Maternal transmission of bacterial symbionts in the desert locust

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May 5, 2020

Abstract

Long-scale, multi-generational migration is instrumental in the devastating potential of locust swarms as a major agricultural pest. Gut bacterial symbionts have been shown to augment locust immunity, and to support locust swarming via the emission of attracting volatile compounds. To date, however, it is unclear whether and how those beneficial symbionts are transmitted vertically through generations. Using comparative 16S rRNA amplicon sequencing and genetically- engineered bacteria, we demonstrate here for the first time transgenerational-transmission of gut bacteria in the desert locust, as well as its underling mechanism: females inoculate bacteria into the egg pod's foam-plug, through which larvae pass upon hatching. in addition to chitin, which is its primarily component, we show the foam to also contain bactericidal proteins, such as lysozyme that select for specific bacteria. These findings reveal for the first time the mode of transgenerational-transmission of symbionts in locusts, potentially contributing to the swarms' migratory success.

Introduction:

Locusts (order: Orthoptera) have affected the lives of people since biblical times (Exodus 10: 4–19), and to date still constitute a serious agricultural threat (FAO 2019). During outbreaks or plagues, these highly polyphagous insects (Chapman and Joern 1990) perform long-distance migrations, devastating large parts of the developing world (Symmons and Cressman 2001; Van Huis et al. 2007; Cease et al. 2015; FAO 2019). The desert locust (*Schistocerca gregaria*) is a well-known locust species, mostly originating in the African Sahel (Symmons and Cressman 2001; Lorenz 2007; Cease et al. 2015). Under the appropriate conditions, *S. gregaria* swarms develop and can potentially reach the Arabian Peninsula, the Middle East, southern Europe, and even south-west Asia (FAO 2019).

These large-scale migrations comprise of several consecutive generations (Skaf et al. 1990; Symmons and Cressman 2001). Each generation will develop through 5 nymphal instars into reproductive adults (Symmons and Cressman 2001). Post-copulation, females oviposit their egg pods within the soil, surrounding the eggs with a fine sheath of a foamy secretion of previously unknown composition. The same secretion appears as a thick foam plug above the egg pods (Fig. 1). Upon hatching the young hatchlings will crawl through the foam plug to reach the soil surface and start a new locust generation (Uvarov 1977; Hägele et al. 1999; Symmons and Cressman 2001).

An important aspect of the desert locust's biology is that of the symbiotic interaction with the bacteria inhabiting its gut. These have been shown to augment the locust immunity through colonization resistance (Dillon and Charnley 1995; Dillon and Charnley 2002; Dillon et al. 2005), and were also suggested to maintain the swarm's integrity through the emission of attractant volatiles (Dillon et al. 2000; Dillon and Charnley 2002; Dillon et al. 2002). Although *S. gregaria* does not engage in an obligatory interaction with any specific bacterial species, it is consistently associated with bacteria of the families*Enterobacteriaceae* and *Enterococcaceae* (Dillon and Charnley 2002, Shi et al. 2014, Lavy et al. 2019; submitted). It has been

traditionally believed that the locust's acquired bacteria are strictly environmentally-determined (Dillon and Charnley 2002, Salem et al. 2015). However, migrating *S. gregaria* in their swarming phenotype encounter very different environnements, plants and oviposition sites (Popov 1958; Uvarov 1977; Pener and Simpson 2009; Cease et al. 2015) that harbor very different bacteria (Soussi et al. 2015). Hence, it is unlikely that the vicinity of the newly-hatched individuals can be the primary source of the bacterial agents that eventually play pivotal physiological roles as noted above. Rather, we hypothesized that the locusts possess a core microbiome that is vertically transmitted across generations.

There are numerous examples of insects that inoculate their offspring with beneficial bacteria, through different mechanisms. For instance, the burying beetle (*Nicrophorus vespilloides*) manipulates the bacterial composition of the carcasses upon which its larvae are reared (Shukla et al. 2017). In addition, it feeds its offspring regurgitated food, thus inoculating the young with advantageous gut bacteria, protecting them through colonization resistance from carcass-originated pathogens (Wang and Rozen 2017a; Wang and Rozen 2017b). Other examples include brood-cell smearing with vertically transmitted*Streptomyces* by solitary digger wasps to protect the larvae from pathogenic fungi and to ensure bacterial transmission to the next generation (Kaltenpoth et al. 2005; Kaltenpoth et al. 2010; Kroiss et al. 2010, Engl et al. 2017); and symbiont-enclosing capsules deposited by Plataspidae (suborder: Heteroptera) females to enable symbiont acquisition by their hatchlings (Fukatsu and Hosokawa 2002). These inoculation mechanisms and many more are thoroughly reviewed by Salem et al. (2015) and Onchuru et al. (2018).

We have recently reported the same operational taxonomic unit (OTU), assigned as *Enterobacter* (*Enterobactercea*), to be present in both laboratory-reared gregarious and solitary locusts across several generations (as well as in field-collected *S. gregaria* ; Lavy et al. 2019). These data suggest a potential for a symbiont transgenerational inoculation mechanism in the desert locust. The current study thus sought to clarify whether locusts vertically transmit beneficial bacterial agents; and to reveal the mechanism by which such bacteria are inoculated across generations, contributing to the successful migration of locusts to new territories.

