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INVERTEBRATE MICROBIOLOGY

Changes in Bacterial Community Structure Across the Different Life Stages of Black Soldier Fly (*Hermetia illucens*)

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Abstract

The digestive capacity of organic compounds by the black soldier fly (BSF, *Hermetia illucens*, Diptera: Stratiomyidae, Linnaeus, 1758) is known to rely on complex larva-microbiota interactions. Although insect development is known to be a driver of changes of bacterial communities, the fluctuations along BSF life cycle in terms of composition and diversity of bacterial communities are still unknown. In this work, we used a metabarcoding approach to explore the differences in bacterial diversity along all four BSF developmental stages: eggs, larvae, pupae, and adult. We detected not only significant differences in bacterial community composition and species richness along the development of BSF, but also nine prevalent amplicon single variants (ASVs) forming the core microbiota. Out of the 2010 ASVs identified, 160 were significantly more abundant in one of the life stages. Moreover, using *PICRUSt2*, we inferred 27 potential metabolic pathways differentially used among the BSF life cycle. This distribution of metabolic pathways was congruent with the bacterial taxonomic distribution among life stages, demonstrating that the functional requirements of each phase of development are drivers of bacterial composition and diversity. This study provides a better understanding of the different metabolic processes occurring during BSF development and their links to changes in bacterial taxa. This information has important implications for improving bio-waste processing in such an economically important insect species.

Keywords Black soldier fly · Bacterial communities · Metabolic pathways · Metabarcoding · Development

Introduction

Insects are the most numerous and diverse group of terrestrial animals [1]. They have successfully diverged into a high diversity of morphotypes and lifestyles, and colonized almost every habitat across the planet [2, 3]. Over evolutionary times, insects have established close relationships with microbes forming complex host-microbiota interactions [4, 5], which can be involved for instance in the digestion of certain food, in helping in the balance of nutrients or, even, in protecting the host against several biotic and abiotic threats [6]. The beneficial role of the microbiota on the development and fitness of insects has important ecological and evolutionary implications [7]. Several insect capacities, which rely on host-microbiota interactions, also have industrial and biotechnological applications. This is the case of termites [8], which are able to degrade lignocellulose, or the black soldier fly (BSF, *Hermetia illucens*, Diptera: Stratiomyidae, Linnaeus, 1758), whose larvae are able to degrade a wide range of organic substrates, and convert them into biomass [9, 10]. As such, it has become one of the most important insect species for industrial bioconversion processes with strong implications in the feed and food industry as well as other insect-based industries [11, 12].

The BSF degradation capacity may alleviate some of the negative consequences of the increasing accumulation of organic solid waste and help achieving sustainable development goals [13]. Studies have explored the larval gut microbial communities in BSF and their potential role in degradation capacity for potential bio-waste processing applications [10, 14, 15]. Many bacterial taxa such as *Dysgonomonas*

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[14, 16], *Proteus*, and *Providencia* are commonly found in BSF microbiota, and may play a role in the degradation of a wide range of organic substrates [10]. Given that BSF larvae are wide-range generalists that feed on a large variety of substrates [10], they might harbor a more diversified microbiota than specialist species [17], conferring an adaptive advantage to colonize new environments. Moreover, the recent discovery that adult BSFs are able to eat [16] may encourage the presence of certain bacterial taxa that are absent in other insect species in which the adults do not feed. Besides diet [4, 7, 18] and phylogeny, development can also shape the microbial communities of insects [7, 19].

BSF, together with other holometabolous insects [20, 21], undergo a complete gut remodeling during the pupal stage as they transform into adults [15]. This, together with the fact that they are sessile, may make them vulnerable to external threats, such as infections, parasitism, or predators [20, 21]. Moreover, the pupa may get rid of the excess metabolic secretions (meconium) before adult emergence [22]. These life history processes suggest possible co-occurring microbiota diversity and composition changes at each developmental stage, potentially linked to variation in digestive functions. In this work, we aim to determine changes in bacterial diversity and community composition along the course of BSF development by addressing the following questions: (1) is there a core microbiota (group of prevalent bacterial taxa) shared among the whole life cycle of BSF and among different populations? If so, are these taxa consistent with the core microbiota previously described in BSF larvae? (2) Is development a driver of composition and diversity of bacterial communities of BSF (focusing on eggs, larvae, pupae, and adult)? And if so, (3) are the bacterial taxa detected linked to specific functions that are essential for each developmental stage?

Improving knowledge of bacterial community dynamics during the entire BSF life cycle might help understanding the succession of metabolic processes taking place, and potentially inform effective microbial biotechnological approaches to improve bio-waste conversion efficiency.

Material and Methods

Experimental Design and Processing of BSF Samples

A total of 105 samples covering four developmental stages of BSF (15 eggs, 46 larvae, 15 pupae, and 29 adults) were included in this study (detailed information in Supplementary information S3; Table S1), as well as 16 negative controls (3 extraction and 13 PCR amplification negative controls). Samples came from two independent populations and were reared from eggs in the same facility but independently in different enclosures. Larvae were fed on a non-sterilized and controlled Gainesville diet [23], which is based on wheat, alfalfa, and corn flour in the proportion 5:3:2.

Samples were preserved in ethanol (96%) and stored in a −20°C freezer until dissection and/or processing. Larvae, pupae, and adult samples were superficially washed with sterile water and dried prior to dissection of the gut under sterile conditions using fine tip forceps. Following the dissection, samples were immediately used for DNA extraction (without further storing). The gene encoding for the cytochrome oxidase I (COI) was sequenced for one BSF of each population, in order to confirm their identity. These sequences are available in NCBI under the accession numbers OP537078 and OP537079.

DNA Extraction and 16S rRNA Gene Metabarcoding Library Preparation and Sequencing

Following DNA extraction, a 16S rRNA gene metabarcoding library was prepared using the V3 and V4 regions of the gene. This library was sequenced on Illumina MiSeq (detailed protocol in Supplementary information S1). Raw sequence data is available in the NCBI Sequence Read Archive under the BioProject PRJNA851044.

16S rRNA Gene Metabarcoding Library Processing

Libraries were filtered using a standard toolbox of software, which included *cutadapt* software [24] for primer trimming, *PEAR* software [25] for read merging, and *vsearch v2.8.2* [26]. software for all the remaining bioinformatic steps until obtaining an amplicon single variant (ASV) table and the respective bacterial sequences. Bacterial sequences were classified taxonomically to genus level using *mothur* software [27] (detailed protocol in Supplementary information S1).

