2016; McCutcheon, 2007). 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 the present 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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4. CONCLUSIONS

In this PhD thesis, two research articles and one review article were presented. The review summarised literature data regarding the bacterial community of moth pests using Next Generation sequencing (NGS) technologies, showing the bacterial communities of two groups of moths: forest and crop pest. Despite their high worldwide economic importance, the informations on the microbiota associated to these moths were very poor, also compared with other insect orders. Despite this, the collected data provided information about the possible shift/change of the moth's bacterial communities in relations with different factors. Among others, three factors are potentially able to affect the bacterial community associated to the moth pests: *i)* the diet; *ii)* the origin of the population, and; *iii)* the developmental stages. In particular, I fitted the bacteria community information of different moth pests (at family level) provided by the literature with a NMDS analysis. Then these data were fitted with the diets factors that might affect the structure of the associated bacterial communities, finding that the bacterial communities of moths were mainly influenced by the group of plants on which they feed (gymnosperms and angiosperms). This result can be explained by the fact that the forest moths generally feed on conifers (gymnosperm), which contain a high variety of compounds such as terpenes (the main component of resins in conifers) (Fürstenberg-Hägg et al., 2013). As example, the host plants of the moth pest *Thaomatopoea pityocampa* (*Pinus halepensis*, *Pinus nigra* and *Pinus pinaster*) contain some specific essential oils such as the monoterpenes myrcene and a-pinene (Macchioni et al., 2003) while *Pancratium maritimum*, the host plant of *Brithys crini* contain potent alkaloids (Hetta et al., 2013). Monoterpenes are known to possess toxic effects on insects, such as *Spodoptera litura* or several beetle species (Trapp et al., 2001; Hummelbrunner et al., 2001). As for other insects (Ceja-Navarro et al., 2015), we can hypothesize that the microbiota of the moths feeding on these conifers may play a role with the detoxification of such toxic compounds. In particular, *Dentroctonous ponderosea* (mountain pine beetle), harbour some bacteria belonging to the genus *Pseudomonas* and *Rahnella*, that are able to degrade terpenes, allowing *D. ponderosea* to feed on terpene-rich trees (Adams et al., 2013). Although there are few studies invesitigating the microbiota of moths, none of these studies focused on stored product pests.

Stored products, as seeds, dried meat and fruit, are infested and damaged by a different type of pest, including arthropods (e.g., insects and mites) and vertebrates (e.g., rodents and birds) (Rees, 2004; Spieksma, 1997). Within insects, five orders of insect were recognised as the major pest of stored products: beetles (Coleoptera), moths (Lepidoptera), psocids or bookilice (Psocoptera), bugs (Hemiptera) and wasps

(Hymenoptera) (Rees, 2004). Insects infestations of these products cause high economic losses (Rees, 2004). Furthermore, these types of insects are also considered as a harmful pest, because they are potentially vector of human and animal pathogens (Mason, 2012). Therefore, an efficient control of these pests is necessary. Since some of these traditional approaches can be dangerous to human health (e.g. chemical insecticide), the manipulation of bacteria symbiont could represent a promising alternative for the control of these pests (El-Aziz, 2011). For this reason, the study of the bacterial community associated to stored product pests is important. The few studies that investigated stored product pest were focused on both insects (e.g., in *Tribolium castaneum*, *Liposcelis bostrychophila*, *Acanthoscelides obtectus*, *Callosobruchus maculatus*, *Sitotroga cerealella*, *Phthorimaea operculella, Sitophilus zeamais, Stegobium paniceum;* Prabha et al., 2011; Behar et al., 2010; Yusuf et al. 2004; Sevim et al., 2016, Lakshmikantha et al., 2006) and mites (e.g., *Tyrophagus putrescentiae*; Erban et al., 2016; Pekas et al., 2017).