Material and Methods:

Egg pods sampling

Gregarious locusts were reared under crowded conditions for many consecutive generations. Food and ambient conditions are detailed in Lavy et al. (2019).

In order to examine the possibility of transgenerational bacterial transmission, we conducted an experiment comparing the bacterial composition of locust mothers and their newly-hatched and unhatched offspring.

Newly-matured females were introduced individually into a 11x12x14.5 cm metal cage containing two mature males and food. A 50 ml centrifuge tube (Corning, NY, United States) filled with autoclaved moistened sand was replaced daily until oviposition occurred and egg pods were detected. The females were then sacrificed and kept individually in 70% absolute ethanol, at -20°C, until tissue sampling.

The tubes containing egg pods were incubated at 37°C. Around day 11 of incubation (the typical period for hatching in these conditions) the tubes were meticulously examined for the presence of hatchlings. Upon detection (within 30 min of their emergence to the sand surface) three hatchlings were collected into a 1.5 plastic tube using sterile forceps. The egg pod was then excavated and three healthy-appearing larvae-bearing eggs were collected in the same manner.

The larvae were sacrificed, washed and vortexed five times x 1 minute in filtered saline (0.9% NaCl) to remove unattached external bacteria (Senderovich et al. 2008), and stored in 70% absolute ethanol at -20°C until further use. Samples of the foam and the surrounding sand were also collected from each tube containing an egg pod and stored in the same manner, allowing to assign hatched larvae to their unhatched siblings and to the foam and the sand from the same tube (egg pod).

With and without foam treatments

Individual sexually-mature females were housed and supplied with sand filled oviposition tubes as described above. Prior to autoclaving, the sand tubes were cut longitudinally and then re-attached together. After the female had laid eggs, the egg pods were incubated at 37°C for five days. At the 5th day post-oviposition, the tubes were opened along the pre-made cut and some of the eggs were removed and placed in a fresh similar tube of autoclaved sand. The original egg-containing tubes were resealed and incubated. The eggs that remained in the original tube gave rise to hatchlings that surfaced through the foam plug (foam treatment), while the hatchlings emerging from the relocated eggs surfaced only through a layer of moistened sand (without foam treatment). Upon surfacing, the hatchlings were collected in groups of three, washed 5 times as described above, and stored in 70% absolute ethanol at -20°C until further use.

Females' gut sampling

The locusts' wings and limbs were removed and their surface was sterilized by submerging in 1% NaOCl solution for 2 min followed by two consecutive washings in fresh double-distilled water. They were then dissected aseptically (detailed in Lavy et al. 2019) and their hind-guts were collected. The excised samples were kept individually in 70% absolute ethanol at -20°C until DNA extraction.

DNA extraction and sequencing

Ethanol was removed and bacterial genomic DNA was extracted using the "Powersoil" DNA isolation Kit (Mo Bio Laboratories Inc., Carlsbad CA, United States), according to the manufacturer's instructions, using 60 µl for final DNA elution. To determine bacterial composition, polymerase chain reaction (PCR) of variable areas V3 and V4 of the prokaryotic 16S rRNA gene was applied on the extracted DNA; using a universal primer containing 5-end common sequences (CS1-341F 5'- ACACTGACGACATGGTTCTACANNNNCC-TACGGGAGGC AGCAG and CS2-806R 5'-TACGGTAGCAGAGACTTGG TCTGGACTACHVGGGTW TCTAAT). PCR conditions: initial 94°C step for 2 min followed by thirty PCR cycles of denaturation at 94°C/ 30s, annealing at 50°C /30s and extension at 72°C /30s; ending with 4 min at 72°C. The reactions were performed using the PCR master mix Go Taq® Green Master Mix (Promega Corporation, Madison, WI, United States). PCR product validation was conducted by agarose gel 1% electrophoresis. Deep sequencing of the amplified amplicons was conducted on an Illumina MiSeq platform at the Chicago Sequencing Center of the University of Illinois.

In vivo foam inoculation

Plasmid electroporation:

Overnight grown, locust-isolated *Klebsiella pneumoniae* (top hit type-strain: ATCC 13884(T), similarity: 99.76%) was inoculated into a fresh lysogeny broth (LB) and incubated at 37°C for 90 min. The cells were then chilled on ice, harvested by 2 rounds of centrifugation (4000 rpm for 10 min) and washed with double-distilled water, followed by resuspension in 10% glycerol. 60 µl of cell suspension was mixed with 2 µl of pUA66 (Zaslaver et al. 2004) and electroporated. The cells were then transferred into 1 ml LB broth and incubated at 37°C for 1 h, followed by plating on a selective substrate to select for the Kanamycin resistant phenotype of pUA66.