Biodiversity Analyses

Rarefaction curves for each developmental stage and for the complete sampling were produced using the R package *vegan* [28]. The core microbiota within the developmental stages was calculated with the R package *microbiome* [29] setting a minimum prevalence of 0.8. The ASVs detected as members of the core microbiota were assigned, when possible, to species level against NCBI Genbank nr/nt using BLAST algorithm [30] and R package *taxonomizr* (https://github.com/sherrillmix/taxonomizr/; last accessed on November 2021). Moreover, taxonomic classification of the core was confirmed by phylogenetic inference using maximum likelihood phylogenetic tree constructed with *PhyML* v3.0 [31] (Supplementary information S2; Figure S2). We calculated the relative read abundance (RRA) for the complete sampling and for each developmental stage, in order to account for ontogenic

variations. Finally, we calculated the core independently for population 1 and for population 2, using the same R package [28] and the same prevalence threshold (0.8).

We explored the effects of development stage on bacterial communities by computing a negative binomial generalized linear model (GLM), using a nested model and applying a log link function (999 iterations) with the *mvabund* R package [32].

We estimated observed ASV richness ($q=0$) and inverse Simpson index ($q=2$), using the *hilldiv* R package [33]. We also analyzed beta diversity (community composition) through a principal coordinate analysis (PCoA) and canonical correspondence analysis (CCA) based on Bray–Curtis dissimilarity matrix using the *phyloseq* R package [34]. Variable differences were tested applying a non-permutational multivariate analysis of variance (PERMANOVA, 999 permutations) on Bray–Curtis dissimilarity matrix using the *vegan* R package [28]. Also, a permutation test was used to test the significance of the CCA using the same R package [28].

Differential Abundance and Functional Profiling

In order to assess differential genera abundance along the course of the four developmental stages, we computed a *DESeq2* negative binomial Wald test between each pair of developmental stages, using the *DESeq2* R package [35]. RRA of significantly differentially abundant ASV (clustered in genera) was normalized using the *Z*-score method. Finally, we visualized these normalized abundances with a heatmap clustered hierarchically using the complete linkage method with the *pheatmap* R package [36].

Functional Profiling Among the Developmental Stages of BSF

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (*PICRUSt2*) was used to infer metabolic pathways of bacterial ASVs across the developmental stages of BSF [37]. The metabolic pathways (third classification level) corresponding to functions were predicted using the *MetaCyc* database (metaCyc.org). First, queried sequences were placed into a reference phylogeny based of 16S rRNA gene sequences using *place_seqs.py*. This step was followed by inferring traits (EC numbers) from unknown lineages applying *hsp.py* and inferring the *MetaCyc* pathways using *pathway_pipeline.py*. Finally, the pathways were added to the prediction using *add_description.py*. Unconstrained and constrained ordinations, PCoA and CCA, respectively [34], were used to visualize potential differences in the inferred metabolic (*MetaCyc*) pathways between life stages. Significance of ordinations was tested through PERMANOVA (999 iterations) [28]. We computed *DESeq2* [35] to select the differentially abundant pathways, whose Z-score were represented in a clustered heatmap [36]. The use of *PICRUSt2* to infer metabolic pathways combined with bacterial community composition, species richness, and differential abundance analysis (*DESeq2*) allows us to draw conclusions on the succession of metabolic pathways along the development of BSF and its correlation with the distribution of bacterial taxa.

Results

Quality Description of 16S rRNA Gene Metabarcoding Library and BSF Microbiota Composition

The *COI* sequences confirmed that populations were BSF and that this gene was 100% identical in both populations. From the 105 samples of BSF included in the 16S rRNA gene metabarcoding library and sequenced in Illumina MiSeq, 6,441,698 raw reads were obtained (61,349 average raw reads per sample). Regarding the completeness of the sampling, the library quality was adequate to study the bacterial diversity along the course of the four developmental stages of BSF, according to the rarefaction curves reaching a plateau and the estimation detection of 90.75% of the ASVs of bacterial communities present in the BSF (Supplementary information S2; Figs. S1A, B and C).

After filtering by read depth, the library consisted of 86 samples (9 eggs, 40 larvae, 15 pupae, and 22 adults) and comprised 2010 ASVs (detailed information in Supplementary information S3; Table S2A). The core microbiota between the four developmental stages, which was defined above as the bacterial taxa shared by at least 80% of the samples present among the four developmental stages and the two populations, included 9 ASVs (13% of total reads) assigned to phyla *Proteobacteria* (5 ASVs), *Firmicutes* (3 ASVs), and *Actinobacteriota* (1 ASV) (Fig. 1; Supplementary information S2; Fig. S2). Moreover, 5 ASVs out of the 9 ASV members of the core microbiota along BSF life cycle were prevalent (> 0.8 of prevalence) in both population 1 and population 2 (detailed information in Supplementary information S3; Table S2B).

Bacterial Dynamics Along the Developmental Stages of BSF

A total of 20 different bacterial phyla (2010 ASVs) were detected with most ASVs belonging to *Proteobacteria* (RRA = 43.69%), *Bacteroidota* (RRA = 24.38%), *Firmicutes* (RRA = 16.96%), and *Actinobacteriota* (RRA = 12.14%). Across all BSF life stages, 201 different bacterial families were identified, with *Dysgonomonadaceae* (*Bacteroidota*,

