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INVERTEBRATE MICROBIOLOGY

The Gut Microbiota Composition of the Moth *Brithys crini* Reflects Insect Metamorphosis

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Abstract

Lepidoptera is a highly diverse insect order with major importance in agriculture as many species are considered pests. The role of the gut microbiota in insect physiology is still poorly understood, despite the research undertaken in recent years. Furthermore, Lepidoptera are holometabolous insects and few studies have addressed the influence of the changes taking place on the gut microbiome composition and diversity during metamorphosis, especially in monophagous species. The V3-V4 region of the 16S rRNA gene was sequenced to investigate the microbiota composition and diversity of the monophagous moth *Brithys crini* during three different life stages: egg, larvae (midgut and hindgut), and adult (gut). Our results showed that the microbiota composition of *B. crini* was stage specific, indicating that the developmental stage is a main factor affecting the gut microbiome in composition and potential functions. Moreover, the diversity of the gut microbiome reflected the developmental process, since a drop in diversity occurred between the larval and the adult phase, when the intestine is completely renewed. In spite of the changes in the gut microbiota during metamorphosis, 29 genera were conserved throughout the three developmental stages, mainly belonging to the Proteobacteria phylum, which define the core microbiome of *B. crini*. These genera seem to contribute to host physiology by participating in food digestion, nutrition, and detoxification mechanisms.

Keywords Gut microbiome · Moth · Holometabolous development · *B. crini* · 16S rRNA sequencing

Introduction

The gut microbiota plays an essential role in animals' growth, development, and adaptation to their environment. In many insect species, it participates in promotion of growth, nutrient supplementation and recycling, resistance to pathogens colonization, production of signaling molecules, and diet/toxin breakdown [1]. The conditions and characteristics of the anatomy and physiology of the host are reflected in the intestinal microbiota and vary between groups of insects, areas of the gut, and stage of the lifecycle [2]. Lepidoptera is a highly diverse order of insects and the most important

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agricultural pest worldwide [3]. The gut microbiome across Lepidoptera is highly variable, and a recent review on the gut microbiota composition among 30 lepidopteran species showed that, in fact, only a few bacterial genera were widespread in all the species, such as *Pseudomonas* and *Enterobacter* (both from Proteobacteria phylum), and *Bacillus*, *Staphylococcus*, and *Enterococcus* (Firmicutes) [4].

As in most insects, the Lepidoptera larvae alimentary canal is divided into three tracts: foregut (stomodeum), midgut (mesodeum), and hindgut (proctodeum). Interestingly, the midgut has an alkaline pH, a large number of digestive enzymes and a peritrophic matrix, resulting in an environment that is difficult to colonize [1, 2]. Although several studies on lepidopteran larvae have found evidence of bacterial activity in the midgut [5–8], the hindgut—where enzyme activity is low and where nitrogen ions and residual metabolites are freely available—represents a more favorable environment for microbial colonization [2].

Some studies have found that microbial populations change during metamorphosis and have tried to address not only the variations in community composition but also the acquisition routes of gut bacteria, which could be horizontal, through diet, or vertical eggs [4, 9–12]. Lepidoptera are holometabolous insects and, consequently, after the last larval instar, the digestive system—especially the fore and hindgut—is completely reorganized, and its content is sometimes completely lost [13]. In addition, the whole system of a chewing phytophagous insect must be rebuilt to adopt the fluid-feeding strategy characteristic of adult lepidopterans. During metamorphosis, the pupa-adult content of the gut lumen and the peritrophic matrix are purged and differentiating pupal midgut epithelial cells synthesize and release antibacterial proteins into the lumen, including lysozymes to prevent septicemic infection [14]. A crop is generated from the foregut and a rectal sac is formed from the hindgut. In the posterior part of the pupa, a wraparound formed of the peritrophic matrix (meconium) is stored in the rectal sac [15] constituting 30% of the insect weight [13]. The meconium is reported to generate new organs for the adult [9, 16]. The results are drastic as follows: the foregut is replaced by the cibariopharyngeal pump, the esophagus, and the crop; the midgut retains its external appearance but is greatly reduced; the hindgut is proportionally longer with a remnant of the rectal sac [12, 17]. This filtering process represents a challenge for both parties, the microorganisms living in the gut and the host trying to retain beneficial bacteria.

Studies on the Lepidoptera gut microbiome have focused mostly on pest species with polyphagous diets. Diet has a huge impact on shaping the gut bacterial community, so one can expect to find microbiota variability between monophagous and polyphagous species [4]. However, the gut microbiome of monophagous species as *B. crini* has received little attention compared with the gut microbiome in polyphagous species [18]. The study of the *B. crini* gut microbiota is also interesting from the biotechnological viewpoint as this species is shows a highly destructive potential on its food plant (*Pancratium maritimum*), which is rich in toxic alkaloids [19]. Recent research has suggested that the microbiota participates in the detoxification process during digestion [8] and some closely related species are known pests [20].

Hence, the goals of this work are as follows: (1) to characterize the gut microbiota of a monophagous Lepidoptera using *Brithys crini* as a model, (2) to analyze the changes in the gut microbiota through three specific life cycle stages (egg, larvae, and adult), and (3) to compare the microbiota composition of the midgut and hindgut of the larvae.

