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THE GUT MICROBIOME ASSOCIATED TO HONEYBEES AND WASTE-REDUCING INSECTS

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Abstract

Insects are the most diverse group of animals on Earth and are adapted to a wide range of habitats. They have a remarkable impact on the human life: they include not only human and animal parasites, crop pests, or vectors of human, animal and plant diseases, but also beneficial insects, such as pollinators (e.g. the honeybee *Apis mellifera*), insects reared to obtain products for the human benefit (e.g. silkworms) or mass-reared insects as food and feed or as biological control agents (e.g. parasitoids or predators). Moreover, since the increase of human population, the growing demand for protein for human and animal consumption is forcing the search of alternative sources: in this scenario insects have been proposed as sustainable rich-protein substrates. For instance, the black soldier fly (BSF, *Hermetia illucens*) is a promising candidate for the sustainable recycling of biological waste into feedstuff for livestock, poultry and aquaculture in the framework of a circular economy approach (Nguyen *et al.,* 2015; van Huis *et al.,* 2013).

The insect evolutionary success and diversification are partially due to the symbiotic relationships that they have established with a wide range of microorganisms. These complex symbiotic interactions include commensal, parasitic and mutualistic relationships (Dale and Moran, 2006). The function commonly attributed to the microorganisms that inhabit the intestinal tract of animals is the depolymerization and breakdown of the diet components, which allow the nutrient supplementation and recycling. Moreover, they also provide detoxification of the toxic diet-components and protection against pathogens and parasites and this underlines the importance of a healthy gut microbiota for the host well-being (Dale and Moran, 2006; Engel and Moran, 2013; Hamdi *et al.,* 2011).

At the beginning of this PhD thesis an introductive chapter offers an overview of the current knowledge on the potential application of microorganisms in relation to the management of the emerging insect farming with agricultural, industrial and environmental interest.

The insect gut microbiota is influenced by many factors, such as the host diet, developmental stage and genetics, and in the last years researchers have been addressed many efforts to elucidate their impact on the host gut microbiota, mainly considering insect pests, parasites or vectors of diseases (Montagna *et al.,* 2015a; Vacchini *et al.,* 2017). Moreover, the importance to characterize factors, such as the oxygen concentration, pH and redox potential status, existing within the insect gut compartments has been only recently recognized and, hence, not so much work has been performed so far in this direction. Indeed, the insect gut includes aerobic and anaerobic niches, passing through microaerophilic habitats, and compartments characterized by acidic, neutral or basic conditions, even in the same digestive tract (Engel and Moran, 2013). Understanding the drivers that shape the

microbial diversity in the insect gut microbiota is pivotal in comprehending the symbiosis interactions and in exploiting insects for agricultural, environmental and feed applications.
Since the increasing interest and limited information on this topic, the aim of this PhD thesis was to evaluate the influence of the diet, developmental stage and physicochemical conditions occurring in the different intestine compartments on the gut microbiota associated to different helpful insects. Specifically, the honeybee *Apis mellifera* and the waste-reducing insect *H. illucens* were considered. These insects are characterized by polyphagous diet, the former more specialized and the latter more variegated for the honeybee and BSF, respectively. Finally, the probiotic effect of some selected bacteria was evaluated to observe a possible bacterial contribution to the BSF performance when insects were exposed to a low-performing diet.

The second chapter of my PhD was devoted to the study of the compartmentalization of the bacterial community along the honeybee intestine, taking into account the variations of the physicochemical conditions of oxygen level, pH and redox potential at a micrometer scale. Indeed, the gut microbiota of the adult honeybee workers has been deeply investigated and shown to include nine dominant bacterial phylotypes, belonging to Proteobacteria, Firmicutes and Actinobacteria (Kwong and Moran, 2016). Although much information is available on the diversity, genomic features and evolution of these phylotypes, a comprehensive study of the bacterial diversity in relation to the physicochemical conditions occurring in the gut portions was missing (Kwong and Moran, 2016). The variation of the oxygen partial pressure (pO2), redox potential and pH in the crop, midgut, ileum and rectum of honeybee foragers was measured taking advantage of microsensors and microelectrodes, while bacterial composition and co-occurrence networks were determined by 16S rRNA gene high-throughput sequencing. Bacterial abundance was measured by quantitative PCR. Results showed that a diagonal oxygen gradient was present in all the compartments that resulted anoxic in their centre, supporting that the previously reported bacterial stratification was linked to the oxygen availability. A progressive pH decrease from the crop to the rectum, presumably associated to the increasing microbial acidogenic activity, was paralleled by an increasing complexity of the network connections. Data provided the evidence of a physicochemically-driven gut compartmentalization of the bacterial communities at the level of the microbiota networking and diversity.

In the third chapter the influence of diet source and developmental stage on BSF bacterial community structure and composition was investigated by applying culture-independent analysis, combining the Automated Ribosomal Intergenic Spacer Analysis (ARISA)-PCR fingerprinting and

16S rRNA high-throughput sequencing. Specifically, insects were reared on three different diets (standard, fruit-waste derived and vegetable-waste derived ones) and sampled at three different developmental stages (i.e. larval, prepupal and adult ones). According to data, the bacterial communities associated to *H. illucens* were characterized by three dominant phyla, Proteobacteria, Firmicutes and Bacteroidetes. Statistical analysis showed significant differences between the bacterial communities considering the three diets and the three developmental stages, except in the case of male and female adults, which did not differ statistically. Regardless the life stage and food source, 10 OTUs were shared among individuals, mainly belonging to the *Enterobacteriaceae* family. Moreover, by using microsensors and microelectrodes it was verified the variation of the physicochemical conditions, in terms of pO2, pH and redox potential within the gut compartments of 4th instar larvae, when reared on the three different diets. Data suggested that the variation of the physicochemical conditions, driven by the modification of the alimentary regimes, possibly affected the bacterial community structure.

Once verified the effect of the diet as a driving force in shaping BSF bacterial community, the last chapter of this PhD thesis was devoted to investigate the potential bacterial contribution to BSF performance when insects were reared on a low-performing diet i.e. the fruit-waste derived one. Bacterial isolations were performed from the dissected guts of BSF larvae reared on standard diet, using selective and enrichment media, resulting in the establishment of a bacterial collection made up of 193 isolates. After the collection dereplication and the strains' identification, the hydrolytic activities of the isolates were characterized in order to evaluate their potential metabolic contribution to the host. Data showed a potential involvement of the bacterial isolates in the breakdown of the diet components and in the nutrient supplementation. Moreover, several isolates showed the ability to produce exopolysaccharides (EPS) that could mediate the bacterial adhesion to the insect epithelia. Finally, based on the results of the metabolic profiles, two isolates were selected and administered, individually and in combination, to BSF larvae reared on nonsterile fruit-waste derived diet to assess their influence on the host performance. Results showed that the administration of selected bacteria could influence the weight gain of the larvae when reared on a nutritionally unbalanced diet.

In conclusion, this work underlines that different factors can modulate the structure, composition and compartmentalization of the bacterial communities associated to non-pest and economically relevant insects. One of the innovative aspects of this study was to have evaluated the physicochemical conditions occurring in the insect gut compartments at the micrometer scale. The combination of such investigations with 16S rRNA gene high-throughput sequencing data provides

useful information to describe the insect-associated microbial community to further uncover its contribution to the host.

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Riassunto

Gli insetti sono il gruppo più diversificato di animali sulla Terra e sono adattati a una vasta gamma di habitat. Essi hanno un notevole impatto sulla vita dell'uomo: includono non solo parassiti e vettori di malattie per uomo, animali e piante, ma anche insetti benefici, come gli impollinatori (per esempio l'ape da miele *Apis mellifera*), insetti allevati al fine di ottenere prodotti per il beneficio umano (ad esempio i bachi da seta), per la produzione di mangimi e alimenti o come agenti di biocontrollo (quali gli insetti parassitoidi o predatori). Inoltre, a seguito dell'aumento della popolazione umana, la crescente domanda di proteine per il consumo umano e animale sta spingendo verso la ricerca di risorse alternative: in questo scenario gli insetti sono stati proposti come una alternativa sostenibile di fonte proteica. Per esempio, la mosca soldato nera (BSF, *Hermetia illucens*) è un promettente candidato per il riciclo dei rifiuti organici e conversione in mangimi per il bestiame, il pollame e l'acquacoltura nell'ottica dell'economia circolare (Nguyen *et al.,* 2015; van Huis *et al.,* 2013).

Il successo evolutivo degli insetti e la loro diversificazione sono dovuti in parte alle relazioni simbiotiche che essi hanno stabilito con una vasta gamma di microrganismi. Queste complesse interazioni simbiotiche includono relazioni di commensalismo, parassitismo e mutualismo (Dale and Moran, 2006).

La funzione comunemente attribuita ai microrganismi che popolano il tratto intestinale degli animali è la destrutturazione e degradazione dei componenti della dieta, che consentono l'integrazione e il riciclo dei nutrienti. Inoltre, essi contribuiscono alla detossificazione dei componenti tossici della dieta e forniscono protezione contro l'invasione di agenti patogeni e parassiti il che evidenzia l'importanza di un bilanciato microbiota intestinale per il corretto sviluppo dell'ospite (Dale and Moran, 2006; Engel and Moran, 2013; Hamdi *et al.,* 2011).

All'inizio di questa tesi di dottorato, un capitolo introduttivo offre una panoramica sulle attuali conoscenze sul potenziale applicativo dei microrganismi in relazione alla gestione dell'emergente "insect farming" in campo agrario, industriale e ambientale. Il microbiota intestinale degli insetti è influenzato da diversi fattori, come la dieta e lo stadio di sviluppo, e negli ultimi anni i ricercatori hanno indirizzato i loro sforzi per delucidare il loro impatto sul microbiota intestinale dell'ospite, principalmente considerando gli insetti dannosi, parassiti o vettori di malattie (Montagna *et al.,* 2015a; Vacchini *et al.,* 2017). Inoltre, l'importanza derivante dalla caratterizzazione dei diversi fattori, come la concentrazione di ossigeno, il pH e lo stato del potenziale redox, esistenti all'interno dei compartimenti dell'intestino degli insetti è stato solo recentemente preso in esame, quindi, non è stato condotto sufficiente lavoro in tale direzione. Infatti, l'intestino degli insetti comprende nicchie caratterizzate da condizioni aerobiche e anaerobiche, passando per habitat microaerofili, e comparti

caratterizzati da condizioni di pH acido, neutro o basico, presenti anche lungo lo stesso tratto intestinale (Engel and Moran, 2013). Comprendere i fattori che modellano la diversità microbica all'interno del microbiota intestinale degli insetti è di centrale importanza per la comprensione delle interazioni simbiotiche e nel loro sfruttamento degli insetti ai fini applicativi in campo agrario, ambientale e industriale.

Dato il crescente interesse e le limitate informazioni su questo argomento, lo scopo di questa tesi di dottorato è stato quello di valutare l'influenza della dieta, lo stadio di sviluppo e le condizioni fisico-chimiche che si presentano nei diversi tratti dell'intestino sull'influenza del microbiota intestinale associato a insetti benefici.

Nello specifico, l'ape da miele *Apis mellifera* e la mosca *H. illucens* sono stati oggetto di studio di questa tesi di dottorato. Questi insetti sono caratterizzati da comportamenti alimentari polifagi: uno in forma più specializzata, mentre l'altro più variegata, rispettivamente per quanto riguarda l'ape e la mosca soldato nero. Infine, l'effetto probiotico di alcuni ceppi batterici selezionati è stato osservato per valutare il possibile contributo batterico sulle performance di BSF quando l'insetto era esposto a una dieta sbilanciata.

Il secondo capitolo della mia tesi di dottorato è stato indirizzato allo studio della compartimentalizzazione della comunità batterica lungo il tratto intestinale dell'ape da miele, tenendo in considerazione le variazioni delle condizioni fisico-chimiche del livello di ossigeno, pH e potenziale redox su scala micrometrica. Infatti, il microbiota intestinale delle api operaie è stato approfonditamente studiato e include nove specie batteriche dominanti, appartenenti ai phylum dei Proteobacteria, Firmicutes e Actinobacteria (Kwong and Moran, 2016). Sebbene diverse informazioni sono disponibili sulla diversità, caratteristiche genomiche e evolutive di queste specie, uno studio comprensivo della diversità batterica in relazione alle condizioni fisico-chimiche che si verificano nelle porzioni del tratto intestinale sono a oggi mancanti (Kwong and Moran, 2016). Le variazioni della pressione parziale di ossigeno (pO2), potenziale redox e pH nelle regioni delle ingluvie, intestino medio, ileo e retto delle api foraggere sono state misurate con l'ausilio di microsensori e microelettrodi, mentre la composizione batterica e i "co-occurrence network" sono stati determinati attraverso il sistema altamente processivo di sequenziamento del gene 16S rRNA. L'abbondanza batterica è stata misurata attraverso PCR quantitativa. I risultati mostrano la presenza di un gradiente radiale per la concentrazione di ossigeno in tutti i comparti intestinali risultando anossici nel loro centro, a sostegno della proposta di una stratificazione batterica, precedentemente riportata in altri studi, come conseguenza della disponibilità di ossigeno. Una diminuzione progressiva del pH dall'ingluvie al retto, presumibilmente dovuta all'incremento di attività

acidogenica da parte dei microrganismi, si è mostrata in parallelo all'aumento della complessità delle interazioni nelle network analisi. I dati hanno fornito prove della evidente compartimentalizzazione intestinale delle comunità microbiche in funzione dei parametri fisico-chimici a livello di diversità e di networking del microbiota.

Nel terzo capitolo è stata esaminata l'influenza della dieta e dello stadio di sviluppo sulla struttura della comunità batterica di BSF applicando metodologie di analisi indipendenti dalla coltivazione, combinando l'Automated Ribosomal Intergenic Spacer Analysis (ARISA)-PCR fingerprinting e il sequenziamento altamente processivo del gene 16S rRNA. Nello specifico, gli insetti sono stati allevati su tre differenti diete (standard, scarto frutta e scarto verdura) e campionati a tre differenti stadi di sviluppo (ovvero larva, prepupa e adulto). In base ai dati, le comunità batteriche associate a *H. illucens* erano caratterizzate da tre dominanti phyla, quali Protebacteria, Firmicutes e Bacteroidetes. L'analisi statistica ha mostrato differenze significative tra le comunità batteriche considerando le tre diete e le tre fasi di sviluppo, tranne nel caso di adulti maschi e adulti femmine che non differivano statisticamente tra loro. Indipendentemente dallo stadio di sviluppo e dalla fonte alimentare, 10 OTUs erano condivisi tra le comunità batteriche degli individui, principalmente appartenenti alla famiglia delle Enterobacteriaceae. Inoltre, attraverso l'utilizzo dei microsensori e microelettrodi è stata verificata la variazione delle condizioni fisico-chimiche, in termini di pO2, pH e potenziale redox all'interno dei tratti intestinali delle larve di quarta età, quando allevate sulle tre differenti diete. I dati suggeriscono che le variazioni dei parametri fisico-chimici, guidata dalle modifiche dei regimi alimentari, probabilmente hanno influenzato la struttura delle comunità batteriche.

Una volta verificato l'effetto della dieta come fattore decisivo nella formazione della comunità batterica di BSF, l'ultimo capitolo di questa tesi di dottorato è stato dedicato allo studio del potenziale contributo batterico alle performance di BSF quando gli insetti venivano allevati su una dieta sbilanciata, ovvero lo scarto frutta. Sono stati eseguiti isolamenti di ceppi batterici dall'intestino estratto da larve di BSF allevate su dieta standard, utilizzando terreni selettivi e di arricchimento, con la conseguente costituzione di una collezione di ceppi batterici composta da 193 isolati. Dopo un processo di dereplicazione dei ceppi e l'identificazione degli isolati, essi sono stati caratterizzati per le loro attività idrolitiche al fine di valutare il potenziale contributo metabolico fornito all'ospite. I risultati hanno mostrato un potenziale coinvolgimento degli isolati batterici nella digestione dei componenti della dieta e apporto di nutrienti per la fisiologia dell'ospite. Inoltre, diversi isolati hanno mostrato la capacità di produrre esopolisaccaridi (EPS) che potrebbero mediare

l’adesione batterica all’epitelio intestinale dell’insetto. Infine, sulla base dei risultati dei profili metabolici, due isolati sono stati selezionati e somministrati, individualmente e in combinazione, alle larve di BSF allevate su una dieta non sterile derivata da scarti di frutta per valutare la loro influenza sulle performance dell’ospite. I risultati hanno mostrato che la somministrazione di batteri selezionati potrebbe influenzare l’aumento di peso delle larve quando allevati con una dieta non bilanciata.

In conclusione, questo lavoro sottolinea come diversi fattori possono modulare la struttura, la composizione e la compartimentazione delle comunità batteriche associate agli insetti benefici ed economicamente rilevanti. Uno degli aspetti innovativi di questo studio è stato quello di valutare le condizioni fisico-chimiche che si verificano nei compartimenti intestinali degli insetti su scala micrometrica. La combinazione di questa tipologia di analisi con i dati di sequenziamento del gene 16S rRNA attraverso le piattaforme di Next-Generation Sequencing (NGS) forniscono informazioni utili per descrivere la comunità microbica associata agli insetti e svelare ulteriormente il loro contributo alla biologia dell’ospite.

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Rational and aim of the work

Due to the increase of human population, the growing demand for protein for human and animal consumption -foreseen to rise over 30% by 2050 compared to 2000 (Godfray *et al.,* 2010)- is forcing the search of alternative sources. Currently, the biggest challenge for agriculture and livestock production is the delivery of secure food for humans, guaranteeing the maintenance of animal welfare and the respect of the environment, and without yield reductions (Davis *et al.,* 2016; Tizard *et al.,* 2016). In this scenario, a serious concern is represented by food waste disposal: since the total volume of solid waste is increasing worldwide, the bioconversion of waste matter into other forms of reusable products has gained a widespread acceptance (Hoornweg and Bhada-Tata, 2012). Insect farming has been recently indicated as one of the most promising solutions to overcome these challenges and provide sustainable protein sources as feed and food (van Huis, 2013).

Insect rearing is a practice that humans have been exploited since long e.g. with apiculture and sericulture that have been carried out to obtain products for human consumption or benefit. Moreover, in many Eastern countries arthropods represent part of the human diet: insect taxa that can be used as food substrates include many species, such as the house cricket *Acheta domesticus*, the two-spotted cricket *Gryllus bimaculatus* and the grasshopper *Locusta migratoria migratorioides*. Currently, the interest is directed towards the acceptance of this alimentary habit in the Western countries and to the introduction of insects as feed. Moreover, insects can be considered as bioconversion agents of organic waste materials into rich-protein substrates: for instance, the black soldier fly (BSF, *Hermetia illucens*) is a promising candidate for the sustainable recycle of biological waste into feedstuff for livestock, poultry (Sheppard *et al.,* 1994) and aquaculture (St. Hilaire *et al.,* 2007) in the framework of a circular economy approach.

Insects establish with microorganisms complex symbiotic interactions, which span both commensal, parasitic and mutualistic relationships (Dale and Moran, 2006). Their capacity to adapt to different terrestrial and aquatic niches is largely provided by the interactions with their microbial partners, which enable the host to thrive on nutritionally imbalanced or limited regimes (e.g. wood, phloem sap or blood). These microbial symbionts can play important roles in many aspects of the host biology and physiology, e.g. being involved in nutrition, development, reproduction, immunity and speciation. Recently, much interest has been directed towards the study of the gut-associated microbial partners and a growing body of evidences is supporting the gut microbiota as a key component for the host well-being (Dale and Moran, 2006; Engel and Moran, 2013). Indeed, when an altered microbiome is present, i.e. in the case of a dysbiosis, an altered physiological status with severe consequences for the host can result or derive (Turnbaugh *et al.,* 2009; Hamdi *et al.,* 2011).

The gut microbiota composition and structure are shaped by many factors, such as the host developmental stage, innate immune system, diet and genetics (Montagna *et al.*, 2015; Vacchini *et al.*, 2017). Among these factors, the oxygen level, pH and redox potential status occurring within the digestive tract play fundamental roles, whose importance has not been so much investigated. Indeed, the insect gut includes aerobic and anaerobic niches, passing through microaerophilic habitats, and compartments characterized by acidic, neutral or basic conditions, even in the same digestive tract (Engel and Moran, 2013). Since the limited information available for the insect species considered in this work, the **aim of this PhD thesis** was to evaluate how the insect gut microbiota is shaped by factors such as the diet source, the developmental stage and the physicochemical conditions occurring in the gut compartments. Specifically, in this PhD thesis the gut microbiota associated to the helpful insects, the honeybee *Apis mellifera* and the waste-reducing insect *H. illucens*, have been considered as models. Finally, the probiotic effect of selected bacteria has been investigated to observe a possible bacterial mitigation on BSF individuals exposed to a low-performing diet.

Intended as an introductive review, the **first chapter** offers an overview of the current knowledge available for the management of the microbial resource associated to reared insects with agricultural, industrial and environmental interest. The manipulation and exploitation of the insect-microbe interactions -in the perspective defined as Microbial Resource Management (MRM)- has been proposed to successfully solve practical problems, even related to human, animal and plant protection and environmental safeguard. In particular, in this chapter the potential biotechnological application of microorganisms is discussed in relation to the emerging insect farming.

The **second chapter** is devoted to the study of the bacterial communities associated to the different gut compartments of honeybees. Specifically, the aim was to evaluate if the variation of the physicochemical conditions of the gut compartments could drive a microbial compartmentalization in the insect gut. 16S rRNA gene high-throughput sequencing has been performed to investigate the bacterial composition and to infer the co-occurrence bacterial networks, while quantitative PCR has been used to determine the abundance of the bacterial communities. Oxygen partial pressure (pO_2), redox potential and pH have been measured in the different honeybee gut compartments (crop, midgut, ileum and rectum) taking advantage of microsensors and microelectrodes.

In the **third chapter**, the variation of the bacterial communities associated to BSF individuals, reared on three different diets (standard, fruit-waste derived and vegetable-waste derived ones) and considering different developmental stages (i.e. larvae, prepupae and adults), has been evaluated by using cultivation-independent techniques. Moreover, pO_2, pH and redox potential occurring in the

digestive tract of 4th instar larvae, when exposed to the three different diets, have been measured by the use of microsensors and microelectrodes.

The **four chapter** is dedicated to the study of the bacterial cultivable fraction associated to BSF larvae to individuate a possible contribution of the isolates to the host physiology. In particular, isolates have been screened for their ability to degrade polymers typically found in organic waste materials, such as cellulose, starch and pectin, to adhere to the host epithelium through the production of exopolysaccharides (EPS) or to be involved in the host carbon and nitrogen metabolisms and phosphorous recycling. Furthermore, based on the results of metabolic profiles, two isolates have been selected and administered, individually and in combination, to BSF larvae into nonsterile fruit-waste diet in order to investigate their influence on the host development.

The **last chapter** presents the conclusions and the future perspectives of this work.

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Chapter 1

Microbial Resource Management in insects for agricultural, industrial and environmental applications

Summary

Despite the introduction of modern agricultural technologies and practices, the world's population is increasing faster than the food production, facing also issues related to food-feed and fuel competition concerning the use of land (Rathmann *et al.,* 2010). Currently, the growing demand of protein for human and animal consumption, which has driven the use of intensive farming systems, is forcing the search for alternative and sustainable sources. Furthermore, the growing environmental problems connected to human activity threaten future levels of agricultural productivity. Recently, the scientific and economic value of insects is rising worldwide due to their biotechnological and ecological applications in different field, from agricultural production to environmental protection and industrial exploitation. Insects establish a wide range of interactions with microorganisms, from mutualistic to parasitic relationships. Many works have highlighted the important role of the gut microbiota for the host physiology and biology which has been primarily involved in the host defense, growth promotion or nutrition, for example making available recalcitrant compounds such as cellulose, hemicellulose and lignin. The manipulation and exploitation of these insect-microbe interactions has been proposed to successfully solve various problems in the framework of the humans, animal and plant protection and environmental safeguard. The present review summarizes and discusses the current knowledge and potential biotechnological applications of microorganisms -with a special focus on commensals- in the perspective of a Microbial Resource Management (MRM) related to the emerging insect farming.

Introduction

Due to the expected increase of human population, the growing demand for protein for human and animal consumption is forcing the search of alternative sources. Currently, the biggest challenge in agriculture and livestock production is the delivery of safe foods for human consumption, with the maintenance of animal welfare and the respect of the environment, and without yield reduction (Godfray *et al.,* 2010). Furthermore, according to the Food and Agriculture Organization of the United Nations (FAO), 1/3 of food production is lost or wasted every year; since the total volume of solid waste is increasing worldwide, the bioconversion of waste matter into other forms of products has gained a widespread acceptance.

Insect farming has been recently indicated as one of the most promising solutions to overcome these challenges and provide sustainable protein sources as feed and food (van Huis, 2013). The high protein content (40-75% of the dry matter) makes insects a promising protein alternative for both human and animal nutrition (Bukkens, 1997). In addition, insects provide lipids, carbohydrates and certain important nutrients, such as vitamins and minerals (Mlček *et al.,* 2014).

Insect rearing is a practice that humans have been exploited since long e.g. with apiculture and sericulture that have been carried out to obtain products for human consumption or benefit, without considering the inestimable value of the pollination service for the biodiversity maintenance. The farming of insects with feed and food purposes comprises other species of interest, such as the black soldier fly *Hermetia illucens*, the yellow mealworm *Tenebrio molitor*, the lesser mealworm *Alphitobius diaperinus*, the wax moth *Galleria mellonella*, the house cricket *Acheta domesticus*, the two-spotted cricket *Gryllus bimaculatus* and the grasshopper *Locusta migratoria migratorioides*. In addition, in the framework of sustainable agriculture and forestry an increasing interest has been directed toward the rearing of predators or parasitoids of insect pests, such as the hymenopteran *Encarsia formosa*, parasitoid of the whiteflies *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) and *Bemisia tabaci* (Gennadius) in many countries throughout the world (Hoddle *et al.,* 1998), or the phytoseiid mites (Knapp *et al.,* 2013). On the other hand, since the ability of saprophagous insects in decomposing the organic material, they have the potential to be used in waste management as the case of the well-known waste-reducer *H. illucens* (Nguyen *et al.,* 2015a; Zhang *et al.,* 2012b). Moreover, in this framework insect farming obviates to a worldwide emerging reality exploiting the natural efficiency of this animal class to digest and reduce organic waste material, converting it into insect biomass available as rich protein feed and food for livestock and human consumption, respectively, with far less greenhouse gas emissions, lower environmental impact, high feed conversion efficiency and lower water input than traditional animal husbandry

(Yen, 2009; Miglietta *et al.,* 2015). Very prolific insects with a high growth rates are of course preferred.

The insect ability to adapt to different environments is provided by the interactions with the microorganisms that inhabit their intestinal tract or are localised in specific insect organs. Arthropods have established a wide array of symbiotic interactions with microorganisms, which involve commensalism, parasitic or mutualistic relationships (Dale and Moran, 2006). Surely, one of the most described aspects is related to the nutritional aspect provided by the symbionts to their host. In fact, during their evolutionary history insects have been adapted to feed on a variety of nutritionally unbalanced substrates and matrices, ranging from wood or phloem sap to blood, exploiting their microbial partners (Dale and Moran, 2006; Dillon and Dillon, 2004). Despite the commensal microorganisms are often transient in the intestinal tract of the animals, they can largely influence the host physiology, considering not only its nutritional status, but also its development (Chouaia *et al*., 2012), mating (Sharon *et al*., 2010) and the modulation of immune systems (Lee *et al.,* 2013).

Aim of this review is to offer an overview of the current knowledge on the management of the microbial resource associated to reared insects with agricultural, industrial and environmental interest. Specifically, we focussed on the commensal symbionts of insects since the increasing attention that they have received in the last years (Engel and Moran, 2013). Commensals can indeed establish a complex network of interactions with the host, even influencing the host systemic growth e.g. by regulating the hormonal signal pathways (Shin *et al.*, 2011; Storelli *et al.,* 2011). For instance, Storelli and collegues (2011) demonstrated the crucial role of *Lactobacillus plantarum*, a commensal bacterium of *Drosophila melanogaster*, for the host growth promotion by acting on TOR-dependent nutrient sensing. From an anthropological point of view, insects can be divided into beneficials and pests, with the first group that includes pollinators, pest parasitoids and entomophagous insects. There is a huge knowledge gap for the insect species exploited in the insect farming from the microbiological point of view, with the exception of honeybees which play an essential service for the agricultural production and the maintenance of plant biodiversity. Moreover, there are several factors that must be taken into account to study the diversity and dynamics of the gut microbiota. The host genotype, developmental stage, gender, physiological conditions and diet source are some of the main driving forces that shape the gut microbial community of the insects (Dethlefsen *et al.,* 2007; Sharon *et al.,* 2010; Montagna *et al.,* 2015; Prosdocimi *et al.,* 2016). With this aim we aimed to summarize the potential biotechnological applications of microorganisms in the perspective of a MRM strategy related to the emerging insect farming.