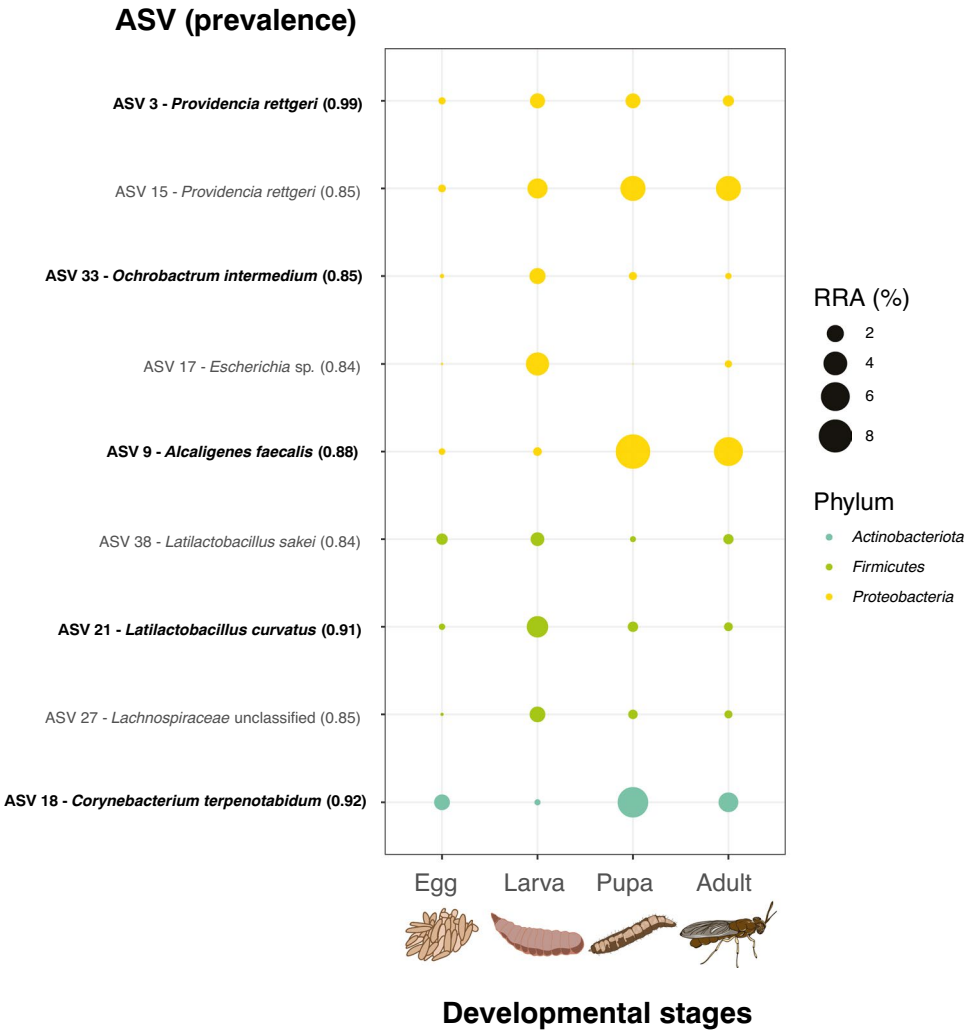


Fig. 1 Relative read abundance (RRA) of the nine ASVs forming the core microbiota across developmental stage (egg, larva, pupa, and adult). Circle size indicates the RRA at each developmental stage; color indicates phylum level taxonomy of the ASVs. Prevalence of each ASV is indicated in parentheses. ASVs marked in bold font correspond to the bacterial taxa prevalent in both BSF populations

Bacteroidales) being the most represented (RRA = 13.01%), followed by *Morganellaceae* (RRA = 8.87%; *Proteobacteria*, *Enterobacterales*), *Flavobacteriaceae* (RRA = 6.65%; *Bacteroidota*, *Flavobacteriales*), and *Lactobacillaceae* (RRA = 6.32%; *Firmicutes*, *Lactobacillales*). Out of the 435 genera identified, *Dysgonomonas* (RRA = 13.01%; *Bacteroidota*, *Bacteroidales*), *Providencia* (RRA = 6.15%; *Proteobacteria*, *Enterobacterales*), *Myroides* (RRA = 5.58%; *Bacteroidota*, *Flavobacteriales*), *Acinetobacter* (RRA = 5.36%; *Proteobacteria*, *Moraxellaceae*), and *Corynebacterium* (RRA = 5.36%; *Actinobacteriota*, *Corynebacteriales*) presented the highest RRA.

Life stages affected bacterial communities at ASV level according to the negative binomial GLM ($Dev_{1,86} = 17{,}356$, $p < 0.001$). The effect of the BSF population on the distribution of bacterial communities was also significant in terms of abundance ($Dev_{1,86} = 10{,}274$, $p < 0.001$). RRA at phylum level varied among the different developmental stages. *Proteobacteria* was the most abundant phylum in the four stages, being considerably more dominant in eggs (RRA = 82.03%) than in the rest of developmental stages (Fig. 2A). In egg and adult stages, *Proteobacteria* was followed in importance by *Actinobacteriota*, *Bacteroidota*, and *Firmicutes* while in the case of larval and pupal stages, the second most abundant phylum was *Bacteroidota*, followed by *Firmicutes* and *Actinobacteriota* (Fig. 2A). Dominant genera were *Acetobacter* (RRA = 19.63%) in eggs, *Dysgonomonas* (RRA = 25.5%) in larvae, and *Myroides* in pupae (RRA = 10.21%) and adults (11.47%) (Fig. 2B).

Across different life stages, ASV richness and diversity (inverse Simpson index) fluctuated (Fig. 3A and B), displaying the lowest value in larval stage and the highest in pupal stage (K-W chi-squared$_{richness} = 8.57$, $p = 0.036$, K-W chi-squared$_{inverse\text{-}Simpson} = 27.29$, $p = 5.129E-06$). The most important fluctuation in ASV richness occurred between larval and pupal stage (post hoc Dunn test, $p = 0.0113$). Among the rest of the stages, there were small and non-significant fluctuations between ASV richness. Regarding diversity, the differences were all significant, with the exception of the differences between pupae and adult, as well as larvae and eggs.

Beta diversity was investigated across developmental stages, showing significant differences between the bacterial communities of egg, larval, pupal, and adult stages (PERMANOVA: $R^2 = 0.167$, $p < 0.001$) (Supplementary information S2; Fig. S3). CCA showed an overlap between bacterial communities in pupal and adult stages and a clear separation between eggs and the rest of life stages ($chi^2 = 3.918$, $p < 0.001$) (Fig. 4A).

Identification of Bacterial Taxa as Indicators of Developmental Stages

Between all developmental stages, 160 ASVs were significantly differentially abundant among life stages and assigned to 73 different genera (detailed information in Supplementary information S3; Table S3). These genera showed a clear clustered structure along the course of the different developmental stages of BSF with various genera being highly specific to one developmental stage (Fig. 4B).

Functional Predictions of Differentially Abundant ASVs Among Developmental Stages

Dissimilarities at the metabolic level were visualized through PCoA and CCA (Fig. S3 and Fig. 5A, respectively) (PERMANOVA: $R^2 = 0.159$, $p = 0.001$; CCA permutation test: $chi^2 = 0.14001$, $p = 0.001$). *PICRUSt2* and Kruskal–Wallis test inferred 188 metabolic pathways (detailed information in Supplementary information S3; Table S4) that were significantly different across BSF life stages according to K-W test among BSF development. These 188 *MetaCyc* third classification pathways were assigned to 27 classification pathways to reduce complexity, whose differences in abundance between the 4 developmental stages are shown in the heatmap (Fig. 5B). The most abundant second classification pathways found in BSF were "Cofactor, Carrier and Vitamin Biosynthesis" and "Amino Acid Biosynthesis." However, "Tetrapyrrole Biosynthesis" was found to be more abundant in adult stages while "Fatty Acid and Lipid Biosynthesis" was more abundant in eggs, "Amino Acid Biosynthesis" within larval stages, and "Glycolysis" during pupal stages.

Discussion

A 16S rRNA gene metabarcoding approach has allowed us to shed light on the core microbiota conserved along BSF life cycle; differences in bacterial composition and diversity between egg, larva, pupa, and adult stages; as well as the dissimilarities in potential functions of the bacterial communities of BSF across these life stages.