Materials and Methods

Insect Collection and Dissection

Eggs of *B. crini* were collected by direct inspection of leaves of *P. maritimum* plants in dune system at Devesa del Saler Beach, València, Spain, at the end of summer (2016). At this location, the larvae are extremely common. Some eggs were placed in Eppendorf tubes of 1.5 ml for DNA extraction to analyze their microbial composition; others were incubated for rearing, at room temperature, in plastic containers with meshes for adequate ventilation. The emerging larvae were fed with leaves of *P. maritimum* obtained from the place of collection. At 15 days of egg hatching, some last instar larvae were used to analyze the midgut and hindgut bacterial composition. The rest of the larvae were left to pupate. One day after pupa emergence, adult guts were dissected; adults were unfed. A total of 15 individuals were selected for the study: 7 last stage larvae, 4 adults, and 4 eggs.

Last instar larvae were incubated for 5 min at 4 °C to reduce their mobility. They were sacrificed by decapitation, superficially cleaned with 90% and, for better dissection, conveniently secured with pins in dishes with paraffin. Dishes were previously sterilized in hood and disinfected with bleach and 90% ethanol. The digestive tract was removed under sterilized conditions; the midgut and hindgut were separated. The intestine was washed with distilled water, and the few plant residues were removed with sterile micro tweezers. The tissues were stored at − 20 °C for further processing. Moths (newly emerged from the pupa) were killed in killing jars, sexed, and similarly dissected. The rectal sac was dissected together with the hindgut under sterilized conditions. Ringer solution (9.1 g NaCl, 0.52 g KCl, 1.2 g $CaCl_2$, 0.8 g $MgCl_2$, 1 l H_2O) was used at the time of dissection in order to maintain organs structure avoiding osmotic stress. The tissues were preserved in Eppendorf tubes for DNA extraction at − 20 °C.

DNA Extraction and 16S rRNA Gene Illumina Sequencing

DNA extraction from tissue samples from eggs (E), larvae (M and H), and adults (A) was performed with the JetFlex ™ Genomic DNA Purification Kit. The DNA quality was checked on a 0.8% (w/v) agarose gel and quantified with Nanodrop-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

All DNA samples were adjusted to the same concentration and the V3-V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with 16SrRNA Illumina sequencing standard primers (forward primer: 5′-CCTA CGGGNGGCWGCAG-3′ and reverse primer: 5′-GACT ACHVGGGTATCTAATCC-3′). For each sample, a 50 μl PCR mix was prepared, containing 0.25 μl of Buffer Taq with MgCl2 (10× 200 mM Tris HCL, 500 mM Kcl, supplied with 1 ml of 50 mN MgCl2), 1 μl of dNTPs (10 mM), 1 μl of each primer (10 mM), 0.25 μl of Taq DNA polymerase (5u/μl), 40.75 μl of nuclease-free water, and 2 μl of DNA template. PCR conditions were 95 °C for 4 min followed by 30 cycles of 94 °C for 50 s, 58 °C for 30 s, and 72 °C for 1 min 30 s and a final extension step of 10 min at 72 °C. A positive control (DNA from the cockroach *Blatella germanica* intestine) and a negative control (PCR mix without DNA) were included. All the amplicons were checked by electrophoresis in agarose gel (0.8%) and sequenced with the Illumina MiSeq technology 300 bp (MiSeq Reagent Kit v3) at the sequencing service of the Foundation for the Promotion of Sanitary and Biomedical Research (FISABIO).

Sequence Data Processing

The sequences were filtered by quality using the prinseq-lite tool [21], a Phred quality cutoff value of 20, and a minimum cut length of 50 nucleotides were used for both strands. Forward and reverse reads were joined using FLASH [22]. Unpaired forward sequences and the paired sequences were concatenated into a single file. The selection of operational taxonomic units (OTUs) was performed with USEARCH by clustering to a 97% identity with -usearch_global [23]. Sequences with low quality were removed with the -fastq_filter command. Unique sequences were selected by avoiding singletons with -fastx_uniques. For the detection and elimination of chimeras, the command -unoise2 was used. The taxonomic assignment was made with the Bayesian RDP classifier [24] that uses as reference the Ribosomal Project Database (RPD) [25].

Ecological and Statistical Analyses

The core microbiota of *B. crini* was determined as the taxa present in all of the stages and in at least three samples from each stage. It was calculated using the QIIME script compute-core-microbiome.py [26] and represented in a Venn diagram by using R.

Rarefaction curves were plotted to check for the sequencing effort and to estimate the threshold of sequences to perform the diversity analyses (30,000 reads). Alpha and beta diversity analyses were performed using QIIME [26]. Richness estimators Chao1 and OTUs observed, as well as diversity indexes Shannon and Simpson, were calculated after resampling with the same number of sequences per sample to avoid bias for differences in depth of sequencing between samples. Rarefaction curves were plotted to estimate the number of taxa in the different stages. A Wilcoxon signed-rank test was applied to compare the alpha diversity metrics between stages.

To study the differences in microbial communities between samples and correlation with the developmental stage, a non-metric multidimensional scaling analysis (NDMS) was performed using Bray-Curtis and Weighted Unifrac distances. In order to test the effect of the stage on the variation in the microbial composition, a non-parametric permutation analysis (Adonis) was carried out with 999 permutations. We also estimated the beta diversity metrics: global β-diversity (βsor), nestedness (βnes), and turnover (βsim) by using the package "betapart" of R [27]. The β-diversity (βsor) can be partitioned in two components: βnes and βsim that measure dissimilarity due to the effect of nestedness (this occurs when a microbial community is a subset of a richer microbial community) and turnover (this occurs when there is a replacement of some species by others as a consequence of environmental and/or historical constrains) [28]. A clustering analysis based on Bray-Curtis dissimilarity was also performed to show the distances between the different groups. These functions are implemented in the R package "Vegan" [29]. The linear discriminant (LDA) effect size (LEfSe) analysis was applied to identify those taxa with statistically significant different abundances between groups of samples [30]. We considered taxa as discriminative of groups when the LDA score was higher than 2 and p value < 0.05.