Probiotic bacteria for social insects

Multicellular organisms are strictly linked to their microbial partners which provide to the host a wide range of metabolic and defensive functions. Vertically transmitted symbionts have evolved several strategies to persist in their hosts, exploiting mechanisms of reproduction alterations such as the cytoplasmic incompatibility and making themselves essential for the host nutrient supply or protection against natural enemies (Hurst and Frost, 2015). For instance, bacteria associated with the eggs of the housefly *Musca domestica* provide an initial food resource for the host and represent a protection against pathogenic fungi on the carrion (Lam *et al.*, 2009). On the other hand, some microbial partners e.g. symbionts of leaf-cutting ants and locusts, are known to produce antibiotics or antagonistic substances to eliminate the microbial invaders of their insect hosts (Dillon *et al.*, 2000; Oh *et al.*, 2009). Recently, Shao *et al.*, (2017) found that *Enterococcus mundtii*, the dominant gut symbiont of the generalist herbivore *Spodoptera littoralis*, actively releases the bacteriocin mundticin KS which, active against the host invading bacteria, does not affect the other gut inhabitants, contributing to the correct development of host gut microbiota.

Social insects share the same environment with the other colony members, which include many individuals at different developmental stages and among which it occurs the opportunity of symbionts' transfer from one generation to the next one and among the nest mates (Engel and Moran, 2013). The role of the colony life in the establishing and shaping of the gut microbiota of *Bombus terrestris* has been investigated by Billiet and colleagues (2017). In their survey bumblebees have been divided in several groups with different rate of contact among the nest mates and with the hive environment; data showed that different insect groups harboured different gut bacterial community profiles with the significantly lower microbial diversity in individuals completed excluded from the colony environment and without contacts with the other nest mates during the adult life stage (Billiet *et al.*, 2017).

Honeybees possess a small, well-defined microbiota made up of nine bacterial phylotypes which account for the 95-99% of the total bacterial community in almost all the adult individuals (Martinson *et al.*, 2011; Kwong and Moran, 2016). These eusocial insects have been recently proposed as a model to study the gut microbial ecology and the symbionts' evolution (Kwong and Moran, 2016), which ultimately could lead to the development of probiotic applications in a contest of MRM strategy. Probiotics, indeed, are a promising alternative to the use of chemicals and antibiotics in the animal breeding to control and prevent pathogen onsets and to promote the animal health (Gaggìa *et al.*, 2010). Thus, a thorough knowledge of the composition and function of the gut microbiota, even in the case of insects, is fundamental for the selection and use of suitable probiotics and prebiotics combination. Although insects are gaining an increasing scientific and

economic value worldwide, there are fewer publications on the probiotic use of microorganisms on these hosts (Berasategui *et al.,* 2016). In addition, when insects enter the food chain, the importance of a comprehensive microbiological analysis is required to avoid or minimise risks for the animal and human health. For instance, larvae of the housefly *Musca domestica* have been suggested as protein supplementation in poultry diet (Zuidhof *et al.,* 2003). Since it is well known that the insect is a vector of foodborne pathogens, like *Salmonella* spp., *Pseudomonas* spp. *Escherichia coli* (Doud *et al.,* 2014; Kobayashi *et al.,* 1999; De Jesús *et al.,* 2004; Doud and Zurek, 2012; Joyner *et al.,* 2013; Macovei *et al.,* 2008), safety issues must be taken into account. Generally, this highlights the consequent requirement of a safety evaluation of insect farming to avoid human and animal health risks.

Pesticides, used in agriculture in order to maximize crop yields, represent a serious problem for non-target species, as demonstrated for the use of neonicotinoid insecticides in the honeybee population decline (Henry *et al.,* 2012; Whitehorn *et al.,* 2012). Indeed, in 2013 the European Commission banned the use of three neonicotinoids on specific crops in Europe (European Commission, Press Release, Brussells 29 April 2013). Recently, researchers showed that by administrating a specific strain of *Lactobacillus plantarum*, the survival rate of fruit flies exposed to the neonicotinoid imidacloprid (IMI) improved significantly when insects became infected by the pathogen *Serratia marcescens* (Daisley *et al.,* 2017). The mechanism involved the stimulation of the host immune system through the immune deficiency (Imd) pathway used by insects to counteract infection, heat and other stresses. Authors suggested that probiotic lactobacilli could mitigate IMI-induced immunosuppressive effects also in honeybee, similarly to what they proved to occur in *D. melanogaster* (Daisley *et al.,* 2017). In addition in a previous work they demonstrated that another probiotic strain of *Lactobacillus rhamnosus* could reduce the absorption and toxicity of organophosphate pesticides in *D. melanogaster* fruit flies (Trinder *et al.,* 2016).

Nowadays, regardless few exceptions the microbial residents within the intestinal tract of arthropods are still poor characterized and explored, although the recent advances in molecular analysis and cultivation-based methods. Understanding the interactions established among microorganisms and with the host and how symbiotic (and probiotic) bacteria can modulate the Imd pathway, will offer novel insights into improving insect health, especially considering the pollinators that in the past two decades are facing a large-scale loss worldwide (Aizen *et al.,* 2009; Klein *et al.,* 2007; Crotti *et al.,* 2013).

Farming of useful insects for productive, waste-reducing, food and feed purposes

In the last decades several studies showed the feasibility to use insects as alternative feed sources for livestock (Bondari and Sheppard, 1981; Nakagaki and Defoliart, 1991; Sheppard, 1999). Despite insects are widely consumed in many regions of the world and currently there is an increasing interest in their exploitation as feed or supplements for livestock and aquaculture, little attention has been addressed to the hygiene, in terms of microbial load and pathogen presence in fresh, processed and stored edible insects (Klunder *et al.*, 2012). Like other food, insects are rich in nutrients and moisture, thus they are a suitable substrate for microorganism survival and growth (Rumpold and Schlüter, 2013). Klunder *et al.*, (2012) gave valuable information concerning the microbiological aspects of farmed mealworms and house crickets in fresh, processed and stored conditions. A more recent study performed by Stoops *et al.,* (2016) investigated the microbial quality of fresh mealworm larvae and grasshoppers sold in the Belgian markets for human consumptions underlining the strong correlation of this kind of food with potential food pathogens. In a further study, performed by using PCR-Denaturant Gradient Gel Electrophoresis (DGGE) on edible insects marketed in the European Union, Osimani *et al.,* (2017) found the presence of both commensal and potential pathogenic bacteria, the latter ascribed to different genera such as *Bacillus*, *Pediococcus*, *Weisella*, *Streptomyces*, *Acinetobacter*, *Agrococcus*, *Arthrobacter*, *Naxibacter*, *Planomicrobium*, *Rufibacter*, *Loktanella*, *Clostridium*, *Vibrio*, *Desulfovibrio* and *Escherichia*. Obviously, the limitation of these works consisted in the lack of a deep microbiological analysis of the insect associated community that is nowadays feasible following the application of a next generation sequencing (NGS) approach.

Agriculture has been estimated to consume 70% of global freshwater (Ridoutt *et al.,* 2012), of which nearly 1/3 is attributed to the livestock production (Mekonnen and Hoekstra, 2012). Conversely, the insect farming shows lower water requirement than traditional animal husbandry. Currently, there is an increasing number of researches on the ability of these invertebrates to convert efficiently the organic substrates into protein-rich animal biomass, with very fast growth rates; these works underlined also the insect ability to utilize all the water they need from just their food (Ghaly and Alkoaik, 2009; Semianowska *et al.,* 2013; Rumpold and Schlüter, 2013). By using WF to compare products obtained from different processes, Miglietta and colleagues (2015) showed that protein could be obtained in a more efficient way, from the freshwater resource framework, from two species of insects (*Tenebrio molitor* and *Zophobas morio* mealworms), reared for human consumption, rather than other traditional farmed animals.

BSF farming has been recently proposed for agricultural and industrial purposes, since it represents a novel management strategy of organic waste disposal, converted in insect biomass that can be

ultimately used as animal feed (Surendra *et al.,* 2016). With the rapid development of the poultry and livestock industry all over the world, the average production of manure is approximately 587 billion tons per year (Afazeli *et al.,* 2014). *H. illucens* has been reported to reduce the poultry manure mass by 50% (Newton *et al.,* 2005). BSF larvae consume a wide range of organic material, ranging from fruits and vegetables to animal remains and manure (Nguyen *et al.,* 2015b); BSF prepupae, containing 44% of protein and 33% of fat on dry weight, are a good additive and substitute of fish meal, whose price is constantly growing, making the production of alternative animal feed protein a lucrative business (Diener *et al.,* 2009; Newton *et al.,* 2005; St-Hilaire *et al.,* 2007). Furthermore, BSF has been shown to reduce the pathogen load of specific substrates (Lalander *et al.,* 2013). For instance, Erickson *et al.,* (2004) found a reduction of *Salmonella enterica* serovar *enteritidis* and *Escherichia coli* O157:H7 in cattle manure treated with BSF, while Liu *et al.,* (2008) reported a significant reduction of *E. coli* in poultry manure. A peptide, effective against Gram-positive bacteria, including the pathogenic *Staphylococcus aureus*, has been isolated from BSF hemolymph (Park *et al.,* 2015). Moreover, it has been demonstrated that BSF presence avoids *M. domestica* oviposition in the feeding substrates, with a reduction of housefly population by 94-100% (Bradley and Sheppard, 1984).

Up to now, few studies have investigated BSF microbial community. By a 16S rRNA high-throughput analysis, Jeon *et al.,* (2011) analysed the composition of the gut bacterial community of BSF larvae reared on three different feeding conditions, reporting that the intestinal microflora is directly influenced by the type of food source. They have also evaluated the potential contribution of the commensal microorganisms to the host digestion of organic compounds that are generally present in food-waste matrices. Zheng and colleagues (2013) analysed the bacterial diversity associated with successive BSF developmental stages when insects have been reared on plant material. Recently, a survey on the intestinal mycobiota associated to BSF larvae fed on different organic substrates at different timings have been performed by Boccazzi *et al.,* (2017). Bacterial strains isolated from BSF can be used as probiotics to improve the larval performances on waste or manure reduction. For instance, Yu *et al.,* (2011) demonstrated that BSF larvae fed on chicken manure inoculated with four indigenous strains of *Bacillus subtilis* and one strain of *B. natto,* isolated from the diet source, could positively influence the growth and development of the host.

The silkworm *Bombix mori* has been domesticated from the wild counterpart *Bombix mandarina* and exploited for the silk production for thousands of years. Due to the economic importance of sericulture in different countries -only in China more than 10 million of silkworm farmers are involved in the silk industry (Li *et al.,* 2011), new efforts have been addressed toward the study of the insect biology and physiology to improve the silk yield and quality production. In a recent

study, Wang and colleagues (2016) have fed silkworm larvae with diets added of single-walled carbon nanotubes (SWNTs) and graphene, obtaining silk fibres with an enhanced mechanical resistance; this could lead to a large-scale production of an improved biomaterial without the use of toxic chemicals and complex multistep procedures. Several factors affect the silkworm growth and larval development, e.g. the presence of pathogen, the pollution and the nutritional conditions -in particular, the availability of vitamin C in the diet source- (Cappezzola *et al.,* 2005; Cui *et al.,* 2003: Ito 1961). Among silkworm pathogens, one of the most aggressive infectious agents is represented by *Bacillus bombysepticus* (Zhang *et al.,* 2015). In the applied entomology field for better understanding the insect biology, including the symbiotic interactions established among the host and the microorganisms, the silkworm gut microbiota still remains poorly understood. Li and colleagues (2016) have investigated the microbial community associated with fluoride tolerance in two *Bombyx mori* strains, providing the evidence that the microbial composition is linked to the insect resistance against the chemical; moreover, data suggested that pH was the main driving force that shaped the intestinal microbial ecology. Also in this case, the potential application of probiotics represents a promising economic and environmentally friendly solution to sustain the host growth and silk production even in presence of contaminant that could affect the insect development.

Insect biorefinery

Biorefinery is the sustainable processing of renewable biomass into bio-based products as food, feed, chemicals, materials and fuel (Bozell *et al.,* 2010; Kamm *et al.,* 2016). These kinds of products could help overcome many of the socio-economic and environmental challenges that humanity is facing nowadays. In any productive process, the generated waste requires disposal proceedings and, in many cases, the waste of one process could be converted into a high economic valuable product (Kiran *et al.,* 2014). Waste biomass, i.e. the residual products generated by crop and animal processing, and forestry waste, i.e. the by-products originated from silviculture or natural environment, can be converted into several high value products through physical and/or chemical treatment or by biological process (Kamm *et al.,* 2016). In this framework, insect could thrive on the biomass disposal producing food, feed, fuel and materials for the green chemistry industries. Insects are indeed very efficient in consuming different types of organic waste, they need few space, consume little water, and their waste is a high-quality fertilizer (Nguyen *et al.,* 2015a; Zhang *et al.,* 2012b; Green and Popa, 2012). For instance, since standard mass-rearing techniques of *H. illucens* already exist (Henry *et al.,* 2015; van Huis *et al.,* 2013), besides the production of feed, BSF can be exploited for the production of biodiesel (Li et al., 2011; Wang et al., 2017; Surendra et al., 2016; Leong *et al.,* 2016). With the aim to obtain biodiesel from farmed insects, one

of the frequent issues to overcome is the low growth rate and lipid amount, as reported by Leong *et al.,* (2016). With the isolation and characterization of bacterial strains possessing microbial activity that could promote protein and organic phosphorous degradation, it has been possible to enhance the growth and development of BSF larvae reared on poultry manure (Yu *et al.,* 2011; Yu *et al.,* 2010a; Yu *et al.,* 2010b; Dong *et al.,* 2009).

Natural products, which are usually secondary metabolites, are produced by plants, fungi, and bacteria and their derivatives constitute the raw materials for different industrial applications, from pharmaceutical- to agro-industrial ones, including the production of bioactive compounds for the green chemistry. Also arthropods represent a valuable source of inexpensive bioactive compounds for the green chemistry and nanotechnology, as review by Lateef and colleagues (2016). Insects are principally made of protein, lipid and chitin. Chitin is a long unbranched polysaccharide, made of β-1,4-linked N-acetylglucosamine and represents one of the major by-products of the insect biorefinery. Chitin has many industrial applications: it is currently extracted from crustaceans from fish industry discards and used in cosmetics, pharmaceuticals, food additives, water treatment and in the production of biopolimers (Khan *et al.,* 2017; Hudson *et al.,* 2015). Regarding to feed production from insects for livestock and aquaculture, chitin digestibility may be problematic (Kroeckel *et al.,* 2012). To overcome this issue, insect meal could be supplemented with probiotic strains selected for the capability to digest chitin or treated with chitinolytic enzymes. Ushakova *et al.,* (2016) have been investigated the effect of an aquaculture feed preparation composed of *B. subtilis* and the biomass of BSF. The authors assumed that the larval chitin could stimulate the fish intestinal microbial community and digestion process. They found a significant improvement of the fish development, underling the synergic probiotic and prebiotic effect of nutrient supplementation with microorganisms and BSF biomass.

When chitin can not be used as a nutrient source, it represents a recalcitrant biomass waste that have to be treated for disposal and recovery of energy. In a recent study, researchers investigated the potential recovery of green energy through chitin degradation in a microbial fuel cells (MFC) powered by *Aeromonas hydrophila* (Li *et al.,* 2017). In their survey, they demonstrated that the microbial anaerobic degradation of chitin through the MFC generated seven-fold more total metabolites compared to the fermentation systems, as well as additional electricity.

Insects could be also exploited as “bioreactors” for the production of high valuable molecules. The silkworm *B. mori* have been engineered for the expression of a fusion protein between the cholera toxin B subunit and the 42-amino acid isoform of the amyloid-β peptide in order to develop a low-cost oral vaccine against Alzheimer’s disease (Li *et al.,* 2014). In this way, several biomedical proteins have been successfully expressed in silkworm pupae (Jin *et al.,* 2008; Chen *et al.,* 2006),

making this animal model one of the high-efficient expression systems suited for the expression of recombinant protein in eukaryotic organisms. Recently, *Lucilia sericata* larvae have been engineered and used to prove the feasibility to produce a variety of growth factors and antimicrobial peptides (Linger *et al.,* 2016). Overall, insects are good candidate for the recycling and production of high valuable compounds. The auspicious of a good quality and yield in breeding this kind of animals can take advantage from an integrative microbial resource management.

Mass rearing of sterile insects and parasitoid insects

Among biological control approaches, the sterile insect technique (SIT) consists in the mass production, sterilization, and release of high density of insects toward a target pest population (Vreysen and Robinson, 2011). SIT has been deployed to counteract several species of insects of agriculture, veterinary and medical importance (Vreysen *et al.,* 2007). Successful mass rearing is crucial for sterile insect technique programs. The sterilization process using gamma radiation has been shown to affect seriously the integrity of gut tissue, microbiota, and cellular organelles, resulting in a lower fitness in the male individuals (Ben Ami *et al., 2010*). Another important factor for the successful and cost-effective application of this technique is the longevity of sterile male individuals (Ben Ami *et al.,* 2010). For instance, females of some species of insects, e.g. *Anastrepha ludens* (Diptera: Tephritidae), preferentially copulate with "old" males rather than younger individuals. Furthermore, sterile males with long life-span have potentially more opportunity to find and mate with wild females than short-lived males, being more effective in inducing sterility into wild population. The supplementation of beneficial bacteria to the larval diet has been demonstrated to improve the host development (Ben Ami *et al.,* 2010).

Whereas MRM has been proposed to enhance a successful SIT, much less efforts have been addressed toward the investigation of the microbial partners -and their contribution to the host growth- of parasitoid insects. Few studies reported the influence of biotic and abiotic factors on the physiology and behaviour of these insects, without taking into account the contribution of their microbial partners (Rousse *et al.,* 2009). For instance, it has been found that the female beewolf digger wasps cultivate symbiotic actinobacteria in their antennal glands (Goettler *et al.,* 2007), which ultimately provide to the wasp offspring a "combination prophylaxis" due to complementary or synergistic effects of different bacteria-produced antibiotics (Kroiss *et al.,* 2010). Streptomycetes are indeed well known for their ability to produce diverse antibiotics and other secondary metabolites (Behal, 2000). This protection is highly efficient against a broader range of pathogenic microorganisms and the mixture of antibiotic compounds, acting simultaneously, can prevent the onset of resistant pathogens. Different studies highlighted that adding probiotics to the insect diet

could significantly improve the effectiveness of SIT (Hamden *et al.,* 2013; Ben Ami *et al.,* 2010), which can be integrated easily in an integrated management of these living resources.

One of the proposed MRM approaches to control insect pests exploits the intracellular bacterium *Wolbachia* through microbial transfections of hosts which are not infected by this microorganism (Zabalou *et al.,* 2009). In tropical regions of Australia, Morrow and colleagues (2014) had identified *Wolbachia* strains shared among different tephritid fruit fly species and, for the first time, in two of their natural parasitoid species. This provides more opportunities to investigate the horizontal transmission and the ecological and evolutionary role of *Wolbachia* in the interactions between host taxa. Several cases of biological control of agricultural pests, by exploitation of natural parasitoids to reduce insect pest populations, take advantage of the knowledge on insect symbiosis. For instance, some *Wolbachia* strains that infect parasitoid insects induce thelytokous parthenogenesis in their hosts (Arakaki *et al.,* 2000) and the thelytokus parthenogenetic phenotype in a parasitoid would have several advantages for the pest control. Since only female individuals kill their hosts, this could overcome the necessity of mass-producing parasitoids for release on field due to the fact that no males individuals are generated and no mating is required for the maintenance of population (Stouthamer, 1993; Bourtzis, 2008). On the other hand, *Spiroplasma*-infected *Drosophila* fruit flies have proven to be more resistant than their uninfected counterparts to parasitic wasps (Ballinger and Perlman, 2017). Further studies are necessary to deepen the knowledge regarding the underlying mechanisms of the microbe-insect interactions in presence of one or more natural enemies. For instance, Ballinger and Perlman (2017) showed that symbiont-encoded toxins that confer protection to flies against a nematode parasite are also implicated in the mediation of resistance to their parasitic wasps.

Insects and bacteria as plastic degrading agents

After more than one century of plastic production, this material represents one of the most anthropogenic waste which pollutes different environments and constitutes a pressing concern. This has led to the necessary transition towards a circular economy (Neufeld *et al.,* 2016). Plastic debris could be easily found at different levels of the food chain due to the trophic activity of several animals, in particular in marine ecosystem (Barnes 2002; Davison 2011; Murray 2011; Cole 2013; Farrell 2013; Setälä 2014; Galloway *et al.,* 2017). Solè and colleagues (2017) have indicated that plastic-polluted environments represent a synthetic ecosystem that could sustain a novel community of organisms. This evidence opens the possibility of a promising strategy in managing plastic waste using microorganisms and insects as suitable biodegrading agents.

The searching for catabolic enzymes that can break down molecular constituents of plastic materials is of great interest. Recently, the larvae of wax moth *Galleria mellonella*, known for causing damage to beehives, have been founded to be able, in absence of other nutrient sources, to eat and breakdown polyethylene (PE) plastic material (Bombelli *et al.*, 2017). PE is a polymer composed of a linear backbone of carbon atoms recalcitrant to biodegradation that requires long time (weeks or months) for a modest reduction which is operated by few microorganisms (Yamada-Onodera *et al.*, 2001; Bonhomme et al. 2003). Bombelli and colleagues (2017) found that around a hundred wax worms started to degrade a plastic bag after 40 minutes and that 12 hours late the plastic bag mass has been reduced of 92 mg. Scientists affirmed that this degradation rate was extremely fast compared to other recent discoveries, where, for example, bacteria biodegrade plastics at a rate of 0.13 mg a day. Previously, bacteria isolated from the intestine of another waxworm, *Plodia interpunctella*, had shown the capacity to breakdown the long carbon chain of PE (Yang *et al.*, 2014). Another polymer widely used in plastic production is the polyethylene terephthalate (PET). Only few fungal species are known as degraders of this synthetic compound, characterized by a high ratio of aromatic residues which confer the recalcitrant behaviour to microbial degradation (Müller *et al.*, 2001; Kint and Munoz-Guerra, 1999). The current state of knowledge on the plastic degradation exerted by microorganisms is still not sufficient for a suitable bioremediation or recycling approach of this environmental issue (Yoshida *et al.*, 2016). In conclusion, insects and microorganisms could be applied as synergistically plastic biodegrading agents.

From the surface of PET contaminated environmental samples with a microbial consortium that induced morphological changes on plastic film, by the use of a culture-dependent approach that requiring enrichment steps, a novel species belonging to the genus *Ideonella* has been found to be nutritionally dependent on PET (Yoshida *et al.*, 2016). Authors have also investigated the genes involved in PET degradation analysing the transcriptome of *I. sakaiensis* cells under different growing conditions including bis(2-hydroxyethyl) terephthalate and PET films. In summary, the synergic and integrated management of insects and microorganisms that could survive in presence of different types of plastic as major carbon source, as currently reported in these studies, could have the potential for significant biotechnological applications.

Conclusions

Insects and microorganisms are emerging protagonists of the novel concept of bioeconomy. The study of insect-microbial symbiosis represents not only a remarkable source of microbial biodiversity and functional novelty, but also a new source of microorganisms and enzymatic activities with a wide range of technological applications. In this review, we have assembled the available information on the current knowledge about reared insects and their microbial ecological aspects related to arthropods in the perspective of a MRM strategy. Insect farming for food and feed production can benefit of MRM since the growth-promoting effect exerted by the gut microbiota. (Storelli *et al.*, 2011). Insect symbionts can be used to promote the host health when the insects are also reared for agricultural purposes, as well as for industrial and environmental applications (Crotti *et al.,* 2012). For instance, the severe decline in the beekeeping sector might find an alleviation with the exploitation of beneficial microorganisms that can improve the overall insect's health (Crotti *et al.,* 2013). The efficacy of probiotic treatments is influenced by several factors such as the dose, timing, duration of the administration and variety of strains and further studies are thus necessary. Moreover, the investigation method needs to be uniformed for a better trial comparison in order to obtain high output accuracy. In this framework, the probiotic strategy meets the requirements of sustainable insect farming as probiotics can enhance the two key factors of insect growth performance and disease resistance. The ultimate goal foresees the realization of microorganism-based products for future MRM applications that should be validated by a reliable scientific literature.

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Chapter 2

Physicochemical conditions explain niche compartmentalization of the honeybee gut bacterial communities

ABSTRACT

The gut microbiome of the adult honeybee workers includes nine dominant bacterial phylotypes. Although considerable information has been accumulated on the diversity, genomic features and evolution of these phylotypes, an inclusive study linking the physicochemical gut conditions with the bacterial diversity and structure in the different gut portions is lacking. Considering that some of the phylotypes are acidophiles, either aerobic, microaerophilic or facultative anaerobic, we hypothesize that a compartmentalization of the bacterial community exists in the honeybee gut and that pH, oxygen concentration and redox potential may contribute to explain such distribution. Variation of the oxygen partial pressure (pO_2), redox potential and pH in the different gut compartments (crop, midgut, ileum and rectum) of honeybee foragers was measured taking advantage of microsensors. Bacterial composition and co-occurrence networks were determined by 16S rRNA gene high-throughput sequencing, while abundance was measured by quantitative PCR. The crop was primarily dominated by *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 and *Parasaccharibacter apium*, whereas the midgut and the ileum resulted inhabited by *Snodgrassella alvi*, *Gilliamella apicola*, and *Lactobacillus* Firm-5. *Bifidobacterium*, *Bartonella apis*, *Lactobacillus* Firm-4, and *Lactobacillus* Firm-5 dominated in the rectum. A diagonal oxygen gradient was measured in all the compartments that resulted anoxic in their centre, thus suggesting that the previously reported bacterial phylotype stratification on the gut epithelia is linked to oxygen availability. A progressive pH decrease from the crop to the rectum, presumably associated to the increasing microbial acidogenic activity, was paralleled by an increasing complexity of network connections. Our data provide evidence of a physicochemically-driven gut compartmentalization of the bacterial communities correlated with a rearrangement of the microbiome networking and diversity of the dominant bacterial phylotypes.

INTRODUCTION

Oxygen availability, pH, diet and host innate immunity have been indicated as driving factors for the gut colonization by its microbial partners (Engel and Moran, 2013; Lee *et al.,* 2013). For instance, the gut pH of insects is finely regulated and can vary from alkaline (e.g. midguts of lepidopteran larvae), to acidic values (e.g. crops of mosquitoes) (Engel and Moran, 2013): in some soil-feeding species also pH gradients ranging from 5 to >12 can be found in the different gut compartments (Köhler *et al.,* 2012). Gut-dwelling microbes can also produce fermentation products, affecting the gut pH and ultimately the microbial colonization (Engel and Moran, 2013).

On the other hand, oxygen is a crucial parameter that drive the gut microbiome structure. Considering the restrictions imposed by the diffusive transport of oxygen through epithelia, it has been estimated that the oxygen influx, per unit of volume, is 500 times larger in the termite gut than in the rumen (Brune, 1998). This makes oxic, or microoxic, the major part of the insect gut, in spite of the efficient oxygen consumption exerted by the epithelial cells in a fraction of millimetre (Brune *et al.,* 2000). Hence, the presence of gut anaerobic niches derives from the high microbial oxygen-consuming activity or is due to particular chemical processes existing in the gut compartments, such as in the case of the highly alkaline P1 segment of a soil-feeding termite (Brune *et al.,* 2000). Experimentally, gut compartments of few insects, which mainly rely on wood diets, have been characterized as anoxic (Köhler *et al.,* 2012; Brune *et al.,* 1995; Ceja-Navarro *et al.,* 2014; Šustr *et al.,* 2014; Brune and Friedrich, 2000), whereas in insects with a sugar-based diet, such as honeybees, the oxygen low content has been deduced from the presence of facultative anaerobic or microaerophilic bacteria (Kwong and Moran, 2016). Recently, a study demonstrated that hypoxia is essential for the larval mosquito development representing a signal for growth and molting. (Coon *et al.,* 2017; Shi *et al.,* 2014).