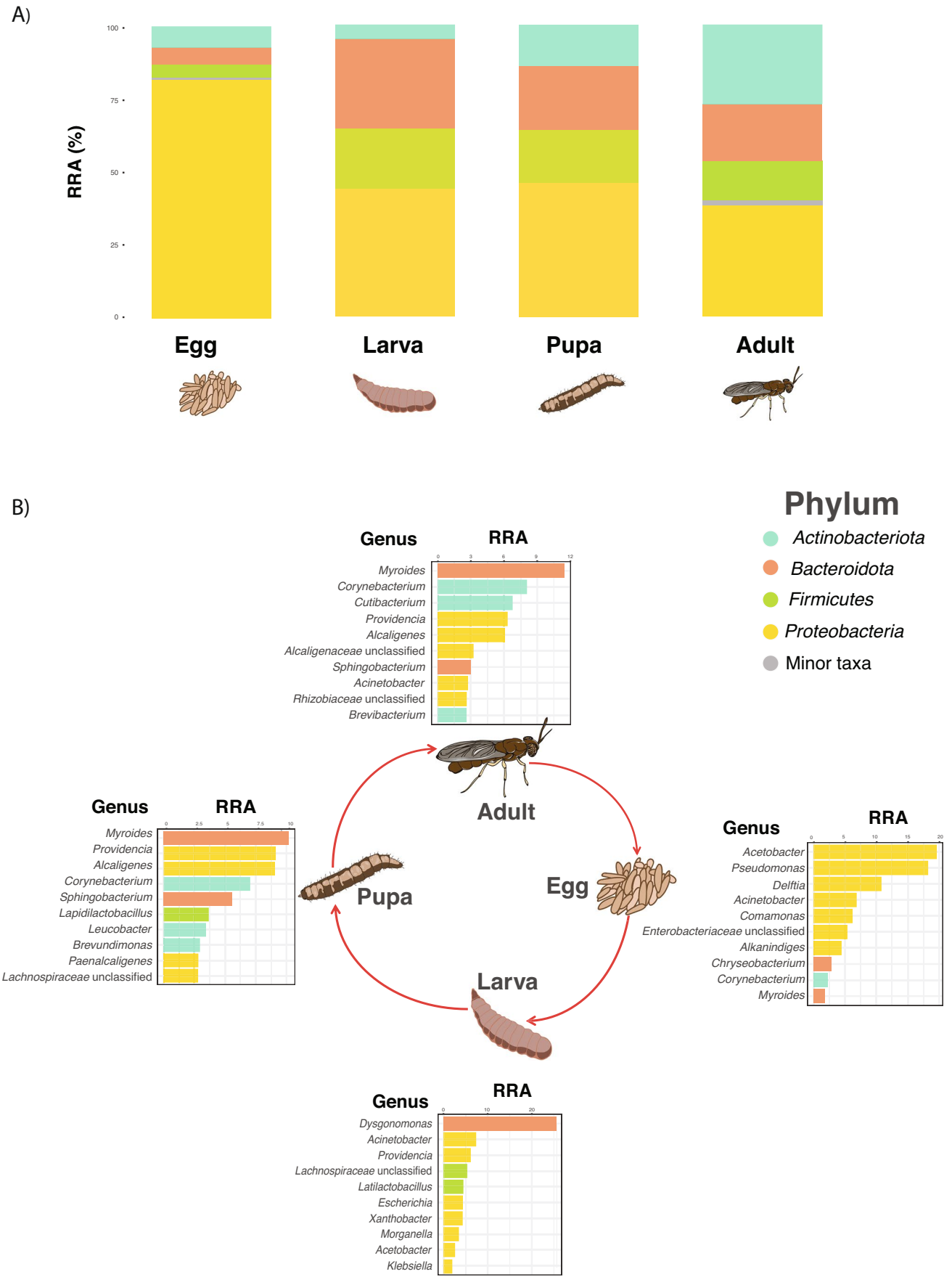
Core Microbiota Along BSF Life Cycle

BSF core microbiota along its life cycle and among two populations (bacterial taxa present in at least 80% of the sampling) was composed by nine ASVs which belonged to bacterial genera *Providencia*, *Alcaligenes*, *Escherichia*, *Latilactobacillus*, *Corynebacterium*, *Ochrobactrum*, and a member from the *Lachnospiraceae* family. Members of *Providencia*, which was already detected within BSF core microbiota [10], have been reported to contribute to the degradation of antibiotic bacterial residues [38] and plant cell walls [39]. Members of the genus *Providencia* have also been suggested to reduce development time on BSF, promoting its growth during pupae and eclosion of adult flies [40]. This goes in line with *Providencia* being more abundant within pupae in this study (Fig. 4B). Moreover, members of *Providencia* are known to be vertically transmitted in the Diptera species *Bactrocera dorsalis* [41]. Members of *Alcaligenes* may contribute to insect protection against certain entomopathogenic fungi [42]. The prevalence of these core bacteria and their potential functions described in previous works suggest that they may carry out essential functions for the fitness and development of BSF.

By looking at the core microbiota across all developmental stages, we obtained a different picture compared to previous studies only focusing on BSF larvae [10, 15, 18]. These differences not only confirm the role of development in structuring bacterial communities [5, 6] but also indicate that certain bacteria previously described as BSF resident taxa and confirmed by our study, such as *Morganella* or *Enterococcus* [14], are only prevalent among larval stage (prevalent ASVs in 80% within only the larval stage).

Moreover, the genus *Dysgonomonas* has been shown to be part of the core microbiota of BSF larva fed on different substrates [43]. This is consistent with the fact that members of *Dysgonomonas* are potentially involved in the function of degradation of plant material [14, 15, 41]. This goes in line with our results, in which *Dysgonomonas* was the most abundant genus along all the development (Fig. 2B) and differentially more abundant in larvae than other stages (Fig. 4B). However, when exploring the members of the core microbiota in our study, *Dysgonomonas* was neither part of the core nor prevalent within the larval stage (< 80%). This suggests that *Dysgonomonas* might be specific to larval stages and potentially environmentally acquired at each generation by the larvae and not a permanent resident of BSF along its development.