Co-Occurrence Network Analysis Based on Bacterial Relative Abundance

We estimated a co-occurrence network of the bacterial composition using the SparCC software [31]. The correlation coefficients were estimated from the OTU-rarefied abundance tables collapsed at genus level (100 iterations). To estimate the "two-sided" p values, 500 bootstraps were used. We considered significant correlations those with a p value lower than 0.01. We used the igraph package implemented in R software to calculate (Newman-Girvan algorithm) and display the network (force-directed layout option) [32].

Prediction and Statistical Analysis of Potential Functions

We used the software phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) to predict computationally the genomic content (based on genomes available in databases) from the 16S rRNA data [33]. We used the GreenGenes database (version 13.5) implemented in the Galaxy Server (http://huttenhower.sph.harvard.edu/galaxy/) and the KEGG Orthologs database for predictions. To analyze the differences in functional profiles between the different stages, we performed an NDMS analysis and the Adonis test (999 permutations) as for the taxonomic analysis. The linear discriminant (LDA) effect size (LEfSe) analysis was also applied to identify those functions statistically significant different between groups of samples [30]. We considered functions as significant when the LDA score was higher than 2 and p value < 0.05.

Results

Composition and Diversity of the Gut Microbiota of *B. crini*

A total of 22 samples were sequenced by Illumina obtaining an average of around 133,000 reads per sample and 131,000 after the quality control (detailed information per sample in the Table S1). The *B. crini* microbiota consists mainly of members of the phyla Proteobacteria, Bacteroidetes, and Firmicutes (Fig. S1). However, the distribution of these phyla varies during development. The egg microbiota contains mostly Proteobacteria (92% on average) and in second place Bacteroidetes (7%). The larval midgut and hindgut microbiota showed similar phyla composition enriched not only in Proteobacteria (52%) and Bacteroidetes (36%) but also in Firmicutes (10%). Interestingly, the adult gut is dominated again by Proteobacteria (96%) followed by Firmicutes (3.7%), whereas Bacteroidetes is almost absent (< 0.1%).

When we examined lower taxonomic levels (Fig. 1a), we found that the most abundant genera in eggs were *Rosenbergiella* (27%), *Serratia* (16%), and *Acinetobacter* (13%), all of them are Proteobacteria (see the relative abundance of all the samples at genus level in Table S2). In larvae midguts, two genera, *Empedobacter* (Bacteroidetes) and *Klebsiella* (Proteobacteria), were the most abundant (22% and 16%, respectively). The same genera and similar values were identified in larvae hindguts (M and H), *Klebsiella* (21%) and *Empedobacter* (21%). An important change was observed in adults, where the most abundant genus was *Serratia* (Proteobacteria) with a high abundance in three of the samples, representing more than 80% of the relative abundance. However, sample A3 showed a different profile with a higher representation of *Providencia* (80%), another Proteobacteria genus, whereas Serratia was only 14%. The overall difference in bacterial composition between stages was evident; however, larvae and eggs showed greater similarity in contrast to adults.

In total, 26 genera were detected in 100% of the samples constituting the core of *B. crini* (Fig. 1b). From the 26 common genera, 20 belonged to Proteobacteria and six to Bacteroidetes phyla. When we used the criterion of considering common genera as those with a representation of at least three replicates in all stages (see M and M), three more genera were added to the core: *Pantoea* (Proteobacteria), *Ralstonia* (Proteobacteria), and *Staphylococcus* (Firmicutes).

Structure and Interaction of the Gut Microbiota of *B. crini*

To identify potential beneficial relationships between bacteria in the gut microbiota of this moth, we performed a co-abundance network analysis based on genus composition of the samples (Fig. 2). We identified four clusters formed by a total of 25 genera (one independent cluster and three interconnected ones). Interestingly, most of the genera (20) identified in the network belonged to the core microbiota (Fig. 1b). The network (similar to the core microbiota) was enriched in Proteobacteria (20 genera), but Bacteroidetes (4) and Firmicutes (1) were also identified. Microorganisms that are connected in abundance networks might be keystone species in the ecosystem, since various members of the community depend on them. In this network, the most connected ones (9 connections) found were *Ochrobactrum* and *Acinetobacter*, followed by *Empedobacter* (8), and *Pseudochrobactrum*, *Comamonas*, and *Klebsiella* with seven edges.

Comparison of the Gut Microbiota Composition of *B. crini* at Different Developmental Stages

The gradually flattening rarefaction curves showed that a great number of bacterial species in all stages were captured with the chosen sequencing depth especially for the adults which were the closest group to reach the plateau (Fig. 3a). However, at OTU level, a fraction of the diversity of the egg and larvae microbiota remained undescribed. Both, egg and larvae samples had the greatest richness of phylotypes, while adult samples showed a considerable reduction. The egg microbiota showed the higher number of OTUs, followed by the hindgut with the midgut in last place (Fig. 3b; Table S3). Chao1 and observed OTUs richness estimators (richness), Shannon and Simpson diversity indexes (evenness), showed that the diversity of adult samples is significantly lower than in the other stages (p value < 0.05). There were no significant differences in diversity metrics between egg and larvae samples (p value > 0.05) nor between hindgut and midgut (p value > 0.05).