The honeybee gut microbiome has been deeply characterized considering its composition (Kwong and Moran, 2016), evolution (Kwong *et al.,* 2017), and genomic features (Engel *et al.,* 2012; Kwong *et al.,* 2014; Ellegaard *et al.,* 2015). Worker honeybees own a gut core microbiome made up of nine bacterial phylotypes, which include aerobic and facultative anaerobic bacteria that account for the 95-99% of the total bacterial community in almost all the adult individuals (Kwong and Moran, 2016). The core microbiome is dominated by five bacterial taxa i.e. the Beta-Proteobacterium *Snodgrassella alvi*, the Gamma-Proteobacterium *Gilliamella apicola*, the two sister clades of *Lactobacillus* Firm-4 and Firm-5 and the Actinobacterium *Bifidobacterium* (Kwong and Moran, 2016; Martinson *et al.,* 2011; Martinson *et al.,* 2012). In addition, the Proteobacteria *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium* and the *Gluconobacter*-related phylotype, indicated as Alpha2.1, are less prevalent but included in the core (Kwong and Moran,

2016). Finally, the Bacteroidetes *Apibacter adventoris* could be found at low abundance (Kwong and Moran, 2016). Variations to this bacterial organization have been described according to the age, caste and season (Kwong and Moran, 2016). These bacterial symbionts can contribute to the breakdown of pollen walls, be involved in the defense and immunity against pathogens or in the host interactions (Kwong and Moran, 2016, Engel *et al.*, 2012). Transmission and acquisition experiments showed that trophallaxis does not represent the principal route for bacterial transmission, underling that a faecal route and a contact with the hive components is required for the establishment and development of the typical honeybee bacterial community (Powell *et al.*, 2014).

Although the efforts performed so far have shown that some of bee phylotypes are restricted to or preferentially colonize certain portions of the gut (Kwong and Moran, 2016), a documented explanation of such a distribution is lacking. No direct measurements of physicochemical conditions occurring in the gut of honeybees have been performed and the experimental verifications of these physicochemical parameters could help in understanding the niche specialization of the gut bacterial partners (Kwong and Moran, 2016). Here, we investigated the variation of oxygen partial pressure (pO_2), pH and redox potential in the different gut compartments (crop, midgut, ileum and rectum) of the honeybee workers of *Apis mellifera ligustica* by using microsensors. Moreover, bacterial composition and abundance were investigated by 16S rRNA gene high-throughput sequencing and quantitative PCR. The community interaction was then evaluated by co-occurrence network analysis in order to assess i) the correlation between physicochemical parameters and the network topology, ii) the size and complexity of the bacterial networks for each gut compartment; and iii) the presence of taxa particularly relevant for the network suggesting their role in shaping the gut community.

MATERIALS AND METHODS

Honeybees and dissections.

Adult foragers (> 23 days old, Johnson 2008) of *Apis mellifera* (Hymenoptera: Apidae) were collected in late summer (September-October 2016) from the experimental apiaries located at DISAFA (Grugliasco, Torino, Italy). Honeybee guts were then dissected using sterile forceps and needles in Ringer's solution and used for the gut physicochemical or microbiome characterisation.

Microsensor measurements.

Microsensor measurements were performed using a setup similar to the one described by Brune *et al.,* (1995). After dissection in Ringer's solution, insect guts were gently leant on the agarose surface of customized plastic chambers, filled with 2% agarose prepared in Ringer's solution, and embedded in Ringer's solution solidified with 0.5% agarose. Microsensors (Unisense, Aarhus, Denmark) were used to measure the oxygen concentration, pH and redox potential within the different compartments of the gut of *A. mellifera*. In particular, 11 digestive tracts in case of pO_2, 12 in case of pH and 16 for the redox potential were dissected. Oxygen measurements were performed using oxygen microsensors with 50 µm-diamenter tip (OX-50), following a calibration at oxygen partial pressures of 0 and 21 kPa as previously described (Brune *et al.,* 1995). The pH microelectrodes (PH-50), with customized very sharp 50 µm-diameter tips, were calibrated using standard solutions of pH 4.0, 7.0, and 9.0 (Brune *et al.,* 1996). The redox microelectrodes (RD-50) with tip diameter of 50 µm were calibrated using saturated quinhydrone solutions in pH standard solutions of pH 4.0 and 7.0 (Ebert and Brune, 1997). In both cases, the electrode potentials were measured against a reference electrode (REF-RM), an open-ended Ag-AgCl electrode with a gel-stabilized electrolyte connected to a high-impedance millivolt-meter.

DNA extraction.

From the whole digestive tracts, organs (i.e. crop, midgut, ileum and rectum) were separated after being frozen at -20°C in order to avoid the release of the internal organ content. Five pools of each gut compartment (containing each 10 organs) were subjected to DNA extraction following the protocol by Sambrook *et al.,* (1989) with modifications: prior to DNA extraction, pools of gut compartments were homogenized in 0.5 ml 1× TE (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8), while after ethanol precipitation, DNA was eluted in 100 µl (midgut and rectum) or 50 µl (crop and ileum) 1×TE and stored at -20°C.

Characterization of Apis mellifera bacterial community through molecular ecology approaches.

In order to study the bacterial diversity associated to the different honeybee organ pools, Illumina libraries were prepared using the Illumina® Nextera XT Sample Prep Kit and amplifying the V3 and V4 variable regions of the 16S rRNA gene, using 341F and 785R primers (Klindworth *et al.*, 2013). Amplicon PCRs were performed in duplicate on all samples in a final reaction volume of 25 µl, which contained 1 U Platinum Taq DNA polymerase High Fidelity (Invitrogen), 1× PCR buffer, 2.0 mM $MgCl_2$, 300 µM dNTPs and 0.3 µM of each primer. Thermal conditions were set as

follows: an initial denaturation at 94 °C for 60 sec, followed by 25 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 68 °C for 30 sec, and a last elongation step at 68 °C for 10 min. The duplicate PCR products were then merged and checked on 1% agarose gel electrophoresis. A cleaning step was performed using Illustra™ ExoProStar™ 1-Step (GE Healthcare Life Sciences). Five µl of PCR product were transferred in a new 96-well plate, where 2 µl of ExoProStar mix were added. The reaction mix was incubated at 37 °C for 15 minutes and then at 80 °C for 15 minutes to inactivate the enzyme. An index PCR was run using Illumina® Nextera XT Index kit (96-indexes), where 5 µl of the enzymatically-treated PCR product were used as a template for the index PCR in a final reaction volume of 50 µl. PCR conditions were as follows: 1 U Platinum Taq DNA polymerase High Fidelity (Invitrogen), 1× PCR buffer, 2.0 mM $MgCl_2$, 300 µM dNTPs with different combinations of Index adapter1 and adapter2 (5 µl each), provided by the above mentioned Illumina® Kit. The thermal cycle was set with an initial denaturation step at 94 °C, followed by 8 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 68 °C for 30 sec, and a last elongation step at 68 °C for 10 min. The PCR products were checked on 1% agarose gel electrophoresis. In order to clean up and normalize the PCR products, the SequalPrep™ Normalization Plate kit (Invitrogen) was used. Finally, all the tagged samples were pooled together and concentrated in a CentriVap DNA Concentrator (Labconco).

The obtained 16S rRNA library pools were sequenced in a MiSeq system, 2 x 300 base pair read length, in the Biological Core Lab at the King Abdullah University of Science and Technology.

Bioinformatics.

The obtained sequences were analysed using a combination of the UPARSE v8 (Edgar, 2013) and the QIIME v1.8 (Caporaso *et al.,* 2010) software. Briefly, raw forward and reverse reads for each sample were assembled into paired-end reads considering a minimum overlapping of 50 nucleotides and a maximum of one mismatch within the region using the fastq-join algorithm (https://code.google.com/p/ea-utils/wiki/FastqJoin). The paired reads were then quality filtered, the primer sequences were removed and the individual sample files were merged in a single fasta file. This file was imported in UPARSE where operational taxonomic units (OTUs) of 97% sequence similarity were formed and chimeras were removed using both de-novo and reference-based detection. For reference chimera detection, the "Gold" database containing the chimera-checked reference database in the Broad Microbiome Utilities (http://microbiomeutil.sourceforge.net/) was used. The taxonomy was assigned to the representative sequences of the OTUs in QIIME using UClust (Edgar *et al.,* 2010) and searching against the latest version of the Greengenes database (McDonald *et al.,* 2012). Rarefaction was assessed and all the sample had a coverage of more than

99%. Finally, an OTU table (i.e., a sample x OTU count matrix with a tab containing the taxonomic affiliation of each OTU) was created. The OTU table and the phylogenetic tree was calculated with FastTree2 (Price *et al.,* 2010) using default parameters and the PyNast-aligned (Caporaso *et al.,* 2010b) representative sequences as an input. The OTU table and the phylogenetic tree were used as inputs for the subsequent analyses of alpha- and beta-diversity. Raw sequences were deposited at the ENA European Read.

Statistical analysis.

To test the bacterial compositional differences along the intestinal tract of the bees, we performed a Permutational multivariate analysis of the variance considering as categorical explanatory variables the gut portions fixed and orthogonal (4 levels: crop, midgut, ileum and rectum). Prior to run PERMANOVA analysis we tested the homogeneity of the dispersions among the categorical variable using PERMDISP ($F_{1,3}$=1.97; p=0.322). The statistical tests were performed by PRIMER v. 6.1, PERMANOVA+ for PRIMER routines (Anderson *et al.,* 2008). With the same experimental design, using the analysis of variance in R, we tested the difference of the number of 16S rRNA gene copies (our response continuous variable) - log transformed for normality - followed by a pairwise comparison with Tukey's HSD test. Linear discriminant analysis effect size (LEfSE, www.huttenhower.sph.harvard.edu/galaxy/) was applied to the OTU table, according to the method of Segata *et al.,* (2011), to identify bacterial taxa that could be detected as discriminant among the gut parts (Wilcoxon p-value: 0.05, LDA>2).

To explore differences in physicochemical parameters along the gut, we used a PERMANOVA considering oxygen concentration, pH and redox potential as our continuous response variables, while the gut portions as our categorical explanatory variables fixed and orthogonal (6 levels: crop, anterior midgut, middle midgut, posterior midgut, ileum and rectum). In order to verify further correlations with the other gut portions, we averaged the values relative to pO_2, pH and redox potential for the 3 midgut sections (i.e. anterior, middle and posterior ones).

Using the routine CoNet in Cytoscape 3.4 (Faust and Raes, 2012), we built a co-occurrence network to find OTUs significantly co-existing or mutually excluded in the different gut tracts. We used as input data the OTU table after removing the rare OTUs (less than 0.1% of sequences per sample). To build the network we combined an ensemble of the Pearson and Spearman correlation coefficients, and the Bray-Curtis (BC) and Kullback-Leibler (KLD) dissimilarity indices. To compute the statistical significance of the co-occurrence/mutual exclusion we first computed edge-specific permutation and bootstrap score distributions with 1,000 iterations (Barberán *et al.,* 2011). The clustering coefficients, neighbourhood connectivity distribution, betweeness centrality and

degree of connection for each node were calculated among the most important statistical descriptors of the network. The modularity of the network was instead calculated with gephi algorithm according to Blondel and collaborators (2008). These structural properties offer the potential for quick and easiest comparisons among complex datasets from different intestinal tracts. Centrality measures, like the degree of connection as descriptor of how much a node (taxon) is connected and the closeness centrality as a descriptor of the extent of influence of a node of the network among the intestinal tracts, were statistically tested using a generalized linear model in R using the package MASS (Venable and Ripley, 2002). As explanatory variables, we considered the 4 main groups that compose the bees gut microbiome (4 levels: Curbiculate core members, *Apis* specific members, other Curbiculate associates, others) in each single gut portion. Since the degree of connection is count data, a negative binomial distribution of the error was used in the model, while a quasipoisson distribution for the closeness centrality was applied. In addition, we tested the difference of degree of connection, closeness centrality and average path length, considering the gut tract explanatory variables (4 levels: crop, midgut, ileum and rectum) and adopting for the degree of connection and closeness centrality a negative binomial and a quasipoisson error distribution respectively while for the average path length we used a normal error distribution on log transformed values. This statistical analysis was carried out in R (R core team, 2017). Correlation between network topological coefficient and physicochemical gut parameters were tested using a combination of three correlation methods (Pearson, Kendall and Spearman) using the R package Performance Analytics (Peterson *et al.*, 2015).

Abundance of bacterial community by qPCR.

Quantitative PCR (qPCR) was performed with a CFX Connect^{TM} Real-Time PCR Detection System (Bio-Rad, Milan, Italy). Concentration of DNA samples was measured with a Nanodrop ND-1000 spectrophotometer. Total bacteria were quantified evaluating the *16S rRNA* gene copies of Bacteria using 357F and 907R primers with a final concentration of 200 nM (Favia *et al.,* 2007). Regarding thermal conditions, an initial denaturation at 98°C for 3 min was followed by 40 cycles consisting of denaturation at 98°C for 15 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min; a final step for melting curve analysis from 65 to 95°C (measuring fluorescence every 0.5°C) was added. To quantify the number of bacterial 16S rRNA gene copies for *S. alvi*, *Lactobacillus* Firm-5 and *G. apicola*, we used the primer pairs Beta-1009-qtF 5'-CTT AGA GAT AGG AGA GTG-3', Beta-1115-qtR 5'-TAA TGA TGG CAA CTA ATG ACA A-3', Firm5-81-qtF 5'-GGA ATA CTT CGG TAG GAA-3', Firm5-183-qtR 5'-CTT ATT TGG TAT TAG CAC C-3', Gamma1-459-qtF 5'-GTA TCT AAT AGG TGC ATC AAT T-3', Gamma1-648-qtR 5'-TCC TCT ACA ATA CTC

TAG TT-3' with a final concentration of 200 nM (Martinson *et al.,* 2012). For thermal conditions, we used an initial denaturation at 98°C for 3 min followed by 40 cycles consisting of denaturation at 98°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 15 min; a final step for melting curve analysis from 65 to 95°C (measuring fluorescence every 0.5°C) was added. 16S rRNA gene fragments cloned in pGEM T-easy Vector Cloning Kit (Promega, Milan, Italy) were used as indicated by Favia *et al.,* (2007).

RESULTS

Honeybee gut structure and weights.

The honeybee gut is constituted by four morphologically distinct compartments (Fig. 1A), i.e. crop, midgut, ileum and rectum (Kwong and Moran., 2016). Adult foragers (i.e. adults older than 23 days, Kwong and Moran., 2016) were sampled and weights of pooled compartments were measured after dissection. The measurements referred to the individual compartments are reported in table 1.

Table 1. Gut compartment weight in *A. mellifera*. Fresh weight of pooled (10) compartments is given with the average weight per pooled organs and the average weight for the individual organ (obtained by dividing per ten).

Pooled compartment	Replicate	Weight (g)	1 organ weight (g)
Crop	1	0.0251	0.0025
	2	0.0370	0.0037
	3	0.0380	0.0038
	Average	**0.0334**	**0.0033**
Midgut	1	0.1347	0.0135
	2	0.1440	0.0144
	3	0.1890	0.0189
	Average	**0.1559**	**0.0156**
Ileum	1	0.0066	0.0007
	2	0.0120	0.0012
	3	0.0146	0.0015
	Average	**0.0111**	**0.0011**
Rectum	1	0.3425	0.0343
	2	0.3790	0.0379
	3	0.3837	0.0384
	Average	**0.3684**	**0.0368**

Evaluation of gut compartments' physicochemical conditions.

Dissected intestinal tracts were prepared in gel-embedded chambers in order to measure the pO_2, pH and redox potential (E_h) values in each different gut portion. Measurements were collected by sampling and profiling the gut portions of several individuals, i.e. 11 digestive tracts in case of pO_2, 12 in case of pH and 16 for the redox potential. Interestingly, we found that oxygen levels were close to 0 μmol/L in the crop, midgut and ileum, whereas a significant increase of oxygen content was found in the rectum (PERMANOVA, $F_{5,59}$=4.53; p=0.001), documenting a value of 11 μmol/L corresponding to 0.8 kPa (Fig. 1B). This indicated a high oxygen-consuming activity in the gut compartments that made the entire digestive tract anoxic at the centre as evaluated during microsensors analysis (Fig. 2). The faint increase in the oxygen content of the rectum may reflect the oxygen possibility to diffuse in the organ through the anus or suggest a slightly different metabolic rate or peculiar physical structures in this compartment.

A significant decrease in the pH values was measured along the digestive tract passing from an average value of 6.1 in the crop, to 5.6 in the midgut, 5.2 in the ileum and 4.8 in the rectum. (Fig. 1C; PERMANOVA, $F_{5,68}$=9.7; p=0.001). Generally, we found sub-acidic pH all along the digestive system of the adult honeybee.

Finally, positive redox potentials were measured all along the anoxic gut portions (Fig. 1D). A significant difference was reported for the three sections of the midgut (i.e. anterior, middle and posterior ones) in relation to the other compartments (PERMANOVA, $F_{5,59}$=4.93; p=0.003). The slight increase of E_h passing from midgut to hindgut (ileum and rectum) was in agreement with the pO_2 increase detected in the respective compartments, where pO_2 showed an increasing trend from posterior midgut to ileum and rectum (Fig. 1D).

Bacterial abundance and diversity in the different gut compartments.

By qPCR (using a universal primer set), we found that each gut compartment contained relatively high numbers of bacteria, with 10^4 - 10^6 copies of 16S rRNA gene per gut portion and with the highest density found in the rectum (Fig. 1F). Specifically, the bacterial abundance increased along the gut from proximal to distal compartments, confirming the results reported by Martinson *et al.*, (2012): an average of 8.58×10^4 copies of 16S rRNA gene in the crop, 8.79×10^4 copies of 16S rRNA gene in the midgut, 6.71×10^5 copies of 16S rRNA gene in the ileum and 1.99×10^6 copies of 16S rRNA gene in the rectum were assessed in the pooled gut compartments (Fig. 1F). No statistical differences were found between the crop and midgut bacterial abundances, as well as between ileum and rectum (ANOVA, $F_{3,16}$ = 27.1 p<0.0001) (Fig. 1F). More abundant bacterial communities were hosted in the distal compartments than in the proximal ones. Furthermore, the

principal bacterial phylotypes described by Martinson *et al.*, (2012), i.e. *S. alvi*, *Lactobacillus* Firm-5 and *G. apicola* (previously known as Beta, Firm-5 and Gamma-1 phylotypes) were quantified by qPCR (Fig. 1F). While there was no difference among the three taxa in the midgut, data showed that *Lactobacillus* Firm-5 resulted a major constituent of the crop and rectum, whereas *S. alvi* and *G. apicola* were significantly more abundant in the ileum, confirming what previously reported (Fig. 1F; Martinson *et al.*, 2012).

High-throughput sequencing of 16S rRNA gene amplicons was performed to describe the bacterial communities inhabiting the gut portions (Fig. 1E, G). Following quality check and chimera removal, a total of 1,238,856 Illumina reads were obtained from the 20 samples which included 5 crop pools, 5 midgut pools, 5 ileum pools and 5 rectum pools. The coverage of the bacterial α-diversity associated with the different gut portions was verified by the construction of rarefaction curves. Alpha diversity indexes (OTU richness, evenness dominance and diversity) did not significantly vary along the whole intestinal tract. However, canonical analysis of principal coordinates (CAP), calculated applying Bray-Curtis distance index, revealed a significant bacterial community composition among crop, midgut, ileum and rectum portions (PERMANOVA; $F_{3,16}$=5.73; p=0.001). It is worth to notice that from the pairwise comparison, midgut and ileum did not present a significant difference of bacterial assemblage (Fig. 1E, G).

The gut microbiota of the worker honeybees collected from apiaries in North Italy resulted dominated by the same 9 bacterial phylotypes reported in previous publications (a summary of the existing literature can be found in the review by Kwong and Moran, 2016). Particularly, we retrieved the five major taxa in the "Corbiculate core", i.e. *S. alvi*, *G. apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 and *Bifidobacterium* (Kwong and Moran, 2016; Martinson *et al.*, 2011; Martinson *et al.*, 2012). On the total bacterial community, they accounted for the 71.06% of the entire community in the crop, 79.07% in the midgut, 71.52% in the ileum and 85.22% in the rectum. In addition, less prevalent were the Proteobacteria *F. perrara*, *B. apis*, *P. apium* and the Alpha2.1 phylotype (Fig. 1G). Collectively, these nine phylotypes accounted for the 86-96% of the total bacterial community (91.95% in the crop, 86.86% in the midgut, 86.05% in the ileum and 96.22% in the rectum). Finally, *A. adventoris* was found at low abundance (0.02% in the crop, 0.26% in the midgut, 0.32% in the ileum and 0.10% in the rectum).

The crop was mainly dominated by *Lactobacillus* Firm-5 and Firm-4, *P. apium* and other *Lactobacillus* sp., while the midgut was characterized by the presence of *S. alvi*, *G. apicola*, *Lactobacillus* Firm-5 and members of Enterobacteriales (Fig. 1G). The ileum mainly showed the presence of *S. alvi*, *G. apicola*, *Lactobacillus* Firm-5 and *B. apis*. Conversely, the rectum is dominated by *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *Bifidobacterium* and *B. apis*.

Interestingly, *B. apis* colonization showed an increase from crop to rectum with percentages that passed from 1.51 in the crop, to 3.98 in the midgut, to 9.79 in the ileum and 9.45 in the rectum. The same ascending, and even more marked, trend was shown by *Bifidobacterium* (4.29% in the midgut, 4.98% in the ileum and 28.31% in the rectum). Summarizing, *S. alvi*, *G. apicola*, *F. perrara* and other members of Enterobacteriales showed a better colonization of the midgut and ileum compartments, with low abundance in the peripheral portions (crop and rectum). Being present in all the compartments, *Lactobacillus* Firm-5 and Firm-4 preferentially colonized the crop and rectum (Fig. 1F,G).

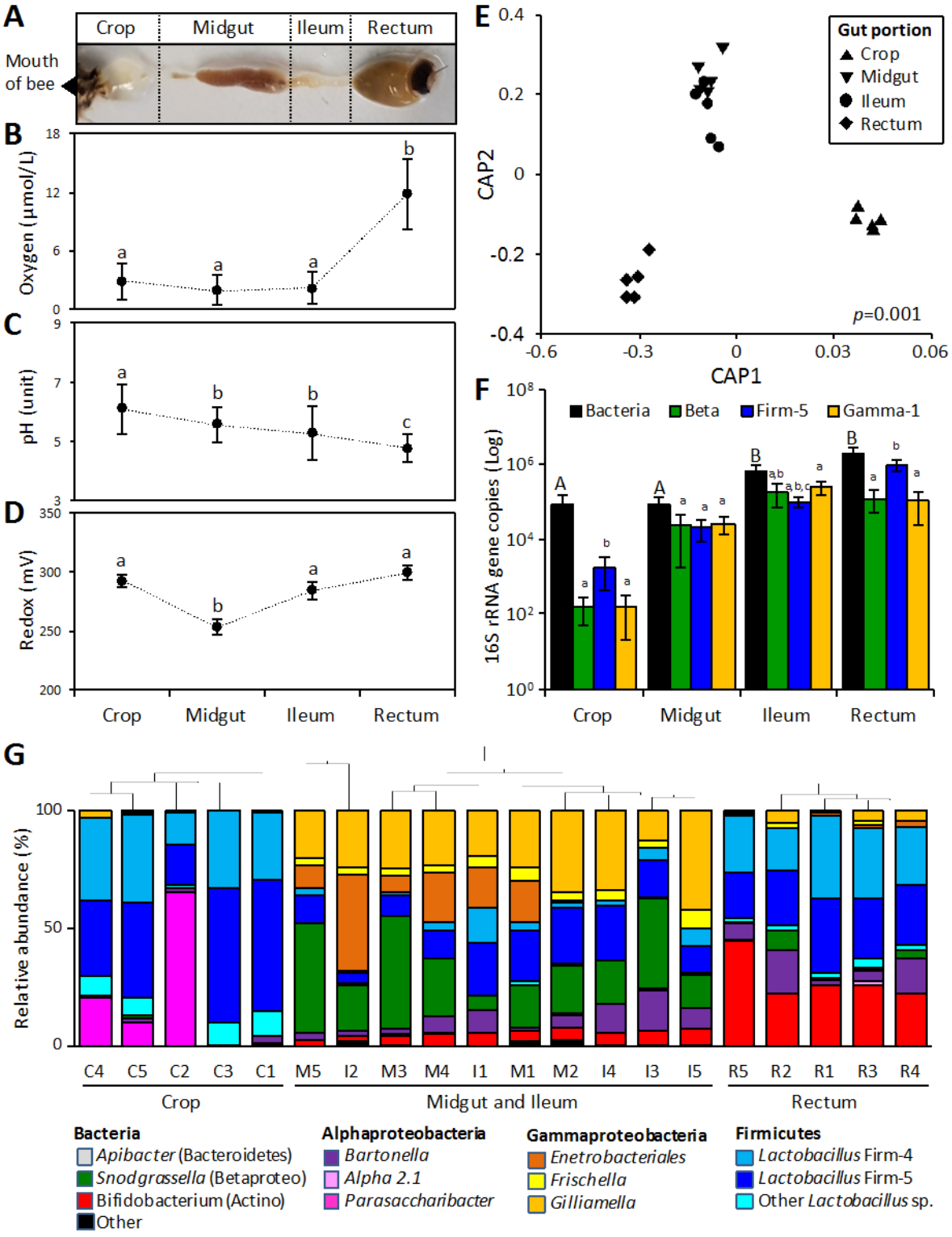

Figure 1. Physicochemical and microbial characterization of the honeybee gut compartments. Honeybee gut compartment are depicted in panel A. Oxygen concentration (µmol/L) (B), pH (C) and redox potential (mV) (D) values in crop, midgut, ileum and rectum. Composition of the bacterial communities inhabiting the different gut sections (determined by Illumina platform analysing the V3-V4 region of the 16S rRNA gene) visualised by canonical analysis of principal coordinates (CAP; for the statistic see the text) (E) and the relative abundance of the bacterial taxa (G). Abundance of the bacterial communities (measured by

quantitative PCR) in the honeybee gut (F) is indicated as Log-transformed number of 16S rRNA gene copies in the different gut compartments. An increase in the bacterial abundance was measured from proximal to distal compartments (black bar; significativity among gut tracts has been indictaed with capital letter). Log transformed numbers of 16S rRNA gene copies specific for Beta, Firm-5 and Gamma-1 in the different gut compartments are also depicted (the significativity among the bacterial copies for each gut tract was indicated by lower case letter; for the statistical paramenter see the text).

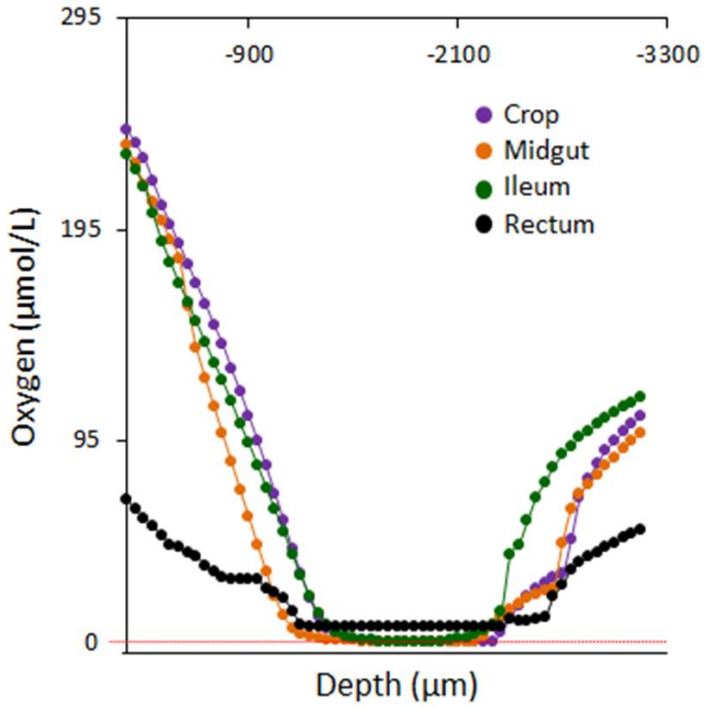

Figure 2. Radial profiles of oxygen concentration in the crop, midgut, ileum and rectum gut compartments of honeybees.