Our study raised the question of the transmission of the microbiota in BSF. Indeed, the existence of a core microbiota across the four main life stages of the insect suggests that some members could be resident of the BSF along the four developmental stages. It is also worth remarking that 5


A)
RRA (%)
100
75
50
25
0
Egg
Larva
Pupa
Adult
B)
Phylum
Actinobacteriota
Bacteroidota
Firmicutes
Proteobacteria
Minor taxa
Genus
RRA
Myroides
Corynebacterium
Cutibacterium
Providencia
Alcaligenes
Alcaligenaceae unclassified
Sphingobacterium
Acinetobacter
Rhizobiaceae unclassified
Brevibacterium
Adult
Egg
Larva
Pupa
Genus
RRA
Myroides
Providencia
Alcaligenes
Corynebacterium
Sphingobacterium
Lapidilactobacillus
Leucobacter
Brevundimonas
Paenalcaligenes
Lachnospiraceae unclassified
Genus
RRA
Acetobacter
Pseudomonas
Delftia
Acinetobacter
Comamonas
Enterobacteriaceae unclassified
Alkanindiges
Chryseobacterium
Corynebacterium
Myroides
Genus
RRA
Dysgonomonas
Acinetobacter
Providencia
Lachnospiraceae unclassified
Latilactobacillus
Escherichia
Xanthobacter
Morganella
Acetobacter
Klebsiella

◂**Fig. 2** Taxonomic composition of the BSF bacterial community across developmental stages (egg, larva, pupa, and adult). **A** Mean relative read abundance (RRA) in percentage of the main bacterial phyla. **B** Mean relative read abundance (RRA) of the 10 most abundant bacterial genera (colored by phylum)

ASVs, which are members of *Corynebacterium*, *Latilactobacillus*, *Providencia*, *Ochrobactrum*, and *Alcaligenes* (which is also a prevalent taxa within larval stage), have shown to be prevalent (> 0.8) in both populations 1 and 2 (detailed information in Supplementary information S3; Table S2B). This observation, even if not a direct proof, suggests that these taxa were potentially vertically transmitted, as they are conserved among both populations and the four developmental stages. Additionally, it reveals a certain stability of the gut microbiota and thus confirms the existence of a strong association between BSF and its microbiome [44]. In contrast, the remaining ASVs, such as members of *Escherichia*, that were not omnipresent in one population but were part of the core of population 1, may have been horizontally transmitted (i.e., environmental origin from the different enclosure). This study shows that some bacterial members are conserved along the course of BSF development, regardless of whether these bacteria are picked up from the environment or transmitted vertically [45]. Altogether, it suggests that the BSF microbiota might follow a mixed-transmission mode [46].

Development as Driver of Change of BSF Bacterial Communities

The development of BSF has shown to be responsible of switches in bacterial composition and diversity in each stage of the life cycle. We found a dynamic of change in ASV richness and diversity during BSF development (Fig. 3 A and B) in which both, richness and diversity, decrease from egg to larval stage while they increase from larval to pupal stage and, finally, decrease to its minimum in adults. Because pupae are sessile and vulnerable to numerous external threats, they require an immune machinery that may rely on host-microbiota interactions [20, 21]. As in all holometabolous insects, after a complex transformation, the pupa blooms into a flying adult [20, 21], while the metabolic waste from the pupa, which is called meconium, is secreted through the anal opening upon emergence [47], potentially decreasing the level of bacterial diversity within adults [47]. In addition, the excretion of meconium may prevent potential pathogens to infect the insect, and it may explain the decrease in bacterial diversity observed in adults. According to Moll et al. (2001) [22], such removal may happen in several holometabolous insects and specially in those where the larvae are generalist feeders, like in the case of BSF, as their diverse food would harbor a wide range of microorganisms.

Bacterial communities inhabiting BSF egg and larval stages are clearly distinct from each other and from those of pupal and adult stages, while these two mature life stages show an overlap (Figs. 2A, B and 4A). The CCA of metabolic pathways (Fig. 5A) also shows different functional potentials in eggs and larvae, and clear similarities between pupae and adults. Several bacterial taxa might have been acquired from the environment, as a consequence of larval feeding behavior, and others lost along the life stages. It also suggests potential functions which are may be more similar among mature stages than within eggs and larvae. Overall, our results suggest a congruence between taxonomic (Fig. 4A) and predicted functional patterns (Fig. 5A).

Proteobacteria clearly prevails throughout the life cycle, but it is more predominant in eggs (Fig. 2A), as observed in other insects such as the oriental fruit fly *Bactrocera dorsalis* [48]. In contrast, *Firmicutes* is less abundant in eggs (RRA = 4.6%) compared to the more mature developmental stages (Fig. 2A). For instance, members of *Firmicutes*, such as *Lacticaseibacillus*, whose lactic acid bacteria metabolism is known to be involved in the degradation of plant material [49], are more important in larvae (Fig. 4B). This goes in line with the capacity of BSF larvae to degrade a wide variety of substrates [10, 14, 15], potentially relying on bacterial taxa. Members of *Lactobacillus*, which belongs also to *Firmicutes*, are more abundant in adults (Fig. 4B), and several species within this genus are known to provide insects a certain degree of protection against pathogens together with digestion and immune-related functions [5, 50]. These differences in composition and metabolic pathways among BSF life stages emphasize that development may be one of the drivers of insect microbiome [5, 7]. This may indicate that BSF microbiota dynamically changes through BSF development and metamorphosis until the adult phase at least partially in relation to the host functional needs. Additionally, these changes in bacterial community composition might reflect changes in the physicochemical conditions experienced by each developmental stage (e.g., oxygen and hydrogen partial pressures, pH, and redox potential).

Bacterial Functions Along BSF Development

The results of the study suggest that bacterial community composition seems to fill the functional gaps of the insect along the BSF life cycle. Within eggs, lipids are the major source of energy for correct embryo formation [51], but the egg itself is only capable of synthesizing de novo 1% of the necessary lipids for early developmental processes. Some of the differentially abundant bacteria in eggs (Figs. 2B and 4B), such as members of the genera *Acetobacter* [52] and *Comamonas* [53], are potentially capable of making lipids accessible when needed for the host and accelerating insect development. Interestingly, the *PICRUSt2* analysis (Fig. 5B)

