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Succession of the gut microbiota in the cockroach *Blattella germanica*

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Summary. The cockroach gut harbors a wide variety of microorganisms that, among other functions, collaborate in digestion and act as a barrier against pathogen colonization. *Blattabacterium,* a primary endosymbiont, lives in the fat body inside bacteriocytes and plays an important role in nitrogen recycling. Little is known about the mode of acquisition of gut bacteria or their ecological succession throughout the insect life cycle. Here we report on the bacterial taxa isolated from different developmental instars of the cockroach *Blattella germanica*. The bacterial load in the gut increased two orders of magnitude from the first to the second nymphal stage, coinciding with the incorporation of the majority of bacterial taxa, but remained similar thereafter. Pyrosequencing of the hypervariable regions V1–V3 of the 16S rRNA genes showed that the microbial composition differed significantly between adults and nymphs. Specifically, a succession was observed in which *Fusobacterium* accumulated with aging, while *Bacteroides* decreased. *Blattabacterium* was the only symbiont found in the ootheca, which makes the vertical transmission of gut bacteria an unlikely mode of acquisition. Scanning electron microscopy disclosed a rich bacterial biofilm in third instar nymphs, while filamentous structures were found exclusively in adults. [**Int Microbiol** 2014; 17(2):99-109]

Keywords: *Blattella germanica* · cockroach gut microbiota · 16S rRNA gene · endosymbionts · ecological succession

Introduction

Many insects harbor gut microbial communities that actively interact with their hosts at several different levels [18]. Gut microbes are involved in many processes, such as the digestion of recalcitrant plant polymers, the provision of nutrients, the stimulation of midgut self-renewal, the diet-dependent duration of developmental stages, resistance to parasite invasion, and host fitness under different environmental regimes [14]. Additionally, some insects have established mutualistic symbiotic associations with intracellular bacteria, which play a metabolic role by providing their hosts with new metabolic pathways that produce nutrients otherwise lacking in their restricted diets [5]. These intracellular symbionts are vertically transmitted from parent to progeny, as revealed by the congruent phylogenies between hosts and endosymbionts [20]. Cockroaches, one of the first insects in which intracellular bodies presumed to be symbionts were recognized [7], harbor the obligate endosymbiont *Blattabacterium cuenoti* (referred to hereinafter as *Blattabacterium*) in bacteriocytes, which are specialized cells in the fat body that are required for host fitness and fertility. Genome sequencing revealed that *Blattabacterium*

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plays a role in host nitrogen metabolism and in the synthesis of essential amino acids [25,36]. In *Blatella germanica, Blattabacterium* enters the oocyte plasma membrane during ovarian development, prior to chorionogenesis [21]. Once inside, it participates in yolk utilization by vitellophages, thus contributing to embryonic development and providing an added advantage to this symbiotic relationship. Phylogenetic co-cladogenesis between *Blattabacterium* strains and their corresponding hosts indicates that the initial infection occurred in a common ancestor of cockroaches and termites [31]. However, the endosymbiont was lost in all termite lineages, except in the lower termite *Mastotermes darwiniensis* [4]. Key changes during the independent evolution of termites include, beside the loss of *Blattabacterium*, a shift from an omnivorous to a wood diet, the acquisition of specialized hindgut microbiota, and a sophisticated social behavior. The absence of *Blattabacterium* in nearly all termites suggests that its nutrient-provisioning role was replaced by gut microbes. In fact, termite gut microbes help fix nitrogen, degrade lignocellulose, and produce nutrients [48].

In the omnivorous cockroach *Periplaneta americana*, growth is retarded after the elimination of gut anaerobes by the antimicrobial agent metronidazole, proof of the essential role played by gut microbiota in host physiology [8]. Recent research has focused on the relative contributions of gut-resident and intracellular symbionts to host metabolism, both in termites and in cockroaches [45].

Several metagenomic studies have been carried out on the gut bacterial community in cockroaches. Comparison of the phylogenetic relationships of symbiotic bacteria in the xylophagous cockroach *Cryptocercus punctulatus* with those in lower termites showed a partial coincidence with host phylogeny [6]. However, in the omnivorous cockroach *P. americana*, a large proportion of sequences proved to be more closely related to environmental sequences than to those of other symbionts represented in current databases [35].

Researchers have also addressed the mode of microbiota acquisition, finding that the gut microbiota of cockroaches and termites is acquired through food or feces. For instance, in *C. punctulatus* and *M. darwiniensis*, the intergenerational transfer of hindgut microbiota occurs via proctodeal trophallaxis [27]. Vertical transmission by fecal contamination during oviposition has also been demonstrated, e.g., in stinkbugs of the family Plataspidae, where a specific gut bacterium (*Candidatus* Ishikawaella capsulata) is vertically transmitted via a symbiont capsule that is laid on the eggs [23]. In *B. germanica*, descriptions of the transmission mechanism of gut microbiota and the dynamics of ecological succession during the developmental stages are lacking. The corresponding scenarios are complex considering that the anterior and posterior cuticles of the intestinal tract (foregut and hindgut) are renewed during each of the 5–6 moultings (males and females, respectively). How they are recolonized after moulting is unknown, although it has been speculated that the mechanism involves either a reservoir of microbiota in the gut or incoming bacteria from the environment.

In this work, we examined the bacterial load and changes in microbial diversity both in the ootheca (embryos) and in the gut of *B. germanica* in each of the five nymphal instars, as well as in adult males. Our results shed light on the mode of gut microbiota acquisition throughout cockroach development.