2. Streptomycin resistant mutation induction:

1 ml of pUA66 harboring *K. pneumoniae* LB culture was centrifuged, washed, and re-suspended in saline 0.9% NaCl. 100 µl was then applied onto LB plates containing both Kanamycin (50 µg/ml) and Streptomycin. (100 µg/ml). A *K. pneumoniae* colony that had been formed during overnight incubation at 37°C was applied onto a fresh selective plate to maintain the resistant strain.

3. Resistance stability

The engineered bacteria were cultured in antibiotic-free LB at 37°C. Every 24 hours 100 µl were transferred to a fresh 2 ml LB medium and an additional 100 µl were applied on selective plates overnight. Diagnostic PCR was then performed with specific pUA66 primers (forward primer, 5'-CATAAGATGAGCCCAAG-3; reverse primer, 5'-GTCAGTACATTCCCAAGG-3) on *Klebsiella* suspected colonies from each plate to verify pUA66

presence. PCR conditions: initial 95°C step for 3 min followed by 35 PCR cycles of denaturation at 95°C/ 15s, annealing at 60°C /15s and extension at 72°C /10s; ending with 2 min at 72°C. The enzyme used in this reaction was PCR master mix KAPA2G Fast^{TM} (KAPA Biosystems, Wilmington, MA, United States).

4. Inoculation

Mature female locusts were force-fed with 50 µl saline containing ~ 615 X 10^6 *K. pneumoniae* cells and moved to a female-only cage. One-week post-inoculation fecal pellets were collected from each individual female, applied on a selective agar MacConkey, incubated for 24 h at 37° C, and diagnostic PCR for pUA66 presence was conducted on *Klebsiella* suspected colonies from each plate to confirm the presence of the engineered bacteria in the females' gut.

The females were then transferred individually to a 11x12x14.5 cm metal cage containing two mature males, and fresh tubes of autoclaved sand and food were replaced daily. Within 24 hours of oviposition, a sample of the foam plug and the surrounding tube-sand was applied on selective agar MacConkey plates, and incubated at 37°C for 24 h. pUA66 diagnostic PCR was performed on *Klebsiella* suspected colonies from each plate. Ten days post-oviposition the foam and sand were sampled again in the same manner to confirm the presence of the engineered *K. pneumoniae.*

Individual hatchlings emerging from these egg pods were homogenized in 500 µl of sterile saline, and pUA66 diagnostic PCR was performed on these samples as well.

Foam analysis

Mature female locusts were kept individually in a metal cage containing two mature males. As oviposition substrate they were given a 50 ml centrifuge tube containing chemically inert glass beads (diameter: 1 mm) (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofe, Germany) saturated with double-distilled water.

The tubes were replaced daily, and when oviposition occurred, the egg pod was collected and its foam plug was removed and washed in double-distilled water to remove excess glass beads. The foam was then left for 24 hours to dry at 4°C and the dry foam was kept at room temperature until chemical analysis.

For elemental analysis measurements, the foam plugs were crushed manually, the glass beads were removed, and the crushed matrix was soaked in 0.5M NaOH in 25°C until complete decomposition (clear yellowish solution). The soluble hydrolysis products were filtered from the beads, lyophilized and analyzed. The nitrogen content was converted to protein according to the Nitrogen-to-Protein conversion factor range for insects reported by Janssen *et al.* (2017).

In order to analyze the foam protein content, 1.5gr of crushed foam plugs (with the glass beads) were mixed with 8 ml ethanol and sealed in a 14 ml glass vial. The mixture was incubated at 60°C for 96 h until the foam was almost completely decomposed. Ethanol evaporation was performed in an 80°C dry bath and the pellet was dissolved in reducing sample buffer (containing β-mercaptoethnol) for SDS-PAGE analysis. The sample was boiled at 100°C for 10 min. Finally, SDS-PAGE analysis was performed with 15% acrylamide gel at 90V for 4 hours. The six main identified bands (Fig. S1 in supporting information) were analyzed by Mass-Spectrometry (MS) in the Smoler Protein Research Center at the Technion, Haifa. The screening of protein results was performed against the *Acrididae* and *Locusta* protein data bases. Only identified peptides who passed False Discovery Rate (FDR) correction with 99% confidence interval were used for further analysis.

Since the samples could not be dissolved in a manner that would enable carbohydrate analysis, we incubated 0.04g of sand-coated foam plugs in 50 mM potassium phosphate (pH 6.0) buffer with decreasing chitinase concentrations (Sigma- Aldrich, St. Louis, MO, USA), to determine chitin or chitin-like presence in the foam.

Data analyses

Demultiplexed raw sequences were quality filtered (bases with a PHRED score < 20 were removed) and merged using PEAR (Zhang et al. 2014). Sequences of less than 380 bp (after merging and trimming) were discarded. Data were then analyzed using the Quantitative Insights Into Microbial Ecology (QIIME)

package (Caporaso et al. 2010). Vsearch (Rognes et al. 2016) was used for chimera detection and elimination; OTU picking (0.99 similarity) and taxonomy assignment were performed using Silva database (version 128). Chloroplast and *E-coli* sequences were excluded from the downstream analysis due to the known gut content, and to our inability to completely avoid Master Mix derived*E-coli* fragments. To ensure data evenness, before analysis the data were rarefied to 980 seqs/sample. Since the with and without foam section was analyzed separately, the data of this part were rarefied to 1100 seqs/sample. All statistical analyses were conducted using "R" v.3.4.1. (R Core Team, 2013). Bray-Curtis based Analysis of similarities-"Anosim," principal coordinate analysis (PCoA), and Spearman's rank correlations were carried out using the "vegan 2.4-3" package (Oksanen et al. 2008).