Due to the fact that the diet has a great impact on the moth-associated bacterial community and because in particular anyone investigate the microbiota associated to a stored products moth, the worldwide pest *Plodia interpunctella* (indian meal moth, IMM) was used as a study model for these bacterial community studies. Furthermore, the bacterial community of this moth was also investigated across their life cycle. In particular, the first study investigated the microbiota of laboratory populations, feeding on different diets (i.e., artificial diet, *Moringa oleifera* leaves and *Vicia faba* beans), as well as that of field populations feeding on *Capsicum annuum* chilli and white-black buckwheat (named pizzoccheri); whereas the second study focused the attention on the bacterial community of the following developmental stages: eggs, first and last instar larvae and adults (both males and females).

In conclusion, the studies showed that the bacterial community associated to *P. interpunctella* specimens was greatly influenced by diet. The results of the first experiment showed the existence of two distinct communities, named entomotypes, based on the protein and carbohydrate content of the diet. Firmicutes was the dominant component of the microbiota associated with moth feeding on artificial diet and pizzoccheri (both carbohydrate-rich diets), while the other groups did not show the presence of dominant taxa. Similarly, it has been observed that crickets' microbiota differed when insects fed on protein-rich diet compared to when they fed on fiber-rich diet (Santo Domingo et al., 1998). Furthermore, if we compare the bacterial community of *P. interpunctella* with the microbiotas of the mite *Tyrophagus putrescentiae* and *Carpoglyphus lactis* (Hubert et al., 2014; Erban et al., 2016; Pekas et al., 2017), that belongs to a different class, but feed on

the same stored products (Garcia, 2004; Chmielewski et al., 1971), no common pattern regarding bacterial compositions emerges. The bacterial communities associated to these two mites genera, were characterized by the presence of *Leuconostoc*, *Elizabethkingia*, *Solitalea*, Bacillus cereus (for *Carpoglyphus lactis;* Hubert et al., 2014*)* and *Bartonella*, *Blattabacterium* and *Solitalea* for *Tyrophagus putrescentiae* (Herban et al., 2016), while the microbiota of P. *interpunctella* was characterized by *Atopococcus*, *Proprionibacterium*, *Pseudomonas* and *Burkholderia*. These results suggest that several factors (including host phylogeny or physiology) could shape the bacterial communities in addition to the food source or composition. Furthermore, 42 OTUs were shared between the different adult populations of *P. interpunctella* feeding on different diets (artificial diet, *Moringa oleifera* leaves, *Capsicuum annum* chilli, pizzoccheri, *Vicia faba* beans). These OTUs were assigned to the genera: *Propionibacterium*, *Corynebacterium*, *Streptococcus*, *Acinetobacter* and *Staphylococcus*. Interestingly, Sevim and colleagues (2016), using a culture-dependent approach, isolated 17 *Staphylococcus* strains from four stored product pests (*Acanthoscelides obtectus*, *Callosobruchus maculatus*, *Sitotroga cerealella* and *Phthorimaea operculella*) reared on different substrate (beans, kidney beans, corn and potatoes respectively). Despite the low number of studies related to the bacterial communities associated with these insects, the results from the reported studies showed that the genus *Staphylococcus* seems to be related to the stored product pests and could represent a possible target for developing some biocontrol strategies, since it is easy to genetically modify bacteria of this genus (Monk et al., 2012).

Regarding some possible shift among the life cycle of *P. interpunctella*, since no differences were detected among all developmental stages, I can affirm that the bacterial community of *P. interpunctella* was not influenced by the developmental stages, as in the cases of *P. xyostella* (Xia et al., 2017), where Enterobacter cloacae, belonging to the phylum of Proteobacteria, was stable across the analysed developmental stages (3rd instar larvae, pupae and adults). In this second study a different artificial diet was adopted compared to the previous and for this reason it was difficult to compare the results of the two studies, but I can assess that the bacterial community changed using these two different artificial diets. The main finding was that the bacterial community remained stable across the analysed life cycle of *P. interpunctella*. In particular, all developmental stages were composed for more than 60% of OTUs, by the same genus, *Burkholderia*. Furthermore, a bacteria core of 139 OTUs was discovered in all developmental stages, of which 112 OTUs were assigned to *Burkholderia*. The presence of *Burkholderia* with these high relative abundances in all the analyzed developmental