Materials and methods

Insect rearing. The experimental *B. germanica* colony originated from a stable laboratory population (started 30 years ago) housed by Xavier Bellés at the "Institut de Biologia Evolutiva", Barcelona, Spain. Culture chambers were adjusted to 26 ± 1 ºC, 70 % humidity, and a photoperiod of 12D:12L. The insects were bred in lunchboxes with aeration and fed on autoclaved dog food composed of cereals, meat and animal by-products (25 % meat), vegetable origin by-products (2 % beet pulp), oils and fats (poultry fat, source of ω3), minerals, and yeasts. The additives (per kg) were: 11,000 IU vitamin A, 825 IU vitamin D3, 66 mg vitamin E, 55 mg Fe, 1.4 mg iodine, 0.3 mg cobalt, 6 mg copper, 28 mg manganese, 45 mg zinc, and 0.1 mg selenium. Antioxidants, preservatives, and dyes were also present. In summary, the analytical components were: 24 % protein, 10 % fat, 2.5 %; gross fiber, 8.5 % inorganic matter, and 10 % moisture. Water was supplied *ad libitum*. The lunchboxes were renewed weekly. A cohort of 40 individuals maximum was maintained per box.

Nymphal instar and adult stage morphological determination. The nymphal instar (n) was identified by measuring head width, as previously described [42], whereas adults were easily identified by their wings. Accordingly, the specimens were classified as n1, n2, n3, n4, n5 and adults (male), corresponding to individuals 2, 11, 15, 22, 34 and 68 days after hatching, respectively.

Gut dissection and ootheca sampling. Two specimens from the same brood were selected as biological replicates for each of the developmental stages. The insects were anesthetized under a stream of CO_2 and placed dorsally on a paraffin plate. After removal of its head, the cockroach was pinned with minute entomological pins through the prothorax and last abdominal segments. The legs were coxally cut. Dissections were carried out under a stereomicroscope using fine forceps (Wild M8, Lawton, GmbH & Co., KG, Fridingen, Germany) and spring scissors. In the tiny young nymphs, the gut was simply stretched through the anus. In the remaining cases, the body cavity was exposed through an incision in the tergal area and the gut removed from the anus to the level of the metathorax. Residual fat body tissue was removed, placed in a tube, and frozen in liquid nitrogen for further studies. The samples were stored at –80 ºC. Ootheca contents were extracted by drilling the external surface with a pipette tip and sucking out the embryo-containing fluid. Surface samples were routinely tested for bacteria by PCR, with negative results (data not shown).

The guts and ootheca contents were ground manually with a glass rod in cell lysis buffer (JETFLEX genomic DNA purification kit, Genomed, Löhne, Germany) and digested with proteinase K overnight.

Bacterial load determination. Absolute quantification of bacterial 16S rRNA gene copies was carried out by quantitative PCR (qPCR) using the universal bacterial 16S rRNA gene primers 8F 5′-AGAGTTTGATCCTGG CTCAG-3′ and 338R 5′-TGCTGCCTCCCGTAG GAGT-3′ [46]. The HOT FIREPol EvaGreen (Solis Biodyne, Tartu, Estonia) qPCR Mix Plus kit was used together with Roche Light Cycler 2.1 thermocycler. The thermal profile was: 95 ºC for 15 min followed by 40 cycles of 95 ºC for 10 s, 55 ºC for 10 s, and 72 ºC for 18 s. Standard curves were constructed with purified and photometrically quantified amplicons (10^3–10^7 copies, or molecules), to interpolate sample crossing points (Cp). The standards were aliquoted and stored at –80 ºC. The equation of the standard curve was lg (copy number) = –3.58Cp + 36.016, $R^2 = 0.999$. Four measurements per stage were used to statistically test the change in bacterial load from one stage to the next. The Wilcoxon signed-rank test, implemented in R software [51], was applied to compare the sequential stages; comparisons with a p-value less than 0.05 were considered significant.

Bacterial 16S rRNA gene amplification and pyrosequencing. The V1–V3 variable regions of the bacterial 16S rRNA genes were PCR-amplified using the universal primers E8F (5′-TAG AGT TTGATCMTGGCTCAG-3′) linked to the adaptor CCATCTCATCCCTGC GTGTCTCCGACTCAG and 530R (5′-TTGCTGCCTCCCGTAGGAGT-3′) and the sample-specific multiplex identifier (MID) for pyrosequencing. PCR was carried out in a total volume of 50 µl. Each reaction contained the TAKARA reagent (0.25 µl of 5 U/µl Ex Taq HS, 1 µl of 2.5 mM dNTP mixture, 5 µl of 10× Ex Taq buffer), 0.2 µl each of the forward and reverse primers (stock 10 µM), and 50 ng of template DNA. The GeneAmp PCR system 9700 thermocycler reaction conditions were: initial denaturation at 94 ºC for 5 min; 25 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s, and elongation at 72 ºC for 1 min; and a final elongation at 72 ºC for 10 min. To avoid PCR bias, we chose the lowest DNA template quantity and the fewest possible PCR amplification cycles [1,49]. The integrity and quantity of the amplicons were checked by agarose gel (1.4 %) electrophoresis. To precipitate and purify the DNA, 2 µl NaOAc and 40 µl cold 95 % ethanol were added, and the DNA was resuspended in 10 µl water. The Qubit dsDNA BR assay kit was used with the Qubit 2.0 fluorometer to accurately quantify the purified amplicons, which were pooled in equimolar amounts for pyrosequencing. Samples were sequenced in a next-generation 454 pyrosequencer (Genome Sequencer FLX system, Roche) available at the FISABIO-Salud Pública, Valencia.

Processing and taxonomic assignment of 16S rRNA reads. An initial quality trimming and MID sorting process was performed at the Ribosomal Database Project website [15] in the pyrosequencing pipeline. Primers, MIDs, and sequences with a Phred quality score less than 20 (Q20) and short length (<250 pb) were removed. Taxonomical annotation was done by aligning the sequences against a subset from the SILVA SSU NR 111 database (ssu_clsref_111_rc1), comprising 200,000 sequences. To do so, we used SINA software [33], first fixing the annotation of the bacterial taxa at the genus level and then classifying the non-annotated sequences at the closest possible level (family, order, class or phylum), with the prefix "uc" indicating that classification at the lowest level (genus) was not possible.