Results:

We utilized 16S rRNA amplicon sequencing for comparing the bacterial composition of gregarious female *S. gregaria* , to that of their offspring, and the immediate environment of the eggs (i.e. foam and sand). As can be seen in figures 2a and 2b

, we observed a significant difference between the bacterial composition of the hatched and unhatched larvae siblings. The main driver of this difference was the higher diversity of bacterial genera in the hatchlings in comparison to the unhatched larvae (mean values of 2.09 and 1.28 respectively, Fig. 2c). This suggests that hatchlings acquire new bacterial genera from the immediate environment while surfacing, either from the foam or from the surrounding sand. Therefore, we also compared the bacterial biodiversity indexes of the sand and the foam plug. The sand demonstrated low diversity while the foam was characterized by a high diversity of bacterial genera (mean values of 1.07 and 1.74 respectively, Fig. 2c), making it a likely source of the hatchling's bacterial inoculum.

Next, we found the common genera *Enterobacter* , *Klebsiella* , and *Corynebacterium* to be shared among the ovipositing female's hindgut, the foam plug and the hatchlings larvae (Fig. 3, S2). In contrast, these genera were absent from the majority of sand samples. Further comparison of *Enterobacter* and *Corynebacterium* in hatched and unhatched siblings, revealed higher levels of these bacteria in the hatchlings. Such higher levels also correlated significantly with the relative abundance of *Corynebacterium* and *Enterobacter*in the specific foam plugs through which these hatchlings had crawled (Spearman rho values: p <0.001, r= 0.89 and$p$ <0.001, r= 0. 78 respectively; Fig. S3). Other bacteria prevalent in the female's hindgut, such as *Weissella* , were not observed in the foam nor in the hatchlings.

Comparing the bacterial biodiversity in hatchlings that had crawled through the foam with that of those that had not, revealed higher diversity indexes in the "with foam" treatment (Fig. 4a). In addition, the relative abundance of *Enterobacter* and *Cornebcaterium*in the "with foam" hatchlings were significantly higher than in the "without foam" hatchlings (Fig. 4b, 4c), reinforcing the hypothesized role of the foam in vertical transmission of these bacteria.

In order to directly test the possibility of the female's gut as a source of foam bacteria, we used females inoculated with an engineered*Klebsiella pneumoniae* that was kanamycin- and streptomycin resistant, enabling efficient selection. The resistant *Klebsiella*strain was found to be present in the inoculated female's feces 7 days post-inoculation. Therefore, we concluded that it is stably maintained in their gut. On day 1 post oviposition this bacterium was found in 11 out of 15 foam plugs secreted by those females on top of their egg pods, but in only 3 samples of the sand surrounding the egg pods. It remained present in five foam plugs and two sand samples after 10 days; yet, upon hatching, the engineered *K. pneumoniae* was found in only 1 hatchling of the 45 examined (3 per egg pod).

To test whether the foam plug could have biochemical properties that select for particular microbes, we analyzed its composition. Determining the foam plugs' total protein content was done by elemental analysis and identification of the protein repertoire was done by SDS-PAGE and MS analysis. Elemental analysis of the foam plug (Fig. 5a) indicated the presence of ˜0.63% nitrogen, which is translated to a 3-3.5% protein fraction. Nevertheless, this small protein component contained at least eight proteins that are attributed to the locust's immune system, such as thaumatin-like protein, prophenoloxidase, and lysozyme (Tables 1, S4),

out of the 42 proteins that could be identified by mass spectrometry. The low protein content of the foam suggests that it mostly comprises carbohydrates. However, the extremely stable and hydrophobic nature of the foam prevented its carbohydrate profile analysis. Since the foam is insect-derived we hypothesized that these carbohydrate molecules are composed of chitin or a chitin-like polysaccharide. Treating the foam with chitinase caused it to lose its hydrophobicity and to change its shape and color (Fig. 5b), thus confirming our hypothesis that chitin was a major component of the plug.

Discussion

The success of locust swarms to cover very large distances and conquer new grounds during an upsurge (Skaf et al. 1990; Symmons and Cressman 2001) may be, at least partly, attributed to the locust's interaction with beneficial gut microbes, which are important for the locust immunity (Dillon and Charnley 1995; Dillon and Charnley 2002; Dillon et al. 2005) and may also be instrumental in maintaining the locust aggregation behavior (Dillon et al. 2000; Dillon and Charnley 2002; Dillon et al. 2002). Though much knowledge has been gained concerning the bacterial symbionts of locusts (Dillon and Charnley 2002; Shi et al. 2014; Lavy et al. 2019, submitted), the mechanism of maintaining these bacteria within the population and, most importantly, transmitting them from one generation to the next remained unclear. The commonly suggested hypothesis was that locusts acquire their endosymbionts from the hatchlings' environment (Dillon and Charnley 2002, Salem et al. 2015). However, the importance of these bacteria and the diversity of feeding and oviposition habitats that females encounter while migrating (Popov 1958; Uvarov 1977; Pener and Simpson 2009, Cease et al. 2015) make this hypothesis unlikely. Here we demonstrate for the first time that *S. gregaria* deposit beneficial bacterial symbionts in the foam on top of their eggs, thus ensuring that their offspring will be inoculated with specific bacteria.