stages, represents the first report describing the presence of this genus in a lepidopteran species. This bacterium has been described before in *Riptortus pedestris* and other heteropterans. In *R. pedestris, Burkholderia* has been found to establish a beneficial symbiosis conferring resistance to insecticides (Kikuchi et al., 2011; Kikuchi et al., 2007). The role played by this bacterium in *P. interpunctella* is still unclear, and further studies on the system *P. interpunctella-Burkholderia* should be conducted in order to unveil its possible function. Furthermore, a phylogenetic analysis clustered these OTUs in the groups of *Burkholderia Cepacia* Complex (BCC), that cause several diseases in human and animals (Lipuma, 2005, Segonds *et al.*, 2009, Ham *et al.*, 2011).

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5. ANNEX

5.1 SUPPLEMENTARY DATA OF THE WORK "THE INFLUENCE OF DIFFERENT DIETS ON MICROBIOTA ASSOCIATED WITH THE STORED PRODUCTS PEST *PLODIA INTERPUNCTELLA* (LEPIDOPTERA, PYRALIDAE)"

FIGURE

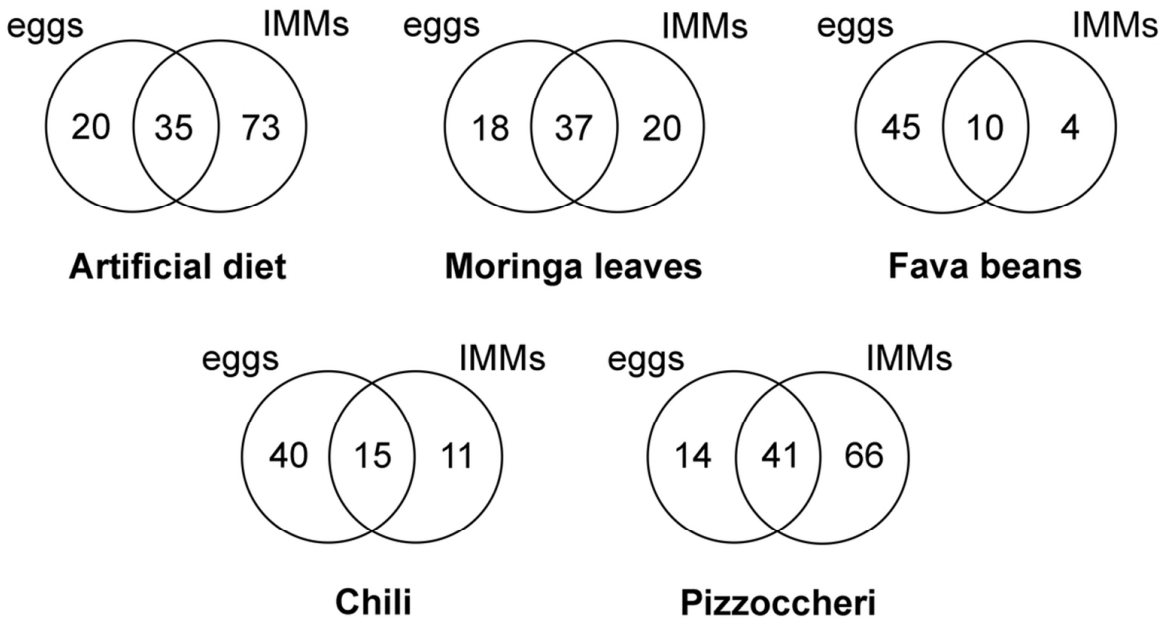

Figure S1. Venn diagram pairs showing the bacterial OTUs (at 97% similarity) shared by eggs and moth groups. The presence of a bacterial OTU is assigned to the group of insects feeding on the same resources when it is reported for at least three specimens of the group.