Peculiar networks in the honeybee gut compartments.

To identify potential interactions and niche-sharing among microbes that inhabited the honeybee digestive tract, we constructed 4 bacterial networks related to the 4 gut compartments (Fig. 3). First, we found that the bacterial networks' topology significantly differed in size and complexity (Fig. 3A). Co-occurrence network analysis allowed the identification of different network modularity, centrality and clusterization for the 4 portions (Tab. 2). Specifically, higher values of modularity have been found in the crop and midgut when compared to the ileum and rectum: the first two sections owned more heterogeneous modules and lower clusterization than the other two distal compartments that presented a more interconnected community (Fig. 3A, Tab. 2). This was paralleled by the value of number of communities: in the first two gut portions the values (46 and 88 for the crop and midgut, respectively) were larger than the ones found in the ileum and rectum (14 and 28, respectively; Tab. 2) (Lambiotte *et al.*, 2009).

Following the same trend reported for the degree of connection, the network centralization had the higher values in the ileum and rectum, supporting once again the tendency of the microbial communities to be more interconnected in the distal tracts than in the proximal ones (Tab. 2). Whereas the number of nodes was similar in the four compartments, the number of interactions (considering both positive and negative ones) increased along the intestinal tract, passing from 1174 in the crop, to 1194 in the midgut, and finally to 2252 in the ileum and 1958 in the rectum (Tab. 2). Particularly, positive interactions, i.e. co-occurrence connections, represented the majority of the interactions in the crop and midgut, while a 1:1 balance of positive (co-occurrence) and negative (i.e. mutual exclusion) interactions occurred in the ileum and rectum (Tab. 2). This suggested that in the more complex communities of the distal compartments, negative interactions of mutual exclusion can be established by the bacterial members, likely competing for space and nutrients. Indeed, we have to remember that these two portions were the most densely inhabited ones, showing the highest values of 16S rRNA gene copy number per compartment (Tab. 2). The other network topological descriptors were consistent with this trend (Tab. 2).

Table 2. Network parameters calculated for each gut compartments, followed by the physico-chemical characteristic measured with the microsensors (expressed as average; see Fig 1A and statistic in the main text).

Network and physiological parameters		**Gut compartment**			
		Crop	**Midgut**	**Ileum**	**Rectum**
Network	Modularity	0.73	0.7	0.61	0.65
	Number of communities	46	88	14	28
	Clustering Coefficient	0.48	0.55	0.56	0.59
	Network centralization	0.17	0.1	0.35	0.38
	Network heterogeneity	0.83	0.71	0.78	0.81
	Number nodes	198	191	201	192
	Network diameter	12	7	6	6
	Network density	0.06	0.07	0.11	0.12
	Interaction	1174	1194	2252	1958
	Co-Occurrence	959	999	1089	1056
	Mutual Exclusion	215	195	1163	902
Microsensors	Oxygen (µmol/L)	2.83	2.01	2.14	11.76
	Redox (mV)	292.38	252.54	283.91	299.16
	pH	6.11	5.63	5.25	4.76

Likewise, the degree of connection of the nodes that characterized each compartment showed a statistically significant difference between crop/midgut and ileum/rectum (GLM, negative binomial family, $p < 0.0001$), as depicted in Fig. 3B. In general, data showed that OTUs from the ileum and rectum portions were significantly more connected than the ones from crop and midgut: box plot graphs depicted a situation in which median values of around 8-10 connections were retrieved for the crop and midgut nodes, while around 20 connections were obtained for ileum and rectum nodes. The 3rd quantile and the whiskers reached around 40 and 70 connections, respectively, in the ileum and rectum, while around 20 and 35 connections, respectively, were retrieved in the crop and midgut (Fig. 3B). Furthermore, we observed in the ileum and rectum an increase of connection of *Bacteroidetes*, *Alphaproteobacteria* and *Gammaproteobacteria* (Fig 3E).

Closeness Centrality varied significantly among the gut compartments (GLM, $\chi^2_{3,777}=30.105$, $p < 0.0001$) with nodes in the crop having less centrality than the ones in the other three compartments (Fig. 3C). Closeness Centrality of the taxa also varied with *Firmicutes* progressively having less contribution from the crop to the rectum (Fig. 3F). Conversely, the average path length significantly decreased from the crop to the rectum (ANOVA, $F_{3,777}=124.98$, $p<0.0001$) (Fig. 3D, G).

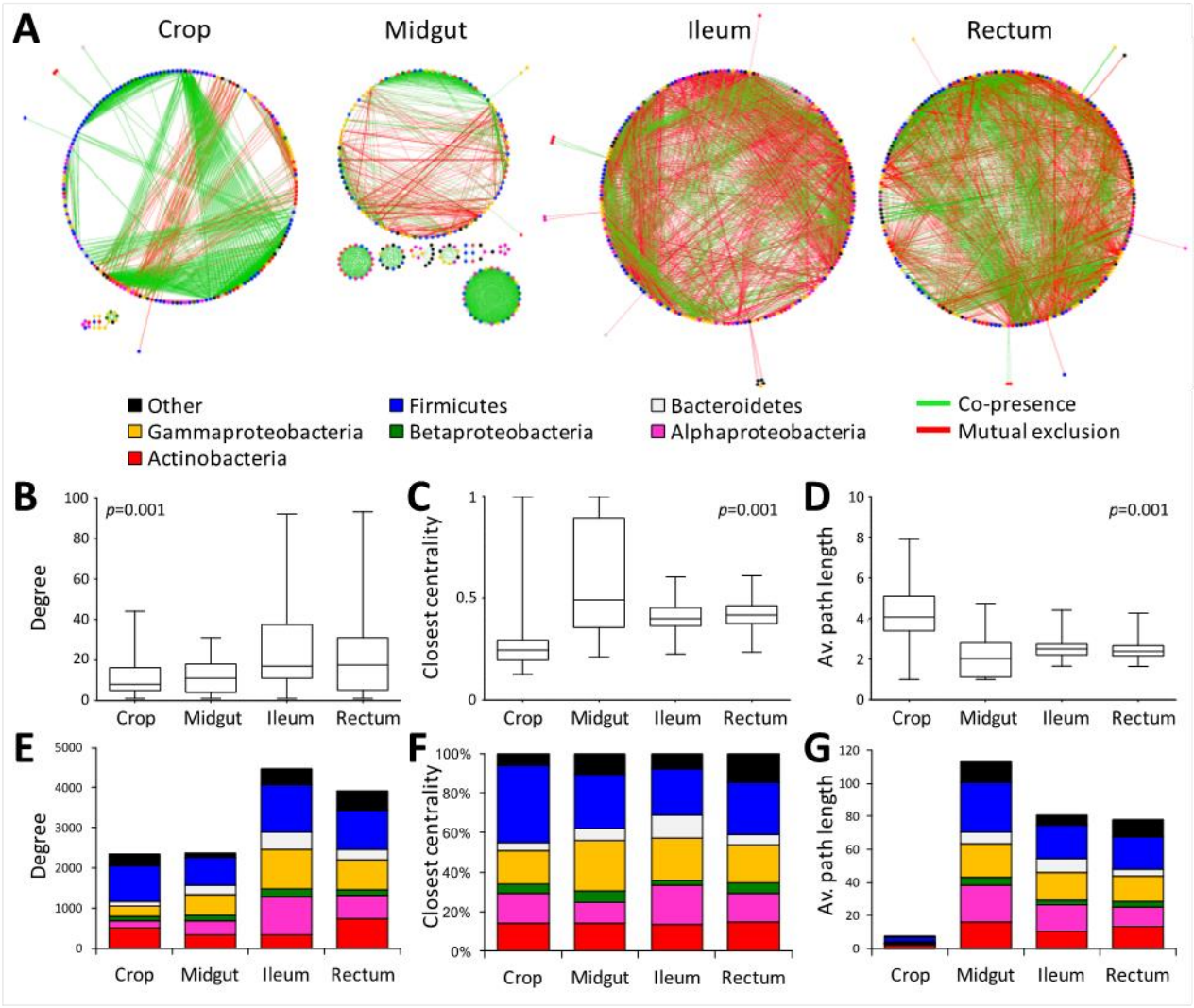

Figure 3. Co-occurrence network diagrams. (A) Network of the four gut tracts investigate, crop, midgut, ileum and rectum. Each node represent taxon significantly interaction with other bacterial groups (colours in the legend). For each gut compartment has been reported: (B) average degree of connection per nodes and (E) taxa total degree connection; (C) average closeness centrality per node and (F) total closeness centrality for each taxon (expressed as percentage); (D) average path length per node and (G) total average path length per each taxon.

In order to assess the centrality in the overall bee gut interactome, the 9 phylotypes (see Kwong *et al.*, 2017) and 3 few other high-frequent taxa were evaluated for their degree of connection and closeness centrality (Fig. 4). Particularly, in the crop the "Other Curbiculate associates" (*Lactobacillus* sp., Alpha 2.1 and *P. apium*) were statistically more connected than the remaining phylotypes (GLM, d.f.=3; Chi-square=49.02 p< 0.001); a similar situation, but considering also *F. perrara* and *Bifidobacterium*, occurred in the midgut (GLM, d.f.=3; Chi-square=45.92 p= 0.007; Fig. 4A-B). In the ileum, we found that the "Corbiculate core" phylotypes (among which *G. apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 and *Bifidobacterium*) increased their level of

degree connection (GLM, d.f.=3; Chi-square=55.22 p= 0.009) (Fig. 4C). Finally, in the rectum, the overall groups increased their degree of connection, even if without any significance difference, when compared to the rest of the gut portion (GLM, d.f.=3; Chi-square=39.81; p= 0.081) (Fig. 4D). On the other hand, *Lactobacillus* Firm-4, *S. alvi* and Enterobacteriales exhibited a higher level of closeness centrality than the other groups in the crop (GLM, d.f.=3; Chi-square=49.01; p= 0.0001), whereas in the midgut *Lactobacillus* Firm-5, *B. apis* and Enterobacteriales showed the highest values (GLM, d.f.=3; Chi-square=1.77; p= 0.03) (Fig. 4E). In the ileum and rectum, it was not recorded a significant difference among the groups and a consistent value of closeness centrality between 0.25 and 0.5 was found (Fig. 4 E-F).

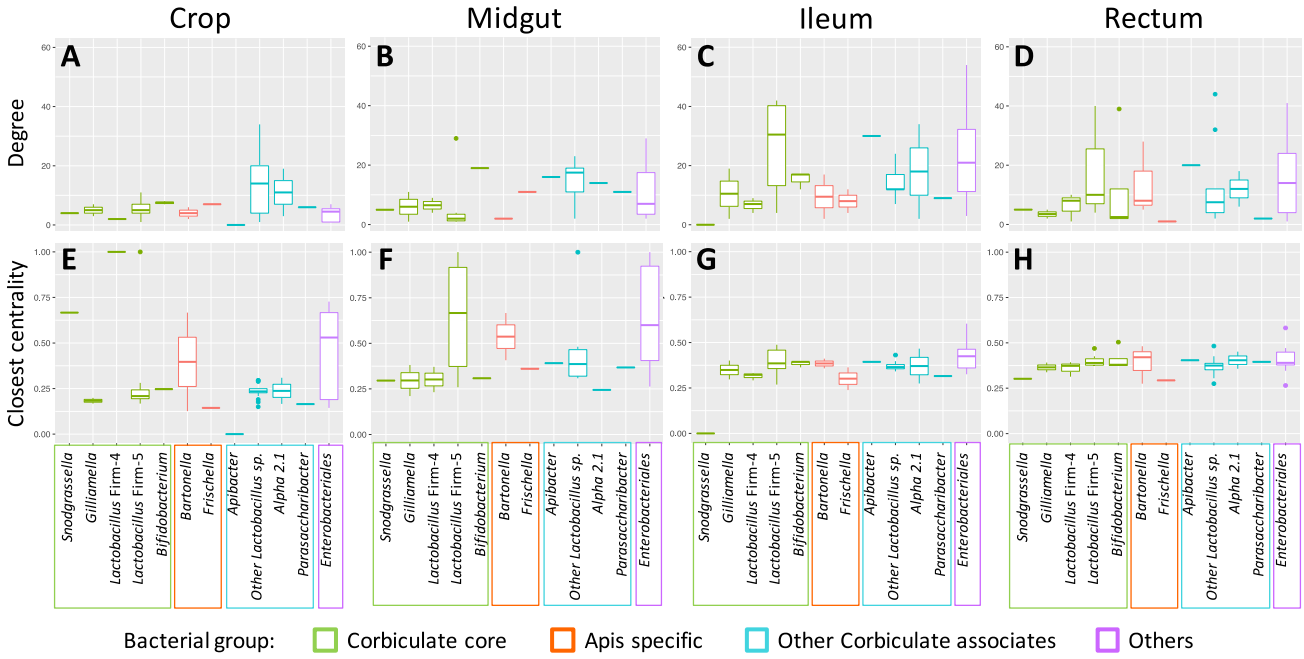

Figure 4. Node degree distribution (A-D) and Node Closeness centrality (E-H) for the main taxa composing the core microbiome of the honeybee gut (see Kwong *et al.*, 2017).

In order to evaluate the influence of the physicochemical parameters (i.e. pO_2, redox potential and pH) on the network characteristics in the different gut compartments, a correlogram was constructed, using three different correlation indexes to validate the analysis (see supplementary materials, Fig. S1). Data suggested a significant correlation of network heterogeneity to the redox potential along the four gut compartments. Conversely, an inverse proportion was found between pH conditions and the network clustering coefficient and the network density (Fig. S1). This highlighted that, passing from proximal to distal compartments, the decreasing conditions of pH influenced not only the density of the network, but also its complexity: the more the pH decreased,

the more the phylotypes were connected one to each other, shaping more complex communities. Interestingly, no statistically significant dependence involving pO_2 was found (Fig. S1).

Summarizing, while the crop and midgut showed a more heterogeneous topological structure of the network, with higher levels of modularity and lower levels of network centralization, the ileum and rectum displayed the opposite trend with a denser network structure and almost a doubled number of interactions among the nodes (phylotypes). Correlogram analysis also showed that modularity is inversely proportional to network density and number of interactions (considering both positive and negative ones), supporting one again that in the simpler communities of the first portions more isolated bacterial members are present. Furthermore, the clustering coefficient was found to be inversely proportional to the network diameter, while increasing values of network centralization are related to increasing values of the network density and number of interactions along the digestive tract.

LEfSe analysis.

We performed LEfSe to identify the discriminant bacterial taxa that significantly characterized the gut compartments (Tab. 3). LEfSE detected in the crop 15 bacterial taxa, among which *Lactobacillus* Firm-4, *P. apium*, *Lactobacillus* Firm-5 and other members of *Lactobacillus* genus characterized the bacterial community of this compartment. In the midgut, 5 taxa were found discriminant, among which the principal ones were *S. alvi*, *Lactobacillus* Firm-5 and members of the Enterobacteriaceae family. On the other hand, *G. apicola*, Enterobacteriales members, *B. apis* and *F. perrara* mainly differentiated the bacterial community inhabiting the ileum, whereas in the rectum the discriminant phylotypes were *Bifidobacterium*, *Lactobacillus* Firm-4, Alpha 2.1 and other members of *Lactobacillus* genus (Tab. 3). Over all, the analysis underlined the presence of some peculiar phylotypes that characterized the bacterial community in each compartment.

Table 3. Linear discriminant analysis (ANOVA p-value: 0.01, Wilcoxon p-value: 0.05, LDA > 2) Effect Size (LEfSe) of bacterial OTUs in gut portions of honeybees

OUT	Gut portion	LDA score	p
Lactobacillus Firm-4	Crop	5.0621152	0.002603
Parasaccharibacter	Crop	5.00644799	0.002822
Lactobacillus Firm-5	Crop	4.63797548	0.0031734
Lactobacillus Firm-5	Crop	4.57435334	0.0028819
Other *Lactobacillus* sp.	Crop	4.46792834	0.0026452
Lactobacillus Firm-5	Crop	4.43273171	0.0041675
Lactobacillus Firm-5	Crop	4.12019797	0.0014968
Lactobacillus Firm-4	Crop	4.04531176	0.0035128
Lactobacillus Firm-5	Crop	3.80044593	0.0039933
Other *Lactobacillus* sp.	Crop	3.79780204	0.0011352
Other *Lactobacillus* sp.	Crop	3.43974298	0.0014649
Other *Lactobacillus* sp.	Crop	3.36533566	0.0051076
Other *Lactobacillus* sp.	Crop	3.28610134	0.0118734
Other *Lactobacillus* sp.	Crop	2.98147719	0.027309
Other *Lactobacillus* sp.	Crop	2.85193888	0.0361368
Snodgrassella	Midgut	5.19377494	0.0013732
Lactobacillus Firm-5	Midgut	4.56387598	0.033341
Enterobacteriales	Midgut	3.98219764	0.0198203
Other (*Pasturellales*)	Midgut	3.71260585	0.048052
Other (*Pasturellales*)	Midgut	3.26992567	0.0032474
Gilliamella	Ileum	5.10092028	0.0015459
Enterobacteriales	Ileum	4.74458884	0.0236681
Bartonella	Ileum	4.64143549	0.0336877
Frischella	Ileum	4.36195548	0.0009828
Other (*Pasturellales*)	Ileum	3.39995089	0.039129
Apibacter	Ileum	3.23270443	0.0285407
Other (*Pasturellales*)	Ileum	3.17418565	0.0032091
Enterobacteriales	Ileum	3.0362398	0.038775
Enterobacteriales	Ileum	3.01217133	0.0227565
Bifidobacterium	Rectum	5.14982935	0.0008727
Lactobacillus Firm-4	Rectum	4.18290782	0.002617
Alpha 2.1	Rectum	3.47449453	0.0152585
Other *Lactobacillus* sp.	Rectum	3.21546745	0.0173335
Other *Lactobacillus* sp.	Rectum	3.17923	0.0377419

DISCUSSION

Previous studies aimed at investigating the diversity of the honeybee bacterial community have just suggested the existence of microaerophilic and anoxic niches in the digestive tract, following the report of bacteria that can grow better in absence of or under oxygen concentrations lower than that of air (Kwong and Moran, 2016). However, no experimental verifications have been performed so far in this perspective. The present work represents the first comprehensive study exploring the physicochemical conditions occurring in the different compartments of the honeybee gut, combining it with a highly resolved analysis of the bacterial communities (Fig. 1). Recently, the profiles of oxygen, pH and redox potential have been measured in few other insects, mainly lower and higher termites (Köhler *et al.*, 2012; Brune *et al.*, 1995; Ceja-Navarro *et al.*, 2014; Šustr *et al.*, 2014; Brune and Friedrich, 2000) and analysed with high-throughput sequencing data, supporting the existence of microbial and functional gut compartmentalisations (Köhler *et al.*, 2012; Ceja-Navarro *et al.*, 2014). For instance, the physicochemical conditions in the gut portions of the wood-feeding termite *Nasutitermes corniger* evidenced strong and peculiar variations of oxygen and hydrogen contents; correlations with the microbiome composition revealed that the termite gut was highly structured with microbial communities -and thus metabolic activities- peculiar for the several gut portions (Köhler *et al.*, 2012). Similarly, niche-dependent differentiation of the gut microbiome was also demonstrated in the wood-feeding beetle *Odontotaenius disjunctus* showing that sharp oxygen gradients may allow aerobic and anaerobic metabolism to occur within the same regions, in close proximity (Ceja-Navarro *et al.*, 2014).

By using a deep sequencing approach we explored the bacterial diversity in the honeybee gut compartments, confirming the presence of the bacterial phylotypes described by Kwong and Moran (2016). The gut microbiome varied in composition and increased in abundance and complexity along the gut (Fig. 1-4). Interestingly, while we found that alpha diversity indices did not diverge along the digestive tract, a modification of the bacterial assemblage (in terms of phylotypes' identification and abundance) and network topology occurred, highlighting that different bacterial communities are selected in the 4 gut portions. Indeed, OTU numbers, evenness, Shannon and dominance values were constant in the different portions, while selected phylotypes showed to inhabit preferentially specific gut sections. Overall, high-throughput sequencing, quantitative PCR and LEfSe data confirmed the presence of a bacterial compartmentalization: in the crop the principal phylotypes were *Lactobacillus* Firm-5, *Lactobacillus* Firm-4 and *P. apium*, while the midgut and the ileum were primarily inhabited by *S. alvi*, *G. apicola*, *Lactobacillus* Firm-5 and *B. apis*; finally, in the rectum the main phylotypes resulted *Lactobacillus* Firm-4, *Lactobacillus* Firm-

5, *Bifidobacterium* and *B. apis* (Fig. 1 and Tab. 3). Only *Lactobacillus* Firm-5 showed to be distributed and abundant in all the compartments, since presumably adapted to the anoxic conditions of the inner part of each gut section (Fig. 1).

By the use of microsensors we showed that anaerobic conditions occurred in the centre of all gut portions: the lower value was measured in the posterior part of the midgut (1.46 μmol/L), which corresponded to 0.11 kPa, while the highest one in the rectum (11.76 μmol/L) corresponding to 0.8 kPa. Oxygen concentrations at the epithelial level resulted to be higher than the values (close to 0 kPa) reported for each section's centre, thus showing the presence of micron-scale radial gradients for oxygen concentration in all the considered gut regions (Fig. 2). The presence of an oxygen gradient toward the gut centre suggests the occurrence of microaerophilic and anoxic niches able to sustain the microaerophilic or anoxic requirements of the bacterial symbionts (Kwong and Moran, 2013). For instance, *S. alvi* is known to be adjacent to the gut wall (Martinson *et al.,* 2012), where it could thrive in the oxic-microaerophilic habitat since equipped with the cytochrome *bo* and *bd* oxidases (Kwong *et al.,* 2014). Other members of the Curbicolate core, i.e. *G. apicola*, *Lactobacillus* Firm-4 and *Bifidobacterium*, are equipped with the hypoxia-induced cytochrome *bd* oxidase (Kwong *et al.,* 2014; Ellegaard *et al.,* 2015), while *Lactobacillus* Firm-5 does not own neither cytochrome *bo* oxidase nor *bd* one (Ellegaard *et al.,* 2015). Interestingly, the previously described stratification of the bacterial partners (Martinson *et al.,* 2012) is likely to occur in consequence to the oxygen availability and terminal cytochrome oxidases' distribution in the honeybee phylotypes. Taking into account the more abundant and discriminant phylotypes, as depicted in Fig. 1G and Tab. 3, i.e. *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *P. apium*, *S. alvi*, *G. apicola*, *Bifidobacterium* and *B. apis*, it is possible to individuate for each gut portion at least one phylotype equipped with the cytochrome *bo* oxidase, supposing its capability to thrive close to the epithelial surface, where oxygen is more abundant. For instance, *P. apium* (in the crop), *S. alvi* (in the midgut and ileum) and *B. apis* (in the ileum and rectum) showed the presence of cytochrome *bo* and *bd* oxidases. Additionally, *B. apis* owns the cytochrome *c* oxidase genes which sustain its growth under aerobic conditions (Segers *et al.,* 2017; Kešnerová *et al.,* 2016). Interestingly, a recent publication reported that its phylogenetic closest neighbours ('*B. tamiae*' Th239 and *B. henselae* Houston-1^{T}) did not grow under aerobic conditions (viz. in air), hence suggesting the adaptation of this species to this particular niche (Kešnerová *et al.,* 2016). Finally, acetic acid bacteria, to which *P. apium* belongs, are well known colonizers of the insect gut epithelia (Vacchini *et al.,* 2017; Crotti *et al.,* 2010): this supports the presence of *P. apium* in close proximity of the crop epithelium where it can flourish taking advantages of cytochrome *bo* and *bd* oxidases (Chouaia *et al.,* 2014). On the other hand, phylotypes equipped with cytochrome *bd* oxidase (*Lactobacillus* Firm-4, *G. apicola* and

Bifidobacterium) or with none of these oxidases (*Lactobacillus* Firm-5) may be found in the central parts of each portion, suggesting the bacterial niche-specialization to an oxygen-depleted microhabitat.

All the anoxic gut portions showed positive redox potentials with a slight, but significant difference for the midgut's values (Fig. 1C). The presence of anoxic gut compartments with positive redox potentials has been previously reported in other arthropods (Zimmer and Brune, 2005; Šustr *et al.*, 2014). As already mentioned by Šustr and co-workers (2014), this feature underlines that the presence of reducing conditions in the absence of oxygen is also dependent on the presence of proper metabolites with negative redox potential and sufficient reactivity. Along the four gut compartments the redox potential was significantly correlated to the network heterogeneity (0.94; Fig. S1) that indicate the tendency of a network to have hub nodes (Dong and Horvath, 2007). We can hence hypothesize that the bacterial network complexity may be driven or influenced by the redox potential conditions present in the different gut compartments, which presumably differ not only in terms of metabolite types or concentrations, but also considering their reduction degree (Kwong and Moran, 2016). Honeybees rely on a carbohydrate-rich diet, made up of nectar, honey and pollen, which are then metabolized to various fermentation products, such as lactic acid and acetate, accordingly to the bacterial species involved (Kwong and Moran, 2016). Thus, the reduction degree of the metabolites increased along the digestive tract, passing from more complex carbohydrates to simpler acids and alcohols. Another parameter strictly linked to the metabolic feature is represented by the pH *status* of the different gut compartments. Data showed a significant decrease of pH passing from the proximal to distal portions of the digestive tract. Particularly, an inverse correlation was found between pH conditions and the network clustering coefficient and density (-0.94 and -0.93, respectively). It is likely that from crop to rectum, the decreasing pH conditions allow the establishment of a more connected, and homogeneus community (Fig. S1). For instance, a dependence interplay has been described between *G. apis* and *S. alvi* (Kwong *et al.*, 2014) which are present in the midgut and the rectum. *G. apis* is a saccharolytic fermentative bacterium that complements the metabolism of *S. alvi*, which shows oxidizing activity of carboxylic acids (Kwong *et al.*, 2014; Kwong *et al.*, 2017). While the analysis of 16S rRNA gene high-throughput sequencing data does not show differences in the bacterial assemblage between the midgut and ileum (Fig. 1G), co-occurrence network revealed statistically significant variations of all the gut compartment considering the number of communities, the number of interactions, the degree of connections and the closeness centrality (Fig. 3-4), significantly detecting differences between ileum and midgut. This results is of particular interest because we can hypothesize that the

interactome -influenced by the redox potential-and the abundance of bacteria more than their assemblage play a crucial role in differentiating these two compartments.

In conclusion, here we show that the bacterial differentiation occurring in the four gut sections is driven by the modification of the physicochemical parameters of pO_2, pH and redox potential, resulting in a niche adaptation of the different bacterial phylotypes. Particularly, the radial availability of oxygen from each gut section's epithelial surface to the centre seems to influence the phylotypes' stratification, whereas the pH and redox potential conditions can drive longitudinally the phylotype diversification and the complexity of the network connections. Interestingly, pH and redox are shown to control the bacterial interactome of the overall community, offering a useful insight for further manipulative investigations to shed light on the community assembly, the degree of its resilience facing the environmental changing and therefore its contribution to the overall host homeostasis.

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Chapter 3

Diet and developmental stage influence the bacterial community of the food waste-reducing insect *Hermetia illucens*

ABSTRACT

The gut microbiome influences several aspects of the insect biology and physiology, such as the development, reproduction and nutrition and, conversely, many factors, such as the diet, developmental stage and host genetics, can influence its structure and composition. In this work, we investigated the variation of the bacterial communities associated to the black soldier fly (BSF) *Hermetia illucens*, when reared on three different diets (standard, fruit-waste derived and vegetable-waste derived ones) and during its development (i.e. larvae, prepupae and adults). BSF is an interesting candidate as an alternative protein source for livestock in the framework of a circular economy approach. Using cultivation-independent techniques, i.e. by combining the Automated Ribosomal Intergenic Spacer Analysis (ARISA)-PCR fingerprinting on 54 individuals and the high-throughput sequencing of 16S rRNA gene amplicons on 36 individuals, we characterized the bacterial communities associated to the insects, revealing that their bacterial inhabitants belonged primarily to three phyla, i.e. Proteobacteria, Firmicutes and Bacteroidetes. Statistical analysis showed that the diet and developmental stage influenced the insect microbiome structure. Particularly, ten OTUs belonging to Enterobacteriaceae were shared among insects reared on the different food sources and of different stages. By the use of microsensors, we also measured the variation of the physicochemical conditions in terms of oxygen partial pressure (pO_2), pH and redox potential occurring in the digestive tract of 4^{th} instar larvae when exposed to the three different diets. Data suggested that this variation of the physicochemical conditions driven by the modification of the alimentary regimes possibly impacted the bacterial community structure. Overall, we demonstrated that the diet source and developmental stage are important factors that shaped the structure and composition of BSF bacterial community.