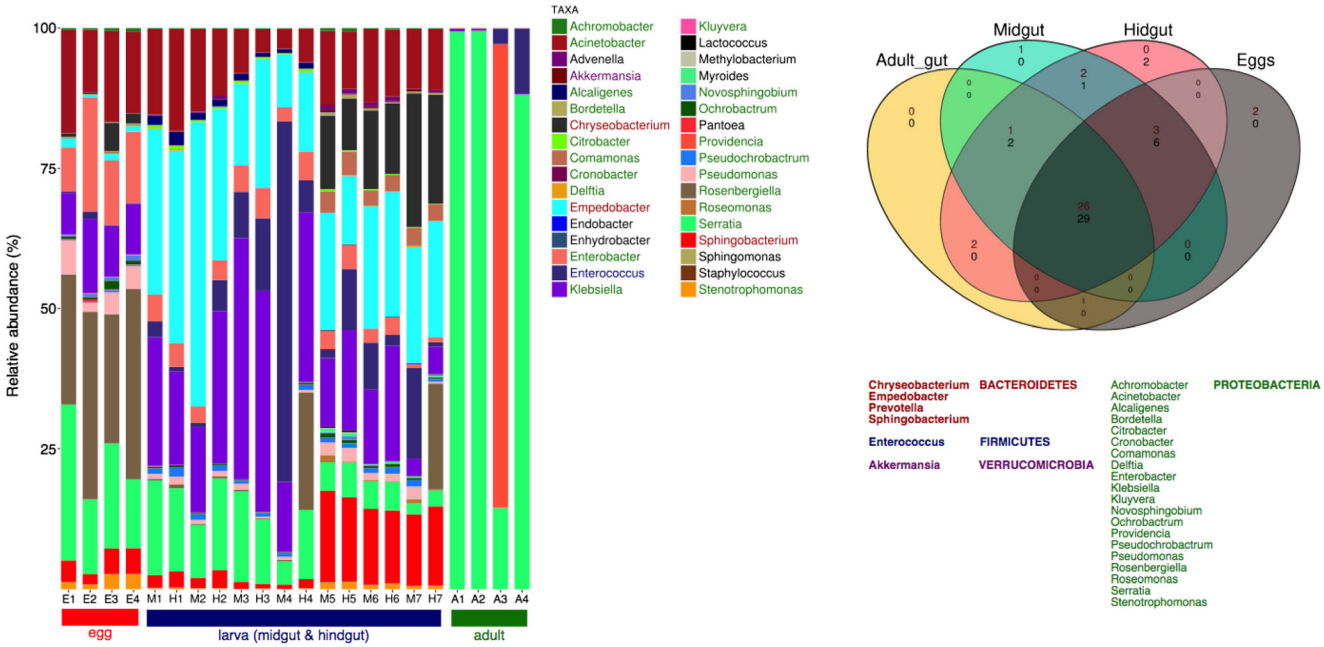

Fig. 1 Gut microbiome composition of the moth *B. crini*. **a** Relative abundance of the most abundant genera identified in the samples. The composition of each sample is based on the taxonomic assignment of the 16S rDNA sequences. **b** Core gut microbiota of *B. crini*. The number of common bacteria between the different groups (egg, midgut, hindgut, adult gut) is represented in a Venn diagram. The core genera and their corresponding phyla are also indicated with colors for the **a** and **b** figures

We performed a NDMS analysis (stress value = 0.09) based on Bray-Curtis dissimilarity to analyze the differences in the microbiota composition between the four groups (egg, larval midgut and hindgut, and adult) of samples (Fig. 4). The analysis showed that the samples were grouped according to their stage, indicating that the *B. crini* gut microbiota is stage specific. Both larval midgut and hindgut cluster broadly overlapped. The adult cluster showed the more different composition compared with the eggs and larvae microbiota. To test these observations (composition variation according to life stages) statistically, we ran an Adonis variance analysis based on the Bray-Curtis matrix. The test showed that 58.22% of variability could be explained by life stage (p value = 0.001). We performed the same analysis (NDMS) based on the weighted Unifrac distance matrix and we observed a similar result, obtaining three composition-based clusters (egg, larvae, and adult) (Fig. S2). The Adonis variance test based on this distance proven to be also significant as with the Bray-Curtis matrix (p value = 0.005). Considering the Unifrac distance, the midgut and hindgut also overlapped. This analysis showed that there is a phylogenetic component between the egg and adult stages since they were not so different in composition as when we considered the ecological distance (Bray-Curtis).

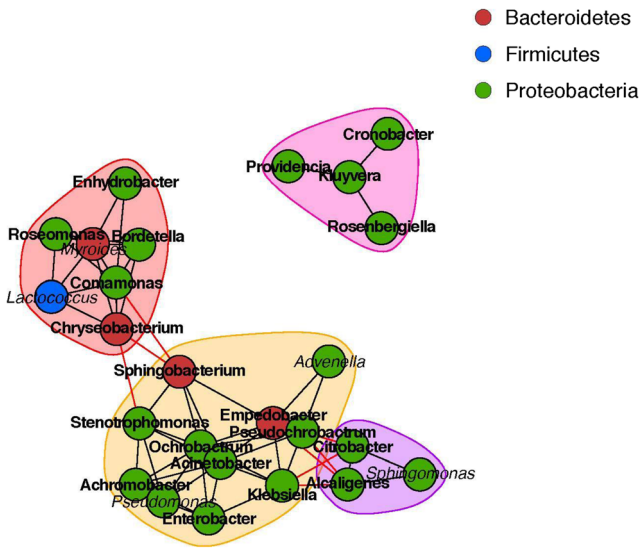

Fig. 2 Co-occurrence network of the gut microbiome of *B. crini*. The network is based on the OTU-table collapsed at genus level and only positive associations are shown (p value < 0.01). The nodes represent the different bacterial genera and are color coded by phylum. Genera belonging to the core microbiome of *B. crini* are written in bold and non-core bacteria in italics

We estimated the beta diversity parameters βnes and βsim to measure the contribution of "nestedness" and "turnover" in explaining the species assembly process of the gut microbial community of *B. crini* along the development. The nestedness (βnes = 0.2) explains more than 70% of the global beta diversity (βsor = 0.28), while the turnover contributed less in the process (βsim = 0.08). In fact, we observed in general a loss of species from the larvae stage to the adult, while there is not a high gaining of species during the adult stage analyzed.