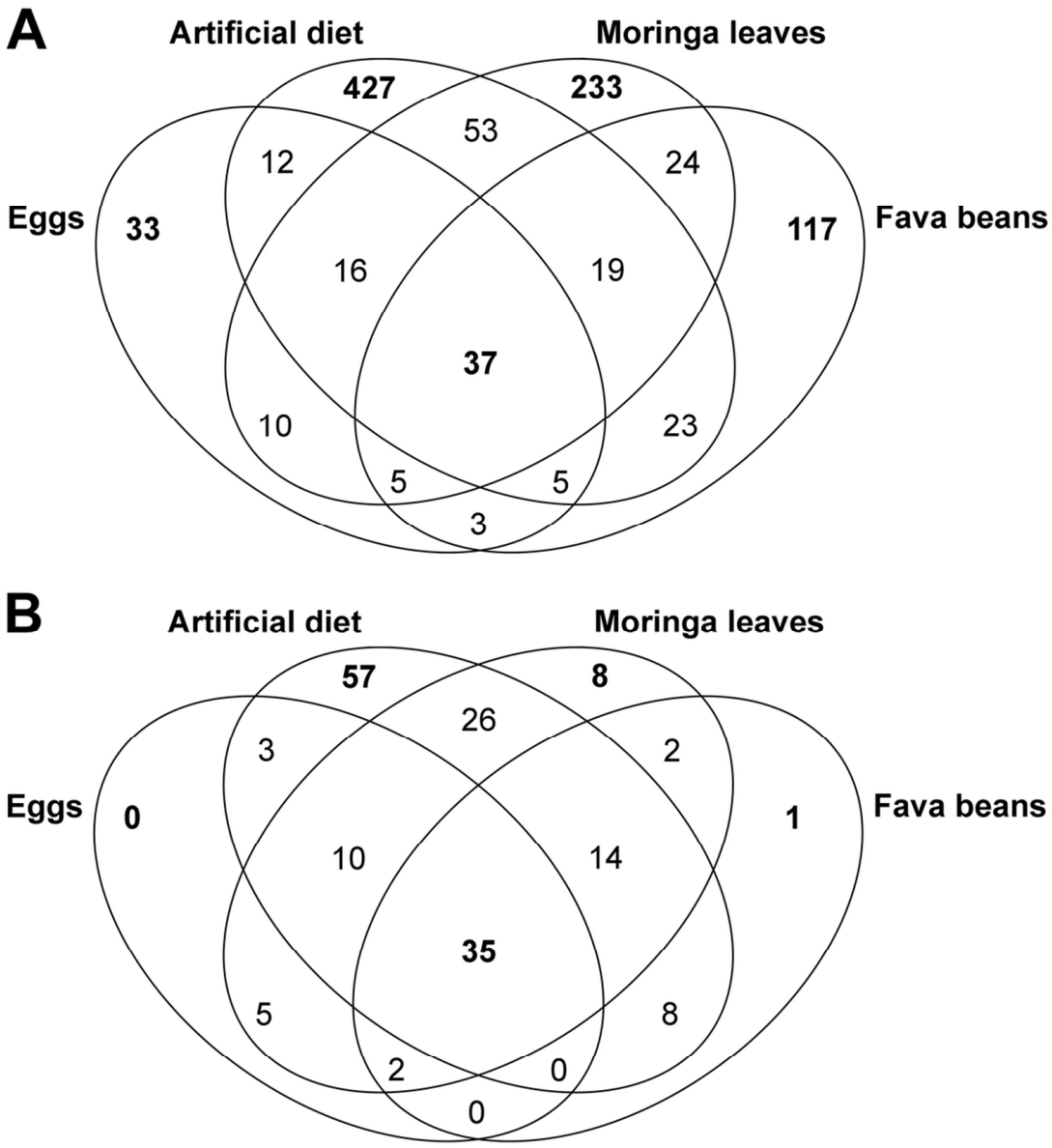

Figure S2. Venn diagrams showing the bacterial OTUs (at 97% similarity) shared by eggs and the three groups of adult moths from the laboratory population. The presence of a bacterial OTU is assigned to the group of insects feeding on the same resources when it is reported for at least one specimen of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