INTRODUCTION

Insects establish with microorganisms a wide array of symbiotic relationships including parasitic, mutualistic and commensal ones. Initial studies have been mostly devoted to investigate the obligate and facultative symbiotic interactions, which provide to their hosts nutritional or defensive services or are involved in host reproductive alterations (Moran and Dale, 2006; Werren *et al*., 2008). Recently, a great interest has been directed to commensal partners, which, despite their transient behaviour in the host intestinal tract, have been demonstrated to influence the host physiology, e.g. in terms of nutrition (Engel and Moran, 2013), modulation of the immune system (Lee *et al.*, 2013), larval development (Chouaia *et al*., 2012) or mating (Sharon *et al*., 2010). Commensals are typically of environmental origin: they can be acquired by the host during its trophic activity or by social contacts with the nestmates or within the nest habitat, such as in the case of honeybees (Engel and Moran, 2014; Powell *et al*., 2014).

Insects that feed on plant and animal debris host a great microbial diversity inside their guts (Carrasco *et al.*, 2014; Gupta *et al.*, 2014; Mikaelyan *et al.*, 2017). For instance, termites, which relying on dead plant matter contribute mainly to the carbon cycle, harbour highly specific gut communities encompassing several hundreds of microbial partners, including bacteria, archaea and protists (Brune and Ohkuma, 2011; Engel and Moran, 2014). These microorganisms are involved in the breakdown of recalcitrant polymers (such as cellulose and hemicellulose) in simpler molecules that are then absorbed and metabolized by the host (Brune *et al.*, 2005; Breznak & Brune 1994). Conversely, the black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomydae) is one of the best known insects that can efficiently reduce vegetable waste or organic refuse such as dairy, poultry, and swine manure (Józefiak *et al.*, 2016; Nguyen *et al.*, 2015; De Marco *et al.*, 2015; Kroeckel *et al.*, 2012). Larvae of this non-pest arthropod can growth on different substrates consuming twice of their weight per day until they reach the prepupal stage with the maximum content of protein and fat (Diener *et al.*, 2009). With the rapid increase of world populations and the resulting growing demand in human food and feed for livestock, insect farming is a sustainable way to overcome these issues, considering that insects are characterized by a rapid growth rate and require few space and water (Rumpold and Schlüter, 2013). BSF has a high natural efficiency in reducing the organic discard without commercial value into insect biomass characterized by high content in protein (over 40%), crude fat (over 30%) and other valuable nutrients (Diener *et al.*, 2009). Moreover, BSF can reduce *Escherichia coli* loads in manure (Liu *et al.*, 2008) and the prepupae stage can be used for the production of biodiesel (Li *et al.*, 2011) or other products such as

chitin and novel antimicrobial peptides (St. Hilarie *et al.,* 2007; Waśko *et al.,* 2016; Park and Yoe, 2017).

BSF bacterial community has been investigated by Jeon *et al.,* (2011) and Zheng *et al.,* (2013). Using pyrosequencing analysis, both authors reported that the bacteria communities associated with *H. illucens* were quite unique compared to the intestinal microflora of other insects. Overall, among six bacterial phyla, the most representative were Bacteroidetes, Proteobacteria and Firmicutes. Moreover, Zheng and colleagues (2013) have shown that bacteria mediate the oviposition preference of BSF by influencing its behavioural responses. In their study, oviposition responses of gravid black soldier flies to bacteria isolated from arthropods that are competitor for nutrient sources, have significantly influence the site of choice where laid eggs, with the resulting preference of sites without the specific bacteria from its competitors, suggesting these microorganisms exert a repellent effect. The preference for oviposition was found for the genera *Providencia* sp. from a niche competitor of recent introduction in North America, where *H. illucens* had limited exposure to it. They also hypothesized that this information could be exploited for the development of approaches to disrupt and manipulate the microbiota to produce communities engineered to repel pest insects, pathogen vectors, or attract beneficial insects (Zheng *et al*., 2013). For instance, the gut microbiota can contribute to the production of pheromones that mediate the insect aggregation as shown in cockroaches (Wada-Katsumata *et al*., 2015).

Here, we aimed to investigate, by the use of a cultivation-independent approach, the variation of the BSF-associated bacterial community when exposed to three different diet conditions and along the insect development. To this aim, three different insect rearing conditions have been established, i.e. based on standard, fruit-waste derived and vegetable-waste derived diets, from which larvae, prepupae and adults have been sampled. Moreover, we also verified within the samples the presence of a shared microbiota, which could play a crucial role during insect development and adaptation to different environmental conditions (i.e. variation in the food substrate). Finally, the gut physicochemical characterization in terms of oxygen partial pressure (pO2), pH and redox potential was also performed on 4^{th} instar larvae and correlated to the variation of the bacterial community structure when insects were exposed to the different diets.

MATERIALS AND METHODS

Insects and surface sterilization procedures.

H. illucens specimens were reared at the facilities of the University of Milan, Italy. BSF larvae were fed *ad libitum* on three different diets, i.e. standard ("SD", 50% wheat germ, 30% alfa alfa as whole plant powder, 20% corn flour, to which an equal volume of water was added, Hosette, 1992), fruit waste-derived ("FW", 1/3 apples, 1/3 pears, 1/3 oranges in a overripe state) and vegetable waste-derived diet ("VW", 1/3 green bean, 1/3 cabbage, 1/3 lettuce in a overripe state) under controlled conditions (25°C, 60-65% R.H.). Microbiological analyses were performed on the insects upon a surface sterilization procedure. After a first washing with 0.1% sodium dodecyl sulphate (SDS) in 50-ml tubes, BSF individuals (larvae, prepupae and adults) were immersed in 1% sodium hypochlorite for 10 minutes, followed by 3 consecutive washings in 70% ethanol and 5 washings in sterilised distilled water. Then, the intestinal tract was dissected from 4th instar larvae and prepupae under sterile conditions, while adults were deprived of the head, legs and wings by using sterile scalpels, since their digestive tracts resulted fragile in consequence of the adult non-feeding status. For cultivation-independent analysis samples were stored in single 1.5 ml tubes containing 98% ethanol at -20°C. In case of bacterial isolation, insect guts were immediately used after dissection.

DNA extraction from insects.

Metagenomic DNA was extracted from BSF specimens using the DNeasy Blood & Tissue Kit (Qiagen, Milano, Italy). Samples stored at -20°C were centrifuged for 5 minutes at 3000 rpm and, after ethanol removal, the tissues were hydrated by adding 1 ml of sterile saline solution (0.9% NaCl). Then, samples were centrifuged for 5 minutes at 3000 rpm, the solution was removed and 180 µl of ATL buffer (provided by the kit) were added. Tissues were smashed in ATL buffer. Alternate incubations at -80°C and 70°C for 10 minutes each were performed. Then, 25 µl lysozyme (20 mg/ml) were added to the homogenate and samples were incubated at 37°C for 30 minutes in order to digest the Gram-positive walls in order to release the bacterial DNA. Last steps were performed following the manufacturer's instructions. Purity of extracted DNA was checked using a Nanodrop 1000 spectrophotometer (BioTek®, PowerWave XS2).

Determination of bacterial community structure using Automated Ribosomal Intergenic Spacer Analysis (ARISA)-PCR.

The variation of BSF bacterial communities was investigated using ARISA-PCR fingerprinting (Mapelli *et al.*, 2013). Primer pairs were ITS-F FAM (5'-GTC GTA ACA AGG TAG CCG TA-3') and ITS-R (5'-GCC AAG GCA TCC ACC-3'). Reaction mixture contained 1.50 U Taq DNA polymerase, 0.20 mM dNTP mixture, 0.30 µM of each primer, 1.5 mM $MgCl_2$ and 1 µl of DNA. The thermal protocol was the following: an initial denaturation step at 94°C for 3 minutes, followed by 30 cycles with a denaturation step at 94°C for 45 seconds, an annealing step at 55°C for 1 minute, an extension step at 72°C for 2 minutes and a final extension at 72°C for 7 minutes. PCR products were initially visualized in 1.5% agarose gel. ABI3730XL genetic analyzer was used to separate ARISA fragments by applying the internal standard 1200-LIZ (Macrogen, Korea). The output peak matrix was transferred to Microsoft Excel for the following analysis. Peaks showing a height value <50 fluorescence units were removed from the output peak matrix before statistical analyses. Each polymorphic ARISA peak was defined as a different operational taxonomic unit (OTU). To account for variability in size associated with standards, ARISA fingerprints were binned ± 1 bp from 150 to 300 bp, ± 3 bp from 300 to 500 bp and ± 10 bp >500 bp. Non-metric multidimensional scaling (nMDS) was carried out in order to explore similarities between OTUs, based on the resemblance matrix generated using Bray-Curtis similarity on the presence/absence of the OTUs within each sample. Significant differences in microbial community composition were scan by permutational analysis of variance (PERMANOVA; Anderson, 2001). Ecological diversity indices were calculated from the matrix of ARISA OTUs. The statistical analysis of the ARISA matrix were performed using PRIMER v. 6.1 (Plymouth Routines In Multivariate Ecological Research) (Clarke *et al.,* 2006), PERMANOVA+ for PRIMER routines (Anderson *et al.,* 2008) and PAST software.

High-throughput sequencing of the bacterial communities.

The bacterial community associated to the insect samples was analyzed through high-throughput sequencing of 16S rRNA gene. Libraries were established using Illumina® Nextera XT Sample Prep kit. The target region was the V3-V4 one of the 16S rRNA gene and primers 341F and 785R were used for the library preparation (Klindworth *et al*., 2013). For each sample, reactions was performed in duplicate using 1U Platinum Taq DNA polymerase High Fidelity (Invitrogen, Dubai, United Arab Emirates), 1× PCR buffer, 2.0 mM $MgCl_2$, 300 µM DNTPs and 0.3 µM of each primer. The PCR conditions were the following: an initial denaturation at 94 °C for 1 minute, followed by 25 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, 68 °C for 30 seconds, and a

last elongation step at 68 °C for 10 minutes. After amplification, the duplicate PCR products were merged and checked in 1% agarose gel. A cleaning step was performing in order to remove nucleotides and primer sequences in excess using the kit Illustra™ ExoProStar™ 1-Step (GE Healthcare Life Sciences, Saudi Arabia): 5 µl of PCR product were transferred into a 96-wells plate and added with 2 µl ExoProStar mixture. The reaction mixture was first incubated at 37°C for 15 minutes followed by 15 minutes at 80°C in order to inactivate the enzyme. PCR index was set up with the kit Illumina® Nextera XT Index (96-indexes): after enzymatic treatment, 5µl of PCR product were added with 1 U Platinum Taq DNA polymerase High Fidelity (Invitrogen, Dubai, United Arab Emirates) for each reaction. Index adapter1 and Index adapter2 were provided by Illumina®. SequalPrep™ Normalization Plate kit (Invitrogen, Dubai, United Arab Emirates) was used to clean up and normalize PCR products. Afterwards, adapters were added to samples and placed in pool in CentriVap DNA (Labconco, Kansas City, USA). Libraries of 16S rRNA gene fragments were sequencing by MiSeq system at the laboratories of King Abdullah University of Science and Technology (KAUST).

Bioinformatic analyses were analyzed by combining UPARSE (Edgar, 2013) and QIIME (Caporaso *et al*., 2010). Forward and reverse sequences were assembled through the script *join_paired_ends.py* in QIIME: this step allowed a minimum overlapping length of 50 nucleotides with a maximum dissimilarity score of about 10%. Quality check control was performed on obtained reads to filter low quality sequences: high-quality sequences (Q > 20) were cleaned from primer sequences and incorporated in a single *.fasta* file. Chimeric sequences were removed by a combination of both de-novo detection and reference-based detection on the Genome Online Database (GOLD) (Reddy *et al.*, 2014) using UPARSE. Operational taxonomic units (OTUs) were defined using a threshold of 97% similarity.

Microsensor measurements.

Oxygen partial pressure (pO_2), pH and redox potential occurring in the gut of 4th instar BSF larvae were measured using microsensors (Unisense, Aarhus, Denmark). Oxygen microsensor (OX-50) had tip diameter of 50 µm; after an overnight polarization it was calibrated in water saturated with air in the CAL 300 calibration chamber (Unisense, Aarhus, Denmark), as well as in an anoxic solution of 0.1 M sodium dithionite.

The pH microelectrode (PH-50) had a tip diameter of 50 µm and a sensitive tip length of 200 to 300 µm; it was calibrated using standard solutions at pH 4.0, 7.0 and 10.0. The redox microelectrode (RD-50) had a tip diameter of 50 µm; it was calibrated using saturated quinhydrone solutions at pH 4.0 and 7.0. In both cases, the electrode potentials were measured against Ag-AgCl reference

electrodes using a high-impedance voltmeter (Ri > 1014 Ω). The current was measured with a Unisense microsensor multimeter and recorded using SensorTracePRO software (Unisense, Aarhus, Denmark). Before measurements, a freshly dissected gut was placed on the layer of 2% (Low Melting Point) agarose prepared with Ringer's solution (7.2 g/L NaCl; 0.37 g/L KCl; 0.17 g/L $CaCl_2$, pH 7.3-7.4) and immediately covered with a second layer of 0.5% agarose prepared with Ringer's solution. Microsensors were positioned using a motorized micromanipulator (Unisense, Aarhus, Denmark). All measurements were carried out at room temperature. For each physicochemical parameter (pO_2, pH and redox potential) and alimentary regime (SD, FW and VW), seven larvae were analyzed.

RESULTS

Diet and developmental stage shaped the insect bacterial community.

In order to estimate the variation of BSF bacterial communities when insects were exposed to different diets and considering different developmental stages, a cultivation-independent approach was performed combining the use of the ARISA-PCR fingerprinting on a larger set of samples (no. 54) with the high-throughput sequencing of the 16S rRNA gene on a smaller selected subset of samples (no. 36). Thus, 6 larvae, 6 prepupae and 6 adults (consisting of 3 males and 3 females) were initially collected from each food source (SD, FW, VW) for a total of 54 samples, the metagenomic DNA was extracted from each sample and then analyzed by ARISA-PCR fingerprinting. nMDS analysis was performed on the ARISA fingerprints, showing that the two factors, i.e. the diet source and the developmental stage, influenced the bacterial community structure (Fig. 1 a-b).

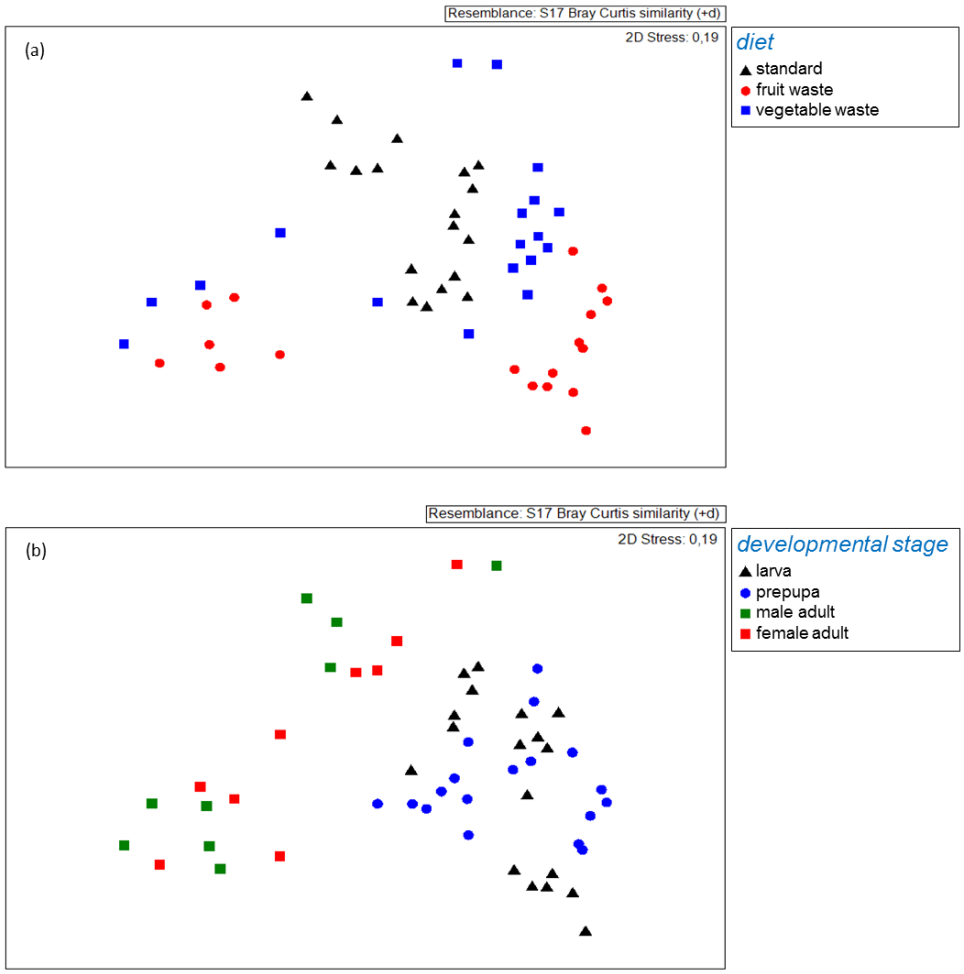

Figure 1. Non-metric multi-dimensional scaling (nMDS) of a Bray-Curtis distance matrix describing the bacterial communities in relation to the insect different diets (a) and developmental stage (b).

In particular, a statistically significant difference in the structure of the bacterial populations hosted by the insects was found considering the rearing diet (PERMANOVA, $F_{2,53}$= 5.29, P = 0.0001; Tab. 1) and the developmental stage (PERMANOVA, $F_{3,53}$= 5.19, P = 0.0001; Tab. 2). Interestingly, within the adult stage the gender (i.e. male or female) did not influence the selection of different bacterial communities (Tab. 2).

Table 1. PERMANOVA pair-wise comparisons of the bacterial community composition based on ARISA profiles considering the food source as test factor. Significant results (P < 0.05) are indicated with asterisks.

Groups	T	P(perm)	Unique perms	P(MC)
Standard, fruit waste	2.703	0.0001	9940	0.0001*
Standard, vegetable waste	2.132	0.0001	9933	0.0001*
fruit waste, vegetable waste	2.037	0.0026	9920	0.0024*

Table 2. PERMANOVA pair-wise comparisons of the bacterial community composition based on ARISA profiles considering the developmental stage as the test factor. Significant results ($P < 0.05$) are indicated with asterisks.

Groups	t	P(perm)	Unique perms	P(MC)
larvae, prepupae	1.766	0.0028	9931	0.0045*
larvae, adult male	2.7676	0.0001	9929	0.0001*
larvae, adult female	2.4716	0.0001	9923	0.0001*
prepupae, adult male	2.62	0.0001	9915	0.0001*
prepupae, adult female	2.4115	0.0001	9913	0.0001*
adult male, adult female	1.0232	0.4032	8154	0.3658

Among the 54 samples subjected to the ARISA-PCR analysis, 36 samples were randomly selected (three representatives for each developmental stage and diet source) and sequenced using high-throughput sequencing of 16S rRNA gene amplicons. After chimera removal and quality check a total of 460533 paired-end reads have been counted (14697±5164, 9039±4550 and 13439±22505 in the larvae, pupae and adults, respectively). All the samples presented Good's coverage values ranging from 0.97 to 0.99, capturing sufficient diversity with an adequate sequencing depth. A total of 262 different OTUs have been identified (53, 63 and 66 of unique OTUs in larvae, pupae and adults, respectively). Overall, we observed that juvenile specimens fed on VW hosted a bacterial community characterized by higher richness (LVW= 54 ± 5 OTU, PVW= 49 ± 11 OTU), diversity (LVW= 3.8237 ± 0.062, PVW= 3.357 ± 0.2304), evenness (LVW= 0.265 ± 0.0164, PVW= 0.2148 ± 0.025) and dominance (LVW= 0.8998 ± 0.0034, PVW= 0.8551 ± 0.0193) if compared to female and male adults (MVW= 42 ± 34 OTU, FVW= 14 ± 5 OTU; diversity MVW= 2.488 ± 1.5741, FVW= 1.9893 ± 0.927; evenness: MVW= 0.1133 ± 0.0868, FVW= 0.3109 ± 0.1164; dominance: MVW= 0.7018 ± 0.192, FVW= 0.6252 ± 0.1511) (Tab. 3). On the contrary, for the insects fed on FW we retrieved a lower richness, diversity and evenness in juvenile specimens (LVW= 11 ± 1 OTU, PVW= 18 ± 2 OTU) compared to the respective adults (richness: MVW= 36 ± 2; FVW= 45 ± 25; diversity: MVW= 2.427 ± 1.0774; FVW= 2.2987 ± 1.0775; and evenness: MVW= 0.187 ± 0.1625; FVW= 0.1487 ± 0.0956; Tab. 3). In the case of SD, we did not reported a significant variation in the alpha-diversity indices analysed passing from juvenile to adult stage (Tab. 3).

Table 3. Diversity indices estimated for the bacterial communities associated with the BSF specimens considering the Illumina dataset.

Growth stage	Diet	Richness (N. OTUS)	Evenness (e^H/S)	Diversity (Shannon H')	Dominance (Simpson λ)
Larvae	Standard	25 ± 3	0.0874 ± 0.0268	1.0606 ± 0.3188	0.3464 ± 0.1435
	Fruit	11 ± 1	0.1477 ± 0.0466	0.6936 ± 0.5045	0.2623 ± 0.2225
	Vegetable	54 ± 5	0.265 ± 0.0164	3.8237 ± 0.062	0.8998 ± 0.0034
Pupae	Standard	30 ± 6	0.1272 ± 0.0309	1.8967 ± 0.1111	0.6326 ± 0.0243
	Fruit	18 ± 2	0.1613 ± 0.0473	1.52 ± 0.2528	0.5179 ± 0.1161
	Vegetable	49 ± 11	0.2148 ± 0.025	3.357 ± 0.2304	0.8551 ± 0.0193
Adult male	Standard	25 ± 11	0.1378 ± 0.1271	1.21 ± 0.8108	0.4428 ± 0.2958
	Fruit	36 ± 2	0.187 ± 0.1625	2.427 ± 1.0774	0.612 ± 0.2335
	Vegetable	42 ± 34	0.1133 ± 0.0868	2.488 ± 1.5741	0.7018 ± 0.192
Adult female	Standard	29 ± 16	0.1593 ± 0.0371	2.036 ± 0.8937	0.5925 ± 0.2424
	Fruit	45 ± 25	0.1487 ± 0.0956	2.2987 ± 1.0775	0.6029 ± 0.1715
	Vegetable	14 ± 5	0.3109 ± 0.1164	1.9893 ± 0.927	0.6252 ± 0.1511

Principal Coordinate Analysis (PCoA) revealed a strong clustering of bacteria at the OTU level (97% identity) according to the 'Developmental Stage' (Figure 2). However, the interaction between 'Developmental Stage' and 'Feeding Condition' significantly determined the diversity of bacterial communities associated to BSF gut (PERMANOVA: df=6,28, F=3.4361, p=0.001).

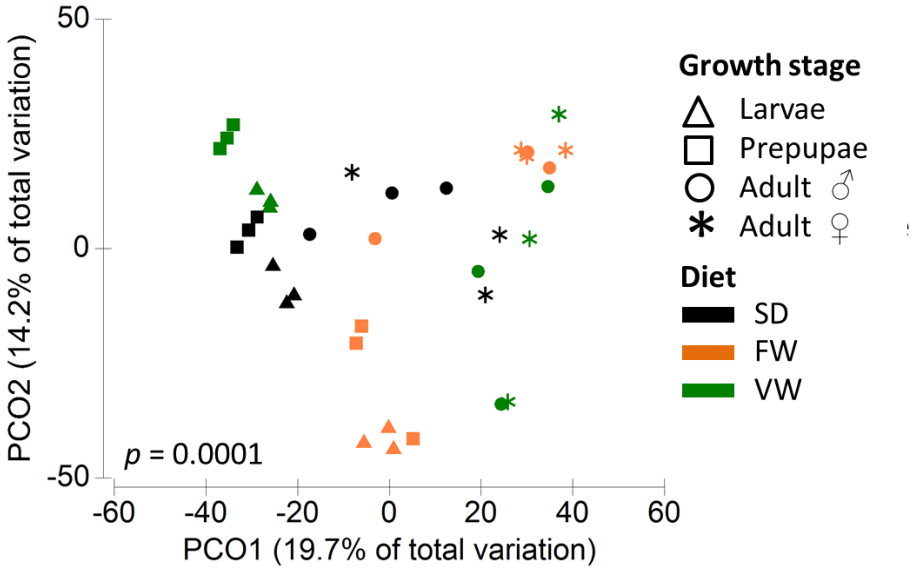

Figure 2. Principal coordinates analysis (PCoA) on the phylogenetic β-diversity matrix on *H. illucens* samples, considering the bacterial OTUs. Different geometric shapes denote the life stage while colors indicate the different diets.

Particularly, by quantifying the contribution of the individual factors to the bacterial community variations determined by PERMANOVA of Bray-Curtis, we observed that not only the two factors "growth stage" and "diet", but also their interaction contributed to the bacterial variation explaining the 15.91%, 18.92% and 29.44% of the variation, respectively (Tab. 4-5).

Table 4. PERMANOVA analysis on the 16S rRNA gene-based Illumina dataset. Main test comparison of the distance matrix generated according to OTUs distribution of bacterial communities associated to insects reared on three different diets and sampled at different growth stages using two-way PERMANOVA. Df = degree of freedom, MS = mean sum of square, F = F-statistic and p is the statistical p value. With asterisks we highlighted the statistical significant terms.

Source	d.f.	MS	F	P value
Growth stage	3	9359.9	5.6655	0.001*
Diet	2	10558	6.391	0.001*
Growth stage X Diet	6	5676.8	3.4361	0.001*

Table 5. Estimates of components of variation (PERMANOVA) between diet and growth stage, with their interaction. With asterisks we highlighted the statistical significant terms (see Table 4).

Factor	Estimate	Sq.root	%
Diet	681.02	26.096	15.91*
Growth stage	792.73	28.155	18.52*
Diet X Growth stage	1260.7	35.506	29.44*
Residual	1547.1	39.333	36.13

Taxonomic composition of the BSF bacterial communities.

Observing the taxonomic affiliation of OTUs, we found that Proteobacteria, Firmicutes and Bacteroidetes were the dominant phyla within the bacterial communities associated to *H. illucens* considering all the different diet conditions and developmental stages. On the other hand, sequences affiliated to Actinobacteria, Acidobacteria, Cyanobacteria and Fusobacteria were less represented in the microbiomes and thus considered as less abundant phyla (Fig. 3). In particular, the phylum Bacteroidetes was the most abundant one in the larval and prepupal microbiomes, whereas adult individuals, including both male and female, were dominated by Proteobacteria. Actinobacteria sequences were mainly retrieved from adult individuals (Fig. 3).