Biodiversity and statistical analysis. The composition and structure of the gut microbiota in each sample were characterized using 16S rRNA gene amplicons. Structure was analyzed by estimating two diversity variables, i.e., the expected number of taxa and the Shannon diversity index [39], and two richness estimators i.e., the Chao1 [12] and the abundance-based coverage estimator (ACE) [13]. All diversity variables were calculated after re-sampling (with the same number of sequences per sample, to avoid sequencing effort differences) using the multiple_rarefactions.py and alpha_diversity.py scripts of the QIIME program [11]. The Wilcoxon signed-rank test was used as above to statistically compare the mean ranks of the Shanon index and the Chao1 and ACE richness estimators between nymphs and adults. Rarefaction curves were generated to estimate the number of expected taxa at the different stages [22], using the Vegan Community Ecology Package [30].

A canonical correspondence analysis (CCA) showed sample variation in terms of taxon abundance and its relationship to host stage. A multivariate ANOVA based on dissimilarity tests (Adonis) was used to test the effect of the variable "developmental stage" in explaining the observed variation in the data. These analyses were also run with Vegan software.

A regression analysis was performed to identify those taxa showing a statistically significant trend over time, considering the sampling time (days). In addition, statistically significant differences between the relative abundances of taxa in n3 nymphal instars vs. adult specimens were determined. These two analyses were performed with the ShotgunFunctionalizeR R package [24]. The function test GeneFamilies.regression was used for the regression analysis, while the test GeneFamilies.dircomp was used for multi-sample comparison. Both tests are based on the Poisson model.

Clustering analysis. A cluster analysis was used to study inter-sample similarity in taxon composition. The pvclust R software [41] was used to calculate the uncertainty of the hierarchical clusters using bootstrap resampling techniques. The approximate unbiased (AU) p-value with 10,000 replicates was chosen to calculate the probability of each cluster.

Scanning electron microscopy (SEM). The guts of third instar nymphs and adults were inspected by SEM. The study required the use of fixed fresh material from between three and ten specimens of *B. germanica*. Before dissection, the cockroaches were starved for three days to obtain a clear gut surface. The specimens were anesthetized by CO_2 in a killing jar. A drop of fixative (paraformaldehyde 2 %/glutaraldehyde 2.5 %) was gently injected through the thorax into the body cavity using a hypodermal syringe (30G needle). The whole specimen was then immersed in the same fixative.

General dissection procedures were modified from standard methods for anatomical preparation for optical microscopy. Dissection and cleaning were carried out under a MZ9.5 Leica stereomicroscope, with the insects placed in 30-mm glass embryo dishes and by using spring micro-scissors, Dumont (Fine Science Tools, GmbH, Heidelberg, Gernany) forceps (number 5), and fine-model brushes (5/0) . After fixation, the abdomen was dissected in insect Ringer solution. Fragments of the foregut, midgut, and hindgut were cleaned and longitudinally opened, then fixed again in the same fixative. Fragments of digestive content and nematodes were removed. Each gut piece was placed inside a microporous specimen capsule (30-µm pore size, Ted Pella, Redding, CA, USA) immersed in absolute ethanol, and then subjected to critical point drying in an Autosamdri 814 critical point dryer (Tousimis, Rockville, MD, USA). The fragments obtained were arranged on SEM stubs using the silver-conducting paint (TAAB, Berks, England) and examined under a Hitachi S-4100 scanning electron microscope. Images were edited with Photoshop CS3.

Sequence data deposition. All sequences obtained in this study were submitted to the European Bioinformatics Institute (EBI), EMBL Nucleotide Sequence Submissions Database ID: ERP002663.

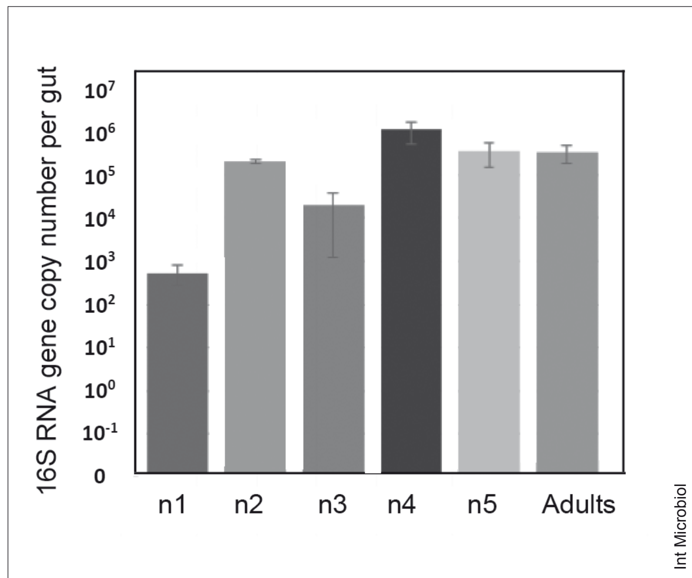

Fig. 1. Time-course changes in gut bacterial load throughout the development of *Blattella germanica* assessed by qPCR. Bacterial abundance was inferred from the copy number of bacterial 16S RNA genes in total DNA extracted from cockroach guts. Four determinations were carried out at each stage.

Results

Time-course changes in the bacterial load. To estimate the bacterial load per gut we determined the bacterial 16S rRNA gene copy number by qPCR, using total DNA extracted from *B. germanica* instars n1–n5 and male adults (2, 11, 15, 22, 34, and 68 days after hatching). As shown in Fig. 1, bacterial load increased from stage n1 to stage n2 but then remained relatively constant through the following moultings, until the adult stage. However, despite some variations between stages, only the change from n1 to n2 was significant (Wilcoxon signed-rank test, $p = 0.03$).