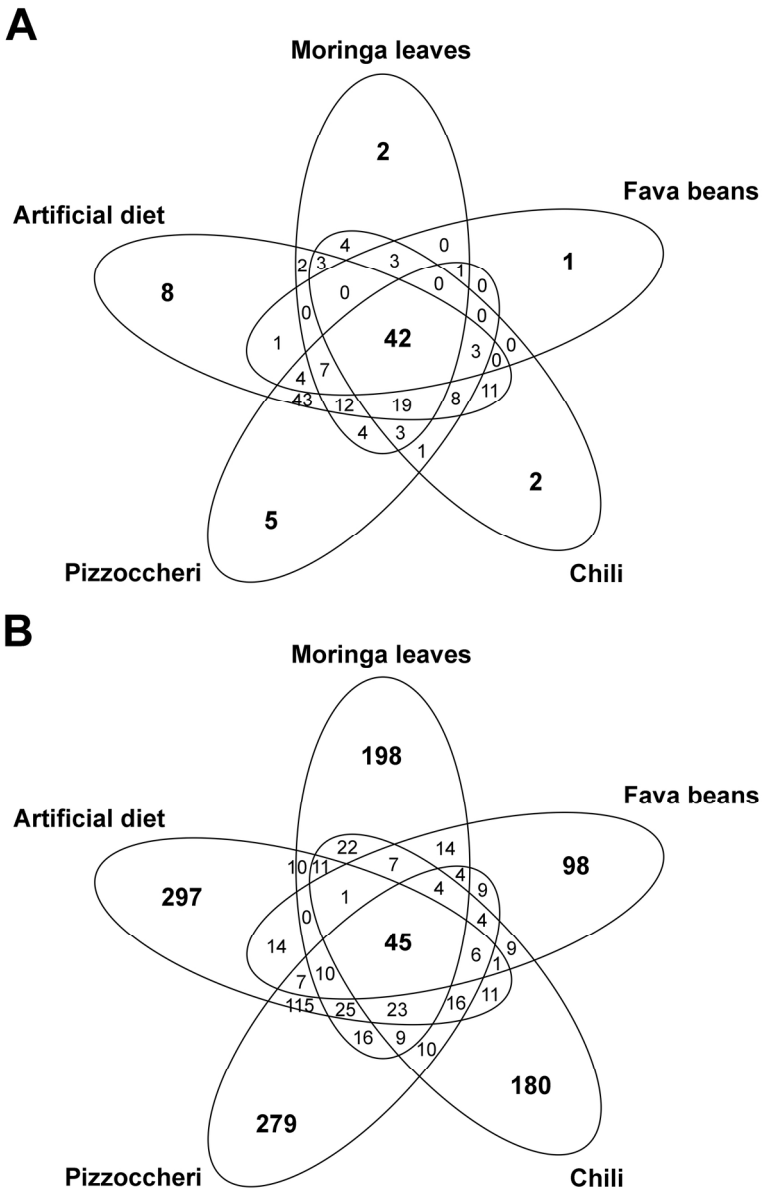

Figure S3. Venn diagrams showing the bacterial OTUs (at 97% similarity) shared by the five groups of adult IMMs. The presence of a bacterial OTU is assigned to the group of insects feeding on the same resources when it is reported for at least three specimens of the group (A) and one specimen of the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

TABLES

See the attacched Excel file, named "Supplementary material_DIET"