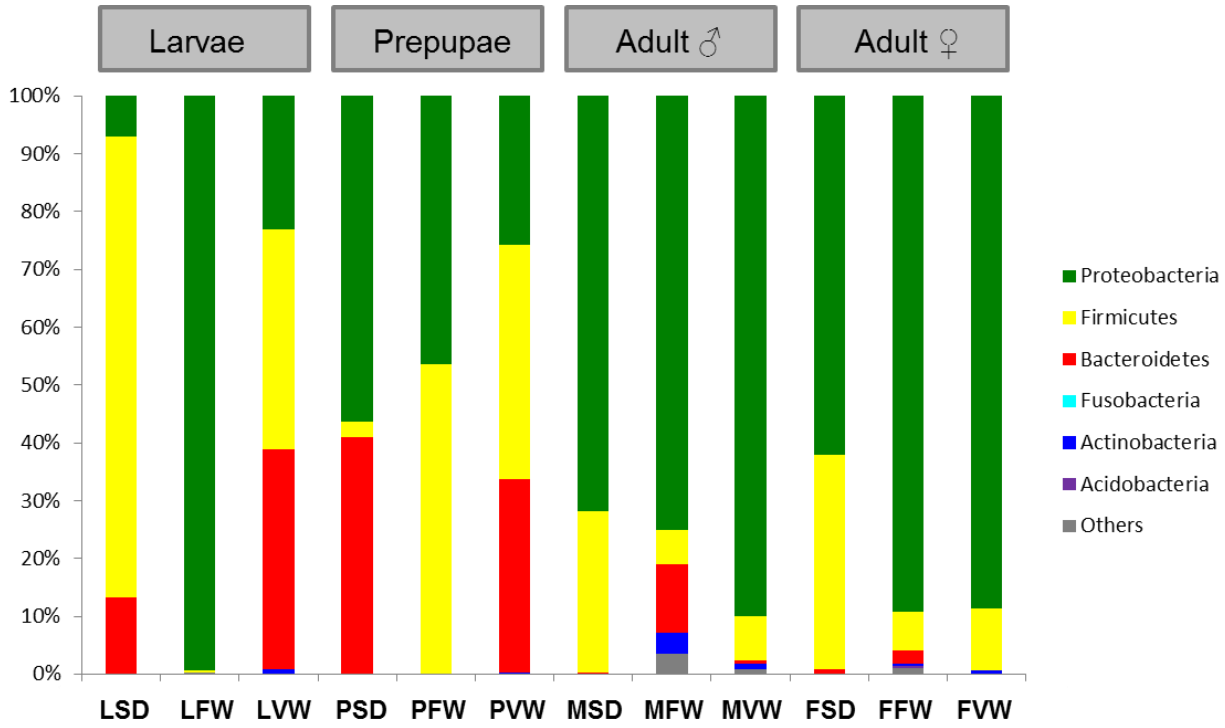

Figure 3. Relative abundance of the bacterial phyla inhabiting BSF intestinal tract. Sample codes are like this: first letter of codes refers to the fly stage (L: larva; P: prepupa; M: male adult; F: female adult); second and third letter of codes refers to feeding system (SD: standard diet; FW: fruit-waste derived diet; VW: vegetable-waste derived diet).

At the order level, five main taxa were found to inhabit BSF bacterial communities, i.e. Enterobacteriales, Lactobacillales, Bacteroidales, Clostridiales and Pseudomonadales (Fig. 4). The most diverse gut environments were observed for the larval and prepupal stages fed with vegetable-waste derived diet, which mainly showed the presence of Bacteroidales (LVW= 37.7%, PVW= 33.4%), Clostridiales (LVW= 27.6%, PVW= 34.0%), Enterobacteriales (LVW= 13.9%, PVW= 1.6%), Lactobacillales (LVW= 9.0%, PVW= 4.1%), Pseudomonadales (LVW= 4.8%, PVW= 0.1%) and Xanthomonadales (LVW= 4.5%, PVW= 21.4%). In the adult stage we found that individuals from fruit-waste feeding conditions had the highest bacterial diversity, with males harboring 24 bacterial orders, whereas the females hosted bacteria of 21 orders, of which 16 were shared between them. In female adults, 15.6% of sequences were classified as Rhodospiralales that were not found in male individuals.

The Illumina dataset was then screened to find the shared OTUs within the bacterial communities associated to the different BSF specimens; results were represented by Venn diagrams in Fig. 5 and 6 (Tab. 8-10).

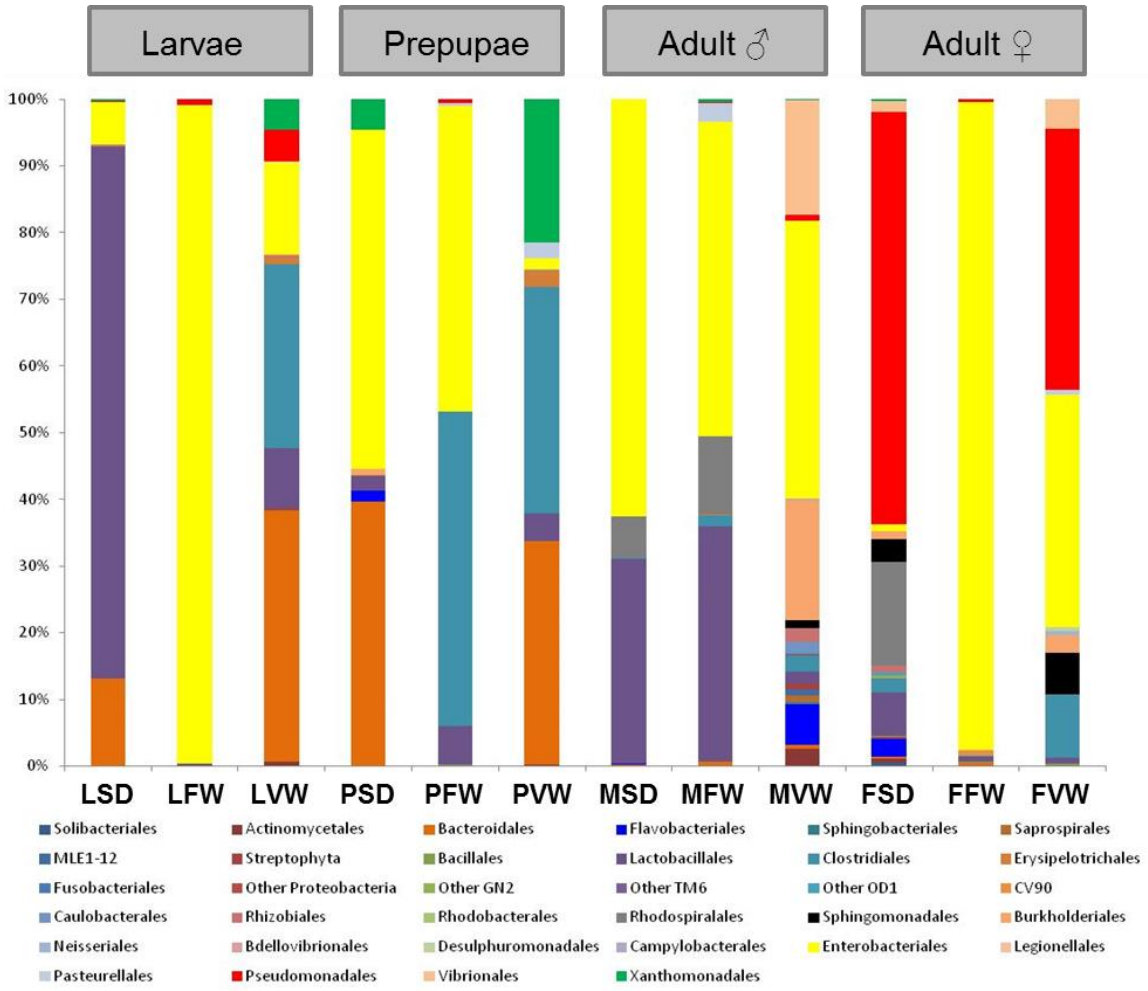

Figure 4. Relative abundance of the bacterial orders inhabiting BSF intestinal tract. For the sample codes see Fig. 3

Table 8. Shared OTUs of *H. illucens* considering a) larval, b) prepupal and c) adult stages.

a) OTU ID	Taxonomy	Rel. Abun. (%)	SD (%)	FW (%)	VW (%)
0112	*Streptococcus* sp.	0.02	0.01	0.02	0.02
0002	Enterobacteriaceae	30.40	0.20	78.53	0.66
0005	*Klebsiella* sp.	0.48	0.08	0.01	1.71
0008	*Enterococcus* sp.	29.02	72.75	0.01	4.15
0080	Enterobacteriaceae	0.38	0.05	0.01	1.39
0009	*Providencia* sp.	11.72	4.27	20.20	8.94
Total shared OTUs = 6		72.02	83.50	98.77	16.87
b) OTU ID	**Taxonomy**	**Rel. Abun. (%)**	**SD (%)**	**FW (%)**	**VW (%)**
0001	*Morganella* sp.	1.88	5.32	0.02	0.21
0108	*Vagococcus* sp.	0.90	0.04	1.88	0.08
0005	*Klebsiella* sp.	9.10	0.02	19.60	0.06
0009	*Providencia* sp.	21.39	45.09	12.34	0.73
Total shared OTUs = 4		33.27	50.46	33.84	1.08
c) OTU ID	**Taxonomy**	**Rel. Abun. (%)**	**SD (%)**	**FW (%)**	**VW (%)**
0013	Enterococcaceae	4.79	16.17	0.2899	0.386
0135	*Acinetobacter* sp.	0.03	0.01	0.0994	0.0078
0019	*Leuconostoc* sp.	1.39	4.88	0.0966	0.0111
0002	Enterobacteriaceae	4.21	0.039	10.23	40.736
0021	Leuconostocaceae	0.69	1.804	0.7041	0.0843
0026	Comamonadaceae	0.76	0.016	3.5646	0.0377
0005	*Klebsiella* sp.	7.69	0.87	0.2319	14.412
0080	Enterobacteriaceae	5.16	2.125	3.0952	7.636
Total shared OTUs = 8		24.72	25.91	18.312	26.649

Table 9. Shared OTUs of *H. illucens* considering the diet: a) SD, b) VW and c) FW.

a) OTU ID	Taxonomy	Rel. Abun. (%)	LSD (%)	PSD (%)	ASD (%)
0001	*Morganella* sp.	25.55	0.916	3.024	60.568
0011	*Dysgonomonas* sp.	5.02	13.251	0.010	0.022
0112	*Streptococcus* sp.	0.02	0.013	0.039	0.004
0013	Enterococcaceae	6.46	0.115	0.008	16.169
0114	*Morganella* sp.	0.10	0.006	0.006	0.236
0017	Xanthomonadaceae	1.25	0.339	2.759	0.079
0005	*Klebsiella* sp.	0.38	0.085	0.010	0.870
0008	*Enterococcus* sp.	30.12	78.529	0.960	0.081
0009	*Providencia* sp.	11.94	4.609	25.638	0.079
Total shared OTUs=9		80.84	97.863	32.454	78.109
b) OTU ID	**Taxonomy**	**Rel. Abun. (%)**	**LFW (%)**	**PFW (%)**	**AFW (%)**
0002	Enterobacteriaceae	35.99	78.53	3.52	10.23
0005	*Klebsiella* sp.	6.00	0.01	19.60	0.23
0080	Enterobacteriaceae	7.41	0.01	21.54	3.10
Total shared OTU=3		49.40	78.55	44.66	13.56
c) OTU ID	**Taxonomy**	**Rel. Abun. (%)**	**LVW (%)**	**PVW (%)**	**AVW (%)**
0135	*Acinetobacter* sp.	0.01	0.004	0.011	0.014
0002	Enterobacteriaceae	2.79	0.486	0.029	7.468
0005	*Klebsiella* sp.	9.70	1.249	0.036	26.423

0080	Enterobacteriaceae	5.26	1.016	0.021	13.999
0009	*Providencia* sp.	2.28	6.539	0.408	0.028
Total shared OTU=5		20.04	9.294	0.504	47.933

L: larva; P: pupa; A: adult.

To perform this analysis, only OTUs present in 2/3 of the samples from the same diet or developmental stage were considered as shared OTUs. Data revealed that the relative abundance of the shared OTUs decreased during BSF development, passing from 72% in larvae, to 33% in prepupae and 25% in adults, respectively. This decreasing trend could be ascribed to physiological changes occurring in holometabolous insects like *H. illucens*, for which, after metamorphosis, the imago possesses an atrophied intestine with likely a smaller community (Tab. 8).

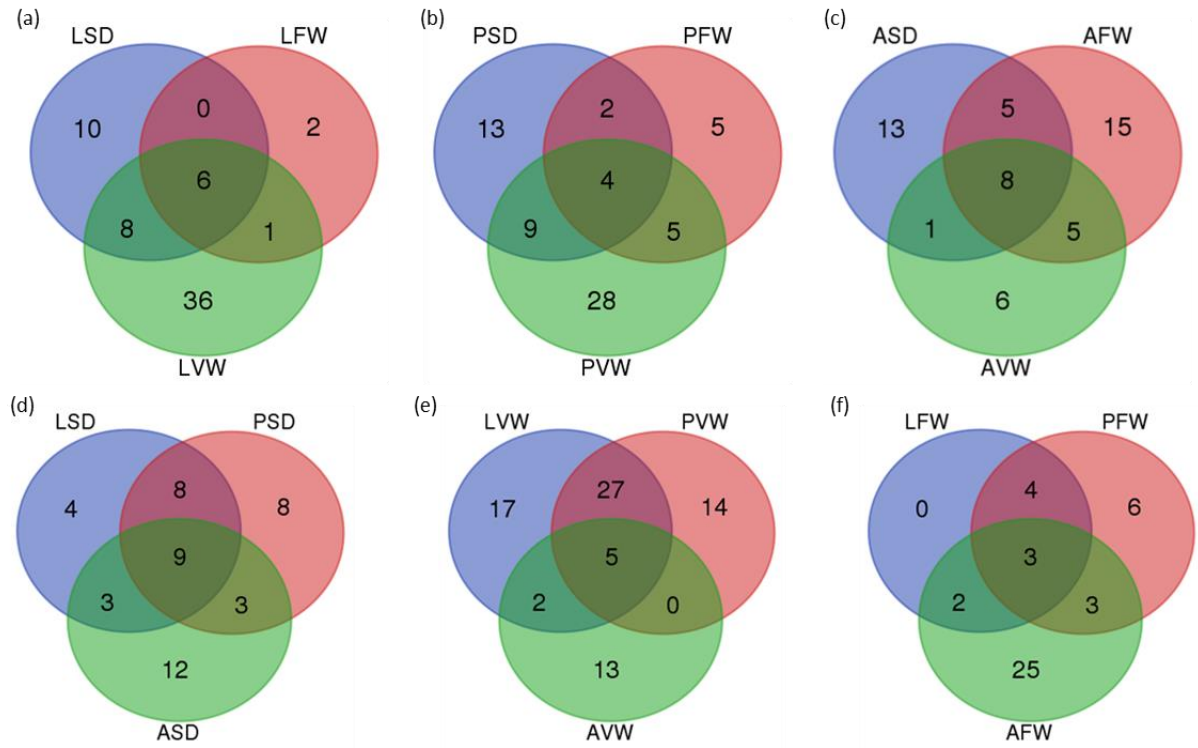

Figure 5. Venn diagrams of shared OTUs among insect individuals, considering the larval (a), prepupal (b) and adult (c) stage and in function of the food substrates: standard (d), fruit-waste derived (e) and vegetable-waste derived diets (f).

The shared OTUs showed a high relative abundance considering the total OTUs, except in the case of larvae and prepupae reared on the vegetable-waste derived diet that presented the lower abundance (Tab. 8). Considering all the stages grown on the same diet, the individuals reared on standard diet retained the highest number of the same shared OTUs (Tab. 9). Finally, we found that only 10 out of 262 OTUs were shared across all the developmental stages and diet sources (Fig. 6).

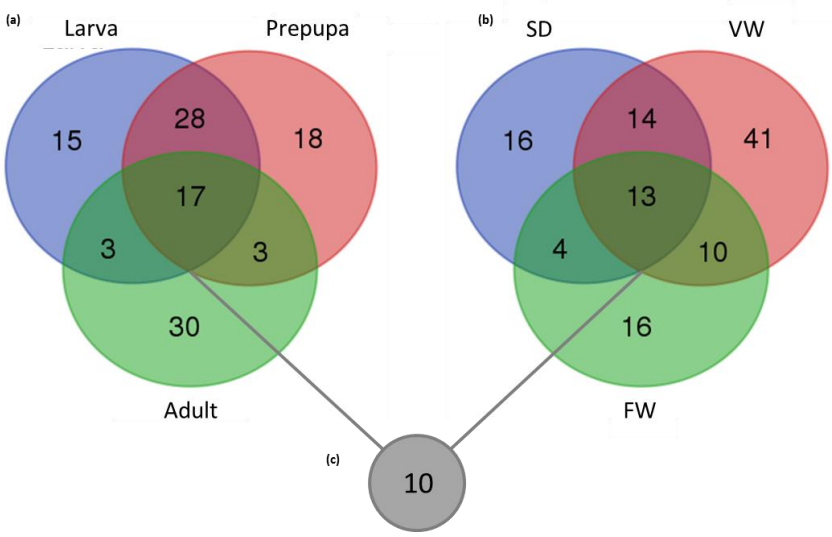

Figure 6. Venn diagrams showing shared OTUs according to the growth stages (a) and diet conditions (b). In (a), 17 OTUs were shared by the three developmental stages. In (b), 13 OTUs were shared by three feeding conditions. In (c) Number of OTUs with constant presence in all the analyzed samples.

These 10 shared OTUs, which were assigned to two phyla, four family and seven genera (Tab. 10), showed strong variations in abundance among diets and developmental stages (Tab. 8-9). Bacteria affiliated to these genera were also found in previous works dedicated to the characterization of the bacterial community associated to *H. illucens* (Jeon *et al.* 2011; Zheng *et al.* 2013).

Evaluation of physicochemical conditions of the gut compartments.

BSF gut is composed of several morphologically different regions, i.e. crop, foregut, midgut and hindgut. Dissected intestinal tracts were prepared in gel-embedded chambers in order to measure the pO_2, pH and redox potential (E_h) values in each different gut portion. Measurements were collected from several individuals reared on three different diet sources (standard, fruit-waste derived and vegetable waste derived ones). In each case, once the tip of the sensor penetrated the gut wall, oxygen concentrations decreased rapidly, until reaching the lumen center then increased, highlighting a radial distribution of oxygen within the organ diagonal section. The faint increase in the oxygen content of the distal parts of the guts may reflect the oxygen possibility to diffuse in the organ or suggest a slightly different metabolic rates affected by diet and microbial composition. The oxygen levels were maintained at values close to 0 μmol/L in the crop for each feeding condition, whereas more variable values were recorded in the foregut, midgut and hindgut, with a significant difference of oxygen content for the midgut of larvae reared on SD compared with larvae reared on VW ($p=0.0319$). A value of 0 μmol/L corresponding to 0 kPa was documented for SD larvae and an average of 24.20 μmol/L corresponding to 1.77 kPa for VW larvae (Fig. 7a). Despite traces of oxygen were found in the center of some gut compartments , the highest value recorded was not above 2.5% of oxygen concentration.

The pH of the gut contents was found to be highly variable along the gut. The intestinal pH of the crop was 7.4 in case of SD and VW, whereas in FW was found slightly higher with a value of 7.8 ($p=0.043$). The lower pH values were found to be similar in the foregut of larvae on the three feeding conditions (pH 5.5 ± 0.4349, n = 21), suggesting that in this compartment the diet source had not an influence. In midgut and hindgut regions the pH increased, with the highest values found in the midgut of larvae reared on VW (pH 9.3 ± 0.4475, n = 7), whereas in case of the other diet conditions the values were significantly lower, with slightly acidic conditions in larvae fed on SD (Fig. 7b). Overall, the distal parts of BSF intestinal tract showed an influence of the diet source, with the most significant differences in the midgut and hindgut regions. The highest differences were found between the midgut and hindgut tracts between larvae fed on SD compared with those fed on VW ($p= 0.0002$ for both cases).

Finally, positive redox potentials were measured all along the anoxic and anoxic-microaerophilic gut portions, with average values between 200 and 300 mV (Fig. 7c). A statistical difference was reported for the midgut of larvae reared on SD and FW diet ($p=0.0277$).

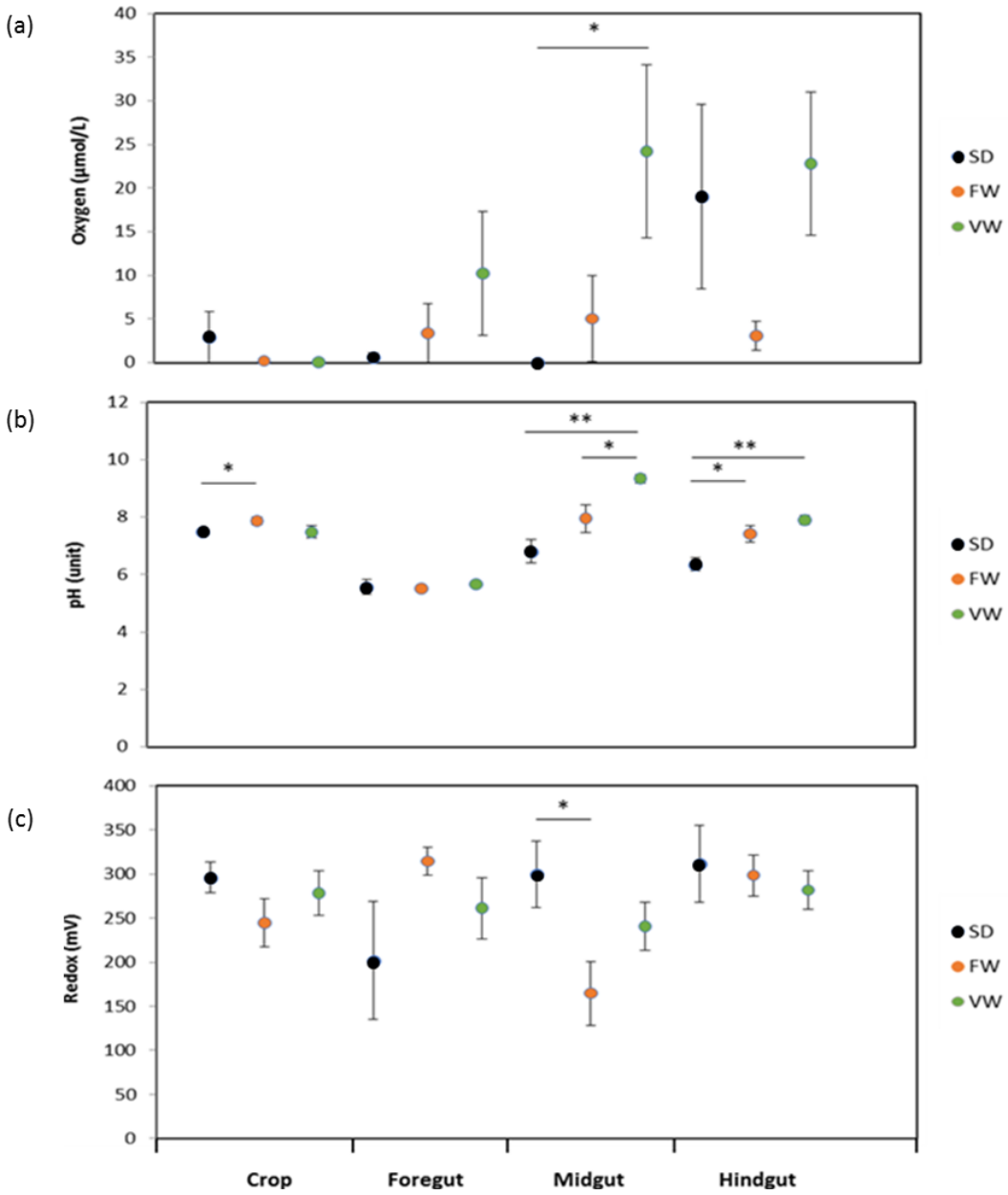

Figure 7. Microsensor profiles of physicochemical conditions in the gut of *H. illucens* larvae. Axial profiles of oxygen partial pressure (a), pH (b) and redox potential (c) along gut compartments of larvae reared on standard diet (SD), fruit-waste (FW) and vegetable-waste (WV). All deviations are given as standard error of the mean.

DISCUSSION

BSF is known to feed on a wide variety of organic matter ranging from agro-industrial waste to livestock manure (Diener *et al.,* 2009; Nguyen *et al.,* 2015). The advantage to use this species compared to many other fly species is that it is not recognize as a pest (Bradley *et al.,* 1984). A large number of reports have identified the important functions exerted by the intestinal microorganisms for their hosts, ranging from humans to nematodes and insects. The microorganisms that inhabit the animal gut plays not only significant roles in digestion, host physiology, development and immune system, but also prevents colonisation of pathogens and is therefore involved in maintaining the host health (Qin *et al.,* 2010; Roh *et al.,* 2008; Warnecke *et al.,* 2007). Different factors, e.g. the host diet and developmental stage, have been shown to modulate the composition and structure of the insect gut microbiota (Montagna *et al.,* 2015a; Vacchini *et al.,* 2017). To investigate the difference and dynamics of the microbial communities associated to BSF in different life stages and reared on their diets, we compared data obtained from the application of different molecular techniques, i.e. the ARISA-PCR fingerprinting and the high-throughput sequencing of 16S rRNA gene. Our data clearly indicated that diet source and developmental stage are driving forces that shaped the bacterial community structure and composition (Fig. 1-2). To our knowledge, this is the first work in which ARISA-PCR fingerprinting analysis is used to estimate changes in structure and composition of the bacterial community associated with insects. ARISA fingerprinting allowed us to cluster the different samples on the basis of the diet and developmental stage in insects. To gain a greater appreciation of the bacterial community diversity present in insects, we also explored the taxonomy composition by 16S rRNA gene barcoding. Interestingly, it was not surprisingly to have found an abundance of plant-soil associated bacteria with BSF commensals, as well as members of the Enterobacteriaceae family that are widely associated with the gut environment of several arthropods, especially in detritivores insects, like BSF.

Insects reared on VW had much more bacterial complexity at the genus level than the individuals fed on SD and FW (Fig. 3). In the prepupae reared on VW, we found sequences affiliated to the genus *Wohlfahrtiimonas*, a genus belonging to the Gammaproteobacteria class. Members of this genus were isolated for the first time from the gut of *Wohlfartia magnifica* (Diptera: Sarcophagidae) larvae and classified as *Wohlfahrtiimonas chitiniclastica* by Tóth *et al.,* (2008). Recently, an additional species in this genus has been isolated from the gut of *H. illucens* for which the name of *W. larvae* has been proposed (Lee *et al.*, 2014). Both these two species have been reported to have strong chitinase activity (Tóth *et al.,* 2008). As shown in table 3, larvae and prepupae from VW had

the highest OTU number and Shannon indices, with few dominant taxa, as indicated by Evenness index. On contrary, larvae reared on standard diet exhibited the lowest Evenness index, suggesting a more even distribution of the bacterial taxa, in particular by Firmicutes (Fig. 4). These results are in agreement with those performed by Jeon *et al.,* (2011) and Zheng *et al*., (2013): by using 16S rRNA gene pyrosequencing, the authors described the bacterial diversity variation of BSF individuals when sampled at different life stages or fed with three different diets. Differently, in the present study we considered both factors as determinant ones in shaping the bacterial community associated to the insects. In particular, Jeon and colleagues (2011) analysed the gut bacterial community of *H. illucens* larvae fed on three different food sources, showing that it was mainly composed of four phyla with different distributions among diets (Jeon *et al.,* 2011). They identified the same phyla that we reported in our survey with different proportions within the larval stages examined. The influence of diet as environmental factor that selects and shapes the intestinal microbiota is a well-known described phenomenon in the scientific literature (Breznak and Brune, 1994; Jeon *et al*., 2011). Our work supports the previous research and expands the findings to different food sources. Moreover, we also found sequences that affiliated to bacterial taxa found by Zheng *et al.* (2013) and we confirmed their previous results according to which the bacterial composition shifted within the insect life cycle (Zheng *et al.*, 2013).

To date, there is little information documenting the physicochemical conditions occurring in the insect gut in combination with the high-resolution profile of the associated bacterial microbiota, with no reports about *H. illucens*. In studies on mammalian systems, the intestinal pH and redox status have showed important physiological effects on Ca^{2+} availability and on the composition of the gut microbial community (Millon *et al.,* 2016). It has also been reported that the gut microbiota also causes both alteration in pH and redox potential reflecting in the microbial activity (Zheng *et al.,* 2017). In this work, microsensors and microelectrodes were used to measure the luminal oxygen level, redox potential and pH within the different gut compartments of *H. illucens* larvae fed on the three different organic matrices to evaluate the influence of the diet regime on the gut physicochemical parameters. The crop, foregut and midgut were anoxic, whereas a slight increase in the oxygen content was found in the center of the rectum consistently for each considered diet condition. The detected trace of oxygen in the distal part of guts remains in any case under 2,5% of concentration, resulting in an anaerobic or microaerophilic niche. Anoxic gut regions with a positive redox potential have been reported in the gut of several other insects (Šustr *et al.,* 2014; Köhler *et al.,* 2012). The pH values differed considerably between the gut compartments: these are also influenced by the diet conditions especially considering the portions of midgut and hindgut.