Our data suggest that the locust hatchlings acquire the bacterial symbionts post-hatching, while climbing up to the surface through the foam plug. The bacterial genera *Enterobacter* , *Klebsiella* , and *Corynebacterium* shared among the female's gut, the foam, and the hatchlings provide further evidence for the maternal origin of these foam-inhabiting bacteria. However, it seems that the inoculation process is also somewhat selective, as, for example, *Weissella* , which is prevalent in the mother's gut, was not detected in the foam or in the offspring samples. The data presented concerning the direct link between the relative abundance of *Corynebacterium* and *Enterobacter*in the foam and in the hatchlings emerging through it (Fig. S3) further support the important role played by the foam in ensuring the offspring's inoculation with these maternally-derived bacteria.

Although we failed to obtain locust-isolated *Enterobacter* stably expressing the antibiotic resistance markers, we were able to demonstrate a vertical transmission of genetically- modified locust-isolated *Klebsiella* from the female gut to the foam plug, and eventually to one of the hatchlings. These results clearly indicate that locust females inoculate the foam with gut bacteria upon oviposition. This also offers some proof of concept for a bacterium originating in the female's gut that eventually reach her offspring. It is possible that the antibiotic resistance agents incur a substantial fitness cost for the bacteria expressing them within the foam, allowing fitter bacteria to dominate the niche.

As noted, both *Enterobacter* and *Klebsiella* are consistently found as gut bacterial symbionts of the desert locust, and species of both genera were found to produce guaiacol and phenol which are considered to be cohesion pheromones that help to maintain the integrity of the swarm (Dillon et al. 2000, Dillon and Charnley 2002). Moreover, in a previous study we showed the consistency of the same *Enterobacter* operational taxonomic unit through several generations of laboratory-reared locusts as well as field-collected *S. gregaria* (Lavy et al. 2019). This suggested that a mechanism exists that is responsible for maintaining these gut bacteria, including a transgenerational inoculation mechanism as we have demonstrated here.

Corynebacterium is known to inhabit insects of different orders (e.g. Zucchi et al. 2012; Segata et al. 2016; Tobias 2016; Park et al. 2019), as well as the reproductive system of locust females (Lavy et al. submitted). Itoh et al. (1996, 1997) identified several *Corynebacterium* species that have the rare ability to utilize gaseous acetophenone as a carbon source sufficient for their proliferation. Acetophenone was also identified in the

gaseous fraction emitted from foam plugs secreted by *S. gregaria* females (Rai et al. 1997). Therefore, it is highly plausible that the acetophenone in the foam enriches for *Corynebacterium* species that in turn can produce antibiotic substances (Gumiel et al. 2015; Tobias 2016). The locusts thereby "feed" bacteria that in turn can protect their eggs.

However, this is not the only mechanism that locusts appear to utilize to ensure the presence of the selected bacteria in the foam. Mass-spectrometry analysis of the protein fraction of the foam revealed several immune-related peptides, such as lysozyme, thaumatin-like protein, and prophenoloxidase. We speculate that the presence of these peptides in the medium surrounding the eggs provides an extra layer of protection in addition to the mechanical barrier of the foam itself. This additional protection, can potentially prevent chitin-degrading pathogens from damaging the eggs, as in the case of the chironomid (*Chironomus* sp.) and *Vibrio cholera* interaction (Halpern and Broza 2001, Laviad et al. 2016). In the locust case, it is plausible that the gram positive targeted lysozyme (Ragland and Criss 2017) in the foam, is responsible for the near absence of maternal-gut *Weissella* and ambient sand *Bacillus* (both are gram-positive bacteria) from the foam.

To the best of our knowledge, such use of foam, to protect an egg-mass, both mechanically and immunologically; is known only from several foam-nesting frog species. These frogs cover their eggs with a protein-rich foam to protect them from desiccation as well as from pathogens (Cooper and Kennedy 2010).

As noted, the extreme stability of the foam prevented us from fully understanding the content of its carbohydrate fraction. Nevertheless, we have demonstrated here for the first time that chitin is a major component of the locust foam plug. The solid, hydrophobic nature of this polysaccharide probably acts as a physical protection layer against predatory organisms as well as against desiccation.

In previous studies we hypothesized that the desert locust employs some mechanism in order to inoculate their offspring with beneficial bacteria, and thus maintain specific bacterial symbionts within the population across generations (Lavy et al. 2019, submitted). We show here for the first time that the foam deposited above the eggs is used as such an inoculation mechanism. Furthermore, we have demonstrated that the foam is more than just a reservoir for the bacteria deposited by the mothers. It is also a selective medium, supporting potentially mutualistic species while inhibiting the proliferation of others, thus maintaining a beneficial bacterial consortium to be transmitted across locust generations.