5.2 SUPPLEMENTARY DATA OF THE WORK "THE MICROBIOTA ACROSS THE DIFFERENT DEVELOPMENTAL STAGES OF *PLODIA INTERPUNCTELLA*"

FIGURE

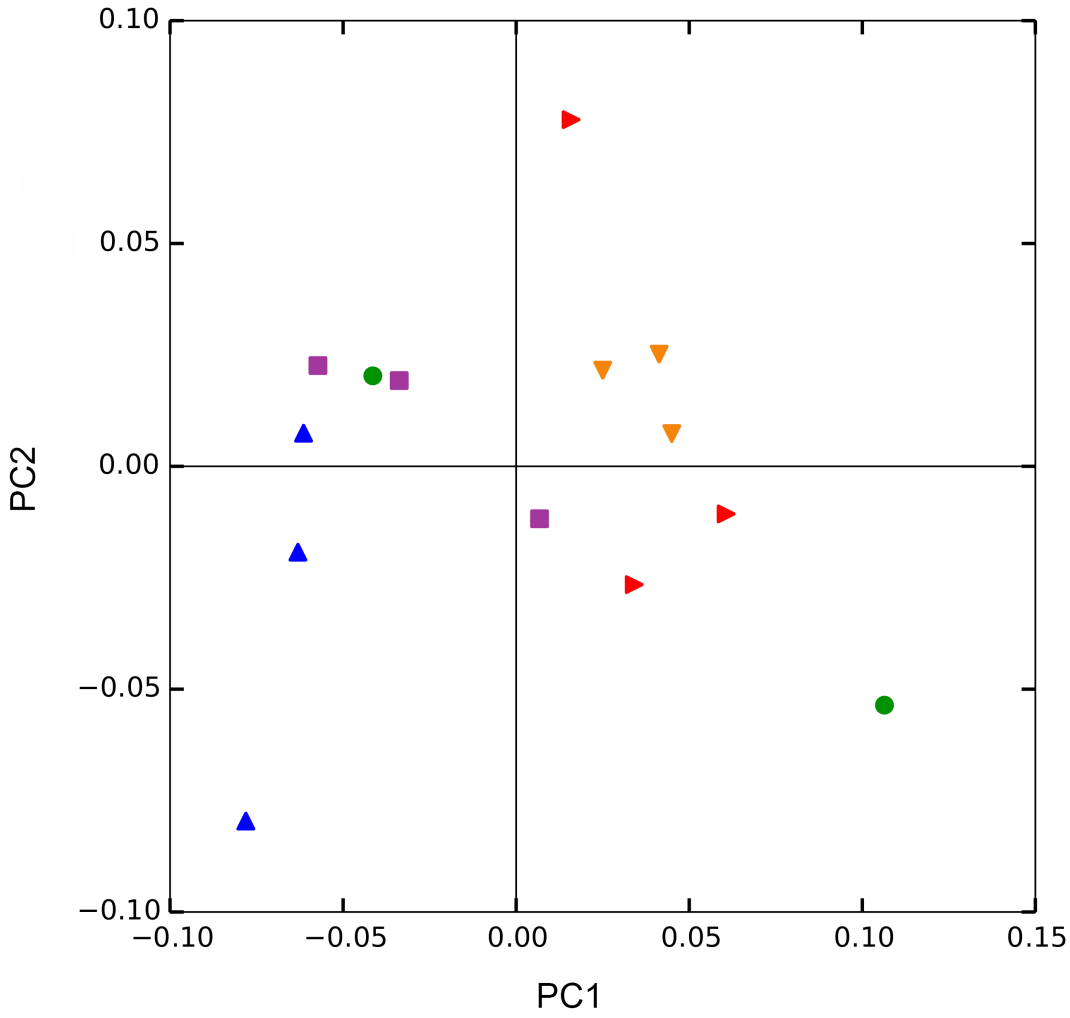

Figure S2. Principal Coordinates Analysis (PCoA) of the bacterial structure in the analyzed stages of IMM. Blue triangle = adult male; purple square = adult female; red triangle = eggs; orange triangle = early instar larvae; green circle = last instar larvae.