16S rRNA gene pyrosequencing. From ootheca and the guts of *B. germanica*, 52,562 sequences belonging to a 700-bp PCR amplicon of bacterial 16S rRNA gene were obtained. As shown in Fig. 2, seven phyla were detected at the highest taxonomic level: Bacteroidetes, Deferribacteres, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria, and Synergistetes. At the family level, there were 22 different taxa, while at the lowest taxonomic level discriminated by this technique (genus), 18 bacterial genera were identified, 16 of which were Gram-negative and 13 anaerobic. Note that, in the ootheca (embryos), only *Blattabacterium* and unclassified Blattabacteriaceae, most likely *Blattabacterium* as well, were detected. In the remaining stages, except n1, *Blattabacterium* was present in all samples only at low abundance. Dissection of the gut without contamination by traceable amounts of fat body is not technically feasible. Thus, the recovery of *Blattabacterium* sequences in the extracts from gut tissue was almost certainly an artefact of the method. In n1 nymphs, removal of the fat body from the gut tissue is practically impossible, such that the amount of *Blattabacterium* recovered may be extraordinarily high (Fig. 2). The dramatic differences observed in ootheca and n1 instar samples with respect to *Blattabacterium* as well as the poor representation of the remaining taxa recommend the exclusion of these stages in further analyses.

Microbial diversity. Figure 3 shows the rarefaction curves for each stage of cockroach development, including the ootheca. While both the ootheca and the n1 samples were expected to have the lowest number of taxa, the number in instar n1 was higher than that in the ootheca samples. This indicated that the first colonizers of the gut ecosystem appeared at this stage, even though the relative abundance of *Blattabacterium* was still high. The remaining nymphs and adults had similar numbers of expected taxa (>40). The rarefaction curves also showed that the most abundant taxa in the cockroach intestinal ecosystem were sequenced, although a higher number of reads would have been required to reach the plateau in all samples.

Estimates of bacterial diversity (expected number of taxa and based on the Shannon index) and richness (Chao1 and ACE) in the gut of nymphal (instars n2–n5) and adult stages (Table 1) after data re-sampling showed similar numbers of expected taxa and a similar Shannon index for all nymphs

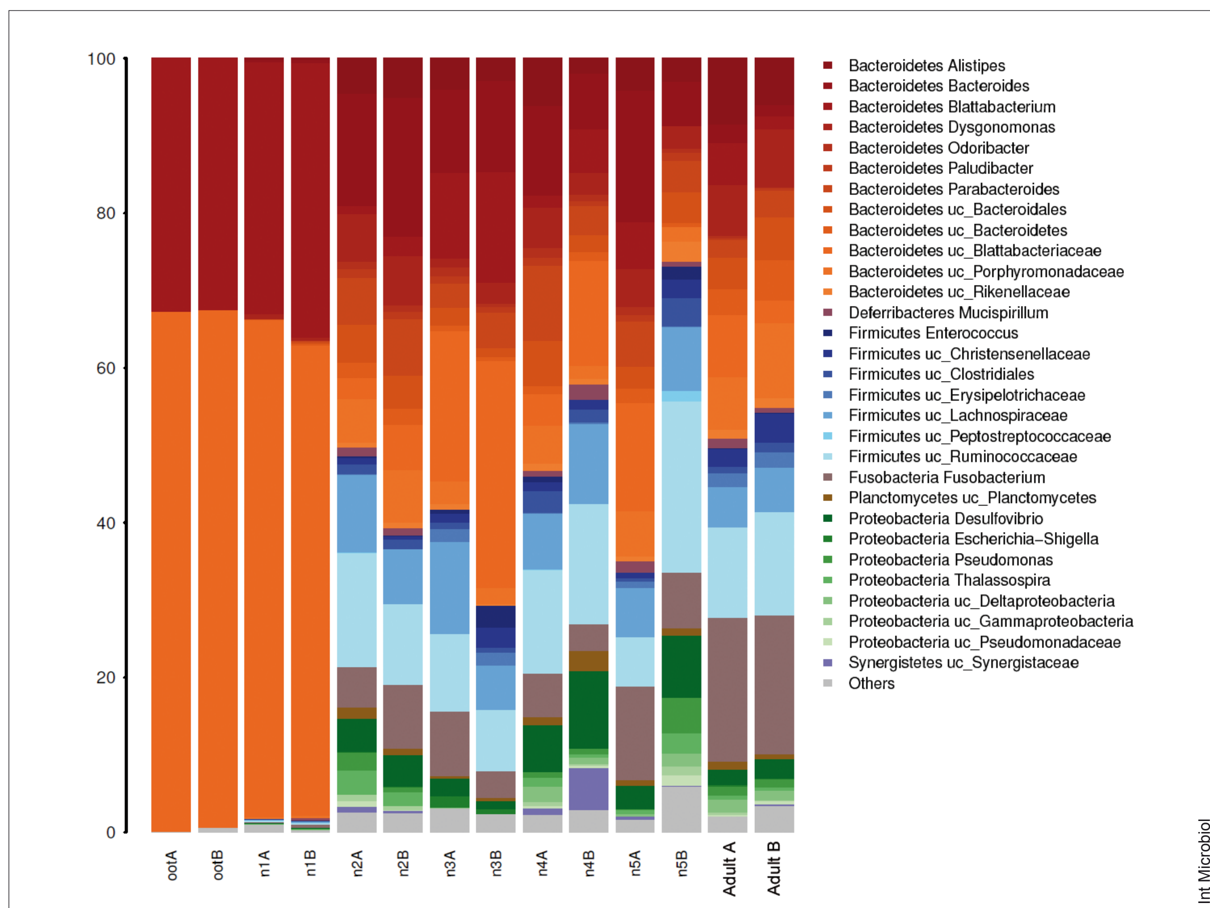

Fig. 2. Bar plot of the relative abundance in bacterial taxa at each of the developmental stages of *Blattella germanica*, including *Blattabacterium* reads. The *y*-axis represents the proportion of pyrosequencing reads belonging to each taxon (genus and unclassified levels "uc"). Taxa with an abundance <1 % are included in "others."