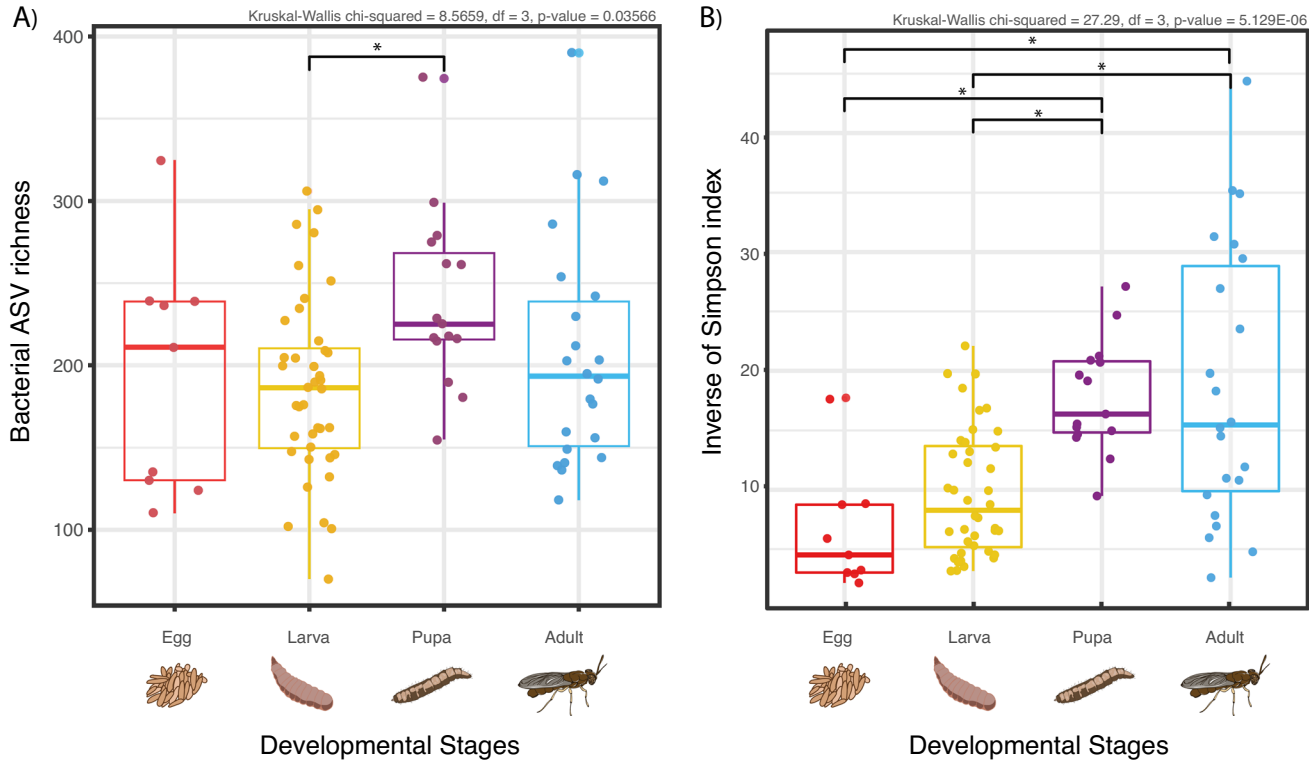


Fig. 3 Boxplot representing the **A** bacterial ASV richness and **B** inverse Simpson index along the course of BSF development (egg, larva, pupa, and adult). Kruskal–Wallis test results (global ASV richness fluctuations) are indicated in the top right of the plots. Moreover, post hoc (Dunn test) significance fluctuations among developmental stages are shown in both plots

found lipid biosynthesis to be significantly more abundant at the egg stage than in the other life stages. Regarding nitrogen (N) fixation, we identified several bacterial genera known to harbour diazotrophic species that were more represented in eggs (Fig. 4B). Among them, *Acinetobacter* [54] and *Azospirillum* [54] could help making N available for the biosynthesis of proteins, nucleotides, and other biomolecules essential for insect growth and development [54, 55]. This is supported by our analysis since "Inorganic Nutrient Metabolism" (which includes iron, manganese, N metabolism, among others) was inferred as significantly more important within eggs (Fig. 5B). Finally, it is worth mentioning that aside from its role in lipid metabolism, *Comamonas*, which was predominant in eggs (Fig. 4B), may also protect against oxidative stress [53].

BSF larvae, as several other insect species, rely on their associated microorganisms to successfully degrade organic waste. Together, they show a powerful detritivore machinery for the bioconversion of a wide spectrum of organic waste, and are able to convert waste into valuable biomass, which can then be used to produce biofuel, animal feed, or other bioproducts of industrial interest [10, 12, 14, 15, 43]. This biodegradation process is performed in association with several beneficial microbes [56, 57], such as presumably *Dysgonomonas*, members of the genera *Clostridium* [58] and the family *Lachnospiraceae* [59] (Fig. 4B), which may be able to degrade plant cell material. The *PICRUSt2* analysis, together with differential bacterial taxa distribution along the BSF life cycle, further emphasizes that the bacterial degradation of plant material is a key process for larvae, as the "Carbohydrate Degradation" and "Fermentation" metabolic pathways are significantly more displayed in larvae than in other stages (Fig. 5B). Larval capacity to degrade plant defensive compounds can also be suggested by increased secondary metabolites degradation processes. The *PICRUSt2* analysis also emphasizes the well-known capacity of BSF larvae to transform biowaste into valuable protein-rich food due to its larval high protein content.

As aforementioned, pupal stages represent a key phase in insect metamorphosis and adult differentiation. However, even if essential, metamorphosis is a challenging physiological process in which the insect undergoes a complete organ reorganization that can be challenging for the insect survival. In addition, BSF pupae are sessile, which makes them vulnerable to potential infections, predators, or parasites [20, 21] and, thus, in need of protection against potential threats and nutrient supply. They probably rely, in certain occasions, on bacterial communities to achieve