We suggested that the pH heterogeneity of the larval gut can be primary reflected in the diversity of the gut microbiota.

This is the first comprehensive analysis of the digestive tract of the food-waste reducing insect, *H. illucens*, which combines a microsensor-based study of the physicochemical gut conditions with a highly resolved analysis of the bacterial community, considering both the diet source and developmental stage as driving forces that shape the microbial community structure and composition.

CONCLUSION

This work contributes to the knowledge of the bacterial community associated to *H. illucens* using cultivation-independent techniques. Our results revealed that the diet source and developmental stage are driving forces that select and shape the microbial consortia associated to BSF. We found that under different diet regimes and during different life stages, a stable core of few OTUs is maintained with changes in abundance. Future research should take into account these findings along the path to identify and characterize the presence of a core microbiome in this animal. Using microsensors and microelectrodes we measured the oxygen level, redox potential and pH within the different gut compartments, characterizing the gut microenvironment of BSF larvae when reared on three different organic substrates. Prior to this survey, no information about the physicochemical parameters occurring in the gut of BSF larvae was available. In conclusion, the digestive tract of BSF harbors a complex bacterial community that is influenced by diet and developmental stage and shows variation of the physicochemical conditions due to diet modification. The combination of different techniques for the characterization of the microbial community and the physicochemical parameters existing in the gut microenvironment provides useful information for future investigation of the microbial interactions with the host.

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Chapter 4

Contribution of the cultivable gut bacterial community of *Hermetia illucens* to the host physiology

ABSTRACT

The larvae of black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomydae), are used to convert organic waste into high valuable products. Insect biology and physiology are primary influenced by the gut microbiota which is involved in many aspects from metabolism and immune system homeostasis, to development and reproduction. However, the diversity and function of the intestinal microbiota in the BSF gut remain largely unknown. This work describes procedures used to isolate pure strains potentially involved in the digestion of food within the intestinal tract of *H. illucens*. A total of 193 bacterial strains were isolated from the dissected gut of BSF larvae reared on standard diet using selective and enrichment media. 16S rRNA gene identification showed that most of the bacteria constituting the cultivable gut community can typically be found in soil, plants and the intestinal tract of insects. Furthermore, the hydrolytic profile of the bacterial collection was assessed in order to evaluate the potential metabolic contribution provided to the host and select candidate strains for augmentation trials. We tested the hypothesis that the addition of the two strains, *Bacillus licheniformis* HI169 and *Stenotrophomonas maltophilia* HI121, isolated form the gut and selected for their complementary metabolic activities, could improve the performance of BSF when reared on unbalanced food source. Data showed a weight increase for larvae reared on fruit waste and augmented with the combination of the selected bacterial strains, following three consecutive treatments. In conclusion, data suggest that commensal bacteria could provide metabolic contribution and the administration of selected indigenous strains, with complementary metabolic capabilities, can influence the host performance.

INTRODUCTION

In the recent years a great interest has been directed towards the idea of exploiting insects to recycle the organic waste into insect biomass, mainly for the production of feed, food and fuel (Makkar *et al.,* 2014; Van Huis *et al.,* 2013 Rumpold and Schlüter, 2013; Li *et al.,* 2011). The black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae), is a promising candidate for waste management and insect biomass production intended as an alternative source of protein. It is regarded as a non pest insect, native of the tropical and warm temperate zone of America, and now it is present in many countries around the world (Tomberlin *et al.,* 2002; Newton *et al.,* 2005; Green and Popa, 2012). This saprophagous insect can consume a wide range of organic material, ranging from food waste to animal remains and manures (Nguyen *et al.,* 2015b; Tomberlin *et al.,* 2005; Sheppard *et al.,* 1994). Cordell *et al*. (2009) estimated that only a small proportion of the nutrients returns into the food cycle from the proportion of organic waste generated in high-income countries. It is well known that bacteria can degrade plant wastes (Miki *et al.,* 2010), animal remains (Burkepile *et al.,* 2006; Barnes *et al.,* 2010), as well as food waste; they are associated with saprophagous animals which also participate to the recycling process of the organic matters. It was shown that BSF larvae can reduce the house fly (*Musca domestica*), populations by 94-100% (Bradley and Sheppard, 1984) and, unlike the common fly, they also reduce *Escherichia coli* and *Salmonella spp.* populations (Liu *et al.,* 2008; Lalander *et al.,* 2015).

Data on the microbial communities associated to BSF are still limited. By the use of high-throughput sequencing (HTS) on the 16S rRNA gene the microbiota associated to this insect was investigated considering different host developmental stages and feeding conditions (Zheng et al., 2013; Jeon *et al.,* 2011). These studies highlighted that both the diet source and life stages directly influenced the host bacterial diversity (Kwong and Moran, 2016; Engel and Moran, 2013; Wong *et al.,* 2013). Zheng *et al.,* (2013) also reported that specific bacteria mediate the oviposition preference of gravid BSF females (Zheng *et al., 2013*), suggesting a potential exploitation of the strains in insect rearings with biotechnological applications.

The positive influence of an indigenous strain of *Bacillus subtilis* on the development of BSF larvae was demonstrated when insects were fed with poultry manure augmented with the strain: data showed, in fact, an enhanced larval weight by 30% and a reduced development time up to 10% in the bacterized insects compared to the non-bacterized ones (Yu *et al.,* 2011). Moreover, since BSF is employed in the conversion of the organic waste for the production of biodiesel, Zheng and colleagues (2012) established a co-conversion strategy using BSF larvae assisted by a commercial formulation of microbes and enzymes to enhance the conversion rate of waste enriched in

lignocellulosic materials. Finally, by isolation Jeon *et al.* (2011) characterized a bacterial collection obtained from BSF to investigate the isolate hydrolytic profile, in order to evaluate the potential contribution provided to the host in the food waste-reducing activity. In the present work, we established a bacterial collection from BSF larvae reared on a standard diet and we evaluated the contribution of the bacterial commensals to the host metabolism through *in vitro* characterization of the isolate hydrolytic activities potentially involved in diet component breakdown and nutrient supplementation. Furthermore, based on the results of isolate hydrolytic profiles, two bacterial strains, *Bacillus licheniformis* HI169 and *Stenotrophomonas maltophilia* HI121, were selected and added (individually and in combination) into nonsterile fruit-waste diet in order to investigate their influence on the host performance.

MATERIALS AND METHODS

Insects.

H. illucens individuals were reared at the facilities of the University of Milan, Italy. BSF larvae were fed *ad libitum* on standard diet (SD) (wheat germ 50%, alfa alfa 30%, corn flour 20% to which is added an equal volume of water according to Hosette, 1992) under controlled conditions (25°C, 60-65% R.H.). In order to exclude the outsider microorganisms, the exterior surface of BSFs was washed according to the following protocol: after a first washing in a 50-ml tube of 0.1% SDS for 5 minutes, larvae were washed in 1% sodium hypochlorite for 10 minutes, then in 70% ethanol for three times, and finally washed in sterilised distilled water for five times. After the exterior surface sterilization, intestinal tract dissection was performed from larvae of 4^{th} instar under sterile condition.

Bacterial isolation.

After dissection, guts were homogenated in 900 µl of 0.9% NaCl, which were used to inoculate liquid enrichment media in order to select uricolytic and cellulolytic strains. Particularly, we used 3 different enrichment media: i) Enrichment Medium (EM) (0.8% uric acid; 0.05% KH_2PO_4; 0.2% K_2HPO_4; 0.01% NaCl; 0.01% $MgSO_4 \cdot 7H_2O$; 0.01% $CaCl_2 \cdot 2H_2O$; pH 7.0 (Gosh and Sarkar, 2014)); ii) CA medium (0.25%$NaNO_3$, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% NaCl, 0.01% $CaCl_2 \cdot 6H_2O$ and Filter paper (Whatman filter paper no. 1, two discs of 2.00 cm^2 per 30 ml)); iii) CMC medium (0.25% $NaNO_3$, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% NaCl, 0.01% $CaCl_2 \cdot 6H_2O$ and 0.2% carboxymethylcellulose (CMC) (Gupta *et al.*, 2012)). Liquid media were then incubated at 30°C in shaking conditions. Subcultures were made every 7 days, for three times,

and finally plated onto Basal-Trace (BT) solid medium (0.3% uric acid, 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.01% $MgSO_4$ $7H_2O$, 0.01% NaCl, 0.01% $CaCl_2$, 1% (v/v) trace element solution (5.0% $FeSO_4$ $7H_2O$, 5% $CuSO_4$ $7H_2O$), pH 7.0, agar 1.5%) in case of the culture from Enrichment medium (EM) (Ghosh and Sarkar, 2014), whereas CA and CMC liquid media were plated on CMC agar media (Gupta *et al.,* 2012). Moreover, serial dilutions (0.1 ml) of the gut homogenates were spread on the surface of different types of plates. In particular, we used i) basal medium (0.1% $[NH_4]NO_3$; 0.1% yeast extract; 50 ml standard salt solution; 1 ml trace element solution and 1.5% agar and final pH 7.0) added with 0.5% Avicel (Sigma-Aldrich) or 0.5% CMC with 0.1% Congo-red (Sigma-Aldrich) for differential isolation of exocellulolytic or endo-cellulolytic, respectively (Ventorino *et al.,* 2015); ii) casein agar (1.5% peptone from casein; 0.5% soy peptone; 0.5% NaCl; 1.5% agar; pH 7.3 ± 0.2); iii) pectin agar (0.4% $[NH_4]SO_4$; 0.01% NaCl; 0.01% $MgSO_4$, $7H_2O$; 0.01% $CaCl_2$ $2H_2O$; 0.05% yeast extract; 0.0052% Fe(III)-citrate; 500 ml potassium phosphate buffer (50% K_2HPO_4 1M + 50% KH_2PO_4 1M); 0.5% pectin; agar 1.5% pH adjusted to 7.0); iv) chitin agar (0.25% $NaNO_3$; 0.2% K_2HPO_4; 0.02% $MgSO_4$, $7H_2O$; 0.02% NaCl; 0.01% $CaCl_2$ $2H_2O$; 0.5% chitin from crab shells; 1.5% agar pH 6.8-7.2); v) nutrient agar-uric acid (NA-UA) medium (0.5% gelatin peptone, 0.3% beef extract, 0.2% NaCl, 0.3% uric acid, pH 7.0, agar 1.5% (Ghosh and Sarkar, 2014)). When colonies appeared on the agar plates, they were picked up and streaked on the same media used for the isolation until pure strains were obtained. The bacterial collection was then conserved in 15% glycerol solution at -80°C.

Bacterial identification.

Total DNA from each isolate was extracted by boiling protocol or using phenol-chloroform DNA extraction protocol in case of failure of boiling lysis (Sambrook *et al.,* 1989). The bacterial collection was dereplicated by ITS-PCR fingerprinting (Daffonchio *et al.,* 1998) and representatives of each ITS-group were identified by near-full lenght 16S rRNA gene sequencing and alignment according to Altshul *et al.,* 1990. Primer pairs sequences for ITS-PCR were: ITS-F (*forward*): 5'-GCC AAG GCA TCC AAC-3' and ITS-R (*reverse*): 5'-GTC GTA ACA AGG TAG CCG TA-3'. Reactions were carried out in 25 µl volume and contained: 1 U Taq polymerase, 0.20 mM dNTP mixture, 0.30 µM of each primer, 1.50 mM $MgCl_2$ and 1 µl of total DNA. If necessary, DNA was properly diluted. Cycling conditions used to amplify the ITS regions were as follows: initial denaturation for 4 minutes at 94°C, 30 cycles with 45 seconds at 94°C, 1 minute at 55°C, 2 minutes at 72°C and a final extension of 10 minutes at 72°C. For each ITS group two candidates were selected and the 16S rRNA gene was amplified using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3').

Reaction mixture was conducted in 50 µl of final volume, containing 1.2 U Taq polymerase, 0.2 mM dNTP mixture, 0.3 µM of each primer, 1.5 mM $MgCl_2$ and 2 µl of total DNA. PCR thermal protocol was as follows: 5 minutes at 94°C, 35 cycles with 45 seconds at 94°C, 1 minute at 55°C, 1 minute at 72°C and a final step of 10 minutes at 72°. PCR fragments were sequenced at Macrogen (South Korea) and sequences were then aligned against the National Center for Biotechnology Information (NCBI) database using BLASTn (Altschul *et al*., 1990; Johnson *et al*., 2008).

Screening of the hydrolytic activities and exopolysaccharide (EPS) production by bacterial isolates.

For amylase-screening, bacterial cultures were spotted onto nutrient broth (NB) agar plate supplemented with starch (1%) and then incubated at 30°C for 48 hours. After incubation, plates were flooded with 1% Lugol's iodine solution (Jacob and Gerstein, 1960) to identify extra-cellular amylase activity.

Cellulase-screening was performed as described by Ventorino *et al.*, (2015) using a medium containing 0.1% $[NH_4]NO_3$, 0.1% yeast extract, 50 ml standard salt solution, 1 ml trace element solution, 0.5% CMC and 1.5% agar at pH 7.

Pectinase-screening medium contained 0.67% Yeast Nitrogen Base, 1.0% polygalacturonic acid and 1.5% agar at pH 7.0 ± 0.2 (Park *et al.*, 2007).

Esterase activity was evaluated using a medium composed of 1.0% peptone, 0.5% NaCl, 0.01% $CaCl_2{\cdot}2H_2O$, 10 ml tween 80 and 2.0% agar at pH 7.4 ± 0.2 (Mazzucotelli *et al.*, 2013). A white precipitate formation around the colonies, resulting from the deposition of crystals of calcium salt, indicated the solubilization of fatty acids due to the esterase activity.

Esterase/lipase activity was detected on tributyrin agar medium which contained 0.8% NB, 10 ml tributyrin, 4 ml tween 20 and 1.5% agar at pH 7.5 ± 0.2. Tributyrin agar plates with spotted isolates were incubated for 72 hours at 30°C. The clear zone of hydrolysis is indicative of either esterase and/or lipase activity according to Gupta *et al.* (2003).

True lipase activity was screened by rhodamine oil agar (ROA) medium containing 0.8% NB, 0.4% NaCl, 3.125% olive oil, 10 ml rhodamine B (1 mg/mL solution) and 2% agar at pH 7, following the protocol described by Kumar *et al.* (2012).

Extracellular protease activity was assessed using milk agar medium composed of 0.5% pancreatic digest of casein, 0.1% glucose, 0.25% yeast extract, 3.5% skim powder milk and 1.5% agar (Jeon *et al.*, 2011 - modified). Plate were examined after 72 hours of incubation at 30°C. The appearance of a clear zone around spotted isolates indicated the production of extracellular protease. Ammonia production was evaluated as described by Cappuccino and Sherman (1992).

To detect urea degradation, isolates were inoculated in tryptic soy broth (TSB) liquid medium and incubated overnight at 30°C in shacking condition; 0.5 ml were then transferred in 1.5 ml tube and washed twice with 0.9% NaCl (5 minutes, 4500 rpm, room temperature) to remove the residual growing medium. Pellet was resuspended with 470 µl of solution B (0.01% KH_2PO_4, 0.01% K_2HPO_4, 0.05% NaCl, 0.013% $NiCl_2$, 1 mL phenol red 0.2% and 100 mL dH_2O) and 30 µl of solution A (0.2% urea, 2 mL ethanol 95% and 4 mL dH_2O). Uric acid breakdown was screened observing the formation of clear haloes around the isolates spotted onto NB-UA plates (0.8% NB, 0.5% uric acid and 1.5% agar), incubated for 48 hours at 30°C (Morales-Jiménez *et al.*, 2013).
Phytase-screening medium (PSM) contained 1% glucose, 0.4% Na-phytate, 0.2% $CaCl_2$, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot H_2O$ and 1.5% agar at pH 7. Degradation of Na-phytate was evaluated after incubation at 30°C for 4 days. The presence of clear zones around the isolates spotted on plates was considered as indication of phytate mineralization (Jorquera *et al*., 2008).
EPS production was estimated according to Santaella *et al.* (2008) using modified Weaver mineral media with the addition of sucrose (2%).

Bacterial strains for augmentation trial.

The strains used in the augmentation trials were: *Bacillus licheniformis* HI169, *Stenotrophomonas maltophilia* HI121 (both obtained from the gut of BSF) and *Escherichia coli* DH5α pKan (DsRed) (Crotti *et al.*, 2009) (considered as an outsider strain). *B. licheniformis* HI169 and *S. maltophilia* HI121 were inoculated in tryptic soy broth (TSB) medium and cultured at 30°C for an overnight, whereas *E. coli* DH5α pKan (DsRed) was inoculated in Luria Bertani (LB) medium and cultured at 37°C for an overnight. The cells were then inoculated at 5% concentration in 100 ml of the appropriate growth media and incubated for 24 h. After growth, cells were centrifuged at 3000 rpm for 15 minutes at 4°C, the supernatants were discarded and pellets washed three times with physiological solution (0.9% NaCl) in order to remove the residual medium nutrient source. Cells were then diluted to a final concentration of 10^8 cfu/ml in 0.9% NaCl.

Augmentation trials.

Bacterial augmentation was performed in 10.5 × 5 cm plastic boxes, containing ~ 60 g of fruit waste, FW (apple 1/3, pear 1/3 and orange 1/3). Different bacterial augmentation treatments (in three replicates) were considered: i) 10^8 cfu/ml of *B. licheniformis* HI169; ii) 10^8 cfu/ml of *S. maltophilia* HI121; iii) 5×10^7 cfu/ml of *B. licheniformis* HI169 + 5×10^7 cfu/ml of *S. maltophilia* HI121; iv) 10^8 cfu/ml of *E. coli* DH5α pKan(DsRed); v) sterile physiological solution (NaCl 0.9%),

as negative control. Fifty 9-days-old BSF larvae were added into each container, covered with breathable caps, and stored in the climate chamber (25°C, 60-65% R.H.). To prevent bacterial cross contamination among the treatment groups, highly hygienic standards were maintained at all times. Additional applications were repeated at the same conditions of the first treatment after 7 and 8 days. Ten larvae from each container were randomly selected and weighed every 2/3 days on an analytical balance (SartoriusCP64, Germany); after measurements, the larvae were replaced in the same container. The time from the beginning of the experiment to the observation of the first prepupa was recorded. All prepupae were removed from the container at the moment of appearance and weighted. Observations continued until all larvae entered the pupal stage or died.

Statistical analysis.

Determination of the sample weight, growth rate and prepupal survivorship was based on statistical analysis comparisons among groups. Results were expressed as mean values of the 3 independent replicates. Differences were calculated using a Student t-test with a two-sided distribution.

RESULTS

Bacterial isolation from the larval guts.

A bacterial collection composed by 193 strains was obtained from the guts of four BSF larvae reared on standard diet, using selective and enrichment media. After dereplication by ITS fingerprinting, the identification of 115 representatives of the 78 ITS groups was performed by partial 16S rRNA gene sequencing. Isolates were assigned to four phyla, namely Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. Proteobacteria phylum resulted the most abundant one with 88.1% of the isolates, which belonged to Alpha (3% of the total isolates), Beta (3% of the total isolates) and Gamma-classes (83% of the total isolates). Firmicutes (8.8% of the total isolates) were mainly composed of *Bacillus* spp., whereas Actinobacteria and Bacteroidetes isolates represented the 2.1% and 1.0% of the total collection, respectively. Bacteroidetes were only represented by the class of Flavobacteria (Fig. 1a). At the family level, within the phylum of Proteobacteria the most abundant family was represented by Enterobacteriaceae (62.2% of the total isolates), while Firmicutes were represented by Enterococcaceae (6.2% of the total isolates), Staphylococcaceae (2.1%) and Bacillaceae (0.5%) (Fig. 1b). Within the entire collection, the most represented genera were *Providencia* (22.3% of the total isolates), *Klebsiella* (17.1%), *Escherichia* (7.3%), *Morganella* (5.7%), *Stenotrophomonas* (8.3%) *Acinetobacter* (7.8%) and *Enterococcus* (5.7%) (Fig. 1c).

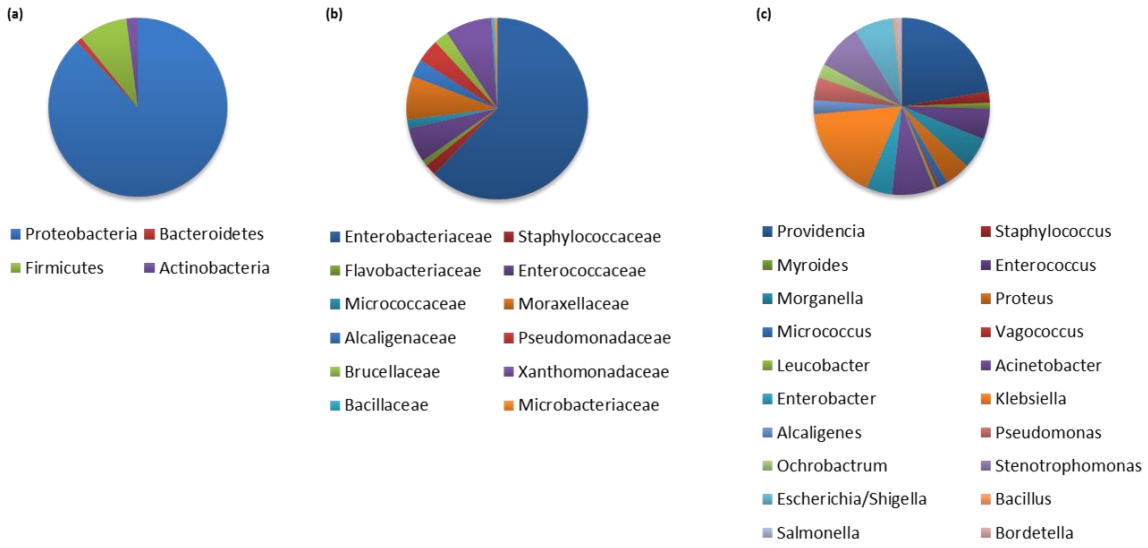

Figure 1. Diversity of the bacterial isolates obtained from BSF. Graphs represent the bacterial taxonomy diversity of our collection. Bacterial diversity at the (a) phylum, (b) family and (c) genus level.

Hydrolytic profiles and EPS production.

All the bacterial isolates were screened for hydrolytic capabilities to investigate the bacterial potential contribution to carbon and nitrogen metabolism in the insect gut. First of all, we found isolates able to produce EPS matrix (33% on the total isolates), which could mediate the bacterial adhesion to the insect epithelium. Degradation ability of polysaccharidic compounds was shown by the 34% of the isolates: 15 isolates, belonging to 4 genera (*Ochrobactrum*, *Klebsiella*, *Bacillus* and *Pantoea*), were able to degrade cellulose, while only 3 isolates (*K. pneumonia* HI105, *K. pneumonia* HI106 and *B. licheniformis* HI169) were amylolytic. Forty-seven isolates belonging to 10 genera were found as pectinase producing bacteria (Fig. 2). 47% of the isolates were able to degrade lipids: in particular, 21 isolates, ascribed to 7 genera, could degrade Tween 80, 26 isolates from 5 genera hydrolized tributyrin and 44 bacteria, belonging to 7 different genera, showed a positive results in the true lipase assay using agarized plates containing olive oil (Fig. 2). The capacity to utilize nitrogen by recycling it from bio-waste molecules was identified in a large part of the collection members: 63 and 31 isolates were able to degrade urea and uric acid, respectively. One-hundred and seventy-five isolates were able to produce ammonia from peptides, whereas 32 isolates belonging to 9 genera released protease (Fig. 2). Phytase activity was found in 119 isolates ascribed to 13 genera (Fig. 2). Finally, we investigated the production of exopolysaccharide (EPS) matrix by the isolates and 59 of them, belonging to 9 genera, were positive (Fig. 2).

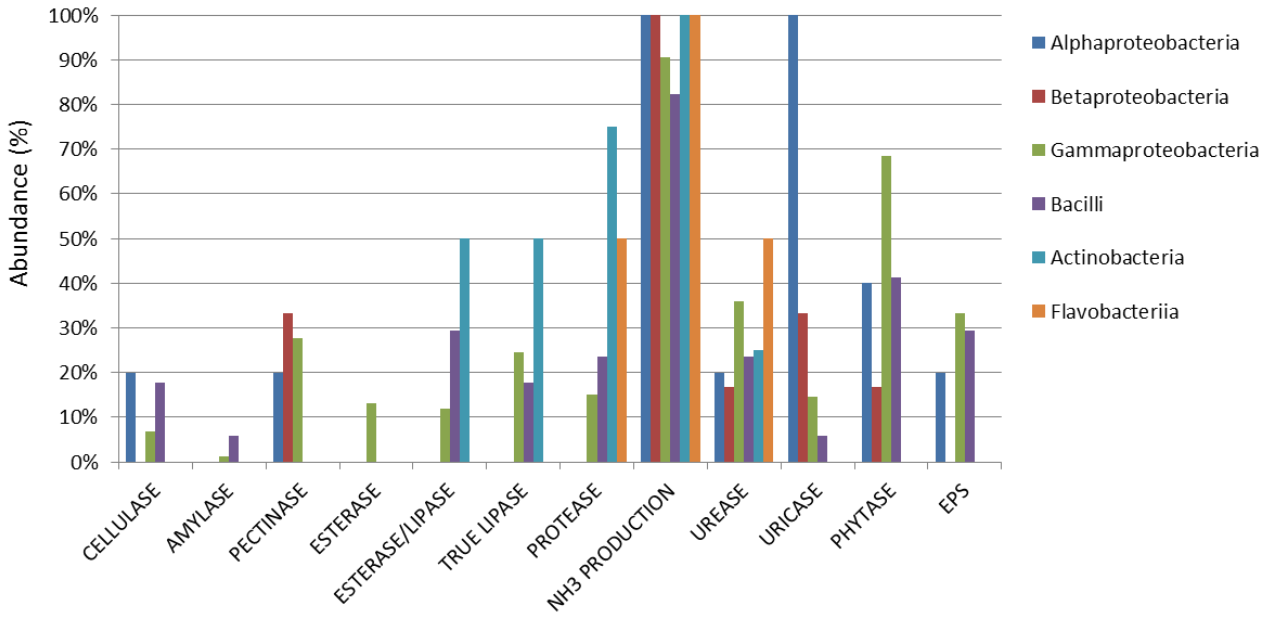

Figure 2. Hydrolytic profiles of the gut bacterial isolates of *H. illucens* larvae reared on standard diet. In the graph the percentage of bacterial isolates capable of hydrolytic activities and production of exopolysaccharides (EPS) are indicated.

Selection of the strains for the augmentation trials.

Following the hydrolytic activity screenings, *Bacillus licheniformis* HI169 and *Stenotrophomonas maltophilia* HI121 were selected for the augmentation trials due to their complementary metabolic activities. Indeed, *B. licheniformis* HI169 showed the ability to breakdown cellulose and starch, possessed uricolytic activity, released ammonia, dissolved Tween 80 and produced EPS. Conversely, *S. maltophilia* HI161 had the ability to digest casein, release ammonia, mineralize the organic phosphorous, breakdown pectin and produce lipase.

Immature life-history traits.

Bacterial augmentation treatments were applied to BSF fed on a FW-based diet and the weight of BFS specimens were measured along the experimental time. We observed that few days before the pupation, the larvae reached the maximum weight (hereinafter reported as “maximum weight”), from which a slight decrease was reported before reaching the final weight (indeed indicated as “final weight”). We verified this behaviour for all the different treatments.

Considering the maximum weight, the mean larval weights of BSF differed significantly for the combination of the two selected strains, *B. licheniformis* HI169 + *S. maltophilia* HI121 in comparison to the control ($p < 0.05$). The administration of the two combined strains allowed the larvae to reach the average maximum value of 178.50 ± 7.15 mg, whereas for the control larvae the average maximum weight value was 149.60 ± 6.50 mg (Tab. 1). For the other treatments, the

maximum weights of larvae augmented with *B. licheniformis* HI169, *S. maltophilia* HI121 and the outsider *E. coli* DH5α pKan (DsRed) were 163.49 ± 6.95, 157.21 ± 11.73 and 161.90 ± 4.03 mg, respectively (Tab. 1).