and adults (range: 37.04–49.74 and 3.59–4.33, respectively). Thus, despite compositional differences between samples, they had the same degree of homogeneity. The richness values were also similar between nymphs and adults. According to Wilcoxon signed-rank tests comparing the indexes between nymphs (n2, n3, n4, n5) and adults, the differences were not significant (p = 0.71; 1.0; 0.71 and 0.89 for expected N, Shannon, Chao1, and ACE, respectively).

Variations in bacterial composition in *Blatella germanica*. A cluster analysis testing sample similarity according to taxa abundance distribution (Fig. 4) yielded two defined clusters, grouping nymphal samples in one and adult samples in the other. In addition, only adults and n3 samples clustered together. To evaluate sample pattern variation in taxon abundance and host developmental stage, an ANOVA was run to define the effect of "stage", formed by stages n2–n5 and the adult stage. The difference between the two proved significant (p = 0.02). A CCA then provided further information on differences in taxon abundance according to developmental stage. As shown in Fig. 5, the first axis explained 20 % of the overall variability, separating the n3 nymphal stage and the adults from all the other stages. The second axis explained 19.5 % of the variability and separated adult samples from nymphs. Both statistical approaches indicated that part of the variation in gut bacterial composition in *B. germanica* is stage-related, and that n3 nymphs and adults differ from the other stages.

To determine whether, during development, the number of taxa significantly accumulated or decreased, we carried out a regression analysis based on a Poisson model. Time served as the independent variable, with samples taken 11, 15, 22, 34, and 68 days after hatching, corresponding to n2–n5 (nymphal stages and adult). The results showed a total of 23 significant

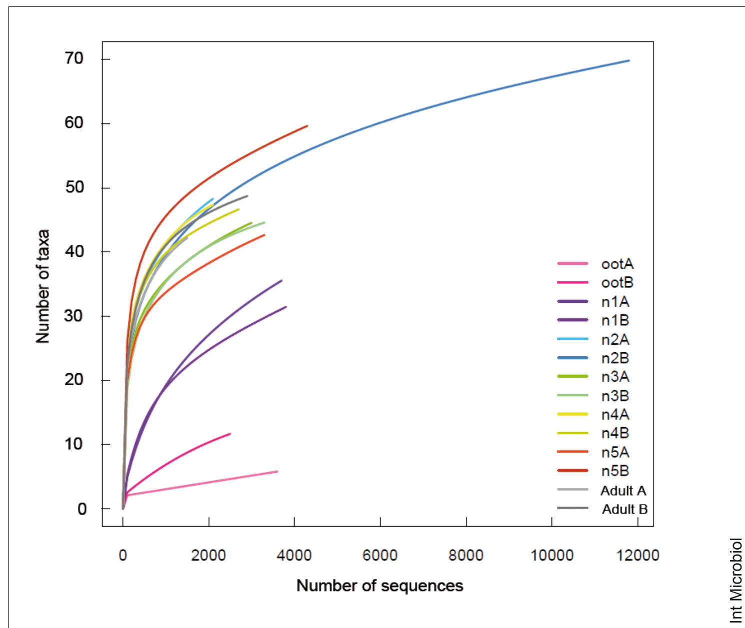

Fig. 3. Rarefaction curves of the sequencing reads for each developmental sample showing the maximum expected number of taxa.

taxa—14 increasing and nine decreasing in abundance between days 11 and 68 and thus depict the microbial ecological succession taking place inside the cockroach gut. Figure 6 shows the dynamics of these significant taxa, excluding those with a level of relative abundance below 0.50 % at all stages. The largest increases over time were in *Fusobacterium*, which was most predominant in adults, and the unclassified Deltaproteobacteria, Christensenellaceae, Erysipelotrichaceae and Desulfobacteraceae (Fig. 6A). Taxa that decreased over time were *Bacteroides*, *Parabacteroides*, *Thalassospira*, and the unclassified Lachnospiraceae (Fig. 6B).

As early as stage n2, 86 % of the total taxa were already present, concomitant with the burst in bacterial abundance per gut (see Fig. 1). Since the n3 nymphal stage and the adult stages had the highest within-stage homogeneity (Fig. 4) and the highest heterogeneity between developmental stages (Fig. 5), and given their behavior and physiology (see Discussion), we carried out a comparative analysis to identify which taxa are particularly enriched at each of these stages. At the adult stage, *Fusobacterium*, uc_Bacteroidetes, uc_Porphyromonadaceae, *Dysgonomonas*, uc_Deltaproteobacteria, *Pseudomonas*, and *Mucispirillum,* were the most abundant, whereas at the nymphal stage, *Bacteroides*, *Enterococcus*, uc_Lachnospiraceae, *Escherichia-Shigella*, *Parabacteroides*, and *Odoribacter* were more abundant.