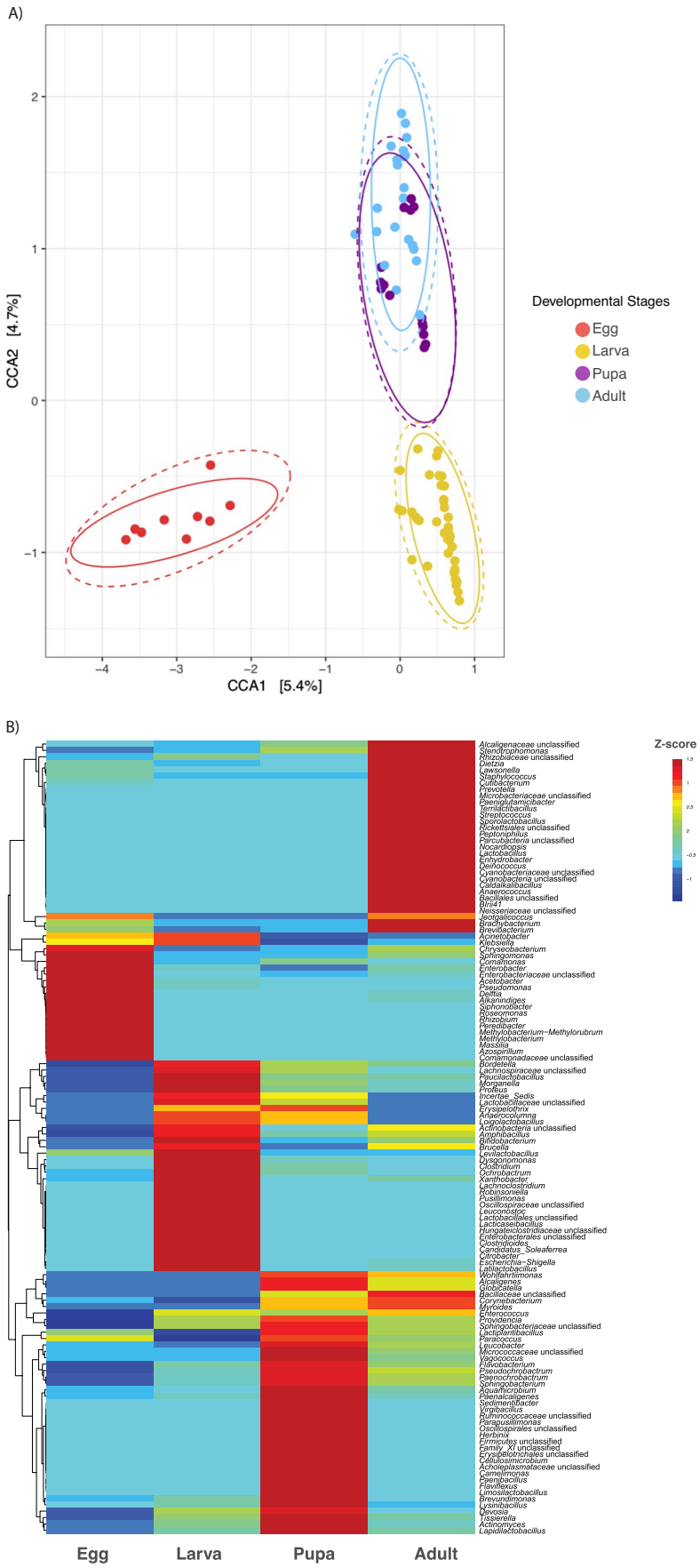
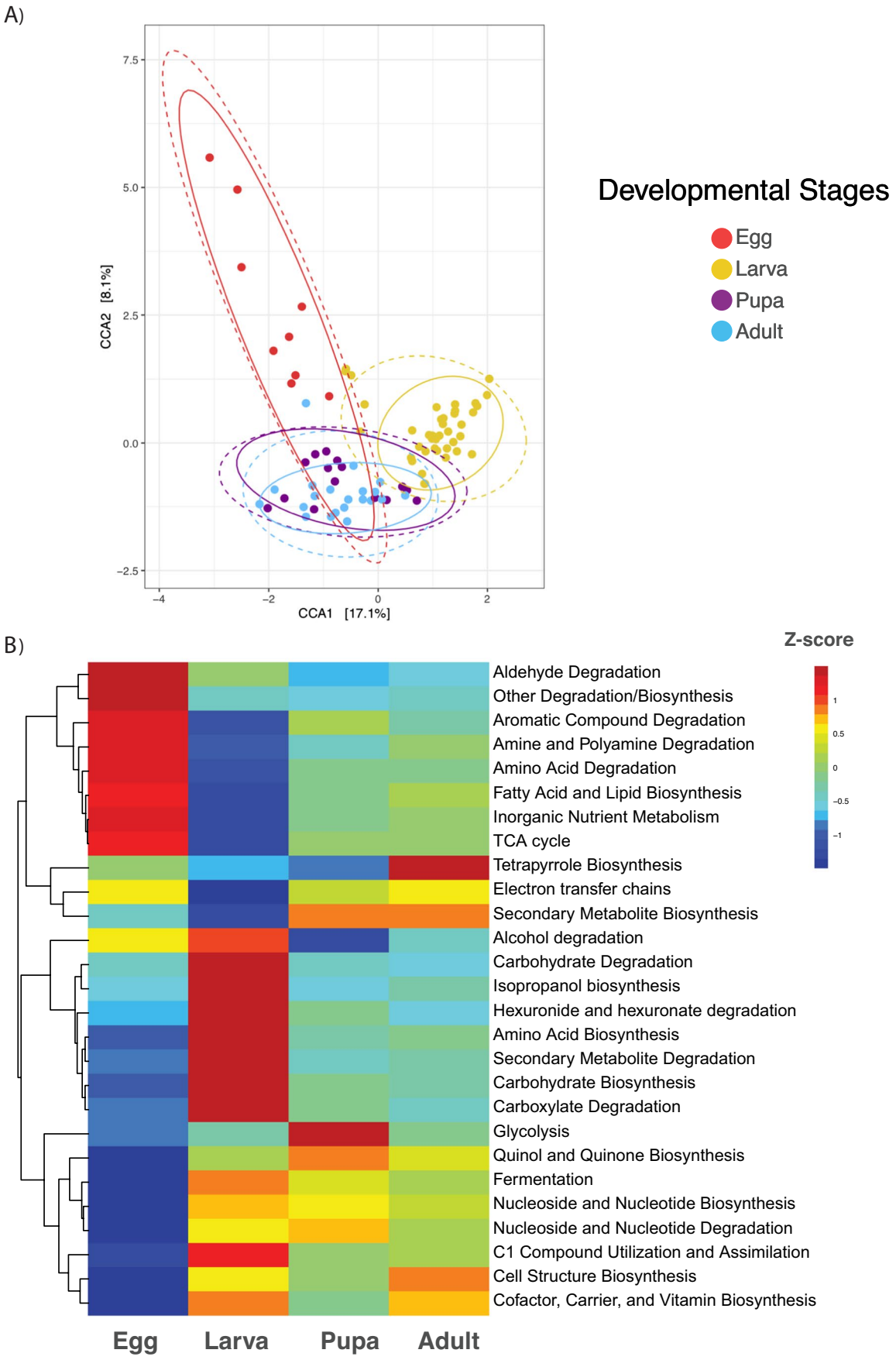


Fig. 4 **A** Canonical correspondence analysis (CCA) plot by developmental stage at ASV level. **B** Clustered heatmap of the *Z*-score of RRA from differentially abundant bacterial genera according to *DESeq2* analysis per developmental stage


A)
7.5
5.0
2.5
0.0
−2.5
CCA2 [8.1%]
−4
−2
0
2
CCA1 [17.1%]
Developmental Stages
Egg
Larva
Pupa
Adult
B)
Z-score
1
0.5
0
−0.5
−1
Aldehyde Degradation
Other Degradation/Biosynthesis
Aromatic Compound Degradation
Amine and Polyamine Degradation
Amino Acid Degradation
Fatty Acid and Lipid Biosynthesis
Inorganic Nutrient Metabolism
TCA cycle
Tetrapyrrole Biosynthesis
Electron transfer chains
Secondary Metabolite Biosynthesis
Alcohol degradation
Carbohydrate Degradation
Isopropanol biosynthesis
Hexuronide and hexuronate degradation
Amino Acid Biosynthesis
Secondary Metabolite Degradation
Carbohydrate Biosynthesis
Carboxylate Degradation
Glycolysis
Quinol and Quinone Biosynthesis
Fermentation
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Degradation
C1 Compound Utilization and Assimilation
Cell Structure Biosynthesis
Cofactor, Carrier, and Vitamin Biosynthesis
Egg
Larva
Pupa
Adult