A clustering analysis based on bacterial composition and abundance of all the samples showed that the distances

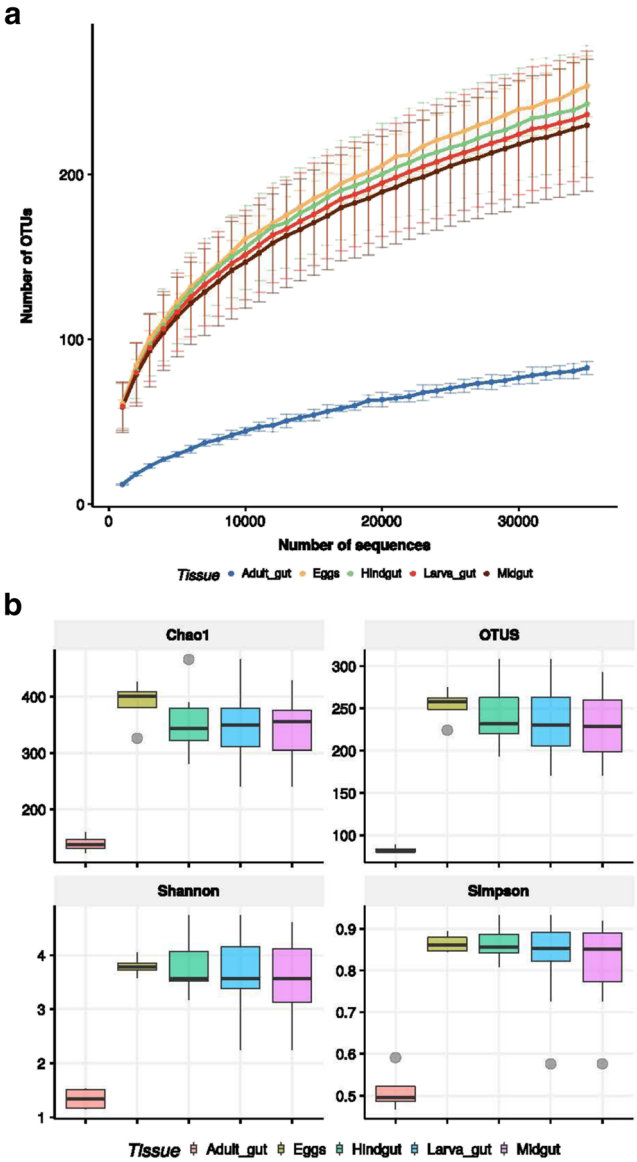

Fig. 3 Alpha diversity of the gut microbiota of *B. crini* at the different developmental stages. **a** Rarefaction curves based on the number of OTUs of the egg, midgut, hindgut, and adult gut samples. **b** The diversity metrics Chao 1 richness estimator, number of OTUs, the Shannon, and the Simpson indexes were estimated for adults, eggs, midguts, hindguts, and larvae (midgut and hindgut together)

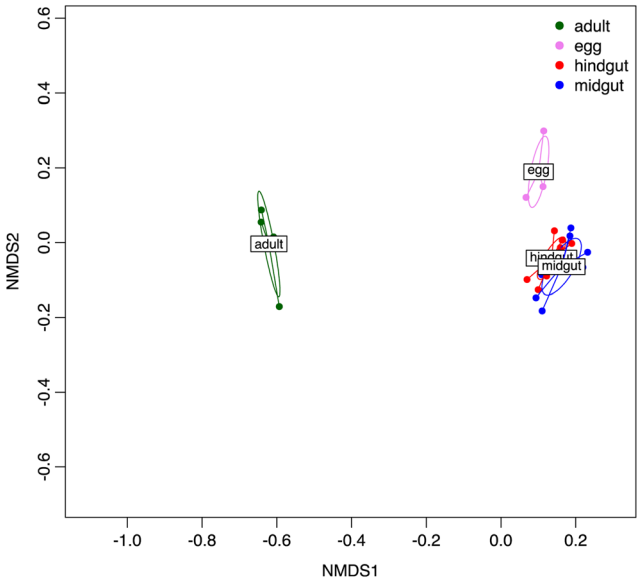

Fig. 4 Comparison of the microbial composition of the different metamorphic stages. NMDS based on the gut microbiota composition of *B. crini*. The samples were clustered by developmental stage in different colors: egg (pink), midgut (blue), hindgut (red), adult gut (green)

between eggs and larvae were lower than they were for adult, reaffirming that this last stage, besides having the greatest variability within individuals, is the one that differs most with respect to the rest (Fig. S3). Interestingly, in the larval group, the midgut and hindgut bacteria taken from the same organism did not cluster; all the samples from midgut and hindgut were shuffled up in the cluster. This result can be explained by the different properties of these two parts of the intestine (midgut and hindgut) and also by inter-individual variation.

We also analyzed the distances between replicates and between different tissues (12 comparisons), and found that the replicates of a given stage are more similar to each other than to the rest of the samples (Fig. S4). This result confirms that the composition of the microbial community is more similar within stages and more varied between stages (Table S4). The variability observed in adult gut replicas is the consequence of A3 sample where *Providencia* appears as the most representative genus while the other samples are dominated by *Serratia*.