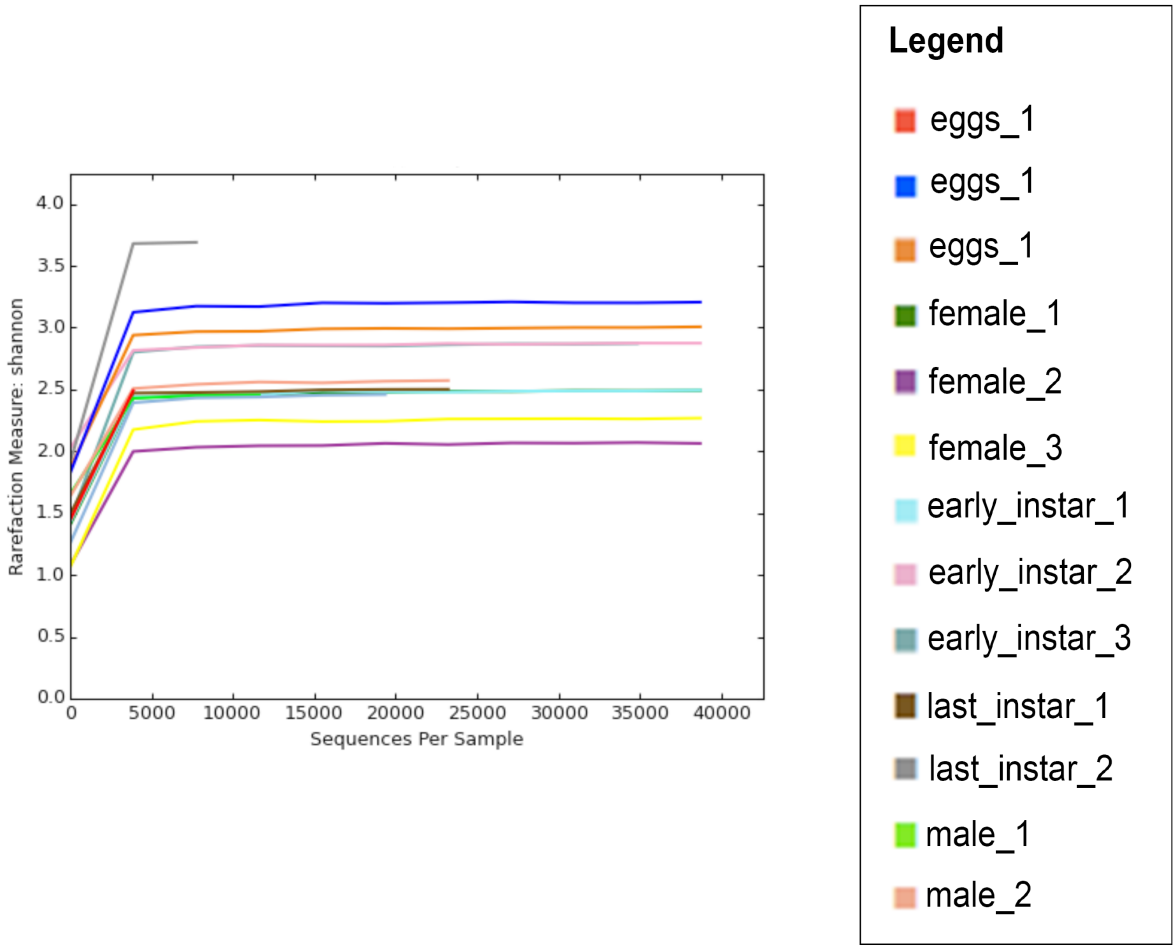

Figure S1. Rarefaction curves obtained using Shannon Index.