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Legends :

Figure 1 : **(a)** Illustration of a locust female laying her eggs in the ground **(b)** the egg pod is enveloped in a foam sheath, and sealed with a foam plug. (**c**) Illustration of the egg pod in the soil with the foam sheath removed, to highlight the position of the foam plug above the eggs.

Figure 2 : **(a)** Weighted UniFraq Principal Coordinate Analysis (PCoA) of the bacterial composition of locust hatchlings (cyan) and their unhatched siblings (red). (ANOSIM: R=0.18, p =0.008).**(b)** Bray Curtis, genus based PCoA of the bacterial composition of locust hatchlings and their unhatched siblings. (ANOSIM: R=0.19,p =0.007). Colors as in (a). **(c)** Genus-level, Shannon biodiversity indexes of the bacterial composition of the hatched and unhatched siblings, in addition to the foam and sand surrounding the eggs. Differences between the groups were analyzed by Kruskal-Wallis test and Dunn's post hoc test. Boxes within the violin represent the range containing 50% of the data points and the black horizontal lines denote the median.

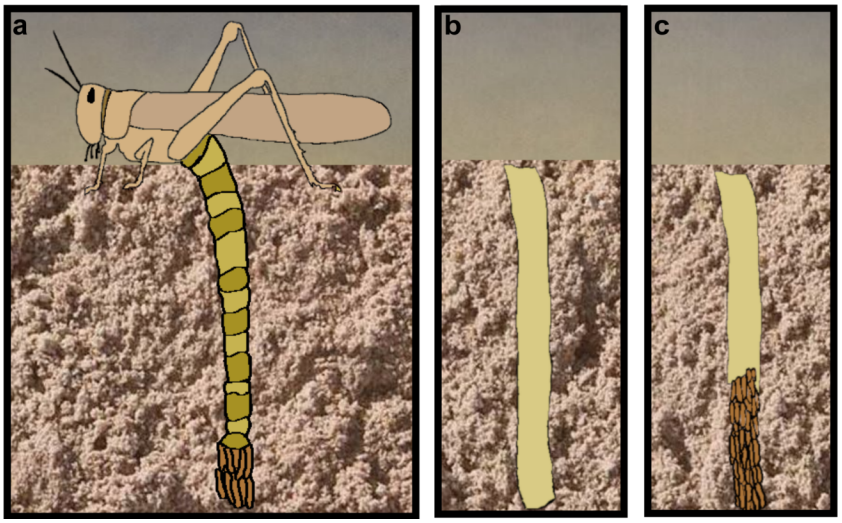
Figure 3 : Venn diagram of the dominant ($>$ 75 reads per sample type) shared OTUs among the female hindgut (left, n=16) (**a**), the hatchlings (right, n=16) (**b**), and the foam (bottom, n=15) (**c**). Inserts show a sketch of the sample origin (highlighted in white), and a bar chart describing the relative abundance of the core bacterial genera in each sample-type (minimum of 80% presence in the tissue samples). Bacteria mentioned in the main text are uniformly colored. The taxonomical assignment of the three common OTUs is listed on the right.