Scanning electron microscopy. For the same reasons, we chose a nymphal stage (n3) and the adult stage to visualize the gut microbiota by SEM (Fig. 7). Microbial

Table1. Number of expected taxa (N) and Shannon Chao 1 and ACE indices calculated from the re-sampled data set

Sample	N (SD)	Shannon (SD)	Chao1 (SD)	ACE (SD)
n2A	45.00 (1.57)	4.21 (0.02)	55.33 (8.11)	59.72 (7.15)
n2B	44.61 (2.65)	4.10 (0.03)	57.61 (13.25)	58.05 (8.76)
n3A	39.45 (1.78)	3.86 (0.03)	49.76 (10.30)	49.00 (5.79)
n3B	39.33 (1.80)	3.59 (0.03)	45.91 (6.45)	47.02 (5.04)
n4A	44.87 (1.26)	4.29 (0.02)	49.51 (4.99)	51.69 (4.08)
n4B	43.25 (1.59)	4.15 (0.02)	50.14 (7.37)	48.66 (3.98)
n5A	37.04 (1.83)	3.95 (0.03)	48.91 (11.88)	43.66 (4.91)
n5B	49.74 (2.16)	4.33 (0.03)	60.01 (10.65)	57.38 (5.50)
Adult A	42.98 (0.15)	4.12 (0.00)	46.52 (0.51)	47.85 (0.41)
Adult B	44.88 (1.59)	4.13 (0.03)	50.93 (6.55)	50.05 (3.55)

SD, standard deviation.

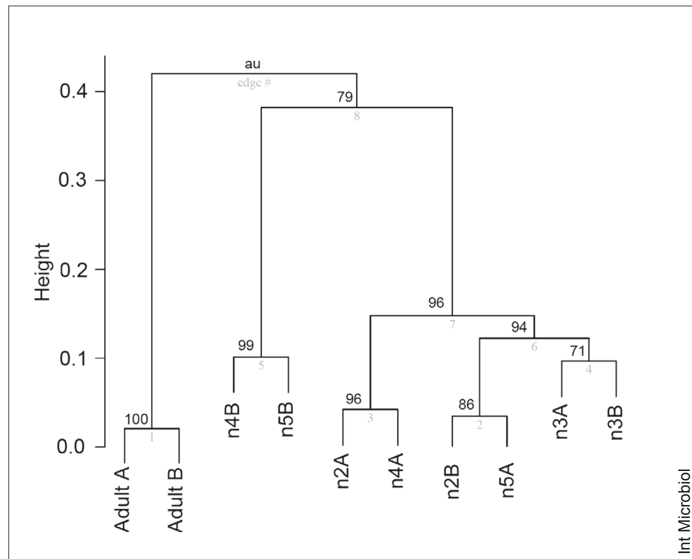

Fig. 4. Clustering analysis based on taxon composition. Bootstrap values appear above each cluster and the order of the clustering (edge #) below. Height indicates the average distance between the corresponding clusters.

colonization was not detected on the foregut or midgut surface, as previously reported for *P. americana* [9]. However, a dense bacterial biofilm formed by rosettes of rods was observed on the hindgut cuticle in both stages. Filamentous structures were present exclusively in adult cockroaches.

Discussion

Cockroaches harbor a rich microbiota in the gut, but how it is acquired after hatching and how it develops are unclear. Our qPCR results showed that in *B. germanica* the bacterial load per gut increased by about two orders of magnitude between hatching and the n2 instar stage, when 86 % of the total detected bacterial taxa appeared. In the other stages, the bacterial load remained more or less constant, with no significant differences between them. This finding is in accordance with those reported for other insects, such as *Zonocerus variegatus,* whose culturable bacterial load increases 80-fold between the first nymphal instar and the adult [2]. Similarly, the bacterial load of *Frankliniella occidentalis* increases by four orders of magnitude between the first instar and adult

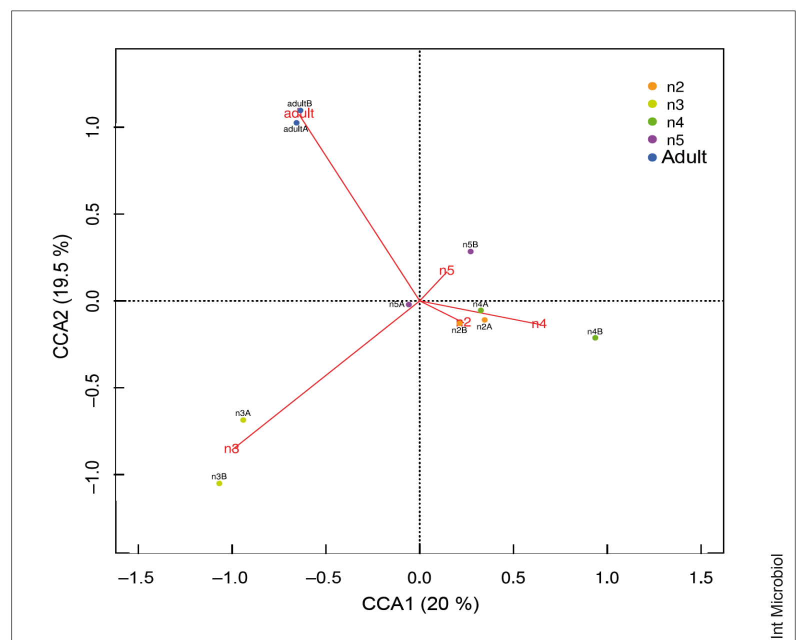

Fig. 5. Canonical correspondence analysis (CCA) of *Blattella germanica* gut microbiota. The developmental stages are represented as five vectors (n2, n3, n4, n5, and adult). The axes represent the percentage of the corresponding total variance explained. The closeness of the points is an indicator of similarity.