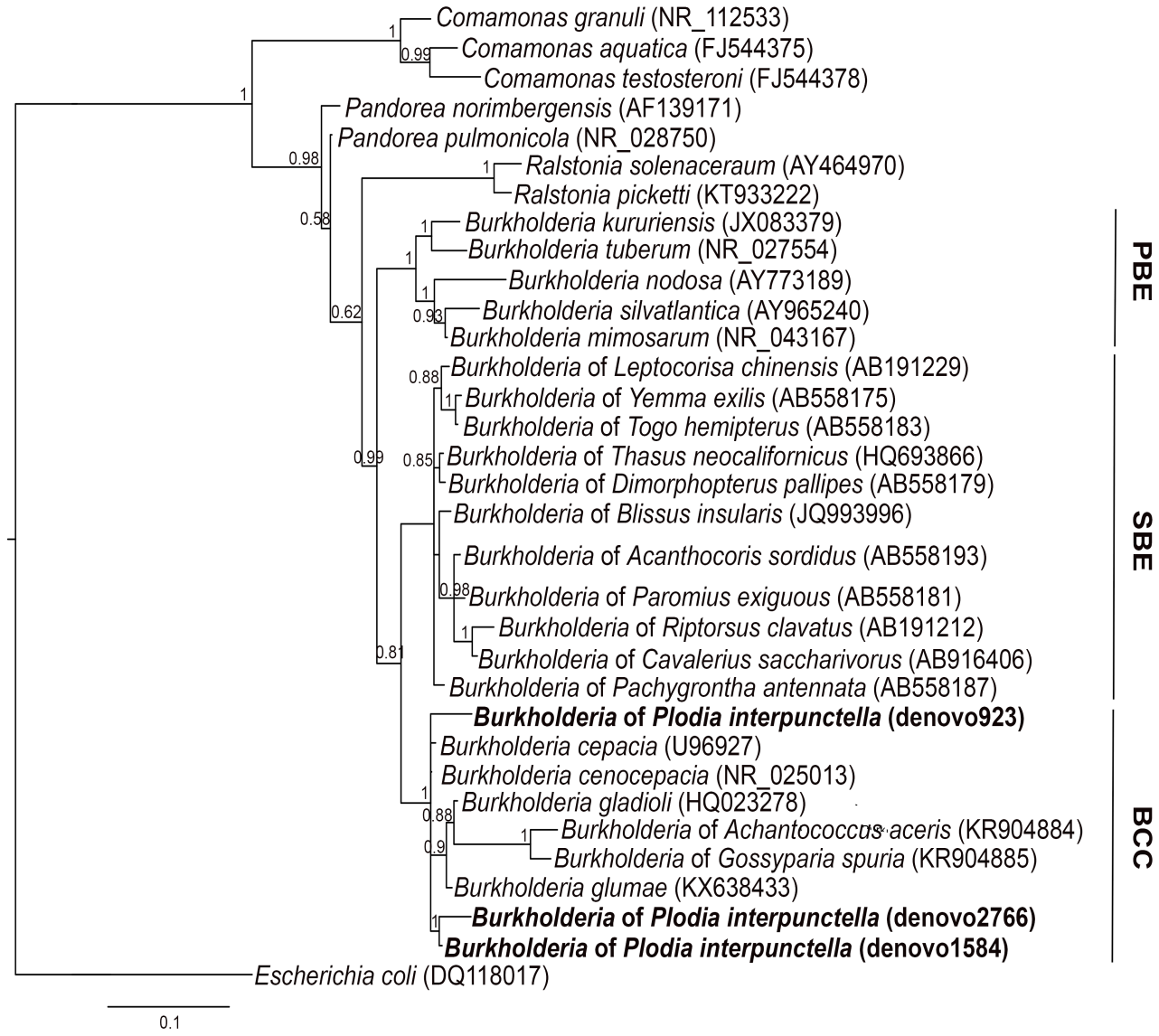

Figure S3. Bayesian phylogram based on 16S rRNA gene sequences. Vertical dashes lines indicate the three different *Burkholderia* groups. SBE: stinkbug associated beneficial and environmental *Burkholderia*; BCC: *Burkholderia cepacia* complex; PBE: plant-associated beneficial and environmental *Burkholderia*. 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 denovo OTUs.

TABLES

See the attacched Excel file, named "Supplementary material_DEVELOMPMENTAL_STAGE"