◂**Fig. 5** **A** Canonical correspondence analysis (CCA) plot by developmental stage at differential abundance functional pathway (inferred by *PICRUSt2*) level. **B** Clustered heatmap of the *Z*-score of relative abundance from differentially abundant functional pathway (inferred by *PICRUSt2*) according to *DESeq2* analysis per developmental stage

these two goals [20, 21]. Consistently, in our study, the main change occurs between the larval to pupal stages with a switch in abundance, composition, richness, and diversity (Figs. 3A, B and 4B) and several bacterial taxa within pupae that could play diverse immunity and defence roles in the host were detected (Fig. 4B). For instance, a *Corynebacterium* strain is able to degrade carbaryl insecticide [60], while *Brevundimonas* strains can produce astaxanthin and other carotenoids, which are antioxidant protecting cells from potential damage [61]. Interestingly, members of the *Lactiplantibacillus* (family *Lactobacillaceae*) are also more represented in pupal stages and have shown the capacity to delay or even inhibit fungal infections [62]. The fact that *Lactiplantibacillus* is a lactic acid bacteria is consistent with "Glycolysis" being potentially more abundant within pupal stages as lactic acid fermentation involved the glycolysis pathway to produce lactate from glucose [63]. These potential protective functions relying in the pupal bacterial communities are consistent with the pathway "Secondary Metabolite Biosynthesis" being more abundant in this life stage and in adults, according to the *PICRUSt2* results. Secondary metabolites include those secreted by bacteria and having defensive functions in insects (e.g., piericidin, streptochlorin, diaphorin, or astaxanthin, which, as stated above, could be produced by member of *Brevundimonas*), protecting them against diverse threats [20, 21, 64]. Even though *PICRUSt2* is an adequate tool that has allowed us to discuss potential correlations between several bacterial taxa and potential functions, it is less accurate when using 16S rRNA gene metabarcoding approaches due to the short length of the fragment of the amplicon used. This type of analysis could be further strengthened by combining shotgun metagenomics with metatranscriptomic approaches, in order to confirm these potential bacterial functions among BSF life cycle by looking at gene expressions at the genomic level (i.e., mapping metatranscriptomic reads on metagenome-assembled genomes).

BSF adults, as for other insects, may not be able to synthesize all B vitamins, which play key roles as cofactor in several enzymatic reactions needed to achieve optimal fitness [65]. That may explain why the metabolic pathway "Cofactor, Carrier, and Vitamin Biosynthesis" is particularly important within larvae and adults (Fig. 5B). This hypothesis is further supported by the highest abundance of the pathway, "Tetrapyrrole Biosynthesis" in this life stage. Indeed, tetrapyrrole forms part of the chemical structure of vitamin B12 complex (i.e., cobalamin) [66]. In fact, members of the genus *Providencia* and *Corynebacterium* have been reported to synthesize vitamins [67, 68] (Fig. 4B). Even if it is more present in pupae, *Providencia* is maintained along the whole BSF life cycle and is part of the core microbiota, thus allowing for the synthesis of the vitamins at the adult stage when it is needed. *Corynebacterium* that is more present in adults can also contribute to meet the vitamin requirement of adults.

Numerous bacterial genera described among the BSF development have members reported to be opportunistic pathogens. Although these bacteria usually coexist with no detrimental impact in a fit host, they can become virulent in the case of a perturbation, such as a prior infection. Indeed since BSF is currently used for animal feed [10–12, 18, 43] and could be used as a sustainable alternative for direct human consumption in the future, the presence of potential pathogens can represent a food security issue. For instance, *Providencia*, whose proven benefits to the physiology and development of BSF have been largely described [10, 69], is also known to present strains that cause infections in humans, provoking diarrhea, or urinary track and eye infections [70]. In terms of risk assessment and food security, attention should be paid also to the presence of genera such as *Campylobacter*, even if its abundance is low (less than 0.5%), as it is an opportunistic pathogen of humans and food production animals [70]. Certain members of *Morganella* have also shown pathogenic activity [70]. Notwithstanding, members of *Morganella* have been shown to be beneficial for BSF [10, 69] rather than a pathogenic threat, as together with *Providencia* strains, they are known to have the capacity to secrete bacteriolytic enzymes that can degrade the cell walls of other pathogens, such as the opportunistic pathogens *Escherichia coli* and *Pseudomonas aeruginosa* [10, 69, 71]. In line with this, our results show that the presence and abundance of *Providencia* negatively correlate with the abundance of *Pseudomonas*, as this genus is only important in terms of abundance within eggs and its abundance decreases as the abundance of *Providencia* increases (Fig. 4B). The same dynamic is observed between *Morganella* and *Pseudomonas*, although the differences in abundance are less contrasted (Fig. 4B). In contrast, we did not find a negative correlation between *Escherichia* and *Providencia* or *Morganella*, as *Escherichia*, *Providencia*, and *Morganella* are all more abundant within larvae. *Escherichia* was most likely acquired from the feeding source and transmitted and conserved (in low RRA) throughout the BSF life cycle. This advocates for strict sanitary rules preventing BSF-feeding substrates to be contaminated by pathogens that larvae can acquire through feeding [70]. Although opportunistic pathogens represent a potential risk, a number of bacteria described in this study can provide resistance against pathogen infections and they may contribute to the

detoxification of environmental contaminants. Our results suggest that BSF has the potential to defend itself against potential threats thanks to high plasticity in the composition of its microbiota. This ability allows this insect to transform a wide range of substrates potentially containing high pathogen loads, which could be disadvantageous or even lethal for other insect species.

Conclusion

The fluctuations of bacterial communities through the BSF life cycle shed light onto the succession of metabolic pathways which are key for the insect development. This emphasizes the fact that development together with environmental conditions is responsible for microbial community switches in insects during its ontogeny. Effective microbial biotechnological approaches aimed to improve bio-waste conversion efficiency will thus require considering this to match the host functional needs though BSF development. Further research through shotgun metagenomics and/or metatranscriptomics is also needed to confirm the potential microbial functionalities inferred in this study as well as to characterize potential pathogens and evaluate their virulence capacities across the BSF entire life cycle.

Conflict of Interest

The authors declare no competing interest.

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Author Contribution All authors contributed to the study conception and design. The first draft of the manuscript was written by Marina Querejeta, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability Raw sequence data is available in the NCBI Sequence Read Archive under the BioProject PRJNA851044. ASV table and ASV sequences are provided in Supplementary information S4 (Tables S5 and S6).

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