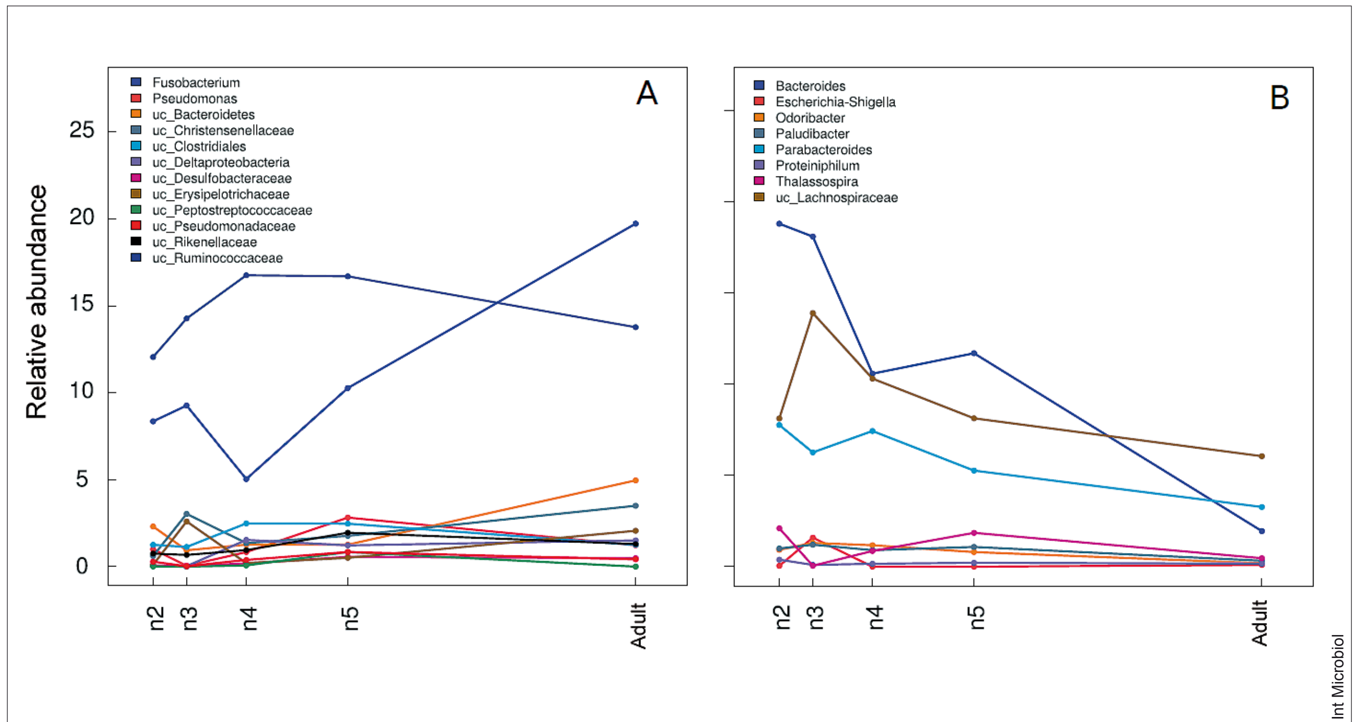

Fig. 6. Temporal trend of taxa that show a statistically significant change during the instar and adult developmental stages. (**A**) Increasing over time; (**B**) decreasing over time.

stage [47]. Nevertheless, the evenness of the gut bacterial yield between n3 and adult stages is intriguing, considering that the gut cuticle of the hindgut is renewed during moulting and given the changes in the microbial composition detected in this work. A microbial reservoir in the midgut (not shed during successive moults) could spread to newly formed tissues, keeping the bacterial population size constant. This is the case in the wax moth *Galleria mellonella*, in which a small microbial reservoir persists in the gut [10].

Bacterial composition underwent important temporal changes during development, with some taxa showing significant changes in abundance. *Blattabacterium* was the only bacteria detected in embryos inside the ootheca, consistent with a lack of vertical transmission of the gut microbiota; instead, horizontal transmission of behaviors such as trophallaxis, coprophagy or body/ootheca licking seems probable. Further experiments with antimicrobial agents and analysis of the gut microbiota of offspring are underway in our laboratory to ascertain whether bacterial taxa eliminated by antibiotics can be restored via environmental sources.

Overall, the gut content among most nymphs is similar but it differs from that of adults, as shown by CCA. This similarity suggests that at these stages the gut habitat is suitable for a specific fraction of early-colonizing microorganisms. Habitats with similar conditions tend to select for similar microbial communities [16]. When the source (reservoir) diversity is low, similar communities are observed [17], as in the case of the nymphal guts after each moulting. Additionally, some species depend on the presence of other microbial community members [18]. However, given that some of the replicates did not cluster together, it may be that the establishment of the microbiota after each moulting event is influenced by stochastic factors, with the final composition determined by the order of the microorganisms' arrival. Similar results were obtained with *P. americana* [37]. In adults, the gut microbiota is no longer disturbed by moulting, which enables ecological succession to continue until an ecosystem equilibrium is reached. This scenario probably explains the differences in microbial composition between nymphs and adults.

Cluster analysis also revealed that samples belonging to the adult and n3 nymphal instar grouped together, although their bacterial compositions were very different. Strikingly, the bacterial assembly of n3 samples differed not only from those of adults but also from those of all other nymphal stages. These differences could be related to the physiological state of the host at that particular developmental stage, corresponding to the social/behavioral transitions of the third instar stage [28]. Stage-specific traits in *B. germanica*, e.g., the specific