Having found that the composition was stage specific, we looked for those bacterial genera associated with the different stages. The midgut and hindgut are different regions of the gut, but they were examined during the same stage. Previous analyses showed that they harbor a similar composition. However, to check whether they differ in specific genera, we compared them statistically by using LEfSe [30]. Only one genus (*Endobacter*) resulted significantly more abundant in the midgut region. However, *Endobacter* is a genus contributing to less than 0.5% of the relative abundance for both groups. Thus, we concluded that they could be treated as just one group (larvae).

We then compared egg and larvae composition to identify bacteria gained or lost during the transition from egg to larvae. We found 12 genera more abundant in the egg microbiota and nine in the larvae (Fig. S5A). Some of the most representative genera in the egg microbiota were highly abundant in the samples, such as *Rosenbergiella* (average of 28%), *Serratia* (18%), *Acinetobacter* (15%), or *Enterobacter* (13%) (Fig. 5). The most abundant genera associated with the larvae microbiota were *Empedobacter* (23%) and *Enterococcus* (10%). We also analyzed how the change from larvae to adult (a complete renewal of the intestine) is reflected in the gut microbiota of *B. crini*. The diversity greatly decreased during this change,

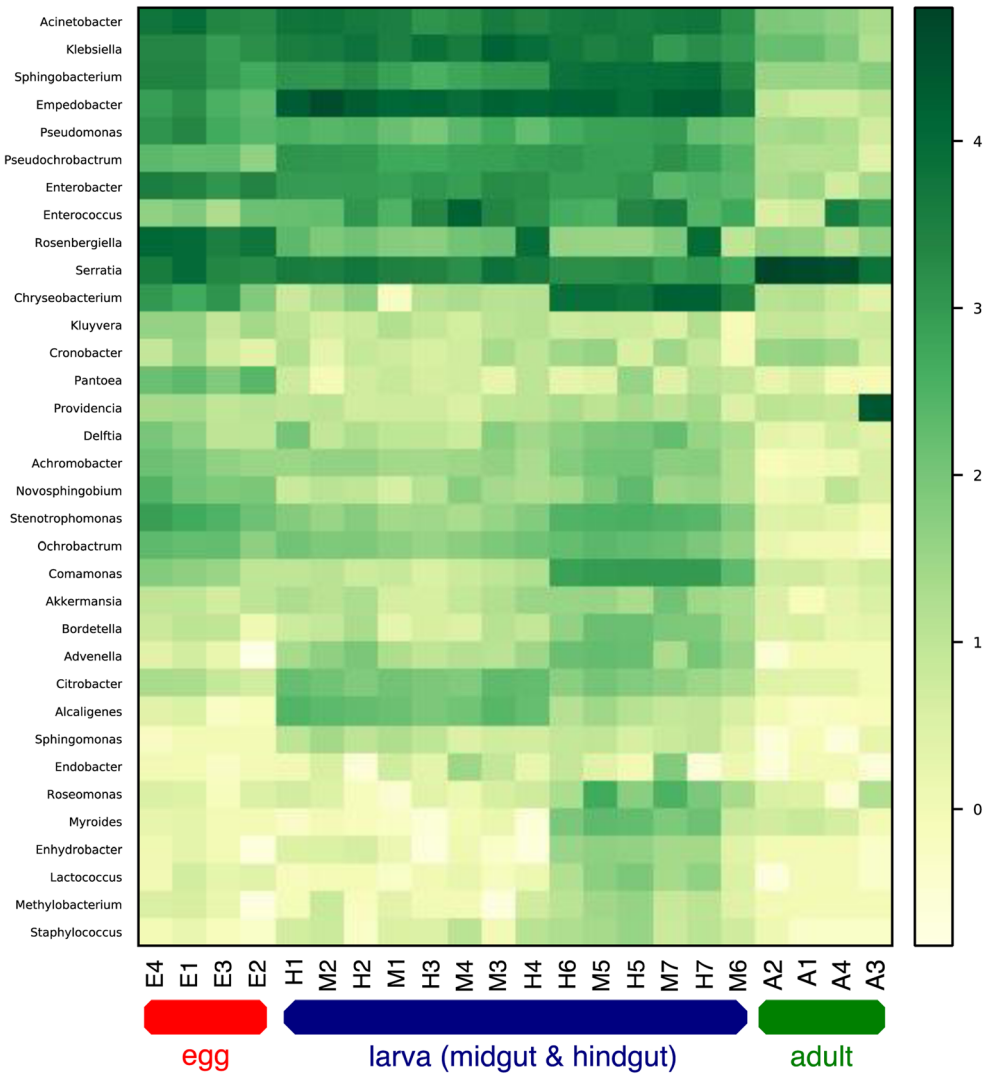

Fig. 5 Heatmap based on taxon composition and abundance of the gut microbiota of *B. crini* considering all the samples. Heatmap color scale represents the proportion of sequences assigned to the taxa

27 genera were statistically more abundant in the larvae while only one genus (*Serratia*) was significantly more abundant in the adult (Fig. S5B). *Serratia*, with a mean of 75% (96% if we exclude A3), appears as a pioneering species in the recolonization of the gut (Fig. 4). Finally, the comparison between adult gut and egg showed a similar result to that of the larvae-adult comparison, with only *Serratia* being overrepresented in the adult gut while 24 genera were more abundant in the egg (Fig. S5C).

Comparison of the Microbiome Potential Functions of *B. crini* at Different Developmental Stages

We used the PICRUSt software to predict the metagenomes (based on KEGG database) of the samples and to find those functions associated with each stage. We performed a NDMS analysis based on the obtained KEGG functional profile (Fig. S6) and we found a similar pattern as the one based on the taxonomic composition, although the differences between clusters were less strong (Adonis test not significant, p value > 0.05).