TABLE 1. Immature life-history traits in the five experimental conditions.

Bacterial treatments	Larval maximum wt (mg)	Growth Rate (GR)	Pupation (%)
Control	149,6 ± 6,5	0,048 ± 0,0014	92,22 ± 0,588
HI121	157,2 ± 11,73	0,044 ± 0,0041	89,78 ± 3,205
HI169	163,5 ± 6,95	0,04 ± 0,0029	88,67 ± 0,666
HI121 + HI169	178,5 ± 7,15	0,045 ± 0,0036	89,56 ± 3,638
E. coli DH5α pKan(DsRed)	161,9 ± 4,03	0,042 ± 0,0045	88,22 ± 6,186

Wt: weight

Interestingly, the larvae with the combination of the two selected strains reached the maximum weight at the same time of the control ones (50 days). On the contrary, the larvae fed with *B. licheniformis* HI169 and *S. maltophilia* HI121, individually, reached the maximal weight 2 days later (52 days), whereas the *E. coli* DH5α pKan (DsRed) treatment shortened this time to 48 days. It is noteworthy to mention that this treatment allowed to reach a final average weight (140.43 ± 4.55) lower than the ones measured for the other treatments (147,33 ± 9; 148,86 ± 11,37; 155,96 ± 9,3; 159,59 ± 7,8) (Fig. 3).

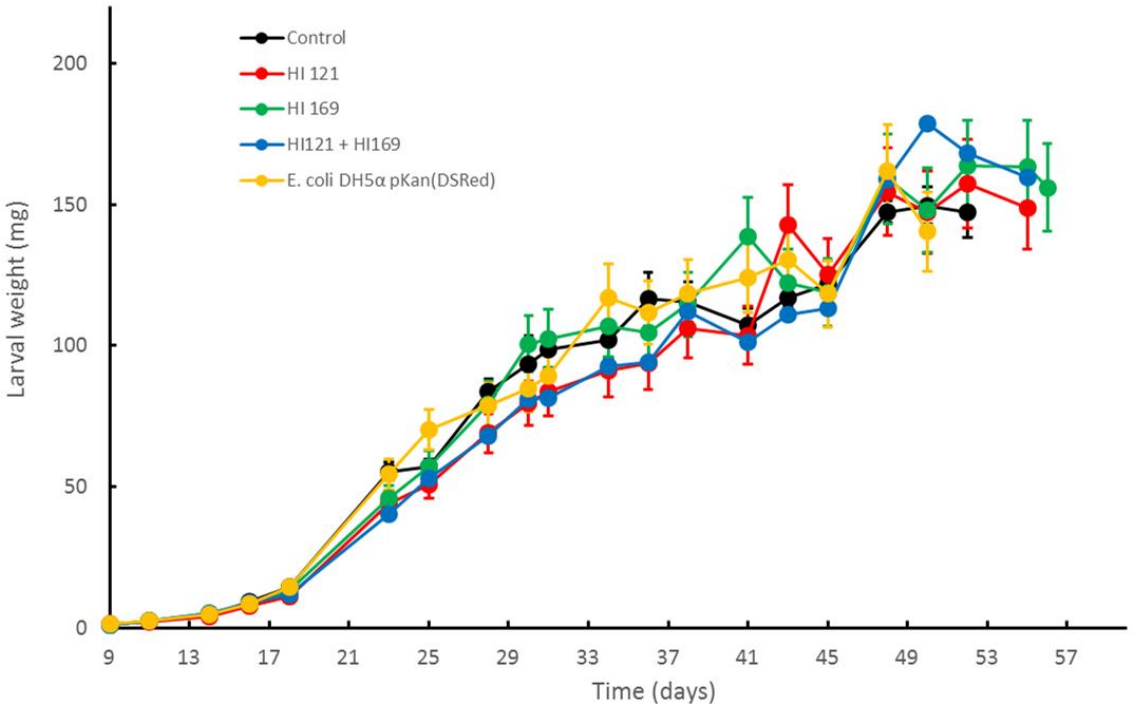

Figure 3. Weights of larvae augmented with selected bacterial strains. Weights of *H. illucens* larvae reared on non-sterile fruit-waste diet with or without the selected bacterial strains. The horizontal axis indicates the time (days); the vertical axis reports the weight (mg). Values in the graphs are means with their standard errors represented by vertical bars. Control: non-sterile fruit-waste diet without inoculation of selected strains; HI121: larvae with the addition of *S. maltophilia* strain HI121; HI169: larvae with the addition of *B. licheniformis* strain HI169; HI121 + HI169: larvae with the addition of the combination of two selected strains HI121 and HI169; *E. coli* DH5α pKan (DsRed): larvae with the addition of the outsider strain, not isolated from the larval gut of BSF.

Time from hatching to pupal stage per treatment ranged from 44 to 91 days (Fig 4) without significant differences among the treatments. The survivorship of prepupae was not significantly different for all the treatments, except for larvae augmented with *B. licheniformis* HI169 compared with the control ($p < 0.05$; Fig. 5).

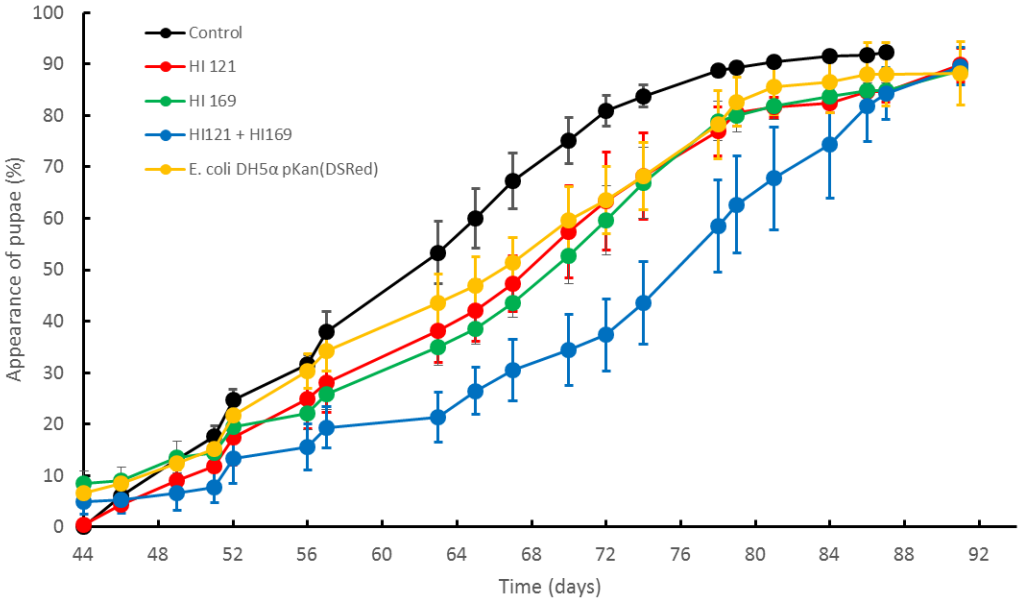

Figure 4. Appearance of pupae in percentage. The appearance of pupal is reported as average percentage within the population of each thesis. Values in the graphs are means with their standard errors represented by vertical bars. Control: non-sterile fruit-waste diet without inoculation of selected strains; HI121: larvae with the addition of *S. maltophilia* strain HI121; HI169: larvae with the addition of *B. licheniformis* strain HI169; HI121 + HI169: larvae with the addition of the combination of two selected strains HI121 and HI169; *E. coli* DH5α pKan (DsRed): larvae with the addition of the outsider strain, not isolated from the larval gut of BSF.

However, in this case (larvae augmented with *B. licheniformis* HI169) there was a lower percentage of individuals that reached the pupal stage in comparison to the control ones. The growth rate (GR) per treatment did not differ significantly ($p > 0.05$; Tab. 1 and Fig. 5).

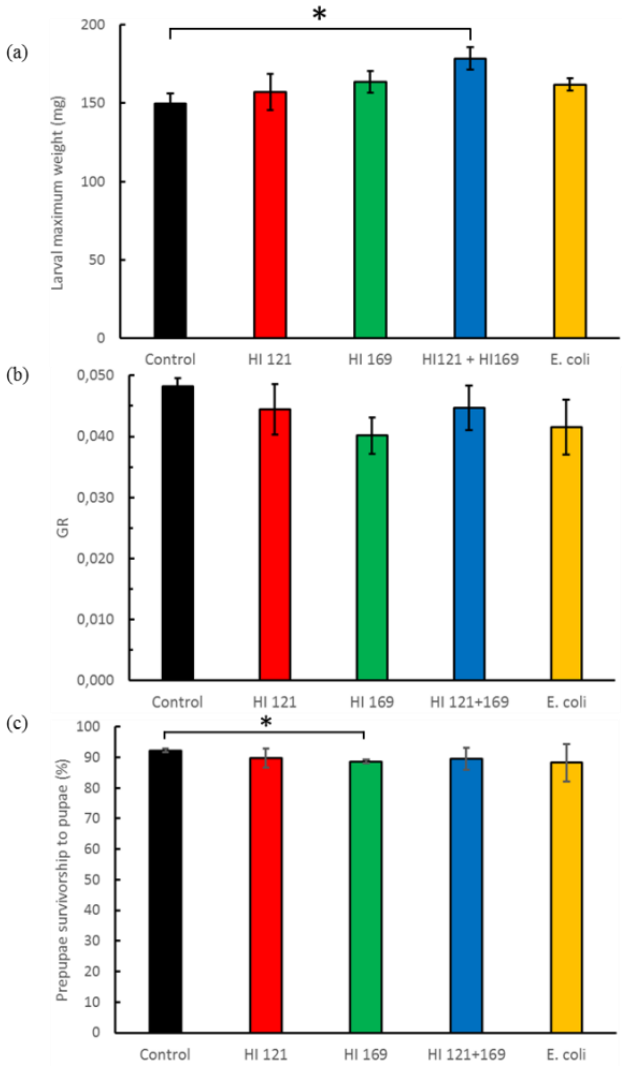

Figure 5. Performance indicators of augmentation trials. (a) Average larval maximum weight reached during development; (b) average growth rate (GR); (c) percentage of survival of larvae to pupal stage. Average values are indicated with standard errors.

DISCUSSION

BSF is known to feed on a wide variety of organic matter ranging from agro-industrial waste to livestock manure (Diener *et al.*, 2009; Nguyen *et al.*, 2015). The advantage to use this species compared to many other fly species is that it is not a pest. Here, to understand the potential contribution provided by the gut microbial consortium to the host physiology and development, a bacterial collection was established and characterized for the hydrolytic capabilities potentially involved in the diet component breakdown and nutrient supplementation. We decided to focus our attention on the bacteria within the gut of larvae fed on the standard diet, on which BSF larvae showed higher performance and fitness hallmarks than on the fruit-waste derived one (Jucker *et al.*

in press). We hypothesized that this could be also related to the contribution of a more efficient intestinal microbiota involved in an improved nutritional status.

Among the isolates, we found an abundance of plant-soil bacteria and members of the Enterobacteriaceae family associated to BSF gut, in agreement with previous findings for other detritivores insects (Jeon *et al.,* 2011; Oravecz *et al., 2004*). By the use of culture-independent techniques, Jeon and colleagues (2011) investigated the gut bacterial community of *H. illucens* larvae fed on three different food sources, showing that it was mainly composed of four phyla with different distributions among the diets. Generally, they identified the same phyla that we reported in our survey. The influence of the diet as an environmental factor that selects and shapes the host intestinal microbiota has been studied in different arthropods (Breznak and Brune, 1994; Jeon *et al*., 2011; Montagna *et al.,* 2015a; Vacchini *et al.,* 2017; see chapther 3 of this PhD thesis).

During isolation trials we found that the genus *Providencia* was often present in association with the gut of larvae reared on different agro-waste, as previously reported by Jeon *et al*. (2011), Zheng *et al.* (2013) and Zheng *et al.* (2015): in this last work the authors demonstrate that gravid black soldier fly female were attracted for the oviposition by *Providencia* sp. (Zheng *et al.* 2015).

By using plate-based assays, the evaluation of hydrolytic profile of the isolates showed that these bacterial strains possessed metabolic traits that can play a role in the breakdown of recalcitrant molecules and complex nutrients. Particularly, the isolates with a wider range of hydrolytic extracellular enzymes mainly belonged to the phylum of Proteobacteria which represented the 88.1% of the entire bacterial collection. Surprisingly, only 3 isolates of the entire collection were able to release amilolytic enzyme, despite the standard diet was supposed to contain starch. Conversely to the results obtained from Yu *et al.* (2011), in which they obtained diverse *Bacillus* strains from BSF larvae reared on poultry manure, in our survey based on 16S rRNA gene high-throughput sequencing we did not find members of the genus *Bacillus* (see chapther 3 of this PhD thesis). However, in the present study a strain of *Bacillus licheniformis* was isolated from intestinal tract of larvae reared on the standard diet. This strain was positive in amylase, esterase, uricase, ammonia and EPS production.

Food waste contains high amount of polysaccharides including complex molecules, such as cellulose, hemicellulose, xylan, pectic substances and lignin (Salisbury and Ross, 2001); it is well known that microbiota play a key role in the breakdown of this compounds, providing an important contribution to the host digestion (Anand *et al.,* 2010). However, Genta *et al.* (2006) reported that enzymes involved in polysaccharide degradation were also present in the midgut of *Tenebrio molitor* larvae treated with antibiotics compared with non-treated controls, suggesting that gut

microorganisms may have an auxiliary, non-essential role. In our survey we reported that starch and cellulose hydrolysis were poor represented in the bacterial collection.

Then we investigated the gut bacterial contribution to recycle bio-waste molecules, such as urea and uric acid. Indeed, we reported the presence of uricolytic bacteria in the gut of BSF larvae and their capability to use uric acid as sole carbon and/nitrogen source *in vitro*. Possibly, the uric acid is released from the Malpighian tubules into the *Hermetia* hindgut, as it was reported in *Reticulotermes flavipes* (Potrikus and Breznak, 1980). It is noteworthy to mention that not all the bacteria isolated from the enrichment cultures with cellulolytic or uricolytic activity showed the expected enzymatic activity, when tested using plate based assays. The isolation of bacteria that did not show cellulolytic or uricolytic activities could be due to their ability to sustain the degrading community.

Moreover, we found that extracellular protease and ammonia production were widespread through bacterial isolates (Fig. 2). These metabolic activities share the same final products, i.e. the ammonia (NH_3) that could be assimilated directly by the insect via glutamine synthetase pathway or could be first incorporated into bacterial biomass and become available later following the lysis of the microbial cells in the gut environment (Sabree *et al., 2009*).

Due to the increasing exploitation of *H. illucens* in disposal of biowaste and its conversion in animal feed, the gut microbiota capability to mineralize the phytic acid is important for improving the host growth rate and physiological status. Indeed, the presence of phytate in feedstuffs leads to deprivation of essential minerals, amino acid and proteins (Wodzinski and Ullah, 1996). Beyond its primary function as phosphorous and energy storage (Reddy *et al.*, 1982), the phytic acid in plant tissues may play a defensive role, as reported in the study of Green and colleagues (2001): the authors showed a correlation between the presence of phytic acid in the diet and the mortality of three species of Lepidoptera. While different studies have evaluated the positive effect of the supplementation of microbial phytase to the diet of broilers chickens and pigs (She *et al.*, 2015; Picón-Rubio *et al.*, 2009; Kemme *et al.*, 1997; Sebastian *et al.*, 1996a and b; Yi *et al.*, 1996; Adeola, 1995), there is still poor information about the influence of phytate on the insect growth and development. In our collection, the phytase activity was widespread among isolates.

Insects are characterized by a high plasticity in the adaptation to varying environmental conditions, and this is primarily reflected in their development (Pöykkö and Hyvärinen, 2012). Developmental time, weight and growth rate are the three parameters that we considered in the performance evaluation of the insects. In our experiments, the insect weights were significantly affected only in presence of the combination of the two selected strains. In this case, the time from hatching to the end of larval development was similar to the one shown by control larvae, but longer than the ones

shown by larvae fed singularly with the bacteria or with *E. coli* (Fig. 3). Generally, longer developmental time may result in bigger body size –and weight– and typically, when the insect reaches the adult stage, a larger body size increases the fecundity of insects (Fox and Czesak, 2000). The diet source quality, especially the nitrogen concentration, is a critical determinant for the insect growth and fecundity (Mattson, 1980; Slansky and Scriber, 1985; Awmack and Leather, 2002). In this study, we showed that the administration of bacteria, isolated from the intestinal tract of larvae reared on a nutritionally balanced diet and selected on the basis of the results of hydrolytic activity screening, could influence the weight of larvae when reared on a nutritionally-unbalanced diet.

CONCLUSION

In this work, we characterized the cultivable fraction associated to BSF and we explored its potential metabolic contribution to the host development and physiology. The study falls in the a microbial resource management (MRM) perspective of BSF with the aim to exploit these waste-reducing insects as an alternative protein-rich and high-energy meal for livestock or in the biodiesel production. Possible applications of characterized and selected microorganisms include several sectors from animal or human food, to waste disposal. In conclusion, we found that at the same density of population, the addition of microorganisms to the FW diet source could slightly increase the weight of BSF larvae.

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General conclusions

Since the increasing economic value and scientific interest towards insects, also due to their biotechnological and ecological applications in different fields -from the agricultural production to the environmental protection and the industrial exploitation-, the study of the insect-microbe interactions and the eventual manipulation of the insect gut microbiota is gaining a great attention (Berasategui *et al.,* 2016). Many evidences support the role of the native gut microbiota as an important component of the host physiology, since, in many cases, is primarily involved in the host defense and growth promotion (Dillon and Dillon, 2004; Kikuchi *et al.,* 2012; Vàsquez *et al.,* 2012). Different factors, e.g. the host diet and developmental stage, have been shown to drive the composition and structure of the insect gut microbiota (Montagna *et al.,* 2015a; Vacchini *et al.,* 2017). However, such information is available for a limited number of insect species (mainly represented by insect pests or vectors of plant, animal and human diseases) or factors. For instance, the studies performed so far did not consider other important parameters, such as the physicochemical conditions existing in the host digestive tract. Hence, this thesis aimed to contribute to this gap of knowledge, investigating how the structure and composition of the gut bacterial communities associated to non-pest insects could vary according to diet, developmental stage and micro-environmental variations. The two insect species considered in this work, the honeybee *Apis mellifera* and the black soldier fly (BSF) *Hermetia illucens*, are characterized by polyphagous diet behavior, the former more specialized and the latter more variegated, respectively; moreover, at different extent, both insects can enter into contact with the food chain. In the case of BSF the variation of the diet source and developmental stage has been considered, while for honeybees the variation of the physicochemical conditions occurring in the different gut compartments has been taken into account. Specifically, one of the innovative aspects of this work was to have considered the physicochemical conditions of the distinct microenvironments (namely the gut compartments) as an important component that could shape the bacterial community. Up to now, very few publications have examined this feature: the attention has been mainly devoted to intestines of lower and higher termites and few other insects, i.e. the omnivorous cockroach *Shelfordella lateralis*, the wood-feeding beetle *Odontotaenius disjunctus* and the saprophagous dipteran *Penthetria holosericea* (Köhler *et al.,* 2012; Brune *et al.,* 1995; Ceja-Navarro *et al.,* 2014; Šustr *et al.,* 2014; Brune and Friedrich, 2000). In the present work, data show that the spatially organized bacterial community in the intestinal tract of honeybees is driven by the modification of the physicochemical parameters of oxygen partial pressure (pO_2), pH and redox potential, resulting in a niche adaptation within the four gut regions. Particularly, the radial availability of oxygen in each gut section, from the epithelial surface to the organ centre, supports the phylotypes' stratification, whereas the pH and redox potential conditions drive longitudinally the phylotype

diversification and the complexity of the network connections. Interestingly, pH and redox show to control the bacterial interactome of the overall community, offering a useful insight for further manipulative investigations to shed light on the community assembly, the degree of its resilience facing the environmental changing and, therefore, its contribution to the overall host homeostasis.

On the other side, the gut of *H. illucens* harbors a diverse bacterial community, which differs considerably among developmental stages and is strongly influenced by the diet source. To date, only few works have characterized the bacterial community of this insect (Zheng *et al.,* 2013; Jeon *et al.,* 2011): the results of the present study provide a further insight in understanding how factors such as diet and developmental stage can drive the bacterial assemblage. Moreover, taking advantage of microsensors and microelectrodes, it has been shown that BSF gut resulted mostly anoxic, as in the case of the honeybee's one, likely due to the microbial and animal activity.

Finally, the probiotic effect of some selected bacteria has been investigated to observe a possible bacterial mitigation when BSF individuals have been exposed to a low-performing diet, such as the fruit waste derived one. The cultivation-based investigation of BSF larvae represents an important step to improve our understanding on the role exerted by the members of the insect microbiota. It is well known that the gut microbiota is involved in the digestion of complex dietary components and in the recycling of bio-waste molecules from the animal metabolism (e.g. urea and uric acid) (Engel and Moran, 2013). For this reason, the cultivation survey has been focused on the identification and characterization of 193 isolates with different hydrolytic activities to investigate their potential "symbiotic contribution" to the host physiology. However, it is noteworthy to mention that the functional roles played by individual members of a community and their interactions are difficult to elucidate, mostly due to their resistance to cultivation. Metagenomic and metatranscriptomic approaches have provided clues into the functional understanding of the gut network (Warnecke *et al.,* 2007; He *et al.,* 2013; Liu *et al., 2013*). In the present study, bacteria selected on the basis of the hydrolytic profile data (*Bacillus licheniformis* HI169 and *Stenotrophomonas maltophilia* HI121) have been administered to BSF individuals exposed to an unbalanced diet source, represented by the fruit-waste derived diet. Growth performance of larvae reared on non-sterile fruit-waste diet, inoculated with or without the selected bacteria (individually and in combination), has been compared with the larval growth on the fruit waste diet, added with an outsider strain of *Escherichia coli,* typically not associated with BSF. Results suggest that the augmentation of bacterial strains to BSF larval diet could influence the animal weight gain. However, the improvement of the BSF developmental rate is not so remarkable. This could be directly related to the selected isolates or to the chosen experimental conditions. As reported in the previous survey based on molecular tool, the bacterial communities of larvae reared on standard and fruit-waste diet

conditions were strongly different, as well as the physicochemical parameters that have been measured. The severity imposed by the diet conditions could be too high to have completely overcome the beneficial effect exerted by the strain administration. Moreover, further investigations could be done to enlarge the number of isolates used in BSF administration trials and observe possible influences on the host development.

In conclusion, the study of factors that modulate the structure and composition of the bacterial community associated to non-pest and economically relevant insects, performed by the use of different techniques, allowed us to draw a more precise picture of the response of the microbiota associated to the insects in changing environments. The combination of such investigations with data related to the physicochemical conditions (pO_2, redox potential and pH) existing in the different gut compartments provides useful information to describe the associated microbial community in the perspective of a further investigation of its contribution for the host.

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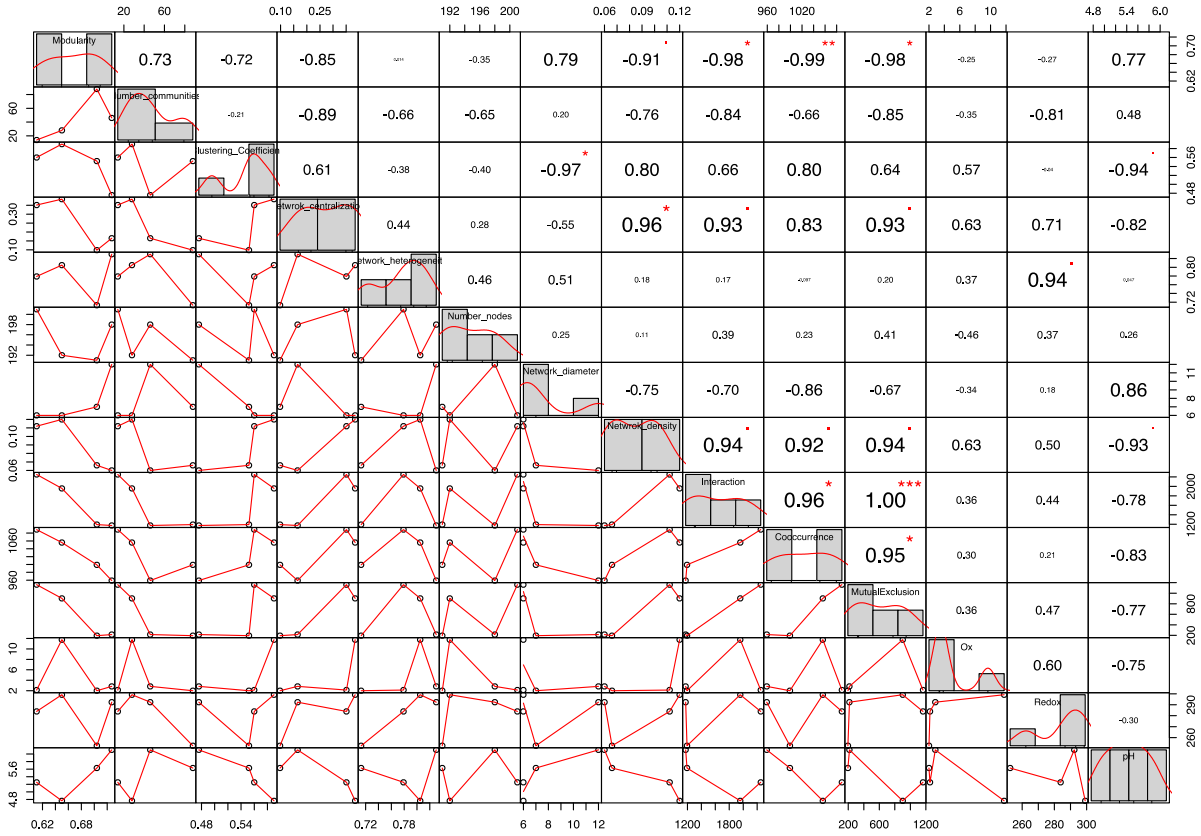
Supplementary materials

Figure S1. Correlogram between the main network topological parameters (see Fig. 3) and the microenvironmental gut condition such as PO_2, redox potential and pH. The correlogram was calcolated using three different statistical methods (Kendall, Spearam and Pearson), using the R package 'Performance Analytics'. Asterisks indicate the presence of statistically significant differences among the considered parameters. Beyond the others, significant correlations are found for pH and network density and for redox potential and network clustering coefficient and network heterogeneity ($p<0.05$).



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Activities performed during the Ph.D.

Paper published in international journals:

Vacchini V., Gonella E., Crotti E., Prosdocimi E.M., Mazzetto F., Chouaia B., Callegari M., Mapelli F., Mandrioli M., Alma A., Daffonchio D. (2017). Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria. Environ. Microbiol. Rep. 9:91-103.

Oral dissertations in national or international meetings:

- **Callegari M.,** Marasco R., Jucker C., Mapelli F., Fusi M., Borin S., Daffonchio D., Savoldelli S., Crotti E.: Developmental stage and diet drive the bacterial community diversity in the food-waste reducing insect *Hermetia illucens.* IV international conference on Microbial Diversity (2017). Bari (Italy), October 24-26, 2017.
- **Callegari M**., Marasco R., Jucker C., Mapelli F., Fusi M., Borin S., Daffonchio D., Savoldelli S., Crotti E.: The bacterial community of the food-waste reducing insect *Hermetia illucens* (L.) is shaped by diet and developmental stage. VII international conference on Environmental, Industrial and Applied Microbiology, BioMicroWorld2017. Madrid (Spain), October 18-20, 2017.

Partecipation at International and National conferences with extended abstract

Callegari M., Marasco R., Jucker C., Mapelli F., Fusi M., Borin S., Daffonchio D., Savoldelli S., Crotti E. Developmental stage and diet drive the bacterial community diversity in the food-waste reducing insect *Hermetia illucens*. Microbial Diversity 2017. Bari (Italy), October 24-26, 2017.

Partecipation at International and National conferences with poster

- **Callegari M**., Fusi M., Marasco R., Gonella E., Borin S., Tsiamis G., Alma A., Crotti E., and Daffonchio D. Bacterial communities in the gut compartments of the honeybee *Apis mellifera* are exposed to different pH and oxygen concentrations. Microbial Diversity 2017. Bari (Italy), October 24-26, 2017.
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