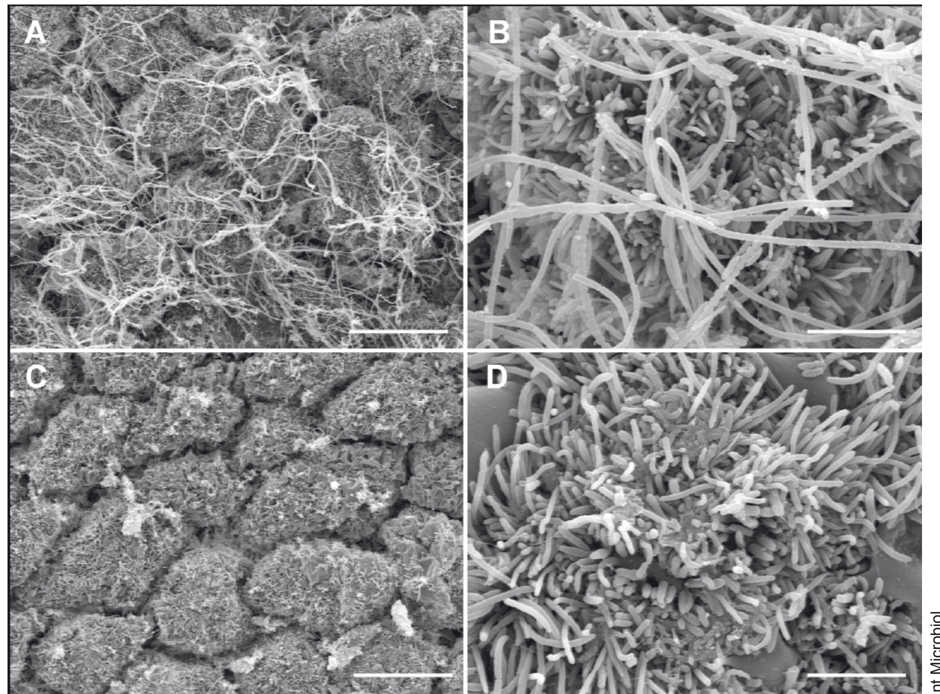

Fig. 7. SEM images of the luminal surface of the hind gut of *Blattella germanica*. (**A,B**) adult; (**C,D**) n3 instar nymph. Scale bars: 50 µm (A,C) and 5 µm (B,D).

activity of acetylcholinesterase, whose levels have been shown to decrease in the n3 instar, have been described [34].

The most abundant phyla in all stages, except embryos and stage n1, were Bacteroidetes (60 %), Firmicutes (30 %), and Proteobacteria (10 %). These phyla are also predominant in the gut of the cockroaches *S. lateralis* [38] and *C. punctulatus* [6]. Among the 18 genera identified (abundance >1 %), the presence of five genera of aerobic bacteria, *Massilia, Porphyrobacter, Pseudomonas, Sphingomonas*, and *Thalassospira*, was noteworthy. Among the taxa identified at the family level, Lachnospiraceae produces acetate, propionate, and butyrate, three potential host energy sources. This bacterial family is also present in other insects, such as termites and locusts, and in omnivorous animals, including mammals [44]. The family Erysipelotrichaceae comprises four different genera that in a study in mice responded differently to diet and host health [50]. Other cases of a host-related relationship between bacterial family and host have been reported, such as the overrepresentation of Peptostreptococcaceae in children living with pets [3]. Moreover, Ruminococcaceae, together with Lachnospiraceae, are the predominant autochthonous families in both human and mouse colon [29].

Changes in relative abundances were observed for *Fusobacterium* and *Bacteroides*, which, respectively, accumulated and decreased during the insect life cycle (Fig. 6). Both are anaerobic Gram-negative bacteria. *Fusobacterium varium*, for instance, is an integral constituent of the human gut microbiota, and, unlike many gut microorganisms, is capable of fermenting both amino acids and glucose [32]; by contrast, simple sugars are not the main energy source of *Bacteroides*. In fact, a large part of the *Bacteroides* proteome includes proteins that hydrolyze polysaccharides. The ability to convert complex polysaccharides into simpler (usable) compounds might allow *Bacteroides* to be more competitive at early stages in cockroach development. We propose that, as *Bacteroides* initially predominates and degrades complex nutrients, the monosacharides and amino acids released would allow *Fusobacterium* to proliferate and progressively accumulate.

SEM images showed a higher abundance of segmented filaments in the adult than in the n3 instar, which suggests an increase in microbial community complexity over time. Some groups would take longer to colonize the ecosystem, ruling out their presence in nymphs because of the successive renewals of the cockroach fore and hindgut cuticle. The name *Arthromitus* has been applied collectively to conspicuous filamentous bacteria found in the hindguts of termites and other arthropods. A recent and meticulous study in the termite *Reticulitermes* definitively clarified the monophyletic origin of *Arthromitus* within the family Lachnospiraceae [43]. Segmented filamentous bacteria (SFB) from vertebrates

form a distinct lineage within the family Clostridiaceae. *Arthromitus* lives in the hindgut of termites and cockroaches such as *Blaberus giganteus* [19], *C. punctulatus*, *Blatta orientalis*, *S. lateralis* [43], and *Gromphodorhina portentosa* [26]. *P. americana* reportedly harbors gut microbiota with a similar morphology [9].

Among the genera detected at the n5 stage that persist in the adult were *Anaerofustis, Cetobacterium, Enterobacter*, and *Hydrogenoanaerobacterium.* The latter consists of thin rods that are 14.5-μm long and usually form pairs [40] and is thus a good bacterial candidate for some of the structures observed in the SEM images, although these could also be attributed to filamentous fungi. It is known that fungi colonize the *B. germanica* gut [37] and some of the morphologies observed are compatible with those of Trichomycetes, which are obligate fungal dwellers in the guts of insects, crustaceans, and millipedes [26]. Methods such as FISH or metagenome analysis are necessary to elucidate the sources of these different structures in adults.

In summary, bacterial community composition in the *B. germanica* gut differs between nymphal instars and adults. Specifically, the former has an additional abundant genus (*Bacteroides*) that decreases in abundance when the insect moults as it becomes an adult, after which there is a progressive accumulation of the genus *Fusobacterium*. The main increase both in bacterial quantity and diversity takes place after the first moult, after which bacterial load and richness remain constant. According to our results, it is more likely that the gut microbiota is horizontally transferred via fecal contents, rather than vertically via transfer to the egg.

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