Despite the fact that overall, there are not significant differences between clusters we looked for those specific functions explaining the difference observed in the NDMS analysis by using the LEfSe test [30]. Similarly, to the taxonomic result, we did not find statistical differences in the potential metagenome between the midgut and hindgut, thus considering both as the group "larvae." We compared the egg and the larvae groups and we identified 32 KEGG pathways associated (more abundant) with the egg stage and 62 to the larvae stage (Table S5). The main roles of 13 of the 32 pathways more abundant in the eggs have unknown functions, while 45 of the 62 more abundant in larvae are related to the bacterial metabolism, including the metabolism of amino acids, carbohydrates, cofactors, vitamins, or terpenoids and polyketides, and different xenobiotic degradation processes. When comparing larvae and adult samples, more functions (58) were overrepresented in the larvae compared with the

adult (23) mainly related again to metabolism, highlighting xenobiotics processes including degradation of some toxic compounds as benzoate, naphthalene, atrazine, bisphenol, ethylbenzene, polycyclic aromatic hydrocarbon, or styrene compounds (Table S6). We finally performed the egg-adult comparison and we found 16 KEGG pathways overrepresented in the adult while 30 were more abundant in the egg (Table S7). In this case, also the adult showed less activity regarding the degradation of xenobiotics. The functional profile of adults in correspondence with the taxonomic results (low diversity) showed a less diverse repertoire of functions compared with the egg or larvae stages.

Discussion

B. crini is a holometabolous insect whose monophagous larvae feed on *P. maritimum*, a plant that produces toxic compounds with antimicrobial activity [8, 34]. In contrast with other oligophagous and polyphagous lepidopteran, we found that *B. crini* harbors a richer and more diverse microbiota (34 genera identified) [7, 9, 10, 35, 36]. We would have expected that species having a wide range of food plants would host a large set of bacteria able to degrade a huge variety of different nutrients in a potentially open habitat. By contrast, species with a restricted diet would host a lower set of bacteria considering the presence of few ecological niches, especially in the case of a toxic plant. However, our results suggest that having a specific but nutritionally poor diet is connected with a diverse microbiota. We conjecture that this restricted diet requires a greater number of organisms to complete the metabolic pathways necessary to supply the demand for essential nutrients.

We have studied the microbiota composition in three different morphological stages: eggs, larvae, and adults, and observed combinations of microorganisms and potential functions associated with the different stages. A similar result was found during the development of the Lepidoptera *S. littoralis* [9]. In the three stages, the most abundant phyla proved to be Proteobacteria; however, the most abundant genera belonging to this phylum varied between the different stages. Similarly, other Lepidopteran microbiomes seem to be dominated by Proteobacteria species [4, 35–37]. In addition to this phylum, the larva and the eggs harbor members of the Bacteroidetes phylum, which are practically absent in adults. Firmicutes was found to be the third most abundant phyla found in larva and adults, but not in eggs. Nevertheless, this is the first study in *B. crini* to identify 26 bacterial genera (mostly Proteobacteria) present in all the stages analyzed, which constitute the core microbiota (29 with a more relaxed criterion). In spite of the low number of samples analyzed in this study, it seems that this core of bacteria might play an important role in *B. crini*. Because these 26 taxa were present in all three stages, there are two possibilities regarding its acquisition: (i) some of them are normally present in the insect's environment and are acquired by the egg when it is in contact with the plant or (ii) some are transferred to the egg by the mother to ensure the transmission to the offspring. Microorganisms able to colonize the gut of a holometabolous organism like this moth must have co-evolved with its host to survive the changes associated with metamorphosis. It is worth mentioning that genera composing the core such as *Enterobacter*, *Enterococcus*, or *Acinetobacter* have also been identified in the gut microbiome of a variety of Lepidoptera [4, 10, 37–39].

A particularly interesting result was that 20 of the 26 genera forming the core participate in the four clusters obtained in the co-occurrence network analysis, which suggests a cooperative interaction between them. In fact, the most connected ones in the network (*Ochrobactrum*, *Acinetobacter*, *Empedobacter*, *Pseudochrobactrum*, *Comamonas*, and *Klebsiella)* belong to the core. Thus, the putative association of these genera indicates that they might contribute to the stability and resilience of the whole microbial community, playing a role in restoring the microbiota, for example, after the complete renewal of the gut during metamorphosis. In total, of the 25 genera that were positively associated, 20 belong to the phylum Proteobacteria. Co-occurrence of phylogenetically related microorganisms suggests cooperation and/or sharing the same biological niche, thus explaining that most of them are present in the different developmental stages.

Some of the core genera we identified have been associated with functions that could benefit the host. For example, *Enterobacter* spp. and *Enterococcus* participate in detoxifying plant toxins and overcoming plant defenses in different Lepidoptera, reviewed by Paniagua Voirol et al. [4]. Confirming the importance of some of these widespread genera in Lepidoptera such as *Enterobacter* or *Acinetobacter* is the fact that they were identified in the network as "keystone" bacteria, which indicates that they might play a central role in the stability of the microbial community in the Lepidoptera gut.

From the bacterial core, *Rosenbergiella* species was highly abundant in eggs. This genus is present in flower nectar [40]. Its overrepresentation in eggs could be due to the fact that this bacterium is abundant in *P. maritimum* and may adhere to the eggs when they are in contact with the plant. We speculate that it might be linked to the degradation of phenolic compounds, as it is the case of some other members of the family Enterobacteriaceae [41] such as *Enterobacter*, which is also an important genus during the egg stage. In fact, the biphenol degradation pathway was more abundant in the egg and in the larvae; this genus was more abundant compared with the adult stage. The mechanism through which the egg acquires the microbiome is still unknown. However, the presence of core bacteria in all the stages suggests vertical transmission as a

possible via, although the eggs may also acquire bacteria from the environment through contact between the egg surface and the adults through the genital tract, as occurs in other insects [9].