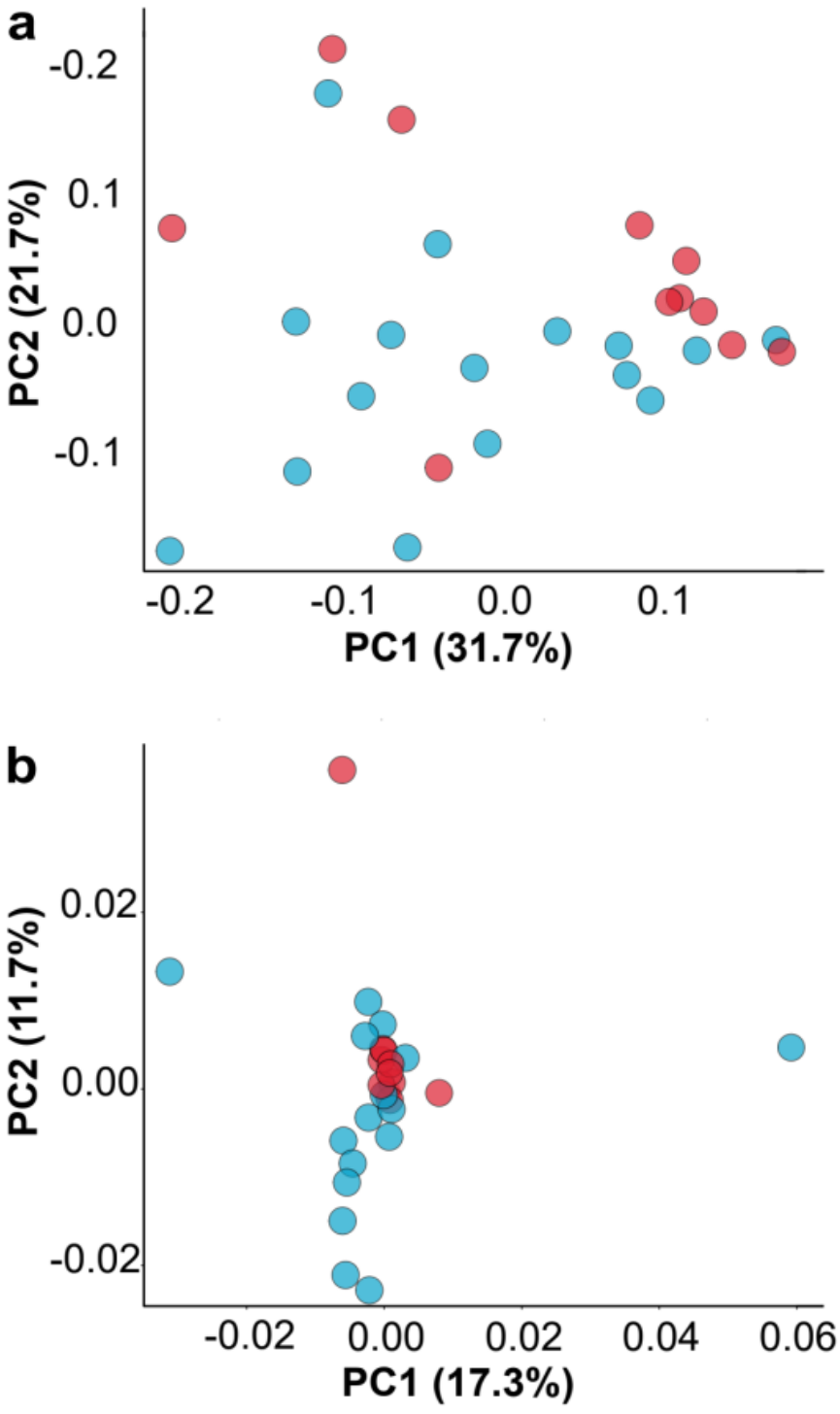
Figure 4 : Violin plots representing data of the hatchlings passing through the foam plug (with foam) and their siblings that did not pass through the foam (without foam). Gray boxes within each violin represent the range containing 50% of the data points and the black horizontal line denotes the median. **(a)** Shannon diversity indexes (T-test. p =0.04). **(b)** Relative abundance comparison of *Corynebacterium* in the with-foam and the without-foam treatments. (Mann Whitney. p =0.01). **(c)**Relative abundance of *Enterobacter* in the with-foam and the without-foam treatments (Mann Whitney. p =0.03).

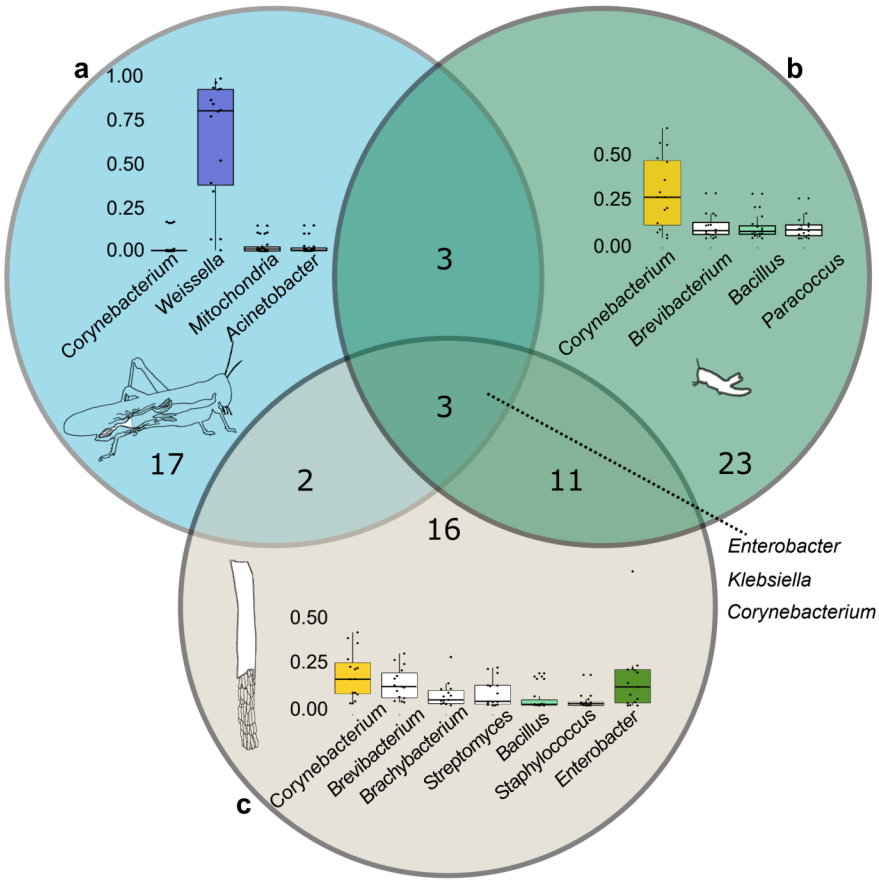
Figure 5 : **(a)** Elemental analysis of locust nest plugs.**(b)** Partial hydrolysis of the foam plug mass by incubation with decreasing order of chitinase active unit (U) concentrations.