A recent study by Hammer et al. [11] indicated that the caterpillar or lepidopteran larvae do not have a resident gut microbiome. However, we found a conserved and diverse gut microbiota in the seven examined gut larvae (seven hindgut and seven midgut) of *B. crini* and a large proportion seemed to be shared with the egg. This indicates that a set of microorganisms are conserved between the transition from the egg to the larval stage. Among them, *Empedobacter* is a core genus that is significantly associated with the larval stage, which is related to fiber feeding in the cockroach *Periplaneta americana* [42]. *Enterococcus* and *Klebsiella*, also highly abundant in the larvae, have been found in the butterfly *Heliconius erato* [10] and in the moth *Busseola fusca*, where it produces enzymes for the digestion of plant-derived compounds such as lignin, cellulose, and xylene, which may aid in host nutrition [35]. Interestingly, *Enterococcus* was also more abundant in the larvae of the moth *S. littoralis* [9]. The larval stage is the main feeding stage of moths [43], thus it is not surprising to find bacteria involved in food digestion. This was confirmed with the predicted metagenome potential functions, since metabolic pathways involved in the metabolism of many essential molecules related to energy (carbohydrates, vitamins, amino acids, etc.) even those involving xenobiotic and toxic compounds, were more abundant compared with the adult. In addition, some species of *Klebsiella* have the ability to fix nitrogen, possibly playing the same role during this stage.

Other important core genera and predominant taxa found in the adult gut microbiota was *Serratia*. This genus is composed of many species present in several hosts including humans and insects [44, 45]; the latter is characterized by the production of a red alkaloid pigment known as prodigiosin [46]. One of its representatives is *S. marcescens*, which has been found in the lepidopteran Noctuidae *Heliothis virescens*. When this bacterium is found in the digestive tract in small amounts, it is not pathogenic; however, when it invades the hemocoel, it is detrimental to its host [45]. The strain *S. marcescens* UFPEDA 398 produces alkaloids that have antimicrobial activity [47], which may be beneficial to the host. On the other hand, *Serratia* is known to degrade aromatic compounds such as purine alkaloids [48, 49]. In *B. crini*, it was observed that a red pigment was stored in the rectal sac, possibly the product of species belonging to *Serratia*. Furthermore, as the diet of *B. crini* is high in aromatic compounds (alkaloids Amaryllidaceae), this genus may provide the necessary pathway for its degradation or decomposition in other beneficial metabolites or simply those less toxic to the host. Only one of the adult gut samples showed an abundance of *Providencia*, which was not identified or detected even in low abundance in the other stages. Since bacteria from this genus are an opportunistic pathogen widely distributed in animals [50], it does not seem to be a normal member of the gut microbiome in this insect but rather an opportunistic one. It would be necessary to study a large series of adults to confirm the real presence of this genus.

Regarding gut microbial composition throughout insect metamorphosis, we found that during the transition from larvae to adult, a significant drop in species richness and diversity occurred. Thus, the microbial community is definitely affected by the complete restructuring of the gut. The associated changes in the microbiota are a direct consequence of the physiological, morphological, and biochemical transformations of the gut associated with the metamorphosis. Similarly, to our results, Hammer et al. [10] described a 50% loss of diversity from the larvae to the adult in the metamorphosis of the butterfly *H. erato*. The same study showed that adults that had recently emerged from pupae presented a lower diversity compared with mature adults [10]. The adult samples analyzed in our study were taken at the beginning of adulthood. Thus, we may speculate that diversity would probably increase after longer feeding and interaction of the animal with the environment.

As stated before, the adult microbiota of *B. crini* was dominated mainly by *Serratia*, a genus forming part of the core but with different abundance in the different developmental stages (eggs, 18%; larvae, 9%; and adults 96%). Thus, this species could be a keystone in the repopulation and succession of the gut microbial ecosystem. However, the possibility that during the renewal of the microbial community *Serratia* acts as an opportunistic species, taking advantage of its higher growth rate, cannot be ruled out. Supporting this hypothesis, *Serratia* was not identified in the network of connected genera of *B. crini*, most of which belonged to the core microbiota (Fig. 2).

In summary, the gut microbiome of *B. crini* reflects the lifecycle of the moth, especially those changes associated with the metamorphosis, with a strong shift in composition related to the restructuration of the gut. Further studies including more samples and time points during the development would confirm the effect of the development on the gut microbiota composition of this species. Besides this, the monophagous diet of this insect leads to higher diversity compared with polyphagous species, indicating that the gut bacteria could complement nutritional deficiencies in the diet. Some of the species detected in the core microbiota of this moth seems to participate in the plant detoxification process as it was show with a higher abundance of toxic compounds degradation pathways in the larvae stage compared with the adult. Future studies based on metagenomics and/or transcriptomics would help to clarify the functional role of the microbiota in host physiology aspects such as nutrition or development.

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Authors' Contributions A. Moya conceived the work. F. González-Serrano, T. A. E. Pérez-Cobas, T. Rosas-Pérez, and J. Baixeras, performed the experiments and the analyses. The manuscript was drafted and assembled by F. González-Serrano, A. E. Pérez-Cobas, and A. Latorre, coordinated by A. Moya and A. Latorre. All authors participated in the editing of the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interest.

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