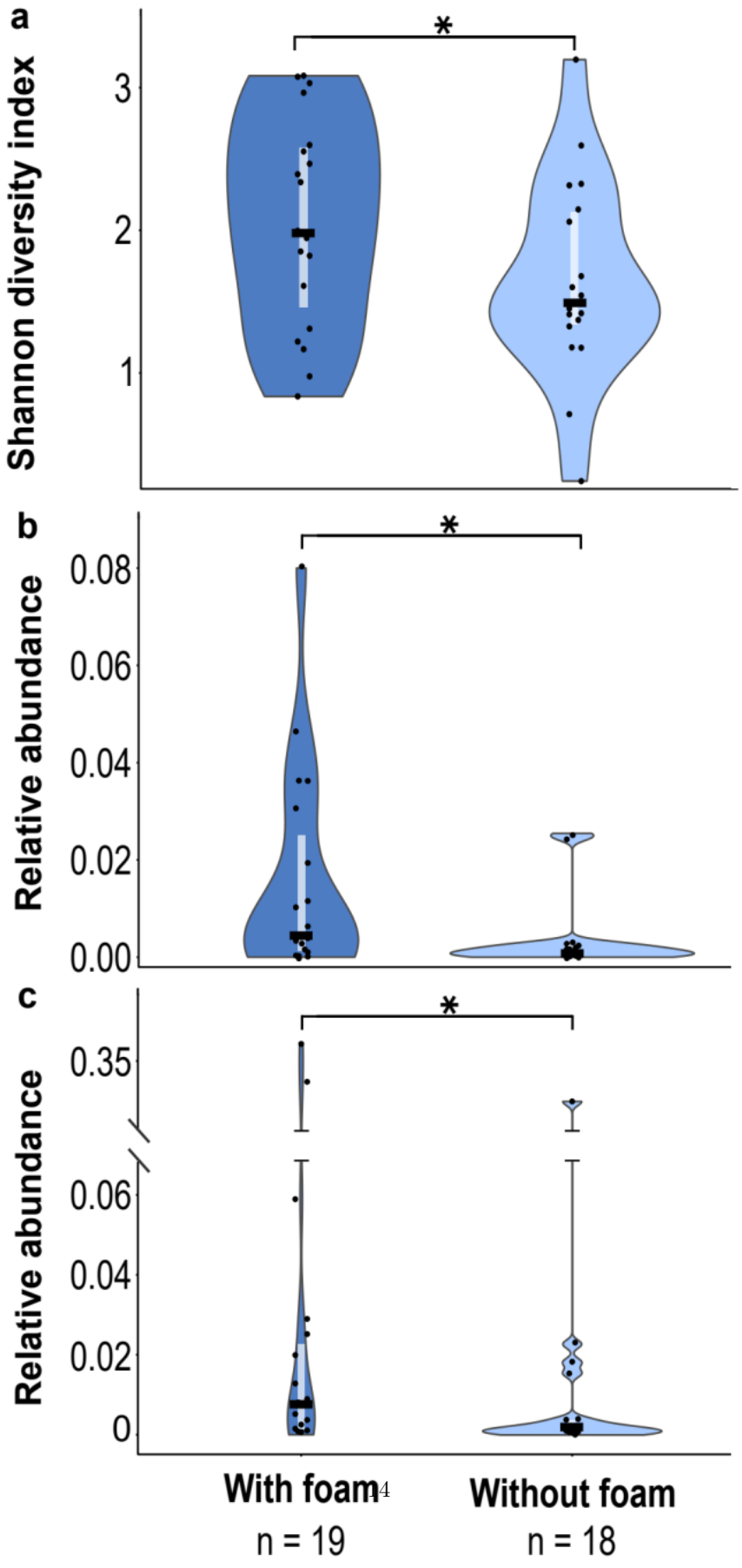
Table 1 : Proteins found in the foam plug and suspected as immune agents that may have a bacterial regulatory role. This is a partial list of 8 out of 40 proteins (only immune-related proteins are presented).

Accession	**Description**	**MW [kDa]**	**Reference**
40949967	Thaumatin like protein	26.00	Brandazza et al. 2004
1120618827	Vitellogenin A	150.71	Song et al. 2013
1394299014	C-type lysozyme	79.99	Unpublished
972988174	Ferritin subunit	26.53	Bonilla et al. 2015
414079973	Prophenoloxidase 1	84.20	Unpublished
1231943145	Vitellogenin B	26.45	Unpublished
414145764	Chain C, Greglin	9.18	Derache et al. 2012
371942914	C-type lysozyme	15.69	Mohamed et al. 2016









a

Repeat	N (%)	C (%)	H (%)	S (%)
A	0.741	2.840	0.358	0.0841
B	0.519	3.540	0.129	0

b